MONASH UNIVERSITY
THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
ON..... 18 November 2003 .....
.....[redacted].....
Sec. Research Graduate School Committee

ADDENDUM

p 62 add to start of last paragraph: "Refer to Appendix A for composition of Solutions I, II and II."

p 63 add to start of last paragraph: "Refer to Appendix A for composition of Solutions I, II and II."

p 107 last paragraph, line 8: insert "Alternatively, the absence of growth factors in C2C12 differentiation culture media may indicate growth factor sensitivity of the *Gabpα* promoter and therefore may be a non-specific effect. Growth factor responsiveness needs to be addressed."

p 111 replace last sentence of paragraph 1: "Mobility shift and site-specific mutation studies are necessary to prove that these binding sites are functional and to identify the transcription factors responsible for basal and cell type-specific *Gabpα* expression."

p 119 second paragraph, line 2: insert "Protein extracts were prepared according to their wet weight (see Chapter 3) to generate samples of equivalent concentration and confirmed by Coomassie staining of SDS-PAGE prior to Western blot."

p 122 second paragraph, line 12: insert "As it is difficult to find a housekeeping gene that is ubiquitously expressed at constant levels in all tissues and β-tubulin expression is known to vary in some tissues, only relative Gabpα protein levels could be determined. Repeating these experiments with additional loading controls would help to confirm these results."

p 124 first paragraph, line 3: insert "The discrepancy of this result and the apparent increase in *Gabpα* promoter activity upon C2C12 differentiation (see Chapter 4), indicates that either the *in vitro* system of C2C12 differentiation does not directly reflect the *in vivo* situation of skeletal muscle formation, or that post-transcriptional mechanisms tightly regulate Gabpα protein levels."

p 141 second paragraph, line 7: replace "However, Gabpα is expressed in all cells of heart and skeletal muscle tissues." with "Gabpα appears to be expressed ubiquitously in heart and skeletal muscle. However, as non-muscle cell types are also present in these tissues, in future experiments muscle cells will be discriminated by antibody staining for the muscle membrane protein Dystrophin."

p 149 last paragraph, line 7: insert "A proven skeletal muscle-specific promoter such as *Muscle Creatine Kinase* or *Skeletal Actin* may have been used to overexpress *Gabpα* in skeletal muscle tissues *in vivo*, however this was not attempted as endogenous regulation was desired to mimic the increased dosage relevant to Down syndrome."

p 160 add to end of first paragraph: "In the generation of conditionally targeted *Gabpα* ES cells it was presumed that *PGK-Cre* and *PGK-puromycin* plasmids did not integrate into ES cell DNA as they were not linearised prior to transfection. In addition, genomic DNA extracted from mice resulting from blastocyst injection did not show presence of the *Cre* gene upon genotyping."

p 197 first paragraph, line 2: replace "longitudinal cryosections" with "longitudinal and transverse cryosections"

p 197 add to the end of first paragraph: "Further analysis of skeletal muscle types may reveal tissue-specific morphological effects of loss of Gabpα expression."

p 202 second paragraph, line 21: insert "In future studies, a more reliable method of assessing skeletal muscle fibre type would use Myosin Heavy Chain isoform-specific antibodies. This would eliminate any subjective element in distinguishing staining intensity, and highlight any discordance in skeletal muscle metabolism and Myosin isoform expression, as seen using NADH staining."

p 213 first paragraph, line 11: insert "More precise measurements could also be obtained with a dynamometer. In addition, an extension of histopathological analysis of forearm muscles may also correlate with this finding, as individual muscle types respond differentially to protein deficiencies."

p 240 replace last sentence of third paragraph: "However, this preliminary analysis demonstrates that Gabpα is likely to be one of the ETS proteins necessary for formation of intact AChR clusters at the NMJ."

Characterisation of Gene Structure and Function of the ETS Transcription Factor Gabpα in Mouse

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

Debra Alison O'Leary
BSc(Biomed)(Hons)

Centre for Functional Genomics and Human Disease
Monash Institute of Reproduction and Development
Monash University, Australia

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List of Abbreviations

aa	Amino acid
α-btx	α-bungarotoxin
ACh	Acetylcholine
AChR	Nicotinic acetylcholine receptor
ADSL	Adenylosuccinate lyase
AMV	Avian myeloblastosis virus
AP	Activator protein
APP	Amyloid precursor protein
ATF	Activating transcription factor
ATP	Adenosine triphosphate
AVSD	Atrioventricular septal defect
BAC	Bacterial artificial chromosome
Bax	Bcl-2-associated x protein
Bcl	B cell lymphoma
BLAST	Basic local alignment search tool
BMD	Beckers muscular dystrophy
bp	Basepairs
Brn	Brain-specific homeobox/POU domain protein
BSA	Bovine serum albumin
cAMP	Adenylate cyclase
CBP	cAMP-responsive element-binding protein
CCCP	Carbonyl cyanide m-chloro phenyl hydrazone
CD	Cluster designation antigen
cDNA	Complementary deoxyribonucleic acid
CMS	Congenital myasthenic syndrome
CMV	Cytomegalovirus
CNS	Central nervous system
COX	Cytochrome-c-oxidase
Cre	Causes recombination
CREB	cAMP-responsive element binding protein B
CREM	cAMP-responsive element modulator
CT	Computer tomography
Cyp	Cytochrome P450
DAG	Dystrophin-associated glycoprotein
DMD	Duchenne muscular dystrophy
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
DS	Down syndrome
E	Embryonic day
E4TF	Adenovirus E4 transcription factor
EDL	Extensor digitorum longus
EF	Elongation factor
EGFP	Enhanced green fluorescent protein
ELF	E74-like factor
Elg	ETS-like gene
ELK	ETS-like protein
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
EPC	Endplate current
ER	ETS-related protein
ErbB	v-erb-b2 erythroblastic leukaemia viral oncogene homologue
ERF	ETS-2 repressing factor
ERG	ETS-related gene
ERK	Extracellular signal-regulated kinase
ERM	ETS-related molecule PEA-like
ERP	ETS-related protein
ES cells	Embryonic stem cells
ESE	Epithelial-specific ETS
EST	Expressed sequenced tag
ETS	E26 transformation-specific
EWS	Ewing's sarcoma protein
FACS	Fluorescence activated cell sorter
FEV	Fifth ewing variant
FITC	Fluorescein isothiocyanate
FLI	Friend leukaemia virus integration
Fos	FBJ murine osteosarcoma viral oncogene homologue
Fra	Fos-related antigen
GABP	GA-binding protein
GAP	Growth associated protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G-CSF	Granulocyte-colony stimulating factor
GDNF	Glial-derived neurotrophic factor
GLI	Glioma-associated oncogene
GPX	Glutathione peroxidase
Gr	Granulocyte marker
hαSA	Human alpha skeletal actin
Hox	Homeobox protein
HRP	Horse radish peroxidase
HSA	Homo sapien chromosome
HSV	Herpes simplex virus
Id	Inhibitor of DNA binding
Ig	Immunoglobulin
IκB	Inhibitor of kappa light polypeptide gene enhancer in B cells
IL	Interleukin
Jun	v-jun sarcoma virus 17 oncogene homologue
JNK	Jun N-terminal kinase
kb	Kilobases

ko	Knockout
lacZ	β-galactosidase
Lck	Lymphocyte-specific protein tyrosine kinase
Lin	Lineage marker
loxP	Locus of crossing (x) over of P1 bacteriophage
Mac	Macrophage marker
Mad	Max dimerisation protein
MAPK	Mitogen activated protein kinase
Max	Myc-associated protein x
Mb	Megabases
MCK	Myosin creatine kinase
MEF	Myeloid Elf-1-like factor
MEPC	Miniature endplate current
MHC	Myosin heavy chain
MLC	Myosin light chain
MMU	Mus musculus chromosome
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial deoxyribonucleic acid
MTF	Mitochondrial transcription factor
MTT	3-(4,5.dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MuSK	Muscle-specific kinase
Mx	Myxovirus (influenza virus) resistance, interferon-inducible protein
Myc	v-myc myelocytomatosis viral oncogene homologue
MyoD	Myogenic determination factor
Mzf	Myeloid zinc finger protein
NADH	Nicotinamide adenine dinucleotide
NBT	Nitro blue tetrazolium
N-CAM	Neural-cell adhesion molecule
NERF	New ETS-related factor
NET	New ETS factor
Neu	Neuroblastoma protein
NF	Neurofilament
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B cells
NIX	Nucleotide identify x
NLS	Nuclear localisation signal
NMJ	Neuromuscular junction
NOS	Nitric oxide synthase
Nrf	Nuclear respiratory factor
NRG	Neuregulin
ORF	Open reading frame
P	predicted promoter region
Pax	Paired box gene
PCR	Polymerase chain reaction
PDEF	Prostate-derived ETS factor
PE1/METS	Mitogenic ETS transcriptional suppressor
PEA	Polyomavirus enhancer activator
PET	PC12 ETS factor
PGK	Phosphoglycerate kinase
PI	Propidium iodide
Pip	PU.1 interacting partner
PNT	Pointed
Poly-A	Polyadenylation
POU	Conserved region in Pit-1, Oct-1, Oct-2 and Unc-86 proteins
PRF	PU.1 related factor
PU	Purine rich box binding protein
RACE	Rapid amplification of cDNA ends
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
SAM	Sterile alpha motif
SAP	SRF-associated protein
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEP	Sterility, ETS-related, polycomb protein
SOD	Superoxide dismutase
Sp	Binding protein of the serotonin receptor promoter
Spi	Spleen focus forming virus (SFFV) proviral integration oncogene
SRF	Serum response factor
Stat	Signal transducer and activator of transcription
Stch	Stress 70 protein chaperone, microsome-associated
SURF	Surfeit
TAF	TBP- associated factor
TBP	TATA-binding protein
Tc	Transchromosomal
TCF	Ternary complex factor
TEL	Translocation ETS leukaemia
TESS	Transcription element search system
TF	Transcription factor
tg	Transgenic
TH	Thyroid hormone
TK	Thymidine kinase
TR	Texas red
TRF	TBP-related factor
Ts	Trisomy
TSHR	Thyroid-stimulating hormone receptor
UTR	Untranslated region
VISTA	Visual tools for alignments
wt	Wildtype
YAC	Yeast artificial chromosome
YAF	YY1-associated factor
YEAF	YY1- and E4TF1/hGABP-associated factor
YY	Ying-yang

Abstract

The GABP transcription factor is implicated in the regulation of a broad range of target genes, encoding proteins of mitochondrial function and proteins necessary for signalling at the neuromuscular junction (NMJ). The GABP complex is composed of an ETS protein (α subunit) necessary for DNA binding and an unrelated ankyrin-repeat protein (β subunit) required for target gene transactivation. GABPα and β proteins are expressed in all tissues, however the question of how cell type and promoter specificity is achieved is yet to be answered. Further, although mutation of the GABP binding site of the *Nicotinic acetylcholine receptor epsilon* (*AChRε*) promoter is implicated in several cases of Congenital Myasthenic Syndrome (CMS), the function of GABP during NMJ formation has not been elucidated. Finally, taken together with the list of GABP gene targets, localisation of *GABPα* to HSA 21 begs the question of whether overexpression of GABPα disrupts the stoichiometric balance of the GABP complex, contributing to the muscular hypotonia and redox instability associated with Down syndrome (DS). Regulatory mechanisms of *Gabpα* expression have been investigated here by analysis of mRNA and protein levels throughout embryogenesis and in adult mouse tissues, and function of *GABPα* assessed by generation of mouse models.

Tissue-specific alternative 5' and 3' UTR transcripts of *Gabpα* identified in this study are thought to help regulate the expression of the Gabp complex by post-transcriptional mechanisms such as altered transcript stability, transcript localisation and translation efficiency. In addition, novel alternative splice forms of *Gabpβ1* were identified; encoding proteins of predicted dominant negative function, preventing formation and/or nuclear localisation of the Gabp complex, and Gabp target gene transactivation. Hence, although *Gabpα* and *Gabpβ1* are ubiquitously expressed, tissue-specific post-transcriptional regulation may enable Gabp to regulate target genes of diverse function.

An increase of *GABPα* mRNA expression does not always correspond with an increase in protein expression. However, skeletal muscle and brain of Ts65Dn partial

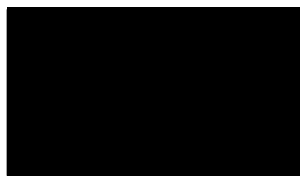
trisomy 16 mice exhibit increased Gabpα protein expression, supporting the hypothesis that increased gene dosage of *GABPα* contributes to tissue-specific characteristics of DS.

Mice carrying a homozygous deletion in the *Gabpα* locus die prior to implantation, indicating that Gabpα function is essential for early embryonic development. In addition, the decrease in number of AChR clusters and simplified NMJ structure in mice lacking *Gabpα* specifically in skeletal muscle supports the idea that Gabp regulates the expression of *AChRδ* and *AChRε*. Furthermore, decreased amplitude of spontaneous endplate currents is suggestive of altered postsynaptic properties of NMJs in the absence of Gabpα. Decreased mRNA expression of another Gabp target in these mice, *Mitochondrial Transcription Factor A*, also correlates with a decrease in the proportion of type I fibres in glycolytic muscles.

Together this data confirms that Gabp is necessary for formation of intact AChR clusters at the NMJ and suggests that Gabp helps maintain the oxidative state of a cell. Therefore, loss of GABPα expression in humans may be a cause of CMS and increased gene dosage of *GABPα* may contribute to the muscular hypotonia and redox imbalance characteristic of DS.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other institution and, to the best of my knowledge, contains no material previously published or written by another person, except where due reference is made in the text. Where the work in this thesis is based on joint research, the relative contributions of the respective persons are acknowledged.



Debra Alison O'Leary

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Part of the work in this thesis was presented orally at the following conferences and symposia:

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* Received a Symposium Gold Award *.

O'Leary D.A., Ristevski S., Kola I., Hertzog P.J. (2001). The Role of Gabpα at the Neuromuscular Junction. Presented at *The Monash Institute of Reproduction and Development Postgraduate Symposium*, Monash IRD, Melbourne, Vic, Australia.

* Received a Symposium Gold Award *.

O'Leary D.A., Ristevski S., Koleski D., Kola I., Hertzog P.J. (2000). The Role of GABP in Down Syndrome. Presented at *The Monash Institute of Reproduction and Development Postgraduate Symposium*, Monash IRD, Melbourne, Vic, Australia.

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O'Leary D.A., Ristevski S., Koleski D., Owen M., Kola I., Hertzog P.J. (2001). Characterisation of the Function of the ETS Gene *GABPα* by use of Mouse Models. Presented at *The 42nd Annual Short Course in Medical and Experimental Mammalian Genetics*, The Jackson Laboratory, Bar Harbor, Maine, USA.

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O'Leary D.A., Ristevski S., Koleski D., Owen M., Kola I. (2000). The Role of GABP in Down Syndrome. Presented at *The Monash University Faculty of Medicine Postgraduate Symposium*, Melbourne, Vic, Australia.

O'Leary D.A., Ristevski S., Koleski D., Owen M., Kola I. (2000). The Role of GABP in Down Syndrome. Presented at *The Lorne Genome Conference*, Lorne, Vic, Australia.

Chapter 1

Introduction:

Transcription Factors and Mammalian Development

1.1 Signal Transduction, Transcription and Development

Mammalian development may be viewed as a progressive series of signal transduction pathways, resulting in the expression of enzymes and structural proteins necessary for organogenesis and cellular homeostasis. Hormones and growth factors binding to their receptors in response to environmental stimuli initiate these signalling pathways. This, in turn, leads to the expression of downstream target genes by transcription of DNA into mRNA, and subsequent translation into a protein product. Regulation of development and cellular homeostasis can therefore be regulated by the availability and activity of transcription factors, mRNA processing and stability, as well as protein production and post-translational modifications. Defects in any stage of signalling pathways can result in genetic disease.

Transcription factors act as switches, capable of turning on or off the processes of proliferation and differentiation within cells. Animal cells are likely to possess 1,000-10,000 different transcription factors (Lemon and Tjian 2000). Some transcription factors are expressed in a very restricted manner, and others are expressed ubiquitously, usually at basal levels. Environmental stimuli, cellular context, and the presence of other transcription factors, can determine the specific effect of a transcription factor on each target gene promoter. Alternative promoter usage may also depend upon which transcription factors are present, resulting in differential protein expression.

Understanding the role of each transcription factor, and its means of regulation, is an important step in understanding the developmental process as a whole. Aberrant expression of ubiquitous transcription factors can result in diseases such as cancer. For example, the gene encoding the cell cycle regulator p53 is mutated in approximately half of all cases of breast cancer (Spandidos et al. 1992), and that encoding pRB is mutated in

30 % of retinoblastoma tumours (Bernards et al. 1989). Overexpression of transcription factors is also implicated in the aetiology of Down syndrome (trisomy 21), as many genes encoded by different chromosomes are overexpressed in Down syndrome individuals, perhaps due to overexpression of their upstream regulators (Pritchard and Kola 1999). Point mutations within transcription factor binding sites can also result in a loss of target gene expression. This has been shown to occur in the rare condition of Congenital Myasthenic Syndrome, where a loss of acetylcholine receptor production leads to severe muscle weakness (Ohno et al. 1999).

1.2 Research Aim

In order to understand the mechanisms of disease that may eventually lead to therapeutics for the treatment of conditions associated with Down syndrome and Congenital Myasthenic Syndrome, it is necessary to firstly identify the function of the basal and tissue-specific transcription factors involved. This research aims to characterise the gene structure, expression pattern and function of one such transcription factor; the GA-binding protein (Gabpα). Gabpα is also known as Nuclear respiratory factor 2 (Nrf-2) and Adenovirus E4 transcription factor-1 (E4TF-1), but throughout the text shall be referred to as Gabpα in mouse, or GABPα in human (or in human and mouse). Of particular interest in this study is the role of Gabpα in skeletal muscle development and function. This was investigated using mouse models.

1.3 Research Approach

Before presenting the research data that has resulted from the study of the gene structure and function of *Gabpα*, it is important to analyse the data that was available at the outset and that which became available during this study.

Chapter 2 brings to your attention the views of current literature in the field of transcription factor biology. A general overview of transcription factors is followed by a more detailed description of the ETS transcription factor family. Gabpα, the ETS factor under investigation, is then reviewed. The role of Gabpα in skeletal muscle and mitochondria in both normal and disease situations is detailed. Together, this chapter

highlights why elucidating the function of Gabpα is an important contribution to the field of transcription factor biology.

Chapter 3 specifically outlines how the research aim was achieved and why particular methods were chosen. Detailed protocols of individual methods are included, with additional case-specific information given in subsequent results chapters.

Chapter 4 describes how the gene structure of *Gabpα* was resolved using genomic cloning, PCR amplification and DNA sequencing. Promoter analysis and characterisation of novel transcripts of mouse *Gabpα*, *Gabpβ1-1* and *Gabpβ1-2* genes is also presented here. Biological implications of these findings are discussed.

Chapter 5 focuses on the expression pattern of Gabpα. The processes of polyclonal antibody generation and validation are presented, enabling the specific identification of Gabpα protein using Western blot analysis. Expression analysis of adult mice using this reagent is included, as is cellular expression analysis by means of the lacZ reporter. The effects of overexpression of *Gabpα* cDNA in NIH3T3 fibroblast cells, Down syndrome fibroblast cell lines and Ts65Dn partial trisomy 16 mice are also presented. Conclusions about Gabpα cellular expression and post-transcriptional regulation are given.

Chapter 6 begins with a background of methodology used to generate mouse lines overexpressing *Cre* recombinase or *Gabpα* cDNA, as well as mice with *Gabpα* targeted for deletion by insertion of *loxP* sites. Results of embryonic stem (ES) cell screening, mouse genotyping and breeding are presented, leading to the generation and expression screening of *Gabpα* total knockout, conditional knockout and minigene transgenic mice.

Chapter 7 describes the phenotype analysis of *Gabpα* total knockout and skeletal muscle specific knockout mice. Analyses of general histology, Gabpα target gene expression and skeletal muscle function are presented.

Chapter 8 discusses the results presented in Chapters 4-7 and areas of future research are highlighted. The implications of this work with regard to elucidation of the cause and treatment of human diseases such as Down syndrome, Congenital Myasthenic Syndrome and mitochondrial disease are also discussed.

Chapter 9 draws together the conclusions that can be made from the results of this study, with regard to what has been learnt about Gabpα function during mouse development, particularly in skeletal muscle function.

Chapter 2

Literature Review:

GABPα - *an ETS Transcription Factor*

The basal transcription factors are expressed in all tissues throughout development and directly interact with components of the transcription machinery (RNA polymerase II, TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH, TATA-binding protein (TBP) and TBP-associated factors (TAFs)), aiding in the initiation of gene transcription (Bhaumik and Green 2001; Smale 2001). Other transcription factors are unique to certain tissues and/or stages of development and act to potentiate or repress basal transcription levels, depending upon their tissue specificity, expression of interacting factors, target specificity and kinetics of action (Sgouras et al. 1995). In any case, to function as a transcription factor, a protein requires a DNA binding domain and a transactivation domain. Alternatively, a domain may be provided by a binding partner (Watson et al. 1992). The functional transcription factor complex binds a consensus DNA sequence present in regulatory regions of target genes, usually within the upstream promoter region. It is the combination of the binding sites present in a promoter region, and the chromatin structure, that determine which transcription factors regulate each gene. Transcription factors can be categorised into families, based upon their primary amino acid sequence conservation, to form functional domains. Examples of transcription factor families include helix-loop-helix, winged helix-loop-helix, basic helix-loop-helix, and leucine zipper proteins. This thesis concentrates on a member of the ETS, winged helix-turn-helix, family of transcription factors, GA binding protein (GABP).

2.1 ETS Transcription Factors

The first ETS transcription factor to be described was v-Ets-1. The DNA binding domain or E26 transformation-specific domain (ETS) is part of a fusion protein product of the replication-defective E26 avian erythroblastosis virus that can lead to acute leukaemia

(Bister et al. 1982; Nunn et al. 1983; Nunn et al. 1984). Cellular ETS transcription factors can also be used to transcribe E26 viral genes, allowing for viral propagation (Janknecht and Nordheim 1993). The cellular proto-oncogenes c-*ETS-1* and c-*ETS-2* encode proteins of 98 % and 95 % identity at the amino acid level with v-Ets, respectively (Watson et al. 1985). However, these two genes have evolved slower than their viral counterpart (Laudet et al. 1999), with over 40 members of the ETS transcription factor family having been identified by virtue of their conserved DNA binding domain (Laudet et al. 1999).

2.1.1 Protein Domains of ETS Transcription Factors

The ETS DNA Binding Domain

The ETS domain is a distinct 84-90 amino acid DNA binding domain that is highly conserved between family members (Nye et al. 1992), and is usually situated at the C-terminus of the protein (Ely and Kodandapani 1998). Similarity of ETS domain primary sequences and tertiary structures of GABPα, ETS-1, FLI-1 and PU.1, as determined by crystal formation, is highlighted in **Figure 2.1**. The ETS domain is a winged helix-turn-helix, consisting of three helices and a four-stranded antiparallel β sheet (Graves et al. 1996). There are two conserved regions within the ETS domain, an amino-terminal aliphatic α helix and a carboxy-terminal basic region, required for DNA binding and/or nuclear localisation of ETS transcription factors (Moreau-Gachelin 1994). Unlike v-Ets, c-ETS proteins can only transactivate target genes by recognition of a specific sequence (Crepieux et al. 1994). An ETS DNA binding site is composed of 10 nucleotides in the major groove of DNA, consisting of a C/AGGAA/T core sequence (Felix Karim et al. 1990; Macleod et al. 1992). Recognition of this consensus sequence, together with flanking interactions in the minor groove of DNA, determines the binding specificities of ETS transcription factors (Nye et al. 1992). Up to 20 nucleotides of DNA can be bound by an ETS domain (Nye et al. 1992), depending upon structural differences of the individual ETS transcription factors (Graves and Petersen 1998; Boyd and Farnham 1999).

The Pointed (PNT) Domain

Pointed (PNT), also referred to as the B or SAM domain, is a conserved domain present in a subset of ETS transcription factors. These include Pnt, ETS-1, ETS-2, GABPα (Slupsky et al. 1998), Elg, ERG, FLI-1, TEL-1, TEL-2 (Potter et al. 2000), SAP-1 (Hassler and Richmond 2001), Yan and ESE-1 (Laudet et al. 1999). PNT is similar in structure to helix-loop-helix domains and is related to that of the yeast sexual

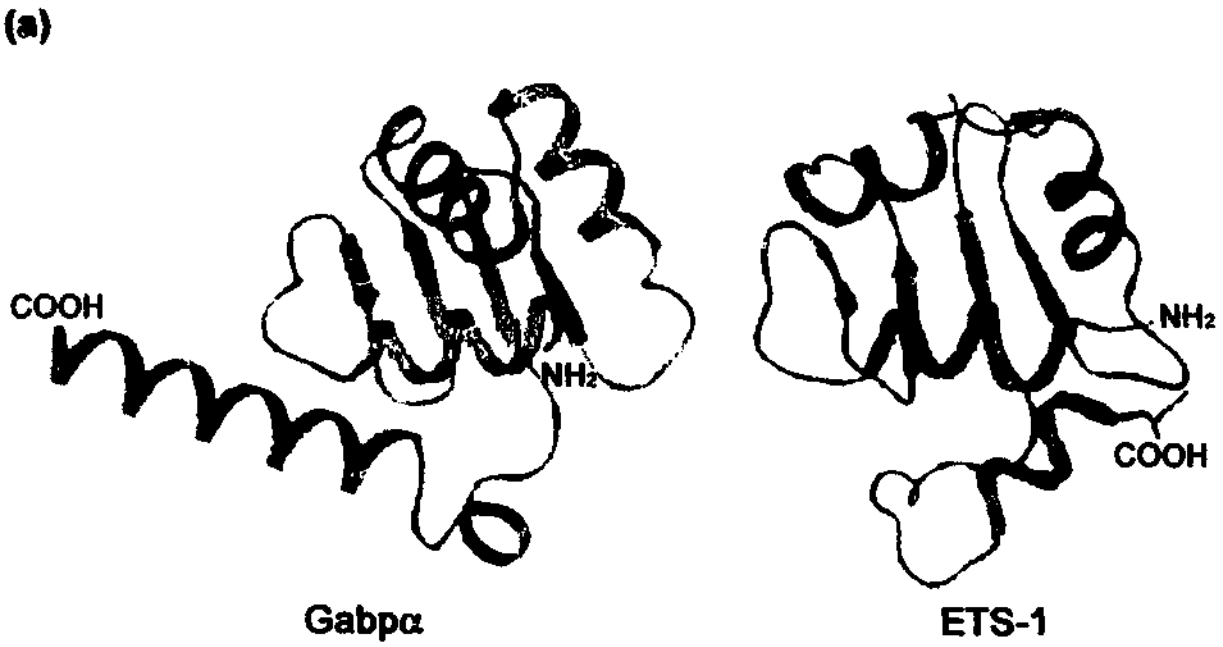


Figure 2.1 - Conservation of the Gabpα ETS Domain.
(a) Similarity of protein structures of Gabpα and ETS-1 DNA binding domains. Amino (NH_2) and carboxy (COOH) terminals are indicated. (b) Amino acid sequence alignment of ETS domains of Gabpα, ETS-1, FLI-1 and Pu.1 proteins. Numbers shown to the left of amino acids indicate positions of ETS domains within the respective proteins, and numbers above sequences refer to Gabpα. Amino acids shown in red are conserved in Gabpα and at least two of the other ETS factors shown, and those in blue are only identical in Gabpα and ETS-1. DNA contacts of the ETS domain are indicated by triangles and regions of Gabpα binding to Gabpβ by hashes. Figure reproduced from Batchelor et al. 1998.

differentiation proteins known as SEPs (yeast sterility, ETS-related, polycomb proteins). This structure has been shown to mediate protein-protein interactions of SEPs, as well as the homodimerisation of Yan (Laudet et al. 1999), ERG (Carrère et al. 1998) and TEL proteins (Lopez et al. 1999), and heterodimerisation of TEL-1 and TEL-2 proteins (Potter et al. 2000). It is the PNT domain that mediates the interaction of SAP-1 with the Serum Response Factor (SRF) in formation of the Ternary Complex Factor (TCF) (Hassler and Richmond 2001), a regulator of immediate early genes, such as c-*Fos* (Masutani et al. 1997). PNT has also been shown to mediate phosphorylation of Yan by Erk of the MAP kinase pathway (Baker et al. 2001). Similarly, fruit fly and human ETS-1 and ETS-2, but not GABPα, use their PNT domains for ERK2 docking, leading to Ras-mediated phosphorylation (Seidel and Graves 2002). Therefore, although many ETS proteins feature both ETS and PNT domains, they are capable of using these regions for different purposes. One reason for this may be the slight differences in secondary structure of the PNT domain. Recently the PNT domains of Gabpα and ERG were compared, and it was found that Gabpα, like Ets-1, contains an additional N-terminal helix within the PNT domain that is lacking in that of ERG and TEL (Mackereth et al. 2002).

Regulatory Domains

Other conserved domains present in ETS transcription factors include autoinhibitory sequences, nuclear localisation signal (NLS) motifs and transactivation domains. Autoinhibitory domains have been identified in murine Ets-1 (Cowley and Graves 2000), chicken Ets-2 (Scheikert et al. 1992), human TEL-1 and TEL-2 (Lopez et al. 1999), GABPα, SAP-1, and ELK-1 (Janknecht and Nordheim 1993; Wasylyk et al. 1993). The inhibitory effect of these domains is mediated by their secondary structures, so alterations in structure caused by association of ETS proteins with other transcription factors, or by binding DNA, often reduces autoinhibition (Tian et al. 1999). Nuclear localisation signals are present in almost all ETS proteins, to allow for their functional role as transcription factors. An exception to this rule is GABPα, which utilises the NLS of its binding partner GABPβ (an unrelated protein), to exert its function in the nucleus. Transactivation domains are also common to the majority of ETS proteins, with the exception of GABPα (which relies on that of β). A subset of ETS proteins contain repression domains and function to disrupt the protein-DNA interactions of activating ETS proteins and other transcription factors; ERF (ETS-2 repressor factor) (Sgouras et al. 1995; Liu et al. 1997), Prf (PU.1 related factor) (Hashimoto et al. 1999), Net (Criqui-Filipe et al. 1999), PE1

(Bidder et al. 2000) and Yan (Lai and Rubin 1992). Therefore, the domains present (or absent) in each ETS transcription factor help determine their specific functions.

2.1.2 ETS Transcription Factor Phylogeny

Conservation of the ETS Domain

The ETS domain has been conserved over at least 600 million years of evolution, from *Caenorhabditis elegans* to *Homo sapiens* (Seth et al. 1992; Wang et al. 1992). The number of ETS genes has also been amplified, with one gene (*Lin1*) present in the earthworm and greater then 30 in humans. **Figure 2.2** shows a homology tree of the known ETS subfamilies, based on amino acid sequence similarity within the ETS domain. The number of ETS proteins can only be estimated, as even since publication of this homology tree, several new family members have been discovered in humans. Within the ERG subfamily, ETS-3 and ETS-6 have been identified, ERP within the ELK subfamily, PDEF within the ETS-4 subfamily, TEL-2 within the TEL subfamily, and ESE-2 (ELF-5) and ESE-3 (EHF) within the ESE subfamily (Kas et al. 2000). ETS transcription factors have now been found across a wide variety of species. *Drosophila melanogaster* (Laudet et al. 1999), *Caenorhabditis elegans* (Hart et al. 2000b), *Xenopus laevis* (Marchioni et al. 1993) and metazoa possess ETS genes, but plants, fungi and protozoa do not (Graves and Petersen 1998). The high level of conservation of the ETS domain between species as diverse as *D. melanogaster* and *H. sapiens* is indicative of conserved protein function.

Conservation of ETS Protein Function

The eight ETS proteins of *D. melanogaster* are essential for every aspect of development (see **Table 2.1**), and Yan (Price and Lai 1999), Pnt (Brunner et al. 1994) and Elg are regulated by mitogen activated protein kinase (MAPK) phosphorylation near or within their transcriptional activation domains (Sharrocks et al. 1997; Wasylyk et al. 1998). MAPK phosphorylation has also been shown to be a key means of regulation for mammalian ETS transcription factors (Yang et al. 1996; Wasylyk et al. 1998; Yang et al. 1998; Yordy and Muise-Helmericks 2000), as well as Lin-1, the only ETS factor identified in *C.elegans* (Sharrocks et al. 1997; Wasylyk et al. 1998; Hart et al. 2000b). Recently, a docking site for MAP kinases has been identified (amino acids FQFP or FQFHP) in Lin-1, as well as Yan of *D. melanogaster* and ELK-1, SAP-1a and NET of *H. sapiens* (Yang et al. 1998). Therefore, ETS transcription factors are effectors of the MAPK pathway across species (Albagli et al. 1996).

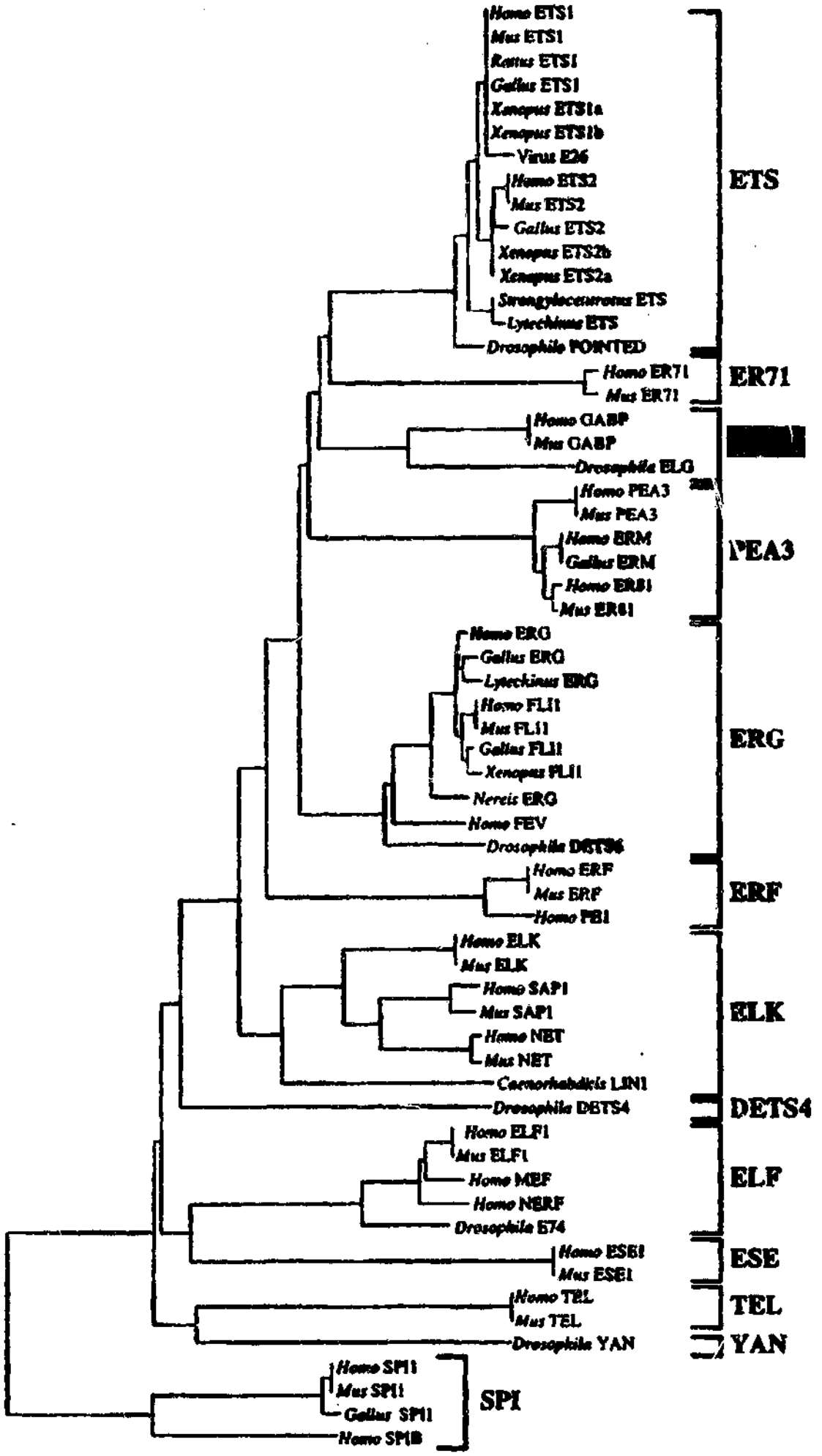


Figure 2.2 - Homology of ETS Proteins Across Species.
Neighbour-Joining tree of ETS family subgroups, based upon similarity within the ETS domain. The length of branches is proportional to the divergence between sequences. The GABP subfamily is highlighted in red. Figure reproduced from Laudet et al. 1999.

Table 2.1 - Drosophila ETS Transcription Factor Functions.

DROSOPHILA ETS PROTEIN	HUMAN / MOUSE ETS PROTEIN	DROSOPHILA ETS PROTEIN FUNCTION	REFERENCES
Pointed	ETS-1	Eye development Neurogenesis Tracheal cell migration Oogenesis	(Klämbt 1993; Albagli et al. 1996)
Yan	TEL	As for Pointed (dimerize)	(Lai and Rubin 1992; Price and Lai 1999)
E74	ELF2	Metamorphosis	(Burtis et al. 1990; Karim and Thummel 1991)
D-elg	GABPα	Oogenesis	(Pribyl et al. 1991; Schulz et al. 1993a; Schulz et al. 1993b; Gajewski and Schulz 1995)
D-ets-3	FLI-1	CNS development	(Chen et al. 1992)
D-ets-4	PDEF/ESF	Germ cell development	(Chen et al. 1992)
D-ets-6	ERG	CNS development	(Chen et al. 1992)
Ets96B	ER81	Undetermined	(Hsu and Schulz 2000)

The most common name of the *D. melanogaster* ETS transcription factor is shown, as well as the corresponding human and/or mouse homologue, the assigned function and the relevant reference. Table adapted from Hsu and Schulz 2000.

2.1.3 ETS Transcription Factor Expression Patterns

As shown in **Table 2.2**, ETS proteins such as Ets-1 and Ets-2 are widely expressed (Maroulakou et al. 1994), while others, such as Elf-3/Ese1, Elf-5/Ese2, PU.1, are restricted to specific tissues (Kas et al. 2000). Therefore, although two transcription factors possess homologous ETS domains, they may not be present in the same tissues or the same cell types within a tissue. Both GABPα and ETS-2 are ubiquitously expressed proteins, containing highly conserved ETS domains, and less conserved PNT domains. However, the two proteins differ across the remainder of their amino acid sequence. Furthermore, differences in transcriptional regulation, alternative splicing, post-translational modifications and protein stability (Bhat et al. 1987; Macleod et al. 1992) are postulated to allow GABPα and ETS-2 proteins to interact with different binding partners, and act upon different target genes. However, overlapping expression patterns of ETS transcription factors makes it difficult to identify which protein acts on which target gene. For example, eight different ETS proteins are expressed in cardiac muscle cells (see **Table 2.2**).

2.1.4 ETS Transcription Factor Functions

It is difficult to determine the roles of specific ETS transcription factors by standard *in vitro* transactivation experiments, due to their highly conserved DNA binding domains and overlapping expression patterns. For example, when the ETS binding sites were first identified in the promoter of mouse *Nicotinic acetylcholine receptor delta* (*AChRδ*) (Baldwin and Burden 1988), it was unknown which ETS protein was responsible for its transactivation, as both Gabp and the highly homologous Ets-2 are expressed ubiquitously. Gabp has since been identified as the ETS protein that recognises the *AChRδ* promoter, by means of electrophoretic mobility shift assay (EMSA) and supershifting with Gabpα and β specific antibodies (Fromm and Burden 1998a). However, the most definitive *in vivo* experiments to assess specific ETS protein functions have been knockout mouse models. Since ETS transcription factors were initially identified as components of an avian erythroblastosis virus, it is not surprising that many ETS proteins play roles in immune cell function. The use of knockout mice has shown that ETS transcription factors are also important for key developmental processes.

Spi-1/Pu.1 and Spi-B are members of the same ETS subfamily, interact with the same binding partners, and recognise Pu.1 binding sites with different affinities (Rao et al. 1999). The difference in function of Pu.1 and Spi-B has been highlighted by the use of

Table 2.2 - Mammalian ETS Transcription Factor Expression in Adult Tissues.

TISSUE	ETS PROTEINS EXPRESSED	REFERENCES
Ectoderm		
Nervous System		
Brain	Elk-1, Pet-1, PEA3, Tel-1, Ets-1, Ets-2, ER81, Gabpα, ERM, ER71, Fli-1, PE1, PEA3	(Bhat et al. 1987; LaMarco et al. 1991; Crepieux et al. 1994; Maroulakou et al. 1994; de Launoit et al. 1997; Wang et al. 1997; Fyodorov et al. 1998; Barbeau et al. 1999; Arber et al. 2000; Bidder et al. 2000)
Nerves	Pet-1	(Fyodorov et al. 1998)
Spinal Cord	Pet-1	(Fyodorov et al. 1998)
Sense Organs		
Eye	Pet-1	(Fyodorov et al. 1998)
Limb Buds		
Tail Bud	Ets-2	(Maroulakou et al. 1994)
Forelimb Bud	Ets-2	(Maroulakou et al. 1994)
Hindlimb Bud	Ets-2	(Maroulakou et al. 1994)
Skin	Ets-1, Ets-2	(Maroulakou et al. 1994)
Mesoderm		
Skeletal System		
Neural Crest	Erg, Fli-1	(Prasad et al. 1992; Vlaeminck-Guillem et al. 2000)
Bones	Ets-1, Ets-2, PE1	(Maroulakou et al. 1994; Anderson et al. 1999; Bidder et al. 2000)
Bone Marrow	Elf-1, Spi-B, PU.1, Erg, Fli-1, Ets-1, Ets-2, Gabpα, Tel-1, Tel-2	(LaMarco et al. 1991; Klemsz et al. 1993; Murakami et al. 1993; Maroulakou et al. 1994; Moreau-Gachelin 1994; Wang et al. 1997; Fisher and Scott 1998; Anderson et al. 1999; Potter et al. 2000)
Cartilage	Erg, Ets-2	(Maroulakou et al. 1994; Vlaeminck-Guillem et al. 2000)
Circulatory System		
Heart	Fli-1, Ets-1, ER81, Gabpα, ERM, Elfr, PEA3, ER71	(Brown and McKnight 1992; Watson et al. 1992; Maroulakou et al. 1994; de Launoit et al. 1997; Aryee et al. 1998; Arber et al. 2000)
Blood Islands	Ets-1	(Maroulakou et al. 1994)
Capillaries	Erg	(Maroulakou et al. 1994; Vlaeminck-Guillem et al. 2000)
Lymph Nodes	Spi-B	(Maroulakou and Bowe 2000)
Placenta	Erg, ERM, Elfr, Elf5, Elf3, Ets-2	(de Launoit et al. 1997; Aryee et al. 1998; Yamamoto et al. 1998; Zhou et al. 1998; Vlaeminck-Guillem et al. 2000)
Urogenital System		
Male External Organs	Erg	(Vlaeminck-Guillem et al. 2000)
Female External Organs	Erg	(Vlaeminck-Guillem et al. 2000)

<u>Excretory System</u>		
Ureter	Erg	(Vlaeminck-Guillem et al. 2000)
<u>Genital System</u>		
Testis	Elk-1, PU.1, PEA3, Gabpα, ER71	(LaMarco et al. 1991; Crepieux et al. 1994; Hromas and Klemsz 1994; de Launoit et al. 1997)
Ovaries	Fli-1, Elfr, Elf5	(Watson et al. 1992; Aryee et al. 1998; Zhou et al. 1998)
Uterus	Gabpα	(LaMarco et al. 1991)
Mammary gland	Elf5, ER81, PEA3, Ese3	(de Launoit et al. 1997; Zhou et al. 1998; Kas et al. 2000)
<u>Muscular System</u>		
Muscle	Gabpα	(LaMarco et al. 1991)
Endoderm		
<u>Digestive System</u>		
Yolk Sac	Tel-1	(Wang et al. 1997)
Gut	Ets-1, Ets-2, Gabpα, Elf3, Ese3	(LaMarco et al. 1991; Maroulakou et al. 1994; Zhou et al. 1998; Kas et al. 2000)
Oral Cavity	Elf5 (tongue)	(Zhou et al. 1998)
Thymus	Elf-1, Spi-B, PU.1, Fli-1, Ets-1, Ets-2, Gabpα, Elfr, Tel-1, Tel-2	(LaMarco et al. 1991; Watson et al. 1992; Wang et al. 1997; Aryee et al. 1998; Anderson et al. 1999; Maroulakou and Bowe 2000; Potter et al. 2000)
Salivary Glands	ERM, Elf5, Ese3	(Kas et al. 2000; Maroulakou and Bowe 2000)
Stomach	Elf5	(Zhou et al. 1998)
Liver	Elf-1, Spi-B, PU.1, Erg, Fli-1, Gabpα, Elf3, Tel-1, Tel-2	(LaMarco et al. 1991; Klemsz et al. 1993; Murakami et al. 1993; Moreau-Gachelin 1994; Wang et al. 1997; Zhou et al. 1998; Maroulakou and Bowe 2000; Potter et al. 2000)
Spleen	Elf-1, Spi-B, PU.1, Fli-1, Ets-1, Ets-2, Gabpα, Elfr, Tel-1, Tel-2	(LaMarco et al. 1991; Watson et al. 1992; Moreau-Gachelin 1994; Wang et al. 1997; Aryee et al. 1998; Anderson et al. 1999; Maroulakou and Bowe 2000; Potter et al. 2000)
Kidney	Gabpα, ERM, Elf5, PEA3, ER81, ER71, Ese3	(LaMarco et al. 1991; de Launoit et al. 1997; Zhou et al. 1998; Kas et al. 2000)
Adrenal Gland	Pet-1	(Fyodorov et al. 1998)
Small Intestines	Pet-1, Gabpα, Elf3	(Brown and McKnight 1992; Fyodorov et al. 1998; Zhou et al. 1998)
Large Intestines	Pet-1, Gabpα	(Brown and McKnight 1992; Fyodorov et al. 1998)
Rectum	Elf5, Ese3 (prostate)	(Zhou et al. 1998; Kas et al. 2000)
Urinary Bladder	Elf5	(Zhou et al. 1998)
<u>Respiratory System</u>		
Lung	Fli-1, Ets-1, Ets-2, ER81, ERM, Elfr, Elf5, PEA3, ER71, Ese3	(Maroulakou et al. 1994; de Launoit et al. 1997; Aryee et al. 1998; Zhou et al. 1998; Arber et al. 2000; Kas et al. 2000; Truong and Ben-David 2000)

Tissue types are subdivided according to their endoderm, mesoderm or ectoderm origin. The ETS transcription factors expressed in each tissue are shown, as are the relevant references.

mouse models. Pu.1 is necessary for multipotential progenitors to progress to lymphoid or myeloid lineages (Hohaus et al. 1995; Fisher and Scott 1998; Iwama et al. 1998), but is not expressed in T cells (Moreau-Gachelin 1994). Spi-B is necessary for normal B cell function and T cell humoral responses (Laudet et al. 1999). Interestingly, when *Pu.1* heterozygous and *Spi-B* knockout mouse models are inter-crossed, a more severe phenotype is observed, suggesting that the two ETS proteins regulate expression of the same gene targets, or genes that function in the same biological pathway (Garrett-Sinha et al. 2001). *Fli-1* knockout mice die at day 11.5 of embryogenesis due to defective vascularisation (Hart et al. 2000a). Conversely, overexpression of Fli-1 in mice results in immunological renal disease (spenomegaly, B cell hyperplasia, hypergammaglobulinemia) (Truong and Ben-David 2000). This highlights the involvement of ETS transcription factors in normal and disease situations.

2.1.5 Aberrant ETS Transcription Factor Functions

As shown by the above example of Fli-1, ETS transcription factors have the capacity to act positively towards healthy development, or negatively when aberrantly expressed in disease. Several ETS proteins are known to be targets of gene capture and retroviral insertion (see **Table 2.3**). Mutations are often induced in the captured sequences, resulting in transactivation and cellular transformation (Blair and Athanasiou 2000). The importance of ETS proteins in cancer is highlighted by the fact that v-Ets-1 was first discovered as part of the fusion protein product of the E26 avian erythroblastoma virus (Bister et al. 1982). Also, fusion of the ETS domain of FLI-1 to the Ewing's sarcoma protein (EWS) is known to result in Ewing's sarcoma (Zhang et al. 1993).

The role of ETS transcription factors in diseases other than cancer is less understood. However, point mutations within the GABP binding site of the *Nicotinic acetylcholine receptor epsilon* (*AChRε*) promoter have been identified as the cause of severe muscle weakness in several cases of Congenital Myasthenic Syndrome (CMS) (Nichols et al. 1999; Ohno et al. 1999). In addition, point mutations within the GABP binding site of the promoter of the purine biosynthetic enzyme *Adenylosuccinate Lyase* (*ADSL*) have been found in three patients suffering from mental retardation, autism and epilepsy caused by ADSL deficiency (Marie et al. 2002). As outlined in **Table 2.3**, the cellular context and interacting transcription factors of ETS proteins implicates them in many important biological pathways, both in healthy and disease situations.

Table 2.3 - Role of ETS Transcription Factors in Human Disease.

ETS PROTEIN	DISEASE	MECHANISM OF ACTION	REFERENCES
ETS-1	Acute Leukemia Cancer metastasis (?) Myeloblastic leukemia Arthritis (?) Osteoporosis/Osteopetrosis (?)	Transposed in (4;11) translocations Regulating MMPs, Osteopontin Upregulation of c-myb Regulating MMPs Regulating Osteopontin	(Sacchi et al. 1986) (Kola et al. 1993; Vandenbunder et al. 1994; Sato et al. 1998; Trojanowska 2000) (Bloch et al. 1995) (Trojanowska 2000) (Sato et al. 1998)
ETS-2	Acute Leukemia Cervical Carcinoma Alzheimer's Disease (?) Down syndrome (?)	Transposed in (8;21) translocations Integration of HPV at gene locus Apoptosis and regulation of APP Regulating bone formation and thymic function	(Sacchi et al. 1986) (Simpson et al. 1997) (Sanij et al. 2001) (Sumarsono et al. 1996; Wolvetang et al. 2003)
GABPα	Congenital Myasthenic Syndrome (CMS) Adenylosuccinate Lyase Deficiency Hepatoma	Mutation of binding site in AChRε Mutation of binding site in ADSL Upregulation of MTFA	(Nichols et al. 1999; Ohno et al. 1999) (Marie et al. 2002) (Dong et al. 2002)
FLI-1	Ewing's sarcoma Erythroleukemia Thrombocytopenia	ETS domain fused to EWS Integration of FmuLV at gene locus LOH at gene locus	(Delattre et al. 1992) (Zhang et al. 1993) (Hart et al. 2000)
ERG	Myeloid leukemia Ewing's sarcoma Cervical Carcinoma	ETS domain fused to TLS/FUS ETS domain fused to EWS Integration of HPV at gene locus	(Shimizu et al. 1993) (Shimizu et al. 1993) (Simpson et al. 1997)
ER81	Ewing's sarcoma	ETS domain fused to EWS	(Jeon et al. 1995; de Launoit et al. 1997)
TEL	Myelomonocytic leukemia Lymphoblastic leukemia Myeloid leukemia	HLH domain fused to PDGFβ HLH domain fused to AML1 HLH domain fused to MN1 or ABL	(Lopez et al. 1999) (Golub et al. 1995) (Golub et al. 1995)
PU.1	Erythroleukemia	Integration of Friend SFFV at gene locus	(Ray et al. 1990)
PEA3	Mammary tumours Ewing's sarcoma	Downstream target of Neu ETS domain fused to EWS	(O'Hagan and Hassell 1998) (de Launoit et al. 1997)
ELK-1	Synovial sarcoma	Disrupted by translocation	(Macleod et al. 1992)
ELF-5	Breast, kidney and prostate cancer	LOH at gene locus	(Zhou et al. 1998)

The most common name of each ETS transcription factor is shown, as well as the human disease to which it is linked, the mechanism of involvement in the disease and the relevant references. (?) represents proposed disease link due to normal gene function and expression pattern.

2.1.6 ETS Transcription Factor Interacting Proteins

Unlike many other transcription factor families, ETS proteins do not usually bind DNA as dimers (Crepieux et al. 1994). The exceptions are ERG (Carrère et al. 1998) and TEL (Lopez et al. 1999), which homodimerise using PNT domains, and TEL, which can heterodimerise with and repress FLI-1 (Kwiatkowski et al. 1998). In general, unrelated protein binding partners help determine the specificity of ETS transcription factors and relieve their autoinhibition (Gu et al. 2000), allowing them to contribute to many different signalling pathways.

The ternary complex factor (TCF) is a complex composed of the serum response factor (SRF), together with the ETS protein SAP-1 (Hassler and Richmond 2001), ELK-1 (Shore and Sharrocks 1994), FLI-1 or PEA3 (Wasylyk et al. 1993; Laudet et al. 1999). The TCF is then able to bind the serum response element (SRE) present in the promoters of immediate early genes. FLI-1 interacts with SRF using two motifs (Dalgleish and Sharrocks 2000), while ELK-1 (Watson et al. 1997) and SAP-1 (Hassler and Richmond 2001) do so using the PNT domain. Expression patterns of these ETS proteins, and the cellular context, determine which proteins form part of the TCF. The ETS repressor protein NET is also capable of binding to SRF, acting as an inhibitor (Yordy and Muise-Helmericks 2000). Similarly, the Id family of proteins allosterically inhibit the TCF by binding the ETS domain (Yates et al. 1999).

ETS binding sites are often in close proximity to basal transcription factor binding sites such as activator protein 1 (AP1) and Sp1 (identified as binding to g/c motifs within the *Serotonin receptor* promoter) (Wasylyk et al. 1990; Verger and Duterque-Coquillaud 2002). As detailed in **Table 2.4**, these and many other transcription factors act as context-dependent binding partners of mammalian ETS proteins. For example, GABP physically interacts with both Sp1 and Sp3 to transactivate the promoter of *Utrophin* (Galvagni et al. 2001), a gene encoding a structural protein of the neuromuscular junction of skeletal muscle. Whereas, ETS-1 and ETS-2 interact with the p300/cAMP-Responsive Element-Binding Protein (CBP) complex, activating the *Stromelysin* promoter (Jayaraman et al. 1999). The DNA recognition helix α3 of the ETS domain is a common contact point of ETS transcription factors with other proteins (Pax5, Pip, AP1 and SRF), generating complexes of similar orientation and framework (Verger and Duterque-Coquillaud 2002), enabling direct enhancement of DNA binding of the ETS transcription factor complexes.

Table 2.4 - ETS Transcription Factor Binding Partners.

ETS PROTEIN	INTERACTING PROTEIN/S	INTERACTING DOMAIN/S OF ETS PROTEIN	REFERENCES
ETS-1	NF-κB, Sp1	ETS	(Krehan et al. 2000; Li et al. 2000a)
	p300, CBP	Transactivation, ETS	(Jayaraman et al. 1999)
	STAT5	C-terminus (including ETS)	(Rameil et al. 2000)
	TFE3, USF	N-terminus inhibitory (NID), ETS	(Tian et al. 1999)
	Tax, Sp1	N-terminus	(Dittmer et al. 1997)
	Pax5	ETS	(Fitzsimmons et al. 1996)
	AP1	ETS	(Wasylyk et al. 1990)
	Pit-1, Pit-1β	N-terminus	(Bradford et al. 1995; Bradford et al. 1996; Day et al. 1998; Bradford et al. 2000)
	Daxx	N-terminus	(Li et al. 2000b)
	Sp100	N- and C-termini	(Wasylyk et al. 2002)
	AML1(CBFα2)	PNT, inhibitory NH_2 end of ETS	(Graves and Petersen 1998; Sato et al. 1998; Mao et al. 1999; Goetz et al. 2000; Gu et al. 2000)
	UBC9, Hus5	ETS, PNT	(Hahn et al. 1997)
	MafB	ETS	(Sieweke et al. 1996)
ETS-2	p300, CBP	Transactivation, ETS	(Jayaraman et al. 1999)
	STAT5	C-terminus (including ETS)	(Rameil et al. 2000)
	AP1	ETS	(Wasylyk et al. 1990)
	Cdk10	PNT, transactivation	(Kasten and Giordano 2001)
GABPα	GABPβ	GABPβ interaction	(LaMarco et al. 1991)
	miTF	GABPβ interaction	(Morii et al. 2001)
	Sp1, Sp3	Undetermined	(Galvagni et al. 2001)
	Pax5	ETS	(Fitzsimmons et al. 1996)
	ATF1, CREB	N-terminus (including PNT and ETS)	(Sawada et al. 1999)

PU.1/SPI-1	MiTF	Undetermined	(Sato et al. 1999)
	STAT1a	Intermediate factor	(Nguyen and Benveniste 2000)
	NF-EM5 (Pip, IRF-4)	PEST	(Pongubala et al. 1992; Pongubala et al. 1993; Eisenbeis et al. 1995; Brass et al. 1996; Fisher and Scott 1998)
	GATA-1, GATA-2	ETS	(Hagemeier et al. 1993; Rekhtman et al. 1999; Zhang et al. 1999; Nerlov et al. 2000; Smale 2001)
	TFIID, RB	Transactivation	(Hagemeier et al. 1993)
	AP1	ETS	(Behre et al. 1999)
	Sp1	Undetermined	(Eichbaum et al. 1997)
	AML-1B, C/EBPα	ETS, transactivation	(Petrovick et al. 1998; Zhang et al. 1999)
	CBP	Transactivation	(Yamamoto et al. 1999; Yamamoto and Oikawa 1999; Yamamoto et al. 2002)
	TBP, HDAC1, mSin3A	PEST, ETS	(Kihara-Negishi et al. 2001)
	ICSBP, IRF-4	PEST, ETS	(Yee et al. 1998; Marecki and Fenton 2000)
	NF-IL6 (CEBPδ, CRP3), HMG I/Y, SRP	ETS	(Nagulapalli et al. 1995)
	UBC9	ETS	(Hahn et al. 1997)
	GSC	N-terminus	(Konishi et al. 1999)
	Rb	N-terminus	(Konishi et al. 1999)
	NFATc	ETS	(Bassuk et al. 1997)
	EBNA-2, EBNA-3C	ETS	(Zhao and Sample 2000)
	AML1(CBFα)	N-terminus	(Mao et al. 1999)
ELF-1	NFATc, NFATp	ETS	(Bassuk et al. 1997)
	Rb	Transactivation	(Crepieux et al. 1994)
	AP1	ETS	(Crepieux et al. 1994)
	NF-κB(p50,c-Rel), HMG-1(Y)	ETS	(John et al. 1995; Graves and Petersen 1998)
	UBC9	ETS	(Hahn et al. 1997)
	NF-κB(p50)	ETS	(Bassuk et al. 1997)
	AML1(CBFα)	N-terminus	(Mao et al. 1999)
SPI-B	STAT1a	Undetermined	(Nguyen and Benveniste 2000)
	CBP	Transactivation	(Yamamoto et al. 2002)
	EBNA-2, EBNA-3C	ETS	(Zhao and Sample 2000)
PEA3	AP1	ETS	(Gutman and Wasylyk 1990; Wasylyk et al. 1990)
ERG	AP1	ETS	(Wasylyk et al. 1990; Basuyaux et al. 1997; Verger et al. 2001)
	ESET (SETDB1)	N-terminus	(Yang et al. 2002)

FLI-1	Sp1, Sp3 AP1 RNA Polymerase II SRF Pax5 UBC9 AML1(CBFα)	Undetermined ETS EWS intermediate N- and C-termini ETS ETS, PNT N-terminus	(Shirasaki et al. 1999) (Wasylyk et al. 1990) (Petermann et al. 1998; Yang et al. 2000) (Dalgleish and Sharrocks 2000) (Fitzsimmons et al. 1996) (Hahn et al. 1997) (Mao et al. 1999)
ERM	TAFII60, TBP, TAFII40 AP1 Androgen receptor	Transactivation (AD1) ETS Undetermined	(Defossez et al. 1997) (Graves and Petersen 1998) (de Launoit et al. 1997)
PE1	AP1	ETS	(Bidder et al. 2000)
NET	CtBP Pax5 UBC9	C-terminus inhibitory (CID) ETS ETS	(Criqui-Filipe et al. 1999) (Fitzsimmons et al. 1996) (Hahn et al. 1997)
ELK-1	Id proteins 1,2,3 SRF Pax5	ETS ETS, PNT ETS	(Yates et al. 1999) (Janknecht and Nordheim 1992; Shore and Sharrocks 1994) (Fitzsimmons et al. 1996)
ERF	Pit-1	Undetermined	(Day et al. 1998)
SAP1 & SAP2	SRF Id proteins 1,2,3	PNT ETS	(Masutani et al. 1997) (Yates et al. 1999)
TEL	SMRT, mSin3A N-CoR, histone deacetylase 3 UBC9	PNT, Central inhibitory Central inhibitory PNT	(Chakrabarti and Nucifora 1999; Wang and Hiebert 2001) (Wang and Hiebert 2001) (Chakrabarti et al. 1999)
MEF	AML1(CBFα), AML1B	N-terminus	(Mao et al. 1999)
NERF-2	AML1(CBFα)	N-terminus	(Mao et al. 1999)

The most common name of each ETS transcription factor is shown, as well as the interacting proteins (alternative names in brackets). Where characterised, the domain of the ETS transcription factor involved in protein binding is indicated. References characterising the protein-protein interactions are shown.

2.2 *The* GABPα/β *Complex*

GABPα is a unique ETS transcription factor, as it requires binding to an unrelated protein, GABPβ, to enable transactivation of target genes. The *H. sapiens GABPα* gene is localised to human chromosome 21 (HSA 21) at q21-22.1 (Goto et al. 1995) and that of *M. musculus* to mouse chromosome 16 (MMU 16) at position 55.0 cM (Chrast et al. 1995). In both instances, *GABPα* is positioned just proximal of *APP* (Tassone et al. 1999). Only humans possess an additional pseudogene of *GABPα* (encoded by HSA 7), lacking introns (Luo et al. 1999), hence this has evolved since separation of the two species. In both human and mouse, one *GABPα* gene locus, producing one transcript and mature protein product, has been described to date. As shown in **Figure 2.3**, the human and mouse GABPα proteins are 60 and 58 kDa in size, respectively. Each protein consists of 454 amino acids, featuring an N-terminal PNT domain, and C-terminal ETS and GABPβ interaction domains (Batchelor et al. 1998).

In humans, two loci for GABPβ, *β1* (7q11.21) and *β2* (15q15.2), each produce two alternative splice forms, β1 and β2, or γ1 and γ2, respectively. GABPβ1 and γ1 differ from their counterparts by a centrally located 12 amino acid insertion (see **Figure 2.4a**) (Gugneja et al. 1995). Only the *β1* locus produces β isoforms capable of homodimerisation, termed β1 and β2, due to presence of a C-terminal leucine zipper motif (Sawa et al. 1996). In mouse, this difference at the C-terminus is seen between the two isoforms β1-1 and β1-2, encoded by the *β1* locus (MMU 2 at 71.0cM) (see **Figure 2.4b**). A second Gabpβ locus, *β2*, also exists in the mouse (MMU 3 at 42.7cM), but is known to produce only one isoform, β2-1, which contains the leucine zipper motif (Thompson et al. 1991; Watanabe et al. 1993). All GABPβ proteins contains five N-terminal ankyrin-like repeats that enable binding to GABPα (LaMarco et al. 1991; Thompson et al. 1991), and possesses the nuclear localisation signal (NLS) and transactivation domains necessary for GABP transcription factor function (Sawa et al. 1996).

2.2.1 GABPα and β Evolutionary Conservation

Outside of the conserved functional domains, β and γ subunits differ more so than the α subunits both within and between species (de la Brousse et al. 1994). This is not surprising, as GABPβ has evolved more recently than α, as *D. melanogaster* features a homologue of GABPα, Elg, but not β (Pribyl et al. 1991). A high level of amino acid

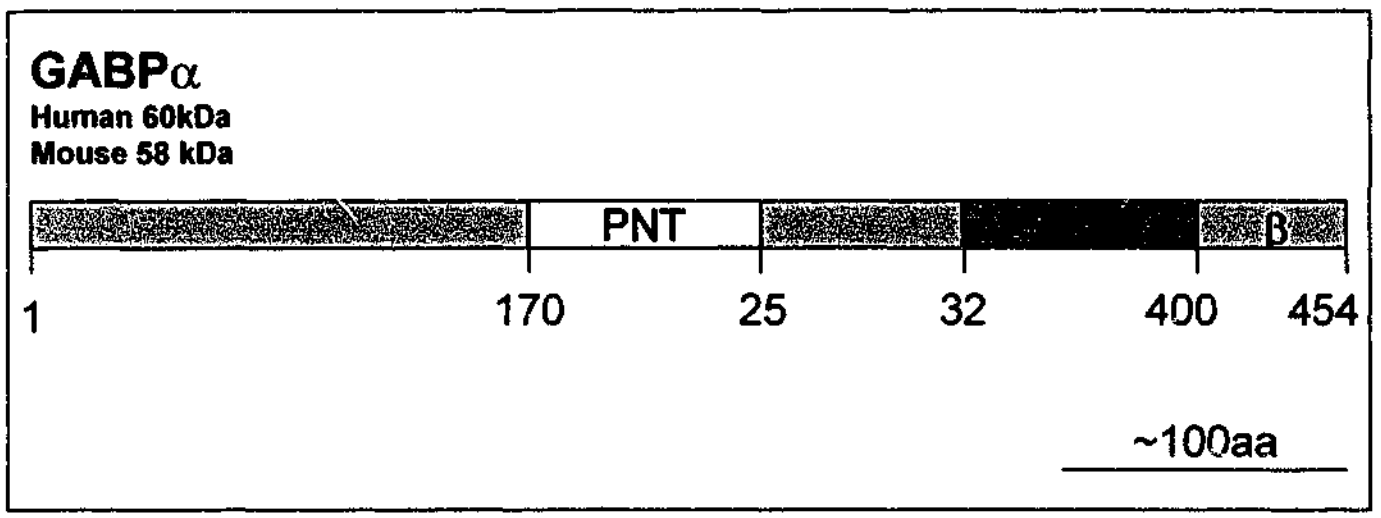
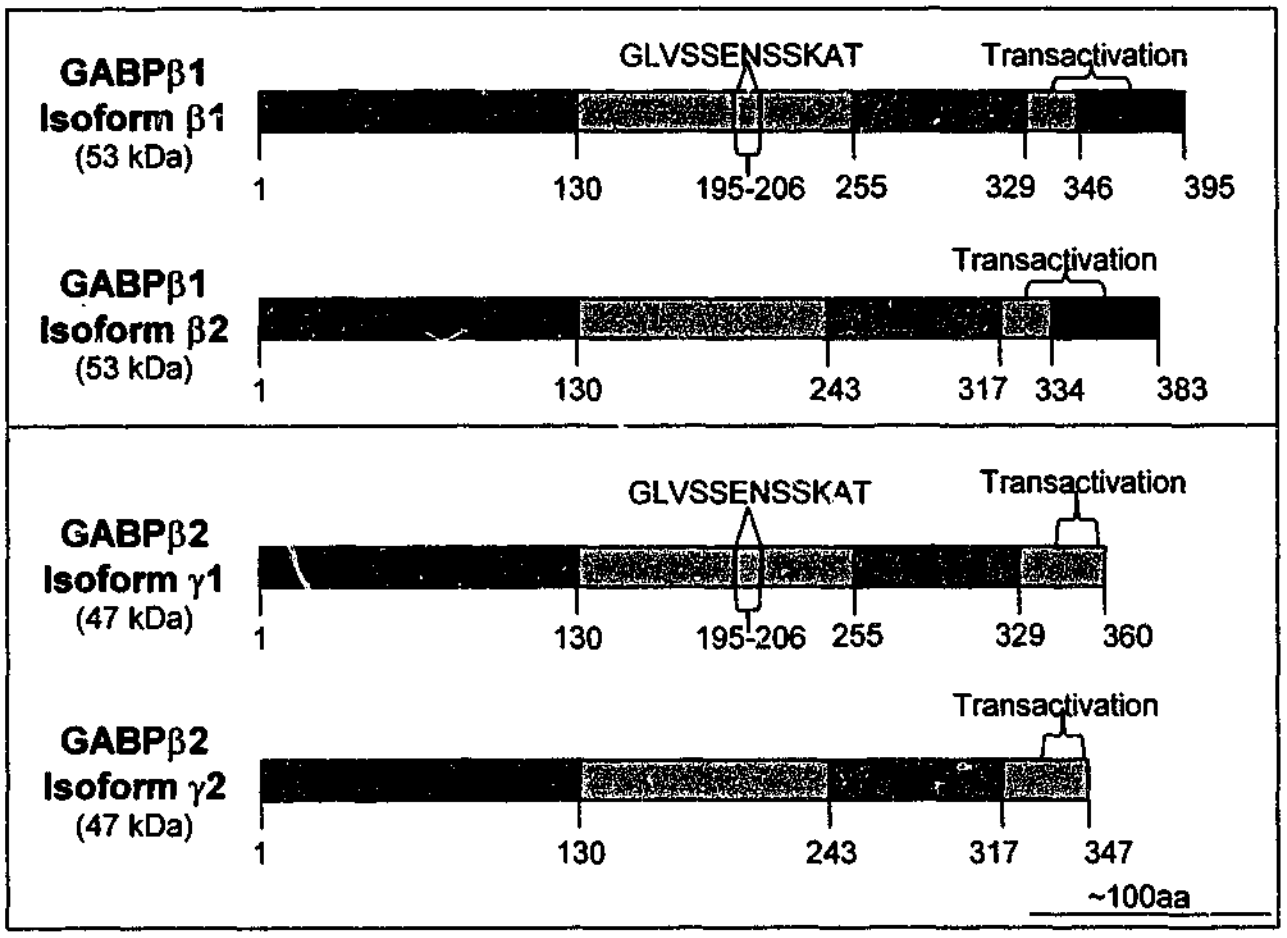


Figure 2.3 - GABPα Protein Structure.
Both human and mouse GABPα proteins are 454 amino acids in length, sharing 96 % amino acid identity. The mouse Gabpα protein is 58 kDa in size and the human GABPα protein is 60 kDa. Both human and mouse proteins feature a centrally located Pointed (PNT) domain, an ETS DNA binding domain towards the C-terminus and a GABPβ interaction domain (β) at the extreme C-terminus.

(a)



(b)

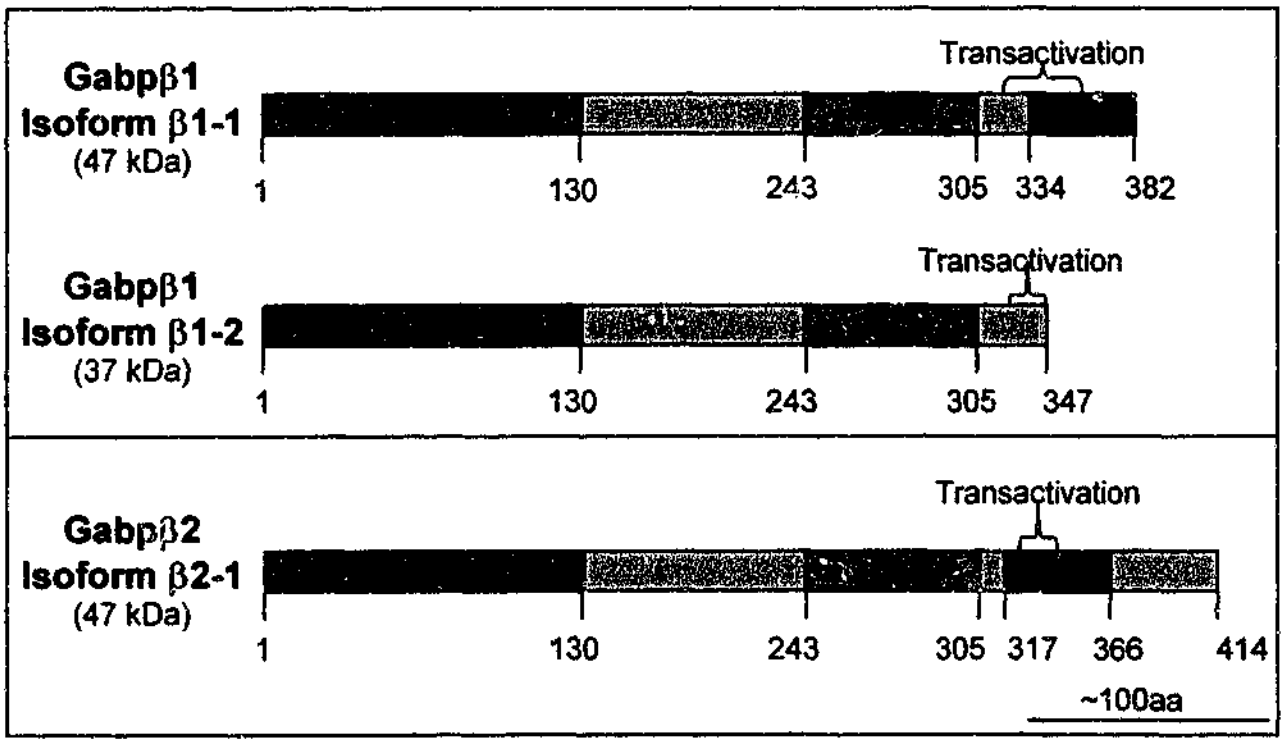


Figure 2.4 - GABPβ and γ Protein Structures.
(a) Each of the two human *GABPβ* loci (*GABPβ1* and *β2*) encode two isoforms of the GABPβ protein: β1, β2 and γ1, γ2, respectively. In each case the first isoform features an additional 12 amino acids in the central region (purple). The β isoforms also possess a leucine zipper homodimerisation motif (β) at their C-termini (blue), which is lacking in the γ isoforms. **(b)** Two loci encode Gabpβ in the mouse, *Gabpβ1* and *β2*. *Gabpβ1* produces two isoforms, β1-1 and β1-2, which differ in the presence or absence of a leucine zipper homodimerisation domain at the C-terminus. *Gabpβ2* produces one isoform, differing to β1-1 in extra sequence at the extreme C-terminus. All mouse and human GABPβ and γ isoforms feature GABPα binding (green), transactivation and nuclear localisation (NLS) (pink) domains.

sequence conservation exists between species throughout the whole GABPα protein (see **Figure 2.5**). Elg of *D. melanogaster* shows the least similarity, with 38 % identity to mouse, and *H. sapiens* shows the highest similarity with 96 % identity. The most conserved region of the GABPα protein is the ETS domain, emphasising its potential role as a DNA binding protein throughout evolution.

2.2.2 GABPα/β Dimers and Tetramers

As shown by the NMR structure determined by crystallisation of the GABP complex bound to DNA (see **Figure 2.6a**), GABP recognises the DNA sequence $^{5'}$CCGGAAGT$^{3'}$ (Thompson et al. 1991; Brown and McKnight 1992), contacting DNA through the α subunit. GABPα is capable of binding DNA as a monomer (see **Figure 2.6b**), however the presence of one or more GABPβ molecules (β or γ isoforms) determines the level of GABP target gene transactivation. *In vitro* studies of GABP complex structure and binding to the *adenovirus E4* promoter demonstrate that GABPγ isoforms result in weak to moderate transactivation, formation of α/β dimers allow moderate transactivation, and $\alpha_2\beta_2$ tetramers (which bind to tandem GGAA sites) enable strong transactivation (Ely and Kodandapani 1998; Suzuki et al. 1998). GABPβ binding reorientates the α subunit to increase its DNA binding affinity (Batchelor et al. 1998), with the $\alpha_2\beta_2$ tetramer-DNA complex being 100-fold more stable than α alone on DNA (Thompson et al. 1991; Graves 1998). GABPα binds both β and γ subunits with equal affinities, so it is the ratio of β/γ subunits in cells that determines the transactivation potential of the GABP complex (Suzuki et al. 1998). *β1-1* is the predominant β transcript expressed in mouse tissue, and its protein product is more readily co-purified with the α subunit, as the α/β1-1 complex is the most stable of all GABP combinations (Yeh et al. 1996).

2.2.3 Regulation of GABP Activity

Once a functional GABP complex has formed, there are many additional factors that can affect its capacity to transactivate a target gene (outlined in **Figure 2.7**). At the transcriptional level, thyroid hormone receptor and basal transcription factors AP2 and Sp1 have been shown to upregulate *Gabpα* expression in the rat liver (Rodríguez-Peña et al. 2002). GABP complex activity is also regulated post-translation. For instance, pro-oxidant conditions have been shown to inhibit GABP binding to DNA *in vitro*, by oxidation of two cysteine residues in the ETS and dimerisation domains of GABPα (Martin et al. 1996; Chinenov et al. 1998).

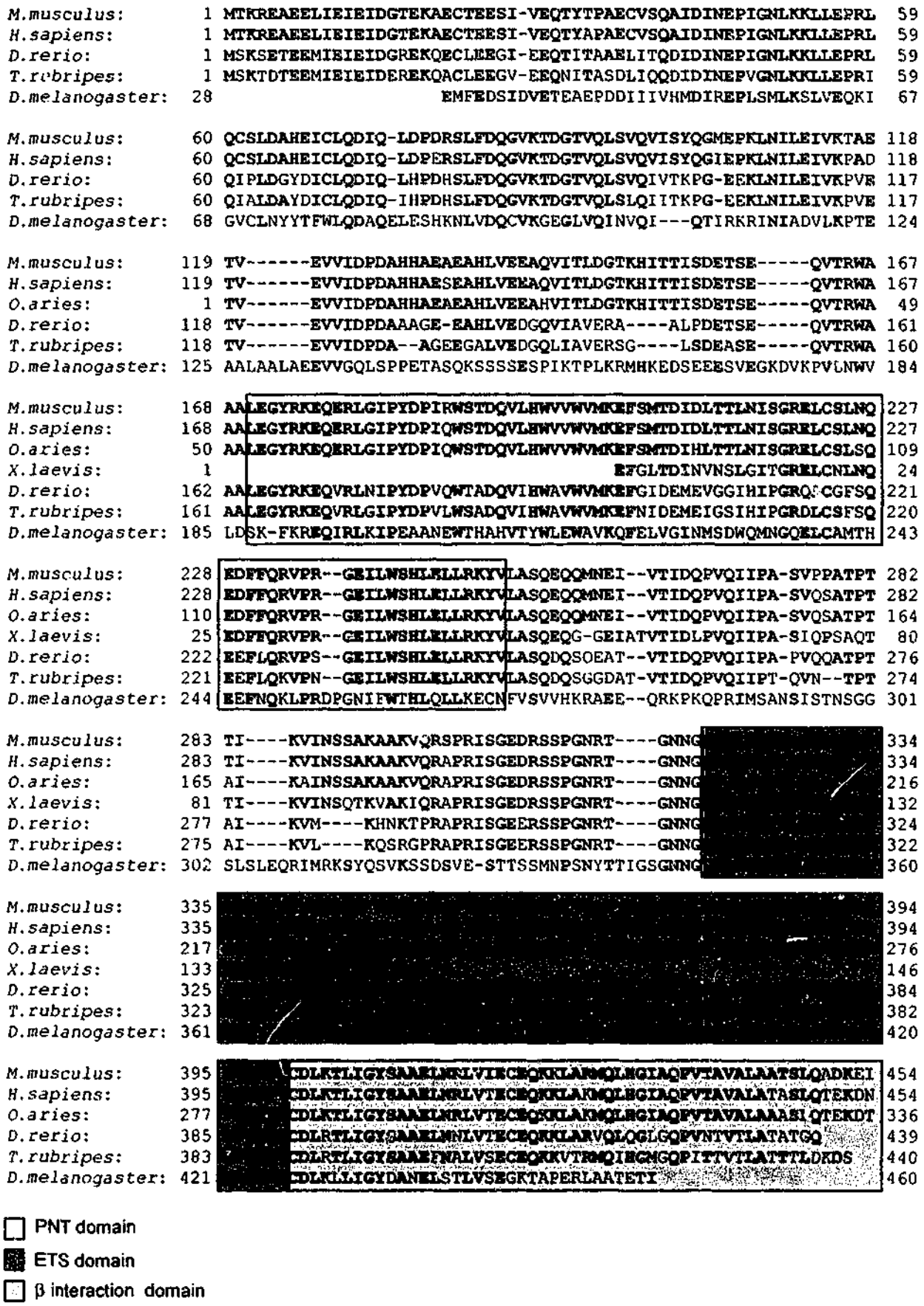
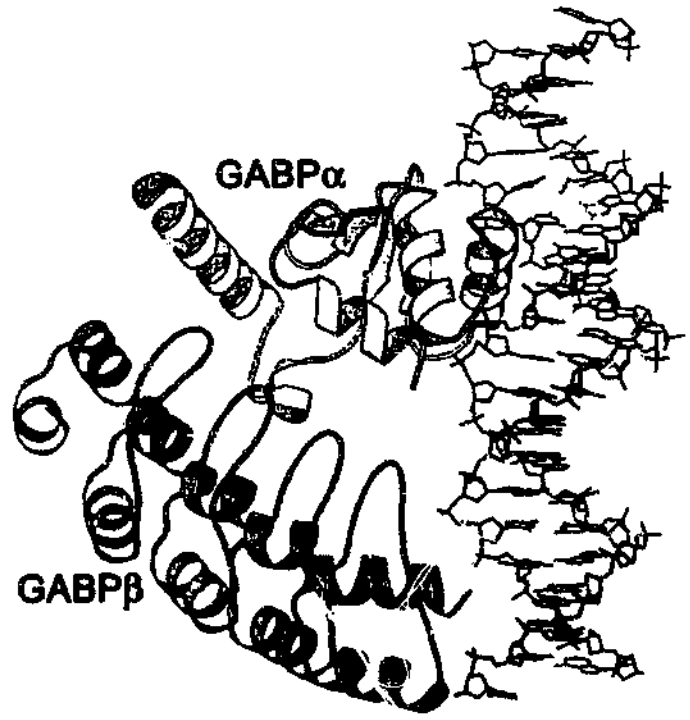


Figure 2.5 – Evolutionary Conservation of the GABPα Protein Sequence.
Amino acid sequence alignment of GABPα proteins, using mouse as the reference sequence. Identical residues are indicated in bold and protein domains are shaded. GI:13646482 *M.musculus* (mouse) 454aa; GI:13646482 *H.sapiens* (human) 454aa, 96 % identity; GI:3046690 *O.aries* (sheep) 336aa, 95 % identity; GI:992622 *X.laevis* (frog) 146aa, 82 % identity; GI:18858577 *D.rerio* (zebrafish) 455aa, 74 % identity; GI:4204471 *T.rubripes* (fugu) 440aa, 70 % identity; GI:544235 *D.melanogaster* (fruit fly) 464aa, 38 % identity.

(a)



(b)

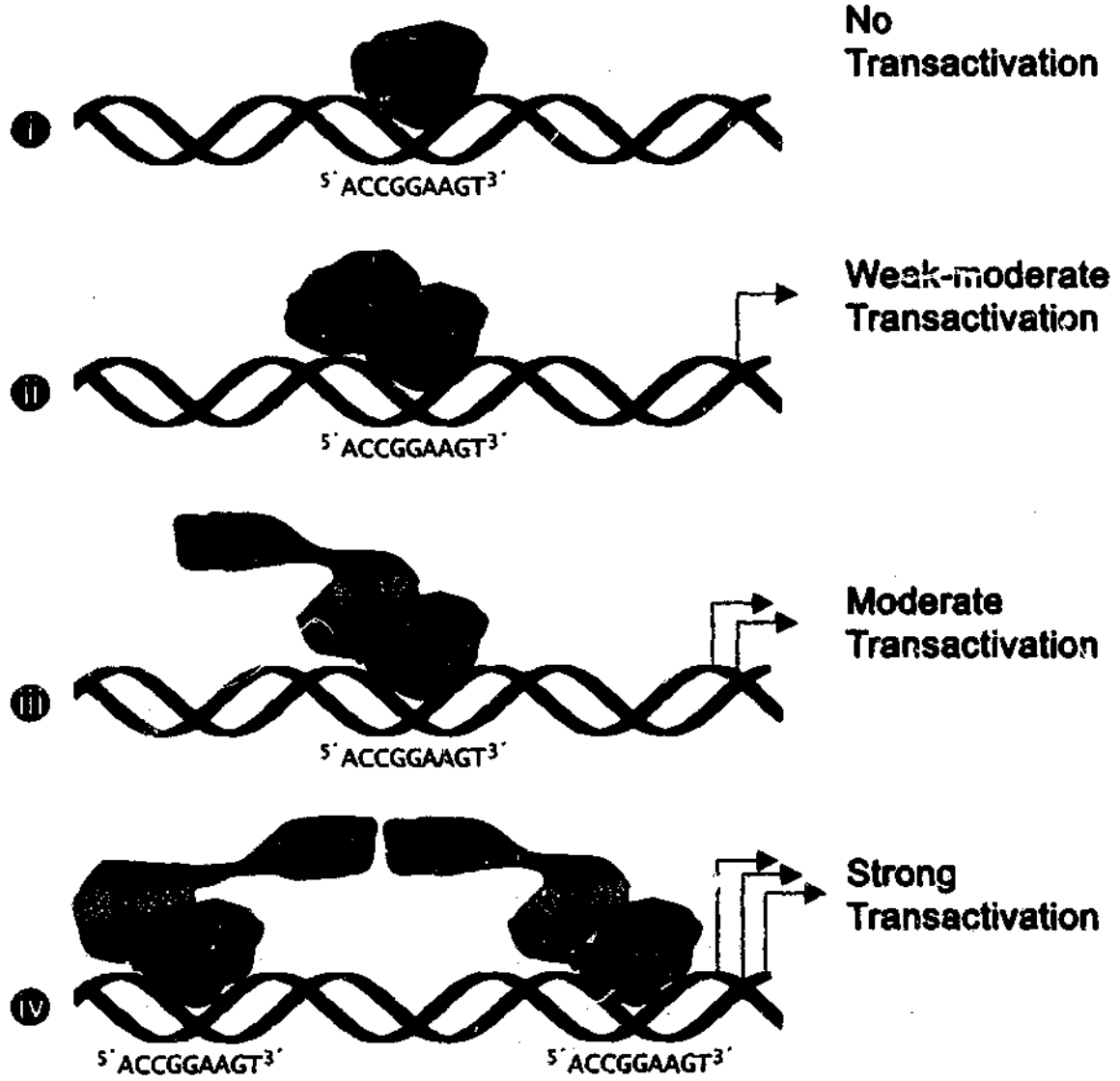


Figure 2.6 - Structure and Transactivation of GABP on DNA.
(a) NMR protein structure of the GABP complex bound to DNA (figure reproduced from Wolberger 1998). **(b)** GABP complexes that can bind DNA. (i) GABPα alone has no transactivation potential. (ii) GABPγ binding to α results in weak transactivation of target genes. GABPβ binding to α results in (iii) moderate to (iv) strong target gene transactivation, determined by presence or absence of a β homodimer.

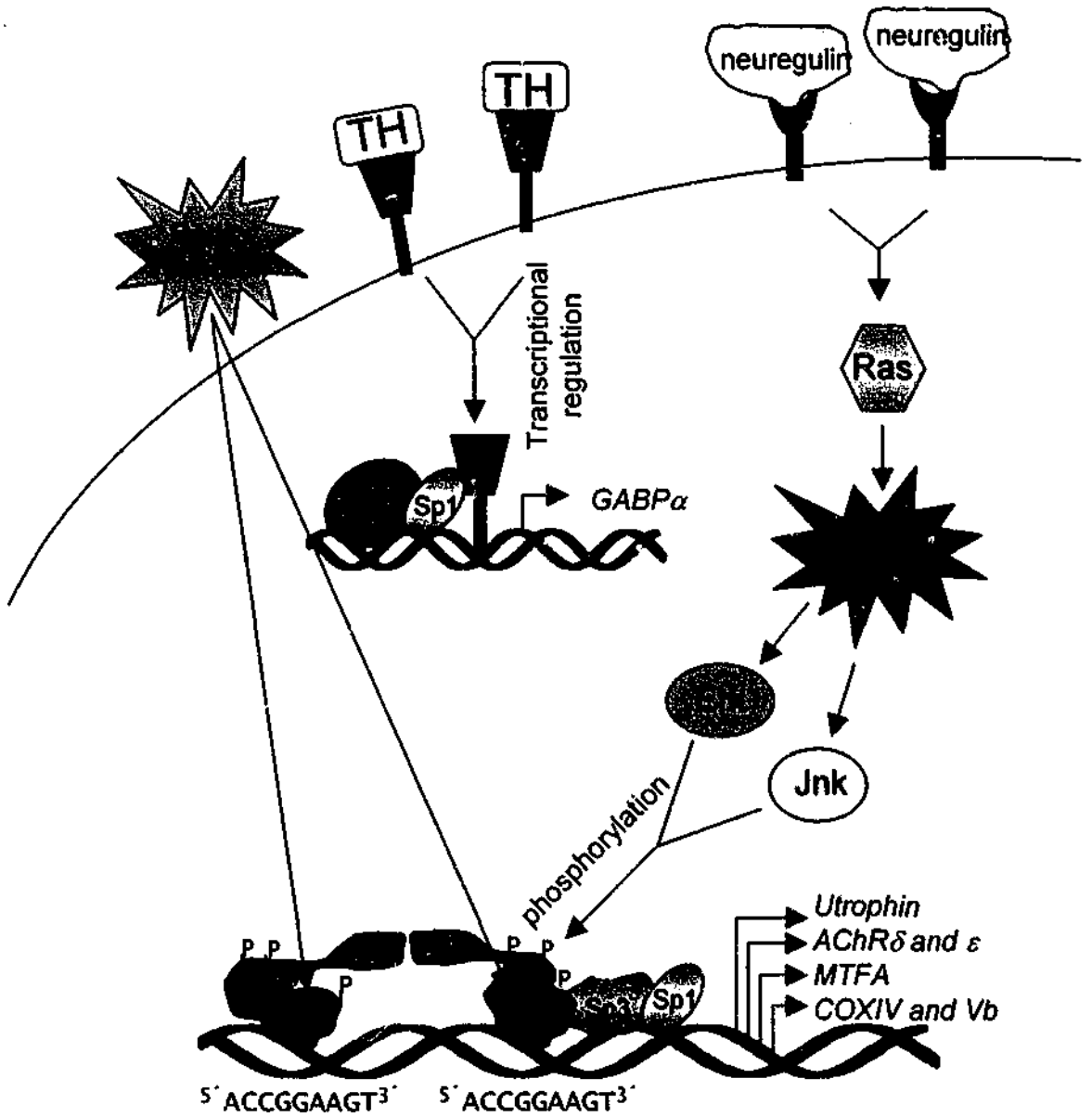


Figure 2.7 - Transcriptional and Post-Translational Control of GABP.
GABP is regulated at transcriptional and post-translational levels. Thyroid hormone (TH) is able to transcriptionally upregulate expression of *GABPα.*, as are the basal transcription factors AP2 and Sp1. Cell signalling molecules, such as Neuregulin, activate GABP via Erk and Jnk phosphorylation and the MAPK pathway, resulting in the up-regulation of GABP target genes (*Utrophin*, *AChRδ* and *ε*, *MTFA*, *COXIV* and *Vb*) together with basal transcription factors Sp1 and Sp3. In contrast, pro-oxidant conditions within a cell inhibit the activity of the GABP complex.

DNA methylation within the GABP binding site can act as a cell type-specific means of regulating GABP function *in vivo*. For example, methylation of a CpG dinucleotide within the Gabp site in the enhancer of *M-lysozyme* (required for differentiation of macrophage/granulocyte cell lineages) in fibroblasts, T cells, myelocytes and immature macrophages prevents Gabp binding. However, the CpG dinucleotide is not methylated in mature macrophages (Nickel et al. 1995; Short et al. 1996). Similarly, the Gabp binding site within the promoter of the Cytochrome P450 gene *Cyp 2d-9* encoding the male-specific steroid 16α-hydroxylase of mouse liver is methylated to a higher degree in female cells than male cells (Yokomori et al. 1995), and methylation state of the Gabp binding site within the rat *Thyroid-Stimulating Hormone Receptor* (*TSHR*) promoter determines gene expression in non-functional versus functional thyroid cells (Yokomori et al. 1998).

The MAPK pathway, mediated by ERK/JNK kinases, is also known to play a key role in the post-transcriptional regulation of GABP complex activity in skeletal muscle (Fromm and Burden 2001). Neuregulin (NRG) is a growth factor that plays a key role in morphogenesis of lung, skeletal muscle and heart, by remodelling epithelium (Chausovsky et al. 1998). NRG binds to ErbB receptors and activates the MAPK kinase pathway, resulting in phosphorylation and activation of both α and β subunits of GABP in skeletal muscle cells *in vitro* (Flory et al. 1996; Fromm and Burden 2001).

As has been found for other ETS proteins, interaction of GABPα with other transcription factors also aids in regulation of its transactivation potential (see **Table 2.4**). For example, the N-terminus of GABPα binds ATF1 and CREB and the three transcription factors synergistically activate the adenovirus E4 promoter *in vitro* (Sawada et al. 1999). GABP also physically interacts (using an undetermined region) with Sp1 and Sp3, resulting in synergistic transactivation of the *Utrophin* promoter in skeletal muscle cells (Galvagni et al. 2001; Gyrd-Hansen et al. 2002).

Physical interactions of GABPβ also affect GABP complex function. Although providing only weak transactivation potential, the γ1 isoform of GABPβ has been shown by yeast-2-hybrid and co-immunoprecipitation experiments to bind (using amino acids 149-347, encompassing the central 12 amino acid insertion and NLS) to the transactivation domain of the cell cycle regulator E2F1 in cardiomyocytes (Hauck et al. 2002). This interaction of GABPγ1 has a synergistic effect on E2F1 transactivation of the histone *H2A.1* promoter *in vitro* through use of the E2F1 DNA binding domain, however GABPγ1

represses the upregulation of proapoptotic E2F1 target genes (Hauck et al. 2002). GABPβ and γ isoforms are also capable of binding to the tissue-specific cofactors of Polycomb proteins, YAF-2 (YY1-associated factor 2) and the closely related YEAF1 protein (YY1- and E4TF1/hGABP- associated factor 1), using amino acids 249-310, overlapping with the NLS (Sawa et al. 2002). YAF-2 and YEAF1 also bind the Ying-Yang -1 (YY1) transcription factor, and can act as cofactors for formation of ternary complexes with YY1 and GABP (Sawa et al. 2002). However, *in vitro* transactivation of the *Retinoblastoma* (*Rb*) promoter by GABP is enhanced by YAF-2 binding, yet repressed by YEAF1 binding (Sawa et al. 2002). Taken together, this data suggests that, although GABPβ does not bind DNA, its functional domains and tissue-specific interactions with other proteins help determine the effect of GABP binding to any given target gene.

2.2.4 GABP Expression

The GABP transcription factor is ubiquitously expressed in mouse, rat and human tissues, and the β and γ isoforms are more readily detected than α (Escrivá et al. 1999). Due to the multi-factorial nature of the GABP complex, it is not surprising that Gabpα and β1-1 levels are concordant across all tissues in the mouse (de la Brousse et al. 1994). However, study of *Gabpα* expression in the rat testes, liver and brain show no correlation between the RNA and protein levels (Vallejo et al. 2000). Therefore, as described above, Gabp is regulated at both transcriptional and post-transcriptional levels.

Alternative Transcripts

Multiple transcripts exist for both the α and β subunits of GABP (as detected by Northern blot analysis), indicating alternative splicing or multiple initiation and polyadenylation sites (LaMarco et al. 1991). However, only one form of GABPα protein has been identified, and the β isoforms characterised do not account for all RNA transcript sizes. Alternative transcripts have been described for other ETS factors: *ETS-1* (Jorcyk et al. 1991; Quéva et al. 1993; Li et al. 1999a), *ETS-2* (Watson et al. 1990), *ERG* (Reddy et al. 1987; Murakami et al. 1993), *FLI-1* (Prasad et al. 1998) and *ELF-5* (Zhou et al. 1998). These transcripts result from the use of alternative promoters, alternative splicing, or alternative polyadenylation sequences. Some ETS proteins resulting from alternative transcripts possess quite different functions. For example, the p42 isoform of ETS-1 lacks the N-terminal inhibitory domain and inhibitory phosphorylation site, and activates an alternative apoptosis pathway in cancer cells by inducing *Caspase-1* expression (Li et al.

1999a). Hence identification of additional transcripts expressed by *Gabpα* and *β* genes would aid in a better understanding of gene and protein function.

Promoter Analysis

The target genes of ETS transcription factors is highly dependent upon their expression patterns, therefore another important means of studying gene function is by analysis of temporal and tissue-specific promoter elements. Recently, the mouse *Gabpα* promoter was cloned and found to be bi-directional, driving expression of the *Mitochondrial ATP Synthase Coupling Factor VI* gene in the opposite direction (Chinenov et al. 2000). Binding sites for ETS proteins, SP1 and YY1 are present in the *Gabpα* promoter (Chinenov et al. 2000), as shown in **Figure 2.8**. ETS sites are common in promoters of other ETS genes, providing a means of competitive inhibition and autoregulatory feedback (Majérus et al. 1992; Liu et al. 1997; Barbeau et al. 1999; Truong and Ben-David 2000). Primer extension and analysis has also shown multiple transcription start sites for both *Gabpα* and *Mitochondrial ATP Synthase Coupling Factor VI* genes, and no TATA box (Chinenov et al. 2000). These features are common to other ETS genes, such as *ETS-1* (Jorcyk et al. 1991) and *ETS-2* (Tymms and Kola 1994).

The arrangement of binding sites within the *Gabpα* promoter is actually quite similar to the bi-directional promoter regulating expression of Surfeit (SURF) inner mitochondrial membrane proteins SURF1/2 (Cole and Gaston 1997), and promoters of GABP target genes *COXIV* and *COXVb* encoding subunits of Cytochrome-c-oxidase, (Lenka et al. 1998), and *Mitochondrial Transcription Factor A* (*MTFA*) (Virbasius and Scarpulla 1994). Taken together, this highlights the housekeeping function of mitochondrial proteins such as Mitochondrial ATP Synthase Coupling Factor VI and correlates with the ubiquitous expression of the Gabpα protein, and function of the Gabp complex to regulate the transcription of many genes necessary for mitochondrial function (discussed below). Heart and muscle-specific transcription factor binding sites present further upstream in the *Gabpα/ Mitochondrial ATP Synthase Coupling Factor VI* promoter region may serve as enhancers of *Gabpα* expression in these tissues of high energy use (Chinenov et al. 2000).

2.2.5 GABP Target Genes

Over 60 potential target genes of GABP have been identified to date (see **Table 2.5**). This number is growing rapidly, as more promoter regions are being sequenced and characterised by deletion and site-specific mutation analysis. To be classed as a valid

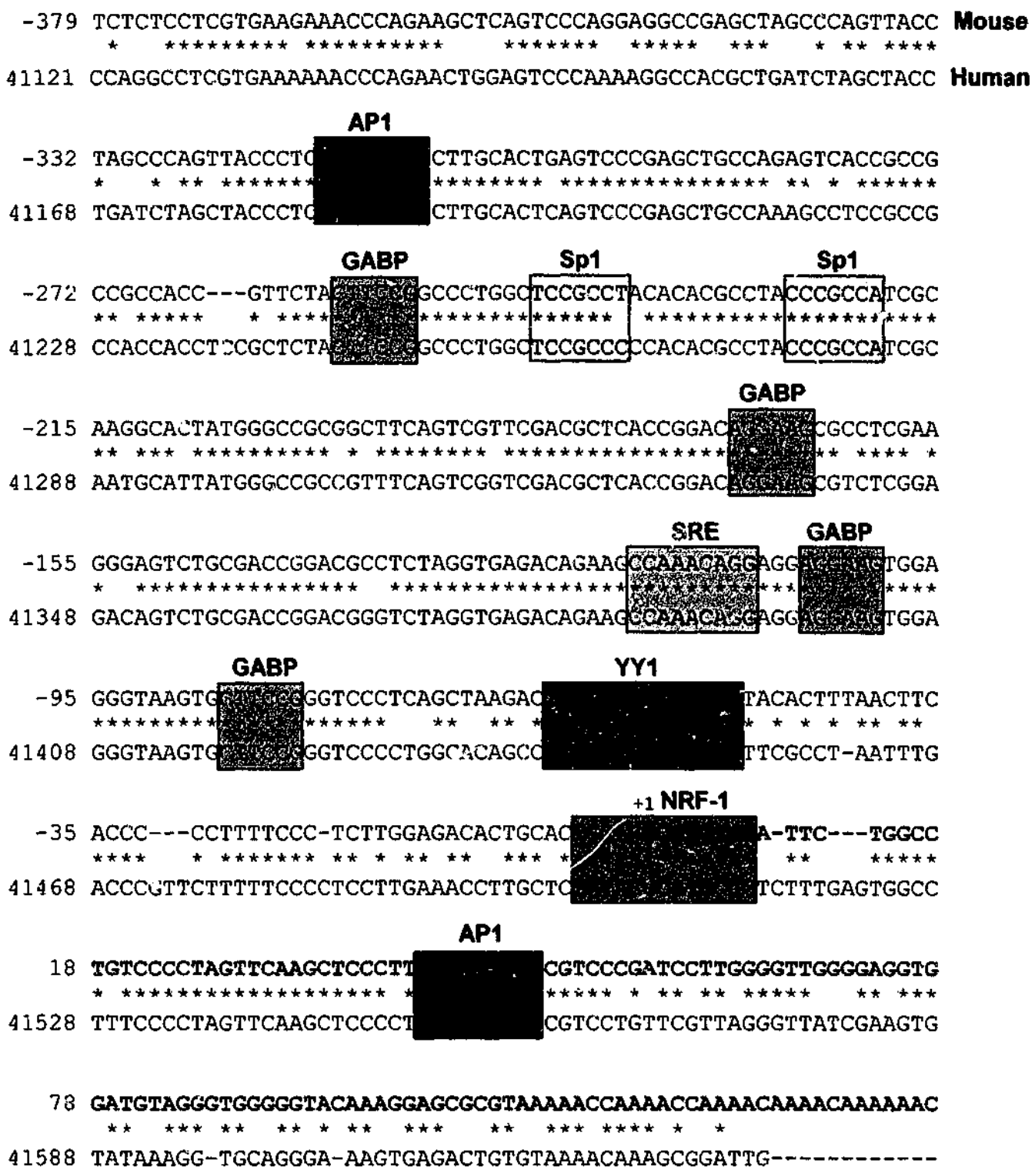


Figure 2.8 – Mouse and Human *GABPα/ATP Synthase Coupling Factor 6* Intergenic Region.

Nucleotide sequence alignment of the mouse and human *GABPαlATP Synthase Coupling Factor 6* intergenic regions. Conserved nucleotides are indicated by asterisks. Conserved regulatory elements are shaded: GABP (blue), Sp1 (yellow), AP1 (green), SRE (orange), YY1 (red), NRF-1 (purple). Mouse *Gabpα* cDNA is indicated in bold, starting at nucleotide +1. Figure reproduced from Chinenov et al. 2000.

Table 2.5 - GABP Target Genes.

TARGET GENE	ASSOCIATED TRANSCRIPTION FACTORS	ASSAYS	REFERENCES
Muscle Structure and Function			
Mouse α4 Integrin	AP1, AP2, Sp1, PU.1 binding sites	DEL, GS, SS	(De Meirsman et al. 1994; Rosen et al. 1994)
Mouse Nicotinic Acetylcholine Receptor δ and ε subunits (AchR δ and ε)	Unknown	DEL, GS, SS, TRAN, NEG, AFF, EXP	(Baldwin and Burden 1988; Gundersen et al. 1993; Koike et al. 1995; Duclert et al. 1996; Si et al. 1997; Fromm and Burden 1998a; Sapru et al. 1998; Schaeffer et al. 1998; Ohno et al. 1999)
Mouse Acetylcholinesterase	EGR-1/Sp1, AP2, NF-κB, GATA-1 binding sites	DEL, GS, SS	(Chan et al. 1999)
Human Neuregulin	Unknown	DEL, GS, SS, MUT, TRAN, DNASE, METH	(Scott et al. 1994; Fromm and Burden 1998a; Fromm and Burden 1998b)
Human and Mouse HER2 heregulin receptor (neu/erbB2)	Sp1 and PU.1 binding sites	GS, SS, MUT, TRAN, METH, UV	(Tal et al. 1987; Scott et al. 1994)
Human and Mouse Utrophin A and B	Sp1 and Sp3	DEL, GS, SS, MUT, TRAN, AFF, IP	(Dennis et al. 1996; Guo et al. 1996; Gramolini et al. 1999; Khurana et al. 1999; Galvagni et al. 2001; Gyrd-Hansen et al. 2002; Briguet et al. 2003)
Mitochondrial Function			
Mouse Cytochrome oxidase subunit Vb (COX Vb)	Sp1, NF-E1 (YY1), NRF-1, AP1, AP2, AP4, GTG, CarG, UAS2, Hap2/Hap3, Mt1, Mt2, Mt3 (rat), Mt4 (human)	DEL, GS, SS, MUT, TRAN	(Carter et al. 1992; Sucharov et al. 1995; Lenka et al. 1998)
Rat and Mouse Cytochrome oxidase subunit IV (COXIV)	Sp1, NF-E1 (YY1), NRF-1, AP1, AP2, AP4, GTG, CarG, UAS2, Hap2/Hap3, Mt1, Mt2, Mt3 (rat), Mt4 (human)	DEL, GS, SS, MUT, TRAN	(Yamada et al. 1990; Carter and Avadhani 1991; Carter et al. 1992; Virbasius et al. 1993; Carter and Avadhani 1994; Scarpulla 1997; Lenka et al. 1998; Bachman et al. 1999; Nie and Wong-Riley 1999)
Human Cytochrome oxidase subunit VIA1 (COX6A1)	NRF-1, YY1(represses), SP1	GS	(Wong-Riley et al. 2000)

Bovine Cytochrome oxidase subunit VIIaL (COX7AL)	Nrf1, Sp1 binding sites	GS, MUT, TRAN, DNASE, METH	(Seelan and Grossman 1993; Seelan et al. 1996; Hüttemann et al. 2000)
Bovine Cytochrome oxidase subunit VIIc (COX7C)	YY1	DEL, GS, TRAN	(Seelan and Grossman 1997)
Mouse Cytochrome oxidase subunit 17 (COX17)	Sp1, Nrf1	DEL, MUT, TRAN	(Takahashi et al. 2002)
Human succinate-ubiquinone oxidoreductase (cybS or SDHD – Succinate Dehydrogenase Ip (Iron sulfur subunit))	Nrf1, cdxA, GATA1 and 2, Lyf-1, Nkx-2, Sox-5, SRY, USF, YY1 binding sites	DEL, GS, MUT	(Au and Scheffler 1998; Hirawake et al. 1999)
Human γ-Glutamylcysteine Synthetase Regulatory Subunit	AP-1 (particularly JunD)	GS, SS	(Moinova and Mulcahy 1999)
Human Mitochondrial ATP Synthase β Subunit	Sp1	DEL, GS, SS, MUT, DNASE	(Villena et al. 1994; Villena et al. 1998)
Human, Rat and Mouse MTFA (mitochondrial transcription factor A)	Sp1 and Nrf1	MUT, TRAN, DNASE, METH	(Tominaga et al. 1992; Virbasius and Scarpulla 1994; Mao and Medeiros 2001; Choi et al. 2002)
Human MTFB (mitochondrial transcription factor B)	Sp1, AP1, NFκB binding sites	-	(McCulloch et al. 2002)
Human Porin isoform I	Sp1 ?/sterol repressor site	-	(Messina et al. 2000)
Human Mitochondrial Glycerol Phosphate Dehydrogenase	Sp1 Sp3, Sp4, PU.1, AP1, AP2	DEL, GS, TRAN	(Gong et al. 2000; Hasan and MacDonald 2002)
Human Mitochondrial Outer Membrane Receptor Tom20	-	-	(Hernández et al. 1999)
Human Mitochondrial Ribosomal S12 Protein	Nrf1 binding site	-	(Johnson et al. 1998)
Immune Cell Function and Haematopoiesis			
Ruminant Interferon Ƴ	Ets-2	MUT, TRAN, Y-1-H	(Ezashi et al. 1998)
Human Thrombopoietin	Unknown	DEL, GS, SS	(Kamura et al. 1997)
Human IL-2	Unknown	DEL, GS, SS, MUT, TRAN	(Avots et al. 1997; Hoffmeyer et al. 1998)
Human IL-16	CREB binding protein (CBP)/p300) (enhancer)	DEL, GS, SS, TRAN	(Bannert et al. 1999)

Human CD18	PU.1 (competitor), Sp1, CREB binding protein (CBP)/p300) (enhancer)	DEL, GS, SS, MUT, TRAN, DNASE, PD	(Böttinger et al. 1994; Rosmarin et al. 1995; Rosmarin et al. 1998; Bush et al. 2003)
Mouse Tumour Necrosis Factor (TNFα)	PU.1	GS, SS, TRAN	(Tomaras et al. 1999)
Human γ_c (subunit of cytokine receptors)	Elf1 (indirectly binds promoter)	DEL, GS, SS, TRAN	(Markiewicz et al. 1996)
Rat Factor IX (coagulation factor)	DBP and HLF C/EBP can co-occupy the ETS site	GS, SS, MUT, TRAN, EXP	(Boccia et al. 1996)
Mouse Neutrophil elastase and Proteinase 3	C/EBP, c-myb, Sp1 binding sites PU.1 and CBF.	DEL, GS, SS, MUT, TRAN	(Nuchprayoon et al. 1999)
Mouse Lysozyme-M	Nf1, C/EBP, AP1 binding sites	GS, SS, TRAN, DNASE, AFF, METH	(Bonifer et al. 1994; Nickel et al. 1995; Short et al. 1996)
Human Heparanase-1 (HPR1)	Sp1	DEL, GS, SS, MUT, TRAN	(Jiang et al. 2002)
Mouse Uroporphyrinogen II Synthase	Sp1, NF1, AP1, Oct1	DEL, TRAN	(Aizencang et al. 2000)
Cell Cycle and Signal Transduction			
Mouse Retinoblastoma Protein (RB)	BCl-3 (promotes GABP tetramer formation) Sp1, ATF/CREB and E2F binding sites	TRAN	(Savoysky et al. 1994; Sowa et al. 1997)
Human BRCA1	Unknown	DEL, GS, SS, MUT, TRAN	(Atlas et al. 2000)
Mouse 180 kDa Subunit of DNA Polymerase α	Sp1, E2F	DEL, GS, MUT, AFF	(Izumi et al. 2000)
Mouse Ribosomal Proteins rpL27A, L30, S16, L32	Sp1 (site in S16 promoter) GABP represses S16 GABP activates L30	GS, SS, MUT, DNASE	(Genuario and Perry 1996; Curcic et al. 1997)
Human Ribosomal S3a	GABP represses S3a	DEL, GS, MUT, TRAN	(Goodin and Rutherford 2002)
Mouse N-deacetylase/N-sulfotransferase 2 (NDST-2)	MITF (*mi* mutant inhibits)	GS, SS, NEG, IP, EXP	(Morii et al. 2001)
Mouse HRS/SRp40	YY1 (repressor), Sp1 (potentiator)	GS, SS, TRAN	(Du et al. 1998)
Mouse Cyp2d9	Nf2d9	GS, SS, TRAN, DNASE, AFF, METH	(Yokomori et al. 1995; Gonzalez and Lee 1996)
Human Fas	AP-1 (synergistic)	GS, SS, MUT	(Li et al. 1999)
Human Oxytocin Receptor	AP1 binding site (c-Fos/c-Jun), Sp1	DEL, GS, SS, TRAN	(Hoare et al. 1999)
Mouse Prolactin	Ets-1, C/EBPα, SAP-1, Elk-1	GS, SS, TRAN, AFF, IP, UV	(Ouyang et al. 1996; Schweppe and Gutierrez-Hartmann 2001)

Human Apolipoprotein A-II (Apo A-II)	C/EBP	GS, AFF, METH	(Cardot et al. 1994)
Mouse 6-phosofructo-2-kinase/ fructose-2-6-bisphosphatase	Sp1	GS, SS, MUT, TRAN	(Dupriez et al. 1993)
Human Folate-binding protein	Sp1	GS, SS, TRAN	(Sadasivan et al. 1994)
Human Aldose Reductase	Sp1 binding sites	DEL, GS, MUT, DNASE	(Wang et al. 1993)
Rat Thyrotrophin-releasing hormone receptor	Unknown	-	(Sellar et al. 1993)
Rat Thyrotropin Receptor Gene (TSHR)	TTF-1, Y-box protein CBP binding site	GS, SS, TRAN, DNASE, METH	(Yokomori et al. 1998)
Human and Rat Thymidylate Synthase	Sp1	DEL, GS, MUT, TRAN, NEG	(Lee and Johnson 2000; Rudge and Johnson 2002)
Human Cyclophilin 40	Sp1, Ap2, Ap1 binding sites	DEL, GS, SS, MUT, TRAN	(Kumar et al. 2001)
Human Tenascin-C	Sp1, Sp3	DEL, GS, TRAN	(Shirasaki et al. 1999)
Human Adenylosuccinate Lyase (ADSL)	Sp1, E2F, C/EBP binding sites	DEL, GS, MUT	(Marie et al. 2002)
Rat Pem Homeobox Gene	Sp1, Sp3, Elf1	DEL, GS, SS, MUT, TRAN	(Rao et al. 2002)
Viral Proteins			
HIV LTR	NFκB and Sp1 binding sites	GS, SS, MUT	(Verhoef et al. 1999)
HSV-1 IE genes (Herpes Simplex Virus immediate early genes)	C1 (co-activator), αTIF, Oct-1, Sp1	DEL, GS, SS, MUT, TRAN, METH, Y-2-H, PD	(Vogel and Kristie 2000)
Adenovirus Early 4 (E4)	ATF/CREB (synergistically)	GS, MUT, TRAN, IP, GST	(Sawada et al. 1999)
Mouse Mammary Tumor Virus LTR (MMTV LTR)	Glucocorticoid receptor (co-operates) CBP and p300 site 19X	GS, SS, MUT, TRAN, DNASE	(Aurrekoetxea-Hernández and Buetti 2000)
Human B19 Parvovirus (P6 Promoter)	Sp1(synergistically), YY1(represses synergy), an ETS factor	GS, SS, TRAN, DNASE	(Vassias et al. 1998)

GABP target genes, grouped according to product function. Transcription factors that interact with GABP, or nearby binding sites, are indicated, as are the assays used to identify the GABP target genes: DEL= promoter deletion, GS= gel shift, SS= supershift, MUT= mutation of ETS site, TRAN= transactivation, DNASE= *DNaseI* footprinting, NEG= dominant negative mutants, AFF= affinity purification, IP= immunoprecipitation, METH= methylation interference, Y-2-H= yeast-2-hybrid, PD= GST pull down, Y-1-H= yeast-1-hybrid, UV= UV crosslinking, EXP= expression pattern. Relevant references are also shown.

target gene, electrophoretic mobility shift assays with competition and supershifting of specific complexes, transactivation assays, promoter deletion analysis and mutagenesis of the ETS binding site should be performed (Sementchenko and Watson 2000).

Classes of genes regulated by GABP include those encoding ribosomal proteins, cell cycle and cell survival regulators, immunophilins, splicing factors and remodelling proteins, mitochondrial proteins, neuromuscular junction (NMJ) proteins, haemopoetic proteins, genes of immune function and viral proteins. Usually, GABP acts as a positive regulator. The ribosomal protein genes *S16* (Genuario and Perry 1996) and *S3a* (Goodin and Rutherford 2002), together with human *Mitochondrial Glycerol Phosphate Dehydrogenase* (Hasan and MacDonald 2002), are the only target genes reported to date that are transcriptionally repressed by GABP. In the case of ribosomal proteins, it is thought that location of the GABP binding sites, relative to the transcriptional start site, determines whether GABP activates or represses the downstream gene (Goodin and Rutherford 2002). ETS-1 has similarly been reported to act as an activator or repressor, depending upon cell type and promoter context (Macleod et al. 1992). This is not surprising, given the dependency of ETS proteins upon interacting transcription factors.

The function of GABP at the NMJ has been extensively characterised, with target genes including *Acetylcholine esterase*, *Acetylcholine receptors* (*AChR*) δ and ε and *Utrophin*. GABP aids in the specific expression profiles of these genes during NMJ formation and retention (Koike et al. 1995; Duclert et al. 1996; Schaeffer et al. 1998). However, GABPα is present in limiting amounts in skeletal muscle (Galvagni et al. 2001), making it necessary for other transcription factors to help regulate target genes. Other well established gene targets of the GABP complex encode proteins of mitochondrial function: MTFA (the major TF of mitochondrial DNA), several subunits of Cytochrome-c-oxidase, Mitochondrial ATP synthase β, Succinate dehydrogenase iron sulfur subunit, and Mitochondrial glycerol phosphate dehydrogenase. Therefore GABP appears to play a key role in the regulation of cellular respiration.

2.3 GABP *Complex Function*

Due to the nature of its target genes, GABP function has been implicated in the processes of skeletal muscle development and cellular respiration, and therefore in the manifestation of muscular and mitochondrial diseases when deregulated.

2.3.1 GABP in Skeletal Muscle Development and Function

Myogenesis

At the onset of myogenesis, mononucleated myoblasts withdraw from the cell cycle and fuse, resulting in formation of multinucleated myotubes, decreased expression of non-muscle isoforms of Actin, Tropomyosin and Myosin proteins, and increased expression of their skeletal muscle-specific isoforms to function as the contractile proteins of muscle fibres (Gunning et al. 2000). The Retinoblastoma (Rb) protein is transcriptionally upregulated during myoblast differentiation (Okuyama et al. 1996). Dissociation of the repressor ATF-1 from the *Rb* promoter exposes overlapping GABP, Sp1 and E2F binding sites, and Bcl-3 acts to increase GABP tetramer formation on the *Rb* promoter resulting in increased *Rb* mRNA expression (Shiio et al. 1996). It is an accumulation of Rb protein, forming complexes with MyoD transcription factors, that signals a cell to permanently withdraw from the cell cycle and activate myogenic differentiation, and inactivation of the *Rb* gene or protein results in an inhibition of myogenesis (Gu et al. 1993). During muscle differentiation, heterodimeric α/β integrin cell surface receptors (particularly $\alpha 4$ integrin, encoded by a GABP target gene) also act to mediate cell-cell and cell-extracellular matrix interactions (De Meirsman et al. 1994; Rosen et al. 1994; De Meirsman et al. 1996).

Following myogenesis, differential expression profiles of the MyoD family of transcription factors (Hughes et al. 1993), and structural proteins such as Utrophin (a GABP target gene) (Jasmin et al. 2002), Neural Cell Adhesion Molecule (N-CAM) (Muller-Felber et al. 1993) and Myosin Heavy Chain (MHC) (Schiaffino and Reggiani 1994) are found in fast twitch/glycolytic/phasic versus slow twitch/oxidative/tonic skeletal muscle fibres. It is the different MHC isoforms that help determine the contractile properties of muscle fibres (Barany 1967), and they are therefore used to identify the various skeletal muscle fibre types: I, IIa, IIb, IIc and IIx. Type I slow twitch fibres are small in diameter and red coloured due to their high density of mitochondria and capillaries, resulting in their high oxidative capacity. Type IIb fast twitch fibres are large in diameter and white coloured due to their low density of mitochondria and capillaries, giving them a low oxidative capacity. Instead, type IIb fibres contain high levels of glycogen, resulting in their high glycolytic enzyme activity. Type IIa fibres are intermediate in colour and display both glycolytic and oxidative properties, and type IIc fibres are undifferentiated, contain MHC I and IIa isoforms, and are more common during gestation than in adulthood, where they constitute ≤ 5 % of skeletal muscle (Fox et al.

1989). Type IIx fibres display properties in between those of type IIa and IIb fibres and are highly abundant in the diaphragm muscle (Schiaffino et al. 1989). While each muscle tissue is composed of a mixture of fibre types, glycolytic fibres are more predominant in tissues used for rapid, forceful movements, such as quadriceps, and oxidative fibres predominate in tonically active muscles, such as the soleus calf muscle (Fox et al. 1989).

Differential expression of isoforms of Myosin heavy chain (MHC) can be detected in undifferentiated satellite cells and primary muscle fibres before specific muscle tissues form, indicating that fibre type is (to some degree) an intrinsic property of each myoblast type (DiMario et al. 1993; Barjot et al. 1995). However, even the fibre type composition (thus contractile properties) of mature skeletal tissues can be altered by changes in innervation (Esser et al. 1993), hormone levels (Yu et al. 1998), nutrition and exercise (Goldspink and Ward 1979). A proposed mechanism of fibre type-specific gene expression involves nerve-dependent determination of intracellular calcium levels and subsequent activation of a calcineurin-dependent signalling pathway (Chin et al. 1998).

One alpha motor neuron (having a cell body in the ventral root of the spinal cord) innervates 10-1700 muscle fibres by extending through the central region of a muscle, perpendicular to the long axis of the myotubes, to form a functional motor unit (Bird 1992). Each motor unit may contain fibres scattered throughout a muscle tissue, but they are always of the same fibre type (Howald 1982), and each small terminal nerve branch innervates only a single muscle fibre (Sanes and Lichtman 1999). The point of contact between each nerve and muscle fibre is termed a neuromuscular junction (NMJ), and termination of motor axon branches adjacent to the main intramuscular nerve results in a narrow and distinct endplate zone rich in NMJs (Burden 2002).

Neuromuscular Junction Formation and Signalling

Each NMJ is composed of three cell types; motor neuron, muscle fibre and Schwann cell (Sanes and Lichtman 1999), as shown in **Figure 2.9**. The motor neuron forms the presynaptic terminal, which has a high density of mitochondria, and synaptic vesicles containing Acetylcholine (ACh). The Schwann cell forms a sheath capping the motor nerve terminal, and Neuregulin is necessary for Schwann cell survival at the NMJ, with its effects being mediated by ETS factors including Gabp, Ets-2 and Net (Parkinson et al. 2002). The skeletal muscle fibre constitutes the complex postsynaptic membrane formation at the NMJ, known as junctional folds. At the NMJ, synaptic vesicles of the motor neuron fuse with the presynaptic membrane, releasing their contents, including

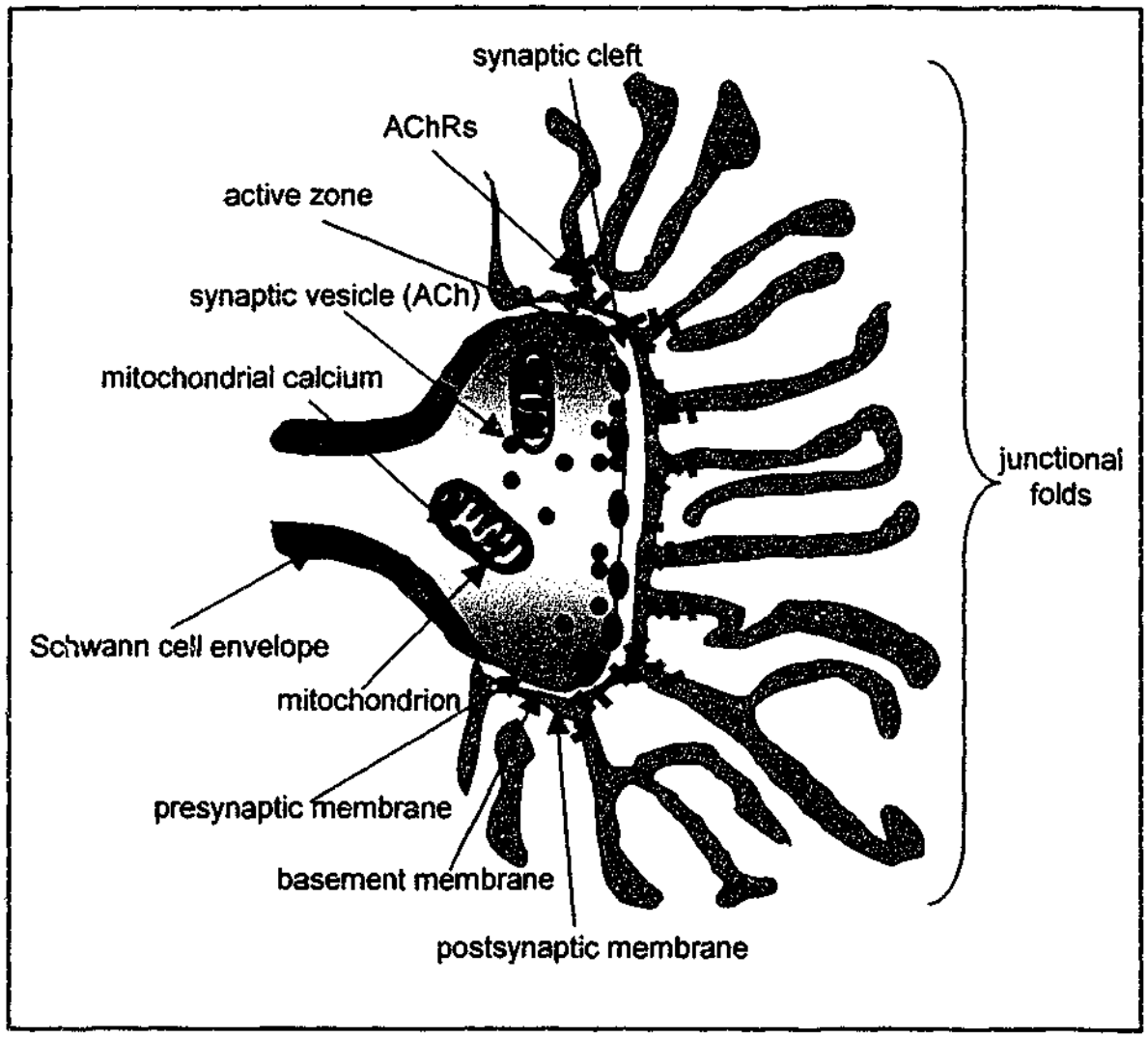


Figure 2.9 – The Neuromuscular Junction.
Schematic representation of a neuromuscular junction. The nerve terminal is coated with a Schwann cell envelope and is filled with mitochondria that release calcium at the endplate. Synaptic vesicles fuse to the presynaptic membrane of the nerve to form active zones. This allows for diffusion of Acetylcholine (ACh) into the synaptic cleft, and uptake of the ACh by receptors (AChRs) located in the postsynaptic junctional folds of skeletal muscle, eliciting a contractile response. Image adapted from Siklos et al. 1995.

Acetylcholine (ACh), into the synaptic cleft. ACh is then taken up by Nicotinic Acetylcholine Receptors (AChRs), located in the top third of junctional folds within the postsynaptic membrane of skeletal muscle (Slater et al. 1992). It is binding of ACh to the AChR ion channel that stimulates skeletal muscle contraction.

It has recently been shown that skeletal muscle can be classified into two groups, independent of fibre type composition, by virtue of how fast NMJs develop, how many AChR clusters are present, and AChR distribution (Pun et al. 2002). Fast synapsing muscles include the adductor, tibialis anterior and intercostals, delayed synapsing muscles include the gracilis anterior, lateral gastrocnemius, diaphragm and sternomastoid, and the medial gastrocnemius exhibits intermediate synapsing (Pun et al. 2002). Therefore changes in protein expression at the NMJ during development need to be studied across both classes of skeletal muscle. Relative position and speed of synapse development of hindlimb muscles is shown in **Figure 2.10**.

AChR components are highly conserved among mammals and mediate synaptic transmission at all vertebrate NMJs (Beeson et al. 1993). The AChR complex is comprised of $\alpha_2\beta\gamma\delta$ subunits in the embryo and $\alpha_2\beta\varepsilon\delta$ in the adult (Sanes and Lichtman 1999). Neuronal innervation, and expression of Agrin, Neuregulin and Acetylcholine stimulate the switch in receptor type in the first postnatal week of development in fast twitch muscle fibres. This process continues until adulthood in slow twitch muscles such as the soleus (Witzemann et al. 1990; Missias et al. 1996; Rimer et al. 1997). In foetal, non-innervated muscle, AChRs are expressed across the myotube surface evenly, and all subunits (including γ) are upregulated in denervated muscle (Boulter et al. 1986; Schaeffer et al. 1998). In mature, innervated skeletal muscle, AChR expression is restricted to the top third of junctional folds (Slater et al. 1992). Disease states of neuromuscular transmission usually result in decreased folding or mislocalised AChR expression (Slater et al. 1992). New clusters of AChRs emerge at sites of synapse formation even after muscle damage and regeneration, by stimulation from neuronal factors such as Agrin (Frank and Fischbach 1979; Slater et al. 1985; Cohen et al. 1997; Meier et al. 1997; Ruegg and Bixby 1998; Bezakova et al. 2001; Bezakova and Lømo 2001).

How important each of the three cell types (motor neuron, Schwann cell and muscle fibre) at a NMJ is to the formation and clustering of AChRs remains controversial (Arber et al. 2002). The neural isoforms of Agrin can stimulate transcription of the mature *AChRε*

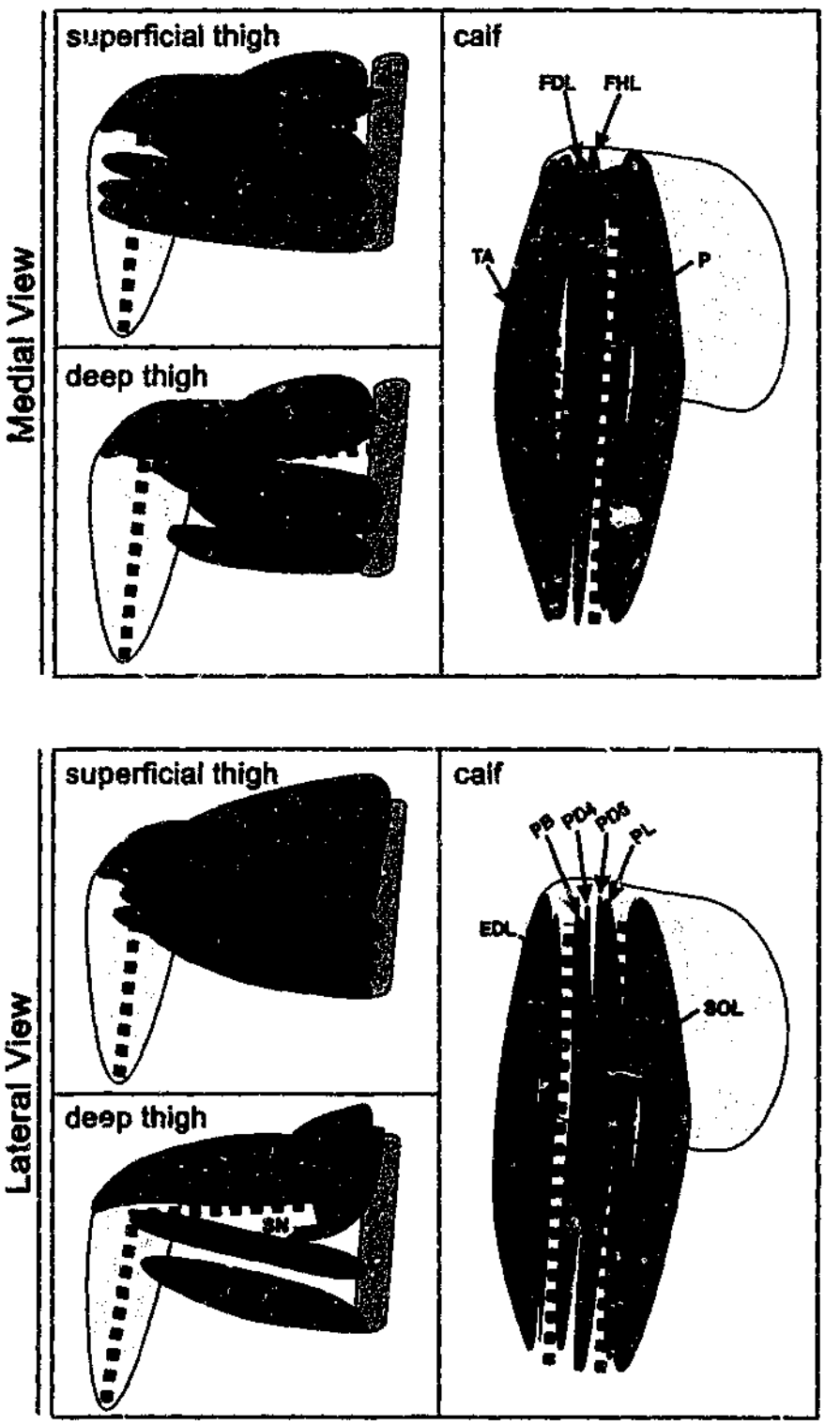


Figure 2.10 - Position of Mouse Hindlimb Muscles.
Schematic representation of the medial (a) and lateral (b) views of mouse hindlimb muscles. Thigh and calf regions are as shown, and the distribution of fast synapsing (green) and delayed synapsing (blue) muscles is indicated. Thigh muscles are: AB-adductor brevis, AL-adductor longus, AM-adductor magnus, BF-biceps femoris, CF-caudofemoralis, GMax, GMed and GMin-gluteus maximus, medius, and minimus, GRA and GRP-gracilis anticus and posticus, P-pectineus, RF-rectus femoris, STd and STv-semitendinosus dorsal and ventral, SM-semimembranosus, TFL-tensor fascia lata, VM and VL-vastus medialis and lateralis. Calf muscles are: EDL and FDL-extensor and flexor digitorum longus, FHL-flexor hallucis longus, LGC and MGC-lateral and medial gastrocnemius, P-plantaris, PB-peroneus longus, PD4 and PD5-peroneus digiti quarti and quinti, PL-peroneus longus, POP-popliteus, SOL-soleus, TA and TP-tibialis anterior and posterior. Dotted lines indicate positions of bones. Figure reproduced from Pun et al. 2002.

subunit (rather than the immature γsubunit), by causing aggregation of Neuregulin and the ErbB receptors of muscle (Meier et al. 1998; Rimer et al. 1998), and both neuronal and muscle Agrin reorganise the cytoskeleton around the synapse (Bezakova and Lømo 2001). However, only the alternatively spliced z-Agrin isoform, expressed specifically by the nerve, potentiates AChR clustering (Sanes et al. 1998). Agrin deficient mice feature no functional neuromuscular synapses, due to loss of AChR clusters and synapse-specific transcription (Gautam et al. 1996). Skeletal muscle cells may also contribute to the decision of which cells form NMJs, as they have been shown to possess heritable positional memory during embryogenesis (Donoghue et al. 1992).

Knockout mice for the receptor (or downstream signalling molecule) of Agrin, Muscle-specific kinase (MuSK), show that MuSK aids in clustering of AChRs and other synapse-specific proteins, as well as synapse-specific transcription (DeChiara et al. 1996; Till et al. 2002). A second unidentified ligand for MuSK is thought to exist, which activates AChR clustering and sodium channel aggregation, independent of Agrin (Jones et al. 1999; Till et al. 2002). However, this process still relies upon Neuregulin/ErbB signalling for the upregulation of AChR transcription, and is potentiated by addition of Agrin. Proteins such as Rapsyn are essential for tethering the AChRs into the cytoskeleton. Rapsyn knockout mice die soon after birth, remaining motionless and unable to breathe (Gautam et al. 1995). These mice feature no AChR clusters, and undetectable levels of Utrophin, Syntrophin, α- and β-Dystroglycan throughout muscles. Re-introduction of Rapsyn into Rapsyn-deficient muscle cells *in vitro* restores their ability to cluster AChRs following Agrin treatment (Han et al. 1999). Hence Rapsyn is crucial for organisation of the synaptic basal lamina and postsynaptic membrane, however it is not essential for synapse-specific transcription. Neuregulin/ErbB signalling through the muscle appears to play an important role in AChR transcription both with and without innervation (Yang et al. 2001).

Mechanisms of Synapse-Specific Gene Expression

Only a small percentage of the total number of nuclei in differentiated myotubes (myonuclei) express postsynaptic proteins to form NMJs (Young et al. 1998). There are two branches of specialisation at the NMJ. The first is local activation of expression of synapse-specific genes, such as *AChR* (Klarsfeld et al. 1991; Sanes et al. 1991; Simon et al. 1992), *Acetylcholine esterase*, *S-laminin*, *N-CAM*, *Agrin*, *Rapsyn* and *Utrophin* (Sanes and Lichtman 1999). The second means of specialisation is the repression of these genes in

non-synaptic regions. The later is due, at least in part, to the presence of electrical activity (Esser et al. 1993; Tang et al. 1994; Bezakova and Lømo 2001; Schaeffer et al. 2001). As the postsynaptic apparatus reaches maturity, Agrin expression is enough to cause protein aggregation, change the conformation of the muscle fibre surface and redistribute organelles (Cohen et al. 1997; Meier et al. 1997).

The E-box, which is the binding site of MyoD transcription factors, is the best characterised element in promoters of AChR subunits and other skeletal muscle-specific genes. However, these myogenic binding sites are dispensable for synapse-specific expression. (Duclert et al. 1996). Recently, the N-box promoter element ($^{5'}$ CCGGAA $^{3'}$) has been characterised and found to control both synapse-specific expression and extrasynaptic gene silencing (Koike et al. 1995). GABP has been shown (by EMSA) to be the transcription factor that binds the N-box, which is present in promoters of *Utrophin* (Khurana et al. 1999), *AChRδ* and *ε* (Schaeffer et al. 1998; Si et al. 1999) and *Acetylcholine esterase* (Chan et al. 1999).

The ε subunit of the AChR, unlike others, needs local nerve-derived signals for its expression (Gundersen et al. 1993). Neuronal growth factors of the Neuregulin (NRG) family (otherwise known as ARIA/Neu/Heregulin) are known to play an important role, as they are released by motor neurons and accumulate in the synaptic basal lamina (Si et al. 1999). Neuregulins have been implicated in development of many organs (Burden and Yarden 1997), such as the trabeculation and atrioventricular valve formation in the heart (Meyer and Birchmeier 1995), differentiation of Schwann cells and oligodendrocytes (Gassmann et al. 1995; Erickson et al. 1997), and lobuloalveloar budding in the mammary gland (Yang et al. 1995). Neuregulin deficient mice (or those lacking the ErbB2, ErbB3 or ErbB4 receptors) die by E10, due to heart, cranial and hindbrain defects, while heterozygous Neuregulin deficient mice present with myasthenia (severe muscle weakness) and have a decreased number of AChR clusters (Lee et al. 1995; Burden and Yarden 1997; Sandrock et al. 1997). Several isoforms of NRG exist, as a result of alternative splicing, and can be grouped into three categories based upon their protein structure; type I NRG proteins contain an Immunoglobulin (Ig)-like loop and a glycosylation domain, type II NRG isoforms contain an Ig loop but no glycosylation domain, and type III NRG proteins contain a cysteine-rich domain (Burden and Yarden 1997). Type II NRG isoforms predominate in skeletal muscle, and the ErbB3 receptor is the key component of the receptor complex in skeletal muscle, its expression being

upregulated during muscle differentiation (Burden and Yarden 1997). Both motor neurons and skeletal muscle cells produce NRG (type III isoforms predominating in sensory and motor neurons), allowing for autocrine actions, such as maintenance of the number and position of Schwann cells in muscle (Burden and Yarden 1997).

NRG proteins are able to activate the Ras-MAPK pathway in muscle fibres, but are either less important or act by different means in Schwann cells (Trachtenberg 1998). In skeletal muscle, Neuregulin receptor binding results in activation of the ERK and JNK serine-threonine kinases, which phosphorylate specific residues in both the α and β subunits of GABP (Fromm and Burden 2001). This phosphorylation of GABP leads to activation of the GABP complex (Fromm and Burden 2001), and subsequent transactivation of target genes such as *Utrophin* (Gramolini et al. 1999) and *AChRδ* and *ε* (Schaeffer et al. 1998; Si et al. 1999). Utrophin deficient mice exhibit a 30 % reduction in the number of AChRs and junctional folds at NMJs (Deconinck et al. 1997a; Grady et al. 1997), indicating the importance of Utrophin in skeletal muscle.

Recently, the proposed function of Gabp in the regulation of synapse-specific gene expression was assessed by generation of a skeletal muscle-specific, Ets dominant-negative mouse model, using the highly homologous DNA binding domain of *Ets-2* linked to a *lacZ* reporter gene (de Kerchove d'Exaerde et al. 2002). These mice exhibit decreased mRNA levels of characterised GABP target genes *AChRε*, *Acetylcholine esterase*, *β2 laminin*, and *Utrophin A*, while no changes in levels of GABP-independent *Utrophin B*, *MuSK* or *Rapsyn* mRNAs were observed (de Kerchove d'Exaerde et al. 2002). The ETS mutant mice also feature correspondingly smaller AChR patches and disorganised junctional folds, when compared to littermate controls (de Kerchove d'Exaerde et al. 2002). The effect of specific loss of Gabp on skeletal muscle development and function is yet to be analysed.

2.3.2 GABP Function in Congenital Myasthenic Syndrome

Congenital Myasthenic Syndrome (CMS) is a term used to describe a collection of heterogeneous disorders of neuromuscular transmission that occur in < 1/500,000 people (Vincent et al. 1997). CMS can be classified as presynaptic (*Acetylcholine* or *Acetylcholine esterase* mutations), synaptic (mutations in the collagen tail, *ColQ*, that anchors Acetylcholine esterase at the NMJ) or postsynaptic (*Acetylcholine receptor* mutations) in origin (Engel et al. 1997; Liyanage et al. 2002). Most subsets of CMS are autosomal recessive in inheritance, but occasionally cases of autosomal dominant

transmission are found. All forms of the syndrome result in muscle weakness (of varying degrees) by two years of age (Slater et al. 1997), with the possibility of becoming wheelchair bound. Symptoms progress slowly throughout life, with neonates suffering from ocular, bulbar or respiratory weakness, exacerbated by crying. By two years of age, children suffer fluctuating ocular palsies, excessive fatigability and delayed motor development (Nichols et al. 1999). Patients can often have normal ultrastructure of NMJs, while possessing decreased release of Acetylcholine and elongated pre-synaptic terminals (Liley 1956; Slater et al. 1997). This subsequently results in decreased expression of Rapsyn or Utrophin post-synaptically (Beeson et al. 1997; Slater et al. 1997). Clinical findings differ from family to family (Gomez et al. 1996), and it has been hypothesised that a mutation in genes encoding these downstream targets could also result in CMS (Deconinck et al. 1997a; Vincent et al. 1997). Unlike Myasthenia Gravis, CMS patients lack antibodies to the NMJ, and do not respond to immunotherapies (Beeson et al. 1997).

Most postsynaptic CMS disorders are due to a detectable decrease in the number of AChRs, due to a decrease in functional AChRε protein or Acetylcholine esterase. Patients with *AChRε* mutations have an increased level of foetal AChRγ protein to compensate for the loss of the ε subunit (Engel et al. 1996; Ohno et al. 1997). Deficiencies of the other AChR subunits result in foetal lethality, due to an inability to compensate (Engel et al. 1996). The mouse model of *AChRε* loss mimics CMS, with persistent AChRγ expression prior to premature death at 2-3 months of age due to progressive muscle weakness (Witzemann et al. 1996). Until recently, the only genetic causes of CMS identified were missense mutations in the coding regions of NMJ proteins, such as *Acetylcholine*, *Acetylcholine esterase* or *AChRε* (Nichols et al. 1999). These mutations result in structural changes of the AChR ion channel and altered affinities for ACh binding (Ohno et al. 1997). However, recent studies have reported a homozygous mutation in the GABP binding site (N box) of the *AChRε* promoter that results in decreased AChRs due to loss of *AChRε* expression (Nichols et al. 1999; Ohno et al. 1999) (see **Figure 2.11**). The same C→ T transition has previously been shown to decrease Gabp binding to the mouse *AChRε* promoter by 90 %, with a 70 % decrease in reporter gene expression (Duclert et al. 1996).

2.3.3 GABP and Muscular Dystrophy

Skeletal muscle hypertrophy and regeneration are adaptive processes. Failure to maintain a balance between the two phases results in aging and myopathies. Several

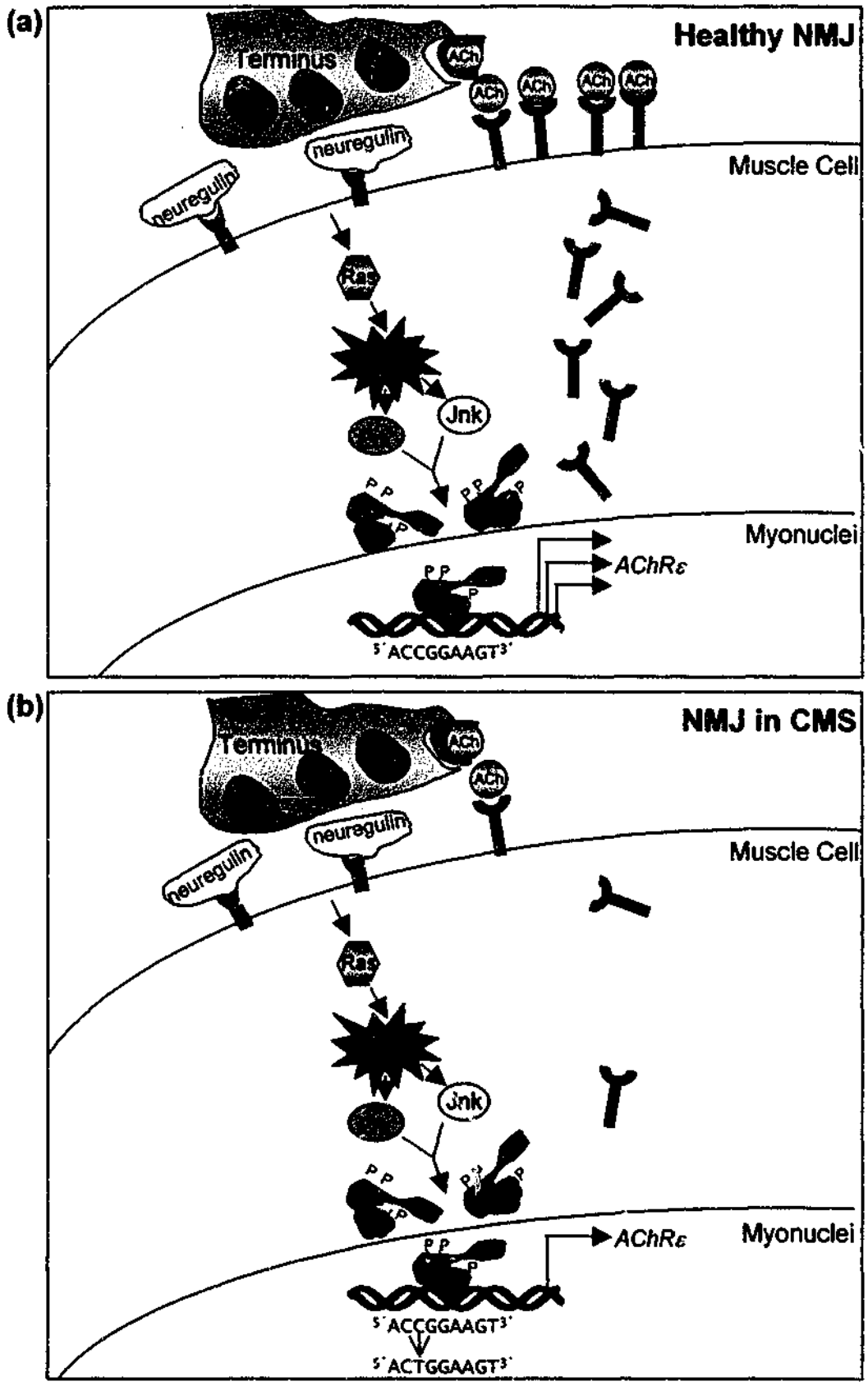


Figure 2.11 - GABP Function at the Neuromuscular Junction.
(a) At a healthy NMJ, Neuregulin signals from the nerve to activate GABP (by means of Erk/Jnk phosphorylation), resulting in upregulation of target genes such as *AChRε*. **(b)** In CMS, a mutation within the *AChRε* promoter prevents GABP binding, resulting in decreased NMJ signalling and muscle weakness.

classes of myopathies exist. X-linked Duchenne Muscular Dystrophy (DMD) is the most prevalent primary myopathy, occurring in 1/3500 male births (Matsumura and Campbell 1994). The protein product reduced or absent in the skeletal muscle of patients suffering from DMD or Beckers Muscular Dystrophy (BMD) is Dystrophin. The position of the mutation within the *Dystrophin* gene determines whether the DMD or BMD phenotype is observed. An intact C-terminus of the protein results in the less severe BMD, as this region of the protein is needed to bind membrane glycoproteins (Straub et al. 1992; Matsumura and Campbell 1994). Dystrophin usually binds Dystroglycan-Sarcoglycan, which links to the extracellular matrix through Laminin (Ahn and Kunkel 1993).

Lack of Dystrophin in the cytoskeleton leads to the production of fragile muscle fibres sensitive to cycles of contraction and relaxation. This is due to a disruption of linkage of Dystrophin Associated Glycoproteins (DAGs) to Actin and the cytoskeleton (Matsumura and Campbell 1994; Gramolini et al. 1999). Patients with DMD are hypotonic from birth, and display decreased tendon reflexes, increased serum Creatine Kinase levels, delayed motor development, and seizures (Barkovich 1998). Current treatment for DMD includes steroids. These molecules divert energy to the muscle, leading to increased strength. However, if used for prolonged periods, steroids can result in weight gain and increased hair growth (Khan 1993).

Fast twitch muscle fibres are preferentially degenerated in DMD, which may be due to their low expression levels of Utrophin, a highly conserved homologue of Dystrophin (Jasmin et al. 2002). *Utrophin* is a 13 kb transcript encoding a structural protein (Love et al. 1989), which is expressed specifically at the NMJ in innervated, mature skeletal muscle (Bewick et al. 1992; Gramolini et al. 1997). Dystrophin is expressed throughout muscle fibres, whereas Utrophin is found specifically at NMJs. At the NMJ, Dystrophin is expressed deep in the junctional folds, where the number of AChRs is low, while Utrophin is found in the receptor-rich crests (Biral et al. 1996). Dystrophin is also upregulated after denervation of skeletal muscle, while Utrophin is not (Biral et al. 1996).

Despite the differences in the normal expression patterns of Dystrophin and Utrophin, DMD patients and Dystrophin knockout (*mdx*) mice both express Utrophin at the sarcolemma as well as at the NMJ (Jasmin et al. 1995; Blake et al. 1996; Gramolini and Jasmin 1998), presumably to compensate for Dystrophin loss. The complementary roles of the two proteins are highlighted in double knockout *Utrophin-Dystrophin* mice, which exhibit exacerbated muscular dystrophy and die prematurely (Deconinck et al. 1997b).

Utrophin has also been shown to compensate for the loss of Dystrophin function in *mdx* mice (Deconinck et al. 1997c), with expression in the correct regions (Tinsley et al. 1996; Tinsley et al. 1998). If loss of Utrophin in the sarcolemma is prevented, new myotubes thrive and degeneration stops, as observed in Utrophin-treated *mdx* mice (Tinsley et al. 1998). Utrophin overexpression also appears to have no side effects in mice (Tinsley et al. 1996), suggesting that increasing the expression and stability of the *Utrophin* transcript, and/or increasing the rate of its transcription and translation, are possible means of DMD therapy (Jasmin et al. 2002). As GABP has been shown to upregulate the *Utrophin* promoter (Gramolini et al. 1999; Khurana et al. 1999) it may be possible to use GABP to upregulate Utrophin in a therapeutic context.

2.3.4 GABP Function in Mitochondria

Mitochondria possess their own genome (mtDNA) and provide the energy for a cell, by means of oxidative phosphorylation of molecules through the respiratory chain. The respiratory chain consists of four multi-subunit enzyme complexes; NADH: Ubiquinone oxidoreductase, Succinate: Ubiquinone oxidoreductase (Succinate dehydrogenase), Ubiquinone: Ferricytochrome-c-oxidoreductase, and Ferrocytochrome-c: oxygen oxidoreductase (Cytochrome-c-oxidase) (Capaldi et al. 1988; Taanman et al. 1997). It is likely that there is co-ordinate regulation of nuclear and mitochondrial gene expression, as many proteins that function within mitochondria are encoded by the nucleus (Scarpulla 1996). Examples include the major transcriptional activators of mitochondrial gene expression, Mitochondrial transcription factors A and B (MTFA and B), as well as subunits of Cytochrome-c-oxidase and Succinate dehydrogenase.

The two transcription factors necessary for transactivation of nuclear-encoded mitochondrial genes are Nuclear respiratory factors 1 and 2 (Nrf-1 and -2) (Scarpulla 2002a). Nrf-2 is an alternative name for GABP. GABP (together with Nrf-1) regulates the expression of *COXIV*, *Vb* (Virbasius et al. 1993), *VIA1* (Wong-Riley et al. 2000), *VIIAL* (Seelan et al. 1996), *VIIC* (Seelan and Grossman 1997) and *XVII* subunits of Cytochrome-c-oxidase (Takahashi et al. 2002), as well as *MTFA* (Virbasius and Scarpulla 1994) and *MTFB* (McCulloch et al. 2002), the key transcription factors of the mitochondrial genome (see **Table 2.5**). Other GABP target genes encode the Succinate dehydrogenase iron sulfur subunit (Hirawake et al. 1999) and Mitochondrial Glycerol Phosphate Dehydrogenase (Gong et al. 2000). Mice lacking Nrf-1 die between embryonic day 3.5-6.5, and blastocysts that lack Nrf-1 possess less mitochondrial DNA than their wildtype littermates

(Huo and Scarpulla 2001). Hence, nuclear factors are necessary to maintain mitochondrial proliferation. Together, this information has lead to the hypothesis that GABP helps mediate the co-ordinate nuclear-mitochondrial communication within a cell (Nie and Wong-Riley 1999).

2.3.5 GABP in Mitochondrial Diseases

With age, there is an increase in total mitochondrial volume due to an increase in their size, and an increased frequency of random defects in complexes III and IV of Cytochrome-c-oxidase in the liver (Müller-Höcker et al. 1997). Mitochondrial DNA mutations affect oxygen-consuming organs such as heart, muscle, renal and endocrine systems (Capaldi et al. 1988; Wallace 1999). A 66-86 % loss of mtDNA results in progressive mitochondrial encephalomyopathy, hypotonia, lactic acidosis, and a deficiency of complex IV of Cytochrome-c-oxidase (Capaldi et al. 1988). However, such conditions are usually Mendelian in inheritance, indicating the importance of nuclear-encoded factors in the causation of mitochondrial diseases (Taanman et al. 1997).

Low levels of MTFA (a GABP target) are found in patients with mitochondrial DNA depletion (Larsson et al. 1994). Cytochrome-c-oxidase deficiency is one of the most common defects in mitochondrial disease, and can result from a defect in any of its three mitochondrial proteins (I, II and III) or ten nuclear proteins (IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc, VIII) (Taanman et al. 1996), many of which are encoded by GABP target genes. Cytochrome-c-oxidase deficiency is directly linked to floppy skeletal muscles in newborn children (Sumegi et al. 1990). This condition leads to the diffuse structure of myofibres and fusion of the mitochondrial cristae membrane (Sumegi et al. 1990).

Some cases of mitochondrial disease may be due to a halt in mtDNA replication and/or absence of transcriptional co-ordination of nuclear and mitochondrial *Cytochrome-c-oxidase* genes (Taanman et al. 1997). However, pathogenic nuclear mutations in oxidative phosphorylation are rare and none have been assigned to specific genes (Scarpulla 1996). GABP may be necessary for the co-ordinate regulation of the nuclear and mitochondrial genomes, and loss of GABP expression could contribute to mitochondrial disease, but this is yet to be shown.

2.3.6 Down Syndrome

Down Syndrome Incidence and Origin

Trisomy 21 is the most commonly identified chromosome abnormality in humans, probably because HSA 21 is the smallest autosome, constituting 1-1.5 % of the haploid genome (Epstein 2001). Down syndrome (DS), which results from trisomy 21, was the first chromosomal disorder to be defined (Lejeune et al. 1959). DS occurs in ~1/700 live births and 25 % of aborted foetuses (Hassold and Jacobs 1984; Network 2000), and results in many different conditions of varying expressivity. Approximately 86 % of DS cases are of maternal origin, with 75 % due to nondisjunction in meiosis I (Hassold and Jacobs 1984; Antonarakis and Group 1991; Antonarakis 1998; Petersen and Mikkelsen 2000; Epstein 2001). The remaining cases result from errors in paternal meiosis I, maternal and paternal meiosis II, and *de novo* balanced translocations (Antonarakis 1998). Mosaic DS patients with *de novo* translocations can survive longer than other DS individuals, but there is no difference in phenotype (Taylor 1968). Cytoplasmic inheritance of mtDNA mutations is one suggested reason for recurrence of aneuploidy in families (Arbuzova et al. 2001). Other factors that increase the risk of a trisomy 21 pregnancy include exposure to abdominal radiation, tobacco smoking and alcohol consumption (Hassold and Jacobs 1984). Polymorphisms in the folate metabolism pathway are also linked to DS incidence (Hobbs et al. 2000; Al-Gazali et al. 2001).

Phenotypic Variation and Penetrance in Down Syndrome

Characteristics of DS include; premature aging (St.Clair and Blackwood 1985) and early onset of Alzheimer-like symptoms (Yates et al. 1980; Van Keuren et al. 1982; Sawa 2001), seizure disorders (Möller et al. 2001), short stature (Cronk et al. 1988), cataracts (Hestnes et al. 1991), skeletal and facial dimorphisms (Kisling 1966), haematopoietic and immune deficiency (Hallam et al. 2000), acute myeloid or lymphoblastic leukemia (Taub et al. 1999; Wan et al. 1999; Savasan and Taub 2000), abnormal lung development (Schloo et al. 1991), decreased fertility (Højager et al. 1978), heart conditions (Hoffman 1987; Cohen 1999), muscular hypotonia (Morris et al. 1982), mental retardation, diabetes, gastrointestinal abnormalities, ear defects and mitochondrial enzyme deficiencies (Prince et al. 1994; Epstein 2001). Nearly every organ system is affected in DS, while it is rare for an individual to have all pathologies (Epstein 2001).

The individual conditions observed in DS all occur throughout the general population, but at a lower frequency (Epstein 2001). For example, congenital heart defects

are observed in 30-60 % of children with DS (Hoffman 1987; Cohen 1999), accounting for 70 % of all atrioventricular septal defects (AVSDs) (Barlow et al. 2001). Some characteristics of DS are linked: congenital heart disease and short stature (Cronk et al. 1988); thymus and heart defects (Bacchus et al. 1987); poor muscle tone, heart disease and poor mental development (Schell 1984). However, external anomalies, such as the distinctive facies of DS, are often more constant than internal traits (Rehder 1981). The expressivity of DS phenotypes varies from one individual to another, due to allelic heterogeneity, epistatic interactions, imprinting, and environment (Korenberg et al. 1994). Culture and psychosocial influences could also play a key role in development of some conditions, such as heart disease (Undén et al. 2001). Cytoplasmic risk factors (due to mtDNA mutations) are another suggested cause of the phenotypic variability seen in individuals with DS (Arbuzova et al. 2001).

Common Features of Down Syndrome

Premature aging and Alzheimer-like pathology is seen in all DS patients over 40 years of age (Wisniewski et al. 1985; Mann 1988; Roth et al. 1996; Anderson et al. 2000; Capone 2001). The next most common pathologies seen in individuals with DS are mental retardation and muscular hypotonia (Davisson and Costa 1999; Epstein 2001). The size of the brain of DS individuals is smaller than that of a diploid person (visible by computer tomography (CT scan)), however brain weight is proportional to body size (Schapiro et al. 1987). Also, DS neurons show increased apoptosis, generation of reactive oxygen species and lipid peroxidation (Busciglio and Yankner 1995). The region of the brain most affected in DS is the cerebellum, which is essential for motor function and learning (Davidoff 1928; Crome et al. 1966; Kisling 1966; Aylward et al. 1997). The cerebellum is highly susceptible to damage from the environment or *in vivo* imbalances such as free radicals, as the cerebellum does not develop completely until several months after birth (Thach 1998; Fonnum and Lock 2000; Rice and Barone 2000; Seidl et al. 2001; Shapiro 2001). Abnormalities in cerebellar structure are, by themselves, non-specific in CT scans of the brain (Ramaekers et al. 1997). However, combined with hypoplasia and frontal lobe shortening, the accuracy of diagnosis of DS from analysis of brain morphology increases (Winter et al. 2000).

Brain development in DS appears to atrophy at ~11 months, indicating incomplete postnatal synaptogenesis (Schmidt-Sidor et al. 1990). Individuals with DS show a decrease in synaptic density in their sensory-motor cortex and decreased synaptic

transmission (Becker et al. 1991). This defect in synaptogenesis contributes to the learning deficits seen in children with DS (Martínez-Cué et al. 1999). A gross loss and atrophy of nerve cells in DS brains is also observed (Mann et al. 1985). Trisomy 21 neuronal cell lines display decreased choline uptake and altered membrane receptor function, resulting in impaired response to neurotransmitters and cytosolic Ca^{2+} buffering (Allen et al. 2000).

Some controversy exists as to whether the hypotonia observed in muscles of young individuals with DS is linked to defects of the CNS (Crome et al. 1966). Respiratory chain disorders such as Cytochrome-c-oxidase deficiency can also result in muscular hypotonia and/or cerebellar hypoplasia (Lincke et al. 1996). In addition, humans with monosomy 21 can feature muscular hypertonia or hypotonia (Nielsen and Tranebjærg 1984; Chettouh et al. 1995), indicating that the gene/s involved is/are extremely dosage sensitive. This highlights the fact that one cannot diagnose DS on the basis of one or two characteristics, karyotyping is required, and microdeletions within the trisomic chromosome may also contribute to the phenotypic variability of DS (Barbi et al. 2000).

Treatment and Prevention of Down Syndrome Phenotypes

Over half of individuals with DS survive into their fifth decade, due to early diagnosis and treatment of symptoms. However, congenital heart disease and susceptibility to infection significantly lower life expectancies, such that individuals with DS have <3 % chance of living to 78 years (Baird and Sadovnick 1989; Prasher et al. 1998; Yang et al. 2002b). No means of prevention currently exists for the development of DS phenotypes, only treatment of symptoms (Epstein 2001).

Trials involving the treatment of DS symptoms have included: human growth hormone to increase growth rate (Torrado et al. 1991); antioxidants to decrease free radicals (Noguchi et al. 2000); megadoses of vitamins and minerals to increase intelligence (Smith et al. 1984); 5-hydroxytryptophan to increase muscle tone (Bazelon et al. 1967; Weise et al. 1974); intervention programmes to improve motor function (Morris et al. 1982); Serotonin to decrease self-injurious behaviours (Gedye 1990); Cholinergic analogues and Cholinesterase inhibitors as treatments of cholinergic deficits (Yates et al. 1980); Folic acid and Betaine to aid in Homocysteine metabolism (Pogribna et al. 2001); Ryanodine receptors to prevent effects of ischemic heart disease (Netticadan et al. 1996; Yu et al. 2000) and brain damage (Sharma et al. 2000); and Enkephalin to strengthen memory (Martinez and Weinberger 1987-1988). All drug trials have resulted in little effect or unwanted side effects (Coleman 1971; Coyle et al. 1986). Therefore, further

characterisation of the functions of genes involved in DS pathogenesis should enable the design and trial of more specific DS therapeutics (Epstein 2001).

Mouse Models of Down Syndrome

Complex disorders are often studied by separating them into a series of phenotypes, each of which can be effected by different genes (Barlow et al. 2001). Animal models are useful to highlight differences in mechanisms of similar phenotypes (Panegyres 1997; Dawson 2000; Rosenberg et al. 2000). For example, oxidative stress, DNA damage and protein oxidation are common pathological features of neurodegenerative diseases of different origins, such as Parkinson's and Alzheimer's disease. Conservation of gene order on HSA 21 exists between, human, pig (Tuggle et al. 2001), cat and mouse (Watson et al. 1986; Cheng et al. 1988; Cabin et al. 1998; Reeves et al. 1998; Reeves and Cabin 1999; Akeson et al. 2001; Pletcher et al. 2001). The mouse is the preferred model species due to size, cost, generation time, and characterised genetics (Watson et al. 1986). MMU 16 is the chromosome most similar to HSA 21, with an identical gene order in the region between *Stch* and *D16Xrf306* (Reeves and Cabin 1999) (see **Figure 2.12**). A break in synteny occurs between the terminal portions of HSA 21 and MMU 16. Hence mouse trisomy 16 is not a true model of DS (Cheng et al. 1988).

Unlike humans with DS, trisomy 16 (Ts16) mice die early in development, perhaps due to overexpression of genes whose homologues are encoded by HSA 17 and 10 (Coyle et al. 1988). Similar to humans, only trisomy of the smallest autosome (trisomy 19) survives to term in mice (Gearhart et al. 1986). However, Ts16 mice do allow for some analysis of DS phenotypes during foetal and embryonic development, such as decreased brain development, thickened and shortened necks, small heads, short snouts, heart disease and severe thymic hyperplasia due to proliferative defects in lymphoid and myeloid cells (Epstein et al. 1985; Epstein et al. 1990). Such analysis is useful, as DS is primarily a developmental problem (most consequences are apparent at birth), and developmental analysis is almost impossible in humans (Baxter et al. 2000). Ts16 chimaeras have been produced to enable postnatal studies, and these mice display a decrease in haematopoietic cell populations but no congenital features (Epstein et al. 1985; Epstein et al. 1990). The cardiac defects of Ts16 mice are more complex than those seen in DS (Webb et al. 1997), perhaps reflecting one of the differences between mouse and human development. Cardiac defects, hypoplasia of the thymus and impaired associative learning have also been

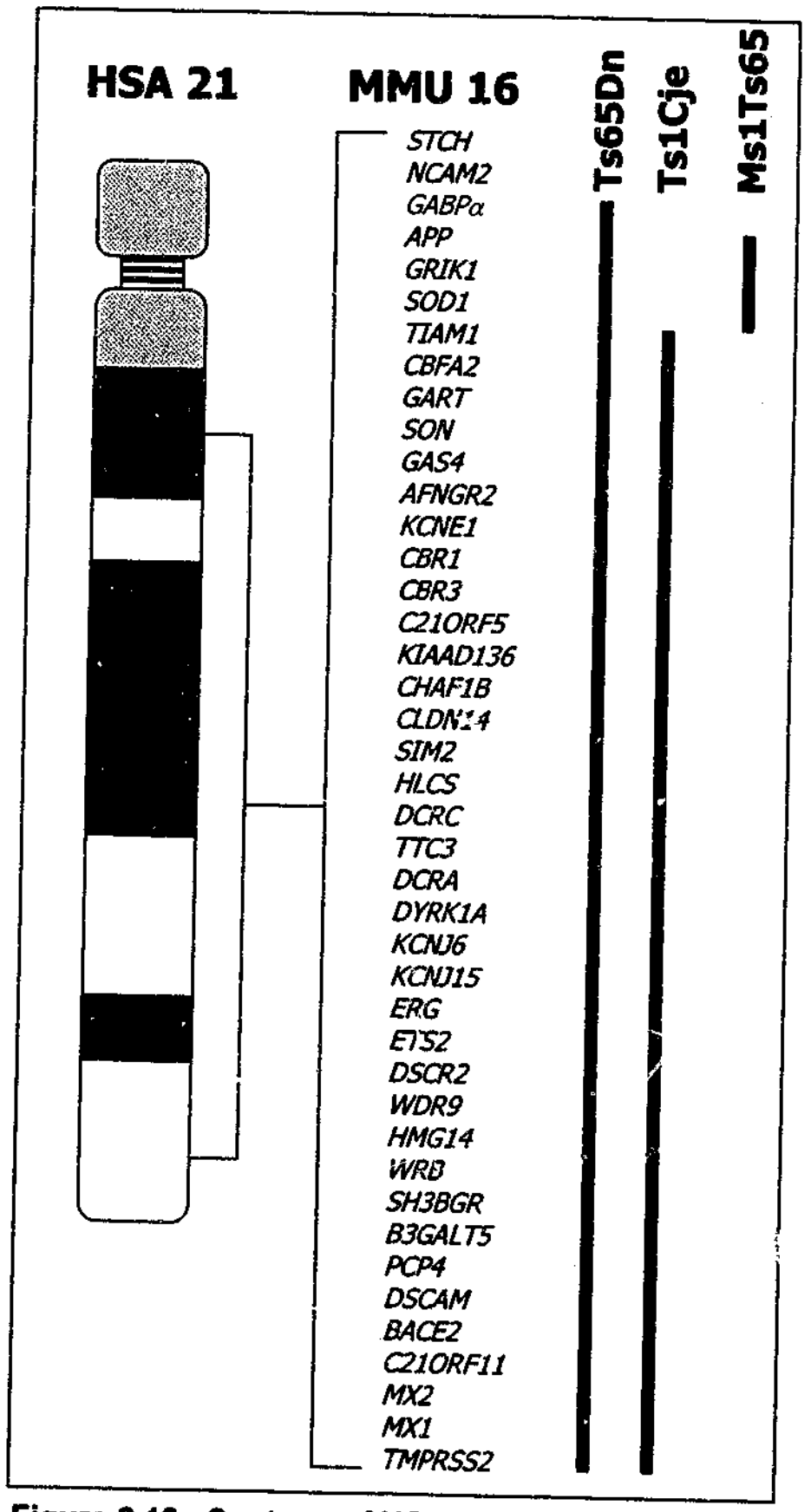


Figure 2.12 - Synteny of HSA 21 and MMU 16.
Conserved gene order of HSA 21 and MMU 16, and trisomic regions present in MMU 16 mouse models. *Gabpα* (red) is trisomic in Ts65Dn mice only. Figure reproduced from Reeves et al. 2001.

observed when chimaeras were generated from mouse ES cells to which HSA 21 had been added (Shinohara et al. 2001).

Partial Ts16 mouse models have since been produced, by means of spontaneous translocation mutations resulting from irradiation of testes (Davisson et al. 1990) (trisomic regions indicated in **Figure 2.12**). Mice trisomic for chromosomal segments smaller then chromosome 19 usually survive (Davisson et al. 1990). Ts65Dn mice carry an extra partial MMU 16, from proximal of *Gabpα* (between *Ncam2* and *Gabpα)* to distal of *Mx-1*, containing >80 % of HSA 21 homologues and <5 cM of MMU 17 euchromatin (Akeson et al. 2001). However, one cannot rule out small deletions within the Ts65Dn chromosome. The Ts1Cje mouse carries a smaller trisomic chromosome, from proximal of *Sod-1* to distal of *Mx*-1 (Davisson et al. 1993).

The Ts65Dn mice have been partially characterised, and found to possess several DS characteristics; male sterility, degeneration of basal forebrain cholinergic brain regions, decreased cerebellar volume (Baxter et al. 2000) leading to poor motor learning (Hyde et al. 2001a), decreased long-term potentiation (Siarey et al. 1997) and distinctive craniofacial abnormalities (Davisson et al. 1993; Escorihuela et al. 1995; Richtsmeier et al. 2000) and decreased sensitivity to nociception (Martínez-Cué et al. 1999). Ts65Dn mice also have an increase in the size of apposition length of excitatory synapses (Kurt et al. 2000), impaired place-learning (Hyde et al. 2001b), learning deficits in habitual behaviour (Escorihuela et al. 1995) and impaired working and long term memory (Epstein et al. 1985; Escorihuela et al. 1995; Reeves et al. 1995; Escorihuela et al. 1998; Nadel et al. 2000; Hyde et al. 2001b). Ts1Cje mice exhibit more subtle learning and behavioural abnormalities (Sago et al. 1998). Effects similar to mental retardation have also been described in Ts65Dn mice (Holtzman et al. 1996). Ts65Dn mice are more active then controls by 6 weeks of age and they develop muscular trembling (Reeves et al. 1995) (Davisson et al. 1993), phenocopying the hypotonia and hyperactivity of DS. Their steps are shorter and more irregular, with a lower running speed and weaker grip force than their littermates (Costa et al. 1999). SAGE analysis of diploid and Ts65Dn mice lead to the discovery that some of the most altered message levels were those of ribosomal proteins (Chrast et al. 2000). Hence, perhaps this deregulation of ribosome function explains the broad range of phenotypes seen in DS. As yet, no heart defects or Alzheimer-like pathology have been found in these mouse models (Antonarakis 1998; Davisson and Costa 1999).

A smaller trisomic region is found in Ms1Ts65 mice (*App-Sod1*), but fewer defects are seen is these mice (Sago et al. 1998). Therefore, further analysis of genes within the trisomic regions of MMU 16 in these partial trisomic mice should aid in the identification of genes causative of DS phenotypes. Also, making these trisomic mice transgenic for BACs containing the remaining HSA 21 homologues (2.4 Mb of MMU 10 and 1.5 Mb of MMU 17) would result in a more complete model for Down syndrome (Akeson et al. 2001; Davisson et al. 2001). Transchromosomal (Tc) mice with HSA 21 fragments inserted into ES cells have been made successfully, with ~5 Mb of this foreign DNA being transmitted to at least third generation mice (Kazuki et al. 2001). These mice have impaired cardiogenesis (Inoue et al. 2000). However, strain variation leads to differences in fragment retention and fragments are lost randomly during development, resulting in phenotypic variation. Future studies are looking into production of artificial chromosomes, using the HSA 21 centromere to perhaps make the fragments more stable in transchromosomal mice (Kazuki et al. 2001).

Overexpression models of single genes encoded by HSA 21 (and MMU 16) should help directly unravel the mechanisms of individual genes in the manifestation of Down syndrome phenotypes (Kola and Pritchard 1999). The *Amyloid Precursor Protein* (*APP*) gene of HSA 21 is increased 3-fold at the mRNA level in DS versus non-DS brain (Coyle et al. 1988). Extracellular β-amyloid containing deposits (formed by APP protein) also become plaques in DS and Alzheimer's disease (Giaccone et al. 1989). It is not surprising that transgenic mice overexpressing the APP Aβ peptide die prematurely, suffering from seizures and an increased level of cortical apoptosis (LaFerla et al. 1995). Transgenic mice overexpressing human APP also exhibit a phenotype similar to human cataracts, which is observed at an increased frequency in DS individuals (Frederikse and Ren 2002).

Superoxide Dismutase 1 (SOD-1) is an enzyme that helps detoxify free radicals. Increased SOD-1 activity and protein levels are observed in DS tissue (Sawa 2001). This resembles the situation of normal aging, and is thought to contribute to the premature aging and/or mental retardation of DS by increasing apoptosis and lipoperoxidation (Feaster et al. 1977; Sinet 1982; Brooksbank and Balazs 1984; de Haan et al. 1992; Cristiano et al. 1995; de Haan et al. 1997; Gulesserian et al. 2001; Turrens 2001). SOD-1 mediates such effects by upregulating genes on other chromosomes, such as the detoxifying enzyme *Glutathione Peroxidase* (*GPX*), *GAP-43*, and *NOS3* (Sawa et al. 1997), the pro-apoptotic genes *Bax*, *CD95* (or *Fas*) and *p53* (de la Monte et al. 1998; de la Monte 1999; Seidl et al. 1999), and

downregulating others, such as the anti-apoptotic *Bcl-2* (Sawa et al. 1997). *Sod-1* transgenic mice suffer from hypoplasia of the thymus and increased apoptosis of thymocytes, together with a 20-30 % reduction in numbers of haematopoietic progenitor cells (Peled-Kamar et al. 1995). Although the lifespan of *Sod-1* transgenic mice is normal (Huang et al. 2000) they do exhibit signs of premature aging (Yarom et al. 1988), including degeneration and withdrawal of terminal axons at NMJs of the tongue (Avraham et al. 1988), resembling that seen in DS individuals (Yarom et al. 1986).

2.3.7 GABP Function in Down Syndrome

The specific roles of the ETS transcription factors encoded by HSA 21 (ETS-2, ERG and GABPα) in the development of DS phenotypes remain unknown, however, *Ets-2* transgenic mice show similar neurocranial, viscerocranial and cervical skeletal abnormalities to those of DS individuals (Sumarsono et al. 1996), indicating that ETS-2 plays a role in skeletal development. In addition, the *Ets-2* transgenic mouse exhibits a smaller thymus and lymphocyte abnormalities similar to those observed in DS (Wolvetang et al. 2003), suggesting that ETS-2 is involved in thymic development. Lack of the 21q21-21q22.1 region of HSA 21, where *GABPα* maps, has been shown to be involved in 10/25 pathogenic features of monosomy 21, including muscular hypertonia and mental retardation (Chettouh et al. 1995). Deletion within this region has also been found in a case of first episode psychosis, where the patient presented with mild mental retardation and dysmorphic features (Takhar et al. 2002). It is a hypothesis of this research that the muscular hypotonia and cholineric signalling defects seen in DS individuals is due to a common means of regulation of the Acetylcholine Receptors in brain and skeletal muscle. GABP is known to regulate *AChRδ* (Baldwin and Burden 1988) and *ε* (Schaeffer et al. 1998) genes in skeletal muscle, and an ETS binding site has been identified in the *Neuronal Nicotinic Receptor* gene cluster (McDonough et al. 2000). **Figure 2.13** summarises how GABP may contribute to DS phenotypes.

Acetylcholine is one of the major neurotransmitter systems affected in Alzheimer's disease and DS (Cairns 2001). Both disorders (and their mouse models) present with loss of cholinergic neurons (Sacks and Smith 1989; Allen et al. 2000; Granholm et al. 2000) and individuals with DS suffer from muscular hypotonia (floppy muscles) during the early years of their lives (Morris et al. 1982; Baxter et al. 2000). This hypotonia may or may not be associated with hyporeflexes and reduced muscle strength (Davisson and Costa 1999). The tongue appears to be selectively hypertrophied in DS individuals, and shows an

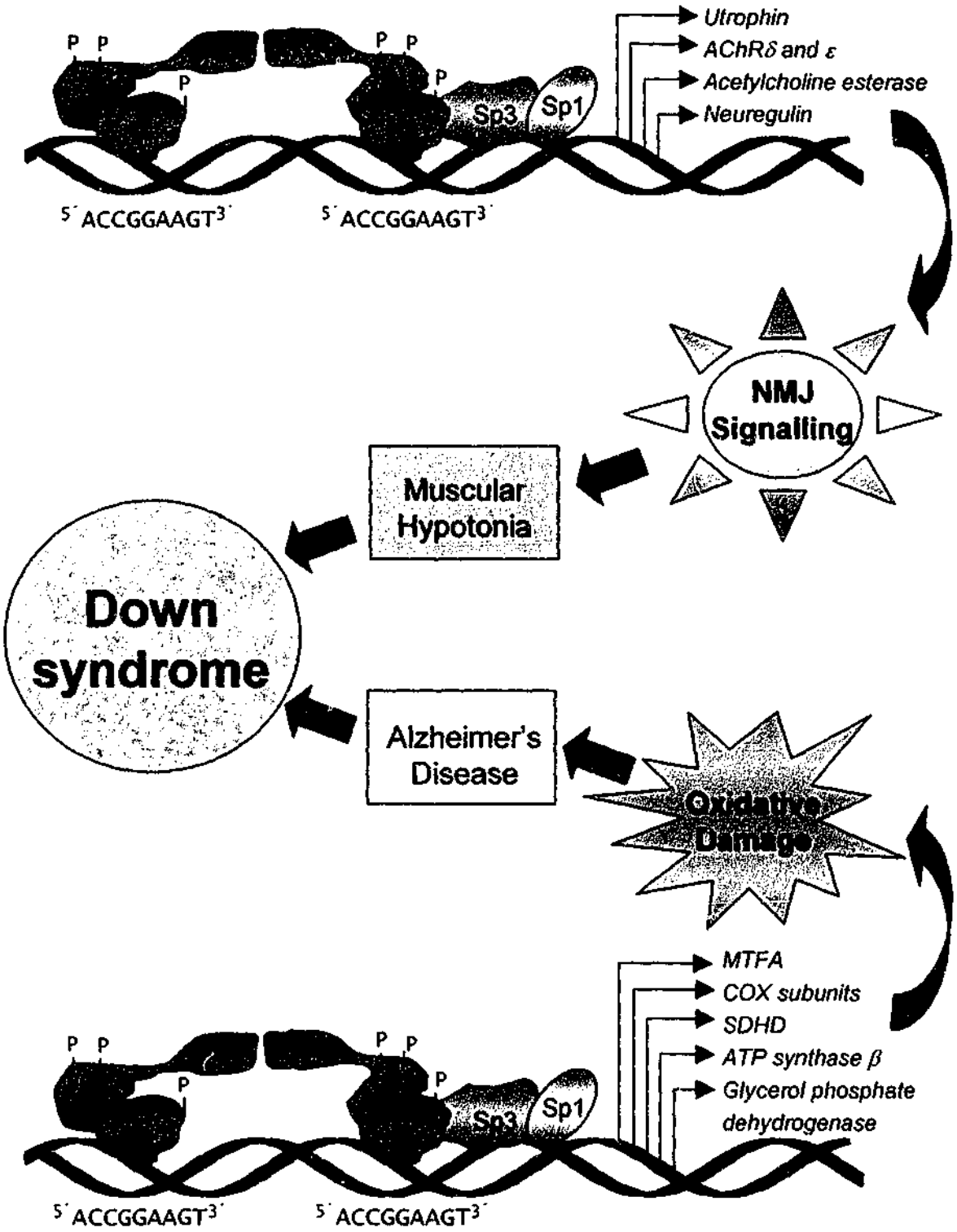


Figure 2.13 - Proposed Role of GABP in Down Syndrome.
Overexpression of *GABPα* leads to deregulated expression of GABP target genes that function at the NMJ and within mitochondria. This may result in the production of free radicals and oxidative damage contributing to Alzheimer's disease and altered neuromuscular transmission resulting in muscular hypotonia.

increase in fast muscle fibres of type IIa (Yarom et al. 1986). GABP is required for the NMJ-specific expression patterns of Utrophin (Fromm and Burden 1998a; Schaeffer et al. 1998; Gramolini et al. 1999), Acetylcholine esterase (Chan et al. 1999), AChRδ (Baldwin and Burden 1988) and ε (Duclert et al. 1993). GABP also regulates the expression of key components of the mitochondrial respiratory chain, COXIV, Vb (Virbasius et al. 1993), VIA1 (Wong-Riley et al. 2000), VIIAL (Seelan et al. 1996), VIIC (Seelan and Grossman 1997) and XVII subunits of Cytochrome-c-oxidase (Takahashi et al. 2002), as well as mitochondria's own transcriptional regulators MTFA (Virbasius and Scarpulla 1994) and MTFB (McCulloch et al. 2002). Therefore, selective overexpression of GABPα in DS may result in deregulated expression of these GABP targets due to altered GABP complex function, contributing to the causation of characteristic traits of DS.

The steady state expression level of *Gabpα* mRNA is lower then that of *Gabpβ* in rat liver, testis and brain (Escrivá et al. 1999), suggesting that GABPα may be the limiting component of the GABP complex. Therefore increased GABPα expression in DS may enable the transcriptional upregulation of GABP target genes, resulting in enhanced mitochondrial activity and the generation of an increased proportion of reactive oxygen species and premature aging characteristic of DS individuals. Increased expression of *AChRδ* and *ε* GABP target genes may also result in the formation of AChRs of unbalanced subunit proportions and decreased AChR function, contributing to muscular hypotonia. Similarly in the brain, formation of receptor complexes of inappropriate composition may result in impaired function of cholinergic neurons.

Alternatively, an increase in GABPα expression may result in the formation of GABP complexes of decreased function. GABP regulates the expression of many proteins required for oxidative phosphorylation in mitochondria, and defects in Cytochrome-c-oxidase (respiratory chain complex IV) have been detected in the blood platelets and brains of Alzheimer's disease patients (Prince et al. 1994). In addition, NADH3 (an enzyme of complex I) is downregulated in DS brain, contributing to oxidative stress (Krapfenbauer et al. 1999). Inhibition of mitochondrial complex I, III or IV results in a decrease in superoxide, whereas a loss of function of more than one mitochondrial gene leads to an increase in superoxide production (Schuchmann and Heinemann 2000). Therefore it has been suggested that mtDNA mutations and mitochondrial deregulation could be a key contributor to the development of Alzheimer-like symptoms and premature aging observed

in DS individuals (Arbuzova et al. 2002). GABP itself is also redox regulated, with pro-oxidant conditions (as in DS) resulting in inactivation (Martin et al. 1996). Therefore, an imbalance in GABP complex stoichiometry and inefficient transactivation of target genes may be observed both in DS or if GABP was lacking.

2.4 *Summary - The Molecular Mechanisms of* GABP *Function*

The GABP complex is a unique ETS transcription factor, in that it is composed of an ETS protein (α subunit) necessary for DNA binding and an unrelated ankyrin-repeat protein (β subunit) required for target gene transactivation (LaMarco et al. 1991). In a healthy mammalian cell, the dimeric or tetrameric GABP complex is thought to enable the co-ordinate regulation of nuclear and mitochondrial genomes (Scarpulla 1996). GABP is also implicated in the differentiation of skeletal muscle fibres and establishment and maintenance of synapse-specific gene expression at the NMJ (Schaeffer et al. 2001).

Disturbing the stoichiometry of the GABP complex, by means of GABPα overexpression in DS, could contribute to the manifestation of muscular hypotonia, by deregulating expression of proteins necessary for neuromuscular signalling. In addition, GABPα overexpression may contribute to the oxidative damage seen in DS individuals, due to a disruption of expression of nuclear-encoded mitochondrial proteins. Inactivation of GABP, due to pro-oxidant conditions of DS cells, may also compound these effects. Loss of GABP transactivation of target genes necessary for neuromuscular signalling results in diseases of severe muscle weakness termed Congenital Myasthenic Syndrome. GABP is also known to upregulate expression of Utrophin, the homologue of Dystrophin (the protein lost in tissue of Muscular Dystrophy patients), and loss of GABP function is implicated in the causation of some mitochondrial myopathies.

Therefore, if the pathway/s of action of GABP can be identified, methods of regulating GABP expression, and hence the expression of downstream gene targets, could be devised in the instance of disease. The research presented herein aims to better understand the role of the DNA binding subunit of GABP, GABPα, in skeletal muscle development and function using mouse models.

Chapter 3

Materials and Methods

3.1 General Methods

Centrifugation steps were performed using the following: Sigma 1-15 microfuge (Quantum Scientific) for volumes up to 1.5 ml, Heraeus Biofuge stratos for 2-50 ml volumes, and Beckman J2-21M/E centrifuge for volumes greater than 50 ml. A Beckman TL-100 ultracentrifuge was used for CsCl gradient separation, and Spintron GT-175S swing-out rotor centrifuge for tissue culture work. Centrifugal force is denoted as x g, except when the microfuge was used, in which case speed in rpm is included. Incubations at 55°C, 65°C or 37°C were performed in Sanyo convection ovens (Quantum Scientific), unless otherwise stated. Other incubation temperatures were achieved by use of an Xtron dry block heater (Bartelt instruments). Enzymes used for molecular biology were supplied by Promega, tissue culture reagents were supplied by Gibco™ Invitrogen Corporation, and chemicals by BDH, unless otherwise stated. A list of all solutions is included in **Appendix A**, a list of suppliers in **Appendix B** and a list of equipment in **Appendix C**.

3.2 DNA Preparation and Methods

3.2.1 Library Screening

A mouse liver genomic library from 4-6 week old female 129SvJ mice, containing *Sau3A*I partially digested DNA (9-22 kb fragments) in the *Xho*I digested λfixII vector (Stratagene) was used. The XL1-Blue MRA(P2) *Escherichia coli* (Stratagene) host strain was used and λ phage DNA extracted using a plate lysis method, as per manufacturer's instructions, following three rounds of purification.

A SvJ129 BAC library (BACPAC Resources), in pBACe3.6 and pTARBAC2 vectors, was screened as per Southern blot, and the co-ordinates of the background grid used to identify positive clones. Positive clones were obtained and DNA was extracted as for plasmid minipreps.

3.2.2 Restriction Endonuclease Digests

The required amount of DNA was supplemented with 10-20 units (U) of the appropriate restriction endonuclease, together with 1 X buffer (Promega or NEB). The total volume was adjusted to 20-50 µl with MQ H_2O. Digests were left for 3 hours to overnight (O/N), at the optimal temperature (37°C in a waterbath and other temperatures in a dry heat block). Reactions were tested for complete digestion by electrophoresis of 2 µl through a 1-2 % (w/v) agarose (Promega) gel.

3.2.3 Cloning of DNA into Plasmid Vectors

Blunt-Ending Restriction Endonuclease Digest Products

Upon restriction endonuclease digestion, DNA was blunt-ended by filling-in, using Klenow DNA polymerase (Promega), or by cutting back, using T4 DNA polymerase (Promega), as per manufacturer's instructions.

Dephosphorylating DNA Fragments

Prior to ligation, the ends of restriction endonuclease digested vector DNA were dephosphorylated, using Calf intestinal alkaline phosphatase (Promega), as per manufacturer's instructions.

Purification of DNA Fragments

Single band PCR products were purified using the QIAquick columns (Qiagen), as per manufacturer's instructions. Multiple band PCR products and restriction endonuclease digest products were electrophoresed through 1-2 % (w/v) agarose gels, bands excised and purified using QiaexII beads (Qiagen), as per manufacturer's instructions. Alternatively, single-band restriction enzyme digest products were resuspended in at least 100 µl and purified using phenol/chloroform extraction and ethanol/salt precipitation. In all cases, DNA was resuspended in 30-50 µl MQ H_2O and concentration determined by 1 µl was electrophoresed through a 1-2 % (w/v) agarose (Promega) gel using 500 ng λ DNA (Promega) digested with *Hind*III and *Eco*RI as a size standard.

Addition of dATPs to PCR Products

Prior to ligation into the pGEM-T or pGEM-T easy T-overhang cloning vectors, blunt-ended PCR products had dATPs added by incubation at 72°C for 15 minutes, in the presence of 0.2 mM dATP, 2 U *Taq* DNA polymerase (Promega), 1 X DNA polymerase buffer and 1.5 mM $MgCl_2$.

Ligation Reaction

In a total volume of 10 µl, 20-30 ng purified vector DNA and the appropriate amount of purified insert DNA were ligated using 5 U T4 DNA Ligase, in 1 X Rapid ligation buffer (Promega), as per the formula;

insert(ng) = [(insert size(kb) x vector(ng)) / vector size(kb)] x insert:vector ratio.

An insert:vector ratio of 3:1 was usually used. Enough MQ H_2O was added to give a total volume of 10µl. Ligations were incubated at room temperature (R/T), for 4 hours for cohesive-ended reactions, or O/N for blunt-ended reactions.

3.2.4 Transformation of Plasmid DNA into Bacterial Cells

Ligation reactions (5-10 µl) were routinely transformed into high efficiency JM109 competent cells (Promega). In cases of repetitive DNA sequences SoloPack Gold competent cells (Stratagene) were used. Epicurian Coli SCS110 competent cells (Stratagene) were employed when methylation sensitive restriction endonuclease sites were required for subsequent cloning steps. In all cases transformations were performed as per manufacturers' instructions. Transformation reactions were then plated out (100-400 µl) on LB agar plates with appropriate antibiotic selection and grown at 37°C O/N.

3.2.5 PCR Screening of Transformed Colonies

A single colony was used to inoculate both 50 µl TE (pH 8.0) and 100 µl LB (containing appropriate antibiotic). Two µl of the inoculated TE solution was then used in a PCR reaction to screen for ligation of insert into the vector, using vector-specific primers. Concurrently, the LB culture was incubated at 37°C, and the cultures of positive colonies were used to inoculate 2 ml miniprep cultures.

3.2.6 Plasmid DNA Minipreps

Three Solution Method

Two ml LB/antibiotic cultures were inoculated and grown at 240 rpm O/N at 37°C. Bacterial cells were pelleted by centrifugation for two minutes at 14000 rpm. Supernatant was discarded and pellets were resuspended in 100 µl Solution I, then tubes were left on ice for 5 minutes. Solution II (200 µl) was added, tubes were mixed by inversion and left on ice for a further 5 minutes. Solution III (150 µl) was then added, tubes were mixed by inversion and left for a final (5 minute) incubation on ice. Samples were centrifuged for 10 minutes at 14000 rpm to collect debris, and supernatants were removed into fresh tubes.

*RNase*A (100 µg/ml) was added to each tube and samples were incubated at 37°C for 30 minutes, or 65°C for 15 minutes. Samples were phenol/chloroform then chloroform extracted (to remove contaminating phenol) using equal volumes, followed by DNA precipitation of the final upper phase with 0.6 volumes of 100 % (v/v) isopropanol. Following centrifugation at 14000 rpm for 10 minutes, DNA pellets were washed with 50 µl 70 % (v/v) ethanol, dried, then resuspended in 50 µl 0.2 X TE.

Purification of Miniprep DNA for Sequencing

Miniprep DNA was re-precipitated by addition of 12 % (w/v) polyethylene glycol (PEG 6000) and 1.5 M NaCl solution, incubation on ice for one hour, then centrifugation for ten minutes at 14000 rpm. After removal of the supernatant, the DNA pellet was washed with 70 % (v/v) ethanol, dried, and resuspended in MQ H_2O. Sequencing was performed with 0.6 pmol of template-specific primer (or T7 and SP6 primers specific to the pGEM-T cloning vector (Promega), sequences 1 and 2 of **Appendix D**), using 400 ng DNA, as determined by quantifying 1 µl of purified DNA by electrophoresis through a 1 % (w/v) agarose (Promega) gel, using 500 ng λ DNA (Promega) digested with *Hind*III and *EcoR*I as a size standard.

3.2.7 Plasmid DNA Maxipreps

Three Solutions Method

A 250 ml culture was inoculated and grown at 240 rpm O/N at 37°C. The culture was centrifuged for 10 minutes at 3000 x g to pellet cells. The pellet was thoroughly resuspended in 10 ml of Solution I by vortexing. After incubation on ice for 5 minutes, 20 ml of Solution II was added, and the sample was mixed by inversion. Following a 5 minute incubation on ice, 15 ml of Solution III was added, and the sample was again mixed by inversion and incubated on ice for 5 minutes. After centrifugation at 3500 x g for 10 minutes, the cleared supernatant was strained through 8-ply gauze (Smith + Nephew). *RNase*A (50 µg/ml) was added and the mixture was incubated at 37°C for 30-60 minutes, or 65°C for 15 minutes. Phenol/chloroform was used to extract DNA twice (centrifuged at 3500 x g for 5 minutes), and the DNA in the final upper phase was precipitated by addition of 0.6 volumes of 100 % (v/v) isopropanol. DNA was collected by centrifugation at 3500 x g for 15 minutes, washed with 70 % (v/v) ethanol, pelleted by centrifugation for 5 minutes at 3500 x g, and air-dried. DNA was then resuspended in 1.2 ml of 0.2 X TE.

CsCl Method

A single bacterial colony was used to inoculate 225 ml Terrific Broth (TB) supplemented with 25ml TB salts and appropriate antibiotic, and the culture was grown at 240 rpm O/N at 37°C. The overnight culture was centrifuged at 4500 x g for 10 minutes at 4°C. The pellet was resuspended in 18 ml Solution I by vortexing. Two ml Solution I containing 100 mg lysozyme was then added, the solution was vortexed and left at R/T for 8 minutes. Forty ml freshly made Solution II was added and the mixture was left at R/T for 10 minutes, following inversion. Twenty ml Solution III was then added, the combined solution was mixed by inversion, and left on ice for 20 minutes. Following centrifugation at 10000 x g for 15 minutes at 4°C, the supernatant was strained through 8-ply gauze (Smith + Nephew), and 0.6 volume of 100 % (v/v) isopropanol was added. DNA was then pelleted by centrifugation at 3500 x g for 25 minutes at 4°C. After removal of the supernatant, DNA was washed with 70 % (v/v) ethanol and the pellet was dried at R/T, before resuspension in 5 ml TE (pH 8.0). To the DNA, 5 g CsCl was added, and mechanical inversion was used to dissolve this prior to addition of 300 µg ethidium bromide. The CsCl mixture was loaded into 2.5 ml heat-seal tubes (Beckman Coulter) using a needle and syringe, tubes were heat-sealed and subjected to ultracentrifugation at 540960 x g O/N.

Following ultracentrifugation, the top of the heat-sealed tube was pierced with a needle to release air, and the middle band of ethidium-stained nucleic acid was withdrawn with a syringe into a sterile 15 ml tube (the remaining upper band being chromosomal DNA and the lower band RNA). Ten extractions with water-saturated butanol (of equal volume) were performed, and 3 volumes of MQ H_2O were added. DNA was then precipitated by addition of an equal volume of 100 % (v/v) isopropanol. This mixture was then centrifuged at 3500 x g for 10 minutes to pellet the DNA. The supernatant was removed and DNA resuspended in 1 ml MQ H_2O. A second round of precipitation was performed by addition of 0.1 volume 7.5 M ammonium acetate and 2.5 volumes 100 % (v/v) ethanol and centrifugation at 3500 x g for 10 minutes. The supernatant was removed, and the DNA washed in 70 % (v/v) ethanol, prior to resuspension in 1 ml TE (pH 8.0).

Endotoxin-Free Method

In order to ensure the absence of any bacterial endotoxins in the plasmid DNA preparation prior to transfection of cells, the EndoFree® Plasmid Maxi Kit (Qiagen) was used, as per manufacturer's instructions.

3.2.8 Preparation of DNA for Pronuclear Injection

Removal of Vector Backbone

The vector backbone of the construct was removed by restriction endonuclease digestion and subsequent QiaexII (Qiagen) gel purification and resuspension in TE buffer (pH 8.0). The pure DNA fragment was then dialysed against TE buffer (pH 8.0) for two hours at R/T. Purified DNA (1 µl) was quantified by electrophoresis through a 1 % (w/v) agarose (Promega) gel, using 500 ng λ DNA (Promega) digested with *Hind*III and *Eco*RI as a size standard.

Linearisation of Vector Construct

The vector was linearised by restriction endonuclease digestion and DNA was purified using a QIAquick (Qiagen) purification column, as per manufacturer's instructions, and resuspended in either TE buffer (pH 8.0) or MQ H_2O. The amount of DNA was quantified by electrophoresis of 1 µl of purified DNA through a 1 % (w/v) agarose (Promega) gel, using 500 ng λ DNA (Promega) digested with *Hind*III and *Eco*RI as a size standard.

3.2.9 Mouse Genomic DNA Preparation

Ear Clip Lysis Method

One hundred µl ear clip lysis buffer containing 10 µg/ml Proteinase K (Roche) was added to tissue from mouse ear clippings, and the mixture was incubated at 55°C for 1 hour. The proteinase K was then inactivated by incubation at 95°C for 10 minutes. Ten µl of this solution was used in a 50 µl PCR reaction to genotype mice.

Mouse Tail Phenol/Chloroform Method

A ~0.5 cm segment of mouse tail was incubated O/N at 55°C in 500 µl tail buffer containing 0.5 mg/ml Proteinase K. The following day, 20 µg/ml *RNase*A was added and the mixture was incubated at 37°C for 1 hour. DNA was extracted twice using an equal volume of phenol/chloroform, and once using chloroform, prior to the addition of 1 ml 100 % (v/v) ethanol to the final upper phase to precipitate the DNA. The melted end of a glass Pasteur pipette was used to spool out genomic DNA, which was then washed in 500 µl 70 % (v/v) ethanol, and left to dry at R/T for 2 minutes. DNA was resuspended in 200 µl 0.2 X TE buffer by incubation at 55°C O/N.

DNA Extraction from Cells

Cells at 80-100 % confluence were washed in PBS and lysed by incubation in cell lysis buffer at 55°C for 4 four hours. DNA was precipitated by addition of 2 volumes 100 % (v/v) isopropanol, and genomic DNA was spooled out using a heat-sealed glass Pasteur pipette, rinsed in 80 % ethanol (v/v), air-dried and resuspended in 0.2 X TE at 55°C O/N.

3.2.10 Thermal Block Cycler PCR

Approximately 250 ng of genomic DNA or 20 ng of plasmid DNA was used as template for PCR. DNA was denatured at 94°C for five minutes, followed by 35-40 cycles of; 94°C for 1 minute, 1 minute at the determined annealing temperature (T_A), and 68°C/72°C for the determined extension time (ET). A final extension at 68°C/72°C for 5 minutes was also performed. $T_A = T_M$ (primer melting temperature) – 5°C and ET = 1 minute per kb of product. PCR reactions were performed in Applied Biosystems Gene Amp PCR System 2400 or 9600 block cyclers.

Taq DNA polymerase (Promega) was used (1.25 U/reaction) for all standard PCRs, however *Elongase* DNA polymerase mix (Invitrogen) (1 U/reaction) was used when genomic fragments >2 kb were being amplified. Blunt-ended or high fidelity PCR products were amplified using *Pfu* DNA polymerase (Promega) (1.5 U/reaction). In all cases 10 pmoles of each oligonucleotide primer and 2 mM Mg^{2+} were used (unless otherwise stated). Where specified, product amplification could only be achieved by addition of 1 M N,N,N-trimethylglycine (Betaine) (Sigma) or 0.3 mg/ml acetylated bovine serum albumin (BSA) (Promega). PCRs were performed as 50 µl reactions, and results assessed by electrophoresis of 10 µl through 1.0-2.0 % (w/v) agarose (Promega) gels.

3.2.11 DNA Electrophoresis

Agarose/TAE Gel Electrophoresis

Agarose (Promega) gels of 0.8-2.0 % (w/v) were made by dissolving agarose (Promega) in 1 X TAE buffer, followed by addition of one drop of 1 mg/ml ethidium bromide (Promega) to visualise DNA. Gels were electrophoresed (BioRad Mini-Sub or Pharmacia Biotech GNA 200 gel tanks) in 1 X TAE buffer at 50-100 Volts (V) (BioRad Power Pac 300 or 3000, or Pharmacia GPS 200/400) for 1-5 hours, depending upon the resolution of DNA fragments required. To determine size and quantity of DNA fragments, 500 ng of a DNA marker was included on each gel; 500 ng λ bacteriophage DNA

(Promega) digested with *Hind*III and *Eco*RI, or 100 bp, 1 kb or 2 log ladders (NEB). Gel photographs were taken under ultraviolet (UV) light using a BioRad GelDoc 1000.

3.2.12 DNA Transfer

Southern Blot

Restriction endonuclease digested genomic DNA, or PCR products, were electrophoresed at 80 V for 2-5 hours through 0.8-1.0 % (w/v) agarose gels. NaOH (0.4 M) was used to transfer DNA to Gene Screen Plus nylon membrane (NEN) by capillary action, according to manufacturer's instructions. After O/N transfer, the membrane was soaked in 2 X SSC for 2 minutes prior to drying O/N at R/T or at 55°C for 1 hour.

Colony Lifts

LB plates of transformed colonies were stored at 4°C prior to use. Hybond-XL circular nylon membranes (Amersham Pharmacia Biotech) were marked for orientation and placed gently over the colonies until membranes were completely wet. Membranes were removed and DNA fixed by autoclaving for 1 minute at 100°C at 350 kPa.

3.2.13 DNA Hybridisation

Pre-Hybridisation

Membranes were pre-hybridised for at least 1 hour prior to radioactive probe addition. A solution of 10 ml dextran sulphate hybridisation mix (for double stranded (ds) DNA probes >100 bp) or 10 ml oligonucleotide hybridisation mix (for oligonucleotide probes of 20-30 bp), containing 0.1 mg/ml denatured (boiled) single-stranded herring sperm DNA (Roche), was used. Incubation of membranes was performed at 65°C for ds DNA probes, or 42°C for oligonucleotide probes, in an Xtron HI 2002 hybridisation oven (Bartelt instruments).

Hybridisation with Random Labelled dsDNA Probes

DNA (25-50 ng) was labelled with 0.5 mCi α ^{32}P-dCTP (Amersham Pharmacia Biotech), using the dry Rediprime labelling kit reagents (Invitrogen) or wet Random Primed DNA Labelling Kit (Boehringer-Mannheim), as per manufacturer's instructions. A Poly-prep chromatography column (BioRad) containing G50 sephadex (Amersham Pharmacia Biotech) was used to rid of any unincorporated α ^{32}P-dCTP. The probe was boiled to denature the ds DNA, and the volume required to achieve $\geq 1 \times 10^7$ counts/ml (determined in a Packard 1900 TR Tri-Carb liquid scintillation analyser) was used to probe

a pre-hybridised membrane O/N at 65°C in an Xtron HI 2002 hybridisation oven (Bartelt instruments).

Hybridisation with End Labelled Oligonucleotide Probes

A 20-30 bp oligonucleotide (10 pmoles) was labelled with 0.2 mCi γ^{32}P-dATP (Amersham Pharmacia Biotech), by incubation with 5 U T4 polynucleotide kinase in 1 X kinase buffer (Promega), for 30 minutes at 37°C waterbath (Ratek instruments). The entire reaction mix was then added to a pre-hybridised membrane and incubated at 42°C O/N in an Xtron HI 2002 hybridisation oven (Bartelt instruments).

Image Development

Following O/N hybridisation, membranes probed with dsDNA were washed at 65°C for 10-15 minutes; once in 2 X SSC/0.1 % SDS (w/v), and twice in 0.1 X SSC/0.1 % SDS (w/v). Membranes probed with an end labelled oligonucleotide were washed sequentially at 42°C for 10-15 minutes once in each of; 2 X SSC/0.1 % SDS (w/v), 1 X SSC/0.1 % SDS (w/v), and 0.1 X SSC/0.1 % SDS (w/v). Results were imaged using a FujiFilm FLA-2000 Phosphorimager with Image Reader FLA-2000 v1.21 and Image Gauge v.3.46 software, or by exposure to Kodak BioMax MR or Amersham Pharmacia Biotech MP or ECL hyperfilm at –80°C (with enhancing screen) for 1-7 days. Film was developed in a Kodak X-OMAT 480 RA processor.

3.2.14 DNA Sequencing and Analysis

Sample Preparation

Gel-purified PCR products (5 ng per 100 bp) or purified plasmid miniprep DNA (400 ng) was sequenced using ABI PRISM BigDye Terminator chemistry (v.3.4.1), and analysed on an ABI 377 DNA sequencer (PE Applied Biosystems), a service provided by Vivien Vasic, Wellcome Trust DNA Sequencing Facility, Monash Medical Centre, Victoria, Australia.

DNA Sequence Analysis

DNA sequence text files were analysed with the Sequencher v.3 sequence alignment program and compared to Genbank database sequence entries using Basic Local Alignment Search Tool (BLAST) analysis (Altschul et al. 1990). Alignment of mouse, rat and human *GABPα* sequences was performed using AVID software (Dubchak et al. 2000b; Bray et al. 2003), using a window size of 100 bp and a conservation level of 50 %, and results were viewed with the Visual Tools for Alignment (VISTA) program (Dubchak

et al. 2000a). Mouse genomic *Gabpα* sequence was analysed via the Nucleotide Identify X (NIX) suite of programs (Williams et al. 1998) for the presence of CpG islands and predicted exons and promoter regions. Transcription factor binding sites were predicted using the University of Pennsylvania Transcription Element Search System (TESS) (Schug and Overton 1997), Baylor College of Medicine (BCM) Search Launcher program (Smith et al. 1996b; Smith et al. 1996a) as well as the MatInspector (Quandt et al. 1995a; Quandt et al. 1995b) and rVISTA programs (Dubchak et al. 2000a; Loots et al. 2002). Secondary structures of transcripts were predicted using mfold (Zuker 1999; Zuker 2003).

3.3 RNA Preparation and Methods

3.3.1 Poly-A+ (mRNA) Preparation

Preparation of Oligo-dT Cellulose

One gram of oligo-dT cellulose (NEB) was used, to which 20 ml 0.4 M NaOH was added. The tube was mixed and left for five minutes, then centrifuged at 150 x g for 5 minutes. The supernatant was removed and 20 ml 1 M Tris-Cl (pH 7.5) was added. The tube was then mixed and left for 5 minutes before centrifugation for 5 minutes at 150 x g. This washing process was repeated twice with 20 ml binding buffer, and this suspension was stored at 4°C (in the dark) until use.

RNA Preparation

Liquid nitrogen frozen cells/tissue was homogenised in 25 ml extraction buffer (containing 10 mg Proteinase K), at 20,500 $^{min\text{-}1}$ for 2 minutes, using a Labortechnik Ultra-Turrax T25 homogeniser (Janke & Kinkel IKA®). This mixture was incubated in a 55°C waterbath (Ratek instruments) for 30 minutes, cooled to R/T, then 2.5 ml of 5 M NaCl was added. To this, 2.5 ml of resuspended oligo-dT beads (equivalent to 0.125 g) were added, and the tube was incubated for 1-2 hours at R/T on a R.S.M.6 rotating wheel (Ratek instruments) or Xtron SH-2004 tilting platform (Bartelt instruments). Following centrifugation at 300 x g for 5 minutes, beads were washed twice with 8 ml binding buffer, and once with 8 ml wash buffer. Poly-A+ RNA was eluted from the beads in 2 ml elution buffer, by incubation in a 60°C water bath (Ratek instruments) for 5 minutes, followed by centrifugation at 300 x g for 2 minutes. This elution process was repeated with 1.5 ml elution buffer, and the total 3.5 ml of eluted RNA was extracted with an equal volume of buffered phenol (Sigma) and centrifugation at 3500 x g for 5 minutes. This was followed

by one chloroform extraction, and RNA was precipitated by addition of 2.5 volumes 100 % (v/v) ethanol and 0.1 volume 3 M sodium acetate (pH 5.2) (Sigma) and incubation of the sample at –20°C O/N. RNA was pelleted by centrifugation at 10000 x g for 1 hour at 4°C and almost all the supernatant was removed. The final ~1 ml of solution was used to resuspend the pellet, which was then transferred to a 1.5 ml microfuge tube. Five hundred µl 100 % (v/v) ethanol was used to remove residual RNA and was also added to the tube. RNA was re-pelleted by centrifugation at 14000 rpm for 10 minutes at 4°C. After drying the pellet, RNA was resuspended in 200 µl DEPC-treated TE (pH 8.0) containing 40 U *RNasin*® Ribonuclease Inhibitor (Promega). Ten µl of this solution was quantified in a Lambda Bio20 UV/Vis Spectrometer (Perkin Elmer), where 1 OD_{260nm} = 40 µg/ml RNA. To the remaining 190 µl of RNA 2.5 volumes 100 % (v/v) ethanol and 0.1 volume 3 M sodium acetate (pH 5.2) were added and this mixture was stored at –80°C.

3.3.2 Total RNA Preparation (AGPC Method)

The rapid AGPC (acid guanidinium thiocyanate-phenol/chloroform) method was used (Chomczynski and Sacchi 1987) for total RNA preparation. Specifically, 500 µl of solution D was added to 0.1-0.2 g of frozen tissue/cells. This mixture was homogenised for ~30 seconds at 20,500 $^{min-1}$ using a Labortechnik Ultra-Turrax T25 homogeniser (Janke & Kinkel IKA®). To this, 50 µl 2 M sodium acetate (pH 4.0) was added, and the sample was mixed by inversion. An equal volume of unbuffered phenol (Sigma) was then added, and the sample was mixed. To, this, an equal volume of chloroform/isoamylalcohol (49:1) was added, and the sample was mixed by inversion, prior to incubation on ice for 15 minutes and centrifugation at 14000 rpm for 15 minutes at 4°C. RNA in the upper phase was precipitated by addition of 0.6 volumes 100 % (v/v) isopropanol and incubation of the sample at –80°C for at least 30 minutes. RNA was pelleted by centrifugation at 14000 rpm for 15 minutes at 4°C, washed in 70 % (v/v) ethanol, air dried, and resuspended in 25 µl DEPC-treated MQ H_2O. RNA was quantified in a Lambda Bio20 UV/Vis Spectrometer (Perkin Elmer), where 1 OD_{260nm} = 40 µg/ml RNA.

3.3.3 *DNase*I Treatment of RNA

Prior to conversion to cDNA, 10 µg total RNA was incubated with 10 U RQ1 *RNase*-Free *DNase*I and 80 U *RNasin*® Ribonuclease Inhibitor (Promega) at 37°C for 30 minutes. The reaction was stopped by addition of 3 mM EDTA and heating the sample at 65°C for 20 minutes.

3.3.4 Reverse Transcription of RNA

If total RNA was being used to prepare first strand cDNA, 5-15 µl aliquots (10 µg) of resuspended RNA were used for reactions with and without reverse transcriptase (RT). If polyA+ RNA was being used, 500 ng aliquots were used. PolyA+ RNA was precipitated by addition of 2 µl of 10 mg/ml tRNA (Sigma) to the ethanol slurry, and centrifugation at 14000 rpm for 20 minutes at 4°C. Pellets were washed in 70 % (v/v) ethanol and air-dried.

First strand cDNA synthesis was performed with Avian Myeloblastosis Virus (AMV) reverse transcriptase (Promega) in most cases, using oligo (dT) primer, as per manufacturer's instructions. For synthesis of high fidelity cDNA for RACE RT-PCR analysis, first strand cDNA synthesis was performed with SuperScript II RNase H reverse transcriptase (Invitrogen), using oligo (dT) primer, as per manufacturer's instructions.

3.3.5 RACE RT-PCR

RACE RT-PCR was performed on a mouse E14 placenta cDNA library (F1: C57Bl6 x CBA) using the Marathon™ RACE kit (Clontech), as per manufacturer's instructions.

3.3.6 Real-Time RT-PCR PCR

The program for real-time RT-PCR reactions was run for 45-55 cycles, using a Light Cycler (Roche) (v3.5 software). An appropriate dilution (neat or 1 in 5) of first strand cDNA was used as template, as determined by optimisation for each primer pair. Fast Start SYBR Green Master Mix (Roche) was used, with 10 pmoles of each primer, in 10 µl reactions. An extension time (seconds) of 1/25 product length (bp) was used and an optimal $MgCl_2$ concentration (2-4 mM) was determined for each primer pair. The entire reaction was electrophoresed through a 1 % (w/v) agarose gel to check for correct PCR product size. To create a standard curve, five 10-fold dilutions of plasmid DNA were used as template (10^{-1}- 10^{-9} ng/µl, depending upon the primers).

3.3.7 RNA Electrophoresis

Agarose/MOPS Gel Electrophoresis

Three µg of poly-A+ RNA was precipitated by addition of 2 µl 10 mg/ml tRNA (Sigma) carrier to the ethanol slurry, and centrifugation at 4°C for 15 minutes at 14000 rpm. The supernatant was removed and pellet dried at 37°C, before being resuspended thoroughly in 10 µl RNA resuspension buffer. The RNA was heated at 65°C for 5 minutes

then placed on ice and 2 µl RNA loading dye was added. RNA was electrophoresed through denaturing 1 % agarose (Promega) gels, containing 0.72 M formaldehyde, 1 X 4-morpholine-propane sulfonic acid (MOPS) (Roche) and 1 µg/ml ethidium bromide (Promega). Electrophoresis was performed in BioRad Mini-Sub gel tanks, containing 1 X MOPS buffer, for 4-5 hours at 70-80 V (using a BioRad Power Pac 300 or 3000). RNA millennium size markers (Ambion) were included on each gel, and photographs were taken under UV light in a BioRad GelDoc 1000.

3.3.8 RNA Transfer

RNA was transferred O/N by capillary action onto Hybond-C extra nylon membrane (Amersham Pharmacia Biotech), according to the manufacturer's instructions, using 20 X SSC as the transfer buffer. Following transfer, the membrane was rinsed in 2 X SSC and dried at R/T, prior to baking at 80°C in a Dynavac V015/200 500 W vacuum oven at –100 kPa for 2 hours.

3.3.9 RNA Hybridisation

Pre-Hybridisation

Membranes were pre-hybridised for at least 1 hour prior to radioactive probe addition. A solution of 10 ml northern pre-hybridisation mix, containing 0.1 mg/ml denatured (boiled) single-stranded herring sperm DNA (Roche), was used. Incubation of membranes was performed at 42°C, in an Xtron HI 2002 hybridisation oven (Bartelt instruments).

Hybridisation with Random Labelled dsDNA Probes

cDNA (25-50 ng) was labelled with 0.5 mCi α ^{32}P-dCTP (Amersham Pharmacia Biotech), as described for DNA hybridisation. The probe was boiled to denature the ds DNA, and the volume required to achieve ≥1 x 10^7 counts/ml (as determined in a Packard 1900 TR Tri-Carb liquid scintillation analyser) was used to probe a pre-hybridised membrane O/N at 42°C in an Xtron HI 2002 hybridisation oven (Bartelt instruments).

Image Development

Following O/N hybridisation, membranes were washed at 65°C for 10-15 minutes in 2 X SSC/0.1 % SDS (w/v) (once), followed by 0.1 X SSC/0.1 % SDS (w/v) (twice). Results were imaged as described for DNA hybridisation.

3.4 Protein Preparation and Methods

3.4.1 Protein Extraction

Protein Extraction from Mouse Tissue

After dissection, tissue was homogenised in 10 mM Tris, 0.1 M EDTA (pH 7.4) supplemented with one Complete Mini EDTA-Free Protease Inhibitor Cocktail Tablet (Roche) per 10 ml (1 µl per mg of tissue), using a plastic pestle. An equal volume of 2 X EDTA sample buffer was added, and the protein extract was boiled for 5-10 minutes. Residual tissue was pelleted by centrifugation at 14000 rpm for 15 minutes at 4°C, and the supernatant containing cytoplasmic and nuclear proteins was stored at –20°C.

Protein Extraction from Cell Lines

Cells from one 80-100 % confluent 175 cm^2 flask were scraped off with a rubber policeman in 1 ml 2 X EDTA sample buffer. This mixture was boiled for 5-10 minutes and passed through a 21-gauge needle (Becton Dickinson) several times, to break down any remaining genomic DNA present. Protein extracts were stored at –20°C.

Bradford Assay

Aliquots of tissue/cell extracts in 10 mM Tris, 0.1 M EDTA (pH 7.4) or phosphate buffered saline (PBS) were serially diluted in PBS, 0.2 volume of Protein Assay Reagent (BioRad) was added, and samples were mixed by pipetting. Known concentrations (0.5-100 µg/ml) of BSA (fraction V) (Sigma) were used as standards, and the coloured reaction was left to develop for 30 minutes. Results were read by absorbance at 595 nm using a BioRad 3550 microplate reader.

Coomassie Spot Test

To confirm results of Bradford Assays, samples were spotted onto a sheet of 135 gsm blotting paper and air-dried. The paper was then dipped into a solution of 0.25 % (w/v) Coomassie brilliant blue in 10 % methanol (v/v), 7 % glacial acetic acid (v/v) for 30 seconds, and washed in 10 % methanol (v/v), 7 % glacial acetic acid (v/v) for 5 minutes. Blotting paper was air-dried and samples quantified by visual comparison with BSA (fraction V) (Sigma) standards.

Coomassie Brilliant Blue Staining

Following SDS-PAGE, gels were soaked in Coomassie blue staining solution for one hour at R/T on a tilting platform (Burtelt instruments). The gels were then washed in

destaining solution O/N at R/T while shaking. Gels were soaked in a mixture of 10 % (v/v) glacial acetic acid, 1 % (v/v) glycerol for at least 2 hours, prior to being dried O/N between two sheets of damp cellophane.

3.4.2 Sodium Dodecyl- Polyacrylamide Gel Electrophoresis

A lower gel mixture (reducing) containing 8-15 % 29:1 Bis/Acrylamide (BioRad), 375 mM Tris-HCl (pH 8.8), 0.1 % (w/v) SDS, 0.1 % (w/v) Ammonium persulphate (APS) (BioRad) and 0.04 % (v/v) TEMED (BioRad) was poured into a Hoefer gel stacker apparatus (Amersham Pharmacia Biotech) and left to polymerise with a layer of 100 % (v/v) butanol covering the surface. Once set, the alcohol layer was removed and upper surface rinsed thoroughly with distilled water. A stacking gel mixture (reducing) containing 5 % 29:1 Bis/Acrylamide (BioRad), 62.5 mM Tris-HCl (pH 6.8), 0.1 % (w/v) SDS, 0.1 % (w/v) APS (BioRad), 0.1 % TEMED (BioRad) was poured onto the lower gels, combs were inserted and gels were left until polymerised (5-10 minutes). Protein samples (10-40 µg) in 2 X EDTA sample buffer were electrophoresed through the polyacrylamide gels in Hoefer AB Mighty Small II tanks (Amersham Pharmacia Biotech), with 1 X Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) running buffer. Samples were electrophoresed at 60 V until they entered the lower phase, then at 80 V until the dye front reached the base of the gel. The Bench Mark Pre-stained Protein Ladder (Invitrogen) was included on each gel as a size standard.

3.4.3 Protein Transfer

Protein gels were transferred onto Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore) after pre-treating the membrane by soaking in 100 % (v/v) methanol for 15 seconds, MQ H_2O for 2 minutes and then anode buffer II for 5 minutes. A Hoefer Semi Phor transfer apparatus (Amersham Pharmacia Biotech) was used, as per manufacturer's instructions. Protein was fixed onto membranes by treatment in 100 % (v/v) methanol for 10 seconds, and membranes were air-dried prior to immunodetection.

3.4.4 Immunodetection of Proteins

Western Blot Immunodetection

Membranes were blocked for at least 2 hours at R/T on a shaking platform (Bartelt instruments) in a solution of 10 % (w/v) semi-skimmed milk protein in 1 X TBST. Membranes were then incubated (with shaking) O/N at 4°C in a solution of 5 % (w/v) semi-skimmed milk protein in 1 X TBST containing the primary antibody (concentrations

detailed in relevant results chapters). Membranes were washed for 45 minutes in 1 X TBST at R/T, changing the solution every 15 minutes, before incubation with the secondary antibody (concentrations detailed in results chapters) in a solution of 5 % (w/v) semi-skimmed milk protein in 1 X TBST for 1 hour at R/T. Membranes were again washed in 1 X TBST, as following primary antibody. Protein bands were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce), and exposure to film (BioMax MR (Kodak) or Hyperfilm ECL (Amersham Pharmacia Biotech)) for 2 seconds to 10 minutes. Film was developed in a Kodak X-OMAT 480 RA processor.

Exposed film was scanned at 100 dots per inch (dpi) using a Canon N1220U, and the intensity of individual bands was determined using FujiFilm MacBAS v2.5 software. Protein levels were quantified as an average ratio to β-tubulin for each sample.

Enzyme-Linked Immunosorbant Assay (ELISA)

Wells of a 96-well immunosorp plate (NUNC) were coated with 1μg recombinant protein, in carbonate coating buffer, and stored at 4°C O/N. Wells were washed with PBS/0.05 % Tween 20 (v/v) three times for 5 minutes each, and blocked with PBS/1 % BSA (w/v) for 1-2 hours at R/T. Dilutions of the relevant antibody were added to each well and incubated for 1-2 hours at R/T. The wells were washed as before, prior to incubation with horse radish peroxidase (HRP)-conjugated secondary antibody (DAKO) in PBS/1 % BSA (w/v) for 30 minutes at R/T. Again the wells were washed, ABST solution containing 2,2'-Azino-di-(3-Ethylbenzthiazoline Sulfonic Acid) was then added to each well for 10 minutes, before stopping the colour reaction by addition of 2 % SDS (w/v). Results were read as absorbance at 405 nm using a microplate reader (BioRad).

3.4.5 Recombinant His-Tag Protein Production

Recombinant protein was expressed from the pQE-31 N-terminal His-tag expression vector (Qiagen) using 0.4 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) induction and purification with TALON nickel resin (Clontech), as per manufacturer's instructions.

3.4.6 Polyclonal Antibody Production

Recombinant protein was used to immunise two rabbits. On day 1, 15 ml pre-immune bleed samples were taken, and 0.8 μg of recombinant protein in Freund's complete adjuvant was used to immunise each rabbit. On days 21, 49 and 77 booster injections of 0.8 μg protein in Freund's incomplete adjuvant were administered, before the rabbits were terminally bled. Sera were stored at –80°C.

3.5 Tissue Culture

3.5.1 General Tissue Culture Methods

Thawing Cells

Frozen cells were removed from liquid nitrogen storage and warmed at 37°C until almost thawed. Pre-warmed growth media was then added and cells were centrifuged at 150 x g for 5 minutes to rid of residual freezing media. Cells were plated out in an appropriate volume of growth media and incubated at 37°C with 5 % CO_2.

Visualisation of Cells

Growth of cells was monitored by visualisation with a Leica DM IRB light microscope. This allowed for an estimation of cell density, cell death and presence of any bacterial or fungal contamination.

Trypan Blue Staining

Cells were diluted 1:5 in 0.4 % (w/v) Trypan blue (Sigma) in PBS, and incubated for 10 minutes at R/T. The number of non-stained viable cells was then counted using a Precicolor haemocytometer (HBG Germany), while Trypan blue staining indicated non-viable cells.

Gelatin Coating of Plates

When growing ES cells, C2C12 mouse myoblast cell line or primary myoblasts, plastic ware was pre-coated in 0.2 % (w/v) gelatin in PBS for 30 minutes at R/T, as gelatin is known to promote adhesion of cells to plastic ware, in particular myoblasts. Excess liquid was removed prior to addition of cell cultures.

Poly-L-lysine Coating of Plates

When growing the neuronal-like PC12 rat adrenal pheochromocytoma cell line, plastic ware was pre-coated in 5 μg/ml poly-L-lysine (Sigma) in PBS for 1 hour at R/T, as poly-L-lysine is known to promote adhesion of certain cell types, in particular neurons. Excess liquid was removed prior to addition of cell cultures.

3.5.2 Culturing of Cell Lines

Maintenance and Freezing

Specific growth media for each cell line used is detailed in **Appendix A.** All cells were maintained at 37°C/5 % CO_2 and passaged when 80 % confluent. Cells were trypsinised by the addition of 0.25 % Trypsin-EDTA (v/v), incubation at 37°C/5 % CO_2 for

5 minutes, and subsequent dilution (up to 1 in 12) in fresh growth media. Cell lines were frozen by trypsinising an 80 % confluent 175cm^2 flask of cells, pelleting cells by centrifugation at 150 x g for 2 minutes, washing in growth media, and resuspension in 1 ml freezing media. Cells were stored for up to 1 month at –80°C, prior to transfer into liquid nitrogen.

Stable Transfection of NIH3T3 Cells

Cells at 80 % confluence were trypsinised, pelleted by centrifugation at 150 x g for 2 minutes, and washed with RPMI containing 10 mM HEPES to rid cells of FCS. Cells were counted using a haemocytometer, and 2 x 10^6 cells were electroporated with 10 µg of linearised DNA in a 0.5 cm glass cuvette (BioRad), at 350 V/960 µF for 10 seconds. Cells were then grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% FCS (v/v), at 37°C/5 % CO_2 for 2 days. After recovery, 3 µg/ml puromycin selection antibiotic was added to the media for several days, until the no DNA electroporation control cells had died. Surviving clones were picked by dislodgement under a dissection microscope and propagated.

Transient Transfection of Cell Lines

The day before transfection, cells were plated out in antibiotic-free media at a density of 2.5-5 x 10^4 cells/well in a 24-well plate or 1-2.5 x 10^5 cells/well in a 6-well plate. For immunohistochemical analysis, coverslips pre-coated with gelatin or poly-L-lysine were added to 6-well plates. Cells were transfected with DNA using Fugene 6 (Roche), as per manufacturer's instructions, the optimised Fugene 6 (µl):DNA (µg) ratio. Media was changed 24 hours post-transfection (supplemented with FCS and antibiotics), and after 48 hours, cells were harvested.

Luciferase and β-Galactosidase Reporter Assays

Cells co-transfected with *Luciferase* and *β-galactosidase* reporter vectors were harvested 48 hours post-transfection, and Luciferase reporter activity was tested using a Luciferase Reporter Gene Assay with constant light signal (Roche), as per manufacturer's instructions. Cell lysates were added to a Costar® 96-well opaque white plate, and relative luciferase activity was measured immediately in a Packard Lumi Count™ luminometer, using a read length of 7 seconds and a gain of 1. One fifth of each cell lysate was added to an equal volume of 2 X β-galactosidase buffer in a 96-well plate and reactions were

incubated at 37°C O/N in order to determine transfection efficiency. β-galactosidase activity was measured by absorbance at 415 nm in a BioRad 3550 microplate reader.

MTT Growth Assay

NIH3T3 cells were plated out in DMEM growth media on replicate 96-well plates (2 x 10^3 - 2 x 10^4 cells/well). Media alone was used as a control. Cells in each well were treated with 0.4 mg/ml 3-(4,5.dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma), incubated at 37°C/5 % CO_2 for 3 hours, then stored at –20°C. This process was repeated on Days 0, 1, 2 and 3 after cells were plated, using replicate plates. On Day 3, all cells were thawed, media was removed, and 150 µl DMSO was added to each well. Following incubation at 37°C/5 % CO_2 for 30 minutes results were read at 595 nm absorbance, using a BioRad 3550 microplate reader.

Oxygen Consumption Assay

NIH3T3 cells were trypsinised, pelleted and resuspended at a concentration of 1 x 10^6 in 20 µl immediately prior to use. A Clark electrode in a water-jacketed chamber, connected to a circulating waterbath (Hofhaus et al. 1996), was used to measure oxygen consumption of cells. The electrode was calibrated with oxygenated water (maximum reading) and addition of several milligrams of sodium dithionite (minimum reading). Media alone was measured prior to each sample addition, as a negative control, and cell samples were added into the 37°C media chamber using a syringe. Readings were recorded as nmol O_2 per second, before and after the addition of 1 mM carbonyl cyanide m-chloro phenyl hydrazone (CCCP) (Sigma), an uncoupling agent of the respiratory chain and phosphorylation system of intact mitochondria.

Cell Synchronisation

Mitotic Shake Off

NIH3T3 cells were seeded at a density of 1 x 10^6 per 175 cm^2 flask in DMEM. Cells were serum starved with 0.5 % FCS (v/v) for 12 hours, prior to the addition of 20 % FCS (v/v) for 14 hours. At this point 0.4 µg/ml nocodozole was added to the media for 6 hours, arresting cells in G_2/M phase (characterised by detachment of cells). After removal of the nocodozole-treated cells by tapping flasks, T=0 hour (late G_2/M) and T=3 hours (early G_0/G_1) timepoint samples were taken; 1 x 10^6 cells were used for fluorescence activated cell sorter (FACS) analysis and 1 x 10^6 cells for protein analysis.

Double Thymidine Block

NIH3T3 cells were seeded at 1 x 10^6 per 175 cm^2 flask in DMEM. Initially, 2 mM thymidine was added to the media for 14 hours to arrest cells at the G_1/S boundary and in S-phase. Cells were released into S-phase by addition of 20 % FCS (v/v) and removal of thymidine for 9 hours, before a second thymidine block of 14 hours allowed the exit of cells from S-phase and entry into the G_1/S boundary. At T=0 hour (late G_0/G_1) and T=3 hours (S-phase), 1 x 10^6 cells were harvested for FACS analysis and protein extraction.

FACS Sample Preparation

Propidium Iodide Staining

Cells were pelleted by centrifugation at 150 x g for 2 minutes, and fixed by resuspension at a density of 1 x 10^6 cells in 0.5 ml by 90 % (v/v) ethanol in PBS. Cells were stored at –20°C for at least 16 hours prior to staining. To stain cells with propidium iodide (PI), cells were centrifuged at 150 x g for 15 minutes at R/T and resuspended in 40 µg/ml PI containing 60 µg/ml *Rnase*A in PBS. To allow for adequate penetration of the dye into DNA, cells were stored at 4°C (in the dark) for at least 2 hours prior to analysis with an EPICS 752 flow cytometer (service provided by Paul Hutchison, Department of Medicine, Monash Medical Centre, Victoria, Australia).

EGFP Staining

NIH3T3, PC12 and C2C12 cells expressing the enhanced green fluorescent protein (EGFP) were pelleted by centrifugation at 150 x g for 2 minutes and resuspended at a density of in 1 x 10^6 cells in 0.5 ml PBS, prior to FACS analysis with an EPICS 752 flow cytometer (service provided by Paul Hutchison, Department of Medicine, Monash Medical Centre, Victoria, Australia).

3.5.3 Isolation of Primary Skeletal Muscle Cells

Mice were sacrificed at day 2-3 after birth and the end of the tail clipped for genotyping (by ear-clip lysis method). After skin removal, all limbs were immediately placed into Hanks buffered saline solution (HBSS) containing no Ca^{2+} or Mg^{2+}, with 20 mM Hepes (Gibco™ Invitrogen Corporation). Skeletal muscle was then teased away from adjoining cartilage and bone and placed into fresh HBSS. In a sterile hood, muscle tissue was transferred into a solution of 0.25 % (w/v) trypsin and 0.5 mg/ml *DNase*I (Sigma), and incubated in a shaking water bath (Ratek instruments) for 40 minutes at 37°C. Cells were pelleted by centrifugation at 150 x g for 5 minutes and resuspended (by 15-20 pipetting actions) in growth media. Residual bone and connective tissue was removed by passage of

cells through a 150 μm stainless steel woven wire mesh (Metal Mesh Pty. Ltd.), prior to plating out onto gelatin coated dishes. Cells were grown at 37°C/5 % CO_2 and media changed after 24 hours (following a PBS wash). Three days after isolation, the media was changed and myoblasts differentiated into myotubes by the addition of differentiation media. This initial solution of differentiation media contained 5μM cytosine arabino-furanoside (Ara-C) (Sigma), to rid of contaminating fibroblasts. Cells were returned to fresh differentiation media without Ara-C 2 days later.

3.5.4 Culturing of MPI Mouse Embryonic Stem (ES) Cells

Maintenance and Freezing of MPI ES Cells

MPI ES cells from mice of an SvJ129 background were grown on a feeder layer of 50 % confluent mouse embryonic fibroblasts, at 37°C with 5 % CO_2, with fresh J1 media added daily. Embryonic fibroblast cells were maintained in Embryonic Feeder Cell Media (EFM) prior to the addition of ES cells. ES cells were passaged onto a fresh feeder cell layer when 80 % confluent, by incubation with a solution of 0.25 % Trypsin-EDTA+HEPES and dilution (up to 1 in 8) in fresh J1 growth media. ES cells were frozen by addition of an equal volume of 2 X Freezing Media following trypsinisation, stored at –80°C for one week, prior to transfer into liquid nitrogen.

Electroporation of MPI ES Cells

Cells were trypsinised and counted prior to the resuspension of 2 x 10^6 cells in 0.5 ml electroporation buffer. DNA was electroporated into cells at 350 V (960 μF) in a 0.4 cm Gene Pulser® cuvette (BioRad), then plated onto embryonic feeder cells for subsequent growth and antibiotic selection.

When electroporating the *Gabpα* conditional knockout construct, cells were selected for 7 days, using geneticin at 15 mg/ml and gancyclovir (GANC) at 2 μM. When subsequently electroporating these clones with the *PGK-Cre* recombinase construct, a *PGK-puromycin* construct was co-transfected, and 3 μg/ml puromycin was used to select cells for 2 days.

Picking MPI ES Cell Clones

ES cells were grown for 3-5 days following electroporation, at 37°C in 5 % CO_2. Clones were then picked using a sterile mouth pipette. Cells were first washed with Hanks/HEPES to remove any traces of foetal calf serum (FCS). ES cell clones were

disaggregated by incubation with 0.25 % (v/v) Trypsin/HEPES for five minutes at 37°C, before being resuspended in J1 media by means of 8-10 pipetting actions. Clones were then grown for several days in a 24-well plate at 37°C in 5 % CO_2, prior to DNA extraction and freezing. Mouth pipetting was performed by Elisabetta De Luca, Centre for Functional Genomics and Human Disease, Monash IRD, Victoria, Australia.

DNA Extraction from MPI ES Cells

Cells were washed with Hanks/HEPES buffer prior to addition of cell lysis buffer and incubation at 55°C for 4 hours. DNA was precipitated by addition of 2 volumes 100 % (v/v) isopropanol, allowing genomic DNA to be spooled out with a heat-sealed glass Pasteur pipette. DNA was washed twice with 80 % (v/v) ethanol, air-dried, and resuspended in 0.2 X TE.

Blastocyst Injection of MPI ES Cells

ES cells were trypsinised and a haemocytometer was used to estimate cell number. A total of 1.5 x 10^5 cells in 1 ml J1 media were injected into blastocysts harvested from C57Bl/6 female mice that had been superovulated by intra-peritoneal injection of 5 U/0.1 ml pregnant mares serum and 48 hours later intra-peritoneal injection of 5 U/0.1 ml human chorionic gonadotrophin. These manipulated blastocysts were subsequently transferred into pseudopregnant (mated with vasectomised male mice) C57Bl/6 female mice. Blastocyst injection and transfer was performed by Susan Tsao, Centre for Functional Genomics and Human Disease, Monash IRD, Victoria, Australia.

3.6 Animal Work

3.6.1 General Animal Husbandry

Mice were housed in windowless rooms with controlled temperature (22 ± 2°C) and a 12-hour light (8:00 am to 8:00 pm) and dark cycle. Pups were weaned 21-28 days after birth, and numbered by ear clipping. Ear clips or 0.5 cm segments of tail tissue was used for genotyping, as previously described.

3.6.2 Behavioural Testing of Mice

Grip Strength

Skeletal muscle strength of mice was analysed by use of the hanging wire test (Crawley 2000). A metal mouse cage lid was edged with masking tape, and mice were

allowed to grip to the cage lid prior to it being shaken 3 times and inverted over a 30 cm deep sink. The time (in seconds) until mice fell from the inverted cage lid was recorded, with a maximum test time of 60 seconds. Tests were repeated over several weeks to assess changes in grip strength with age.

Gait Analysis

Gait analysis was performed by dipping the hindlegs of mice in Higgins® Indian ink (Eberhard Faber) and allowing them to walk though a 5 cm x 60 cm cardboard tunnel, lined with white paper (Baxter et al. 2000). The angle between each set of footprints and regularity of movement was measured (Sango et al. 1995; Crawley 2000).

3.6.3 Collection of Mouse Tissues

Mice were humanely killed by CO_2 asphyxiation if older than 21 days, or by decapitation if younger. To collect embryonic tissues, pregnant females were killed by CO_2 asphyxiation, their embryos dissected from the uterus and subsequently killed by decapitation. Tissues collected for RNA or protein extraction were snap frozen in liquid nitrogen and those to be used for histology were processed immediately.

3.7 Histology

3.7.1 Tissue Fixation and Sectioning

Formalin Fixed Tissue-Paraffin Sectioning

Dissected organs were rinsed in PBS and transferred to 10 % (v/v) formalin in PBS O/N at R/T. Organs were rinsed in 70 % (v/v) ethanol three times over a 24 hour period, processed through a 70-100 % ethanol series in a Leica TP1020 processor and embedded in paraffin wax using a Leica EG1160 apparatus. Tissue blocks were stored at R/T.

Paraffin blocks were chilled in iced-water for 1 hour prior to sectioning at 5-6 μm with a Leica RM 2135 microtome. Sections were floated onto a 54°C water bath and mounted onto Superfrost Plus® (Menzel-Glaser) glass slides. Mounted sections were dried O/N at 37°C prior to storage at R/T.

Prior to staining of paraffin tissue sections, slides were dewaxed with solvent 3B2026 (HiChem) and dehydrated through an ethanol series (2 x 5 minute washes in 100 % (v/v) ethanol, 5 minute wash in 70 % (v/v) ethanol), then rinsed in tap water.

Fresh Tissue-Cryosectioning

Dissected organs were rinsed in PBS and embedded directly into Tissue Tek® Optimal cutting temperature compound (OCT) (Sakura) in foil moulds. OCT (Sakura) blocks were slowly frozen by placement into a plastic tray filled with 100 % (v/v) isopentane, resting on a bed of dry ice. OCT (Sakura) tissue blocks were stored at –80°C. Prior to use, tissue blocks were thawed to –20°C in the cryostat chamber for 30 minutes. Tissue was sectioned at 10-16 µm at –17 to –18°C using a Leica CM 3050 cryostat, and tissue was collected onto Superfrost Plus® (Menzel-Glaser) glass slides. Mounted sections were air-dried prior to storage at –80°C. Prior to tissue staining, mounted slides were thawed to R/T and washed in PBS for 5 minutes to rid of excess OCT (Sakura) compound.

3.7.2 Tissue Staining

Haematoxylin and Eosin Staining

Slides were incubated in Harris haematoxylin (Amber scientific) for 5 minutes, rinsed in tap water for 5 minutes, dipped in acid ethanol, and again rinsed in tap water for 5 minutes. Next, slides were incubated in Scott's blue solution (Amber scientific) for 1 minute, rinsed with tap water for 5 minutes and counterstained with eosin (Amber scientific) for 2 minutes. After dipping in tap water, slides were dehydrated through an ethanol series (5 minutes in 70 % (v/v) ethanol, 2 x 2 minute washes in 100 % (v/v) ethanol). Finally, slides were soaked in histolene (Amber scientific) for 5 minutes, prior to addition of DePex (BDH) mounting solution and a glass cover slip. Staining was imaged using a MPS60 digital camera, on a DMR microscope (Leica Instruments).

X-Gal Staining of Whole Organs or Tissue Sections

Organs to be stained with X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) were dissected in cold PBS prior to fixation. When whole organ staining was performed, tissues were fixed in 0.2 % (v/v) gluteraldehyde (BDH) in PBS for 1 hour 4°C, whereas cryosections of fresh tissue were fixed in 4 % (w/v) paraformaldehyde in PBS at R/T for 10 minutes prior to staining. Following fixation, samples were rinsed three times for 15 minutes at R/T in X-gal rinse buffer. Samples were incubated for 4 hours to O/N at 37°C (in the dark) with X-gal staining buffer, rinsed thoroughly in X-gal rinse buffer, and then PBS. Following staining of whole organs, a series of glycerol washes was used to clear background. Organs were incubated in 25 % (v/v) glycerol, 50 % (v/v) glycerol, 75 % (v/v) glycerol and finally 100 % (v/v) glycerol, each for 2 hours at 4°C, prior to being photographed. Tissue sections were counterstained with eosin (Amber scientific),

dehydrated and mounted in histolene (Amber scientific) and DePex (BDH), prior to viewing on a Leica MPS60 digital camera, using a Leica DMR microscope.

NADH Staining

Slides of transverse cryosections (16 μm) of skeletal muscle were submerged in Nicotinamide adenine dinucleotide (NADH) staining solution and incubated at 37°C for 25 minutes (in the dark). Slides were then rinsed in distilled water for 5 minutes, prior to dehydration and mounting in histolene (Amber scientific) and DePex (BDH) solution, and viewing with a Leica MPS60 digital camera, on a Leica DMR microscope.

α-Bungarotoxin Staining of Tissue Sections

Longitudinal cryosections (16 μm) of skeletal muscle were outlined using a pap pen (Daido Sango) and acetylcholine receptor (AChR) staining was performed O/N at 4°C (in the dark) with 1 μg/ml Texas red-conjugated α-bungarotoxin (Molecular Probes) in PBS. Sections were rinsed and mounted in PBS immediately prior to imaging on a MPS60 digital camera using a DMR microscope (Leica Instruments).

Staining of Wholemount Diaphragms and Teased Muscle Fibres

Intact diaphragm, sternomastoid and soleus muscles were dissected out and rinsed in PBS, prior to being pinned out on Sylgard cured silicone rubber (Dow Corning), and fixed in 2 % paraformaldehyde for 1-2 hours at R/T. After removal of excess fixative by rinsing tissue several times in PBS, muscle fibres from sternomasteid and soleus were teased into small bundles using fine forceps. Intact diaphragm muscles and teased fibre bundles were soaked in 0.1 M glycine for 4 hours at R/T, and excessive connective tissue was then removed under a dissection microscope. Tissue was made permeable by incubation in a blocking solution of 4 % BSA (w/v), 0.5 % Tween 20 (v/v) in PBS for 2-3 hours at R/T (or O/N at 4°C). Muscle tissue was then incubated O/N at 4°C in blocking solution, to which primary antibody was added (concentrations detailed in **Chapter 7**). Muscle tissue was washed 3 times in 0.5 % (v/v) Triton X-100 in PBS, for 1 hour each. Secondary antibody (diluted in blocking solution) was added to tissues and incubated in the dark at 4°C O/N, prior to washing of muscle tissue as before mentioned. Teased muscle fibre bundles of the sternomastoid and soleus were further separated into groups of 1-3 muscle fibres under a dissection microscope, at low light conditions, using fine tungsten needles. Diaphragms were mounted in Vectorshield® Hardset Mounting Medium for fluorescence (Vector Laboratories) and teased muscle fibres were mounted in antifade aqueous mounting

medium (BioRad). Stained fibres and wholemount diaphragms were imaged with a TCS NT digital camera on a DMRXE upright confocal microscope (Leica Instruments).

3.7.3 Electron Microscopy

Diaphragm and soleus muscles were dissected out, rinsed in PBS and pinned out on Sylgard cured silicone rubber (Dow Corning), prior to their fixation in 2.5 % gluteraldehyde solution in PBS O/N at 4°C. Tissue was rinsed in PBS 5 times to remove excess fixative, and diaphragm muscles were further dissected into cubes of approximately 2 mm. The soleus muscle was cut once, transversely ~2 mm from the point of entry of the central nerve. Muscles were then returned to 2.5 % gluteraldehyde in PBS and stored at 4°C before being processed on a Lynx automatic processor through buffer washes, 1 % Osmium tetroxide, 2 % Uranyl acetate in 0.1 M Maleate buffer, then dehydrated through a graded series of acetone and into epoxy resin (ProSciTech). Samples were infiltrated in resin firstly on a rotating wheel, then in a vacuum oven (Dynavac) for a minimum of 48 hours, before being blocked and polymerised at 60°C for a minimum of 24 hours. Survey sections were cut at 0.5 μm and stained with 1 % Toluidine blue in 1 % Sodium tetraborate, then areas were chosen for thin sectioning. Thin sections were cut at 70 nm, and collected on copper/palladium mesh grids. Sections were stained with Reynolds lead citrate (Reynolds 1963) and neuromuscular junctions were examined and photographed using a JEOL 1010 transmission electron microscope.

3.8 Electrophysiology

Whole mouse diaphragm and soleus muscles with intact nerves were dissected free and pinned to the bottom of a 3 ml waterbath, on a bed of Sylgard cured silicone rubber (Dow Corning). Preparations were continuously perfused at the rate of 3 ml per minute with Tyrode solution, and the waterbath was maintained at 32-34°C, pH 7.3, and gassed with 95 % O_2 and 5 % CO_2. The nerve supplying either muscle was gently sucked into a glass pipette filled with Tyrode solution. Two Ag-AgCl wires, one within the pipette and the other outside, were used as the anode and cathode, respectively, to stimulate the axons innervating the muscle. The nerve was stimulated with square wave pulses of 0.08 millisecond duration and 10-15 V strength at a frequency of 0.1 Hertz using a Grass Instruments stimulator (SD48) coupled to a Grass stimulus isolator (SIU5).

Extracellular recordings of spontaneous/miniature endplate currents (MEPCs) and evoked endplate currents (EPCs) were obtained using micropipettes (20 μm diameter) filled with Tyrode solution. Focal extracellular recordings were obtained by adjusting the position of the electrode until both EPCs and MEPCs with rise times of less than 1 millisecond were detected. Once the neuromuscular junction was located, stimulation was halted for 5 minutes before recording MEPCs and EPCs to allow the terminals to replenish vesicle pools.

3.9 Statistical Analysis

Data analysis was performed using Microsoft Excel, unless otherwise stated. The sampled values of two populations (e.g. wildtype and knockout) were deemed to be statistically significant if a two-tailed t-test gave a p value of <0.05.

Chapter 4

Results:

Characterisation of Gabpα and β1 Gene Structure

4.1 Introduction

GABP complex function has been shown to vary depending on subunit composition; in particular which β isoform is present. Northern blot analysis identifies the existence of multiple transcripts of both *α* and *β1*, some of which are tissue-specific (Brown and McKnight 1992). Presented herein is the characterisation of the gene structure and promoter of *Gabpα*, and various transcripts of *Gabpα* and *β1*.

4.2 Gene Structure of Gabpα

4.2.1 Genomic Library Screening

An SvJ129 mouse genomic library (Stratagene) was screened for *Gabpα* genomic clones. In order to obtain clones representative of the 5' end of the gene, an *Nco*I-*Bam*HI restriction digest product spanning 413-678 bp of the *Gabpα* cDNA (GI:M74515) was used as a probe (library screen performed by Dr. Sika Ristevski, Centre for Functional Genomics and Human Disease, Monash IRD, Victoria, Australia). DNA was extracted from three positive λ phage clones, and subsequent restriction endonuclease digestion and oligonucleotide hybridisation confirmed the presence of exons 1-3 (primers 52, 117 and 118) (see **Figure 4.1a**). From this analysis, a restriction endonuclease map of the 5' end of the mouse *Gabpα* locus could be established (see **Figure 4.1b**). A 3.2 kb *Bam*HI-*Eco*RI fragment was identified as encompassing exon 2, the first coding exon of *Gabpα*, and was used as the basis of a conditional knockout construct (see **Chapter 6**). A 6.7 kb *Bam*HI fragment encompassing exon 1 was also isolated for promoter analysis and transgenic construct assembly (see **Chapter 6**).

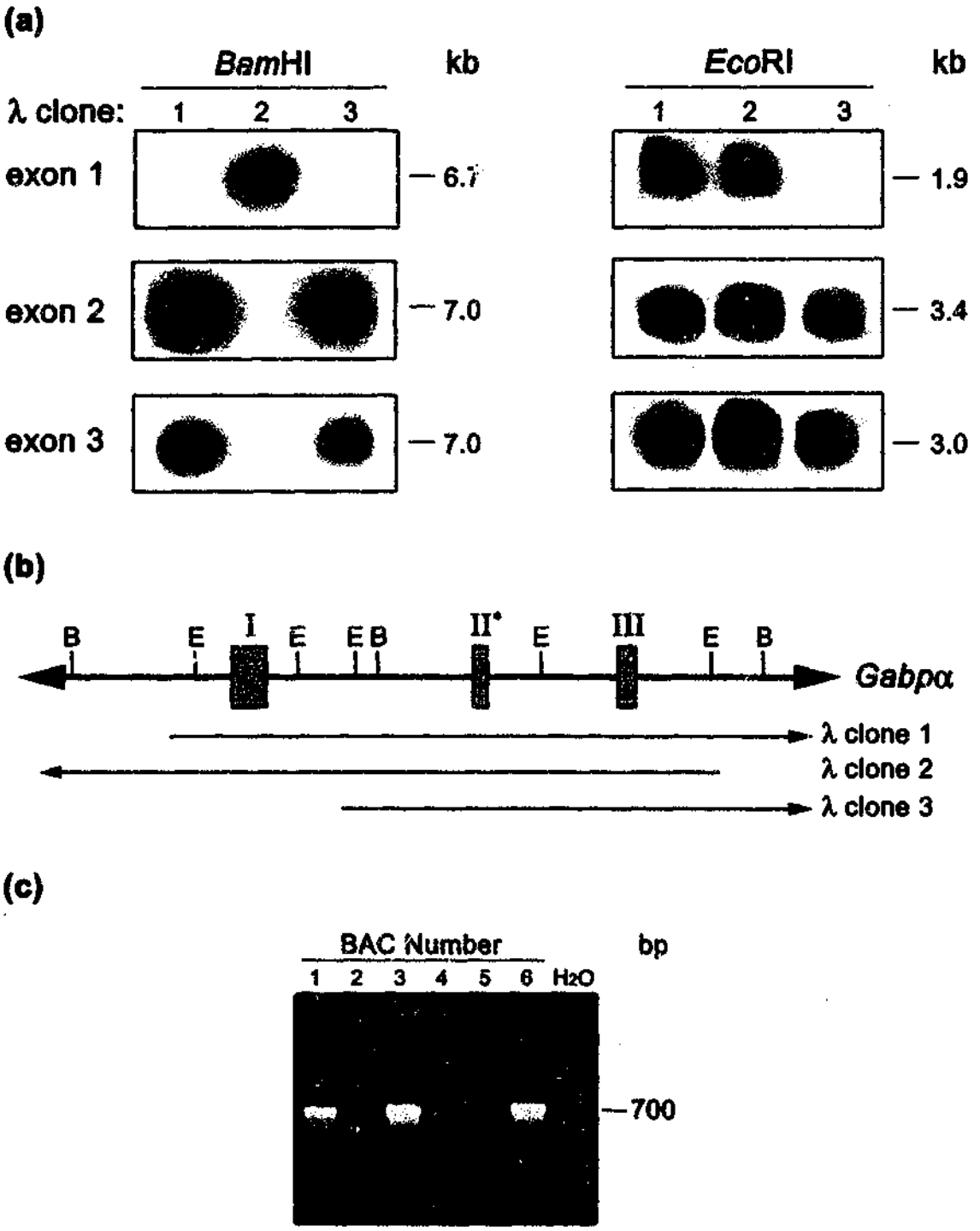


Figure 4.1 - Characterisation of the Mouse *Gabpα* Gene Locus. **(a)** Oligonucleotide mapping of *Bam*HI or *Eco*RI digested DNA extracted from λ phage genomic clones, hybridised with oligonucleotides within *Gabpα* exon 1, exon 2 and exon 3. Sizes of genomic fragments (kb) and λ clone numbers are indicated. **(b)** The resultant *Bam*HI (B) and *Eco*RI (E) restriction endonuclease map of the *Gabpα* locus, with the position of λ phage clones indicated. The initiating methionine is encoded by exon 2, as indicated by *. **(c)** PCR screen of BAC DNA for presence of intron 7, by amplification of a 700 bp product spanning *Gabpα* exons 7 and 8. BAC numbers are indicated.

In order to obtain 3' *Gabpα* genomic sequence, an SvJ129 bacterial artificial chromosome (BAC) library (BACPAC Resources) was screened. A 977 bp *Sac*II-*Spe*I fragment within *Gabpα* intron 3, spanning 12695-13672 bp of GI:27960443 (see **Appendix E**), was used as a probe. Six *Gabpα* positive BACs were identified by hybridisation, three of which were confirmed to contain *Gabpα* intron 7 by PCR of purified BAC DNA template with primers 151 and 152 (see **Figure 4.1c**). These *Gabpα* BACs were used for further genomic analysis.

4.2.2 Mouse *Gabpα* Genomic Structure

The mouse *Gabpα* intron-exon boundaries were predicted by alignment of mouse (GI:193382) and human (GI:286026) cDNA sequences, as those of the human gene had been defined previously (Goto et al. 1995). Primers were designed 50-100 bp either side of the predicted boundaries and PCR reactions were performed using *Elongase* DNA polymerase (Invitrogen). Template genomic DNA used was of the SvJ129 strain from λ phage clones, BAC clones and embryonic stem (ES) cells. As shown in **Figure 4.2a**, sequences 5' and 3' of the intron-exon boundaries of *Gabpα* conform to the gt-ag splice rule (Shapiro and Senapathy 1987). It is of interest to note that *Gabpα* introns 5 and 6 are the largest intervening sequences, at 6.3 and 4.2 kb, respectively.

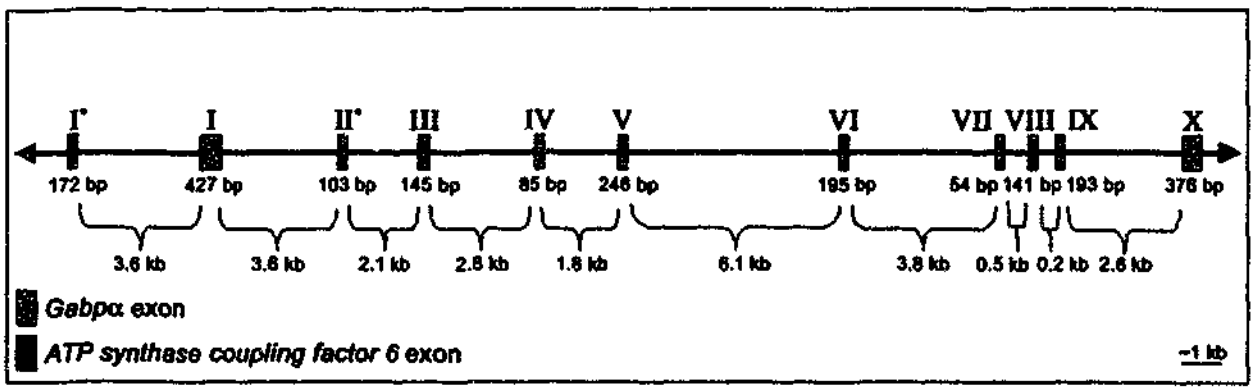
Sequence analysis (Sequencher v3.0 software) generated three genomic fragments (gaps within introns 5 and 6 due to their large size). These have been submitted to Genbank (GI:27960443-27960445) and the full sequences are attached in **Appendix E**. Subsequent to this analysis, a complete 26595 bp genomic sequence of *Gabpα* has been assembled, through comparison of the three genomic fragments of *Gabpα* with the public genome-sequencing database. This assembled sequence is also included in **Appendix E**. As shown in **Figure 4.2b**, *Gabpα* consists of 10 exons, separated by 9 introns. The total genomic region spans ~26 kb in length, and as shown in **Figure 4.2c**, the relative intron sizes of the mouse, while smaller, are comparable to those of the human *GABPα* gene (Goto et al. 1995).

The 5' UTR of *Mitochondrial ATP Synthase Coupling Factor VI* is found in the reverse orientation to *Gabpα*, and is located 165-240 bp upstream of the 5' UTR of *Gabpα* (Chinenov et al. 2000). This gene arrangement is conserved in humans. The first coding exon of *Mitochondrial ATP Synthase Coupling Factor VI* is 3.6 kb away from exon 1 of *Gabpα* (**Figure 4.2b**). This confirms that the human and mouse gene structures and

(a)

Exon	Size (bp)	cDNA (bp)	5' Splice Donor	Intron (Primers)	Size (bp)	3' Splice Acceptor
1	427	1-427	ATTCCGGAGCTG**gt**gagttgcgga	1 (52/81)	3552	tacctcttcc**ag**GACTGAACTTTT
2*	103	428-530	CACAGAAGAAA**gg**ttgatgtttct	2 (117/118)	2146	attaacatttt**ag**CATTGTGGAACA
3	145	531-675	CAAGATATTCAG**gt**aaggctgatc	3 (102/119)	2800	acctatgtta**ag**CTGGATCCAGAC
4	85	676-760	TTTCTTACCAAG**gt**aagttacctt	4 (103/120)	1757	atgtgctgtt**ag**GAATGGAGCCAA
5	246	761-1006	GCATCCCCTATG**gt**aatgagacag	5 (177/121)	6090	ttttctttct**ag**ATCCTATACACT
6	195	1007-1201	TTCTTCGAAAAT**gt**ataaaattag	6 (179/180)	3807	tgtgttcttc**ag**ATGTTTTGGCCA
7	54	1202-1255	CCATTGACCAGC**gt**gagtattcat	7 (151/152)	518	ttttccttttt**ag**CTGTGCAGATTA
8	141	1256-1396	GGAACAGAACAG**gt**acttttgcat	8 (153/154)	205	ttacggattt**ag**GAAACAATGGTC
9	193	1397-1589	CCGTGCATTACG**gt**aagaatttgt	9 (155/160)	2809	ctctttttaa**ag**GTATTATTATGA
10	376	1590-1965	-	-	-	-

(b)



(c)

Intron	Size in Human (kb)	Size in Mouse (kb)
1	6.9	3.6
2	2.8	2.1
3	3.5	2.8
4	3.1	1.8
5	6.8	6.1
6	4.1	3.8
7	1.9	0.5
8	0.2	0.2
9	4.0	2.8

Figure 4.2 - The Gene Structure of Mouse *Gabpα*.
The intron-exon boundaries (a) and resulting gene structure (b) of mouse *Gabpα*. The first coding exon is indicated by *, coding sequences are shown in upper case, introns in lower case, and splice sites (gt-ag) are in bold typeface. Primer numbers used to amplify introns are shown (). Intronic sizes of mouse and human *GABPα* are also compared (c), adapted from Goto et al. 1995.

promoter sequences are highly conserved, establishing the mouse as an ideal model for studying *GABPα* gene function. In addition, the presence of bi-directional promoter elements between the two genes (Chinenov et al. 2000) is suggestive of coupled regulation.

4.3 *Identification of Gabpα and β1 Alternative Transcripts*

Alternative splicing of pre-mRNAs is an important means of regulation of gene expression, and the production of diverse protein products. Heterogeneity of mRNAs observed by Northern blot analysis or identification of multiple protein isoforms are prime indicators of involvement of alternative promoters, alternative splice donor-acceptor sites, and/or alternative 5' and 3' UTRs. As multiple transcripts are detected by Northern blot analysis for both *GABPα* and *β* (LaMarco et al. 1991; Brown and McKnight 1992; Escrivá et al. 1999), we investigated their identity.

4.3.1 *Gabpα* and *β1-1* Transcript Expression Analysis

The expression of *Gabpα* and *β1-1* mRNA between mouse embryonic day (E) 10.5 and E15.5 was examined by Northern blot analysis (kindly performed by Daniela Koleski, Centre for Functional Genomics and Human Disease, Monash IRD, Victoria, Australia), as shown in **Figure 4.3a**. Expression of both transcripts is detected at all stages in placenta, yolk sac and embryo and levels appear to increase slightly with gestational age. *Gabpα* is expressed as a major transcript of 5.4 kb and minor transcripts of 2.8 and 2.5 kb, and *Gabpβ1-1* is expressed as a 2.8 kb transcript. *Gabpα* mRNA was detected using a probe spanning 413-1884 bp of sequence GI:193382 (primers 52 and 53) representing the full length open reading frame (ORF). *Gabpβ1-1* was detected by hybridisation with a cDNA probe spanning 1150-1418 bp of the unique *β1-1* exon 9 region of sequence GI:193384 (primers 87 and 88). Levels of *Glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) were determined to indicate loading, by hybridisation with a probe spanning 585-985 bp of cDNA sequence GI:193423 (primers 58 and 59).

In the adult, the major 5.4 kb *Gabpα* transcript is detected in all tissues, at varying levels, although is barely detectable in small intestine, spleen and epididymis (see **Figure 4.3b**). The minor 2.8 kb and 2.5 kb *Gabpα* transcripts are expressed in a tissue-specific manner, with highest levels observed in testis, seminal vesicles, thymus, liver, lung and large intestine. In addition to the 2.8 kb form of *Gabpβ1-1* that is detected in all tissues at

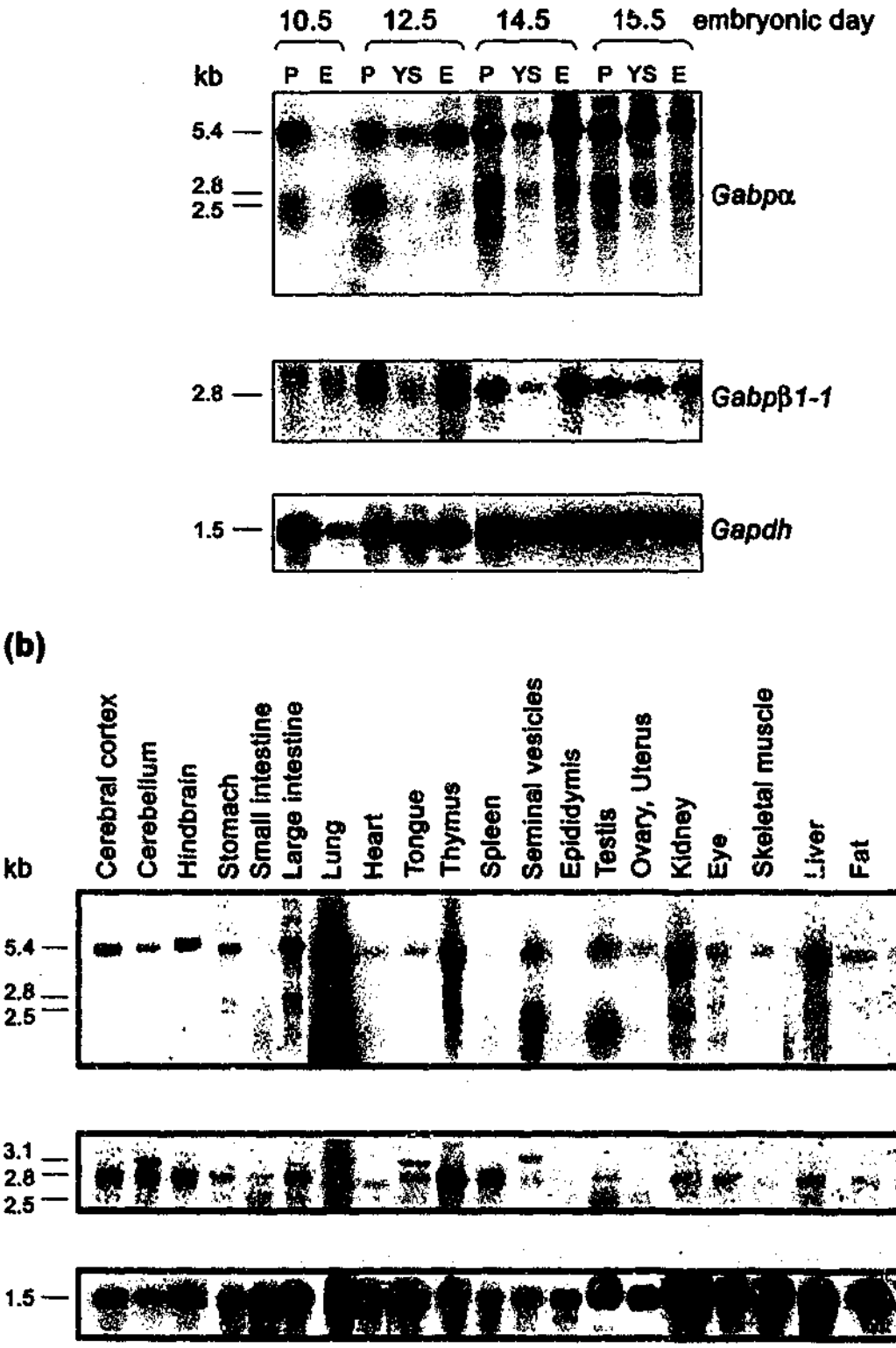


Figure 4.3 - Expression Analysis of *Gabpα* and β*1-1* Transcripts.
Gabpα and β*1-1* transcripts were detected by Northern blot analysis of mRNA derived from (**a**) embryonic and (**b**) adult mouse tissues. Embryonic mRNA derived from placenta (P), yolk sac (YS) and embryo (E) of mouse embryonic day 10.5, 12.5, 14.5 and 15.5 was examined. *Gabpα* transcripts of 5.4 kb, 2.8 kb and 2.5 kb, and *Gabp* β*1-1* transcripts of 3.1 kb, 2.8 kb and 2.5 kb are shown. The 1.5 kb transcript of *Gapdh* indicates loading in both panels.

varying levels, there are two additional transcripts of 3.1 and 2.5 kb, respectively, expressed in a restricted manner. The 3.1 kb transcript is detected in cerebellum, lung, tongue, seminal vesicles and skin, whereas the 2.5 kb form is found in small intestine, lung, testis, ovary/uterus and skin.

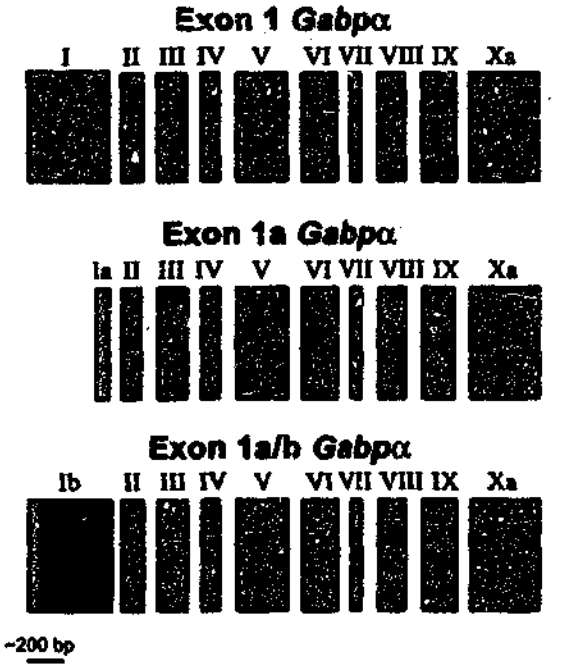
Alternative transcripts have been found for other ETS proteins; Ets-1 (Jorcyk et al. 1991; Quéva et al. 1993), Ets-2 (Watson et al. 1990), Erg (Reddy et al. 1987) and Elf-5 (Zhou, J., PhD thesis 2001). Some of these alternative splice products possess distinct functions to those of the predominant product. For example, the p42 isoform of Ets-1 lacks the N-terminal inhibitory domain and inhibitory phosphorylation site, and activates an alternative apoptosis pathway in cancer cells by inducing *Caspase-1* expression (Li et al. 1999a). Therefore, to better understand how the expression pattern and function of the multi-subunit ETS transcription factor Gabp is regulated, the nature of the various *Gabpα* and *β1-1* transcripts was investigated further.

4.3.2 Identification of Alternative 5' and 3' UTR Transcripts of *Gabpα*

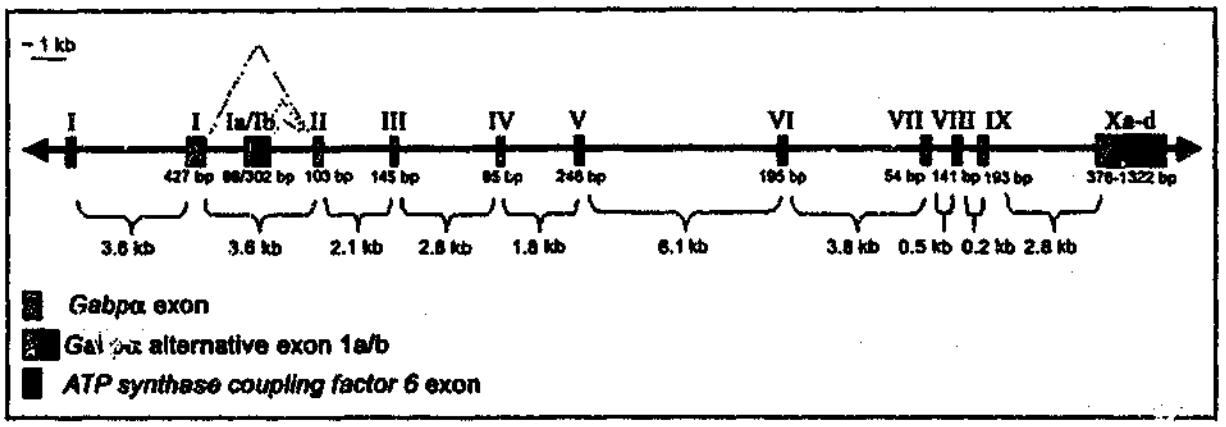
Template cDNA from kidney, thymus and testis was used in RT-PCR analysis of *Gabpα* expression, as these tissues express minor transcripts detectable by Northern blot analysis (see **Figure 4.3b**). RT-PCR amplification was performed across the entire coding region of *Gabpα* with primers spanning 5' and 3' UTR sequence (primers 52 and 53), using *Elongase* DNA polymerase (Invitrogen). In each of the three tissues, only the transcript representing the full length ORF was detected by this method. Therefore, 5' and 3' RACE RT-PCR was used to identify alternative non-coding regions. An E14.5 mouse placental cDNA library (kindly provided by Dr. Melanie Pritchard, Centre for Functional Genomics and Human Disease, Monash IRD, Victoria, Australia) was screened using the universal AP1 primer (primer 6) and various *Gabpα* primers (exon 2r-primer 227, exon 5f-primer 229, exon 7f-primer 231 and exon 9f-primer 232).

Amplification from exon 2 of *Gabpα* yielded multiple RACE RT-PCR products. Sequencing of these products, after sub-cloning into the pGEM-T vector (Promega), lead to the identification of two alternative *Gabpα* exons 1 (exon 1a and 1b), within intron 1 (see **Figure 4.4a**). Both exon 1a and 1b are capable of splicing into exon 2, producing identical coding regions to that of the exon 1, as the translation initiation site is located in exon 2. Exon 1b begins at the same 5' site as exon 1a and includes an additional 216 bp downstream of exon 1a. See **Figure 4.4b** for a diagram of the alternative splicing of

(a)



(b)



(c)

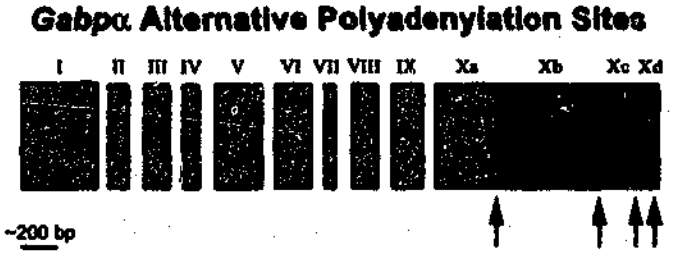


Figure 4.4 - Alternative Transcripts of Mouse *Gabpα*.
(a) Three mouse *Gabpα* transcripts are generated by use of alternative first exons. (b) The mouse *Gabpα* locus, with the position of exon 1 shown in relation to the closely linked gene *ATP synthase coupling factor 6*. Alternative first exons of *Gabpα* within intron 1 (exon 1a/b) are depicted, and stippled lines indicate splicing of the three first exons into exon 2 (the first coding exon). Varying sizes of exon 10 due to alternative polyadenylation sites are also shown in varying shades of blue. (c) The four alternative polyadenylation sites for mouse *Gabpα*. The exon 1 5' UTR transcript is represented and relative sizes of the 3' UTRs indicated (exons Xa-d), with arrows representing polyadenylation sites.

Gabpα, and **Appendix E** for the exact location of the alternative exons 1. Sequences of the three 5' UTRs of *Gabpα* have been submitted to Genbank (accession no:AY282793, AY282794 and AY282795) and are attached in **Appendix F**. Visual inspection of the sequence of exons 1a and 1b revealed that exon 1a contains a start codon followed immediately by a stop codon. In addition, exon 1b is predicted to encode three short ORFs of 40-50 bp in length, one of which extends into exon 2 and terminates 3 bp downstream of the initiating ATG of the translated Gabpα protein. This third potential translation initiation site within *Gabpα* exon 1b may compete with the site within exon 2 for ribosome binding, as both regions resemble the $^{5'}$GCC(A/G)CCAUGG$^{3'}$ Kozak consensus sequence to a similar degree (Kozak 1987). Therefore alternative 5' UTRs may play a role in translational regulation of *Gabpα* expression.

RACE RT-PCR products of exon 5f-AP1, 7f-AP1 and 9f-AP1 reactions varied in size, due to use of alternative polyadenylation signals within exon 10. Sequence analysis (Sequencher) yielded identification of four alternative polyadenylation signals at 25628 bp, 26191 bp, 26472 bp and 26539 bp of the complete *Gabpα* genomic sequence (**Appendix E**). These alternative polyadenylation signals result in mRNA sequences of 1965 bp (GI:193382) as previously described (LaMarco et al. 1991), 2528 bp, 2809 bp or 2911 bp. The last coding exons of *Gabpα* therefore start at the same 5' point, but continue for varying lengths following the termination codon. These alternative last exons have been termed exons 10a, 10b, 10c, 10d, in order of increasing size (see **Figure 4.4c** for relative lengths of 3' UTRs). Sequences of the four *Gabpα* 3' UTRs have been submitted to Genbank (accession no:AY282796, AY282797, AY282798 and AY282799) and are attached in **Appendix F**. It is important to note that only two of the four *Gabpα* polyadenylation signals conform to the $^{5'}$AAUAAA$^{3'}$ consensus sequence (those at 26191 bp and 26472 bp), suggesting that transcripts utilising these polyadenylation sites may be more stable than those with the alternative $^{5'}$AUAAAUA$^{3'}$ or $^{5'}$UAAAAAAUA$^{3'}$ polyadenylation signals (Wickens and Stephenson 1984).

Taken together, this data suggests that a total of twelve *Gabpα* transcripts may exist, with various combinations of exon 1, 1a or 1b, and exon 10a, 10b, 10c or 10d. Transcripts containing exons 1a and 1b may result from alternative promoter usage. Therefore, prior to the design of a *Gabpα* overexpression (transgenic) construct, the tissue distribution of

Gabpα exon 1a and 1b transcripts and promoter activity of the *Gabpα* upstream region were investigated further.

Expression Patterns of Exon 1a/b-containing *Gabpα* Transcripts

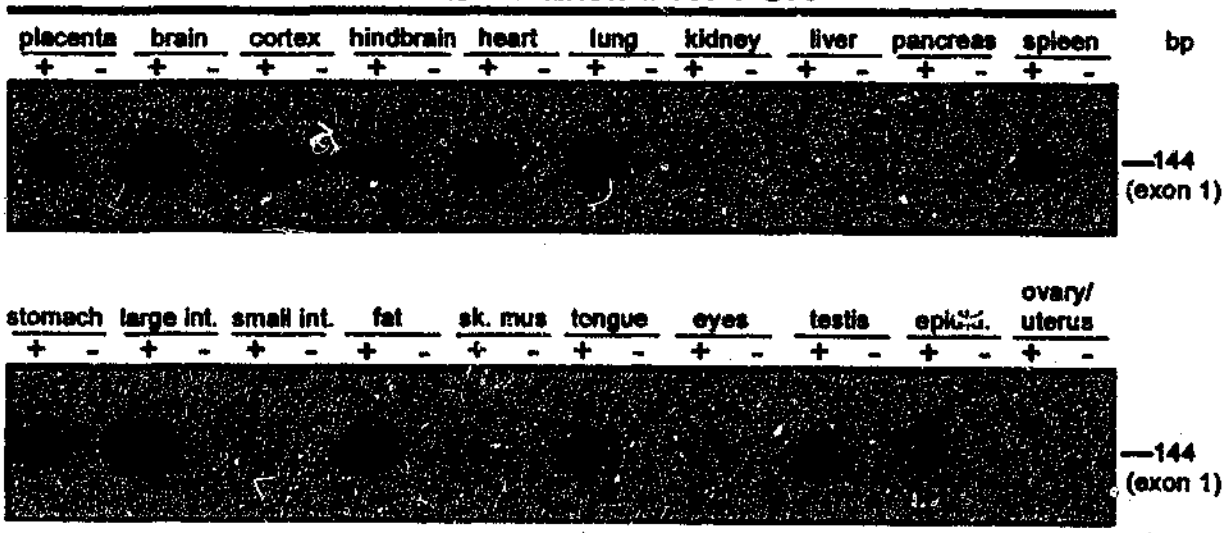
In order to confirm that *Gabpα* exon 1a/b transcripts are expressed, RT-PCR was performed using cDNA of a panel of F1 (C57Bl/6 x CBA) mouse tissues as template. The exon 1 transcript was amplified with primers 52 and 118 to give a 144 bp product spanning 413-557 bp of cDNA sequence GI:193382 (see **Appendix F**) from exon 1 to exon 3 of *Gabpα*. Only one product was ever generated from this reaction, indicating that exons 1a and 1b are alternative exons 1, never found between exons 1 and 2 in cDNA products (see **Figure 4.5a**). The exon 1 *Gabpα* transcript is expressed in all tissues, ranging from almost undetectable levels in liver and pancreas, to relatively high levels in brain, lung and large intestine.

The exon 1a and 1b transcripts were amplified with primers spanning *Gabpα* exon 1a to exon 3 (primers 246 and 118), to give a 201 bp exon 1a product (15-216 bp of partial cDNA sequence AY282794, see **Appendix F**) and a 417 bp exon 1b product (15-432 bp of partial cDNA sequence AY282795, see **Appendix F**). As shown in **Figure 4.5b**, internal oligonucleotide hybridisation within exon 2 (primer 61) demonstrated that both exon 1 and exon 1b RT-PCR products were *Gabpα*-specific. An additional 600 bp product cross-reactive with *Gabpα* was also amplified from some tissues, the identity of which is yet to be found. Exon 1a and 1b transcripts appear to be lowly expressed in most mouse tissues, with highest expression of exon 1a in placenta, hindbrain, heart, kidney, spleen and large intestine, and exon 1b in hindbrain, lung, kidney, liver and testis. However, given that oligonucleotide hybridisation was necessary to visualise RT-PCR products containing exon 1a and 1b indicates that they are expressed at a low level relative to the exon 1 transcript.

Implications of *Gabpα* Alternative Transcripts

A possible explanation for the existence of alternative 5' UTR transcripts of *Gabpα* described here is alternative promoter usage. An ETS gene that has been shown to possess two promoters, resulting in two transcripts of differential expression, is *Fli-1* (Prasad et al. 1998). Use of the second *Fli-1* promoter results in an alternative 5' UTR producing a shorter *Fli-1b* transcript and protein product (Prasad et al. 1998). Transcripts of a more restricted expression pattern to the full length form are known to exist for many other genes. For example, the mitochondrial transcription factor *MTFA* is known to exist as

(a)



(b)

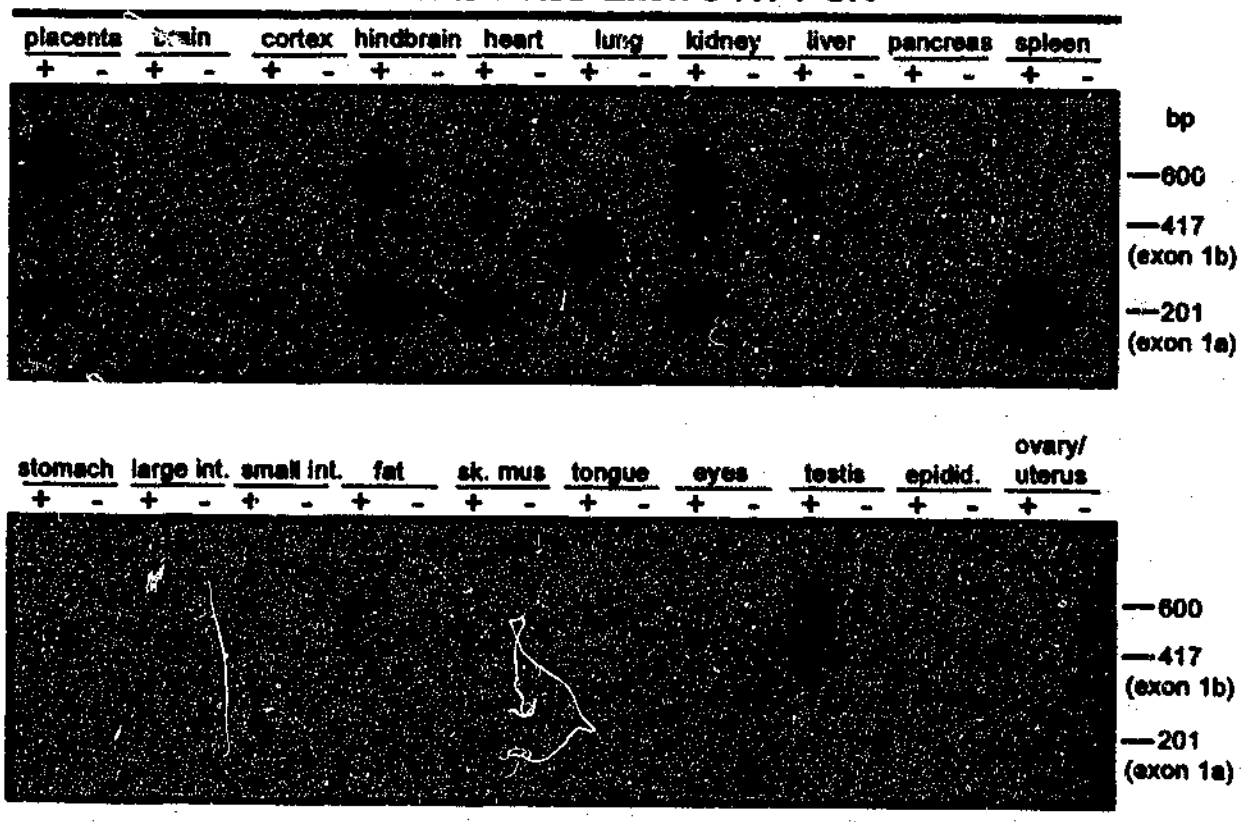


Figure 4.5 - Expression of *Gabpα* 5' UTR Transcripts in Adult Mouse.
Gabpα 5' UTR transcripts were detected by RT-PCR, using 5' primers specific for exon 1 or exon 1a/b and a common 3' primer within exon 3. Products were hybridised with an internal oligonucleotide within exon 2. The 144 bp product representing the exon 1 transcript is shown in **(a)**. The 201 bp and 417 bp RT-PCR products representing exon 1a and exon 1b transcripts are shown in **(b)**, and an uncharacterised 600 bp product.

multiple transcripts, one of which encodes a testis-specific isoform of unknown function (Larsson et al. 1996). Initiation of transcription at several sites, generating transcripts that differ only in their 5' UTRs, can render gene expression less vulnerable to gene silencing by means of promoter mutation (Ayoubi and Van De Ven 1996). Therefore, *Gabpα* alternative 5' UTR transcripts may have evolved as a means of ensuring Gabpα protein is expressed in all tissues.

The presence of three predicted upstream ORFs within exon 1b indicates that the alternative exons 1 of *Gabpα* may also play a negative role in translational regulation of *Gabpα*. Upstream open reading frames often lead to interference of ribosome scanning (Kozak 1996; van der Velden and Thomas 1999). Long 5' UTR sequences can also be inhibitory, due to the presence of stable mRNA secondary structures, making shorter 5' UTRs (such as exon 1a) more favourable for translation (Willis 1999). Sequences contained within the 3' UTR and poly-A tail of mRNA are thought to regulate translation also, by way of protein-protein interactions causing functional interactions between the 5' and 3' UTR (Sonenberg 1994). In addition, a consensus polyadenylation signal is known to be important for transport of transcripts into the cytoplasm (Wickens and Stephenson 1984). It has been suggested that use of alternative 3' UTRs, lacking consensus polyadenylation signals and containing A-T rich sequences, results in *ETS-2* transcripts of lesser stability than the major transcript (Watson et al. 1990), and the 3' UTR of the GABP target gene *Utrophin* is known to be necessary for regulating mRNA stability and transcript localisation within skeletal muscle cells (Gramolini et al. 2001a). Hence, alternative *Gabpα* 3' UTR usage could contribute to differences in translation efficiency.

4.3.3 Identification of *Gabpβ1-1* and *β1-2* Alternative Transcripts

As alternative isoforms of GABPβ influence the function of the GABP transcription factor complex (Suzuki et al. 1998) and multiple *Gabpβ1-1* transcripts were detected by Northern blot (see **Figure 4.3**), the identity of novel *Gabpβ1* transcripts was explored. The intron-exon boundaries of the mouse *Gabpβ1* gene locus were determined by Basic Local Alignment Search Tool (BLAST) analysis (Altschul et al. 1990) of mouse *β1-1* (GI:193384) and *β1-2* (GI:6753933) cDNA sequences against the mouse genome. This confirmed that the *β1-1* transcript differs from *β1-2* by splicing from within the common exon 8 to a unique exon 9 further downstream. RT-PCR analysis was performed using *Elongase* DNA polymerase (Invitrogen) and cDNA from adult mouse brain, lung

and skin, as minor transcripts are detected in these tissues by Northern blot. *Gabpβ1-1* transcripts were amplified with primers 242 and 243 spanning 244-1197 bp of GI:193384, from exon 3 to exon 9. Alternatively, a *Gabpβ1-1* RT-PCR product from 49-1418 bp of GI:193384, spanning exon 1 to exon 9 (primers 114 and 88) was amplified. *Gabpβ1-2* transcripts were amplified with primers 242 and 245 spanning 243-1171 bp of GI:6753933, from exon 3 to exon 8, or with primers 114 and 245, spanning exon 1 to exon 8, from 49-1171 bp of GI:6753933. Several RT-PCR products smaller than those of the full length *Gabpβ1* transcripts were identified and sub-cloned into the pGEM-T vector (Promega). Sequence analysis (BLAST) identified these products as novel splice variants of *Gabpβ1*; three alternatively spliced *Gabpβ1-1* transcripts and three alternatively spliced *Gabpβ1-2* transcripts. Partial cDNA sequences of each splice variant have been submitted to Genbank (see **Appendix G** for sequences), and novel transcripts have been named according to the splice sites used. **Figure 4.6** details all previously identified and novel *Gabpβ1* transcripts, and the protein domains they encode.

The novel exon 7-9 spliced transcript of *Gabpβ1-1* (accession no:AY282802) would result in loss of translational reading frame, premature termination, and therefore loss of functional transactivation and homodimerisation domains (see **Figure 4.6a**). This transcript was also identified as a partial cDNA sequence on the Genbank mouse EST database (GI:9813503), isolated from a 5 month old DH10B mouse infiltrating ductal cell carcinoma of the mammary gland. If produced, this Gabpβ1-1 protein product would be able to bind Gabpα and transport the Gabp complex to the nucleus, allowing α to bind DNA of target genes, but not exert any transactivation, therefore acting as a dominant negative. The second novel splice form of *Gabpβ1-1* from exon 3-5 (accession no:AY282800) would produce a Gabpβ1-1 protein lacking only the C-terminal end of the α binding domain. This region of the domain is known to contain the amino acids M102, L103, E113, K135, F136, K138, six of the ten residues essential for Gabpα binding (Batchelor et al. 1998). Therefore the exon 3-5 Gabpβ1-1 protein product may not be able to bind Gabpα, or may bind at a greatly decreased affinity, leading to decreased Gabp complex activity. The third novel splice form of *Gabpβ1-1* from exon 3-9 (accession no:AY282801) would translate into a protein lacking the C-terminal end of the α binding domain, as well as the NLS. Therefore the resultant decrease in binding to Gabpα and lack of import into the nucleus would cause this isoform to act as a dominant negative.

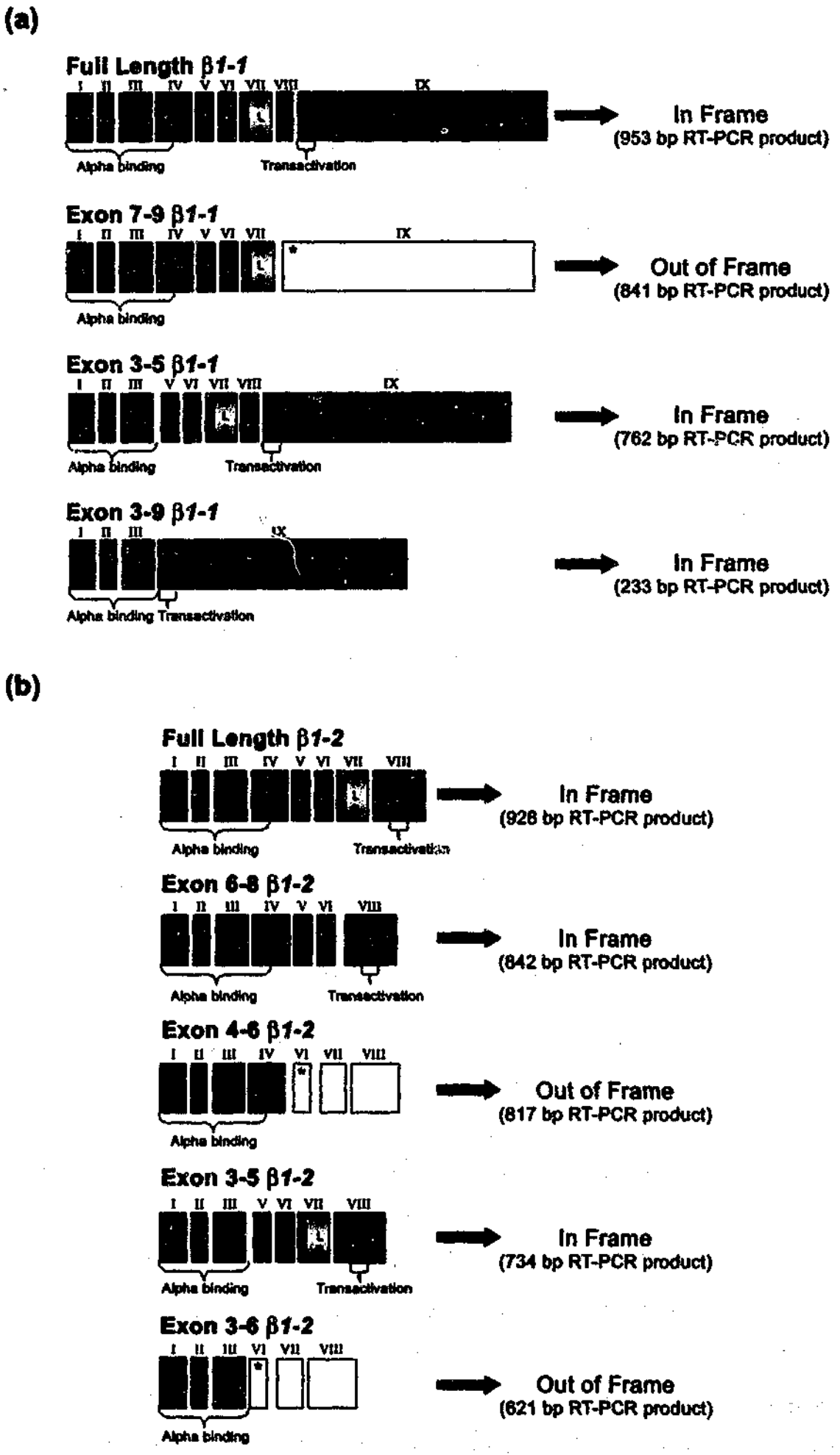


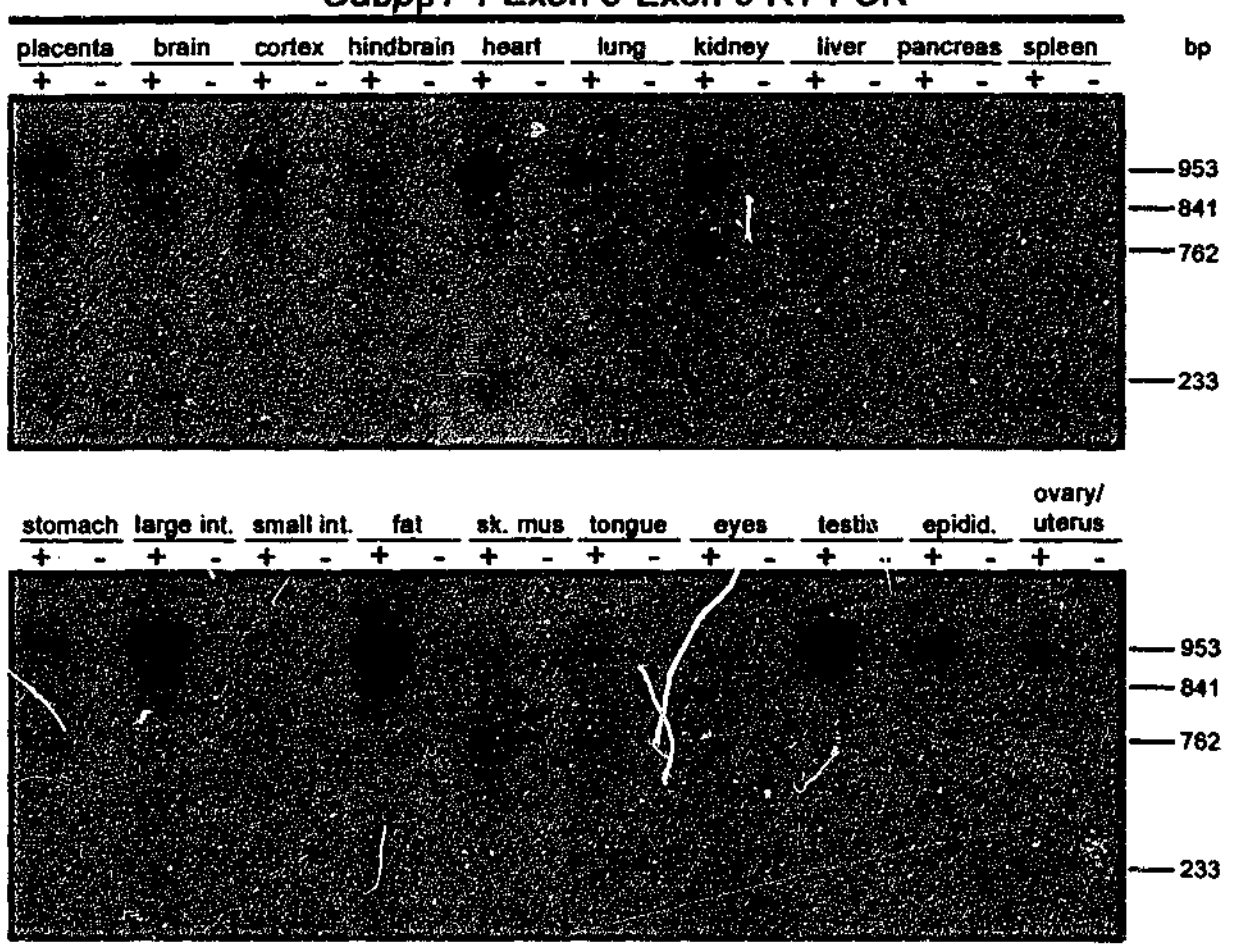
Figure 4.6 - Alternative Transcripts of Mouse *Gabpβ1*.
Protein domains encoded by full length and alternatively spliced transcripts of mouse *Gabpβ1-1* (a) and *Gabpβ1-2* (b). The sizes of RT-PCR products amplified with primers spanning exon 3 to 9 (*β1-1*) or exon 3 to 8 (*β1-2*) of novel *β1* transcripts are indicated, as are reading frames of putative protein products. * indicates premature stop codons, in frame regions are shown in red, out of frame regions in white, NLS in yellow and β homodimerisation domain (Beta) in blue.

We detected more splice variants for *Gabpβ1-2* than for *β1-1* (see **Figure 4.6b**). The novel exon 4-6 splice form of *Gabpβ1-2* (accession no:AY282805) would translate out of frame, terminating prematurely within exon 6, and would therefore lack functional NLS and transactivation domains. This would trap Gabpα in the cytoplasm and result in lack of transcription factor function, acting as a dominant negative. Translation of the second novel *Gabpβ1-2* splice form, from exon 3-5 (accession no:AY282803), would result in a Gabpβ1-2 protein lacking just the C-terminal end of the α binding domain. As in the case of the Gabpβ1-1 exon 3-5 isoform, this protein would either bind inefficiently or not at all to Gabpα, resulting in a decrease or loss of transactivation of target genes. The third novel *Gabpβ1-2* transcript identified, splicing out the region between exons 3 and 6 (accession no:AY282804), would translate into an out of frame protein lacking the C-terminal end of the α binding domain, as well as functional NLS and transactivation domains, due to premature termination within exon 6. Examination of the Genbank mouse EST database revealed an additional exon 6-8 splice variant of *Gabpβ1-2* (GI:4058535), isolated from C57Bl/6 mouse E14.5 placenta cDNA. If translated, this would form a Gabpβ1-2 protein lacking only the NLS. Therefore a Gabp complex containing this subunit would not localise to the nucleus, or transactivate target genes, acting as a dominant negative. Hence, the most likely role for all novel alternative splice forms of *Gabpβ1* is to interfere with the activation of target genes, in a dominant negative manner.

Expression Patterns of Alternative *Gabpβ1-1* and *β1-2* Transcripts

The expression of all *Gabpβ1-1* transcripts was confirmed by RT-PCR using primers spanning 244-1197 bp of GI:193384, from exon 3-9 (primers 242 and 243). Tissues from adult F1 (C57Bl/6 x CBA) mice were used to generate template cDNA, and all RT-PCR products were confirmed to be specific by hybridisation with an oligonucleotide within the unique *Gabpβ1-1* exon 9 region (primer 87). As shown in **Figure 4.7a**, the full length *Gabpβ1-1* transcript (953 bp product) was found in all tissues examined, except for pancreas and spleen. Conversely, the exon 7-9 transcript (841 bp product, see AY282802 in **Appendix G**) was only detected in brain, heart, small intestine, skeletal muscle and tongue. The exon 3-5 transcript (762 bp product, see AY282800 in **Appendix G**) is also restricted in its expression, being expressed in hindbrain, kidney, liver, small intestine, skeletal muscle, tongue and eyes. The smallest *Gabpβ1-1* transcript, spliced from exon 3-9 (233 bp product spanning 195-428 bp of AY282801), was only detected at high levels in

(a)



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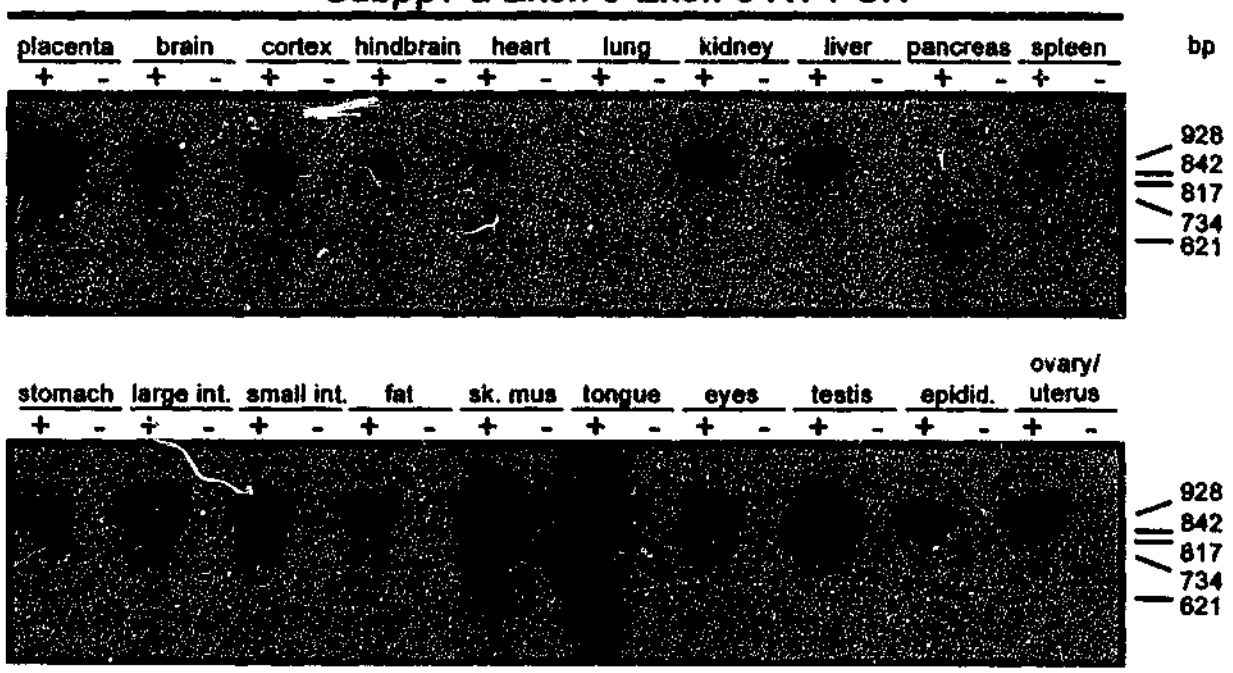


Figure 4.7 - Expression of *Gabpβ1* Transcripts in Adult Mouse.
RT-PCR products representing *Gabpβ1-1* **(a)** and *Gabpβ1-2* **(b)** transcripts, amplified with primers spanning exons 3 to 9 and exons 3 to 8, respectively, and hybridised with specific oligonucleotides. The 953 bp band represents full length *β1-1*, and 841 bp, 762 bp and 233 bp bands represent exon 7-9, 3-5 and 3-9 splice forms, respectively. The 928 bp band represents full length *β1-2*, while 842 bp, 817 bp, 734 bp and 621 bp bands represent exon 6-8, 4-6, 3-5 and 3-6 splice forms, respectively.

heart and spleen. Due to their potential role as dominant negative proteins, these alternative isoforms of Gabpβ1-1 may play a role in tissue-specific regulation of Gabp complex activity.

Abundance of the various *Gabpβ1-1* splice forms relative to the full length transcript could be estimated by intensity of bands on exposed x-ray film, as all products were amplified in the same RT-PCR reaction. In brain, the exon 7-9 splice form (841 bp product) is expressed at equivalent levels to that of the full length transcript. Similarly, the exon 3-5 *β1-1* splice form (762 bp product) is expressed highly in kidney, liver, small intestine, skeletal muscle, tongue and eyes. The exon 3-9 splice form (233 bp product) is the only *β1-1* transcript detected in spleen, and is also highly expressed in the heart. Hence, *Gabpβ1-1* possesses alternative splice forms that are expressed in a tissue-specific manner, at varying levels.

The expression of all *Gabpβ1-2* transcripts was confirmed by RT-PCR with primers 242 and 245, spanning 243-1171 bp of GI:6753933, from exon 3-8. A panel of F1 (C57Bl/6 x CBA) adult mouse tissues were used to generate cDNA, and RT-PCR products were confirmed to be specific by hybridisation with an oligonucleotide within the unique *Gabpβ1-2* exon 8 region (primer 89). As shown in **Figure 4.7b**, the expression pattern of the *Gabpβ1-2* transcripts differs quite remarkably from that of *Gabpβ1-1*. Full length *Gabpβ1-2* (928 bp product) is detected in all tissues except pancreas, and barely detected in lung. Exon 6-8 (842 bp product, GI:4058535) and exon 4-6 (817 bp product, see AY282805 in **Appendix G**) transcripts were expressed at very low levels in brain, skeletal muscle and tongue. Similarly, the exon 3-5 transcript (734 bp product, see AY282803 in **Appendix G**) was detected at low levels, in brain, tongue and eyes. Interestingly, the expression pattern of the *β1-2* exon 3-5 transcript differs from that of the *β1-1* exon 3-5 transcript. The most abundant of *Gabpβ1-2* alternative splice forms is the exon 3-6 transcript (621 bp product spanning 195-816 bp of AY282804), expressed in brain, heart, lung, pancreas, stomach, fat, skeletal muscle and tongue. Therefore, it is likely that these transcripts, being expressed in a tissue-specific manner, may function as negative regulators of Gabp complex function in a cell-specific manner.

Unlike *β1-1*, the abundance of *Gabpβ1-2* alternative transcripts relative to the major transcript is quite low. However, the exon 3-6 splice form (621 bp product) predominates in pancreas, and the exon 6-8 and 4-6 transcripts (842 bp and 817 bp products) are found at

equivalent levels to full length *β1-2* in the cortex and hindbrain. All other tissues express the full length *Gabpβ1-2* transcript at higher levels than the alternative splice forms. This suggests that expression of *Gabpβ1-1* is regulated in a more complex manner than *β1-2*, and has important implications upon transcription factor function, as β1-1 is the predominant protein found in Gabp complexes.

Implications of Novel *Gabpβ1-1 and β1-2* Splice Forms

The alternative splice forms of *Gabpβ1* described here may produce dominant negative isoforms. Protein deletion and cell transfection experiments have previously been used to determine the PPAKR region necessary for nuclear localisation and amino acids 153-267 for transactivation (Sawa et al. 1996). Therefore, if the alternative transcripts of *β1-1* exon 7-9, exon 3-9 and *β1-2* exon 6-8, exon 4-6, exon 3-6 were translated into proteins, this would result in loss of Gabp transcription factor function. Retardation of Gabp within the cell cytoplasm would also allow other ETS family members an opportunity to act on genes normally regulated by Gabp, resulting in different levels and/or temporal expression. Therefore Gabp function may be negatively regulated in a similar manner to NF-κB, to the extent that the binding protein IκB prevents NF-κB from translocating to the nucleus, and hence prevents target gene regulation by NF-κB (Baeuerle and Baltimore 1988).

The amino acids essential for Gabpβ binding to α have been identified as; W35, L37, K69, V70, M102, L103, E113, K135, F136, K138 (Batchelor et al. 1998). Two of these residues are positioned at the tip of each ankyrin repeat loop of Gabpβ and interact with the carboxy terminal extension of the ETS domain of Gabpα via hydrophobic and hydrogen bonds (Ely and Kodandapani 1998). It is this Gabpα/β interaction that results in the complex being significantly more stable than α binding DNA alone (Graves 1998). In those Gabpβ1 isoforms missing exon 4 (produced from transcripts *β1-1* exon 3-5, exon 3-9 and *β1-2* exon 3-5, exon 3-6 splicing), amino acids M102, L103, E113, K135, F136, K138 would be absent. Therefore the Gabpα/β1 interaction would be weaker, if at all possible, in tissues where these transcripts are highly expressed, such as heart, kidney and pancreas.

The inability of some isoforms of Gabpβ1 to bind Gabpα introduces the possibility of β being available to bind other proteins, utilising its transactivation domain and NLS for purposes other than transactivation of Gabp complex targets. Recently, yeast-2-hybrid

experiments identified the Polycomb protein cofactors YAF-2 (YY1-associated factor 2) and the closely related YEAF1 protein (YY1- and E4TF1/hGABP- associated factor 1) as being able to bind to the NLS of GABPβ and γ proteins (Sawa et al. 2002). Whether lack of Gabpβ1 binding α affects these and other protein interactions, by means of altered protein structure remains to be tested, however lack of the NLS may prevent binding to YAF-2 or YEAF-1. The net effect of any alternative protein interactions of Gabpβ1 would be altered transactivation of Gabp target genes.

4.4 Gabpα Promoter Analysis

Some genes are transcribed in tissues from a promoter that yields a 5' UTR full of upstream ORFs, while in the tissue that constitutes the major site of gene expression, a different promoter is used to produce a 5' UTR which is less encumbered (Kozak 1991). Promoter analysis of the *Gabpα* upstream region was performed to identify regulatory elements, and to address the question of exon 1a and exon 1b transcript function. This regulatory information was subsequently used to produce *in vitro* and *in vivo* models of *Gabpα* overexpression, to mimic the increased gene dosage of *GABPα* in Down syndrome (see **Chapters 5** and **6**).

4.4.1 Analysis of *Gabpα* Upstream Sequence

Comparative analysis of the human (GI:14861133), mouse (see **Appendix E**) and rat (GI:25006870) *GABPα* sequences was performed using the Visualisation Tools for Alignments (VISTA) program (Dubchak et al. 2000b) (see **Figure 4.8**). This analysis demonstrates a high conservation level between mouse and rat *Gabpα* across the whole locus. High sequence similarity is also maintained within the coding regions of the human gene. This is not the case for exons 1a and 1b, which are unique to the mouse, suggesting a species-specific means of regulation. The only non-coding regions of *GABPα* which are highly conserved (≥ 75 %) between mouse and human are the CpG island flanking exon 1, a 100 bp region within intron 3, and a 750 bp region within intron 9, immediately upstream of the last coding exon. These areas may contain important regulatory sequences that have been conserved during evolution.

Three putative promoter regions (P1, P2 and P3) were predicted within the *Gabpα* 5' UTR and intronic regions (see **Appendix E** for their sequence and location), using the

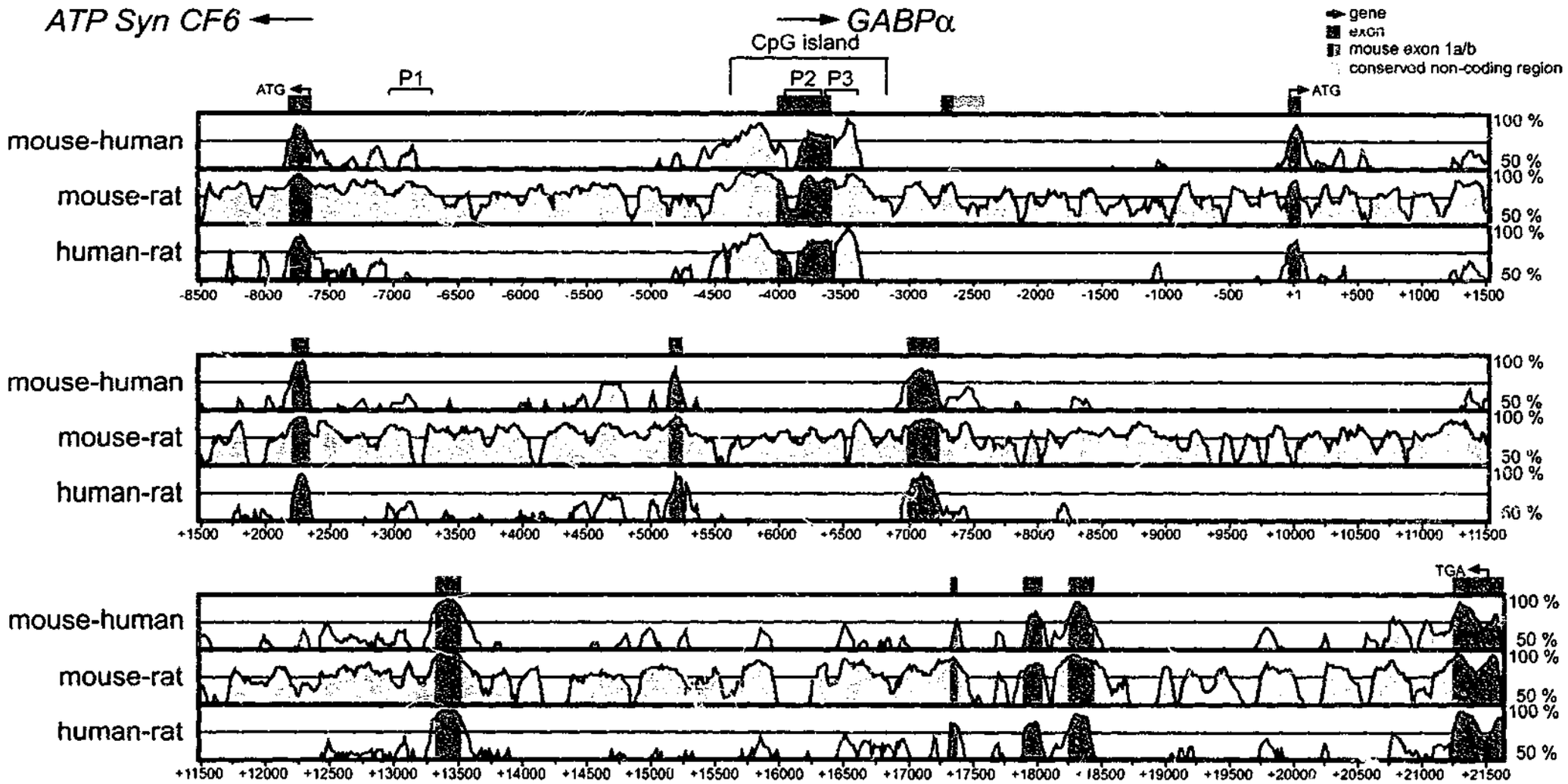


Figure 4.8 - Conservation of *GABP*α Genomic Structure Between Species.
VISTA alignment of human, mouse and rat *GABP*α genomic sequences. The first exon of the closely linked gene *ATP Synthase Coupling Factor* 6 is included, as are the predicted CpG island and promoter regions (P1, P2 and P3). Positions of start and stop codons are indicated and numbering is relative to the *GABP*α translation start site (+1). The midline represents a threshold of 75 % conservation.

Nucleotide Identify X (NIX) application (Williams et al. 1998). The most distal promoter region (P1) is downstream of *ATP synthase coupling factor 6* (-6996 to -6700 relative to the translation start site of *Gabpα* +1). The second predicted promoter region (P2) lies within exon 1 of *Gabpα* (-3927 to -3627), and the third (P3) immediately downstream of this (-3601 to -3310), within the flanking CpG island of -4314 to -3162. Therefore, we examined whether these predicted promoter regions, as well as exons 1, 1a and 1b of the mouse, possess promoter function.

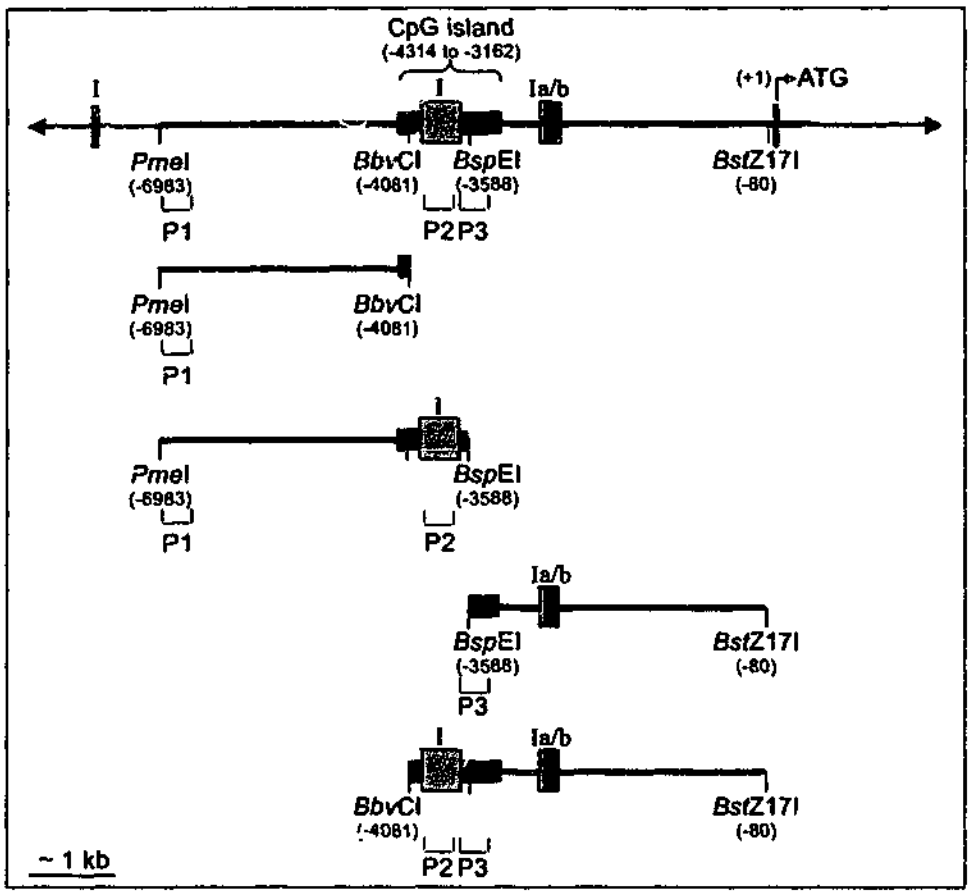
4.4.2 *In vitro* Analysis of *Gabpα* Promoter Activity

The promoter activity of the *Gabpα* 5' region was assessed using the Luciferase reporter assay, which has conventionally been used for measuring transcriptional activity in mammalian cell lines (Nordeen 1988; Brasier et al. 1989). Four reporter constructs were created, using unique restriction endonuclease sites that flank the three predicted *Gabpα* promoter regions P1, P2 and P3, and sub-cloning upstream of *Luciferase* in the pGL-3 basic reporter vector (Promega) (see **Figure 4.9a**) Constructs were sequenced (with primer 3, spanning 4760-4779 bp of GI:U47295), then transiently co-transfected with the *β-galactosidase* reporter vector (Promega) into NIH3T3 mouse fibroblast, PC12 neuronal-like rat adrenal pheochromocytoma and C2C12 mouse myoblast cell lines, and assayed for cell type-specific promoter activity.

Expression Analysis of *Gabpα-Luciferase* Reporter Constructs

As shown in **Figure 4.9b**, when Luciferase expression level was adjusted to account for transfection efficiency (β-galactosidase expression level) and expressed as fold induction above the empty pGL3 basic vector, the highest level of *Gabpα* promoter activity was observed in the C2C12 skeletal muscle cell line, consistent with the proposed role of Gabp in the regulation of neuromuscular signalling proteins. *Gabpα* promoter activity was enhanced in C2C12 cells by addition of differentiation media immediately following transfection, indicating that Gabp may function by regulating genes involved in skeletal muscle differentiation. The same relative pattern of promoter activity was seen across 3T3, C2C12 and differentiated C2C12 cell lines. Induction of *Luciferase* activity was observed across the three cell lines (10 to 40-fold) with the *Pme*I-*Bbv*CI construct (-6983 to -4081), which encompasses P1 and sequence upstream of *Gabpα* exon 1. This activity was enhanced (up to 80-fold induction in differentiated C2C12 cells) when cells were instead transfected with the *Pme*I-*Bsp*EI construct (-6983 to -3588), which also

(a)



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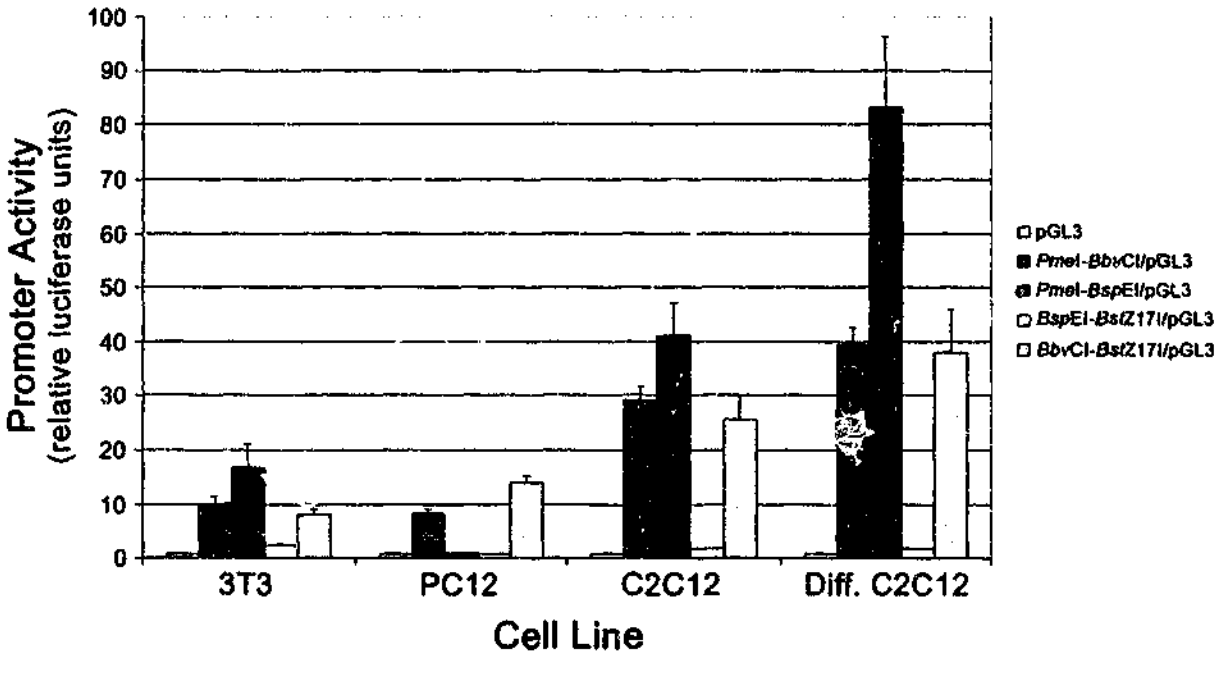


Figure 4.9 - Analysis of *in vitro Gabpα* Promoter Activity.
The *Gabpα* promoter and relevant 5' restriction fragments are shown in **(a)**. Restriction endonuclease sites, exons, predicted promoter elements (P1, P2 and P3), and CpG island encompassing exon 1 of *Gabpα* are indicated. **(b)** Resulting *Luciferase* activities of each construct are shown for 3T3, PC12, C2C12 and differentiated C2C12 cell lines, expressed as fold induction above background (empty pGL3 vector). Error bars show SEM, where n=16 for differentiated C2C12s, n=19 for PC12s and n=23 for 3T3s and C2C12s. Nucleotide numbering is relative to the *Gabpα* translation start site (+1).

includes *Gabpα* exon 1 (P2). No *Luciferase* activity was detected when cells were transfected with construct *Bsp*EI-*Bst*Z17I (-3588 to -80), which contains *Gabpα* intron 1, P3 and exons 1a and 1b. This indicates that the alternative 5' UTRs of mouse *Gabpα* do not function as alternative promoters. However, when exon 1 was added to this intronic region, in the form of construct *Bbv*CI-*Bst*Z17I (-4081 to -80), promoter activity was obtained at equal levels (10 to 40-fold induction) to those seen with construct *Pme*I-*Bbv*CI. Therefore the exon 1 region, and sequence immediately upstream, possesses most of the promoter activity of *Gabpα*.

The exception to this trend of results was seen in PC12 cells (see **Figure 4.9b**), where the addition of construct *Pme*I-*Bsp*EI, which gave highest *Luciferase* induction in all other cell lines, yielded no promoter activity. In PC12 cells the *Pme*I-*Bbv*CI construct, containing promoter elements upstream of *Gabpα* exon 1, gave similar *Luciferase* induction (8-fold) as seen in 3T3 cells. The highest promoter activity in PC12 cells was observed when transfected with the *Bbv*CI-*Bst*Z17I construct, containing *Gabpα* exons 1, 1a, 1b and intron 1 (encompassing predicted promoters P2 and P3). However, as with the other cell lines tested, transfection of PC12 cells with the *Bsp*EI-*Bst*Z17I construct, containing only *Gabpα* exons 1a and 1b and intron 1 (P3), resulted in no *Luciferase* induction. This indicates the presence of a unique repressing transcription factor or absence of an activator in the PC12 cell line, and highlights the ability of *Gabpα* expression to be regulated in a cell type-specific manner. Together, the results obtained from the *Luciferase* assay are consistent with the presence of a CpG island encompassing exon 1, as these structures often contain functional promoter elements (Gardiner-Garden and Frommer 1987). We examined this sequence further, for the presence of transcription factor binding sites.

Transcription Factor Binding Sites within the *Gabpα* Promoter

Transcription factor binding sites within the sequence encompassed by the CpG island flanking exon 1 of *Gabpα* (-4314 to -3162) were predicted using the Transcription Element Search System (TESS) (Schug and Overton 1997), BCM Search Launcher (Smith et al. 1996b), MatInspector (Quandt et al. 1995b) and rVISTA (Loots et al. 2002) computer programs. Several basal and skeletal muscle-specific initiation sites were identified in this region by the prediction programs, as shown in **Figure 4.10**. Few transcription factor binding sites lie within the P2 region of exon 1, with most clustering immediately upstream

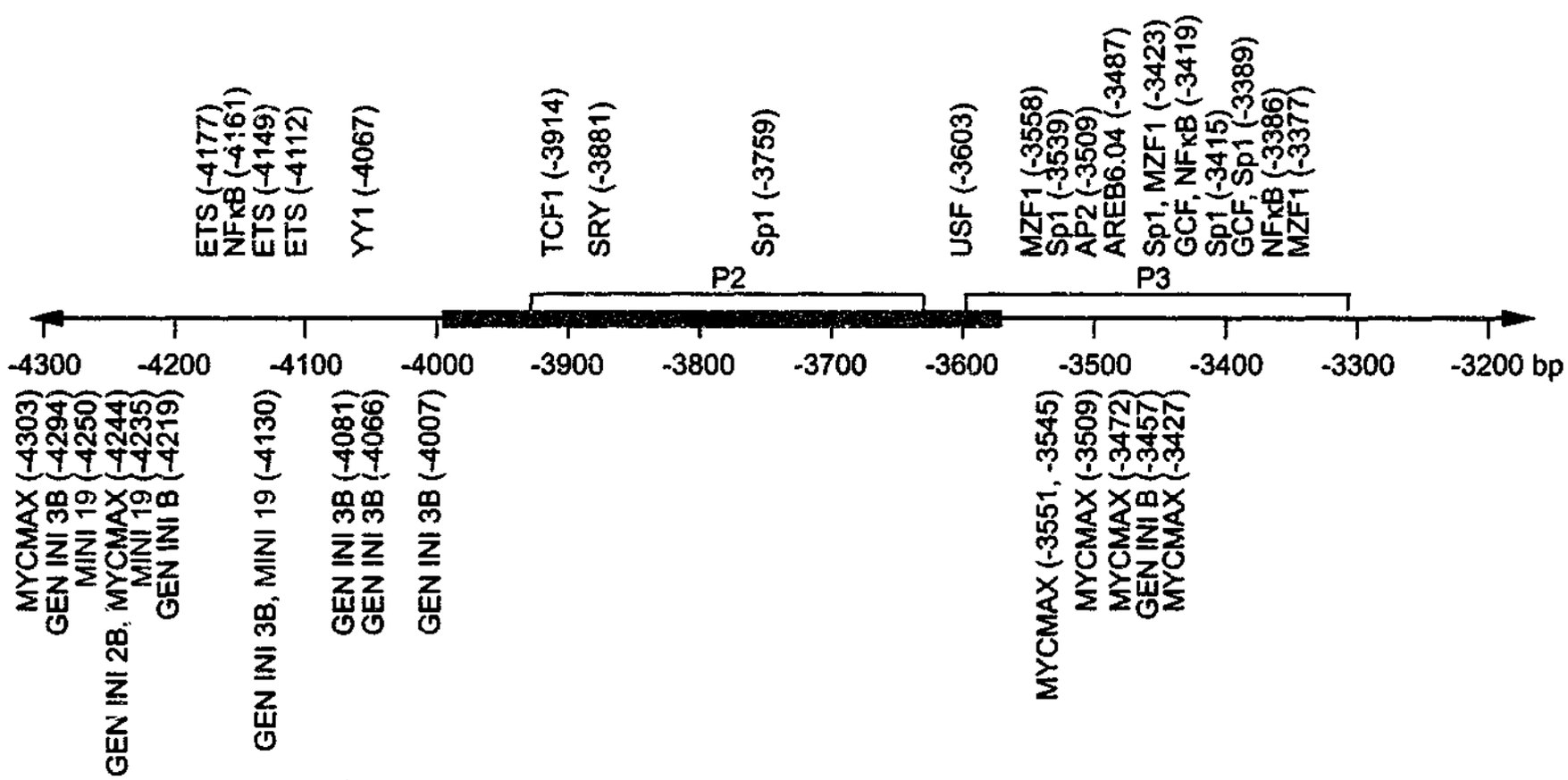


Figure 4.10 - Transcription Factor Binding Sites of the *Gabpα* Promoter.
Transcription factor binding sites within the CpG island region of *Gabpα*, as predicted by TESS, MatInspector, Search Launcher and rVISTA computer programs. ETS binding sites are shown in blue, muscle-specific initiation sites (MINI) in red, and general initiation (GEN INI) and transcription factor binding sites in black. NIX analysis predicted promoter regions P2 and P3 are shown, as is exon 1. Nucleotide numbering is shown relative to the translation start site (+1).

or downstream of exon 1. The three ETS binding sites and the Ying-Yang 1 (YY1) repressor binding sites in the region upstream of P2 were previously described (Chinenov et al. 2000), and this upstream region has been shown to possess bi-directional promoter activity, driving expression of *Gabpα* and the closely linked gene *ATP synthase coupling factor 6*. This is consistent with our transactivation data, however successive 5' promoter deletion is necessary to determine if this region of *Gabpα* (within the CpG island) is the minimal promoter. The general transcription initiation sequences and binding sites for ETS proteins, Sp1, YY1, NFκB, Myc/Max and AP2 present within the CpG island promoter region of *Gabpα* correlate with the ubiquitous expression of the Gabpα protein. However, presence of muscle-specific transcription initiation sequences and binding sites for the haematopoietic transcription factor Mzf1 reflect the ability of Gabp to transactivate tissue-specific genes. Site-specific mutation studies are necessary to identify the transcription factors responsible for basal *Gabpα* expression, and the cell type-specific effects seen in PC12 cells.

4.4.3 *Gabpα* 5' UTR Transcript Secondary Structures

The alternative 5' UTRs of *Gabpα*, exons 1a and 1b, do not contain any functional promoter elements. Therefore, to assess whether the alternative exons 1 may play a role in *Gabpα* translational efficiency, secondary structures and the corresponding free energy values of *Gabpα* transcripts containing the three different 5' UTRs (together with the previously characterised exon 10a polyadenylation signal) were predicted using the mfold program (Zuker 2003). As shown in **Figure 4.11**, a *Gabpα* transcript containing exon 1a has a less branched secondary structure than transcripts containing exons 1 or 1b, and is the least stable structure, with the highest free energy value of -433.91 kcal/mol. The exon 1 *Gabpα* transcript is the most stable, with a free energy of -612.79 kcal/mol and lengthy stem-loop structures. The exon 1b *Gabpα* transcript has a free energy of -480.16 kcal/mol and a predicted secondary structure with shorter branches than the exon 1 transcript.

During the course of investigation of the *Gabpα* 5' UTR transcripts, it was generally noted that RT-PCR products spanning exon 1 to the initiation codon within exon 2 were less efficiently amplified than those spanning exon 1a or exon 1b to exon 2, even though amplification of larger RT-PCR products shows the exon 1 transcript to be more abundant than exon 1a and exon 1b *Gabpα* transcripts (see **Figure 4.5**). This correlates with the predicted central location of the initiation codon within the exon 1 *Gabpα* transcript

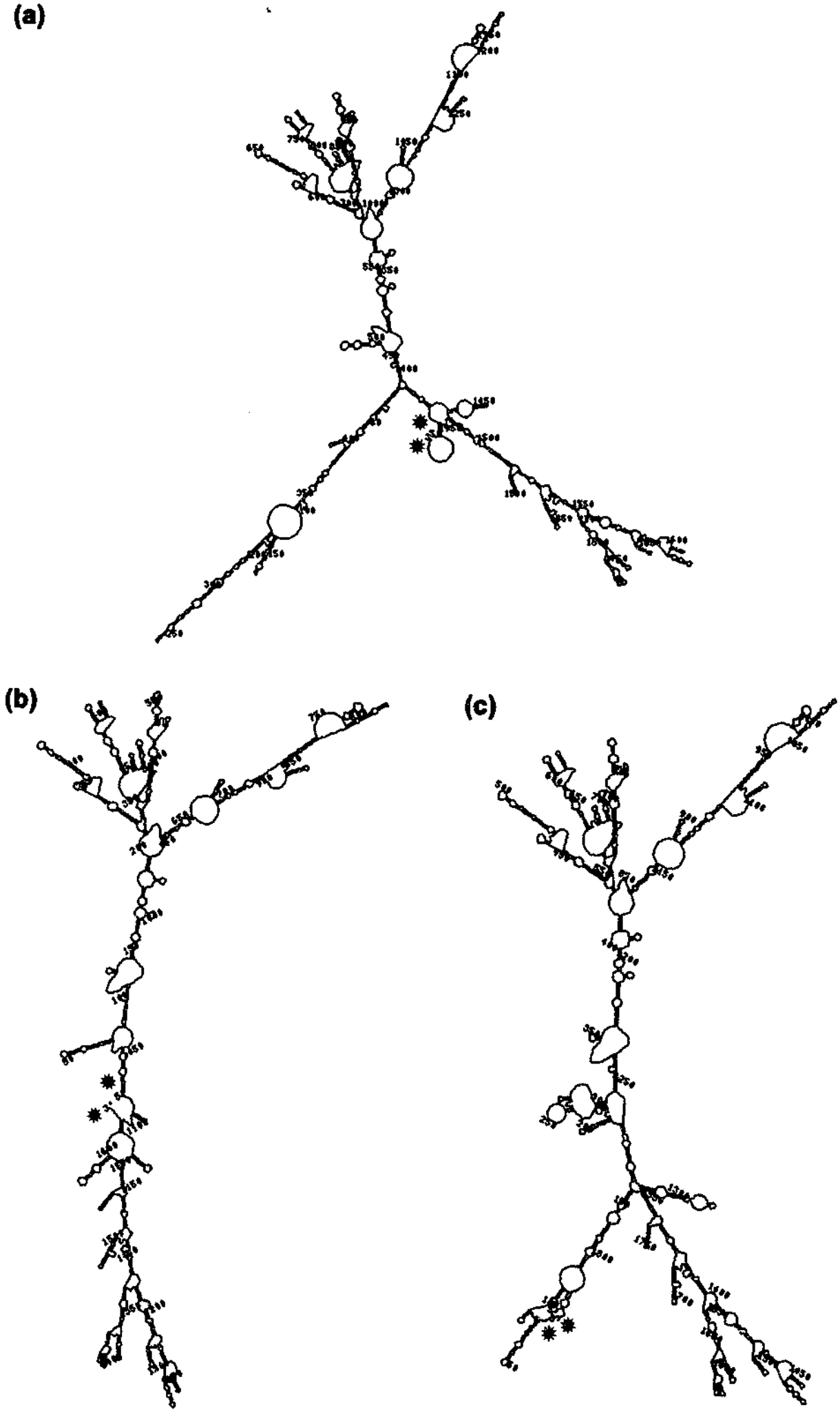


Figure 4.11 - Secondary Structures of *Gabpα* 5' UTR Transcripts.
The stem-loop secondary structures of the mouse *Gabpα* **(a)** exon 1, **(b)** exon 1a, and **(c)** exon 1b 5' UTR transcripts (containing exon 10a), as predicted by the mfold program. The 5' end of each transcript is indicated in green and the 3' end in red.

branched secondary structure. Together with the low free energy of the exon 1 *Gabpα* transcript, the location of the initiation codon may result in decreased translational efficiency, as more free energy would be required to melt the secondary structure to reach the initiating AUG. This has been shown *in vitro* to be the case for the bovine liver mitochondrial *Aldehyde dehydrogenase* mRNA, versus that of the rat, by means of translation of chimaeric mRNAs in rabbit reticulocytes (Guan and Weiner 1989). Therefore, *Gabpα* transcripts containing the less structured exon 1a may be favoured for translation due to the ease of ribosomal binding, and transcripts containing exon 1 may be less efficiently translated due to increased secondary structure.

Repressors and activators that bind to the 5' UTR can also influence where ribosomes bind to initiate translation, resulting in variable protein expression due use of alternative 5' UTRs (Sonenberg 1994). The expression pattern of the three *Gabpα* 5' UTR transcripts suggests that the translation efficiency of *Gabpα* may be regulated in a cell type-specific manner. This is the case for the *Gli-1* oncogene, where expression of the usually lowly abundant γ-5' UTR variant correlates with increased cell proliferation in basal cell carcinomas (Wang and Rothnagel 2001). The translational efficiencies and stabilities of the three 5' UTR *Gabpα* transcripts are yet to be tested experimentally. Only then can a definitive conclusion be made in regard to their function.

4.5 Summary - Transcriptional Regulation of the GABP *Complex*

The genomic structure and 5' regulatory region of *GABPα* is highly conserved between human and mouse, suggesting conserved protein product function. However, the alternative exons 1 within intron 1 of *Gabpα* are unique to the mouse, indicating a species-specific means of transcript regulation. Alternative transcripts of the *Gabpα* and *β1* subunits may be the mechanism behind the Gabp complex achieving specificity of function, in order to regulate such a large number of diverse target genes. Use of alternative transcripts may be an important regulatory mechanism of gene function, only now becoming apparent with the high-throughput sequencing of genomes and expression libraries. For example, a recent genome-wide analysis of 2.2 million human expressed sequence tags (ESTs) identified 27790 alternative splice forms, 667 of which are tissue-specific (Xu et al. 2002).

Gabpα and *β1* genes appear to be subject to tissue-specific modes of regulation. Production of tissue-specific dominant negative splice forms of Gabpβ1 may aid in rapid post-translational regulation of Gabp complex activity in response to environmental stimuli. Expression of alternative *Gabpα* 5' UTRs may be the result of an as yet unidentified alternative promoter. Alternative promoter usage is a suggested means of ensuring ubiquitous expression of a protein, and prevention of gene silencing due to promoter methylation (Ayoubi and Van De Ven 1996). A second possibility is that the alternative 5' and 3' UTRs of *Gabpα* aid in the translational regulation of *Gabpα*, due to differences in mRNA stability and accessibility of ribosome binding sites. Thirdly, the 5' and 3' UTRs of *Gabpα* may determine the intracellular localisation of *Gabpα* transcripts.

Chapter 5

Results:

Characterisation of Gabpα Expression

5.1 Introduction

Gabpα mRNA is expressed in all mouse tissues examined to date (LaMarco et al. 1991), however the quantified expression levels and cellular distribution of Gabpα protein have not been determined. As Gabpα forms a complex with β, the expression level of each subunit may have functional significance for the Gabp complex. Here the generation of polyclonal antibodies raised against Gabpα is described, and characterisation of Gabpα protein levels in the adult mouse. To further our understanding of Gabpα function, *in vitro* overexpression of *Gabpα* was performed and its effect on cellular respiration, cell cycle and target gene expression evaluated. The effect of *in vivo Gabpα* overexpression was also assessed, using Down syndrome fibroblast cell lines and tissues of Ts65Dn partial trisomy 16 mice.

5.2 Characterisation of Gabpα Expression

We have examined *Gabpα* expression at the mRNA and protein level. This is important, as another ETS factor, *PEA3*, is expressed in some human cell types at the mRNA level, but not at the protein level (de Launoit et al. 1997). In the few rat tissues studied for *Gabpα* expression (testes, liver and brain), no correlation was found between mRNA and protein levels (Vallejo et al. 2000). This indicates that, at least in the rat, gene regulation of *Gabpα* occurs at both the transcriptional and post-transcriptional levels. We investigated this further in the mouse.

5.2.1 Production of Gabpα Polyclonal Antibodies

Sequence analysis (BLAST) of the Gabpα mouse (GI:6679899) and human (GI:13646482) protein sequences revealed 96 % amino acid identity (see **Figure 5.1**),

Figure 5.1 – Conservation of GABPα Protein Sequence.

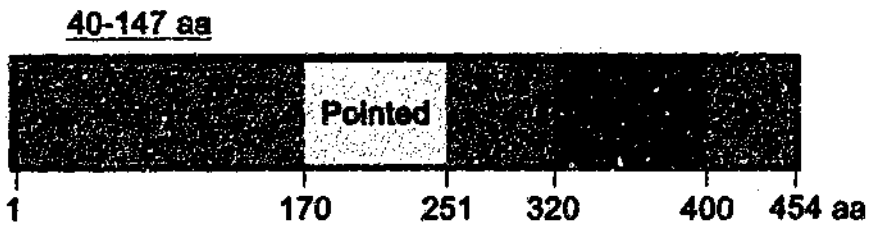
BLAST alignment of the 454 amino acid sequences of human and mouse GABPα proteins, showing 96 % amino acid identity and 98 % amino acid similarity between the two species. The consensus amino acid sequence is shown between those of human and mouse, and identical amino acids are in bold typeface. Residue numbers are indicated on either side of the sequence. Conserved protein domains are highlighted; Pointed in yellow, ETS in blue and Gabpβ interaction domain in pink.

therefore any antibody generated should be capable of recognising GABPα from both species. Antibodies to Gabpβ were not generated, due to lack of a unique sequence of sufficient length between different isoforms of the protein, as well as the presence of repetitive ankyrin repeats.

Gabpα contains ETS and Pointed domains that are conserved in other ETS factors, therefore a unique N-terminal region of 108 amino acids of Gabpα was chosen for antibody production (see **Figure 5.2a**). Bases 572-895 of *Gabpα* cDNA GI:193382, corresponding to amino acids 40-147 of GI:6679899, were cloned into the *Sma*I site of the pQE-31 His-tag expression vector (Qiagen), using a *Bst*OI-*Ban*I digest and the construct was sequenced with primer 51, spanning 3371-3388 bp of the pQE-31 vector sequence (Qiagen) (cloning performed by Dr. Sika Ristevski, Centre for Functional Genomics and Human Disease, Monash IRD, Victoria, Australia). Recombinant protein expression was performed using BL21 DE3 (pLysS) bacterial cells (Stratagene) and eluted on TALON nickel resin (Clontech), as per manufacturer's instructions. An induction series with 0.4 mM IPTG was performed and a plateau of protein production was found to occur after 1 hour (see **Figure 5.2b**). The protein concentration resulting from a large-scale preparation under these conditions was estimated using a Coomassie Spot Test, and Bradford assay, and 0.8 μg of recombinant protein was injected into each of two rabbits (in Freund's complete adjuvant (Sigma)), with two subsequent booster injections. Sera samples taken following the second booster injection were tested for specificity towards Gabpα prior to a final booster injection.

Antibody titres of sera from the two immunised rabbits were determined by ELISA against the recombinant Gabpα immunogen. As shown in **Figure 5.2c**, pre-immune sera from both rabbits showed no innate reactivity to Gabpα. The titre of rabbit 1 was deemed to be 1/10000 and that of rabbit 2 was 1/5000. Pre-absorbing the antisera overnight at 4°C with 1 μg Gabpα recombinant protein prior to use did not, however, result in any decrease in reactivity. This could have been due to addition of too little recombinant protein and/or use of too high a concentration of antisera. Therefore 10 μg Gabpα recombinant protein was subsequently used to pre-absorb antisera of higher dilution prior to Western blot immunodetection of Gabpα.

(a)



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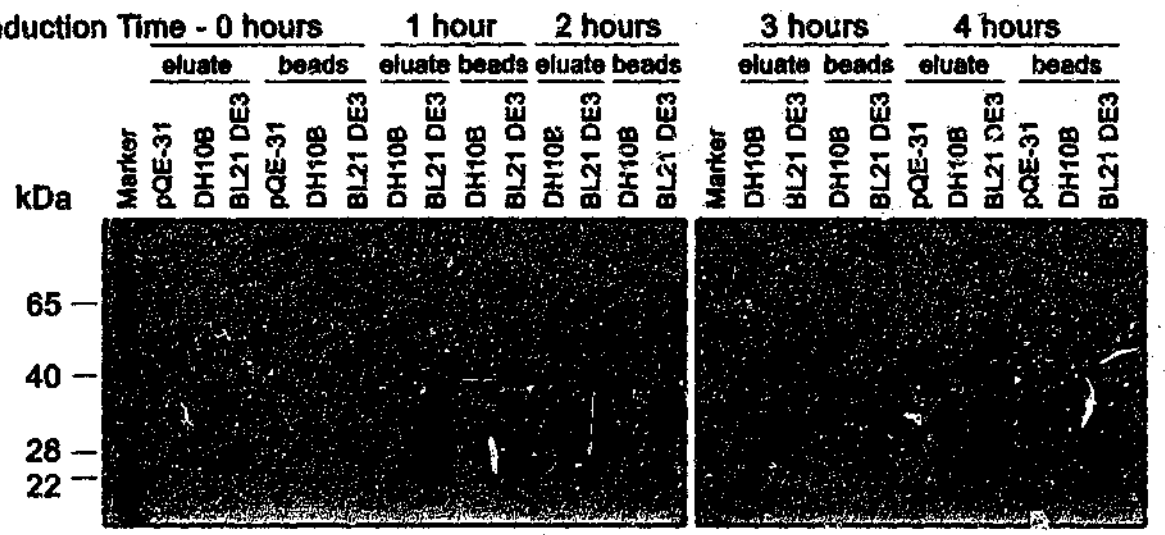

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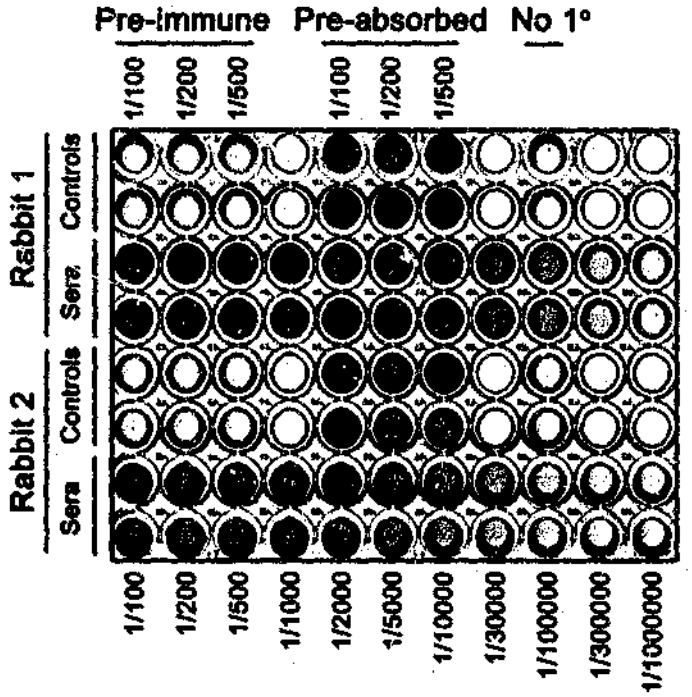


Figure 5.2 - Production of Polyclonal Gabpα Antibodies.
Protein domains of mouse Gabpα are shown in (a); Pointed in yellow, ETS in blue and Gabpβ interaction domain in pink. The 40-147 amino acid (aa) region used for Gabpα polyclonal antibody production is indicated. (b) Coomassie stained SDS-PAGE of the purified 30 kDa His-tagged Gabpα recombinant protein 0-4 hours post-IPTG induction in DH10B or BL21 DE3 bacterial cells. Empty vector (pQE-31) was also included as a negative control. (c) ELISA testing the reactivity and titre of rabbit antisera against the 30 kDa Gabpα recombinant protein. Dilutions are indicated for test bleed, pre-immune and pre-absorbed sera. No primary antibody (No 1°) was used as a negative control.

5.2.2 Validation of Gabpα Polyclonal Antibodies

The specificity of the antisera derived from the two rabbits was examined by Western blot analysis using Gabpα recombinant protein, quadricep skeletal muscle extract and lysate from NIH3T3 cells. Initial testing showed that both antisera detect a 58 kDa band representing full length Gabpα (in combination with 0.3 μg/ml goat anti-rabbit horse radish peroxidase (HRP) conjugated IgG (DAKO) secondary antibody), which can be successfully pre-absorbed with 10 μg of recombinant Gabpα protein (see **Figures 5.3a** and **5.3c**). Other bands were deemed non-specific, as they did not disappear after pre-absorption of the antisera with Gabpα recombinant protein. Titration of primary and secondary antibodies demonstrated that the antiserum from rabbit 2 shows more non-specific immunoreactivity than that of rabbit 1 (see **Figures 5.3b** and **5.3d**). Therefore, in all Gabpα protein analyses shown in the following chapters, 8 % reducing SDS-PAGE and Western blot were followed by incubation of membranes with a 1/5000 dilution of rabbit 1 antisera, followed by 0.15 μg/ml goat anti-rabbit HRP conjugated IgG (DAKO), as this displayed few non-specific bands and detected Gabpα at low concentrations.

5.2.3 Analysis of Gabpα Expression in the Adult Mouse

Tissues from 3 male and 6 female 8-9 week old mice of an F1 (C57/Bl6 x CBA) background were analysed for Gabpα protein expression levels (see **Figure 5.4a**). Pooled protein extracts were used to reduce any effects of natural variation between animals. Western blot analysis was performed and duplicate membranes were probed with 0.05 μg/ml anti-β-tubulin mouse monoclonal antibody (Chemicon), followed by 0.3 μg/ml rabbit anti-mouse HRP IgG (DAKO), to indicate protein loading. Gabpα was found to be expressed in all tissue examined, although barely detected in small intestine and liver. Gabpα protein levels were quantified relative to β-tubulin. In order to average the results of several experiments, ratios of Gabpα to β-tubulin were adjusted such that the tissue of highest expression had a ratio equal to 1. As shown in **Figure 5.4b**, Gabpα is most abundant in the bone marrow, spleen, thymus, lung, ovary/uterus and seminal vesicles. The high levels of expression seen in haematopoietic and immune tissues reflect Gabp function in regulating expression of *IL-2*, *IL-16*, *CD18*, *thrombopoietin* and the γ_c chain of cytokine receptors (see **Table 2.5**). High levels of Gabpα protein in reproductive organs may reflect the fast proliferation rate and high density of mitochondria in these cells, as other target genes of GABP include the cell cycle regulator Rb, components of

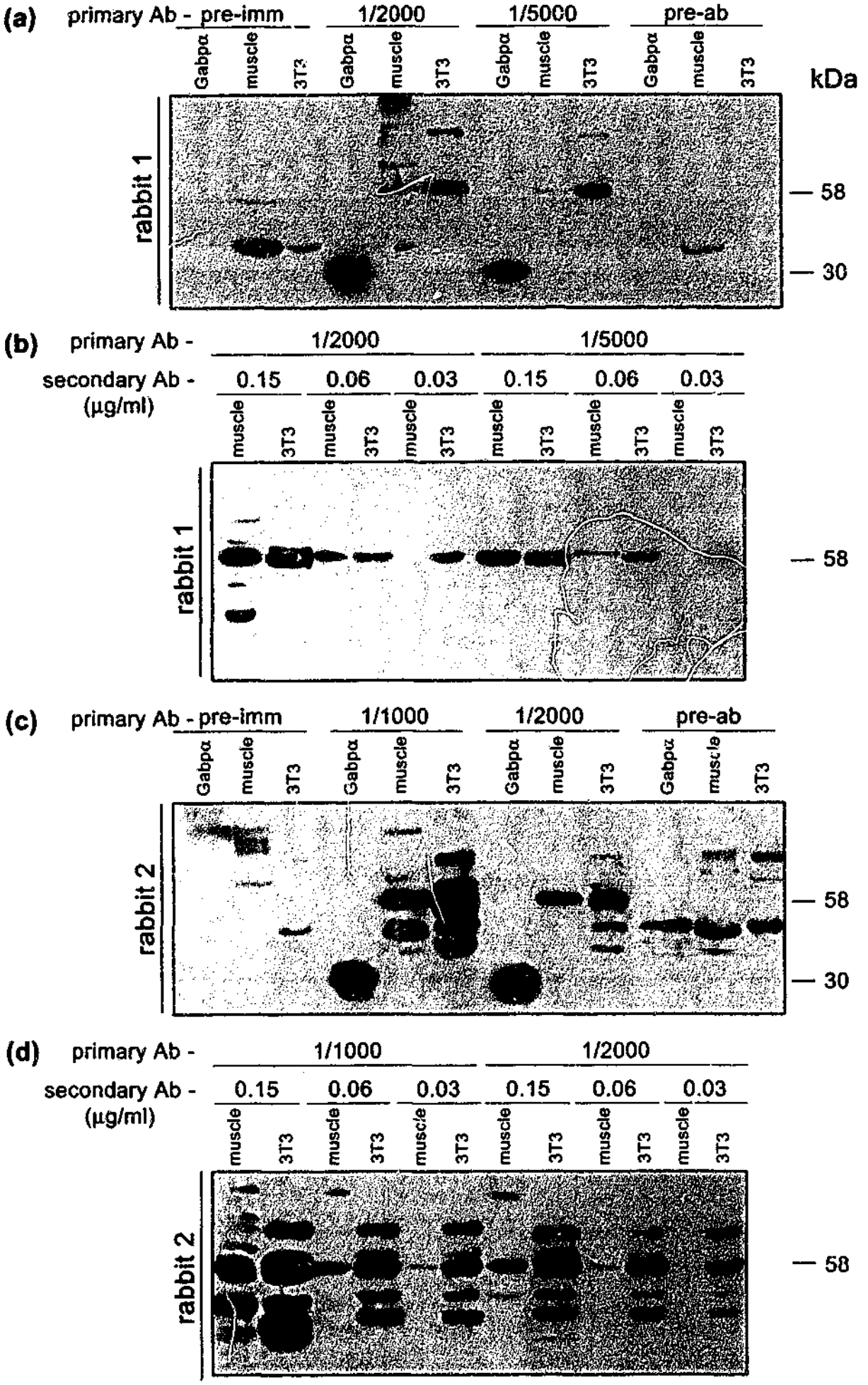
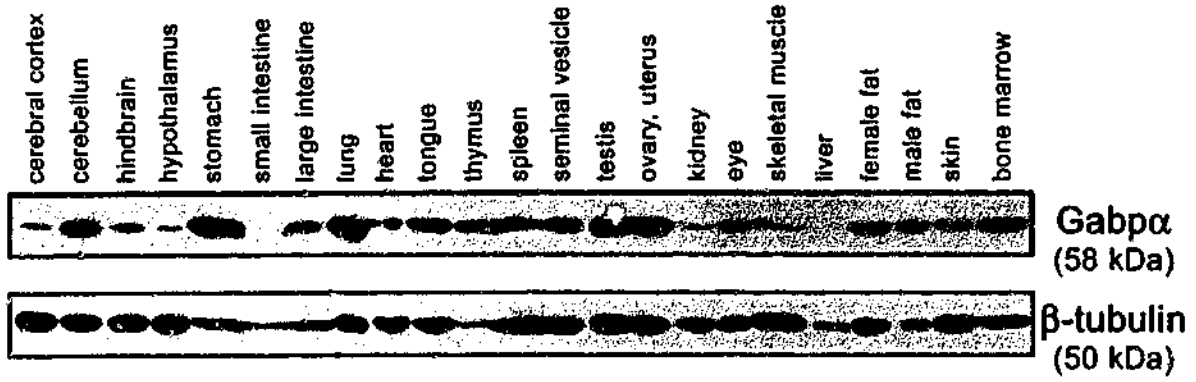


Figure 5.3 - Western Blot Immunodetection with Gabpα Antisera.
Rabbit antisera were tested for Gabpα immunoreactivity using samples of recombinant Gabpα protein (Gabpα), and extracts from mouse quadricep muscle and 3T3 fibroblast cells. Dilutions of **(a)** rabbit 1 and **(c)** rabbit 2 antisera (primary Ab) recognise the 30 kDa recombinant polypeptide immunogen, as well as an endogenous 58 kDa protein band. Lack of immunoreactivity of pre-immune and pre-absorbed antisera with these two bands confirms their specificity. The concentration of secondary antibody (Ab) was titrated with two dilutions of **(b)** rabbit 1 and **(d)** rabbit 2 antisera.

(a)



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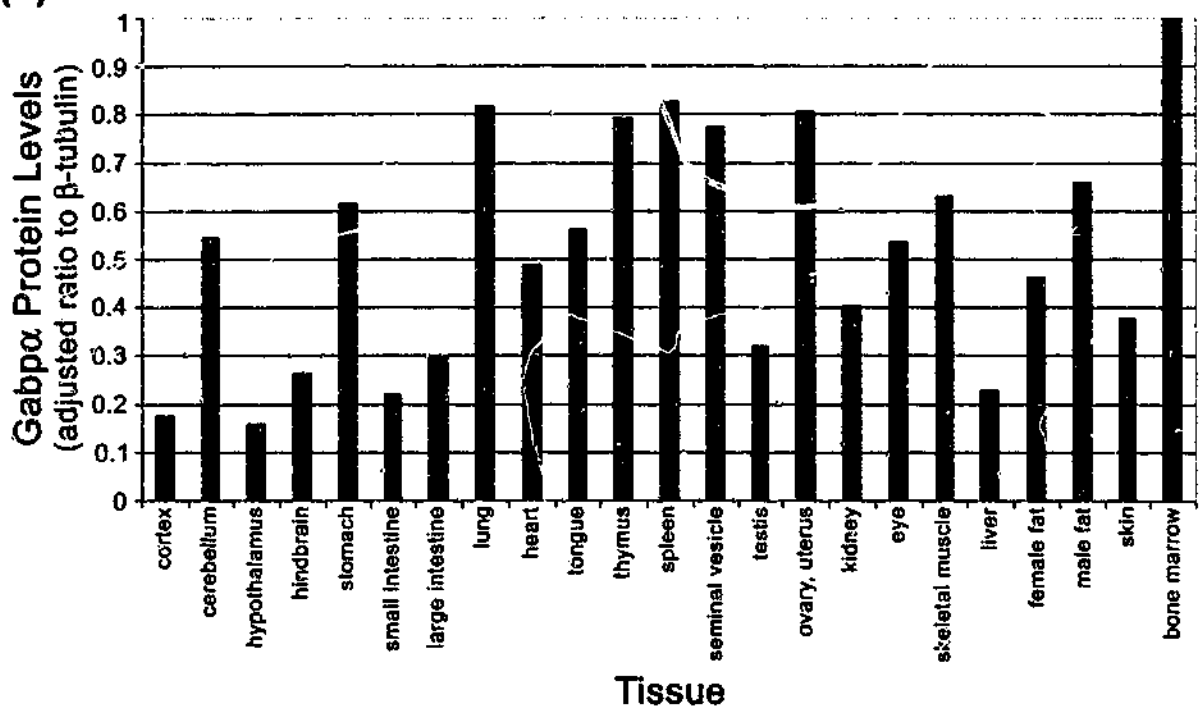


(c)

Tissue	RNA Level	Protein Level
cerebral cortex	+ +	+
cerebellum	+ +	+ + +
hypothalamus	N/D	+
hindbrain	+	+
stomach	+	+ + +
small intestine	+	+ +
large intestine	+	+ + +
lung	+ + + +	+ + + +
heart	+	+ +
tongue	+	+ + +
thymus	+ +	+ + +
spleen	+ +	+ + +
seminal vesicle	+ +	+ + +
epididymis	+	N/D
testis	+ +	+ + +
ovary, uterus	+ +	+ + +
kidney	+	+ + +
eye	+	+ + +
skeletal muscle	+	+ + +
liver	+ +	+ + +
female fat	+	+ + +
male fat	+	+ + +
skin	+	+ + +
bone marrow	N/D	+ + +

Figure 5.4 - Expression Levels of Gabpα Protein in Adult Mouse.
Western blot analysis of adult mouse tissues was performed for Gabpα and β-tubulin proteins **(a)**, allowing Gabpα levels to be expressed as a ratio to β-tubulin **(b)**. Ratios were adjusted across experiments such that the expression in the bone marrow was equal to 1. **(c)** Comparison of *Gabpα* mRNA and protein levels in adult mouse tissues, relative to the housekeeping *Gapdh* mRNA or β-tubulin protein, where + to +++++ represents relative expression level and N/D indicates not determined.

Cytochrome-c-oxidase, and mitochondrial transcription factors MTFA and B (see **Table 2.5**). The reason for Gabpα abundance in the lung is less clear, but could reflect the high abundance of macrophages in this tissue.

Northern blot analysis of *Gabpα* mRNA levels was also performed on poly-A+ purified RNA from adult F1 (C57Bl/6 x CBA) mice. As described in **Chapter 4**, RNA filters were hybridised with a full length *Gabpα* cDNA probe, and relative *Gabpα* transcript levels were determined by subsequent hybridisation of filters with a probe specific for the housekeeping mRNA *Gapdh* (see **Figure 4.3b**). In summary, *Gabpα* mRNA is expressed in all tissues, but at varying levels. As shown in **Figure 5.4c**, in most tissues, the relative Gabpα protein levels reflect *Gabpα* mRNA levels. However, when only two or three tissues are studied (i.e. testes, liver and brain of the rat (Vallejo et al. 2000)) this pattern may be missed, as some tissues appear to exhibit post-transcriptional regulation of *Gabpα*. For example, *Gabpα* mRNA is expressed lowly in tongue, quadricep skeletal muscle and skin, yet relatively highly at the protein level. Conversely, *Gabpα* mRNA is highly expressed in liver, yet the protein level is low. One means of tissue-specific post-transcriptional regulation of *Gabpα* may be the expression of alternative transcripts differing in their stability or translational efficiency, as discussed in **Chapter 4**.

5.2.4 Gabpα Expression in Skeletal Muscle

The expression level of Gabpα in skeletal muscle is of particular interest, as numerous GABP gene targets are expressed at the NMJ. In addition, several of these protein products are expressed at higher levels in differentiated myoblasts compared to undifferentiated myotubes. Utrophin is expressed at two-fold higher levels in differentiated myotubes, due to transcriptional upregulation (Gramolini and Jasmin 1999). Levels of AChRs on the surface of C2 mouse skeletal muscle cells also increases ten to one hundred-fold during their differentiation (Buonanno and Merlie 1986), with a specific eight-fold increase in transcription of the δ subunit. Gabpα protein levels were examined in undifferentiated and differentiated C2C12 mouse skeletal muscle cells, as well as in adult mouse skeletal muscle tissues of different fibre type proportions. Western blot analysis was performed in triplicate on whole-tissue lysates of soleus, gastrocnemius, diaphragm and quadricep muscles from nine 6 week old male C57Bl/6 x SvJ129 mice (see **Figure 5.5a** for a representative image). Gabpα levels were also assessed in cell lysates from undifferentiated C2C12 cells and 72 hours post-differentiation.

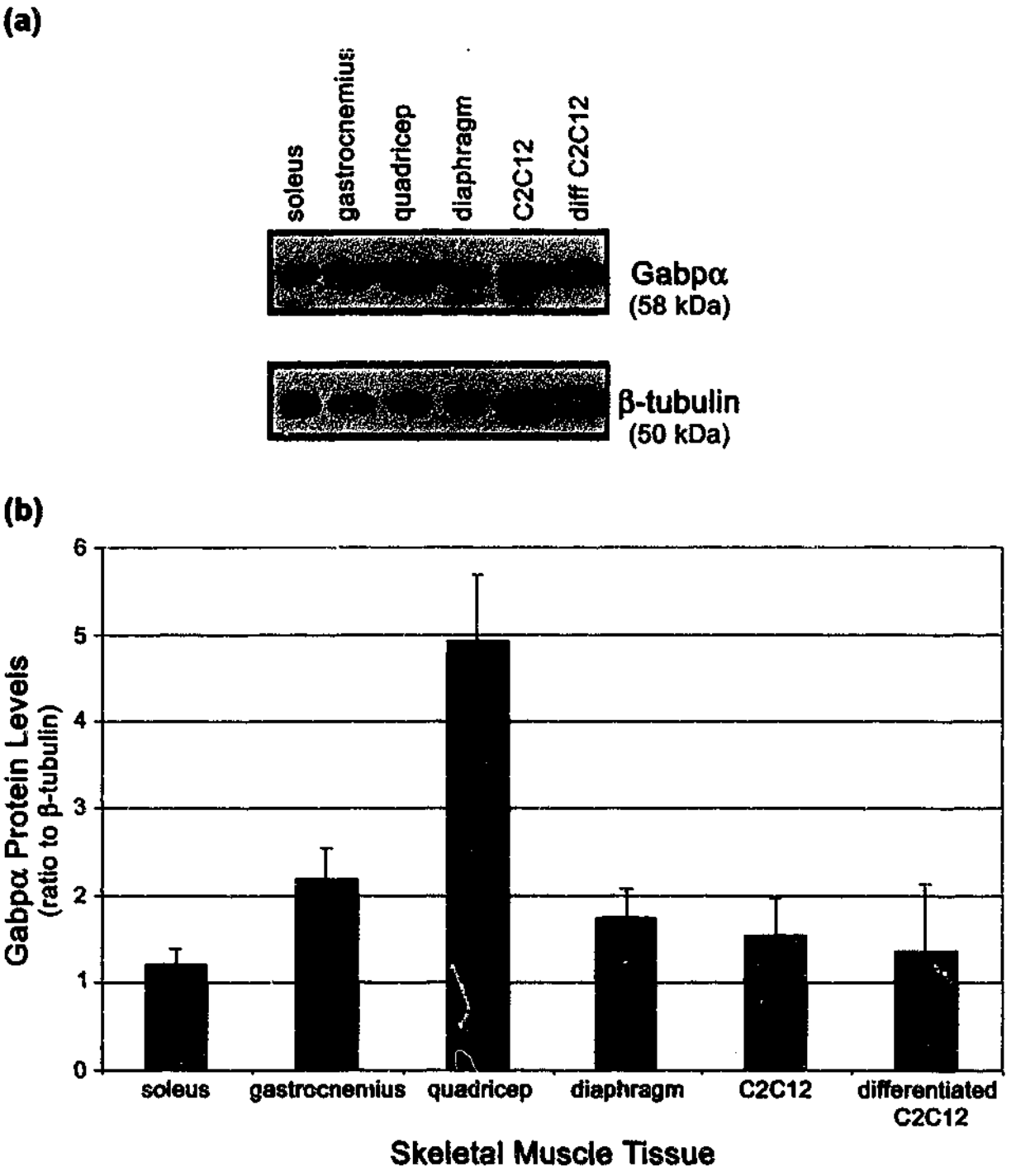


Figure 5.5 - Gabpα Expression Levels in Skeletal Muscle Tissues. **(a)** Western blot analysis of Gabpα and β-tubulin protein levels in skeletal muscle of the soleus, gastrocnemius, quadricep and diaphragm of adult mice, and cell lysates of undifferentiated and differentiated C2C12 cells. **(b)** Quantification of Gabpα protein levels in skeletal muscle tissue and cells, expressed relative to β-tubulin. Error bars represent SEM, where n= 21 for soleus, n= 26 for gastrocnemius, n= 27 for quadricep, n= 24 for diaphragm, and n= 9 for undifferentiated and differentiated C2C12 cells.

When quantified relative to the β-tubulin housekeeping gene (see **Figure 5.5b**), no significant difference was found in Gabpα protein levels of undifferentiated or differentiated C2C12 cells. This suggests that post-translational regulatory mechanisms such as phosphorylation may result in increased Gabp complex activity and subsequent transcriptional activation of target genes (Fromm and Burden 2001). Skeletal muscle tissues of the adult mouse display varying Gabpα protein levels. Quadricep and gastrocnemius muscles, which consist predominantly fast glycolytic type IIb fibres, express higher levels of Gabpα protein than the fast oxidative diaphragm and slow oxidative soleus muscles. This result was unexpected, as many target genes of Gabp function in the oxidative phosphorylation pathway of mitochondria. However, why the level of Gabpα protein in the quadricep is double that of other skeletal muscle tissues is unknown, and taken together, these results suggest that factors other than differentiation and oxidative capacity help determine Gabpα expression levels in skeletal muscle.

5.2.5 Cellular Expression of Gabpα

The polyclonal antibody generated here was not suitable for detection of Gabpα by immunohistochemistry. The polypeptide used as the immunogen for antibody production may therefore not reflect the conformation of the full length Gabpα protein. However, the *β-galactosidase* reporter gene inserted into exon 2 of *Gabpα* during creation of the total knockout mouse model (see **Chapter 6**) was successfully used to determine the cellular localisation of Gabpα in adult mouse tissues. β-galactosidase (lacZ) staining was performed on both formalin-fixed paraffin sections and fresh cryosections of tissue from heterozygous *Gabpα* mice and tissues from wildtype littermates were stained as controls (see **Figure 5.6** for representative images). The *β-galactosidase* reporter gene used contains an NLS, although staining was not always nuclear, indicating that the NLS may not be functional. In heart and skeletal muscle Gabpα is expressed broadly throughout all muscle fibres, whereas it displays a fairly restricted expression pattern in all other tissues examined. In lung and seminal vesicles Gabpα is expressed in the epithelial cells lining the bronchioles and tubules, respectively. Gabpα is expressed in subsets of developing spermatocytes of the testis, and neurons in the brain, and select cells within blood vessels. Gabpα is also expressed in the proliferative cells of cartilage and in the epithelium of the eye. Therefore, although expressed in all tissues, Gabpα is only detected in a subset of cell types. Unfortunately, the high level of background staining observed in wildtype thymus,

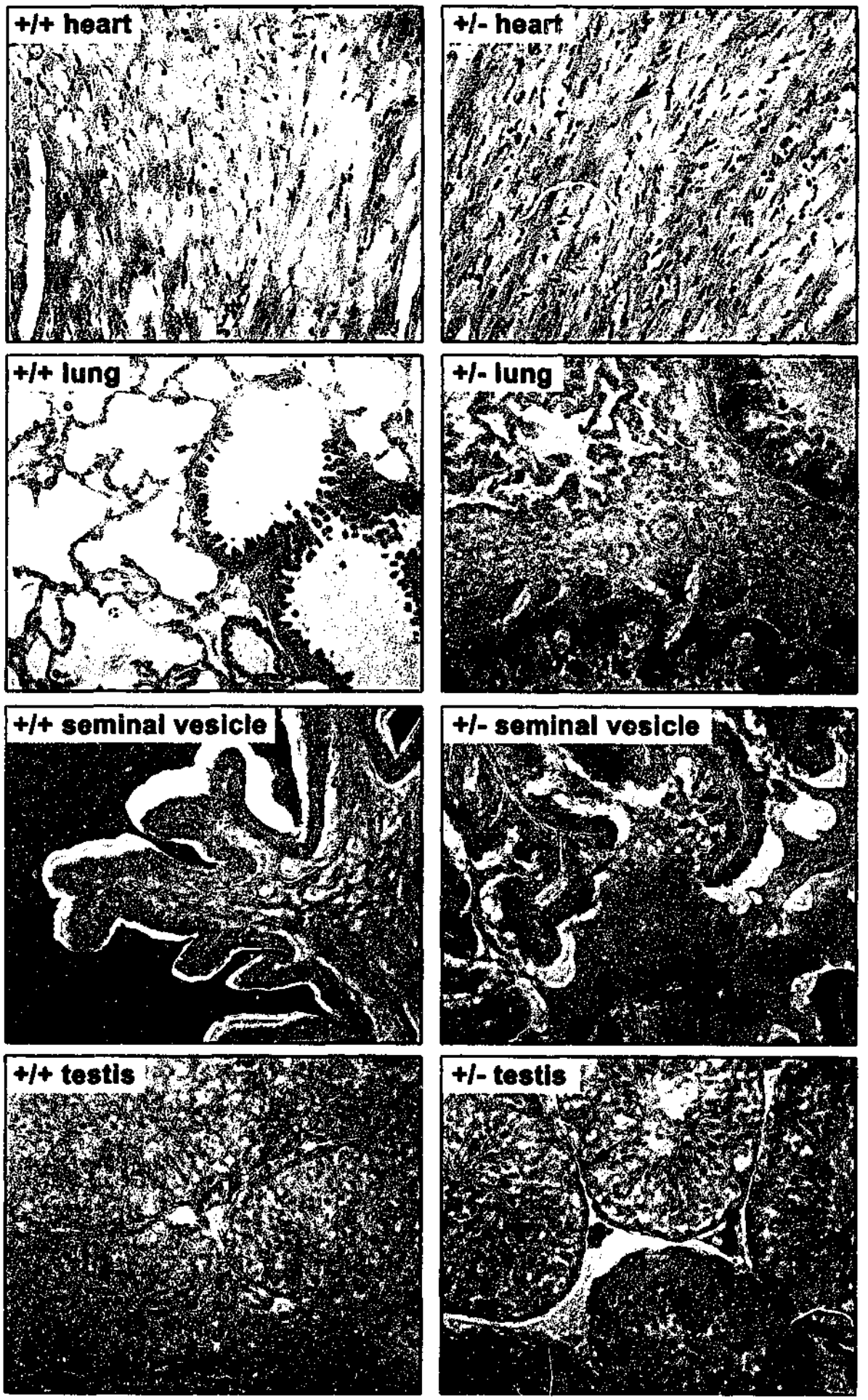


Figure 5.6 - Gabpα Expression in Adult Mouse Tissues.

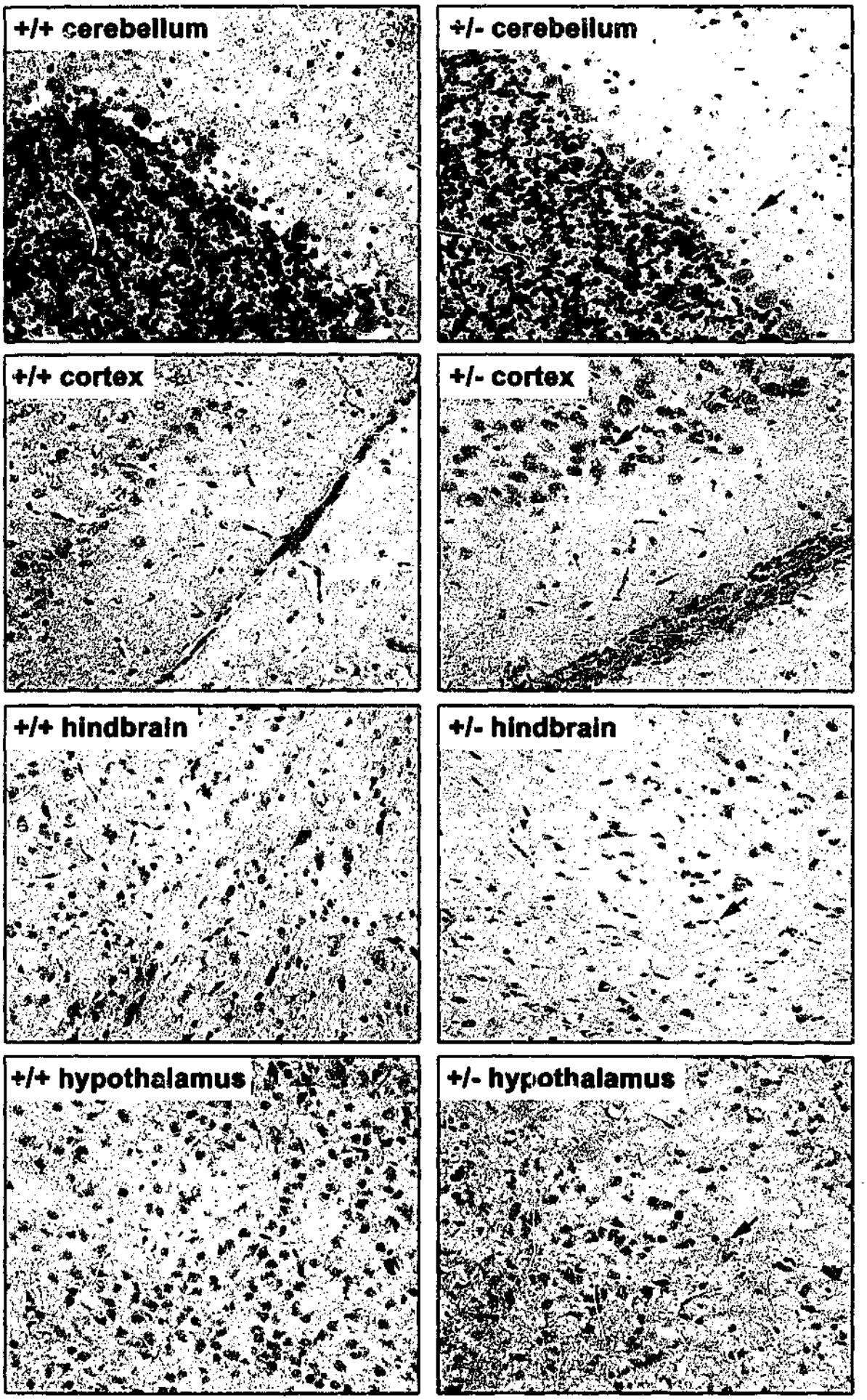


Figure 5.6 - Gabpα Expression in Adult Mouse Tissues.

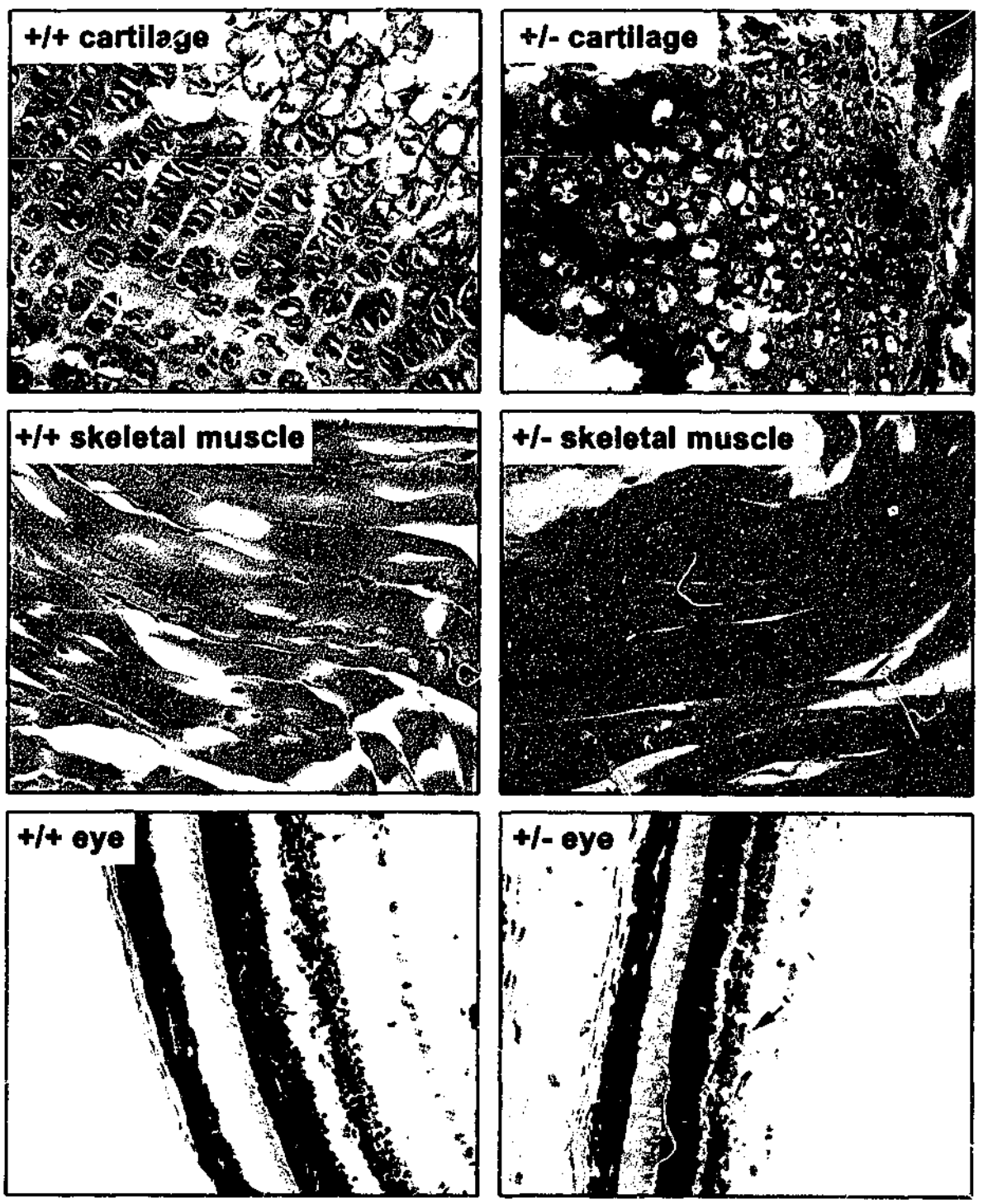


Figure 5.6 - Gabpα Expression Pattern in Adult Mouse Tissues.
LacZ staining of equivalent formalin-fixed tissue sections of wildtype (+/+) and *Gabpα* heterozygous (+/-) mice. Tissues are indicated, and arrows highlight lacZ positive cells, counterstained with haematoxylin and eosin. Images were taken at 400x magnification.

spleen, kidney, liver and ovary/uterus eliminated these tissues from the analysis. Together this indicates that genomic *Gabpα* regulatory sequences allow for cell type-specific expression of Gabpα protein.

Further analysis of Gabpα expression in skeletal muscle was performed, by lacZ and α-bungarotoxin staining (to detect NMJs) of serial longitudinal cryosections of soleus, gastrocnemius and diaphragm skeletal muscle tissues of *Gabpα* heterozygous mice (see **Figure 5.7**). Equivalent *β-galactosidase* reporter gene expression was detected in all muscle fibres, with no increase in staining intensity or number of positive myonuclei surrounding the NMJs. This suggests that it is a characteristic of the *Gabpα* mRNA sequence that is responsible for the preferential expression of *Gabpα* mRNA in sub-synaptic myonuclei, as previously shown by RNA *in situ* hybridisation (Schaeffer et al. 1998). The alternative 5' UTRs of *Gabpα* do not appear to be involved in this process, as exon 1a and 1b transcripts are not expressed in skeletal muscle (refer to **Figure 4.5**). Whether the exon 1 5' UTR or choice of *Gabpα* 3' UTR (by alternative polyadenylation signals) accounts for the sub-cellular localisation of the *Gabpα* transcript within skeletal muscle cells remains to be examined.

5.3 Overexpression of Gabpα in vitro

5.3.1 Generation of NIH3T3 Fibroblasts Overexpressing *Gabpα*

Prior to the generation of a *Gabpα* overexpression transgenic mouse model, *in vitro* overexpression of *Gabpα* cDNA was performed. The NIH3T3 mouse fibroblast cell line was used, to allow for later comparison of transfected cells with immortalised DS fibroblast cell lines obtained from the ATCC. The full length 1.5 kb *Gabpα* cDNA fragment spanning 413-1884 bp of GI:193382 (primers 52 and 53) was removed from the pGEM-T vector backbone by *Nco*I-*Not*I restriction endonuclease digestion, and subsequently cloned into the *Xba*I site of the pEF-*BOS-Puromycin* vector, and the construct was sequenced with primer 201 spanning 1990-2009 bp of the vector backbone (see **Figure 5.8a**). This vector drives expression through the human polypeptide chain elongation factor 1α (*EF-1α*) promoter (Mizushima and Nagata 1990), utilises the polyadenylation signal of *G-CSF* (granulocyte-colony stimulating factor) to stabilise the exogenous transcript, and contains a *Puromycin* resistance cassette for antibiotic selection

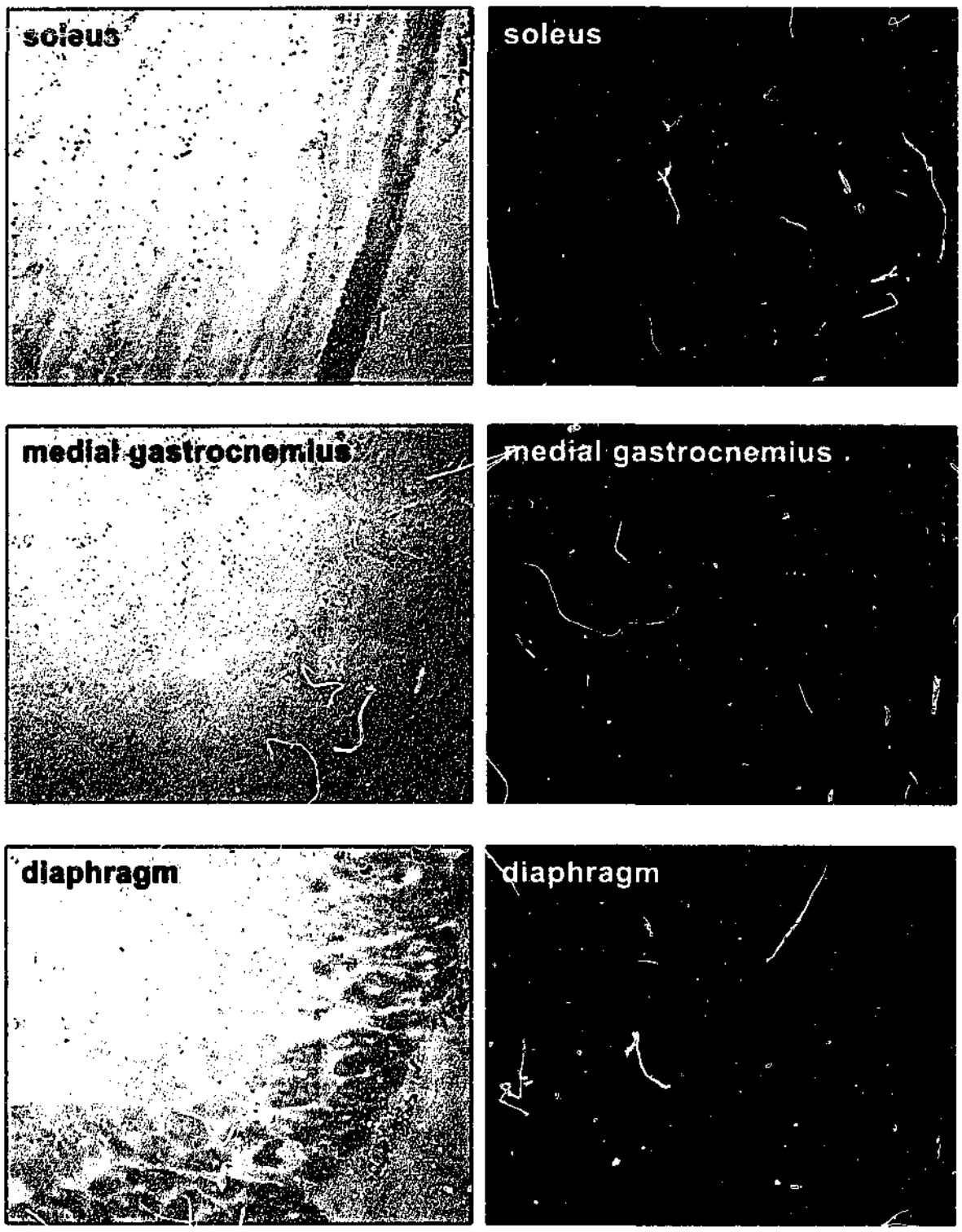
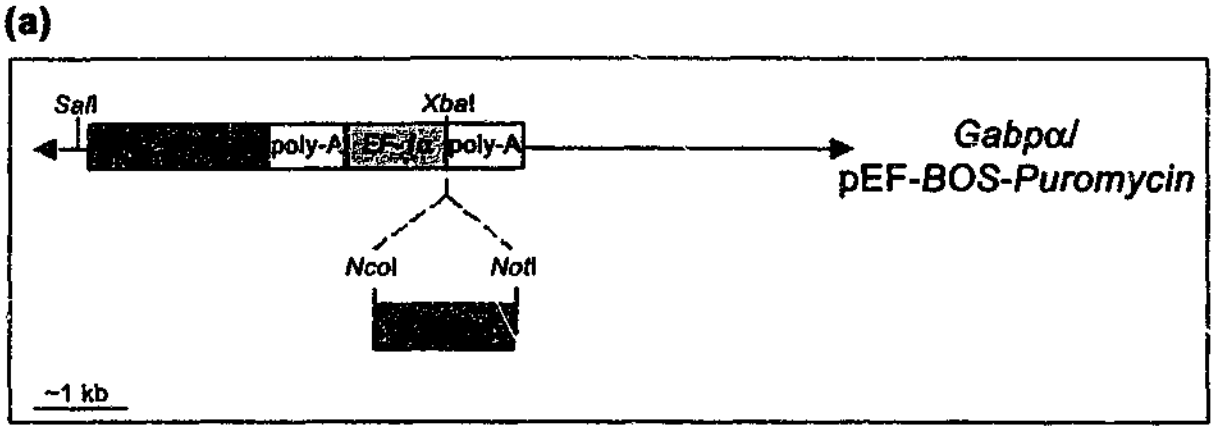
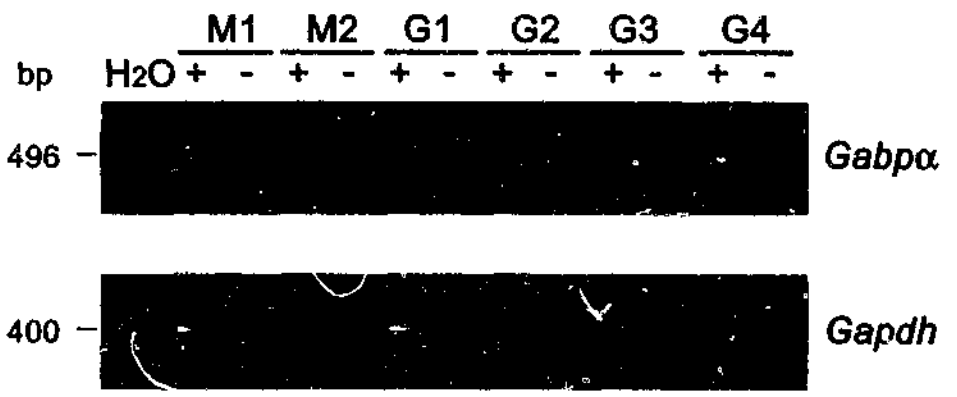


Figure 5.7 - Gabpα and AChR Distribution in Skeletal Muscle.
Serial sections of skeletal muscle from *Gabpα* heterozygous mice were analysed for Gabpα expression by lacZ staining (cells were counterstained with eosin), and AChR expression by staining with Texas red-conjugated α-bungarotoxin. Tissues are as indicated, and images were taken at 200x magnification.





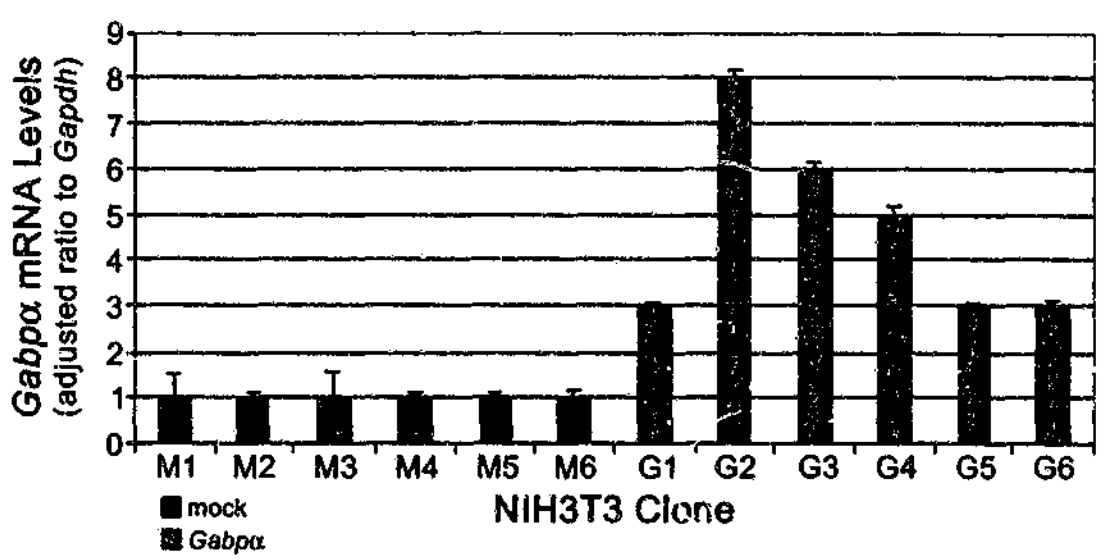


Figure 5.8 - *Gabpα in vitro* Overexpression in NIH3T3 Cells.
(a) Overexpression construct of the *Gabpα* open reading frame (red) under control of the *EF-1α* promoter (orange). The vector also contains the polyadenylation (poly-A) signal of *G-CSF* (yellow) and a *Puromycin* resistance cassette (green). Restriction endonuclease sites are shown. (b) RT-PCR analysis for exogenous *Gabpα* expression, using cDNA from 3T3 clones transfected with mock (M) or *Gabpα* (G) constructs. PCR was performed with primers spanning *Gabpα* exon 9 to within the vector poly-A signal, resulting in a 496 bp product. A 400 bp *Gapdh* RT-PCR product was also amplified to ensure cDNA integrity. (c) Results of real-time RT-PCR analysis for total *Gabpα* mRNA expression using primers spanning *Gabpα* exon 9 to exon 10, expressed as a ratio to *Gapdh*. Ratios were adjusted such that those of mock clones were equal to 1.

in the pEF-*BOS* backbone . The pEF-*BOS-Puromycin* vector has successfully been used to overexpress *ETS-2* cDNA in NIH3T3 cells, resulting in increased ETS-2 protein levels (Sanij et al. 2001). Following *SalI* linearisation of *Gabpα* cDNA and mock (vector alone) constructs, NIH3T3 cells were stably transfected using electroporation and puromycin selection. Individual clones were selected manually and propagated for analysis.

Expression of the exogenous *Gabpα* cDNA in 3T3 clones was confirmed by RT-PCR with a forward 5' primer within exon 9, 1441-1460 bp of *Gabpα* cDNA sequence GI:193382 (primer 155), and a 3' primer at 1990-2009 bp within the polyadenylation signal of the vector backbone (primer 201). Products of 496 bp resulted from cDNA of clones expressing exogenous *Gabpα*, not mock controls (see **Figure 5.8b** for a representative image). The integrity of cDNA samples was confirmed by RT-PCR amplification of a 400 bp *Gapdh* product (primers 58 and 59), spanning 585-985 bp of GI:193423.

The total (exogenous plus endogenous) levels of *Gabpα* mRNA expression were also determined in mock and *Gabpα* 3T3 clones, by means of real-time RT-PCR analysis. Primers spanning *Gabpα* exon 9 to exon 10 (primers 155 and 160) from 1441-1707 bp of GI:193382 were used to generate a 266 bp PCR product. *Gabpα* expression levels were expressed relative to a *Gapdh*, by amplification of a 400 bp product (primers 58 and 59) spanning 585-985 bp of GI:193423. Values were adjusted such that the *Gabpα* to *Gapdh* ratio of mock clones was equal to 1. As shown in **Figure 5.8c**, *Gabpα* 3T3 clones showed a 3 to 8-fold increase in total *Gabpα* mRNA expression levels when compared to mock clones. Eight mock and eight *Gabpα* 3T3 clones were characterised further.

5.3.2 Mitochondrial Function of *Gabpα* NIH3T3 Clones

The affect of *Gabpα* overexpression upon Gabp targets necessary for mitochondrial respiration was assessed in 3T3 clones. Western blot analysis was performed on cell lysates using COXIV (0.2 μg/ml - Molecular Probes) and COXVb (3 μg/ml - Molecular Probes) -specific mouse monoclonal antibodies (Taanman et al. 1993; Capaldi et al. 1995; Taanman et al. 1996) and 0.3 μg/ml rabbit anti-mouse HRP IgG (DAKO). As shown by the representative images and quantification graphs, when levels of COXIV (**Figure 5.9a**) and COXVb (**Figure 5.9b**) proteins were quantified relative to β-tubulin, no significant differences were observed between mock and *Gabpα* clones.

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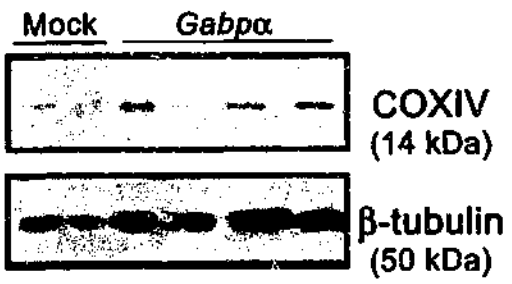

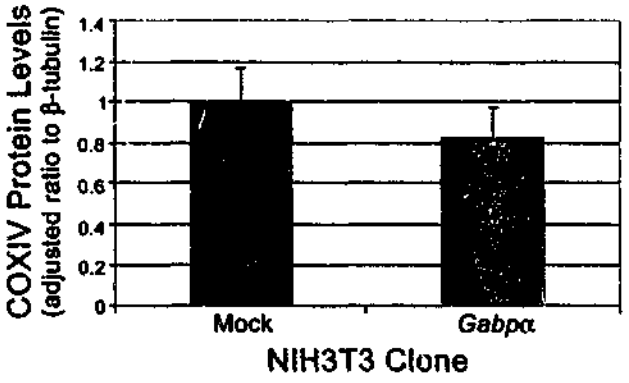

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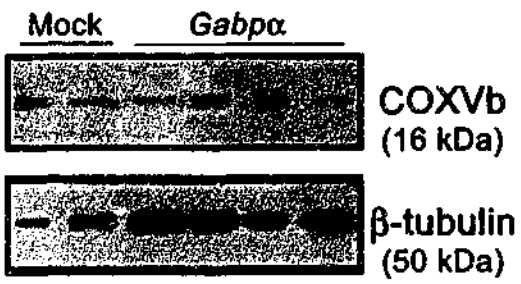

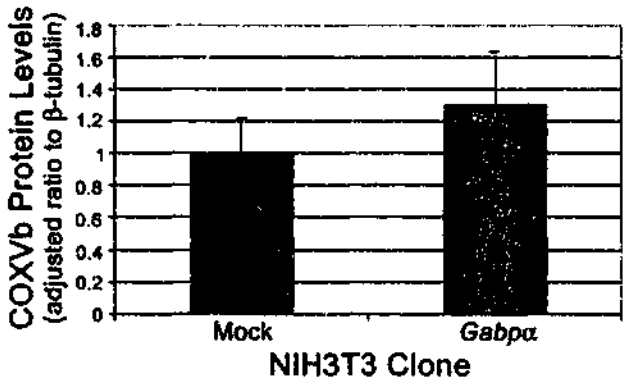

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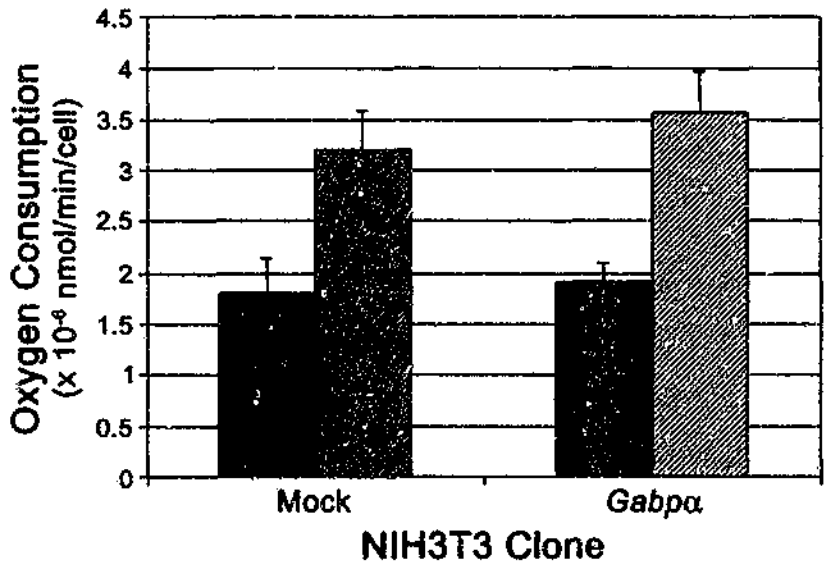


Figure 5.9 - Mitochondrial Protein Expression and Function in NIH3T3 Cells Overexpressing *Gabpα*.

(a) Western blot analysis and quantification of COXIV and β-tubulin protein levels in NIH3T3 cells transfected with mock or *Gabpα* cDNA constructs. Error bars show SEM, where n=11 for mock and n=17 for *Gabpα* clones. **(b)** Western blot analysis and quantification of COXVb and β-tubulin protein levels in mock and *Gabpα* 3T3 clones. Error bars show SEM, where n=5 for mock and n=15 for *Gabpα* clones. **(c)** Oxygen consumption of mock and *Gabpα* 3T3 clones, before (shaded bars) and after (hatched bars) treatment with a mitochondrial membrane uncoupler, CCCP. Error bars show SEM, where n=7 for mock and n=8 for *Gabpα* clones.

Gabpα transfected 3T3 clones were tested for mitochondrial function, by measuring oxygen consumption with a Clarke electrode (Hofhaus et al. 1996). Oxygen consumption of 1 x 10^6 mock or *Gabpα* 3T3 cells was measured both before and after treatment with CCCP (carbonyl cyanide m-chloro phenyl hydrazone) (Sigma), which uncouples the mitochondrial respiratory chain and phosphorylation system. Therefore, after CCCP treatment, cells exhibit their greatest oxygen consumption. However, as shown graphically in **Figure 5.9c**, no differences in oxygen uptake were observed between mock and *Gabpα* 3T3 clones, before or after CCCP addition.

This analysis demonstrates that, although the *Gabpα* 3T3 clones overexpress *Gabpα* mRNA, this does not result in increased expression of target genes of mitochondrial function, or have any corresponding effect upon cellular respiration. The effect of *Gabpα* overexpression upon another Gabp target gene, *Rb*, was assessed.

5.3.3 Growth Characteristics of *Gabpα* NIH3T3 Clones

The cell cycle kinetics of mock and *Gabpα* 3T3 clones were measured, as transcription of the cell cycle regulator Retinoblastoma protein (Rb) is regulated by Gabp (Savoysky et al. 1994). Rb overexpression in human breast cancer implicates it as an oncogene (Spandidos et al. 1992), however Rb action to transcriptionally repress the oncogene *Neu* suggests *Rb* may act as a tumour suppressor gene in some cases of human lung, breast and ovarian cancer (Matin and Hung 1994). Two methods of cell synchronisation were performed concurrently on mock and *Gabpα* transfected clones, generating samples of cells at various stages of the cell cycle. The double thymidine block retarded cells in the G_0/G_1 phase (Lukas et al. 1995; Futcher 1999; Shinomiya et al. 2000), and nocodozole treatment blocked cells in G_2/M-phase (Chou and Chou 1999). Samples were then harvested at T=0 and 3 hours following thymidine and nocodozole treatment, to yield cells in S-phase and G_0/G_1-G_2/M, respectively. FACS analysis of 1 x 10^6 mock and *Gabpα* 3T3 cells stained with propidium iodide showed that one round of synchronisation achieved adequate purity (≥ 86 %) of the cells at different cell stages, as shown by the representative graphs in **Figure 5.10**. From this analysis it was concluded that the timing of the cell cycle was unaltered by *Gabpα* overexpression in 3T3 fibroblasts.

Levels of Gabpα expression throughout the cell cycle were also compared, by Western blot analysis of Gabpα and β-tubulin protein levels (see **Figure 5.11a** for a representative image). When quantified relative to β-tubulin, no difference in the

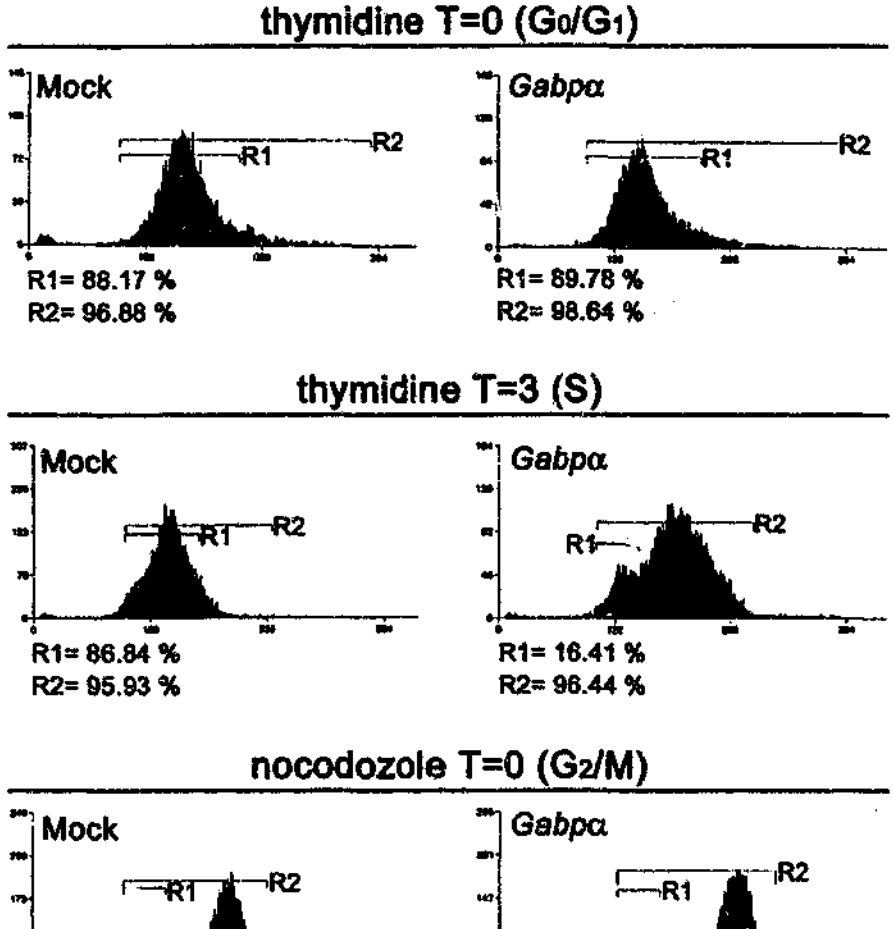


Figure 5.10 - Cell Cycle of NIH3T3 Cells Overexpressing *Gabpα*. FACS analysis of mock and *Gabpα* transfected 3T3 cells, detecting DNA stained with propidium iodide. Increased fluorescence shown by x-axes reflects the duplication of DNA during cell division, and relative cell number is represented as counts on the y-axes. Samples were harvested following double thymidine block treatment, to give cells synchronised in G_0/G_1 (T=0 hours) and S-phase (T=3 hours) of the cell cycle. Samples were also harvested 0 and 3 hours after nocodozole treatment, yielding cells synchronised in G_2/M and at the G_0/G_1-G_2/M boundary, respectively. The percentage of cells in each phase was estimated by comparing cell numbers in R1 and R2 regions.

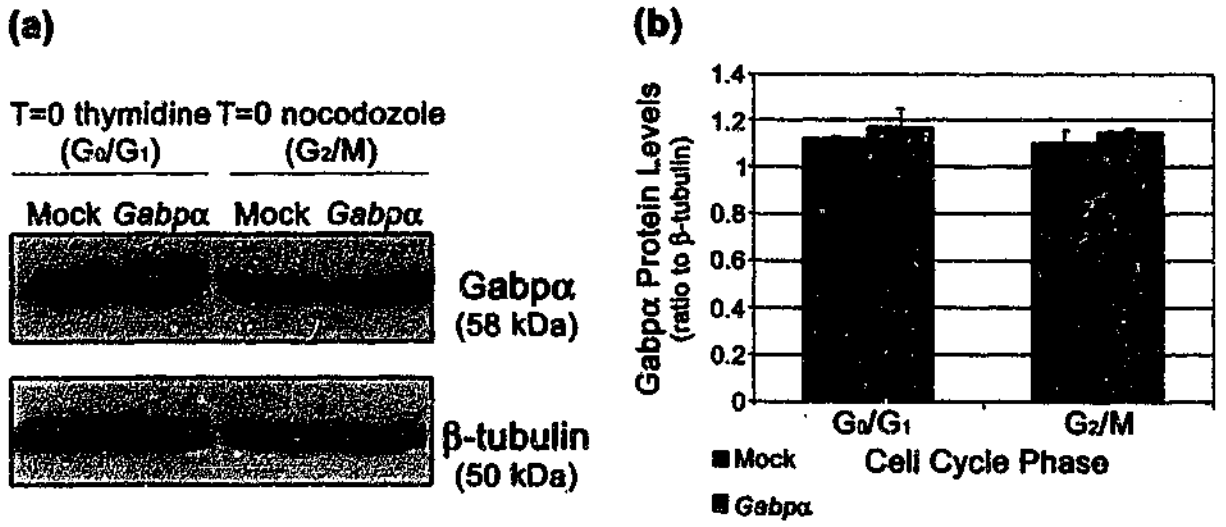


Figure 5.11 - Growth of NIH3T3 Cells Overexpressing *Gabpα*.
(a) Western blot analysis of Gabpα and β-tubulin protein levels in mock and *Gabpα* 3T3 clones synchronised in G2/M (T=0 hr nocodozole) and G0/G1 (T=0 hr thymidine). **(b)** Quantification of Gabpα protein levels, relative to β-tubulin, in mock and *Gabpα* 3T3 clones synchronised in G0/G1 and G2/M. Error bars represent SD, where n=2. **(c)** Average growth curves of mock and *Gabpα* 3T3 clones. Error bars show SEM, where n= 7 for mock and n= 6 for *Gabpα* clones.

expression of Gabpα protein was seen between mock and *Gabpα* 3T3 clones (see **Figure 5.11b**). The proliferation rate of mock and *Gabpα* transfected 3T3 cells was also determined by use of the 3-(4,5.dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) growth assay, which measures the number of mitochondria within a cell population, reflecting the amount of cell division. In agreement with the FACS analysis data, no significant difference in growth rate was observed between mock and *Gabpα* clones (see **Figure 5.11c**).

This data indicates that *Gabpα* mRNA overexpression in 3T3 cells does not result in increased expression of the Gabp target Rb, and therefore does not alter the kinetics of the cell cycle. Gabpα protein levels are also tightly regulated in a cell cycle independent manner, and appear unchanged in 3T3 clones overexpressing *Gabpα* cDNA. Gabpα protein levels in 3T3 clones overexpressing *Gabpα* mRNA were investigated further.

5.3.4 Gabpα Protein Expression in *Gabpα* NIH3T3 Clones

Cell lysates from each characterised mock and *Gabpα* NIH3T3 clone were analysed by Western blot for total (endogenous and exogenous) Gabpα protein expression levels, using the polyclonal antibody sera that was unavailable at the onset of this study (see **Figure 5.12a** for a representative image). As shown in **Figure 5.12b**, quantification of Gabpα protein expression levels relative to β-tubulin showed no significant difference in Gabpα expression levels in mock and *Gabpα* overexpressing 3T3 clones, although the total level of *Gabpα* mRNA was increased 3-8 fold in *Gabpα* transfected clones.

The lack of alteration in Gabpα protein levels explains the indifference in target gene expression levels, cell growth properties and mitochondrial function of *Gabpα* 3T3 clones. These results indicate that, at least in NIH3T3 cells, the levels of Gabpα protein are tightly regulated at a post-transcriptional level. This has important implications for situations of *in vivo* overexpression of *Gabpα*, such as Down syndrome, where the *Gabpα* gene is present in three copies. If Gabpα protein levels are securely regulated *in vivo*, presence of an extra gene copy may be of no consequence. Therefore we examined whether post-transcriptional regulation maintains stable Gabpα protein levels when *Gabpα* is overexpressed *in vivo*.

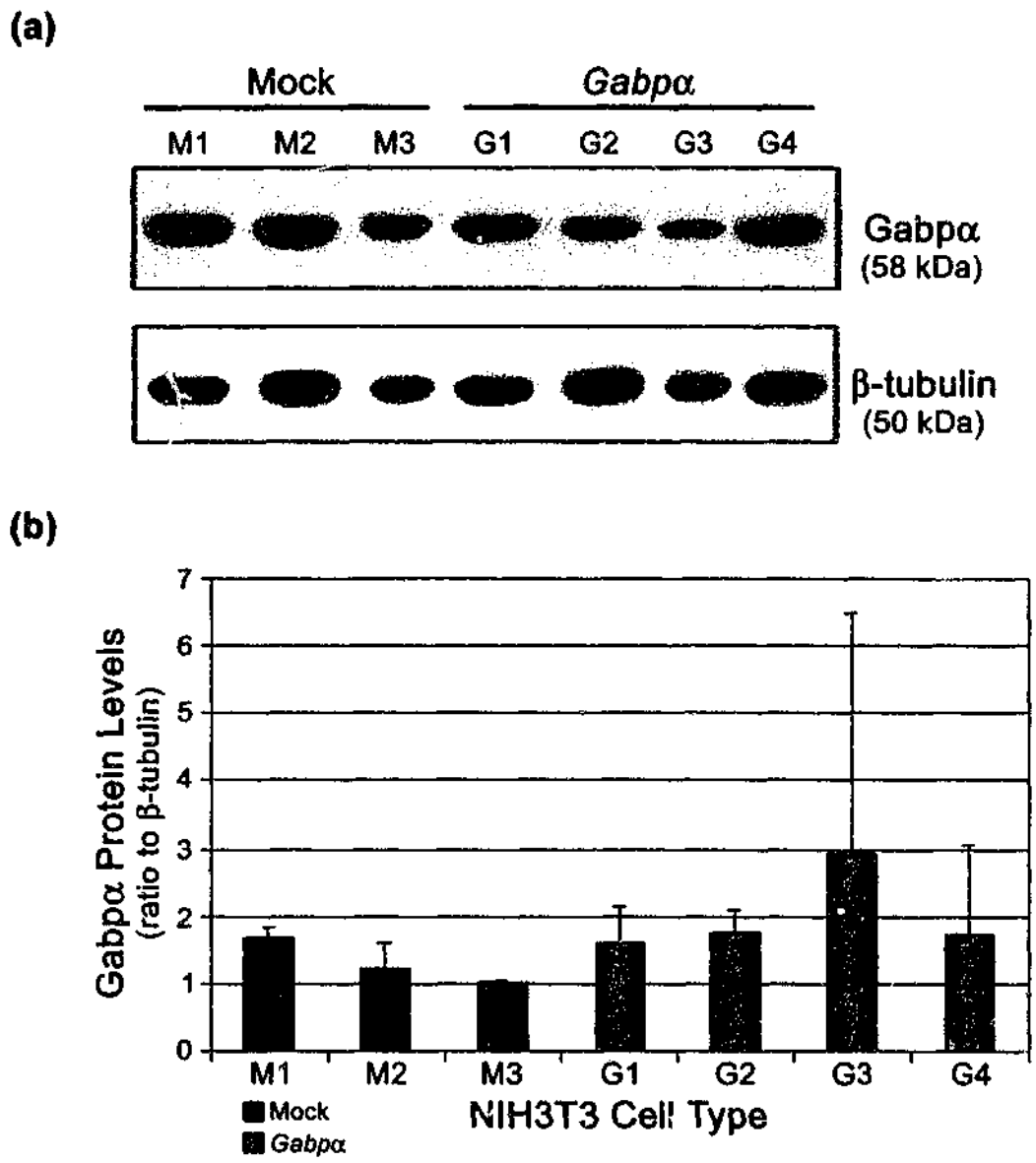


Figure 5.12 - Gabpα Protein Levels in NIH3T3 Clones.
(a) Western blot analysis of protein lysates from 3T3 clones expressing *Gabpα* cDNA, compared to mock transfected clones. Both Gabpα and β-tubulin protein levels were determined. **(b)** Quantification of Gabpα protein levels relative to β-tubulin. Error bars represent SD, where n= 3.

5.4 *Overexpression of GABPα in vivo*

As *GABPα* is localised to HSA 21 and the homologous region of MMU 16, we examined the effect of gene dosage in two model systems of Down syndrome (DS). The first of these were immortalised fibroblast cell lines isolated from DS individuals (obtained from the ATCC). The second model system of Down syndrome analysed was the Ts65Dn mouse (obtained from The Jackson Laboratories), which features a partial MMU 16 trisomy in the region between *Gabpα* and *Mx1* (see **Figure 2.12**).

5.4.1 Expression Levels of *GABPα* in Down syndrome Fibroblasts

Northern blot analysis was performed and mRNA levels of *GABPα* and *GAPDH* assessed in two DS fibroblast cell lines and matched diploid cell lines (kindly performed by Dr. Sika Ristevski, Centre for Functional Genomics and Human Disease, Monash IRD, Victoria, Australia), using hybridisation probes described in **Chapter 4** (see **Figure 5.13a**). When *GABPα* levels were quantified relative to the *GAPDH* loading control, a 1.75-fold increase in *GABPα* mRNA was apparent in Down syndrome fibroblasts, relative to diploid fibroblasts (see **Figure 5.13b**). This is as expected from the increased *GABPα* gene dosage of 1.5-fold in trisomy 21. Only the 5.4 kb transcript of *GABPα* could be detected in these cell lines, highlighting the cell type-specific expression pattern of the minor transcripts. Western blot analysis was used to determine GABPα protein levels in the Down syndrome and diploid fibroblast cell lines, as shown by the representative image in **Figure 5.13c**. No increase in GABPα protein levels was observed in DS fibroblasts relative to diploid cells, when quantified relative to β-tubulin (see **Figure 5.13d**).

This indicates that, as in the mouse, *GABPα* is tightly regulated at a post-transcriptional level in human fibroblast cells. As DS is known to affect all organ systems, we next sought to address the question as to whether *GABPα* expression is regulated by a tissue-specific post-transcriptional mechanism. The Ts65Dn partial trisomy 16 mouse, which has three copies of *Gabpα*, was used.

5.4.2 Expression Levels of Gabpα in Ts65Dn Mouse Tissues

Western blot analysis of Gabpα and β-tubulin protein expression levels was performed using tissue lysates from three wildtype and three Ts65Dn mice (see **Figure 5.14a** for a representative image). When quantified relative to β-tubulin, Gabpα protein levels were found to be elevated 2-fold in brain and 1.4-fold in skeletal muscle of Ts65Dn

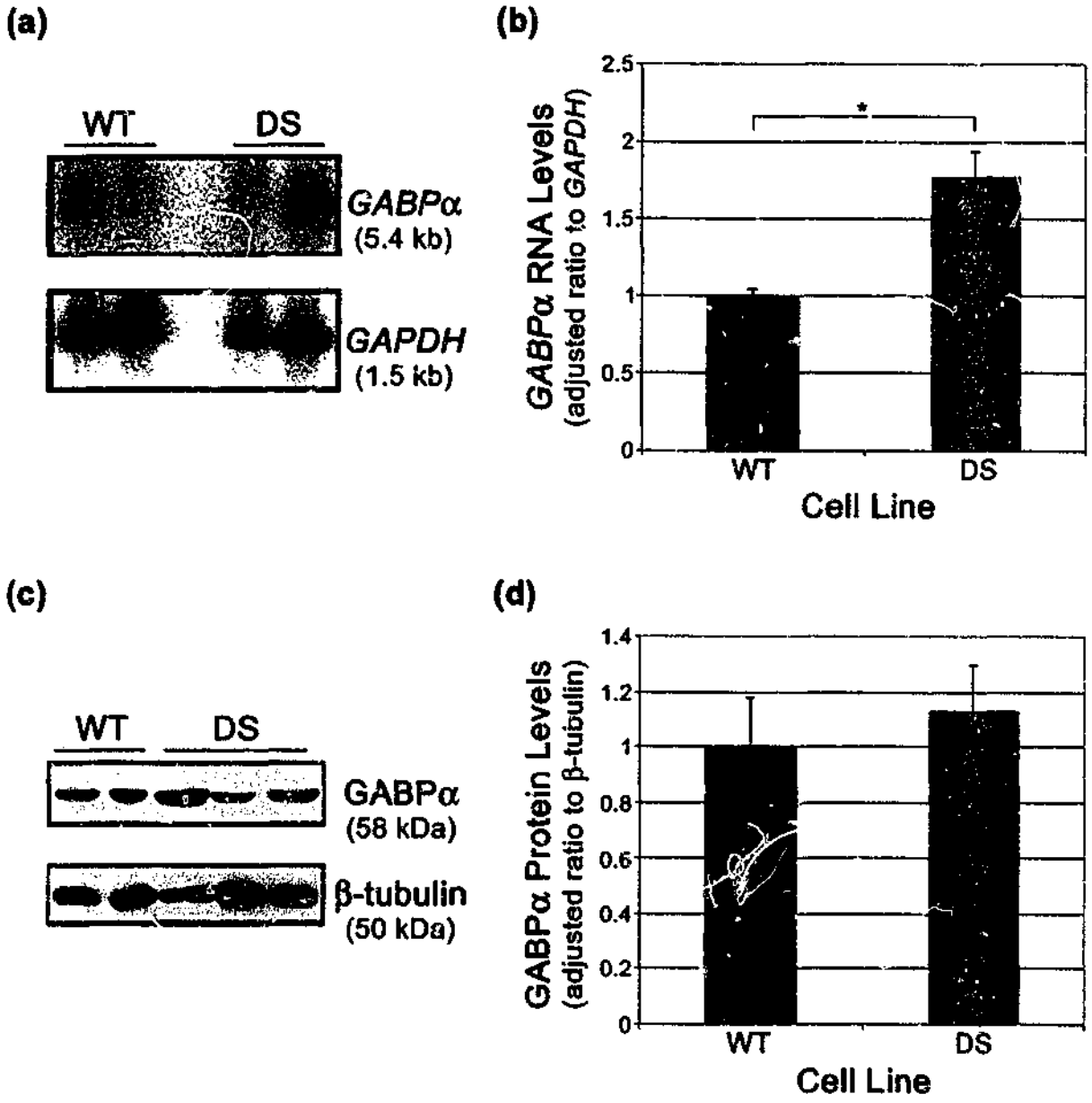


Figure 5.13 - *GABPα* Expression in Down Syndrome Fibroblasts.
(a) Northern blot analysis of *GABPα* and *GAPDH* mRNA levels in Down syndrome (DS) and wildtype (WT) fibroblast cell lines, and **(b)** quantification of Northern blot analysis. Ratios were adjusted so that the wildtype value was equal to 1. Error bars show SD, where n=2. * represents statistical significance, as determined by two-tailed t-test, where p=<0.05. **(c)** Western blot analysis of GABPα and β-tubulin levels in WT and DS fibroblast cell lines, and **(d)** quantification of Western blot analysis. Error bars show SEM, where n=9.

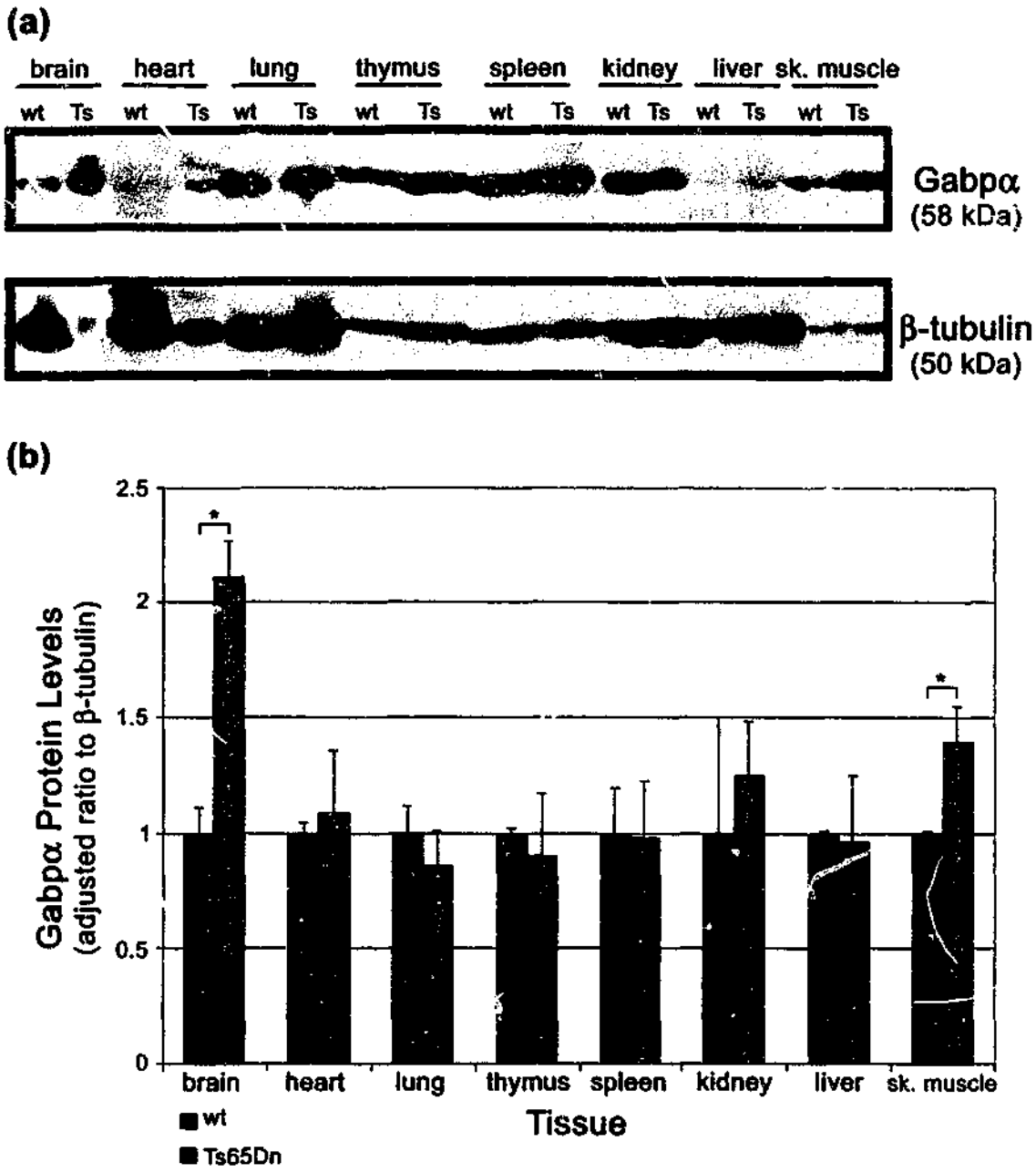


Figure 5.14 - Gabpα Protein Expression in Ts65Dn Mice.
(a) Western blot analysis of Gabpα and β-tubulin levels in tissues from adult Ts65Dn (Ts) and wildtype (wt) littermate mice. **(b)** Quantification of Western blot analysis. Protein levels are expressed as a ratio of Gabpα to β-tubulin, adjusted such that the ratio is equal to 1.0 in wildtype tissues. Error bars represent SD, where n=3 for wildtype tissues and Ts65Dn brain, heart, liver and skeletal muscle, and n=6 for Ts65Dn lung, thymus, spleen and kidney. * represents statistical significance, as determined by a two-tailed t-test, where p=<0.05.

mice, compared to wildtype littermates (see **Figure 5.14b**). However, all other tissues showed unaltered Gabpα levels, suggestive of a tissue-specific mechanism of post-transcriptional regulation.

5.5 Summary – Post-transcriptional Regulation of Gabpα Expression

Production of a Gabpα-specific polyclonal antibody allowed for detection of Gabpα in all mouse tissues examined, at varying levels. However, Gabpα protein is not found in all cell types within each tissue, as demonstrated by lacZ staining of tissue sections from *Gabpα* heterozygous mice, where the *β-galactosidase* reporter gene has been inserted within *Gabpα* exon 2. Gabpα is expressed predominantly in epithelial and haematopoietic cell types, as is the case for many other ETS factors (see Table 2.2). However, Gabpα is expressed in all cells of heart and skeletal muscle tissues. How Gabp acts to specifically upregulate genes expressed at the NMJ of skeletal muscle remains to be determined, however the even distribution of lacZ staining in myonuclei throughout skeletal muscle tissues of *Gabpα* heterozygous mice suggests that it is a property of the *Gabpα* transcript, such as choice of 5' and 3' UTRs, that leads to accumulation of *Gabpα* mRNA in sub-synaptic myonuclei.

Differential levels of Gabpα expression are also observed between different skeletal muscle tissues. Higher levels of Gabpα protein are found in quadricep and gastrocnemius muscles, tissues consisting of a low level of oxidative fibres, relative to that observed in oxidative soleus and diaphragm muscles. However, factors other than fibre type must also contribute to the control of Gabpα protein expression in skeletal muscle, as 2-fold higher levels of Gabpα protein are detected in quadricep compared to gastrocnemius. This highlights the need to study a variety of tissues to obtain a realistic idea of Gabpα function in skeletal muscle.

Comparison of Western blot and Northern blot analysis of *Gabpα* expression in adult mouse tissues shows that *Gabpα* mRNA levels correlate with relative Gabpα protein levels in most tissues. The exceptions to this are cerebellum, tongue, skeletal muscle and skin, which express relatively high levels of Gabpα protein, relative to corresponding *Gabpα* mRNA levels. Conversely, liver exhibits lower levels of Gabpα protein than would be expected from the relative *Gabpα* mRNA levels found in this tissue. This implies that a

tissue-specific means of regulation of *Gabpα* at a post-transcriptional level exists. Such mechanisms may include the expression of alternative transcripts, differing in their stability and/or translation efficiency. *Gabpα* transcripts containing alternative 5' and 3' UTRs have been identified (see **Chapter 4**), however intrinsic properties of the various transcripts are yet to be determined. It is interesting to note that cerebellum, liver, tongue and skeletal muscle tissues display low levels of expression of the exon 1a and 1b-containing transcripts of *Gabpα* (see **Figure 4.5**), suggesting that the alternative *Gabpα* transcripts may play a role in maintaining a constant level of Gabpα protein expression. Other modes of regulation of Gabpα protein levels may include post-translational mechanisms, such as phosphorylation and cellular oxidative state.

Overexpression of *Gabpα* mRNA *in vitro* in NIH3T3 fibroblast cells did not result in overexpression of Gabpα protein, and therefore did not perturb expression levels or function of transcriptional targets of the Gabp complex. Furthermore, Northern and Western blot analysis shows that overexpression of *Gabpα* mRNA *in vivo* in human DS fibroblasts does not correlate with an increased expression of Gabpα protein. However, this tight regulation of Gabpα protein levels does not occur in all tissues. Western blot analysis of Gabpα protein levels in Ts65Dn mouse tissues demonstrates that brain and skeletal muscle are unable to buffer the effect of overexpression of *Gabpα* mRNA, resulting in an elevation in Gabpα protein levels as would be expected from a 1.5-fold increase in gene dosage. Oxidation is of particular importance in tissues of high mitochondrial load, such as brain and skeletal muscle. Therefore, perhaps a relatively high level of Gabpα protein expression in needed in these tissues in order to compensate for the detrimental affects of pro-oxidant conditions in Down syndrome cells upon mitochondrial function. However, pro-oxidant conditions also result in the oxidation of cysteine residues within the ETS and dimerisation domains of GABPα, leading to decreased GABP complex function (Martin et al. 1996; Chinenov et al. 1998). Therefore it is not known whether overexpression of GABPα contributes to some of the tissue-specific phenotypes seen in Down syndrome individuals, such as muscular hypotonia (Morris et al. 1982) and early onset Alzheimer-like symptoms (Wisniewski et al. 1985), by means of deregulated target gene expression. In an attempt to address this question, mouse models of Gabpα overexpression and loss of expression were generated, as described in the following chapter.

Chapter 6

Results:

Generation of Mouse Models of GABPα *Function*

6.1 Introduction

A large number of proposed GABP target genes encode proteins that function at the neuromuscular junction and in mitochondria. As detailed in **Chapter 2**, deregulated expression of these target genes may be involved in the causation of human diseases such as Down syndrome, Congenital Myasthenic Syndrome and mitochondrial disease. In addition, mice expression a dominant negative fusion protein of the ETS-2 DNA binding domain and β-galactosidase reporter protein specifically in skeletal muscle exhibit decreased mRNA expression levels of proposed Gabp gene targets *AChRε* and *Utrophin*, and show altered NMJ morphology (de Kerchove d'Exaerde et al. 2002). Therefore, mouse models of GABPα overexpression and loss of expression were generated to analyse the contribution of the GABP complex to the muscular hypotonia and redox imbalance of Down syndrome, and severe muscle weakness of Congenital Myasthenic Syndrome. Conventional *Gabpα* knockout mice die early during embryogenesis, so *Gabpα* heterozygous mice were studied for changes in skeletal muscle structure and function. Gene structure and promoter analysis information obtained in this study (see **Chapter 4**) allowed for the generation of skeletal muscle-specific knockout and minigene *Gabpα* transgenic mice.

6.2 Gabpα Minigene Transgenic Mice

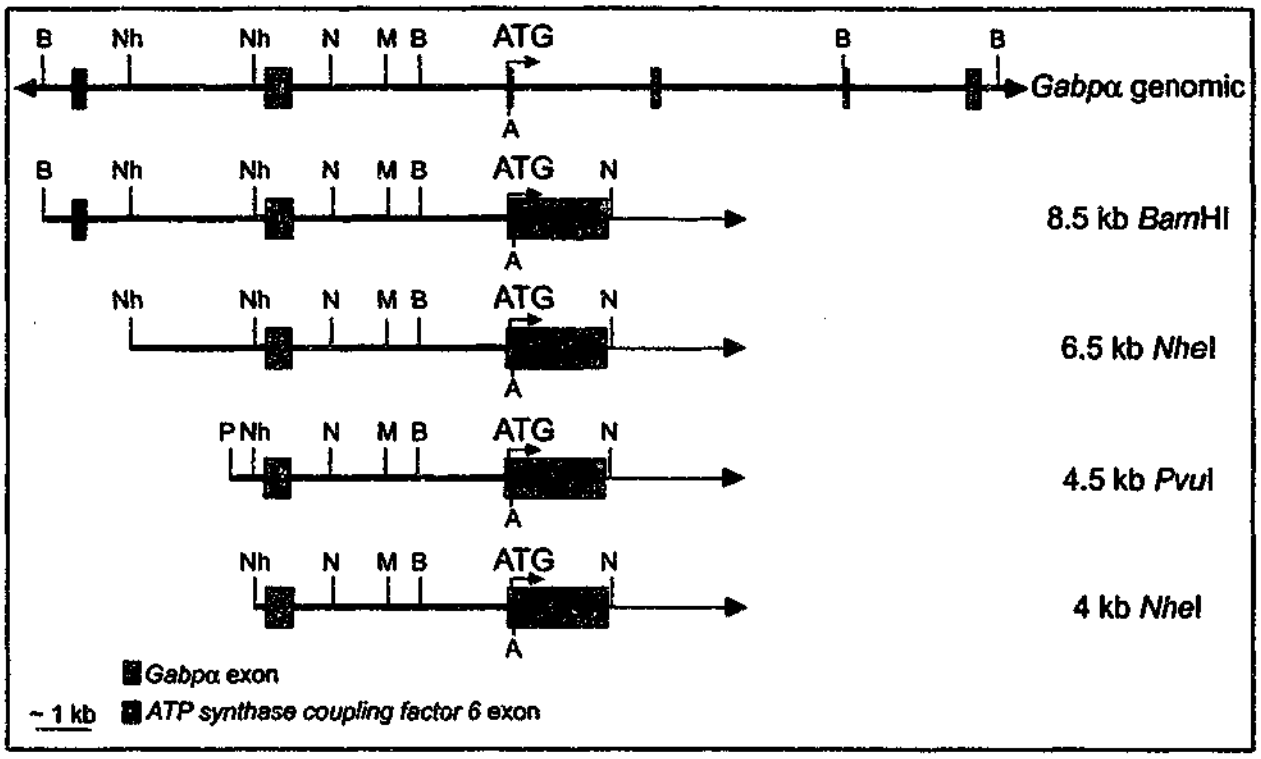
6.2.1 *In vitro* Characterisation of the *Gabpα* Promoter

Promoter function of serial deletions of the *Gabpα* 5' region was assessed *in vitro* using the Enhanced Green Fluorescence Protein (EGFP) reporter system, as this is a non-invasive marker and no substrate is required for its visualisation by excitation with

UV/blue light (Hadjantonakis et al. 1998). The EGFP sequence is adapted from that of the auto-fluorescent protein of the jellyfish *Aequora victoria*, such that codon sequences reflect those preferentially used in highly expressed human proteins, allowing for enhanced transgene expression (Haas et al. 1996). Four reporter constructs were created, using unique restriction endonuclease sites flanking the first exons of *ATP Synthase Coupling Factor VI* and *Gabpα*, and sub-cloning into the p*EGFP*-1 promoterless *EGFP* expression vector (Clontech) (see **Figure 6.1a**). The intron 1 region of *Gabpα* was included, to ensure that exon 1a and exon 1b alternative 5' UTRs could be expressed in *Gabpα* transgenic mice, as well as that of exon 1. A 8.5 kb *Bam*HI-*Age*I genomic fragment spanning the 5' region to within exon 2 of *Gabpα* (1-8490 bp of GI:27960443, as in **Appendix E**) was generated by fusion of a 3' *Age*I tagged PCR product (primers 140 and 209 spanning 5019-8490 bp of GI:27960443, amplified with *Pfu* DNA polymerase (Promega)) to the 5' genomic region at a unique *Mlu*I site (6146 bp of GI:27960443). This *Bam*HI-*Age*I fragment was then directionally cloned into the p*EGFP*-1 vector, fusing the coding sequence of *EGFP* in frame, 10 bp downstream of the endogenous *Gabpα* start codon. The remaining three 5' deletion constructs were created by restriction endonuclease digestion with *Xho*I of the vector and either *Nhe*I (2009 or 4140 bp sites of GI:27960443) or *Pvu*I (3829 bp) of *Gabpα*, followed by re-ligation. Following sequencing with primer 277 spanning 725-745 bp of GI:1377908, these four constructs, as well as the negative control p*EGFP*-1 vector and positive control p*EGFP*-N1 vector (*EGFP* under the control of the ubiquitous *CMV* promoter) (Clontech), were transiently transfected into C2C12, NIH3T3, and PC12 cell lines and assessed for promoter activity.

Levels of fluorescence were determined by FACS analysis, and showed that ~3 % of cells expressed the transfected EGFP constructs. As shown in **Figure 6.1b**, all four *Gabpα* promoter constructs gave basal EGFP expression compared to that of the *CMV* promoter, with greatest expression (1.75 to 4.36-fold increase above background) achieved with the largest construct of 8.5 kb in each of the three cell lines tested. Examination of transfected cells by immunofluorescence confirmed the FACS results, as shown by the representative images in **Figure 6.2**. As there was no significant difference between the levels of EGFP expression induced by each of the four *Gabpα* promoter constructs, the promoter fragment chosen for generation of the *Gabpα* minigene transgenic construct was the 6.5 kb *Nhe*I region. This is the largest *Gabpα* promoter fragment that does not include any of the coding region of the closely linked *ATP Synthase Coupling Factor VI* gene.

(a)



(b)

Construct \ Cell Line	Promoter Activity		
	C2C12	NIH3T3	PC12
CMV	14.74	13.99	35.12
8.5 kb *Bam*HI	1.75	3.82	4.36
6.5 kb *Nhe*I	1.39	1.74	5.22
4.5 kb *Pvu*I	1.77	3.06	1.31
4 kb *Nhe*I	1.42	0.79	1.58

Figure 6.1 - *Gabpα* Promoter-*EGFP* Reporter Expression.
(a) *Gabpα* genomic structure and promoter-*EGFP* constructs, named according to promoter length and 5' restriction endonuclease site used in their construction. *Bam*HI (B), *Nhe*I (Nh), *Pvu*I (P), *Not*I (N), *Mlu*I (M) and introduced *Age*I (A) sites are shown. **(b)** Average promoter activity of *Gabpα-EGFP* reporter constructs in transiently transfected C2C12, NIH3T3 and PC12 cell lines, expressed as fold-induction of EGFP fluorescence above that of the empty p*EGFP*-1 vector, as determined by FACS analysis.

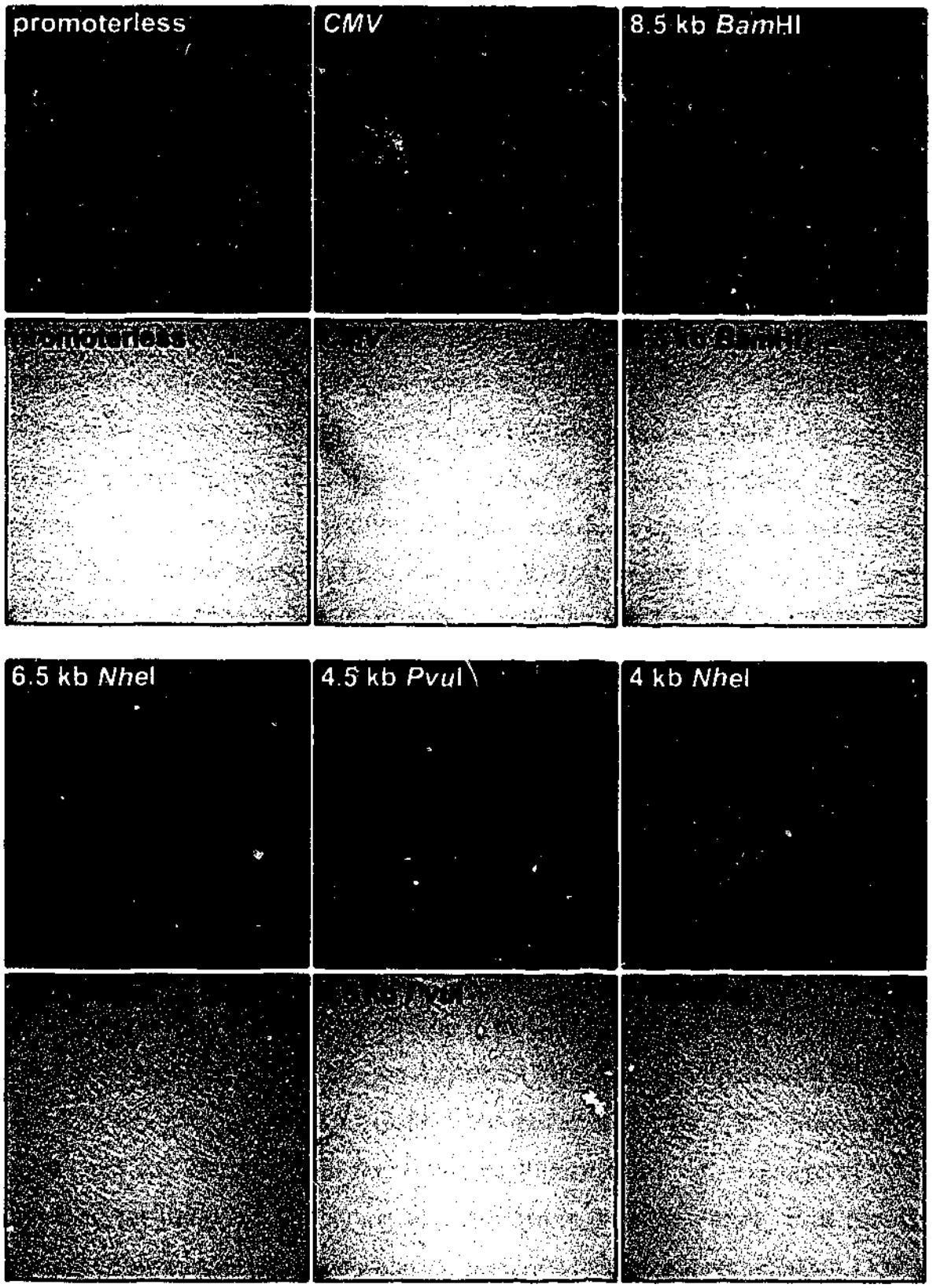


Figure 6.2 - Visualisation of *Gabpα* Promoter-*EGFP* Expression.
Immunofluorescent and bright field images of C2C12 cells transiently transfected with *EGFP* reporter constructs. No promoter, the *CMV* promoter, or 8.5 kb *Bam*HI, 6.5 kb *Nhe*I, 4.5 kb *Pvu*I or 4 kb *Nhe*I *Gabpα* promoter fragments to drive expression of *EGFP*. Images were taken at 200x magnification, and fluorescence intensity was kept constant.

6.2.2 Generation of *Gabpα* Minigene Transgenic Mice

Gabpα Minigene Transgenic Construct Generation

A *Gabpα* minigene transgenic construct was generated by fusing the 6.5 kb *Nhe*I genomic fragment to exon 2 (the first coding exon) of the *Gabpα* cDNA (see **Figure 6.3a**). A 6.5 kb *Nhe*I-*Sap*I genomic fragment of *Gabpα* (2009-8503 bp of GI:27960443, **Appendix E**) was ligated into the corresponding *Sap*I site within exon 2 of the full length *Gabpα* cDNA sequence (bp 478 of GI:193382) in the pBS KS^+ vector backbone (Stratagene). Unique *Not*I sites within *Gabpα* intron 1 (4976 bp of GI:27960443) and the pBS vector site were then used to clone the fused *Gabpα* genomic-cDNA fragment into the p*EGFP*-1 mammalian expression vector backbone (Clontech) in place of the *EGFP* sequence. Following sequencing of the minigene transgenic construct with primers 61, 52 and 270, the 11.9 kb plasmid was linearised at a *Nar*I site within the *Neomycin* cassette of the NI vector, and DNA was purified prior to injection into F1 (CBA x C57Bl/6) mouse pronuclei at a concentration of 2 ng/μl in TE buffer. Microinjection was performed by Susan Tsao and Dr. Anna Michalska, Centre for Functional Genomics and Human Disease, Monash IRD, Victoria, Australia.

Genotyping _Gabpα_ Transgenic Mice

Mice generated from pronuclear injection were screened for presence of the *Gabpα* minigene construct by Southern blot and PCR. Genomic DNA was extracted from mouse tails, digested with *Bam*HI, and transferred onto nylon membranes by Southern blot. Membranes were hybridised with a 200 bp cDNA fragment spanning exon 1a to exon 3 (15-216 bp of sequence AY282794, see **Appendix F**), amplified with primers 246 and 118. As exon 1a lies within intron 1 of *Gabpα*, this probe detected a 7 kb wildtype genomic fragment and a 2 kb transgenic fragment (see **Figure 6.3b**). A fragment of ~7.5 kb, representing the p*EGFP*-1 vector, was also detected in transgenic mice. PCR genotyping of potential *Gabpα* transgenic founders was performed using primers spanning 1330-1464 bp of the *Gabpα* cDNA sequence GI:193382 from exon 8 to exon 9 (primers 153 and 154). The small size of *Gabpα* intron 8 allowed for detection of both wildtype genomic and transgenic cDNA alleles, producing 339 bp and 133 bp products, respectively (see **Figure 6.3c**). A total of 10 of the 44 mice generated from pronuclear injection were found to have integrated the *Gabpα* transgene. These mice were bred with wildtype F1 (CBA x C57Bl/6) mice and assessed for transmission and expression of the *Gabpα* transgene.

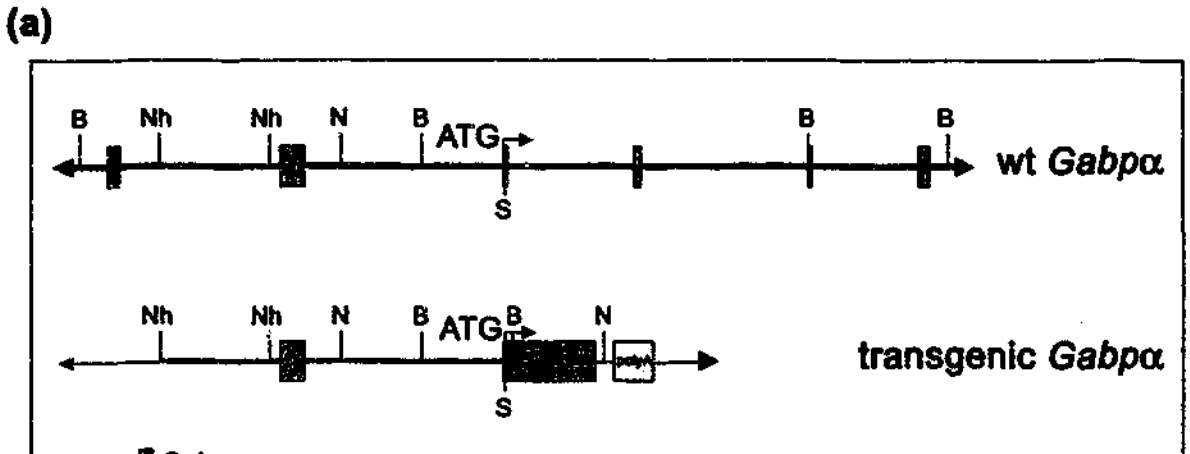


Figure 6.3 - Generation of *Gabpα* Minigene Transgenic Mice.
(a) Wildtype (wt) *Gabpα* genomic structure and minigene transgenic construct. The 6.5 kb *Nhe*I *Gabpα* promoter fragment was fused to the *Gabpα* cDNA within exon 2 and cloned in place of the *EGFP* coding sequence in the p*EGFP*-1 expression vector, upstream of the SV40 polyadenylation signal. *Bam*HI (B), *Nhe*I (Nh), *Not*I (N) and *Sap*I (S) sites are shown. **(b)** Progeny from the microinjected pronuclei were screened for integration of the *Gabpα* transgene by Southern blot of *Bam*HI digested genomic DNA and hybridisation with a probe spanning exon 1a-3 of *Gabpα* cDNA, identifying a 7 kb wildtype (wt), and 2 kb and 7.5 kb transgenic (tg) fragments. **(c)** PCR analysis of genomic DNA with primers spanning exons 8 and 9 yielded 339 bp wildtype and 133 bp transgenic bands.

6.2.3 Assessment of *Gabpα* Transgene Expression

Progeny of the ten founding *Gabpα* transgenic mice were genotyped by PCR and 7 of the 10 lines were shown to be capable of *Gabpα* transgene transmission. Mice from each of these 7 lines were screened for mRNA and protein expression of *Gabpα*. Gabpα and β-tubulin protein levels were assessed in brain, heart, lung, thymus, spleen, kidney, liver, and gastrocnemius, soleus and quadricep muscles of 1 month old wildtype and *Gabpα* minigene transgenic mice from all 7 lines by Western blot analysis. As shown by the representative Western blot image of three transgenic lines in **Figure 6.4a**, no significant difference in Gabpα protein levels was observed between wildtype and *Gabpα* transgenic mouse tissues, when quantified relative to β-tubulin. Therefore *Gabpα* mRNA expression levels of these mice were assessed by Northern blot analysis, using a probe spanning 413-1884 bp of GI:193382 (primers 52 and 53), as shown in **Figure 6.4b**. Although adequate amounts of RNA were present (see **Figure 6.4c**), and the endogenous *Gabpα* transcripts of 5.4 kb, 2.8 kb and 2.5 kb were detected, the 2 kb transgene mRNA was not visible, even upon exposure to film for several days.

To ensure low-level *Gabpα* transgene expression was not missed, RT-PCR was performed using primers spanning *Gabpα* exon 8 to within the polyadenylation signal of the cloning vector (primers 153 and 270), using cDNA from various tissues. However, as shown by the representative image in **Figure 6.4d**, the expected 600 bp product was only ever amplified from positive control plasmid DNA. Resulting RT-PCR products were also hybridised with an oligonucleotide within *Gabpα* exon 9 (primer 154) to ensure low-level transgene expression was not missed (see **Figure 6.4e**). The integrity of cDNA used for RT-PCR was confirmed by amplification of a 400 bp *Gapdh* product spanning 585-985 bp of GI:193423 (primers 58 and 59) (see **Figure 6.4f**).

Gabpα transgene expression was not detected in any of the 7 lines tested. One possible explanation for this is unfavourable integration site. It has been shown that the orientation of transgene integration, with respect to the flanking region (Feng et al. 2001), and the specific chromosomal sequences flanking a transgene, greatly effect its expression (Cranston et al. 2001). An alternative explanation is that the selected *Gabpα* promoter fragment cannot function *in vivo* in mouse tissues, even though it can induce reporter gene expression in cell lines. As overexpression of *Gabpα* was not achieved, these mice were not analysed any further.

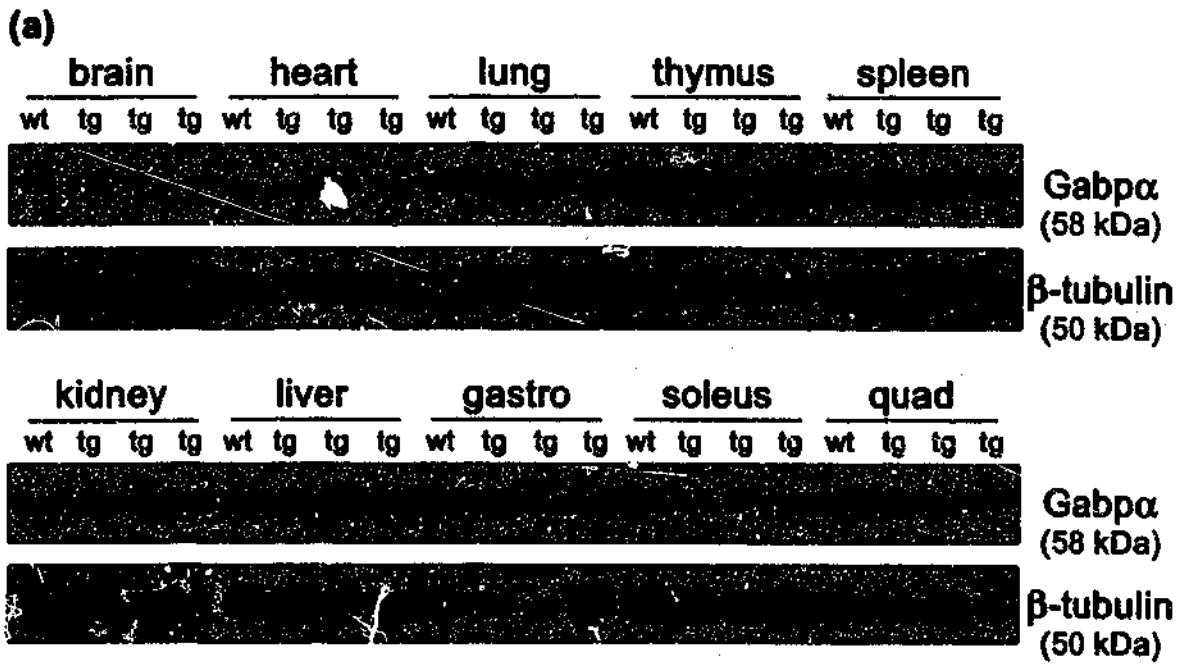


Figure 6.4 - *Gabpα* Transgene Expression Analysis.
(a) Western blot analysis of Gabpα and β-tubulin protein levels in wildtype (wt) and *Gabpα* transgenic (tg) tissues. **(b)** Northern blot analysis of *Gabpα* mRNA levels in tissues from wt and *Gabpα* tg littermates. Endogenous *Gabpα* mRNAs of 5.4 kb, 2.8 kb and 2.5 kb were detected, and loading is indicated by the intensity of 18 S (1.9 kb) and 28 S (4.7 kb) ribosomal RNA fragments after ethidium bromide staining of the agarose gel **(c)**. RT-PCR analysis of *Gabpα* transgene expression using a 5' primer within *Gabpα* exon 8 and a 3' primer within the polyadenylation signal of the expression vector to yield a 600 bp product **(d)**. These products were hybridised with an oligonucleotide within *Gabpα* exon 9 **(e)**. Integrity of the cDNA template was confirmed by amplification of a 400 bp *Gapdh* RT-PCR product **(f)**.

6.3 *Gabpα Total Knockout Mice*

6.3.1 Generation of *Gabpα* Total Knockout Mice

We obtained *Gabpα* heterozygous mice (on a SvJ129 x C57Bl/6 mixed background) from our collaborator Michael Owen at the Imperial Cancer Research Fund Laboratories, London. Exon 2, the first coding exon of *Gabpα*, has been targeted in these mice by insertion of a *neomycin-β-galactosidase* cassette, resulting in replacement of *Gabpα* expression with that of the *β-galactosidase* (*lacZ*) reporter gene (see **Figure 6.5a**).

Genotyping of *Gabpα* Total Knockout Mice

Progeny generated from matings of *Gabpα* heterozygous mice were genotyped by Southern blot and PCR. Genomic DNA was extracted from mouse tails, digested with *Bam*HI, and transferred onto nylon membranes by Southern blot. Membranes were hybridised with a 977 bp *Sac*II-*Spe*I probe within intron 3 (as described in **Chapter 4**), yielding 7 kb wildtype and 13 kb targeted fragments (see **Figure 6.5b**). PCR genotyping was performed (in the presence of 1 M Betaine (Sigma)), using a 5' primer within exon 2 (primer 192 at 3973-3996 bp of the complete *Gabpα* genomic sequence, see **Appendix E**) or within the *Neomycin* gene (primer 194 at 1043-1063 bp of sequence GI:1244765), and a common 3' primer within intron 2 (primer 193 at 4142-4165 bp of the complete *Gabpα* genomic sequence), producing 192 bp and 700 bp wildtype and targeted products, respectively (see **Figure 6.5c**).

***Gabpα* Knockout Mouse Viability**

Gabpα homozygous knockout mice were not obtained from breeding of heterozygous *Gabpα* mice (Owen, M., unpublished data). As shown in **Figure 6.6a**, of a total of 274 mice genotyped at weaning (4 weeks of age), no homozygous knockout mice were identified by PCR genotyping. In order to determine the stage of embryonic lethality, litters from heterozygous *Gabpα* mice were killed prior to birth and embryos from E7.5-9.5 were genotyped by PCR. As shown in **Figure 6.6b**, no *Gabpα* knockout mice were identified from E7.5-9.5. Furthermore, 36 blastocysts were harvested from heterozygous *Gabpα* matings. The targeted allele was not reliably detectable in all litters by PCR, so the exact ratio of wildtype to heterozygous mice at this stage could not be determined. However, all blastocysts were shown to possess the wildtype *Gabpα* allele by PCR and internal oligonucleotide hybridisation (see **Figure 6.6c**). This indicates that Gabpα

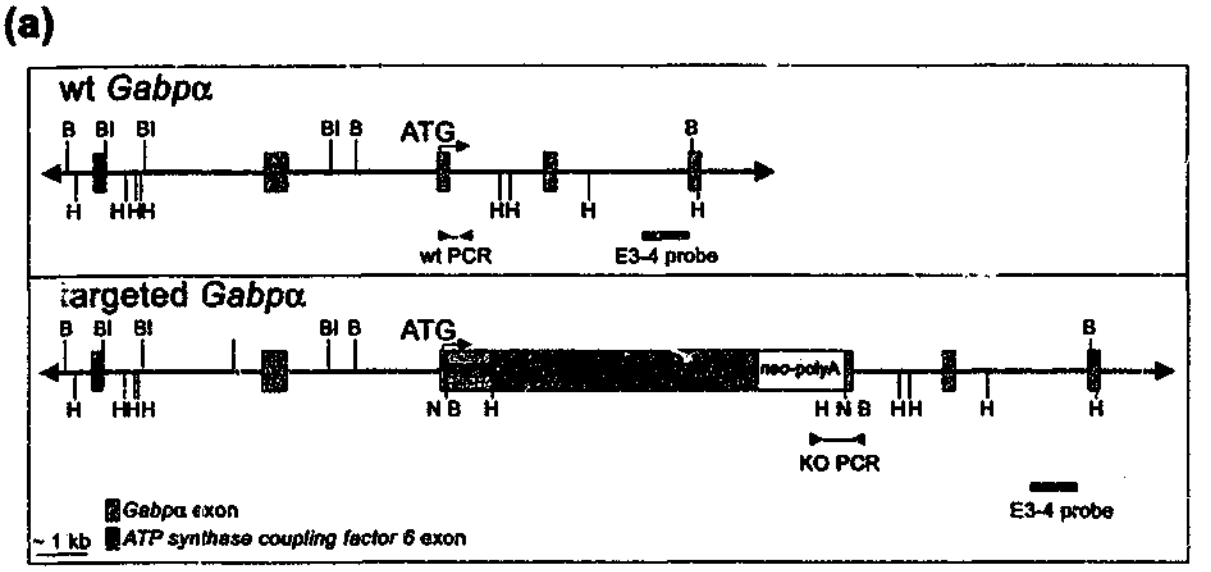


Figure 6.5 - *Gabpα* Total Knockout Mouse Genotyping.
(a) Wildtype (wt) genomic structure of mouse *Gabpα* and the targeted allele, featuring insertion of an IRES-*LacZ-neomycin* cassette into exon 2, immediately downstream of the start codon. Restriction endonuclease sites are indicated: *Bam*HI (B), *Bal*I (BI), *Hin*dIII (H) and *Not*I (N). **(b)** Southern blot analysis by *Bam*HI digestion and hybridisation with the E3-4 probe was used to identify 13 kb targeted and 7 kb wildtype genomic fragments in *Gabpα* heterozygous (+/-) and wildtype (+/+) mice. **(c)** PCR screening of wildtype and *Gabpα* heterozygous mice was performed using a 5' primer with intron 1 (wt PCR) or the neomycin cassette (KO PCR) and a common 3' primer within intron 2. Resulting PCR products were 192 bp wildtype and 700 bp KO fragments.

(a)

	Wildtype	Heterozygous	Knockout
Observed No.	111	163	0
Expected Ratio	1	2	1
Observed Ratio	1	1.5	0

(b)

	Age	Wildtype	Heterozygous	Knockout
Observed No.	E7.5	12 (6)	11 (11)	0 (6)
	E8.5	25 (12)	28 (27)	0 (13)
	E9.5	18 (12)	29 (23)	0 (12)

(c)

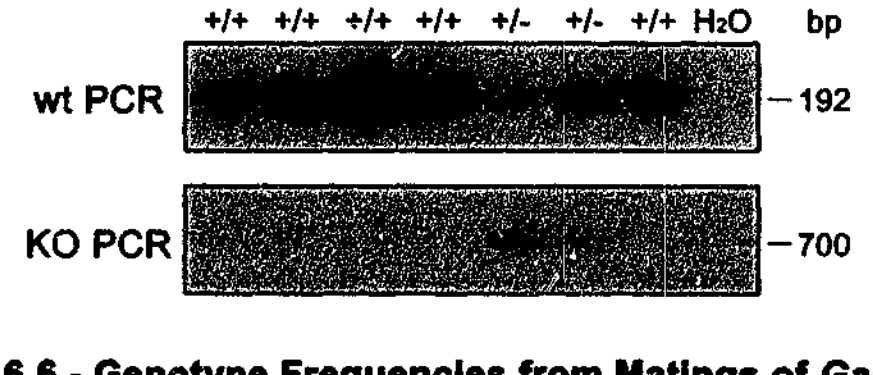


Figure 6.6 - Genotype Frequencies from Matings of *Gabpα* Heterozygous Mice.

Gabpα PCR genotyping results of mice at weaning (4 weeks of age) **(a)**, and at various stages of embryogenesis **(b)**, resulting from matings of mice heterozygous for *Gabpα*. The expected and observed genotype frequencies are expressed as ratios at weaning age, or expected number for each genotype in parentheses for embryos. **(c)** Oligonucleotide hybridisation of 192 bp and 700 bp wildtype (wt) and targeted (KO) PCR products generated from wildtype (+/+) and heterozygous (+/-) *Gabpα* mice, respectively, using DNA from blastocysts of matings of *Gabpα* heterozygous mice.

function is essential for early embryonic development, and that mice lacking *Gabpα* die prior to implantation.

Embryonic lethality is a consequence of deletion of several other ETS genes in mice, implicating them in key developmental processes. For example, *Ets-2* knockout mice die prior to E8.5 due to defects in trophoblast function (Yamamoto et al. 1998), and *Fli-1* knockout mice die at E11.5 due to cerebral haemorrhage leading to defective vascularisation (Hart et al. 2000a). Gabp is also known as Nuclear respiratory factor-2 (Nrf-2), and co-operates with Nuclear respiratory factor-1 (Nrf-1) to transactivate the expression of many genes encoding protein products that function in the mitochondrial respiratory chain (Scarpulla 2002a). Homozygous deletion of the gene encoding Nrf-1 results in a decrease in the number of mitochondria in blastocysts, and subsequent pre-implantation lethality of mice (Huo and Scarpulla 2001). This is hypothesised to be due to decreased expression of Nrf-1 gene targets, such as the mitochondrial gene regulator MTFA, resulting in decreased transcription of mitochondrial DNA (Huo and Scarpulla 2001). As Gabp acts with Nrf-1 to transactivate the *MTFA* promoter, a decrease in the number of mitochondria may cause the early embryonic lethality of *Gabpα* knockout mice. This possibility could be investigated in future studies by staining mitochondria in cultured blastocysts resulting for matings of *Gabpα* heterozygous mice. The early embryonic lethality of *Gabpα* knockout mice meant that only ubiquitous heterozygous *Gabpα* mice were available for phenotype analysis in this study.

6.3.2 Assessment of Gabpα Expression in Heterozygous Mice

The expression levels of Gabpα protein in pooled tissue lysates from 4 male and 4 female 6-8 week old *Gabpα* heterozygous and wildtype mice were determined by Western blot analysis (see **Figure 6.7a** for a representative image). Relative abundance of Gabpα in each tissue was calculated as a ratio relative to β-tubulin protein levels, and ratios were adjusted such that wildtype values were equal to 1. Results of male and female mice were averaged, as no difference in Gabpα protein expression levels was observed between the two sexes. However, as seen in **Figure 6.7b**, a high level of variation was observed across experiments in the pooled tissue lysates of both wildtype and *Gabpα* heterozygous mice. No significant difference in the average level of Gabpα protein was seen between the two genotypes, in any of the tissues tested. This could be partly due to the variation in protein levels of mice on a mixed genetic background, and it would be interesting to re-assess

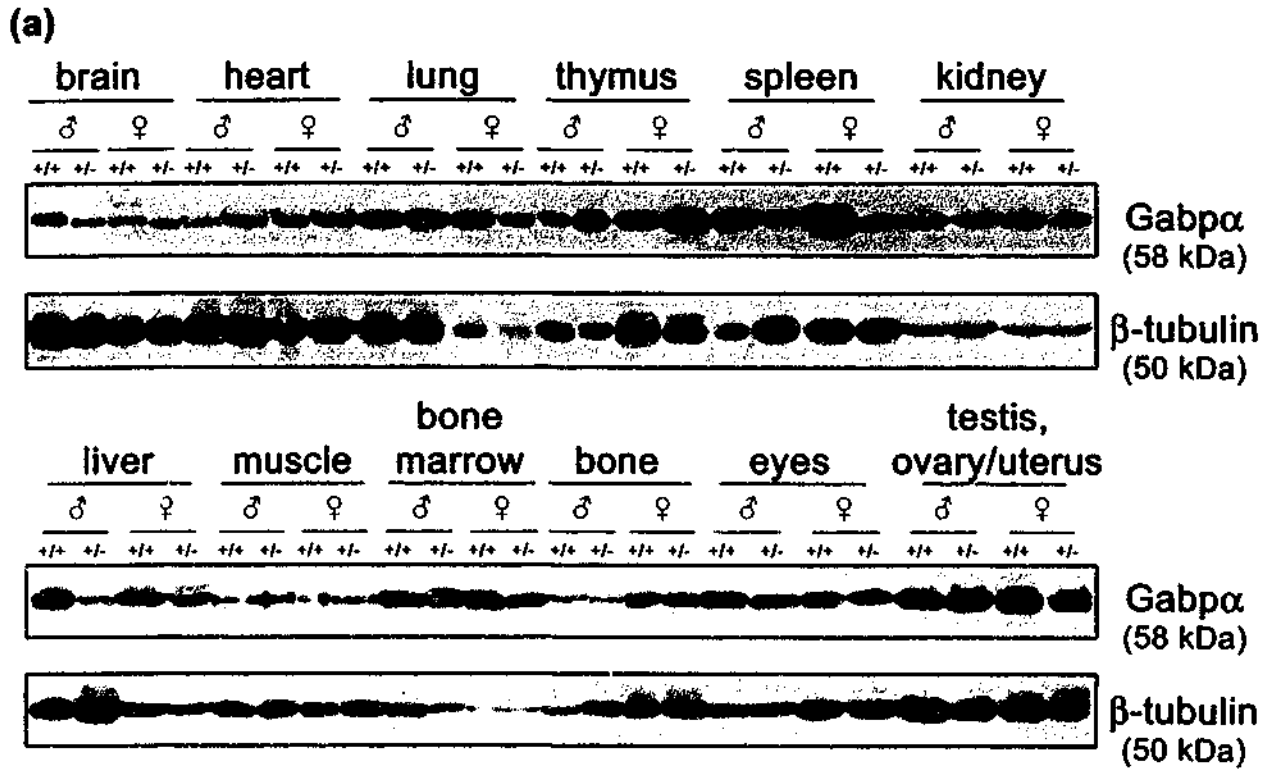


Figure 6.7 - Gabpα Protein Expression Levels in Heterozygous Mice.
(a) Western blot analysis of Gabpα and β-tubulin protein levels in pooled tissue samples from 6-8 week old male (♂) and female (♀) wildtype (+/+) and heterozygous (+/-) *Gabpα* mice. **(b)** Quantification of Gabpα protein levels in tissues of wildtype and heterozygous mice, expressed relative to β-tubulin. Ratios were adjusted such that those of wildtype mice were equal to 1. Error bars represent SEM, where n=8 for all tissues, except testis and ovary/uterus, where n=4.

Gabpα protein expression once heterozygous mice are backcrossed onto a pure genetic background, using samples from individual mice. However, these results are suggestive of tight transcriptional and/or post-transcriptional levels of regulation of *Gabpα* expression.

Mice ubiquitously heterozygous for *Gabpα* expression were analysed alongside skeletal muscle-specific *Gabpα* knockout mice for any subtle defects in skeletal muscle structure or function resulting from slight reductions in Gabpα protein levels not detected in this analysis (see **Chapter 7**).

6.4 Gabpα Conditionally Targeted Mice

6.4.1 Conventional Versus Conditional Knockout Technology

Gene knockout technology is a powerful method for generating animal models with defined genetic changes. Conventional gene targeting methods involve deletion of several or all exons of a gene, or gene substitution with the coding sequence for a selectable marker, such as *Neomycin*. This provides a means of identification of embryonic stem (ES) cells that have incorporated the targeted allele. However, *in vivo* experiments have shown that regulatory sequences of a selectable marker can alter expression levels of surrounding genes more than 100 kb away from the site of integration (Pham et al. 1996). Therefore, the phenotypes of many conventional knockout models are difficult to interpret, as more than one gene may be affected. In addition, certain gene products are essential for early development and the function of these genes can be difficult to ascertain, as knockout mice die during embryogenesis (Tsien et al. 1996).

This has lead to the development of conditional knockout methods, whereby the function of a gene can be examined in a specific tissue and/or at a specific time. One of the best characterised of the tissue-specific methods utilises the *Cre-loxP* system of the P1 bacteriophage (Zheng et al. 2000). Cre (causes recombination) allows for cell type-, tissue- or developmental stage-specific recombination in mammalian cells, on any kind of DNA, and is non-toxic to cells (Hamilton and Abremski 1984). Cre is a 38kDa DNA recombinase of the integrase family that (in the absence of bacterial recA and recBC functions) specifically recombines DNA within flanking A-T repetitive sequences known as *loxP* sites (locus of x-ing over) (Sternberg and Hamilton 1981). A *loxP* site consists of 34 bp, with a core of 8 bp and two 13 bp palindromic flanking sequences, and recombination between *loxP* sites is mediated by formation of a Holliday junction structure

(Sternberg and Hamilton 1981; Hamilton and Abremski 1984; Zheng et al. 2000). The *Cre-loxP* system of conditional gene targeting involves the insertion of *loxP* sites flanking exons of the coding sequence, and subsequent breeding of homozygous targeted mice to *Cre* transgenic mice, resulting in deletion of the *loxP* flanked (floxed) sequence. *Cre* transgenic mice have been used successfully to create tissue-specific knockout mice. For example, the *lck* promoter was used to drive Cre expression resulting in *Stat3* deletion specifically in T-cells (Takeda et al. 1998). The 'flox-and-delete' strategy used in this study involves three *loxP* sites. This enables the floxed selectable marker to be removed prior to construct injection into mouse blastocysts (Torres and Kühn 1997).

6.4.2 Generation of Mice Heterozygous for the Floxed *Gabpα* Allele

Gabpα Conditional Targeting Construct Generation

Exon 2, the first coding exon of *Gabpα*, was targeted for deletion by insertion of a single *loxP* site immediately upstream of exon 2, as well as a floxed *Neomycin* selectable marker within intron 2 (as shown in **Figure 6.8a**). A 3.2 kb *Bam*HI-*Eco*RI genomic fragment, spanning from intron 1 to intron 2 of *Gabpα* (6662-9873 bp of GI:27960443, see **Appendix E**), was inserted into the pBS KS^+ cloning vector (Stratagene) and constituted the 5' and 3' homologous sequences of the targeting construct. Exon 2 of *Gabpα* was removed by *Bcl*I restriction endonuclease digestion (8212-8572 bp of GI:27960443) and replaced with a 1.4 kb *Apa*I-*Nsi*I fragment encompassing the floxed p*MCI-Neomycin* cassette (a kind gift from Prof. Klaus Rajewsky, Institute for Genetics, University of Cologne, Cologne, Germany). Finally, a 380 bp PCR product containing *Gabpα* exon 2 fused to a *loxP* site at its 5' end (primers 169 and 170 spanning 8220-8562 bp of GI: 27960443, generated with *Pfu* DNA polymerase (Promega)) was inserted at a unique *Aat*II site at the 5' end of the *Neomycin* cassette. A negative selection marker, *Thymidine kinase* (*TK*), under the control of the *Herpes simplex virus* (*HSV*) promoter (a kind gift from Dr. Trevor Wilson, Monash Institute of Reproduction and Development, Melbourne, Australia) was introduced into the pBS KS^+ vector backbone at a unique *Xho*I site. Together, this allowed for positive selection (by Geneticin-G418 treatment) of ES cell clones expressing *Neomycin*, and negative selection of clones expressing *TK* (by Gancyclovir treatment), indicative of random integration (Capecchi 1989).

Following sequencing of the targeting construct with primers 206, 117, and 61, Cre-mediated excision of *Gabpα* exon 2 was confirmed *in vitro* by digestion of DNA with

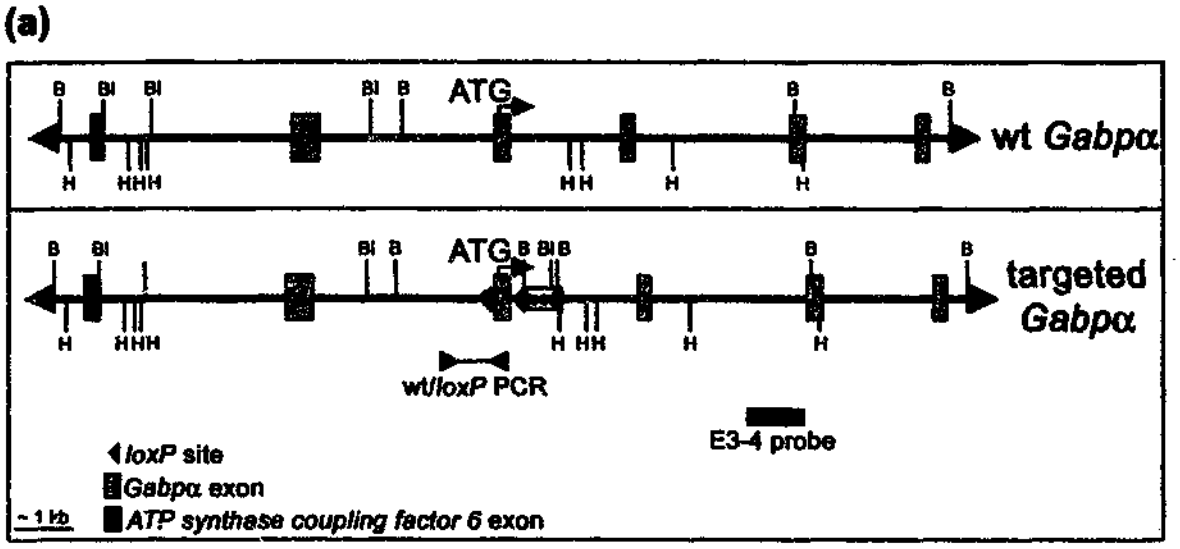


Figure 6.8 - Targeting of ES Cells for Conditional Loss of *Gabpα*. Wildtype (wt) and targeted *Gabpα* alleles are shown in **(a)**. The targeted allele has a floxed exon 2 and *Neomycin* cassette (*neo*) within intron 2. *Bam*HI (B), *Bal*I (Bl) and *Hin*dIII (H) sites are shown. **(b)** *In vitro* testing of *Gabpα* exon 2 deletion, by *Bam*HI digestion of the targeting construct before (targ) and after transformation into *Cre+ E.Coli* (+cre), to give 7.0, 2.0 and 0.7 kb or 7.0 and 1.5 kb fragments, respectively. **(c)** Southern blot analysis of genomic DNA from G418 resistent ES cell clones (numbers shown), using a *Bam*HI digest and E3-4 probe to detect 7 kb wildtype and 5 kb targeted fragments. *Bal*I and *Hin*dIII digestion and hybridisation with a *Neomycin* probe detected 2.5 and 6.5 kb fragments in targeted ES cells **(d)**. **(e)** PCR screening of targeted ES cell clones, using primers flanking the *loxP* site within intron 1, and hybridisation of the 1.3 kb product with the *loxP* sequence.

*Bam*HI before and after transformation of the 9.7 kb targeting vector into Cre$^+$ *E.Coli* (Clontech) (see **Figure 6.8b**). This generated 7 kb, 2 kb and 0.7 kb fragments prior to excision and 7 kb and 1.5 kb fragments following Cre-mediated excision of 1.2 kb of DNA, including a *Bam*HI site within the *Neomycin* cassette. The validated *Gabpα* conditional targeting construct was then linearised at a *Sac*II site within the pBS vector backbone and electroporated into SvJ129 ES cells.

Genotyping *Gabpα* Targeted ES Cells

Genomic DNA was extracted from 235 ES cell clones, digested with *Bam*HI, and transferred onto nylon membrane by Southern blot. A 977 bp *Sac*II-*Spe*I probe within intron 3 (as described in **Chapter 4**) was used to detect a 7 kb wildtype fragment and a 5 kb targeted fragment (see **Figure 6.8c**). DNA from four potentially positive clones (numbers 11, 28, 70 and 231) was analysed further. Presence of the *Neomycin* cassette was confirmed by digestion of genomic DNA with *Hind*III or *Bal*I and Southern blot hybridisation with an 800 bp *Bam*HI-*Mlu*I fragment of *Neomycin*, detecting 6.5 kb or 2.5 kb targeted fragments, respectively. As shown in **Figure 6.8d** all four potential positive clones were found positive by this analysis. Presence of the single *loxP* site upstream of exon 2 was confirmed by *loxP* oligonucleotide hybridisation (primer 4) of 1.3 kb PCR products spanning intron 1 to exon 2 (primers 128 and 61 at 2745-4055 bp of the complete *Gabpα* genomic sequence) (see **Figure 6.8e**). It was concluded from this analysis that clones 11, 28, 70 and 231 were correctly targeted for conditional deletion of *Gabpα* exon 2. Two clones (numbers 28 and 70) were chosen to be electroporated with Cre recombinase *in vitro*, to remove the *Neomycin* cassette prior to blastocyst injection.

Genotyping of *Cre* Transfected *Gabpα* Targeted ES Cells

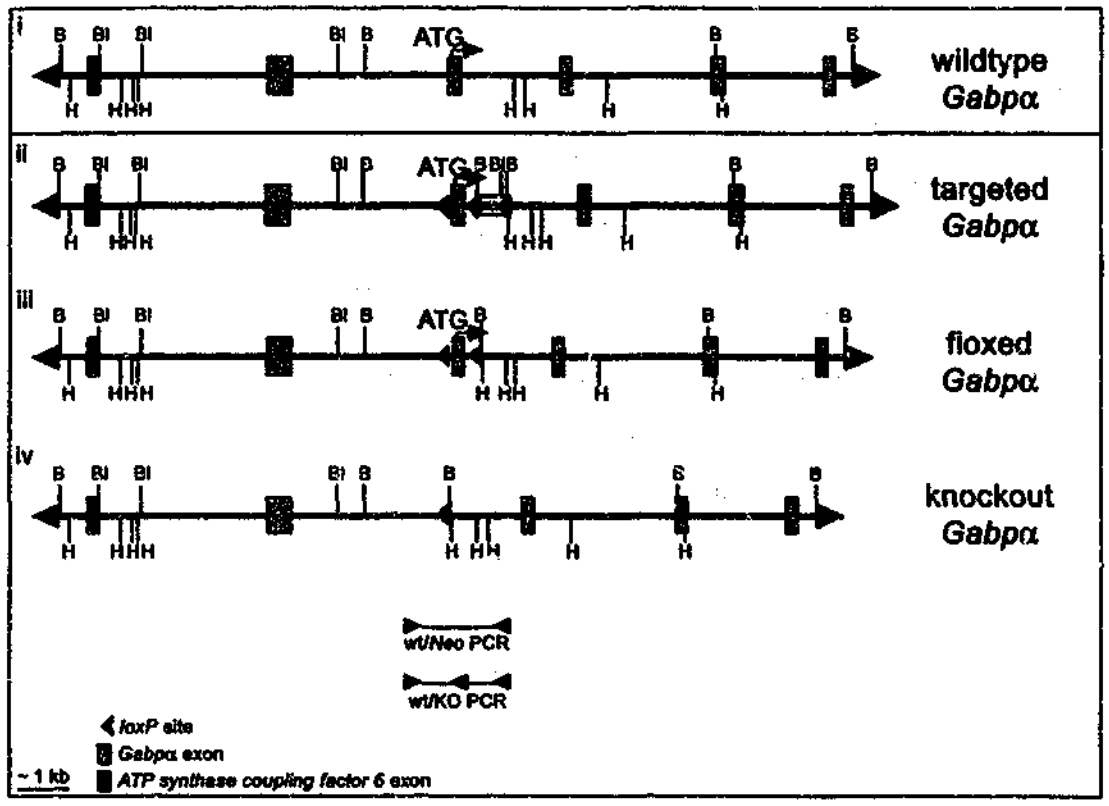
Removal of the *Neomycin* cassette was achieved by transient transfection of *Gabpα* targeted clones with Cre recombinase. The ES cells were electroporated with 2 μg of a plasmid expressing *Cre* recombinase under the control of the *Phosphoglycerate kinase* (*PGK*) promoter, in the pBS KS$^+$ (Stratagene) vector backbone, together with 1 μg of a *PGK-puromycin*-pBS KS$^+$ plasmid (both vectors supplied by Dr. Trevor Wilson, Centre for Functional Genomics and Human Disease, Monash IRD, Victoria, Australia). This allowed for antibiotic selection of 96 positive ES cell clones, 48 from each of the two parental ES cell clones.

As outlined in **Figure 6.9a**, these 96 clones were screened for presence of **(i)** wildtype, **(ii)** targeted, **(iii)** floxed and **(iv)** knockout alleles of *Gabpα*. A PCR product spanning intron 1 to intron 2 (primers 128 and 135 at 2745-4503 bp of the complete *Gabpα* genomic sequence, see **Appendix E**) was amplified, generating 1.5 kb, 1.8 kb and 1.9 kb fragments from the knockout, wildtype and floxed alleles, respectively (see **Figure 6.9b**). Presence of the single *loxP* site immediately upstream of exon 2 was confirmed by multiplex PCR, using a common 5' primer within intron 1 (primer 134 at 3316-3335 bp of the complete *Gabpα* genomic sequence) and 3' primers within exon 2 and intron 2 (primer 61 at 4036-4055 bp and primer 135 at 4484-4503 bp of the complete *Gabpα* genomic sequence). This reaction generated 620 bp, 660 bp and 740 bp fragments representing wildtype, floxed and knockout *Gabpα* alleles, respectively (see **Figure 6.9c**). Cells from the single ES cell clone heterozygous for the floxed *Gabpα* allele (+/loxP) were injected at a concentration of 1.5 x 10^5 cell/ml into blastocysts of C57Bl/6 female mice and transferred into pseudopregnant C57Bl/6 female mice (kindly performed by Susan Tsao, Centre for Functional Genomics and Human Disease, Monash IRD, Victoria, Australia). Resulting chimaeric pups were genotyped for presence of the floxed *Gabpα* allele.

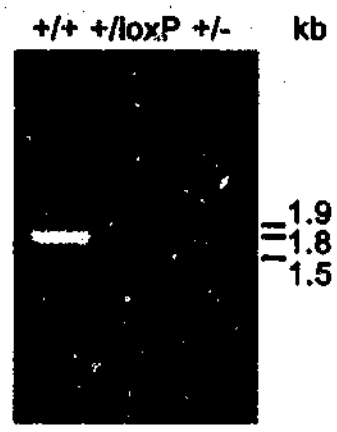
Genotyping of Mice Heterozygous for the *Gabpα* Floxed Allele

The percentage contribution of the SvJ129 ES cells featuring the *Gabpα* floxed allele to the chimaeric pups was estimated upon visual inspection of coat colour one week after birth. As shown in **Figure 6.10a**, mice born from blastocyst transfer into C57Bl/6 females (which are black) ranged in their degree of agouti coat colour (due to SvJ129 ES cells). In total, from the 29 mice obtained, 7 mice featured a coat of >90 % agouti colour and another 11 mice featured a coat of 10-60 % agouti colour. Chimaeras were genotyped by PCR with primers spanning intron 1 to exon 2 (primers 134 and 61 as described above), yielding 660 bp and 620 bp products representing floxed and wildtype alleles, respectively (see **Figure 6.10b**). Genotypes of chimaeras were confirmed by Southern blot analysis of *Bam*HI digested genomic DNA and hybridisation with a 977 bp *Sac*II-*Spe*I probe within intron 3 (see **Chapter 4**), detecting 7 kb wildtype and 5 kb floxed fragments (see **Figure 6.10c**). Mice heterozygous for the floxed *Gabpα* allele were bred with SvJ129 mice to generate a pure SvJ129 background, and C57Bl/6 mice to generate a mixed SvJ129 x C57Bl/6 line. Subsequent breeding of the floxed *Gabpα* mice with mice transgenic for *Cre* recombinase generated conditional knockout *Gabpα* mice (see section **6.7.1**).

(a)



(b)



(c)

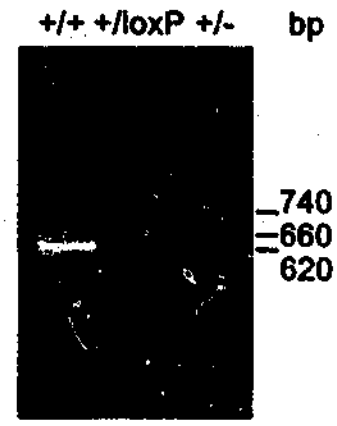
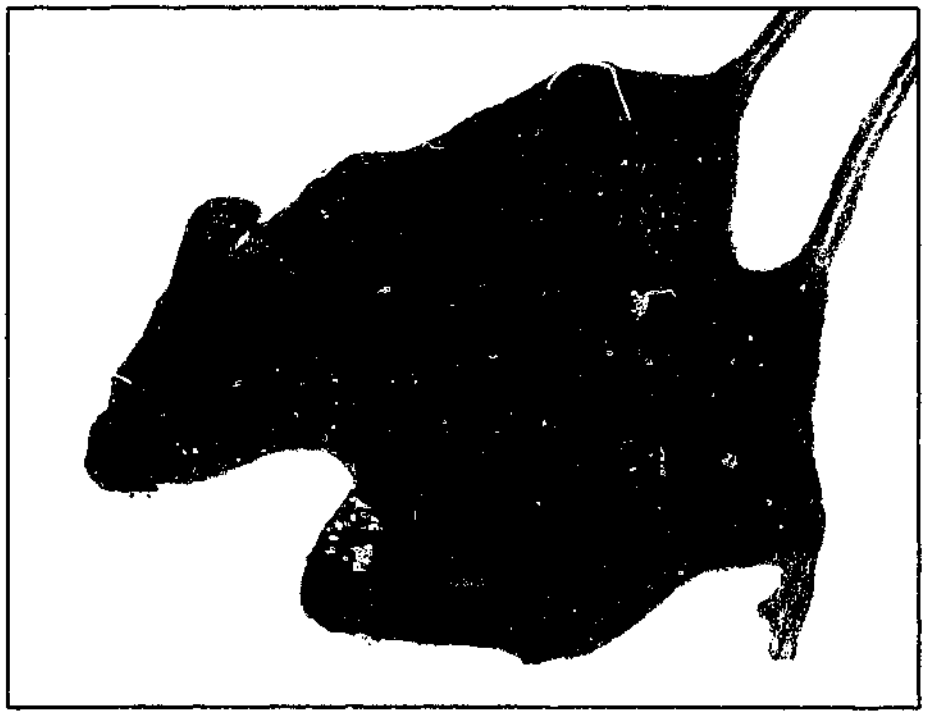


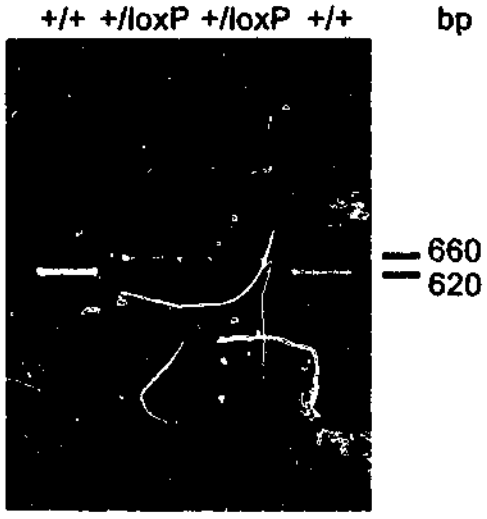
Figure 6.9 - *In vitro Cre* Transfection of *Gabpα* Targeted ES Cells.
The four possible *Gabpα* alleles following transient transfection of *Gabpα* targeted ES cells with *Cre* and *Puromycin* resistance vectors: (i) wildtype (wt or +/+), (ii) targeted (targ), (iii) floxed (loxP), and (iv) knockout (KO or -/-) are shown in **(a)**. PCR screening of resulting Puromycin resistant ES cell clones was performed with primers spanning *Gabpα* introns 1 and 2, flanking the site of insertion of the *Neomycin* cassette (wt/*Neo* PCR) **(b)**, or with three primers spanning *Gabpα* intron 1, to either exon 2 (wt or loxP alleles) or intron 2 (KO allele) (wt/KO PCR) **(c)**.

(a)



(b)

wt/loxP PCR



(c)

*Bam*HI

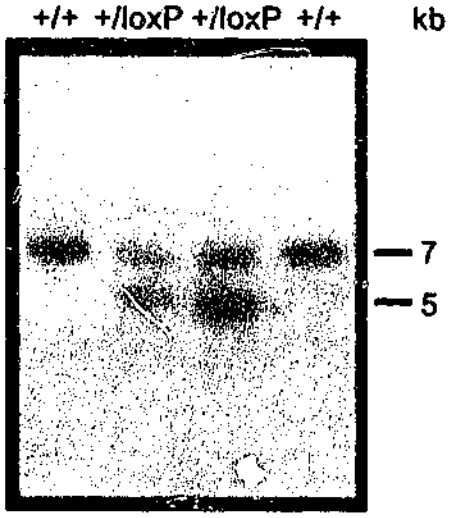


Figure 6.10 - Genotyping of Mice Heterozygous for the Floxed *Gabpα* Allele.

Mice resulting from injections of SvJ129 ES cells heterozygous for the floxed *Gabpα* allele into blastocysts of C57Bl/6 mice were chimaeric, varying in the percentage of agouti coat colour **(a)**. Chimaeras were genotyped for presence of the wildtype (+) and floxed (loxP) *Gabpα* alleles by genomic PCR **(b)**, using primers spanning *Gabpα* intron 1 to intron 2, to generate 620 bp wildtype and 660 bp floxed fragments. Southern blot analysis by *Bam*HI digestion of genomic DNA and hybridisation with an intron 3 probe was used to confirm these results **(c)**, detecting 7 kb wildtype and 5 kb floxed fragments.

6.5 *Myogenin-Cre Transgenic Mice*

6.5.1 Existing Skeletal Muscle-Specific *Cre* Transgenic Mice

Creation of a skeletal muscle-specific knockout *Gabpα* mouse relies upon use of a skeletal muscle-specific promoter to drive expression of Cre recombinase. Choice of a suitable promoter is limited, as many genes are expressed in both cardiac and skeletal muscle, and deletion of genes necessary for heart development often results in embryonic lethality. The most efficient of the characterised skeletal muscle-specific *Cre* transgenic lines utilises the human *α-Skeletal Actin* (*hαSA*) promoter (Miniou et al. 1999). The *hαSA-Cre* mouse line has been successfully used to generate a mouse model of spinal muscular atrophy that closely resembles the human condition (Frugier et al. 2000). Another skeletal muscle-specific *Cre* transgenic mouse line has been generated using the *Muscle Creatine Kinase* (*MCK*) promoter to drive *Cre* expression (Brüning et al. 1998). These mice show a higher level of background expression of Cre in the heart, reflecting the natural expression pattern of Muscle Creatine Kinase (Trask and Billadello 1990). However, the *MCK-Cre* mouse line has successfully been used to study the role of skeletal muscle insulin receptors on the phenotype of NIDDM (Brüning et al. 1998). The third successful use of skeletal muscle-specific targeting of Cre expression is that driven by the *Myosin Light Chain 1f* (*mlc1f*) promoter (Bothe et al. 2000). This *mlc1f-Cre* mouse line has been used to specifically delete the *ErbB4* gene, however the *mlc1f* gene is the fast twitch isoform of the myosin light chain (Rao et al. 1996), so excision may favour fast twitch muscle fibres. The promoter chosen to drive *Cre* expression in this study was that of the MyoD transcription factor *Myogenin*.

6.5.2 *Myogenin* Expression

Myogenin is a member of the MyoD family of transcription factors, involved in the differentiation of myoblasts to myotubes (Venuti et al. 1995) and activation of muscle structural genes, by binding to the E-box in target genes (Wright et al. 1989). *Myogenin* knockout mice die within minutes of birth, indicating the importance of Myogenin function during myogenesis. Mice lacking *Myogenin* have a decreased number of muscle fibres and do not express AChRα or γ proteins, resulting in lack of AChR clusters and nerve connection to muscle (Venuti et al. 1995). *Myogenin* mRNA is expressed from as early as E8.5, in the developing myotome of somites (Wright et al. 1989; Cheng et al. 1992; Yee and Rigby 1993), showing specificity to skeletal myogenesis (Sassoon et al. 1989).

Myogenin is expressed throughout developing myofibres and muscle of newborn mice, but not in innervated adult muscle (Simon et al. 1992). In accordance with this, expression of *Myogenin* mRNA is 20-fold higher in rat foetal thigh muscle compared to that of an adult (Wright et al. 1989). A 133 bp fragment upstream of the *Myogenin* transcription start site is enough to provide expression of *Myogenin* in the mouse (Yee and Rigby 1993), however an element between 1092 and 133 bp upstream is needed for maximal expression. The *Myogenin* promoter has previously been used to generate transgenic mice overexpressing Glial-derived neurotrophic factor (GDNF) in skeletal muscle (Nguyen et al. 1998).

6.5.3 Generation of *Myogenin-Cre* Transgenic Mice

Myogenin-Cre Transgenic Construct Generation

A 1.6 kb fragment of the *Myogenin* promoter spanning 27-1621 bp of genomic sequence GI:200003, amplified with *Elongase* DNA polymerase (Invitrogen) in the presence of 0.3 mg/ml BSA (Promega) (primers 189 and 190), was cloned upstream of a *Cre* recombinase cDNA RT-PCR product in the pGEM-T (Promega) cloning vector (see **Figure 6.11a**). *Cre* PCR primers 187 and 188 were used to amplify a product spanning 574-1950 bp of the *PGK-Cre*-pBS KS$^+$ sequence with *Pfu* DNA polymerase (Promega) in the presence of 1 M Betaine (Sigma)). Following sequencing of the transgenic construct with primer 214, the 3 kb *Myogenin-Cre* transgene was removed from the vector backbone by *Sac*II, *Sal*I and *Sca*I restriction endonuclease digestion, purified by dialysis and microinjected into F1 (CBA x C57Bl/6) mouse pronuclei at 2 ng/µl in TE buffer. Microinjection was performed by Linda Weiss, Centre for Functional Genomics and Human Disease, Monash IRD, Victoria, Australia.

Genotyping of _Myogenin-Cre_ Transgenic Mice

Mice generated from pronuclear injection were genotyped by Southern blot and PCR for integration of the *Myogenin-Cre* transgene. Genomic DNA was digested with *Nco*I and transferred onto nylon membrane by Southern blot. Membranes were hybridised with a 246 bp *Bgl*I-*Sac*II *Myogenin* promoter probe (spanning 1375-1621 bp of GI:200003), detecting an 800 bp wildtype fragment and a 3 kb transgenic fragment (see **Figure 6.11b**). In addition, membranes were hybridised with a 1.4 kb *Sal*I-*Not*I *Cre* cDNA probe (spanning 567-1968 bp of *PGK-Cre*-pBS) detecting a 3 kb transgenic fragment (see **Figure 6.11c**). Southern blot results were confirmed by PCR genotyping using PCR primers spanning 626-1043 bp of *PGK-Cre*-pBS (primers 213 and 214). A 416 bp product was

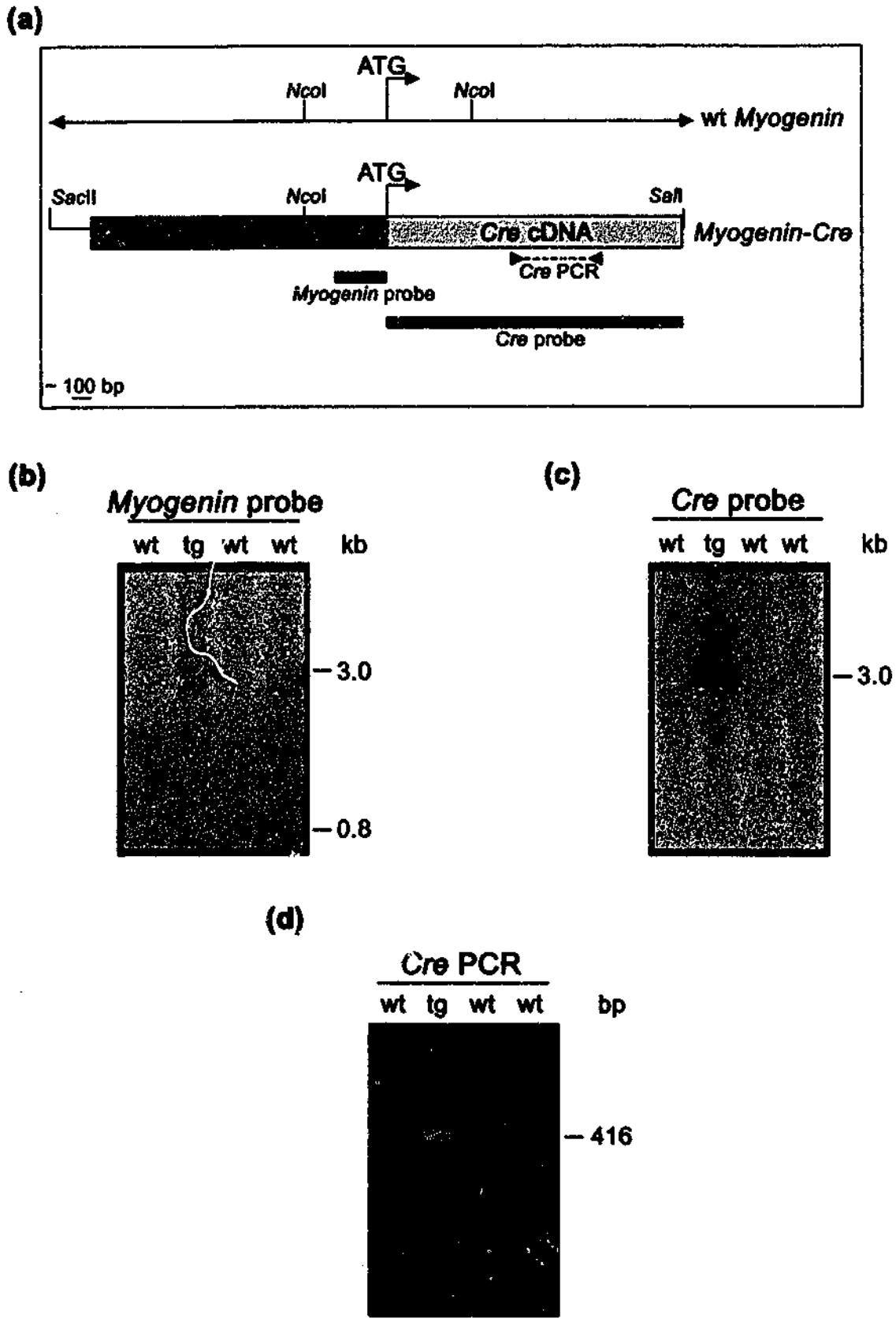


Figure 6.11 - Generation of *Myogenin-Cre* Transgenic Mice.
The wildtype (wt) *Myogenin* locus and the *Myogenin-Cre* transgenic construct, fusing the *Cre* cDNA to the start codon of *Myogenin*, are shown in **(a)**. Restriction endonuclease sites are indicated. *Myogenin-Cre* transgenic mice (tg) were identified by Southern blot, using an *Nco*I digest and hybridisation with a probe within the *Myogenin* promoter region **(b)**, detecting an endogenous *Myogenin* fragment of 0.8 kb and the 3 kb *Myogenin-Cre* transgene. Hybridisation of the same *Nco*I digest products with a *Cre* cDNA probe detected the 3 kb transgene only **(c)**. Genomic PCR analysis using primers within the *Cre* cDNA confirmed Southern blot results, generating a 416 bp transgene product **(d)**.

amplified from transgenic mice (see **Figure 6.11d**). Together, this analysis identified 6 *Myogenin-Cre* transgenic mice from a total of 39 born from pronuclear injection. These *Myogenin-Cre* transgenic founders were bred with wildtype F1 (CBA x C57Bl/6) mice and assessed for their ability to transmit and express the *Myogenin-Cre* transgene.

6.5.4 Assessment of *Myogenin-Cre* Transgene Expression

Progeny from 4 of the 6 potential *Myogenin-Cre* founding lines were capable of transmitting the *Myogenin-Cre* transgene, as determined by PCR genotyping. One line was terminated due to premature death of pups, presumed to be due to insertion of the transgene at an unfavourable site. The 3 transmitting *Myogenin-Cre* lines were tested for transgene mRNA expression by Northern blot analysis of poly-A+ RNA from skeletal muscle of the quadricep. Hybridisation was performed with a 1.4 kb *SalI-NotI Cre* cDNA probe (as described above), however the expected fragment of 1.5 kb was never seen (see **Figure 6.12a** for a representative image). RNA was visible by ethidium bromide staining of the agarose gel prior to transfer (see **Figure 6.12b**) and subsequent hybridisation of the filter with a *Gabpα* cDNA probe spanning 413-1884 bp of GI:193382 (primers 52 and 53) detected the full length 5.4 kb transcript (see **Figure 6.12c**), confirming RNA transfer. Endogenous mRNA levels of *Myogenin* in skeletal muscle of the adult mouse are 20-fold lower than those seen during embryogenesis (Wright et al. 1989), so it was thought that the sensitivity of Northern blot analysis could be too low to detect the *Myogenin-Cre* transgene. RT-PCR amplification of a 416 bp *Cre* product (primers 213 and 214) spanning 626-1043 bp of *PGK-Cre*-pBS was performed using cDNA template from quadricep skeletal muscle of 4 week old wildtype and *Myogenin-Cre* transgenic mice from each of the 3 transmitting lines. However, as shown in **Figure 6.12d**, the *Cre* product was only ever amplified from positive control plasmid DNA of the *Myogenin-Cre* transgenic construct. Amplification of a 400 bp *Gapdh* RT-PCR product spanning 585-985 bp of sequence GI:193423 (primers 58 and 59) verified that the cDNA template was not degraded (see **Figure 6.12e**). The final test for *Myogenin-Cre* transgene expression was the analysis of embryos resulting from matings of the 3 transmitting transgenic lines with the *ROSA26-lacZ* reporter line (obtained from The Jackson Laboratories, Maine, USA).

ROSA26-lacZ mice allow for testing of the correct temporal and/or spatial expression pattern of Cre, by means of expressing β-galactosidase following Cre-mediated excision of a stop codon within the *β-galactosidase* (*lacZ*) coding sequence (Mao et al. 1999b).

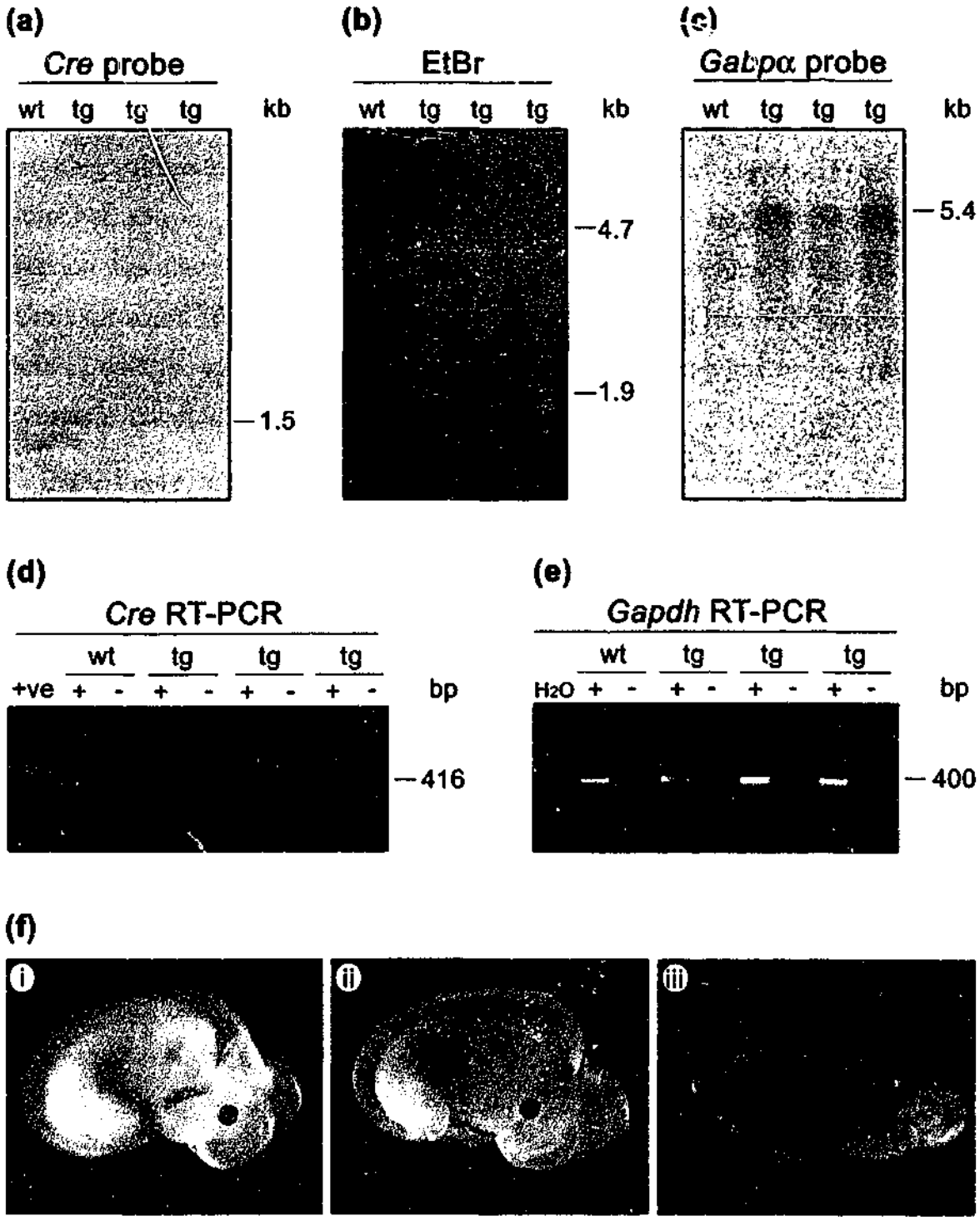


Figure 6.12 - *Myogenin-Cre* Transgene Expression Analysis.
Expression of the *Myogenin-Cre* transgene in transgenic (tg) and wildtype (wt) mice was assessed by Northen blot using a *Cre* cDNA probe to detect a 1.5 kb transgenic fragment **(a)**. Loading of RNA is indicated by the relative intensity of 18 S (1.9 kb) and 28 S (4.7 kb) ribosomal RNA fragments stained with ethidium bromide **(b)**, and RNA integrity was confirmed by reprobing with a *Gabpα* cDNA probe to detect the predominant 5.4 kb *Gabpα* transcript **(c)**. RT-PCR analysis of *Cre* expression was performed using primers within the *Cre* cDNA sequence, to generated a 416 bp product **(d)**. Integrity of the cDNA template was confirmed by amplification of a 400 bp *Gapdh* RT-PCR product **(e)**. LacZ staining of E12.5 embryos from *Myogenin-Cre* x *ROSA26-lacZ* matings **(f)** revealed lack of expression in two *Myogenin-Cre* lines (i) and ectopic expression of Cre, associated with the spinal cord, in the third transmitting line (ii and iii).

Embryos of E9.5-12.5 from *Myogenin-Cre* x *ROSA26-lacZ* matings were analysed for Cre expression by lacZ staining. Presence of the *lacZ* transgene was confirmed by PCR genotyping with primers 267 and 268, spanning 927-1274 bp of cDNA GI:41901, and *Cre* as described above. As shown in **Figure 6.12f**, no skeletal muscle-specific reporter gene expression was observed. Two of the three *Myogenin-Cre* transgenic lines tested exhibited no Cre expression visible by lacZ staining, and the third line showed expression of Cre associated with the spinal cord rather than the myotome, presumably an artefact of the site of transgene integration.

In the future, integration effects could be avoided by targeting the *Myogenin* locus or a defined locus by homologous recombination, which has been shown to result in single-copy integration and similar expression levels in different mouse lines (Bronson et al. 1996). Homologous recombination may be similarly used to produce mice expressing the *Gabpα* transgene (see section 6.2). Flanking the transgene with insulator sequences such as HS4 of the chicken *β-globin* gene, that are known to prevent DNA silencing, should also assure transgene expression (Mutskov et al. 2002).

Myogenin-Cre transgenic mice were not used for the tissue-specific deletion of *Gabpα*, due to a lack of evidence of skeletal muscle-specific Cre expression. Instead, a breeding pair of the validated skeletal muscle-specific human *α Skeletal Actin-Cre* (*hαSA-Cre*) mouse line (Miniou et al. 1999) was kindly provided by Dr. Judith Melki, Institut de Génétique et de Biologie Moléculaire et Cellulaire, C.U. de Strasbourg, France.

6.6 Human α Skeletal Actin-Cre Transgenic Mice

6.6.1 *Human α Skeletal Actin* Expression

Actin is a thin filament protein that slides over the thick filament protein Myosin, and is essential for muscle contraction. Several isoforms of Actin exist: skeletal muscle, cardiac muscle, smooth muscle and non-muscle isoforms (McHugh et al. 1991). Unlike other Actin isoforms, genes encoding α skeletal and α cardiac Actin are present in only one copy in the human genome (Ponte et al. 1983; Gunning et al. 1984; Mogensen et al. 1998). Mutations in the human *α Skeletal Actin* gene (*hαSA*) are known to be associated with myopathies of varying severity (Nowak et al. 1999), highlighting its important role in skeletal muscle. The promoter of *hαSA* has been characterised and elements within 153 to

1300 bp of the transcription start site that are required for skeletal muscle-specific expression (Muscat and Kedes 1987; Muscat et al. 1992; Brennan and Hardeman 1993). This expression pattern has previously been confirmed for the *hαSA-Cre* mouse line by breeding with the *ROSA 26-lacZ* reporter line and staining for β-galactosidase expression (Miniou et al. 1999).

6.6.2 Generation of *Human α Skeletal Actin-Cre* Transgenic Mice

A breeding pair of *hαSA-Cre* mice (on the C57Bl/6J x SJL F1 background) were provided by Dr. Judith Melki, Institut de Génétique et de Biologie Moléculaire et Cellulaire, C.U. de Strasbourg, France. As previously described (Miniou et al. 1999), a 2.2 kb fragment of the *hαSA* promoter was cloned upstream of a *Chicken β-globin* intronic region, followed by the *Cre* recombinase cDNA sequence (GI:12965137) (**Figure 6.13a**).

Genotyping of *Human α Skeletal Actin-Cre* Transgenic Mice

Progeny of *hαSA-Cre* mice were genotyped for presence of the transgene by PCR of genomic DNA with primers spanning 1808-2605 bp of the *Cre* cDNA sequence GI:12965137 (primers 265 and 266, using *Taq* DNA polymerase (Promega) with 1.5 mM $MgCl_2$), generating a 797 bp product in transgenic mice (see **Figure 6.13b**).

6.6.3 Validation of *Human α Skeletal Actin-Cre* Transgene Expression

Skeletal muscle-specific expression of the *hαSA-Cre* transgene was confirmed by use of the *ROSA 26-lacZ* reporter line, genotyping mice with primers 267 and 268 (spanning 927-1274 bp of GI:41901) for presence of the *lacZ* transgene, and as above for the *hαSA-Cre* transgene. Mice from *hαSA-Cre* x *ROSA 26-lacZ* matings were killed at E12.5-13.5 and stained for β-galactosidase expression (see **Figure 6.13c**), as the *hαSA-Cre* transgene has been shown previously to enable Cre-mediated DNA excision from as early as E9 (Miniou et al. 1999; Frugier et al. 2000). Myotome-specific lacZ staining was observed in approximately 25 % of all embryos tested, as expected from Mendelian inheritance of the two transgenes. Male and female *hαSA-Cre* transgenic mice were subsequently bred with mice carrying the floxed *Gabpα* allele to generate skeletal muscle-specific *Gabpα* knockout mice.

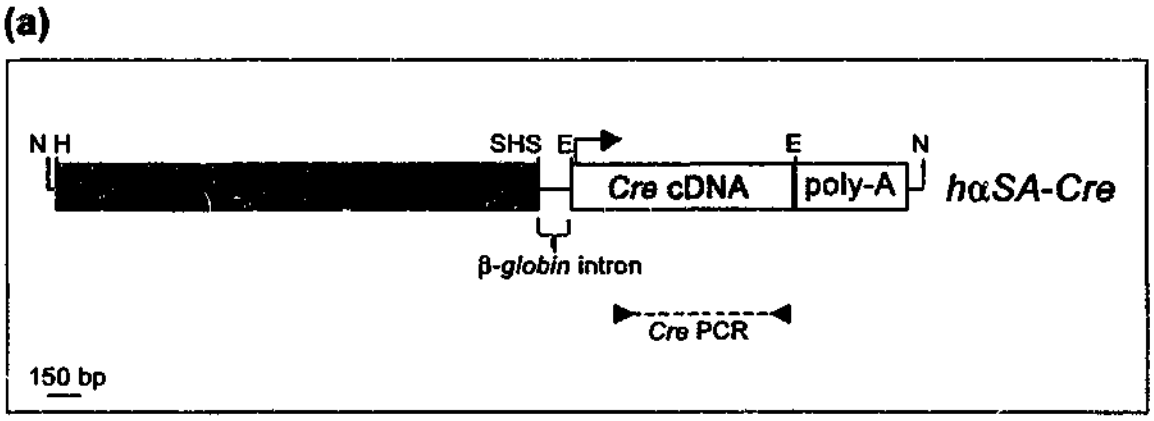


Figure 6.13 - Validation of *hαSA-Cre* Transgene Expression.
(a) The *hαSA-Cre* transgenic construct, featuring 2.2 kb of the human α-*Skeletal Actin* promoter, upstream of the rabbit β-*globin* intron, *Cre* cDNA and SV40 poly-adenylation signal. *Hind*III (H), *Sal*I (S) *Eco*RI (E) and *Not*I (N) restriction sites are shown. Figure reproduced from Miniou et al. 1999. **(b)** Wildtype (wt) and transgenic (tg) progeny of *hαSA-Cre* interbred mice were genotyped by PCR using primers within the *Cre* cDNA sequence, to yield a 797 bp product in transgenic mice only. **(c)** LacZ staining of E13.5 embryos from *hαSA-Cre* x *ROSA 26-lacZ* matings, showing positive staining in myotome blocks and developing limb buds of mice carrying both *hαSA-Cre* and *lacZ* transgenes (i and ii), while no staining was observed in wildtype littermates (right side of panel i).

6.7 *Gabpα Skeletal Muscle-Specific Knockout Mice*

Loss of ubiquitous Gabpα expression in the mouse results in early embryonic lethality, indicating its essential function in early development. Given the number of GABP target genes that are necessary for neuromuscular signalling (Schaeffer et al. 2001) and mitochondrial respiration (Scarpulla 2002b) (see **Table 2.5**), together with the documented involvement of GABP in the generation of some cases of Congenital Myasthenic Syndrome (Nichols et al. 1999; Ohno et al. 1999), the role of GABPα in skeletal muscle development was assessed by creation of a skeletal muscle-specific knockout mouse model.

6.7.1 Generation of *Gabpα* Skeletal Muscle-Specific Knockout Mice

Genotyping *Gabpα* Skeletal Muscle-Specific Knockout Mice

Progeny resulting from breeding of mice heterozygous for the floxed *Gabpα* allele and carrying the *hαSA -Cre* transgene (heterozygous conditional knockout mice) were genotyped for *Gabpα* by PCR with a 5' primer within intron 1 and 3' primer within intron 2 (primers 134 and 135, detailed in section **6.4.2**), yielding 620 bp wildtype and 660 bp floxed allele products. Mice were concurrently genotyped for presence of the *Cre* transgene by PCR with primers spanning 1808-2605 bp of the *Cre* cDNA (primers 265 and 266, see section **6.6.2**), generating a 797 bp transgene product. As shown in **Figure 6.14a**, the 6 possible genotypes are: wildtype (+/+), heterozygous floxed (+/loxP), homozygous floxed (loxP/loxP), *hαSA-Cre* transgenic (+/cre), heterozygous conditional knockout (+/-), and homozygous conditional knockout (-/-). Note that the heterozygous and homozygous conditional knockout mice carry floxed *Gabpα* and *Cre* transgene alleles, but are represented as +/- and -/- for simplicity.

Cre-mediated excision of *Gabpα* exon 2 was confirmed to be skeletal-muscle specific by *Gabpα* PCR genotyping (primers 134, 61 and 135, see section **6.4.2**) using genomic DNA extracted from brain, heart, intestine and skeletal muscle of the gastrocnemius, soleus, diaphragm and quadricep from 4 weeks old wildtype, heterozygous and homozygous conditional knockout mice (see **Figure 6.14b**). Sequencing of PCR products confirmed loss of exon 2 in skeletal muscle only. However, not all DNA extracted from skeletal muscle of homozygous conditional knockout mice was recombined, as demonstrated by presence of the 660 bp floxed PCR product in addition to

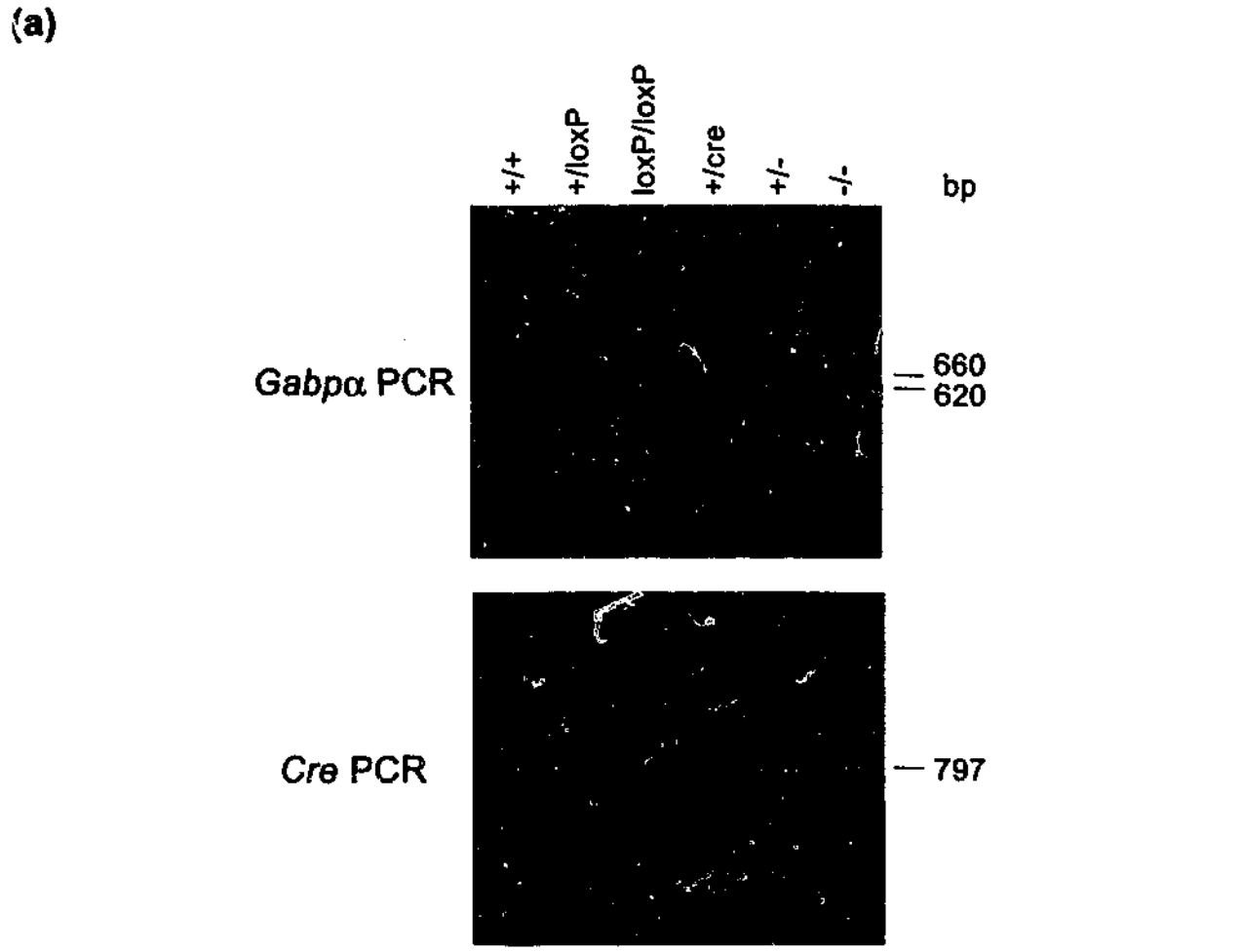


Figure 6.14 - *Gabpα* Skeletal Muscle-Specific Knockout Genotyping.
(a) Progeny resulting from intercrosses of mice carrying the *Gabpα* floxed allele and the *hαSA-Cre* transgene were genotyped by PCR. *Gabpα* products were amplified with primers spanning introns 1 and 2, generating 660 bp floxed (loxP) and 620 bp wildtype (+) products. The *hαSA-Cre* transgene (cre) was detected by PCR with primers within the *Cre* cDNA sequence, generating a 797 bp product in transgenic mice. **(b)** Deletion of *Gabpα* exon 2 was shown to be specific to skeletal muscle by *Gabpα* PCR genotyping of DNA extracted from organs of wildtype (+/+), heterozygous (+/-) and homozygous knockout (+/-) mice. Primers used were a common 5' primer within intron 1 and 3' primers within exon 2 and intron 2, to generate 740 bp, 660 bp and 620 bp products, representing knockout, floxed and wildtype alleles, respectively.

the 740 bp knockout product. This reflects the composition of skeletal muscle tissue; approximately equal proportions of muscle cells and connective tissue (Bothe et al. 2000).

Viability of *Gabpα* Skeletal Muscle-Specific Knockout Mice

Gabpα skeletal-muscle specific knockout mice were expected to be viable, as knockout mice completely lacking skeletal muscle are able to survive until several weeks after birth (Grieshammer et al. 1998). In addition, some mouse models of neuromuscular disease do not demonstrate obvious muscle weakness (Vincent et al. 1997), because mice and rats have a greater safety factor of neuromuscular transmission than humans (Gomez et al. 1996). The safety factor of neurotransmission is the ratio between the endplate potential generated under normal conditions and that required to generate an action potential (Liyanage et al. 2002). The high safety factor of mice makes them less susceptible to neuromuscular disease than humans. Genotyping at weaning (4 weeks of age) showed that the expected Mendelian ratio was observed from intercrosses of heterozygous *Gabpα* conditional knockout mice (as shown in **Table 6.1**).

6.7.2 Confirmation of Loss of Gabpα Expression

The loss of Gabpα protein expression in skeletal muscle of conditional knockout mice was confirmed by Western blot analysis. Lysates from lateral and medial gastrocnemius, soleus, vastus lateralis and diaphragm muscles of three 3 month old floxed, heterozygous and homozygous *Gabpα* skeletal muscle-specific knockout mice were assessed for Gabpα and β-tubulin expression (see **Figure 6.15a**). Gabpα protein levels were quantified relative to β-tubulin, and ratios were adjusted such that those of wildtype tissues were equal to 1 (see **Figure 6.15b**). A significant reduction in Gabpα protein levels was observed in all muscles of homozygous *Gabpα* skeletal muscle-specific knockout mice (as determined by a two-tailed t-test), ranging from 8 to 43 % of floxed levels in soleus and diaphragm, respectively. Gabpα expression levels in heterozygous mice also varied in a tissue-specific manner, from 20 to 84 % of floxed levels in medial gastrocnemius and diaphragm, respectively. The variability between mice meant that no significant difference in Gabpα protein levels in muscles of floxed and heterozygous conditional knockout mice was detected, as was also observed when quadricep muscles of ubiquitous *Gabpα* heterozygous and wildtype littermate mice were analysed (**Figure 6.7**). The presence of other cell types in skeletal muscle tissue is presumed to account for the remaining Gabpα protein seen in homozygous conditional knockout tissues.

Table 6.1 – Genotype Frequencies from *Gabpα* Heterozygous Conditional Knockout Matings.

Genotype	Observed No.	Expected No.
Wildtype	26	26
Heterozygous floxed	43	51
Homozygous floxed	32	26
hαSA-Cre transgenic	78	77
Heterozygous conditional knockout	153	154
Homozygous conditional knockout	78	77

Gabpα and *hαSA-Cre* PCR genotyping results of mice at weaning (4 weeks of age) resulting from matings of heterozygous skeletal muscle-specific knockout mice. The observed and expected numbers for each genotype are shown.

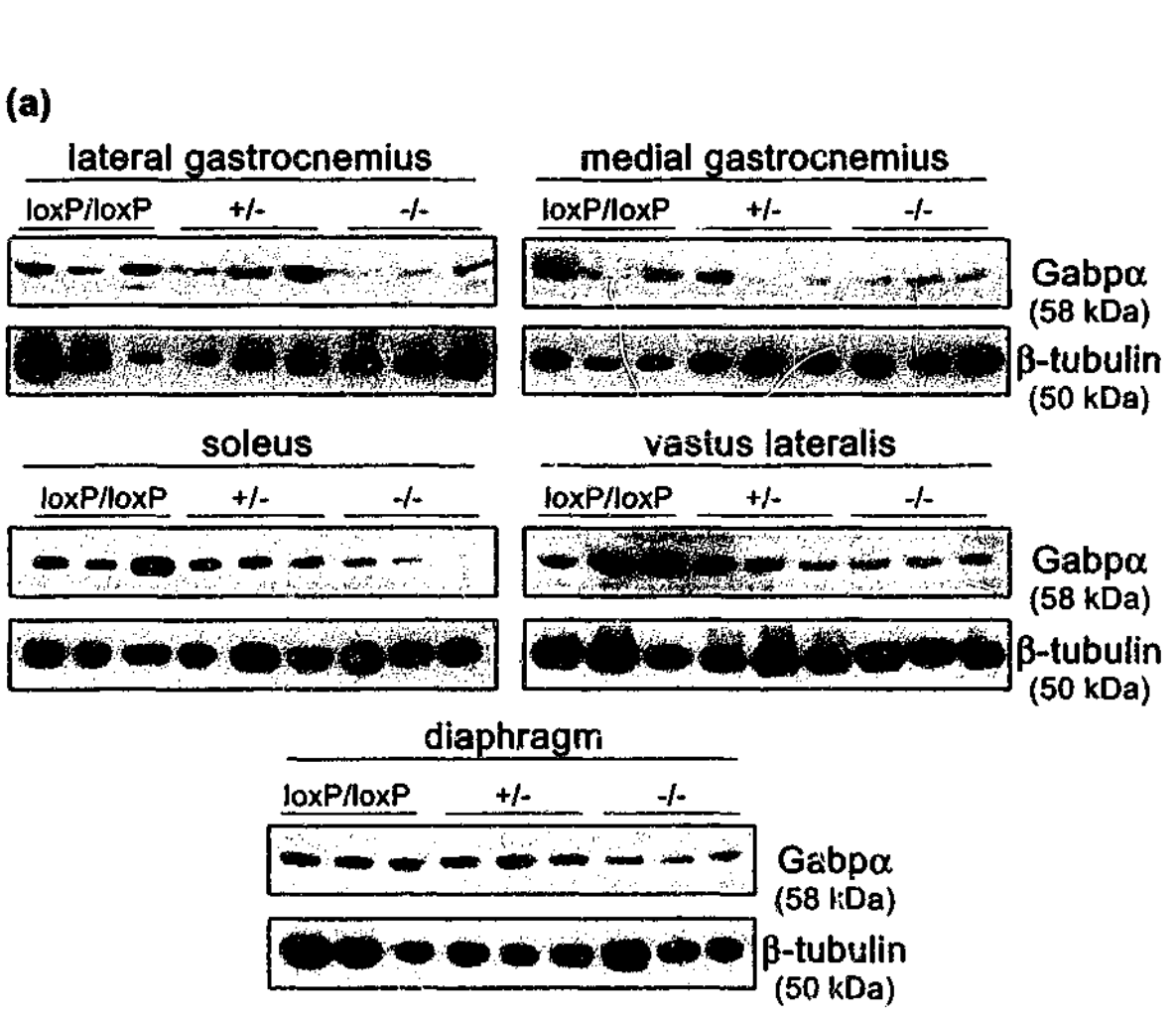


Figure 6.15 - Validation of *Gabpα* Conditional Knockout Mice.
(a) Levels of Gabpα and β-tubulin proteins in tissues from *Gabpα* skeletal muscle-specific knockout mice (-/-) relative to wildtype (loxP/loxP) and heterozygous (+/-) littermates were determined by Western blot analysis. **(b)** Quantification of Western blot analysis, where Gabpα protein levels are expressed as a ratio to β-tubulin. Ratios were adjusted such that those of wildtype tissues were equal to 1. Error bars represent SD, where n=3. * and ** represent statistical significance in a two-tailed t-test, where p=<0.05 and <0.005, respectively.

In order to assess Gabpα protein levels specifically in skeletal muscle cells, extracts from primary skeletal muscle cultures from 2-3 day old wildtype, heterozygous and homozygous conditional knockout mice were analysed (see **Figure 6.16**). Approximately ~50 % of conditional knockout primary skeletal muscle cell cultures show complete absence of Gabpα protein. This variability could be due in part to the presence of contaminating fibroblast cells within the skeletal muscle cultures, or could simply reflect heterogeneous Cre expression. LacZ staining of skeletal muscle tissue from *hαSA -Cre* x *ROSA 26-lacZ* mice has previously been used to estimate a Cre splicing efficiency of 99 % in the *hαSA -Cre* mouse line (Miniou et al. 1999), however this does not account for the fact that there may be heterogeneous excision within a multinucleated muscle cell. Together this analysis shows that skeletal muscle cell-specific loss of Gabpα protein expression was achieved, but there may not be homogeneous *Gabpα* deletion within muscle cells. The only means of confirming this would be immunohistochemistry with a Gabpα-specific antibody, which was unavailable at the time of this study.

6.8 Summary

Although a *Gabpα* promoter fragment was shown to be functional *in vitro*, this did not allow for expression of a *Gabpα* minigene transgenic construct in tissues of the 7 transmitting mouse lines tested. Therefore a different strategy, such as targeting a defined locus by homologous recombination, will be used in the future to create an overexpressing *Gabpα* mouse model, to investigate the role of GABPα in the phenotypes characteristic of Down syndrome. Complete loss of Gabpα expression, by means of gene substitution, results in embryonic lethality prior to implantation, and is probably the result of down-regulation of target genes necessary for mitochondrial transcription and cellular respiration, such as *MTFA*, *COXIV* and *COXVb*. However, *Gabpα* heterozygous mice show no significant reduction in Gabpα protein levels. In order to address the role of Gabpα (and the Gabp complex) in skeletal muscle function, particularly at the NMJ, skeletal muscle-specific *Gabpα* knockout mice were generated using the *Cre-loxP* system. *Myogenin-Cre* transgenic mice were generated, yet the three lines tested did not express the transgene. This is thought to be due to integration effects and may be prevented in the future by use of homologous recombination, and flanking the transgene with insulator

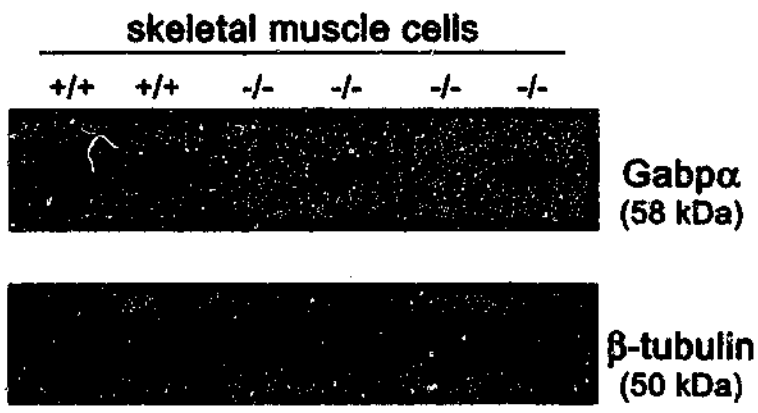


Figure 6.16 - Confirmation of Loss of Gabpα Protein Expression in Skeletal Muscle Cells of Conditional Knockout Mice.
Western blot analysis of Gabpα and β-tubulin protein levels in lysates of primary skeletal muscle cells isolated from 2-3 day old wildtype (+/+) and *Gabpα* skeletal muscle-specific knockout (-/-) pups.

sequences to prevent gene silencing. Conditional deletion of *Gabpα* in skeletal muscle was achieved by use of the 'flox-and-delete' strategy of gene targeting, splicing out the first coding exon of *Gabpα* by use of the previously validated *hαSA -Cre* mouse line. The reduction in Gabpα protein levels varies in a tissue-specific manner, and may be due to heterogeneous Cre expression and/or the innate properties of different skeletal muscle tissues. In order to detect any effects of heterogeneous Cre expression upon the phenotype of *Gabpα* conditional knockout mice, several skeletal muscle tissues were analysed, from both conditional knockout mice and *Gabpα* ubiquitous heterozygous mice, as described in the following chapter.

Chapter 7

Results:

Phenotype Analysis of Mouse Models of GABPα *Function*

7.1 Introduction

Gabpα is expressed in all tissues examined (see **Chapter 5**), yet many proposed GABP target genes are highly specialised, functioning in neuromuscular signalling at the NMJ of skeletal muscle and in mitochondrial respiration (see **Table 2.5**). Given that GABP is implicated in the regulation of genes of diverse functions, we have examined a mouse model heterozygous for *Gabpα* throughout all tissues, and mice homozygous for deletion of *Gabpα* in skeletal muscle cells only. Preliminary results of these phenotype analyses are presented here. Mice were analysed for; body weight, skeletal muscle weight, expression level of proposed Gabp target genes, proportions of muscle fibre types, AChR number and distribution, ultrastructure of the NMJ, gait, grip strength, and biophysical properties of endplate currents.

7.2 Phenotype Analysis of Gabpα Heterozygous Mice

Homozygous deletion of *Gabpα* in all tissues results in early embryonic lethality (see **Chapter 6**), therefore wildtype and heterozygous *Gabpα* mice were compared for general differences in body and organ weight, and tissue histology, and more specifically for differences in skeletal muscle structure and function.

7.2.1 Body and Organ Weight of *Gabpα* Heterozygous Mice

Body weight of male heterozygous *Gabpα* mice was monitored over 6 months and compared to that of wildtype littermates (see **Figure 7.1**). No significant difference was observed between the two genotypes, and both wildtype and heterozygous mice show a gradual increase in body mass with increasing age.

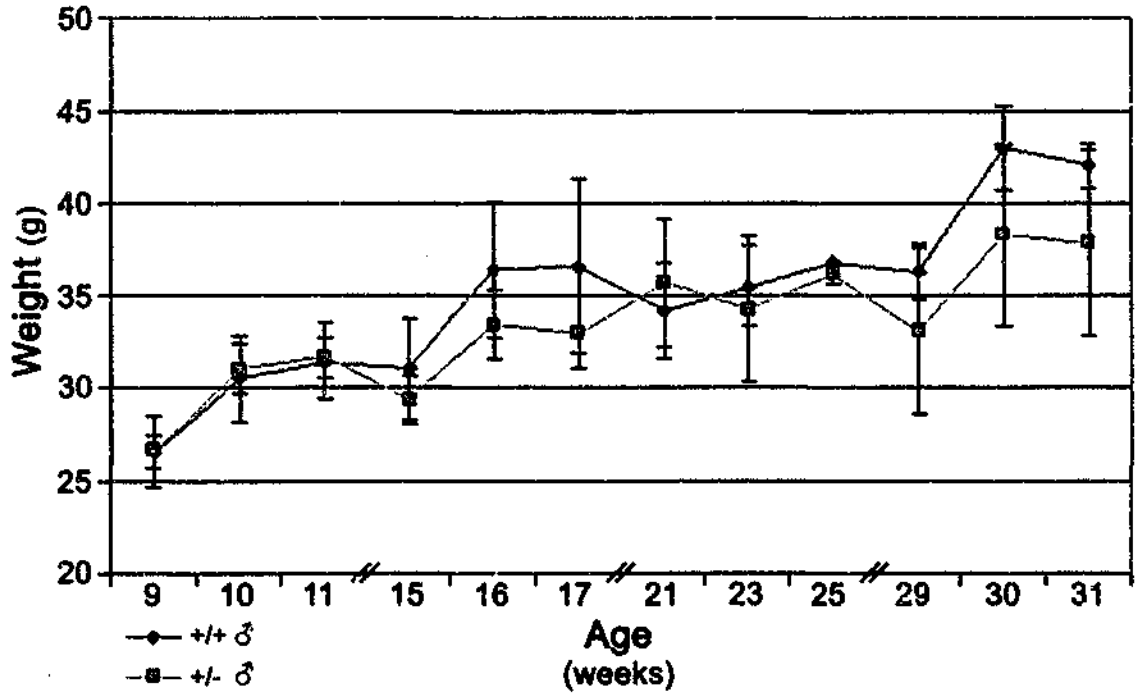


Figure 7.1 - Body Weight of *Gabpα* Heterozygous Mice.
The body weight of male (♂) mice heterozygous for *Gabpα* (+/-), compared to wildtype littermates (+/+), from 9 to 31 weeks of age. Error bars represent SD, where n ranges from 2 to 7 for each animal group at each time point.

Weights of individual organs from mice of each genotype were also compared. Tissues from four 6-8 week old female and male wildtype and *Gabpα* heterozygous mice were analysed, and no significant difference was seen in organ mass between the two genotypes (**Figure 7.2**). Therefore, loss of one allele of *Gabpα* does not affect tissue growth.

7.2.2 Immunophenotype of *Gabpα* Heterozygous Mice

Several proposed GABP target genes encode proteins that function in the immune system, such as IL-2, IL-16, CD18, and the γc cytokine receptor subunit (see **Table 2.5**). Therefore the thymus, spleen, bone marrow and peripheral blood of 3 wildtype and 3 *Gabpα* heterozygous mice (at 15 weeks of age) were analysed for the percentage of T cells, B cells, granulocytes and macrophages present. Immunophenotyping was performed on samples of peripheral blood, bone marrow and single cell suspensions from freshly removed thymus and spleen, by teasing through a steel mesh. Subsequent FACS analysis was performed for CD3, CD4, CD8, B220, IgD, IgM, Gr-1 and Mac-1 cell surface markers (service provided by Ivan Bertoncello, Peter MacCallum Cancer Centre, Victoria, Australia). As shown in **Figure 7.3a**, the cellularity of thymus, spleen, bone marrow and peripheral blood is equivalent between the two genotypes, and no significant difference in the proportion of T cell (CD3, CD4, CD8), B cell (B220, IgD, IgM), granulocyte (Gr-1) or macrophage (Mac-1) cell populations was observed in any of the tissues examined (see **Figure 7.3b**). This indicates that the immune system is able to compensate for any small reduction in Gabp target gene expression caused by loss of one allele of *Gabpα*.

7.2.3 Tissue Histology of *Gabpα* Heterozygous Mice

Gabpα heterozygous mice were examined by histopathology for any tissue abnormalities. Organs were harvested from two wildtype and two heterozygous 8 week old mice of each sex. As shown in **Figure 7.4**, no difference in cell morphology or distribution was observed in brain, heart, lung, quadricep muscle, thymus, spleen, kidney, liver, testis, vas deferens, epididymis, seminal vesicle, ovary/uterus, or eye.

The Gabpα polyclonal antibody was not suitable for immunohistochemistry, however, lacZ staining of tissue sections from *Gabpα* heterozygous mice did not show any obvious cell type-specific effects of decreased Gabpα expression (see **Figure 5.6**). The effects of *Gabpα* gene dosage upon skeletal muscle structure and function were investigated in more detail.

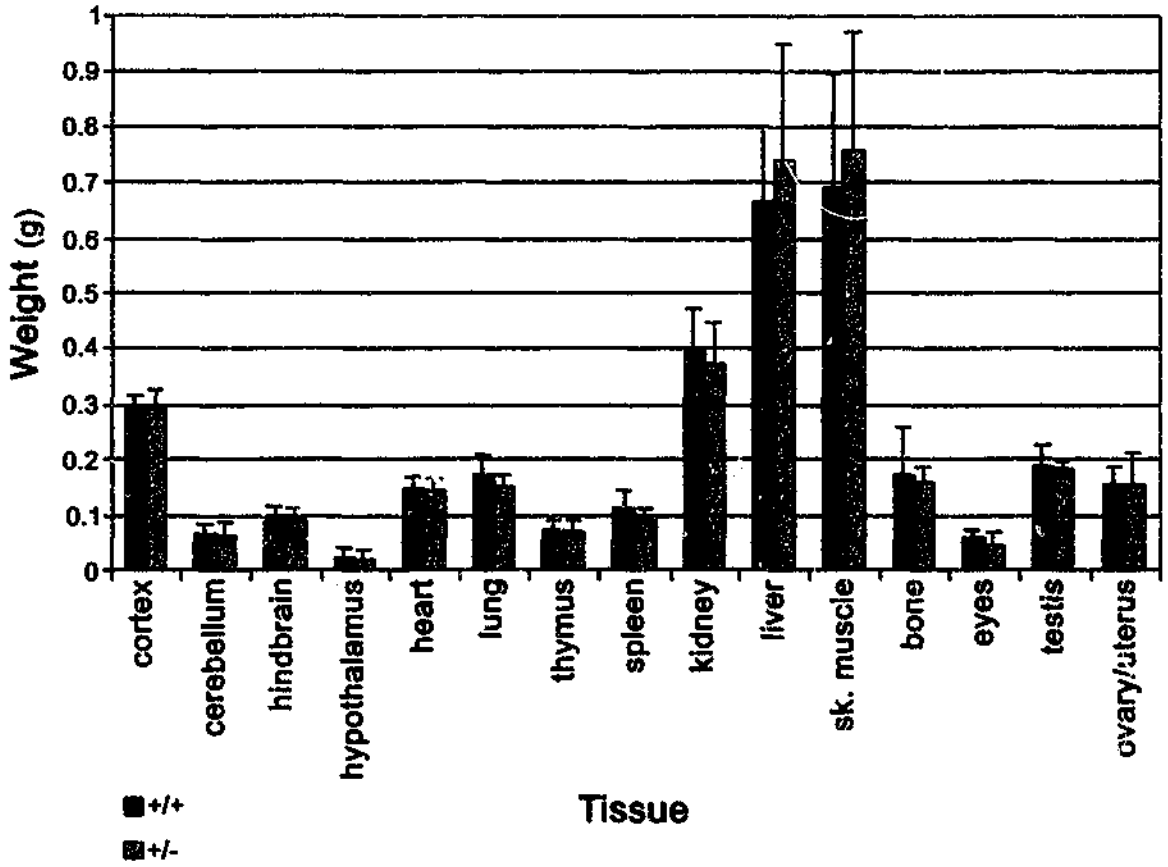


Figure 7.2 - Organ Weights of *Gabpα* Heterozygous Mice.
The wet weight of organs from adult mice heterozygous for *Gabpα* (+/-) were compared to those of wildtype littermates (+/+). The quadricep muscle and femur bone are represented as sk. muscle and bone, respectively. Error bars represent SD, where n=8 for each genotype, except testis and ovary/uterus, where n=4.

(a)

Tissue \ Genotype	Cell Count (x10^6) ± SD	
	+/+	+/-
thymus	34.1 ± 3.6	30.7 ± 9.0
spleen	77.9 ± 7.0	59.6 ± 18.8
bone marrow	12.0 ± 1.0	10.3 ± 2.5
peripheral blood:		
white blood cells/ml	7.2 ± 1.7	6.3 ± 2.0
red blood cells/ml	8.3 ± 1.0	9.4 ± 1.1

(b)

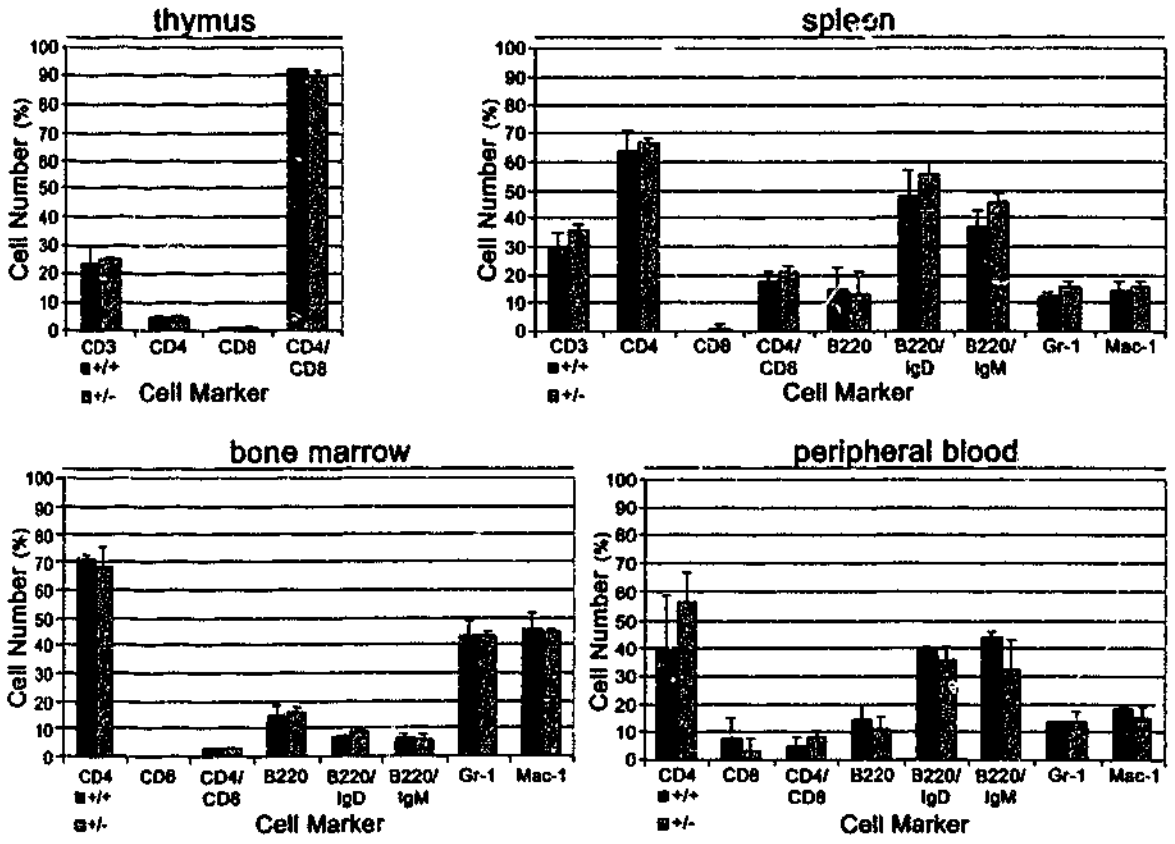


Figure 7.3 - Immunophenotyping of *Gabpα* Heterozygous Mice.
(a) Average cell number in thymus, spleen, bone marrow and peripheral blood of wildtype (+/+) and *Gabpα* heterozygous mice (+/-) at 15 weeks of age. **(b)** Results of FACS analysis for the proportion of T cells (CD3, CD4, CD8), B cells (B220, IgD, IgM), granulocytes (Gr-1) and macrophages (Mac-1) in each tissue from the two genotypes. Error bars and ± represent SD, where n=3 for each genotype.

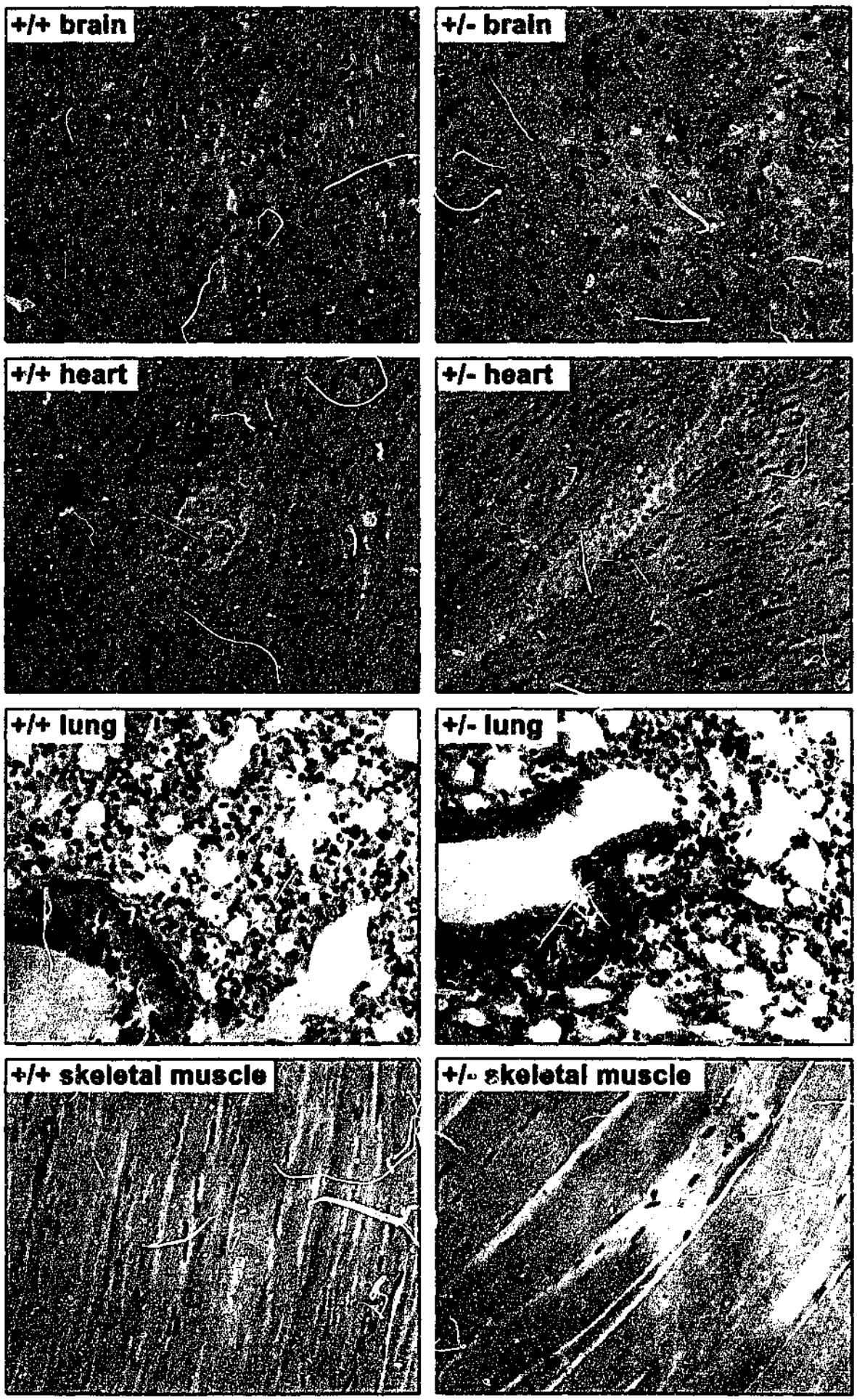


Figure 7.4 - Histopathology of *Gabpα* Heterozygous Mice.

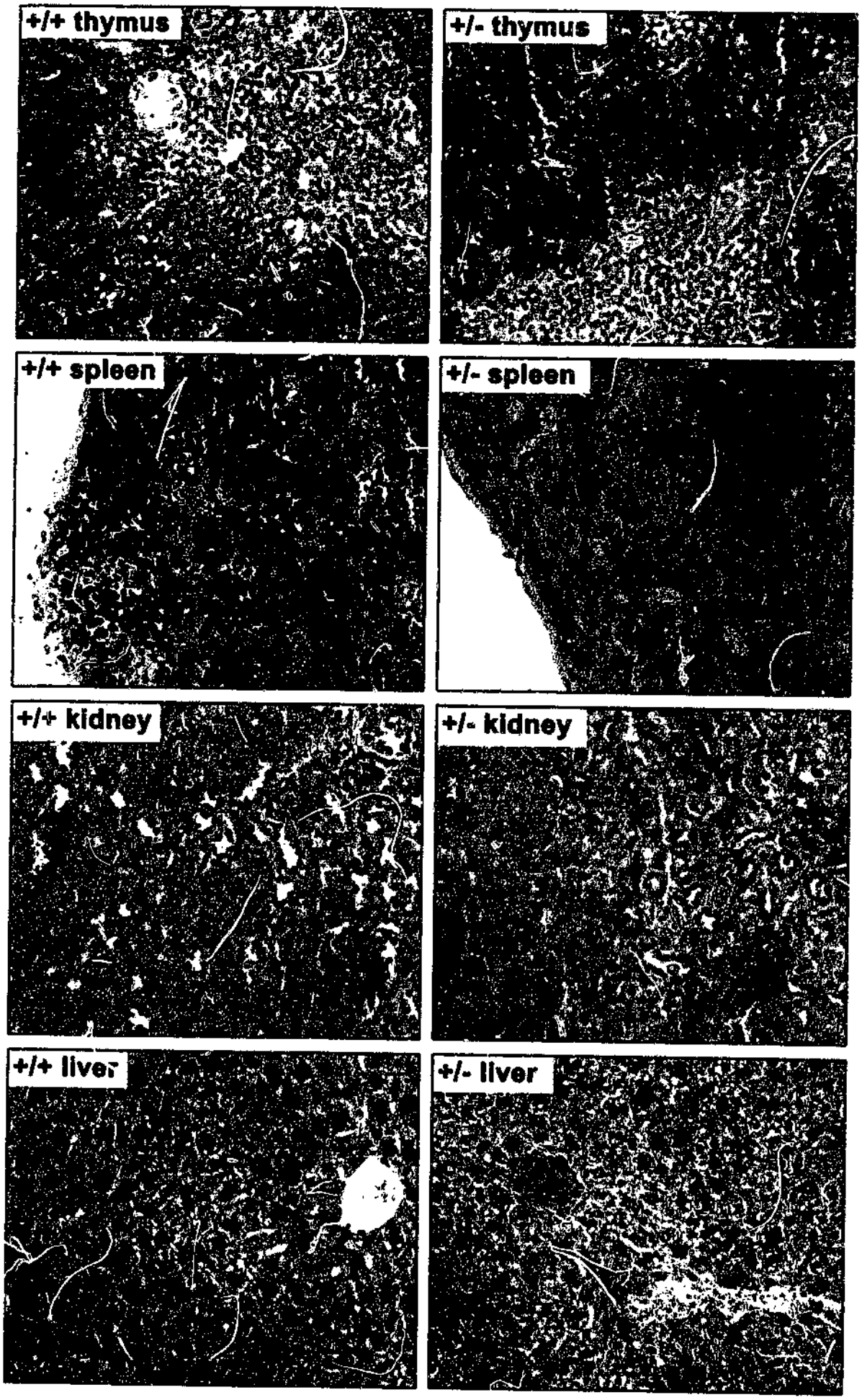


Figure 7.4 - Histopathology of *Gabpα* Heterozygous Mice.

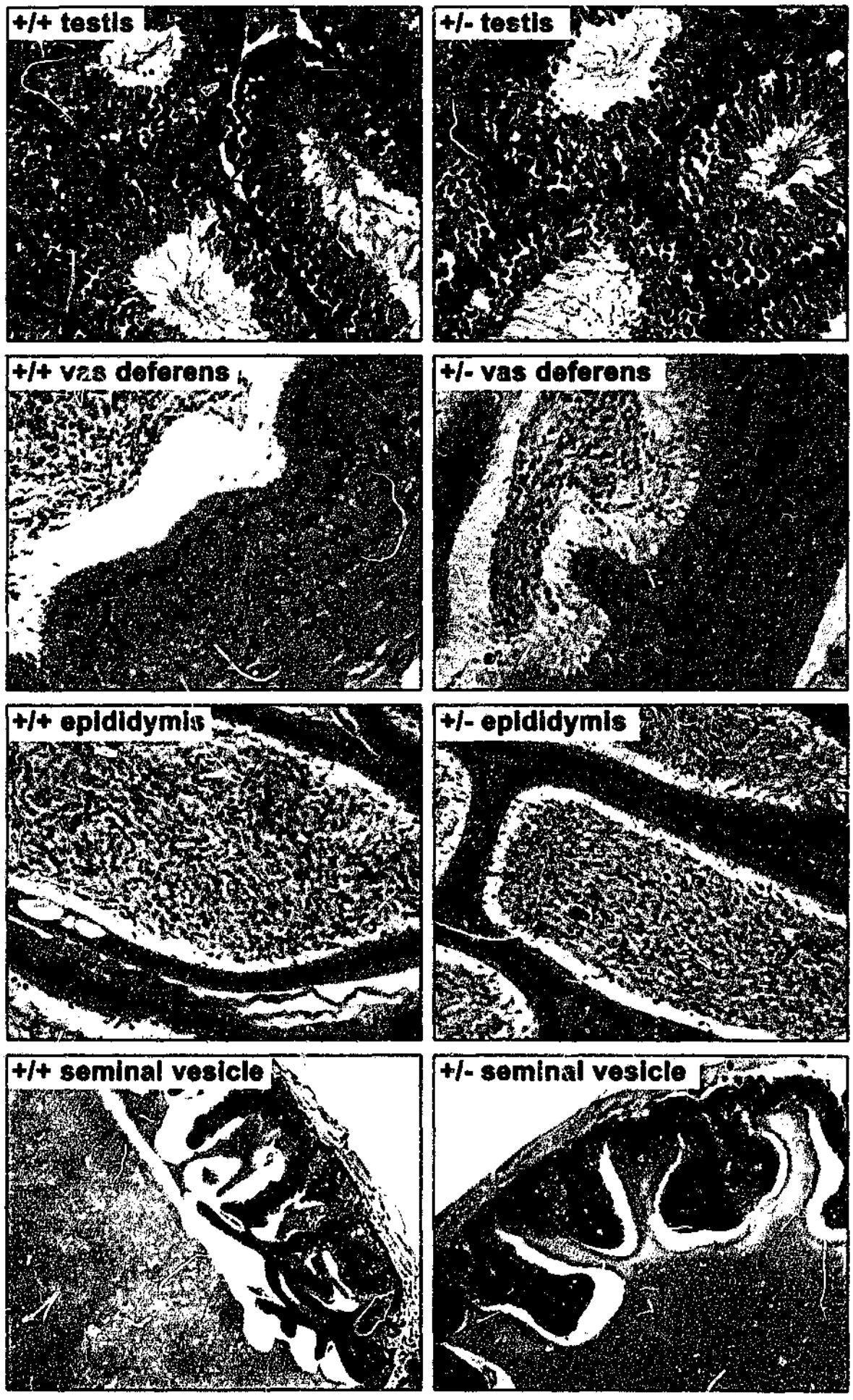


Figure 7.4 - Histopathology of *Gabpα* Heterozygous Mice.

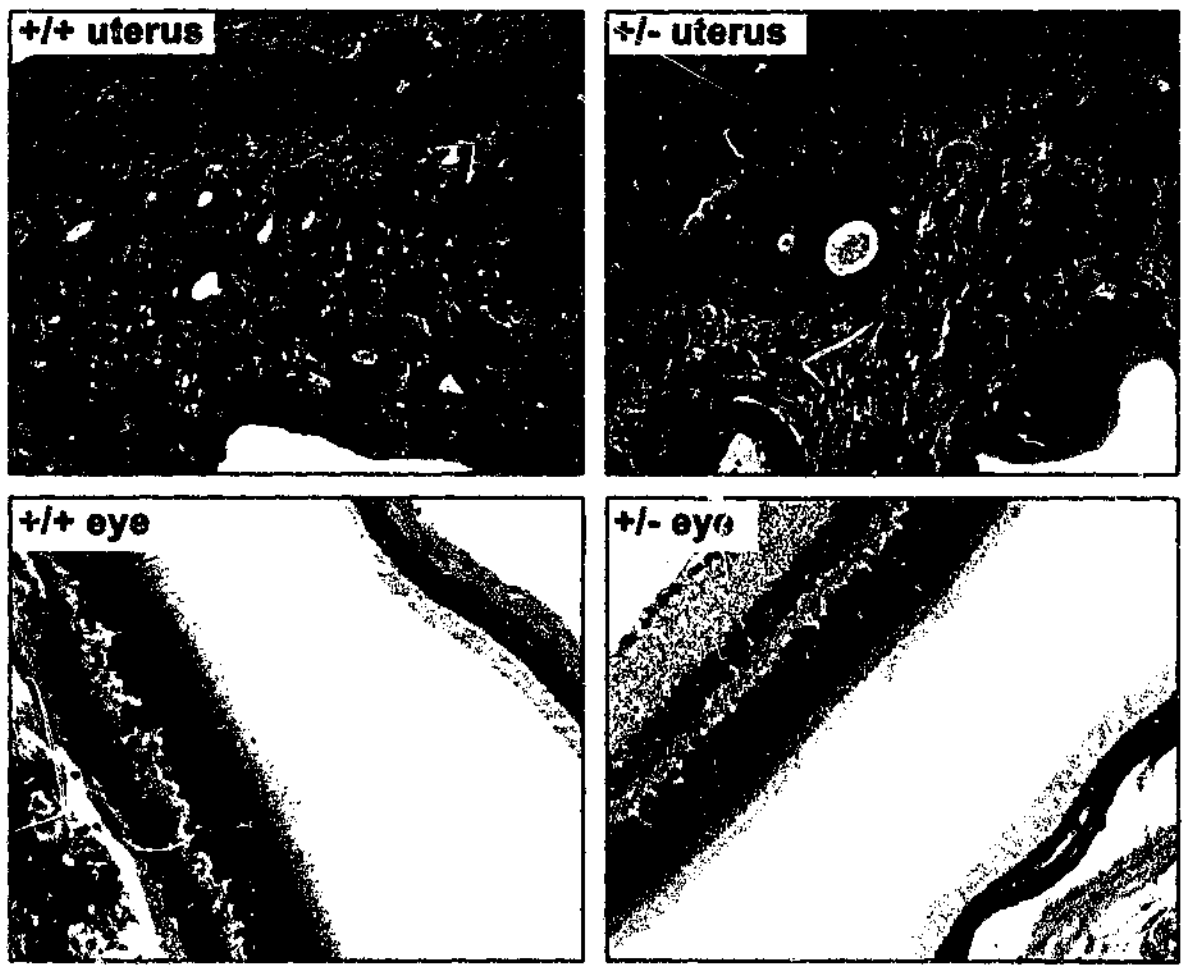


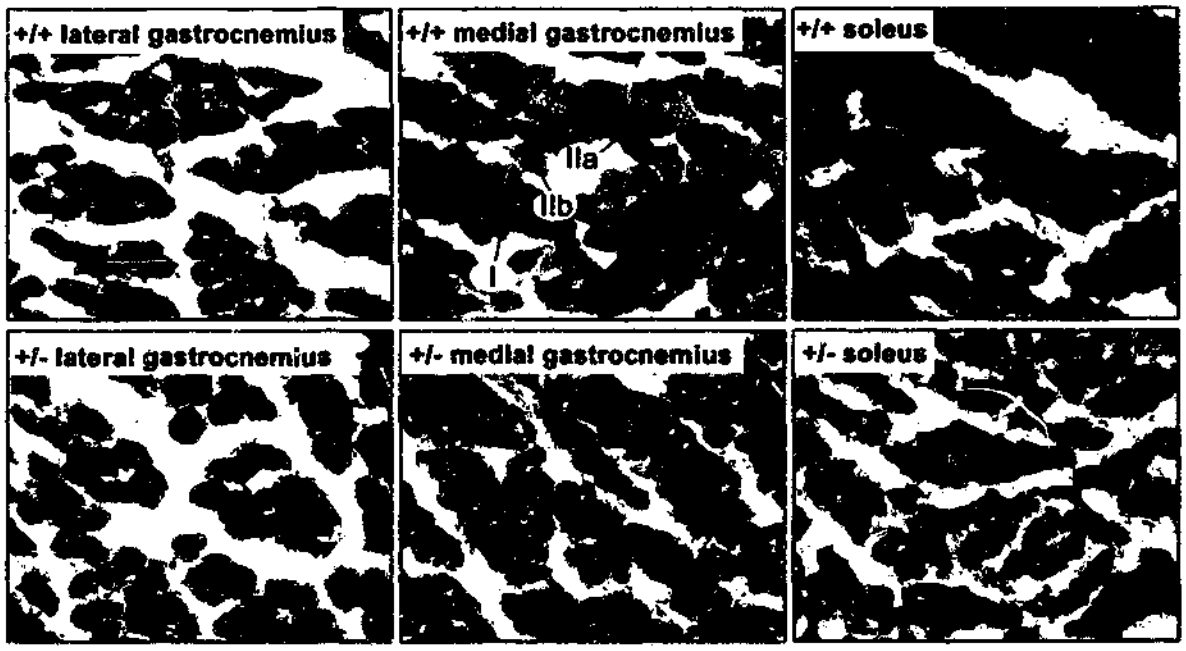
Figure 7.4 - Histopathology of *Gabpα* Heterozygous Mice.
Haematoxylin and eosin stained tissue sections of adult *Gabpα* heterozygous (+/-) and wildtype (+/+) littermates. Tissues are indicated; brain, heart, lung, quadricep skeletal muscle, thymus, spleen, kidney, liver, testis, vas deferens, epididymis, seminal vesicle, uterus and eye. All images were taken at 400x magnification.

7.2.4 Skeletal Muscle Analysis of *Gabpα* Heterozygous Mice

The specific effect of loss of one *Gabpα* allele upon skeletal muscle fibre composition, as a reflection of mitochondrial function, was determined by NADH staining (see **Figure 7.5a** for representative images). This staining reaction measures the ability of flavoproteins to transfer hydrogen from reduced nicotinamide dinucleotide (NADH) to the nitro blue tetrazolium (NBT) dye, and is hence a measure of mitochondrial content. Type I slow oxidative fibres stain dark blue, while type IIb fast glycolytic fibres stain pale blue, and type IIa fast oxidative fibres stain an intermediate blue. The proportion of type I fibres was assessed in transverse cryosections of lateral and medial gastrocnemius, soleus and vastus lateraiis hindlimb muscles, as these muscles feature different fibre type characteristics. Three wildtype and two *Gabpα* heterozygous mice of 1 month of age were examined, and no difference in the distribution of fast and slow fibres was seen in any of the muscles studied. However, the proportion of type I fibres is significantly reduced in the medial gastrocnemius of *Gabpα* heterozygous mice, as determined by a two-tailed t-test (see **Figure 7.5b**). This is surprising, as this muscle normally features a low proportion of such fibres. Whereas the soleus, a muscle consisting of only type I fibres, appears to be unaffected. This suggests that there may be slight loss of type I fibres in *Gabpα* heterozygous mice, and that muscles that usually contain a high proportion of such fibres are resistant to any resultant effects upon mitochondrial function. However, why a similar decrease in type I fibres was not seen in the vastus lateralis, another muscle consisting of predominantly fast type IIb muscle fibres, is unknown. This may reflect a difference in function of the two muscles.

The same muscle tissues were further analysed for differences in AChR distribution, as a measure of NMJ assembly. Longitudinal cryosections of skeletal muscle from three wildtype and two *Gabpα* heterozygous mice of 1 month of age were studied. A Texas red-conjugate of α-bungarotoxin (Molecular Probes) was used to visualise the relative number and position of AChR clusters (see **Figure 7.6a** for a representative image). The extract from *Bungarus multicinctus* venom, the α-bungarotoxin, specifically binds to the AChRα subunit of mature AChR receptors (Gu et al. 1991). No difference in the distribution of AChR clusters was observed between the two genotypes, indicating that loss of one allele of *Gabpα* does not inhibit the synapse-specific expression of the *AChRδ* and *ε* gene targets of Gabp.

(a)



(b)

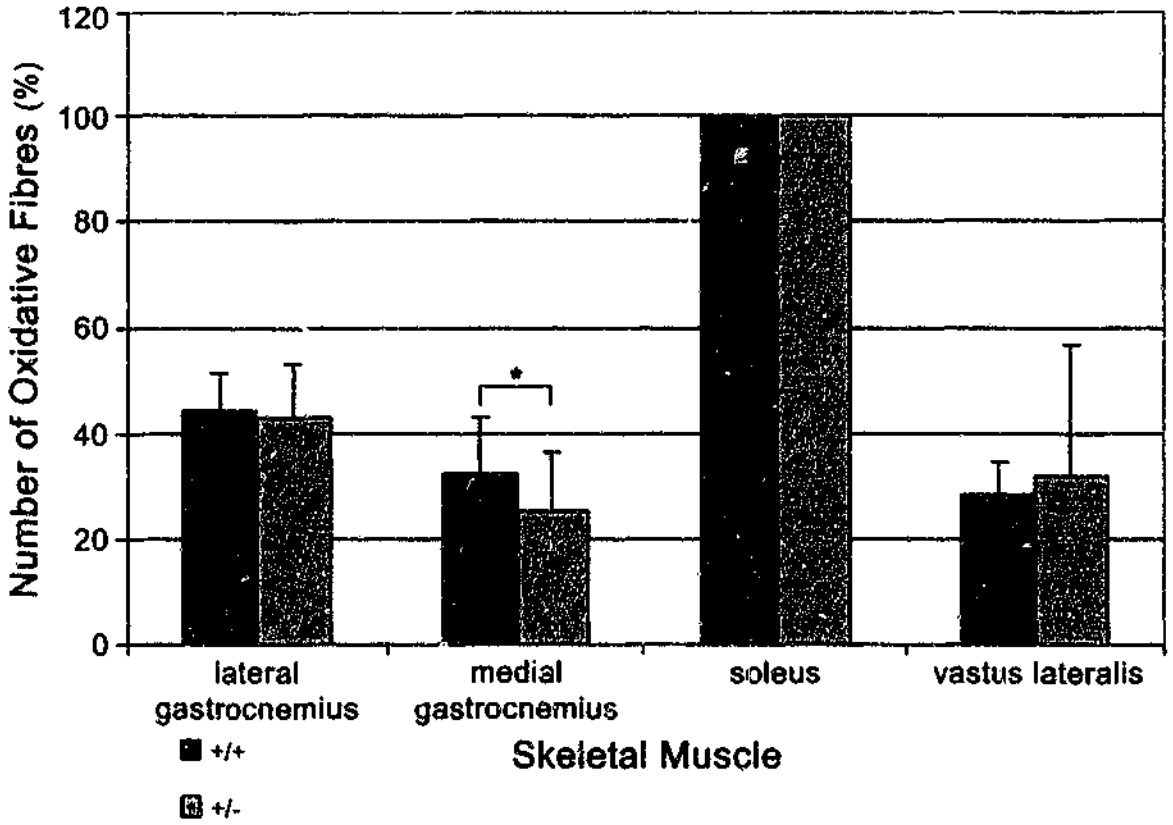


Figure 7.5 - Muscle Fibre Typing of *Gabpα* Heterozygous Mice.
Transverse cryosections of skeletal muscle tissues from 1 month old *Gabpα* heterozygous (+/-) and wildtype mice (+/+) stained with NADH are shown in **(a)**. Type I, IIb and IIa fibres are indicated by dark, light and intermediate staining, respectively, as shown in the top centre panel. The percentage of type I oxidative fibres in each skeletal muscle tissue is shown in **(b)**. Error bars represent SD, where n=3 for wildtype and n=2 for heterozygous mice. * shows statistical significance in a two-tailed t-test, where p=<0.05. Images are at 200x magnification.

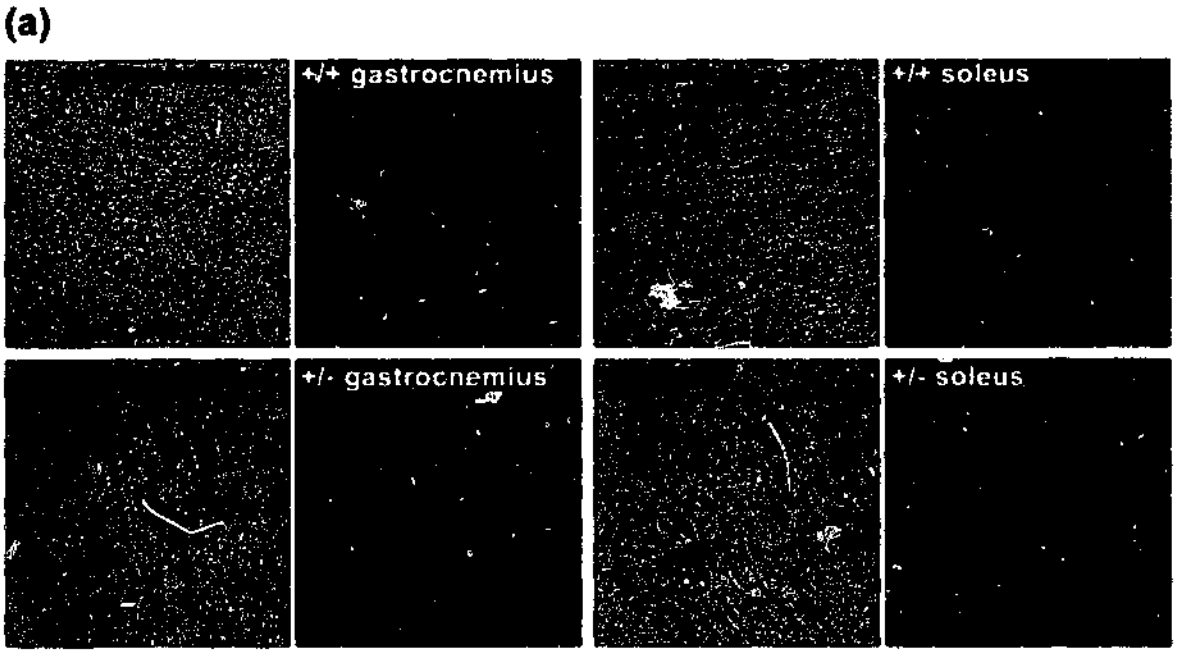


Figure 7.6 - AChR Clustering in *Gabpα* Heterozygous Mice.
(a) Bright field and fluorescent images of Texas red-conjugated α-bungarotoxin stained longitudinal cryosections of skeletal muscle from 1 month old wildtype (+/+) and *Gabpα* heterozygous (+/-) mice showing AChR distribution. **(b)** The number of AChR clusters per skeletal muscle tissue was quantified for both genotypes. Error bars represent SD, where n=6 for wildtype and n=4 for *Gabpα* heterozygous mice. *** represents statistical significance by two-tailed t-test, where p=<0.0005. Images were taken at 100x magnification.

However, as shown in **Figure 7.6b**, the number of AChR clusters per muscle tissue is significantly reduced in the medial gastrocnemius of *Gabpα* heterozygous mice (as determined by a two-tailed t-test). A slight reduction was also observed in the vastus lateralis muscle, however this was not found to be significant (p=0.081). This is in agreement with the NADH staining, in that the medial gastrocnemius, a fast glycolytic muscle tissue, is the most affected by loss of one allele of *Gabpα*. Skeletal muscle tissue may also be classified according to the speed of synapse development during embryogenesis. Fast synapsing muscles obtain compact AChR clusters in alignment with the presynaptic nerve and Schwann cells substantially faster (1 day, from E13.75 to E15) than delayed synapsing muscles (3 days, from E16.5 to birth) (see **Figure 2.10**) (Pun et al. 2002). The medial gastrocnemius is unusual, in that it exhibits intermediate synapsing speed (E16 to E18), whereas the lateral gastrocnemius, soleus and vastus lateralis all exhibit delayed synapse development. Therefore, perhaps Gabp is necessary for expression of AChR genes during a particular stage of development (E15-E16). Further analysis of AChR cluster number and distribution in fast synapsing muscles of *Gabpα* heterozygous mice during development may enable the critical time of Gabp function to be determined. The effect of these slight skeletal muscle abnormalities of the *Gabpα* heterozygous mice upon muscle function was investigated further.

7.2.5 Skeletal Muscle Function of *Gabpα* Heterozygous Mice

The grip strength of male wildtype and *Gabpα* heterozygous littermates was measured from 4 to 31 weeks of age. Assessment involved measuring the time to fall from an inverted wire cage lid, for a maximum testing time of 60 seconds. This method has previously been shown to be a good indicator of neuromuscular function, with a deficit in neuromuscular signalling resulting in decreased grip strength (Sango et al. 1996; Rogers et al. 1997; Crawley 2000). As shown in **Figure 7.7**, no significant difference was seen in the grip strength of wildtype or *Gabpα* heterozygous mice in any age group, and mice of both genotypes displayed reduced grip strength with age. However, any slight neuromuscular defect in *Gabpα* heterozygous mice may be masked by the high degree of variability within each animal group.

Gait of eight male *Gabpα* heterozygous and eight wildtype mice was evaluated at 1 and 6 months of age. Footprint analysis was used, as this is a measure of skeletal muscle weakness and poor coordination, visible as wobbling, stumbling or weaving

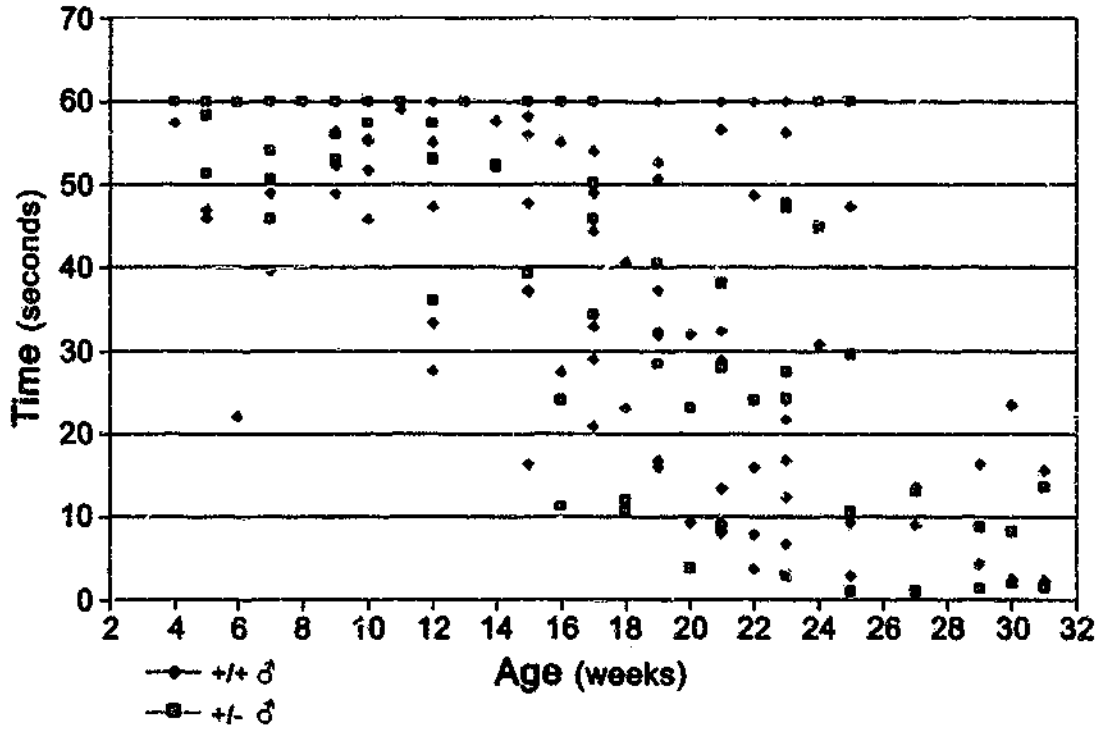


Figure 7.7 - Grip Strength of *Gabpα* Heterozygous Mice.
The grip strength of male *Gabpα* heterozygous (+/-) and wildtype (+/+) mice from 4 to 31 weeks of age, as measured by time taken to fall from an inverted wire cage lid. Maximum testing time was 60 seconds.

(Sango et al. 1995; Crawley 2000). The representative images in **Figure 7.8** demonstrate that no difference in the regularity or angle between footprints was observed between wildtype and *Gabpα* heterozygous mice. Therefore, although loss of one allele of *Gabpα* results in a muscle type-specific decrease in mitochondrial number and number of AChR clusters, this does not result in any overt impairment of skeletal muscle function. Whether other muscle tissues, particularly tonic oxidative muscles, are affected by homozygous deletion of *Gabpα* was studied in more detail using the *Gabpα* skeletal muscle-specific knockout mouse model.

7.3 Phenotype Analysis of Gabpα Conditional Knockout Mice

Gabpα exon 2 was deleted specifically in skeletal muscle by breeding mice carrying floxed *Gabpα* and *hαSA-Cre* alleles (see **Chapter 6**). The effect of loss of Gabpα protein expression upon body and organ weight, Gabp target gene expression levels, and resulting skeletal muscle strength and function, were studied.

7.3.1 Body and Organ Weight of *Gabpα* Conditional Knockout Mice

Body weight of skeletal muscle-specific *Gabpα* knockout mice was monitored from 4 to 28 weeks of age and compared to that of wildtype, floxed and *hαSA-Cre* littermates. Female (**Figure 7.9a**) and male (**Figure 7.9b**) mice were analysed separately, as male mice tend to be heavier than females. No difference was observed between the two animal groups, and both groups of mice show a gradual increase in body mass over time.

The weights of lateral and medial gastrocnemius, soleus, vastus lateralis and diaphragm skeletal muscle tissues, from 3 month old homozygous floxed, heterozygous and homozygous skeletal muscle-specific *Gabpα* knockout mice were compared (see **Figure 7.10**). Heart weight was also measured as a control, as *Gabpα* is not deleted in this tissue. No difference in organ mass was seen across the three genotypes. Therefore loss of Gabpα expression does not affect skeletal muscle growth.

7.3.2 Skeletal Muscle Histology of *Gabpα* Conditional Knockout Mice

Skeletal muscle tissues from homozygous and heterozygous *Gabpα* skeletal muscle-specific knockout, and homozygous floxed mice were analysed by histopathology. The lateral and medial gastrocnemius, soleus and vastus lateralis hindlimb muscles were

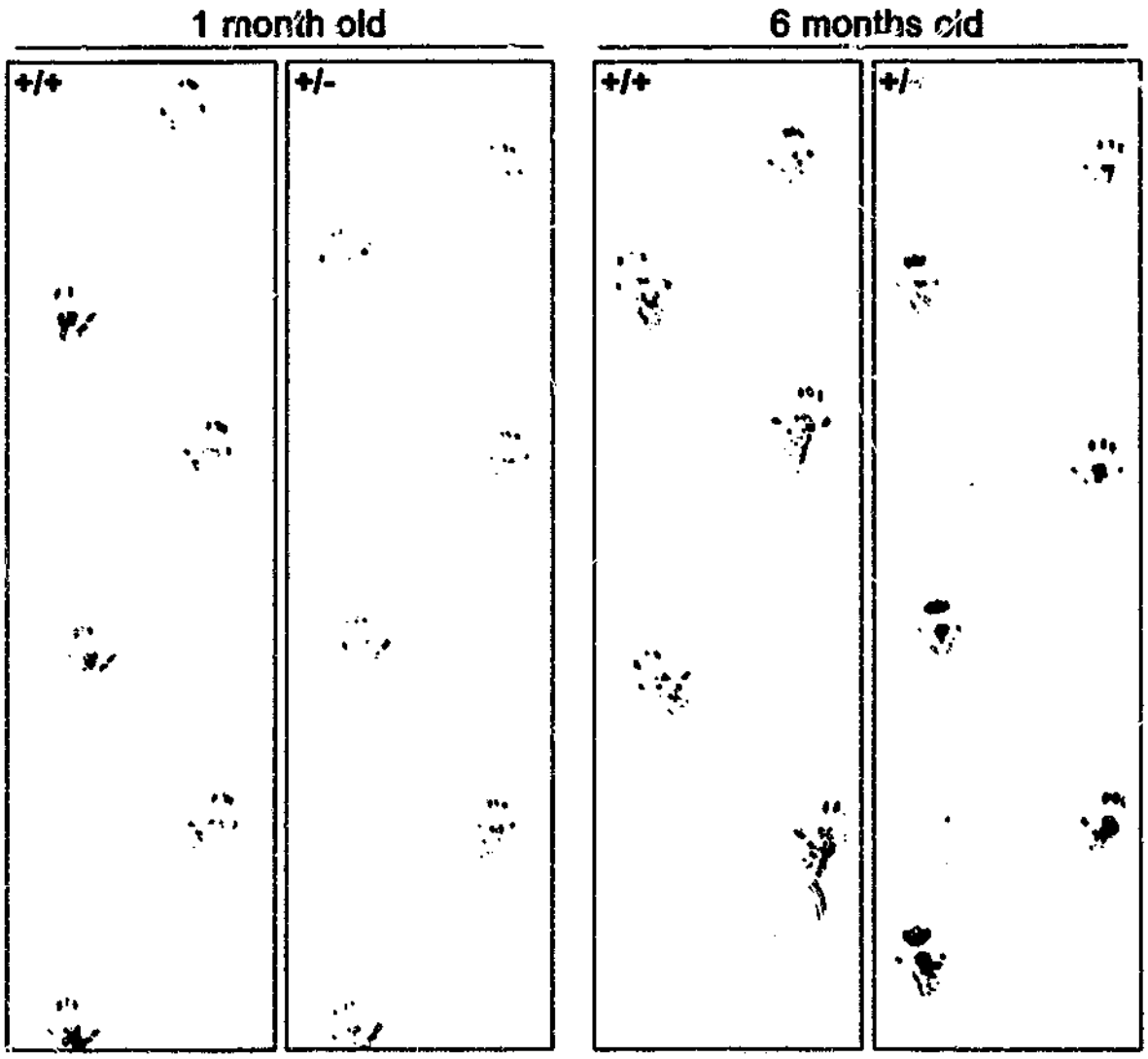
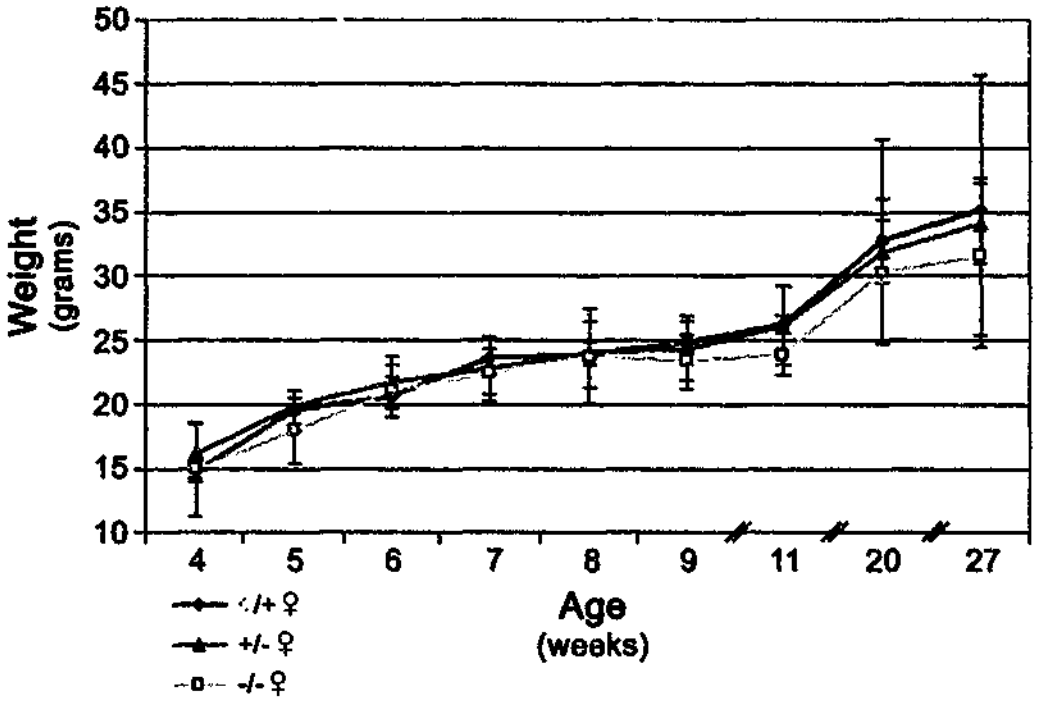


Figure 7.8 - Gait Analysis of *Gabpα* Heterozygous Mice.
Footprints of *Gabpα* heterozygous (+/-) and wildtype (+/+) mice, at 1 and 6 months of age, indicating balanced gait.

(a)



(b)

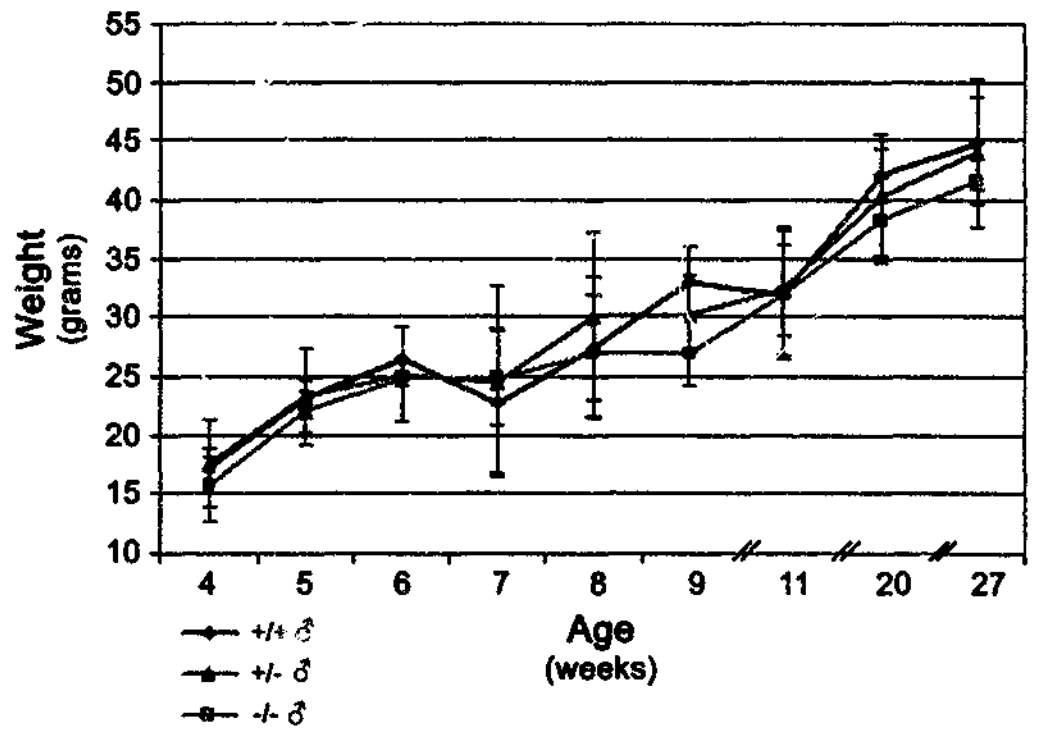


Figure 7.9 - Body Weight of *Gabpα* Conditional Knockout Mice.
The body weight of **(a)** female (♀), and **(b)** male (♂), wildtype (+/+), heterozygous (+/-) and homozygous (-/-) *Gabpα* skeletal muscle-specific knockout mice, from 4 to 27 weeks of age. Error bars represent SD, where n ranges from 2 to 12 for each animal group at each time point. Wildtype animal groups were comprised of mice genotypically +/+, +/loxP, loxP/loxP and +/cre.

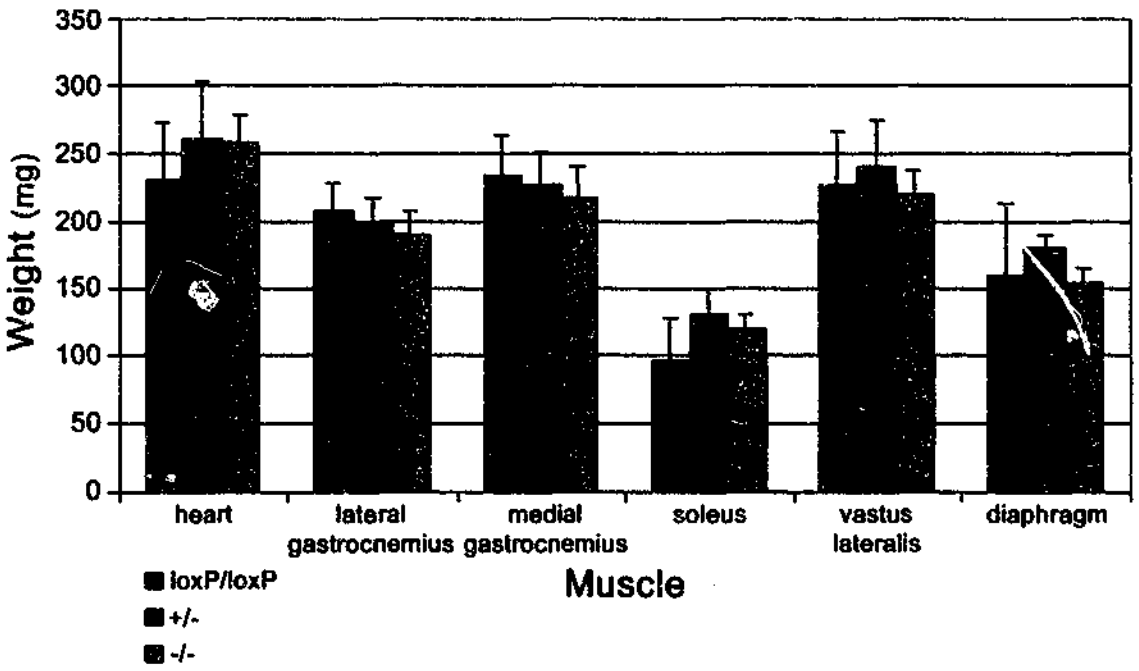


Figure 7.10 - Muscle Weight of *Gabpα* Conditional Knockout Mice.

The wet weight of skeletal and heart muscle tissues from 3 month old wildtype (loxP/loxP), heterozygous (+/-) and homozygous (-/-) *Gabpα* skeletal muscle-specific knockout mice. Error bars represent SD, where n=3 for each genotype.

harvested from two male and two female 1 and 3 month old mice of each genotype, and longitudinal cryosections were stained with haematoxylin and eosin. As shown by the representative images in **Figure 7.11**, no difference in cell density or morphology was observed between the three genotypes.

7.3.3 Gabp Target Gene Expression Levels in *Gabpα* Conditional Knockout Mice

The specific effect of Gabpα loss was assessed by evaluation of expression levels of proposed Gabp target genes. Transcript levels of Gabp target genes *AChRδ* and *AChRε*, *Utrophin*, and *MTFA*, in skeletal muscle of homozygous floxed, heterozygous and homozygous *Gabpα* skeletal muscle-specific knockout mice were assessed by real-time RT-PCR analysis. A 809 bp *AChRδ* product was amplified with primers spanning 1021-1830 bp of cDNA sequence GI:191609 (primers 217 and 218), as well as a 255 bp *AChRε* product spanning 3-258 bp of cDNA sequence GI:2660742 (primers 223 and 224). *MTFA* expression was measured by amplification of a 710 bp product spanning 351-1061 bp of cDNA sequence GI:1575122 (primers 225 and 226), and a 1 kb *Utrophin* product spanning 3014-4016 bp of cDNA sequence GI:1934962 was amplified (primers 219 and 220). *Rapsyn* expression was also monitored as a negative control, as it encodes a skeletal muscle structural protein and is not a known Gabp target. A 91 bp *Rapsyn* RT-PCR product spanning 827-918 bp of cDNA sequence GI:53804 was amplified (primers 273 and 274). Transcript expression levels were quantified relative to *β-actin*, by amplification of a 102 bp *β-actin* product spanning 344-446 bp of cDNA sequence GI:49867 (primers 280 and 281). Template cDNA was generated from diaphragm and soleus muscles of two homozygous floxed, three heterozygous and four homozygous *Gabpα* skeletal muscle-specific knockout mice of 3 months of age. RT-PCR reactions were repeated 3-4 times on each sample for each primer pair, and the average results of successful reactions are shown in **Figure 7.12**. These muscles were chosen as they are predominately composed of oxidative fibres, and any impairment in mitochondrial function resulting from loss of Gabpα expression may be more evident in these tissues.

As shown in **Figure 7.12a**, both *AChRε* and *MTFA* expression levels are significantly decreased in the diaphragm of heterozygous and homozygous *Gabpα* skeletal muscle-specific knockout mice, as determined by two-tailed t-tests. A similar trend is seen for *AChRδ*, but due to the variation between *Gabpα* floxed samples this is not

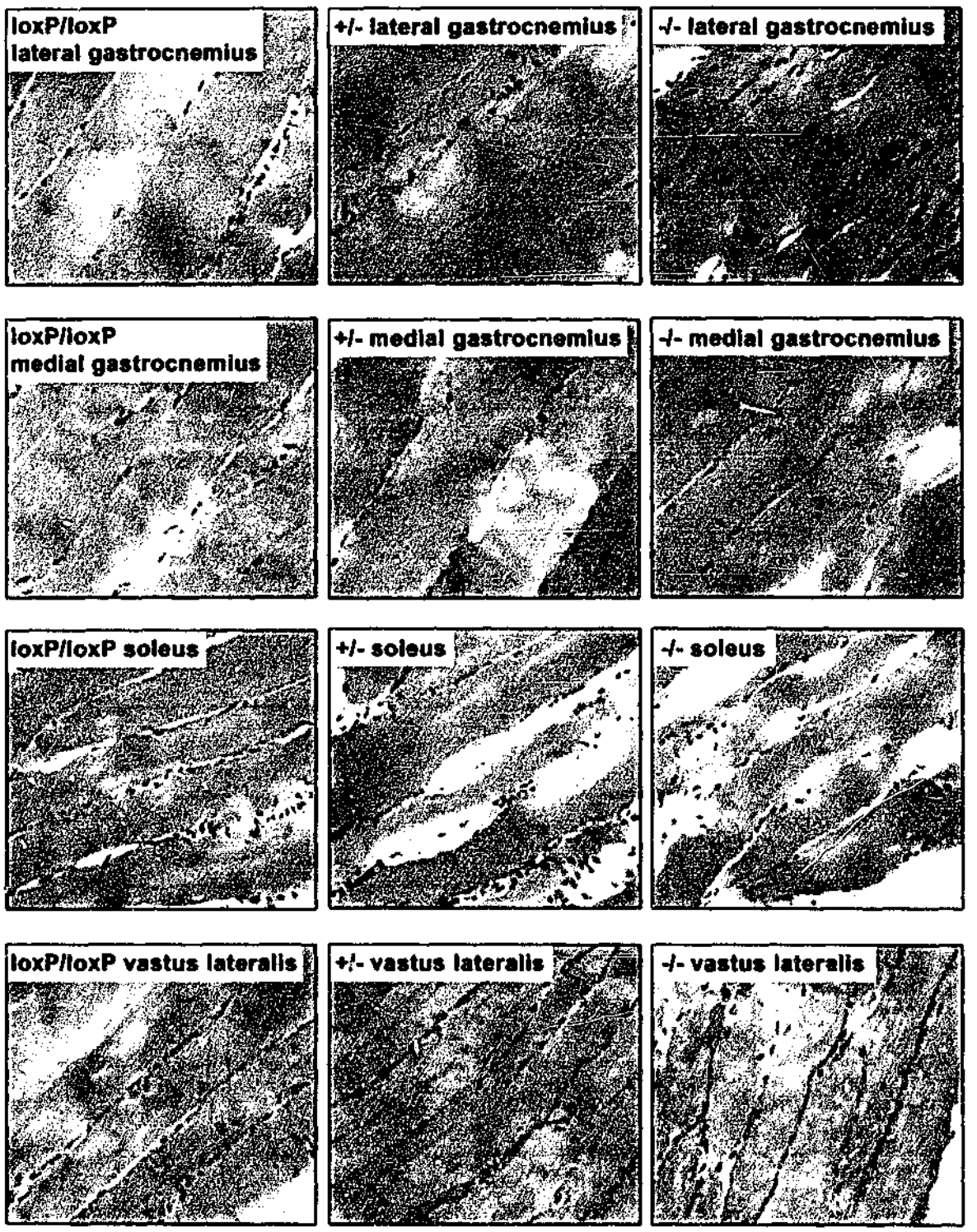


Figure 7.11 - Histopathology of *Gabpα* Conditional Knockout Mice.
Haematoxylin and eosin stained longitudinal cryosections of skeletal muscle from 1 month old wildtype (loxP/loxP), heterozygous (+/-) and homozygous (-/-) *Gabpα* skeletal muscle-specific knockout mice. Images were taken at 400x magnification.

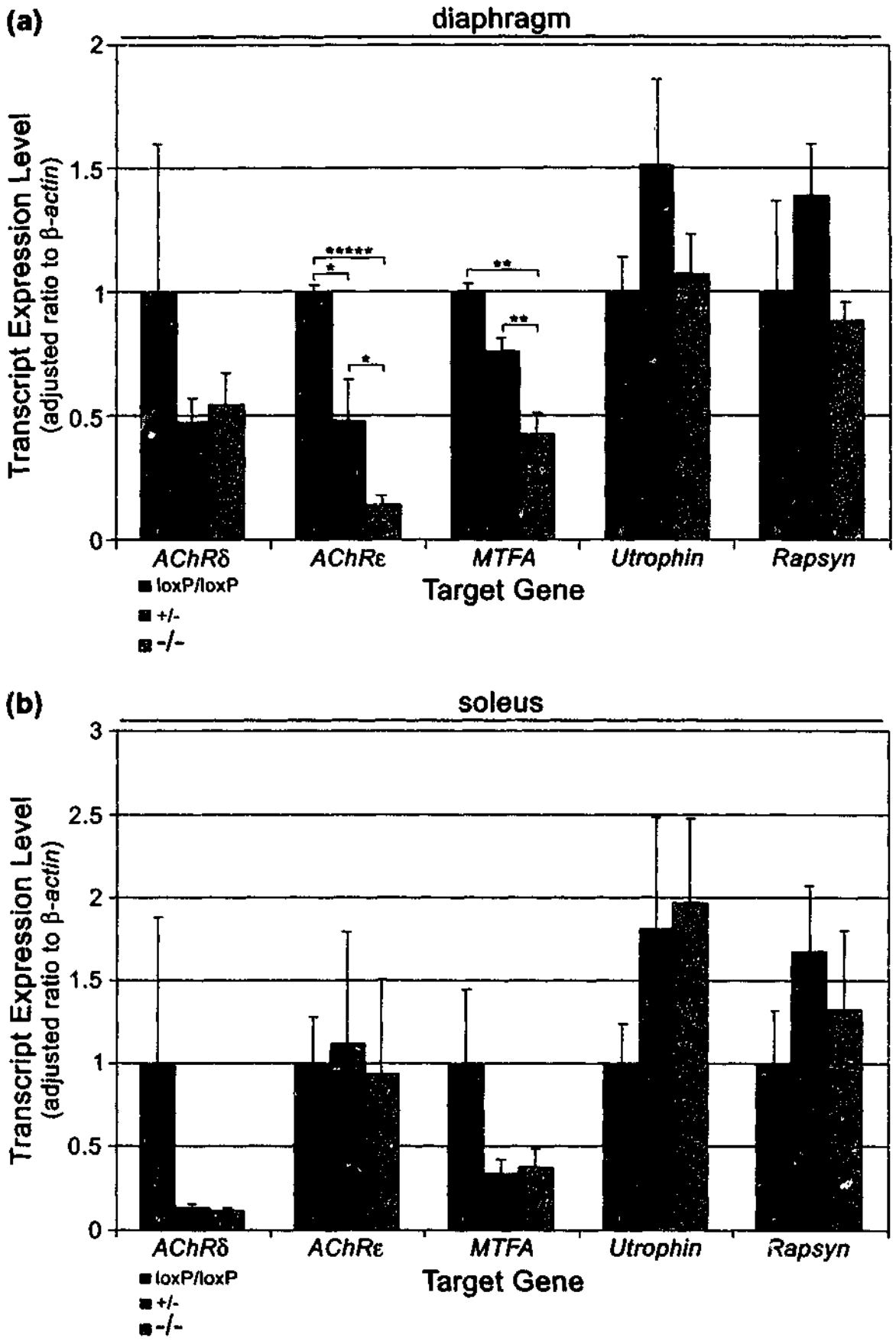


Figure 7.12 - Transcript Expression Levels of Gabp Target Genes in *Gabpα* Conditional Knockout Mice.

Real-time RT-PCR analysis of Gabp target genes, using template cDNA from **(a)** diaphragm and **(b)** soleus of 3 month old wildtype (loxP/loxP), heterozygous (+/-) and homozygous (-/-) *Gabpα* skeletal muscle-specific knockout mice. Expression levels of each transcript are expressed as a ratio to β-*actin*. Ratios were adjusted such that those of wildtype mice were equal to 1. Error bars represent SEM, and n values are shown on each column. *, ** and ***** show statistical significance by two-tailed t-tests, where p=<0.05, <0.005 and <0.000005, respectively.

statistically significant. In the soleus of *Gabpα* conditional knockout mice a trend toward reduced *AChRδ* and *MTFA* levels is also apparent (see **Figure 7.12b**), however *AChRε* levels remain unaltered. In both diaphragm and soleus muscles, the levels of *Utrophin* are comparable between all three genotypes, and as expected, that of *Rapsyn* (as this is not regulated by Gabp).

This data shows that loss of Gabpα expression results in decreased mRNA expression levels of target genes, in a tissue-specific manner. Another ETS transcription factor present in the soleus may be able to compensate for loss of Gabpα expression upon *AChRε* transcript levels. In addition, an ETS protein, and/or other transcription factors are able to maintain normal *Utrophin* expression levels in the absence of Gabpα in both tissues studied. Further analysis of different muscle tissues, from mice of different ages, is necessary before a complete understanding of the role of Gabpα upon target gene expression can be gained. Variation between samples may also be reduced by extraction of RNA from the synaptic region of skeletal muscle tissues, or by analysis of mice crossed onto a pure genetic background.

The protein expression levels of two Gabp targets of mitochondrial function, COXIV and Vb, were also assessed. Western blot analysis was performed using protein lysates of lateral gastrocnemius, soleus and diaphragm muscles from three homozygous floxed, heterozygous and homozygous *Gabpα* skeletal muscle-specific knockout mice of 3 months of age. Medial gastrocnemius and vastus lateralis were not included in this analysis, as basal COXIV and COXVb levels were too low for quantification. Specific monoclonal antibodies allowed for the detection of COXIV, COXVb (Molecular Probes) and β-tubulin (Chemicon) proteins, as shown by the representative image in **Figure 7.13a** (refer to **Chapter 5** for antibody concentrations). Unexpectedly, quantification of COXIV and COXVb protein levels relative to β-tubulin, revealed a significant increase in COXVb expression in the soleus of homozygous *Gabpα* conditional knockout mice, as determined by a two-tailed t-test (see **Figure 7.13b**). A similar trend is also seen in the lateral gastrocnemius, however this is not significant ($p=0.16$). No other differences in COXIV or COXVb protein levels were observed. This suggests that another nuclear regulator of proteins that function in mitochondria, such as nuclear respiratory factor-1 (Nrf-1), may be compensating for loss of Gabpα expression. To investigate mitochondrial function further,

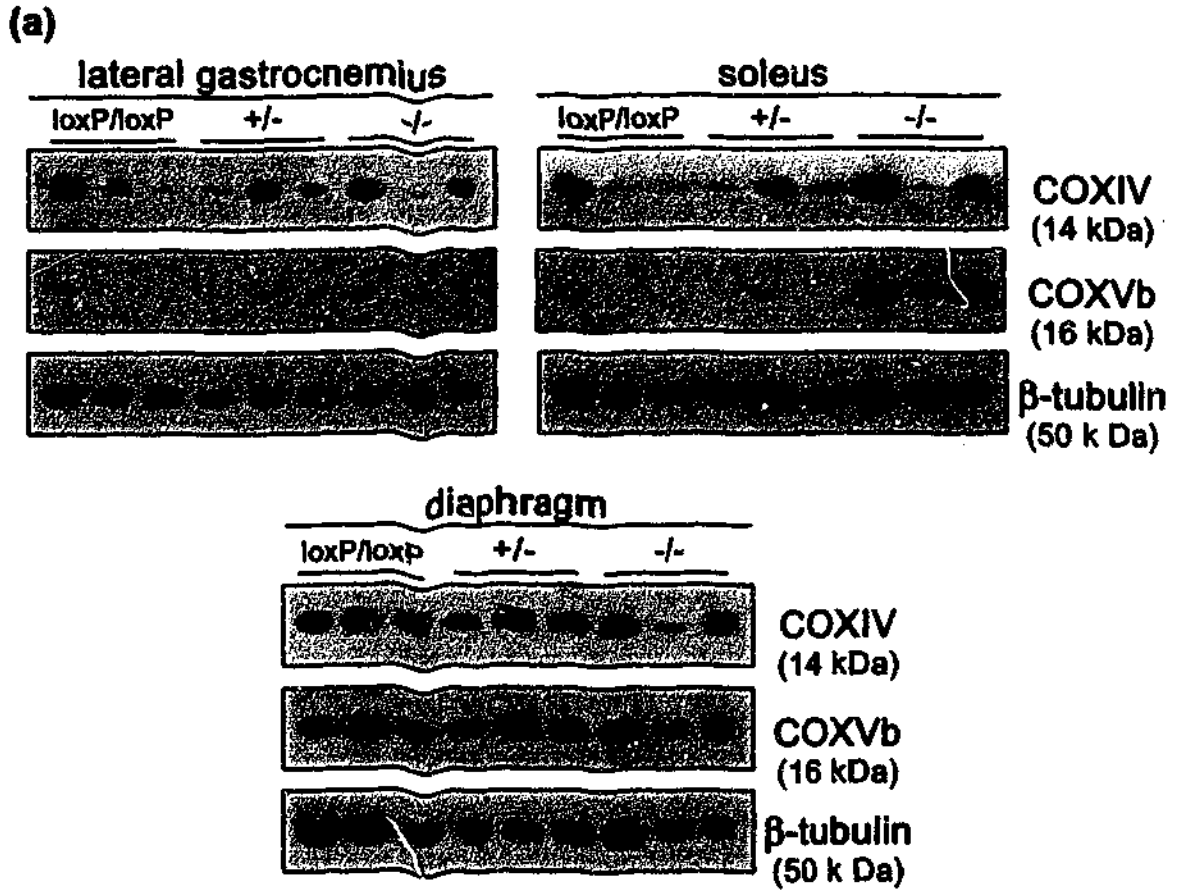


Figure 7.13 - COXIV and COXVb Protein Expression Levels in *Gabpα* Conditional Knockout Mice.

(a) Western blot analysis of COXIV, COXVb and β-tubulin protein levels in skeletal muscle tissues of wildtype (*loxP/loxP*), heterozygous (+/-) and homozygous (-/-) *Gabpα* skeletal muscle-specific knockout mice. **(b)** Quantification of COXIV and COXVb protein levels, expressed as a ratio to β-tubulin. Ratios were adjusted such that those of wildtype mice were equal to 1. Error bars show SD, where n=3 for each genotype. * indicates statistical significance by two-tailed t-test, p=<0.05.

the fibre composition of skeletal muscle from floxed, heterozygous, and homozygous *Gabpα* skeletal muscle-specific knockout mice was determined.

7.3.4 Skeletal Muscle Analysis of *Gabpα* Conditional Knockout Mice

The effect of loss of Gabpα expression upon skeletal muscle fibre composition was measured by NADH staining (see **Figure 7.14** for representative images). Transverse cryosections of lateral and medial gastrocnemius, soleus and vastus lateralis hindlimb muscles of two mice of each sex of homozygous floxed, heterozygous and homozygous *Gabpα* skeletal muscle-specific knockout genotypes were analysed. No difference in the distribution of the fast and slow fibres was seen in any of the muscles studied, at 1, 3 or 6 months of age. However, the proportion of type I slow oxidative fibres is significantly reduced in both the lateral gastrocnemius and vastus lateralis of 3 month old homozygous *Gabpα* conditional knockout mice, as determined by two-tailed t-tests (see **Figure 7.15**). A trend towards reduced type I fibres is also apparent in lateral and medial gastrocnemius of 1 month old *Gabpα* conditional knockout mice, and in medial gastrocnemius of 3 month old mice, however these differences are not statistically significant. Interestingly, no difference in fibre type proportions between the three genotypes is seen at 6 months of age. However, any small difference may be masked by a general reduction in type I fibres in the lateral and medial gastrocnemius with age (see **Figure 7.15**). This suggests that compensatory mechanisms may ensure a minimum proportion of type I fibres is maintained in *Gabpα* conditional knockout mice, as for aging wildtype mice. Ubiquitous heterozygous *Gabpα* mice only have a decreased proportion of type I fibres in medial gastrocnemius at 1 month of age. Therefore study of older mice, and a greater total number of mice, may be necessary before similar results are seen in both *Gabpα* knockout mouse models. It would also be interesting to study levels of other mitochondrial enzymes, such as the Gabp target Succinate dehydrogenase, to determine if a similar tissue-specific phenotype is apparent.

AChR distribution was also analysed in lateral and medial gastrocnemius, soleus and vastus lateralis muscle tissues from two mice of each sex of each of homozygous floxed, heterozygous and homozygous *Gabpα* skeletal muscle-specific knockout genotypes at 1, 3 and 6 months of age. A Texas red conjugate of α-bungarotoxin (Molecular Probes) was used to visualise the relative number and position of AChR clusters in longitudinal cryosections of skeletal muscle. No difference in the distribution of AChR clusters

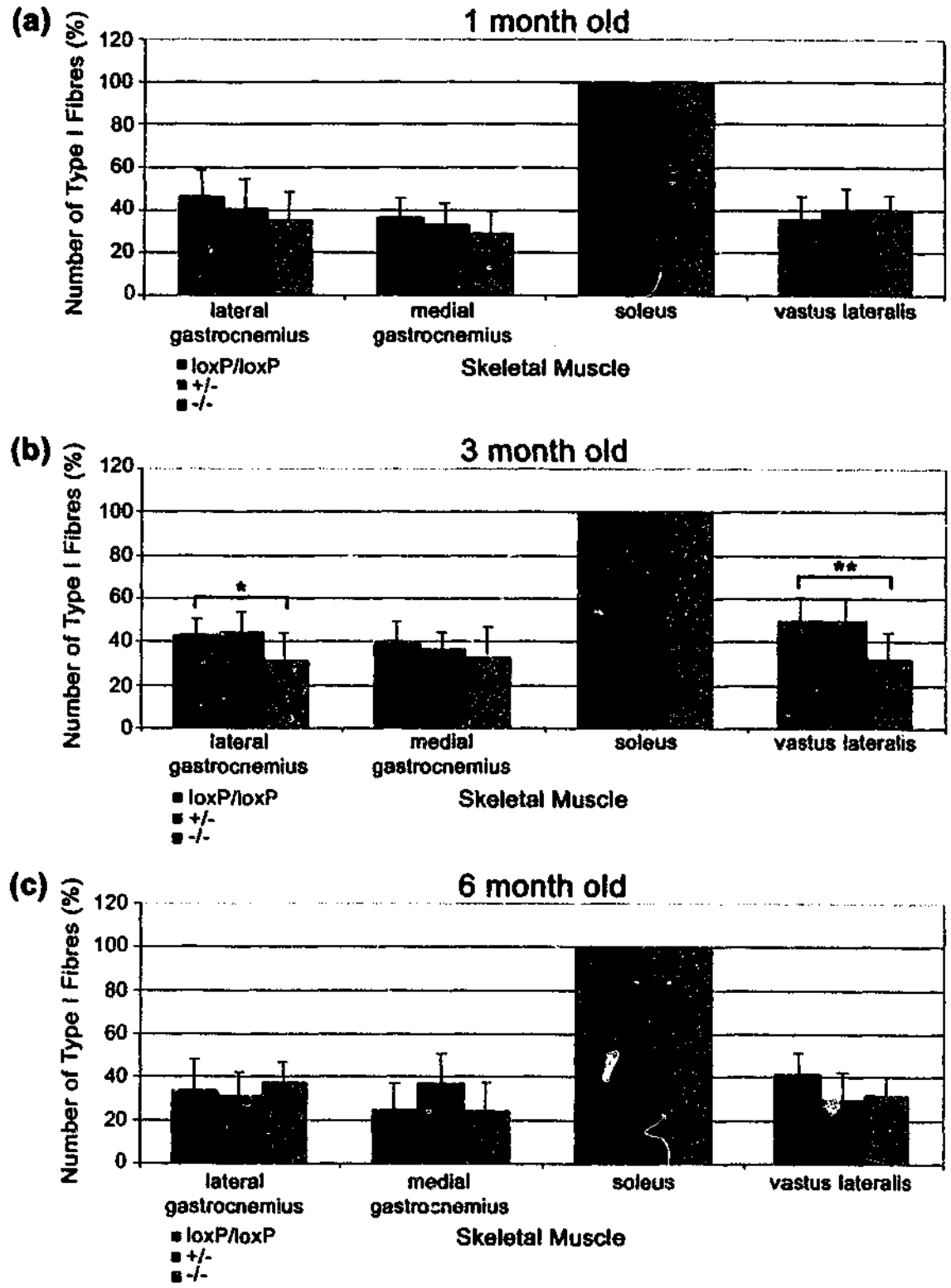


Figure 7.15 - Proportion of Type I Fibres in Skeletal Muscle of *Gabpα* Conditional Knockout Mice.

The percentage of type I oxidate fibres in skeletal muscle tissue of **(a)** 1, **(b)** 3, and **(c)** 6 month old wildtype (loxP/loxP), heterozygous (+/-) and homozygous (-/-) *Gabpα* skeletal muscle-specific knockout mice. Error bars represent SD, where n=8 for each animal group. * and ** represent statistical significance, as determined by two-tailed t-tests, where p=<0.05 and <0.005, respectively.

is apparent between the three genotypes (see **Figure 7.16** for representative images), indicating that Gabpα expression is not essential for the synapse-specific expression of the *AChRδ* and ε gene targets of Gabp. However, as shown in **Figure 7.17**, the number of AChR clusters is significantly reduced in the lateral gastrocnemius of both heterozygous and homozygous *Gabpα* skeletal muscle-specific knockout mice at 1 and 3 months of age, as determined by two-tailed t-tests (see **Figure 7.17a** and **b**). A trend towards reduced AChR numbers was also observed in medial gastrocnemius of 6 month old conditional knockout mice, however this was not found to be statistically significant (p=0.063). The dramatic reduction in AChR cluster number with age in wildtype mice results in an equivalent number of AChRs in 6 month old mice of all three genotypes (see **Figure 7.17c**). This suggests that one form of compensation for the lower AChR cluster number in *Gabpα* conditional knockout mice may be a slower turnover rate. A significant decrease in AChR cluster number is seen in medial gastrocnemius only in 1 month old ubiquitous *Gabpα* heterozygous mice. While study of a greater number of animals may lead to a similar finding in both *Gabpα* knockout mouse models, one explanation for differences in the skeletal muscle tissues affected is the level of *hαSA-Cre* expression in different muscle tissues. Perhaps the *Gabpα* gene is spliced in more myonuclei in lateral gastrocnemius than medial gastrocnemius, due to the normal expression levels of human α skeletal actin. Irrespective of this, further analysis of conditional knockout mice was performed, to assess whether a decrease in AChR expression results in altered NMJ shape.

7.3.5 Ultrastructure of NMJs of *Gabpα* Conditional Knockout Mice

The three-dimensional shape of NMJs was determined by wholemount AChR staining of single muscle fibres of sternomastoid or soleus muscles, or intact diaphragm muscles, from homozygous floxed, heterozygous and homozygous *Gabpα* skeletal muscle-specific knockout mice. Single muscle fibres were isolated from sternomastoid (situated within the neck) and soleus muscles of two mice of each sex of homozygous floxed and homozygous *Gabpα* skeletal muscle-specific knockout genotypes at 3 months of age. The sternomastoid muscle was studied because its lack of connective tissue allows for easy separation of single muscle fibres, and the soleus was chosen to enable study of a highly oxidative muscle. Intact diaphragm muscles were also isolated from four mice of homozygous floxed and homozygous *Gabpα* skeletal muscle-specific knockout genotypes, at 6 months of age, as this muscle shows reduced levels of *AChRδ* and ε transcripts upon

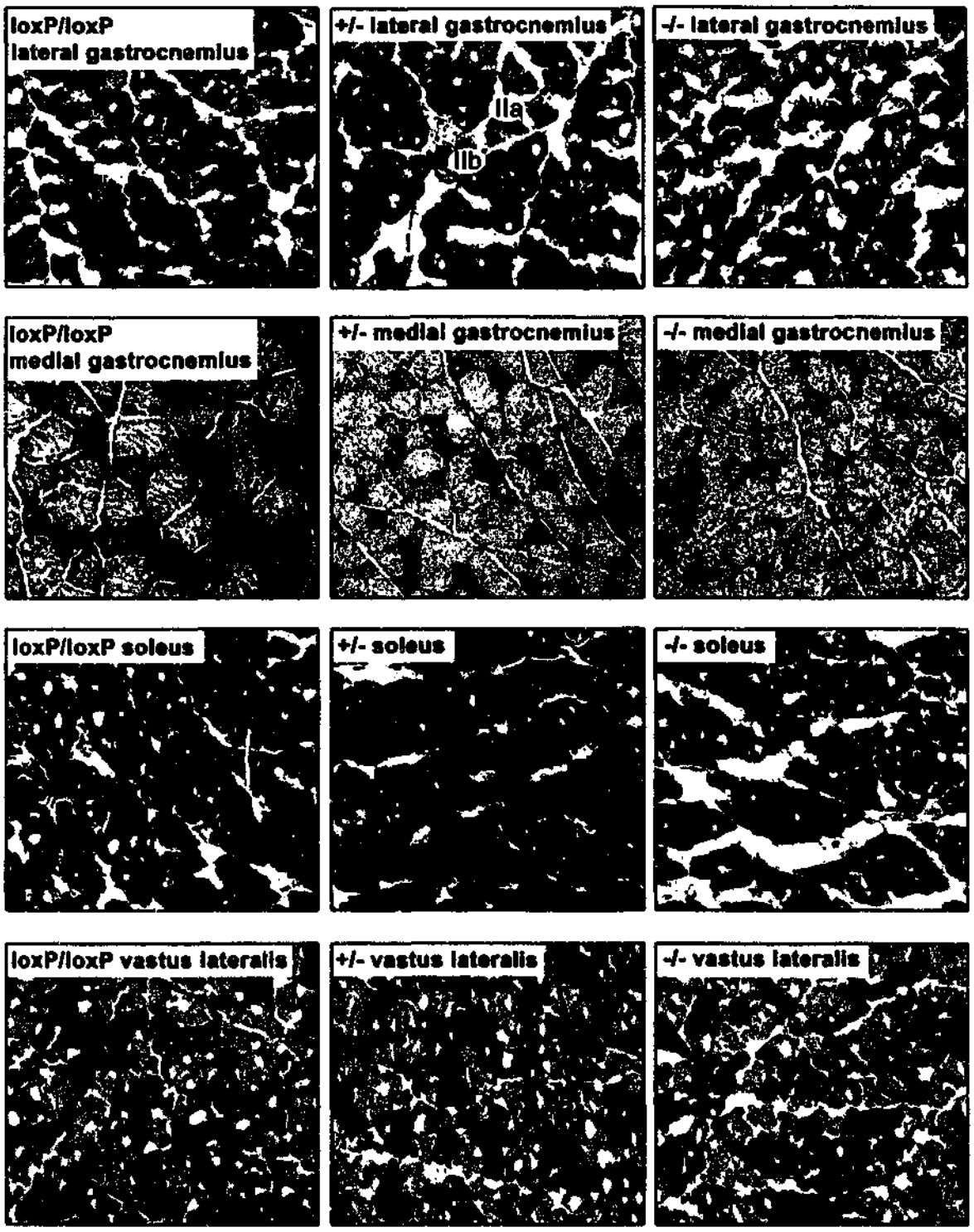


Figure 7.14 - Skeletal Muscle Fibre Typing of *Gabpα* Conditional Knockout Mice.

Transverse cryosections of skeletal muscle from 1 month old wildtype (loxP/loxP), heterozygous (+/-) and homozygous (-/-) *Gabpα* skeletal muscle-specific knockout mice stained with NADH. Type I, IIb and IIa fibres are indicated by dark, light and intermediate staining, respectively, as shown in the top centre panel. Images were taken at 200x magnification.

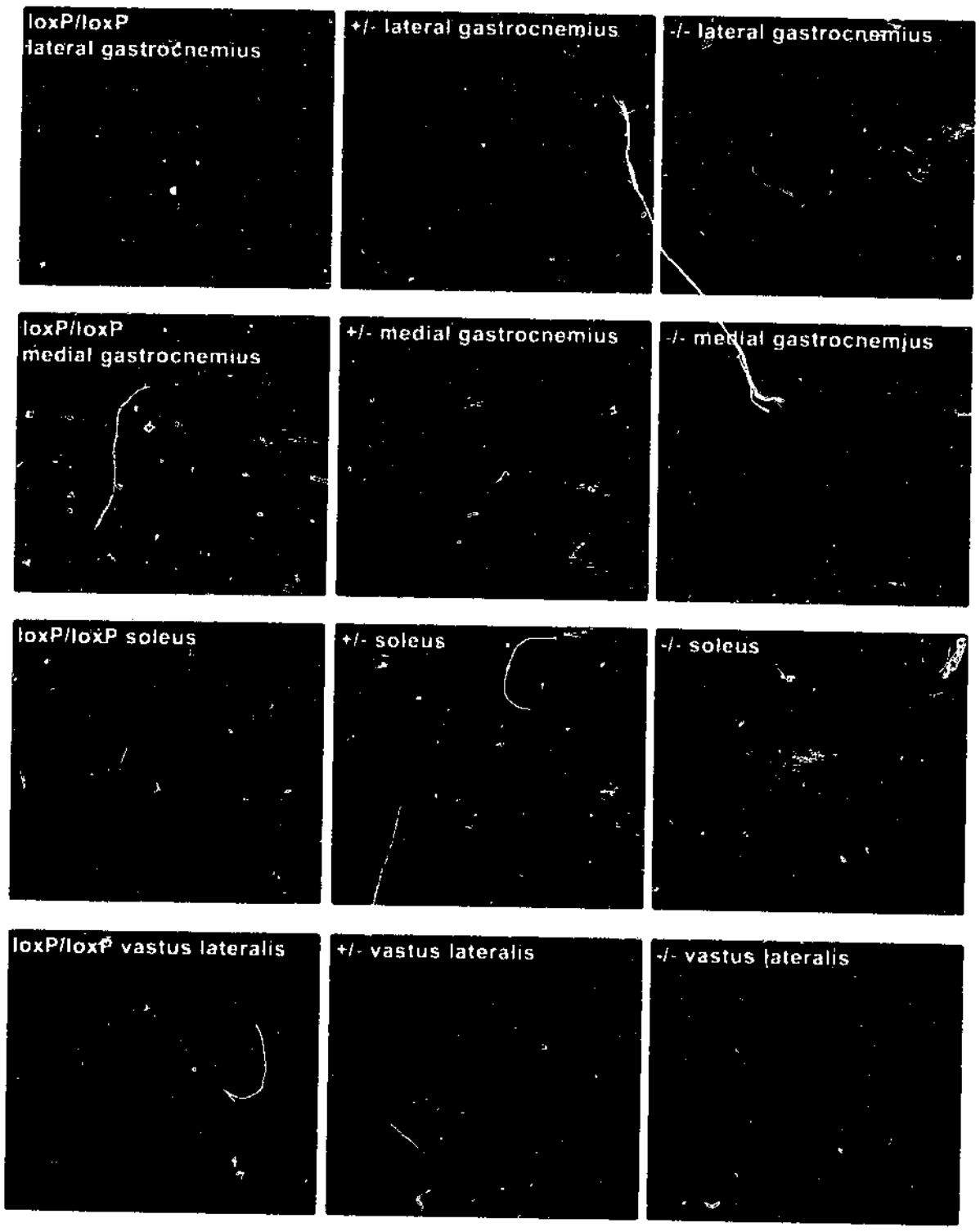
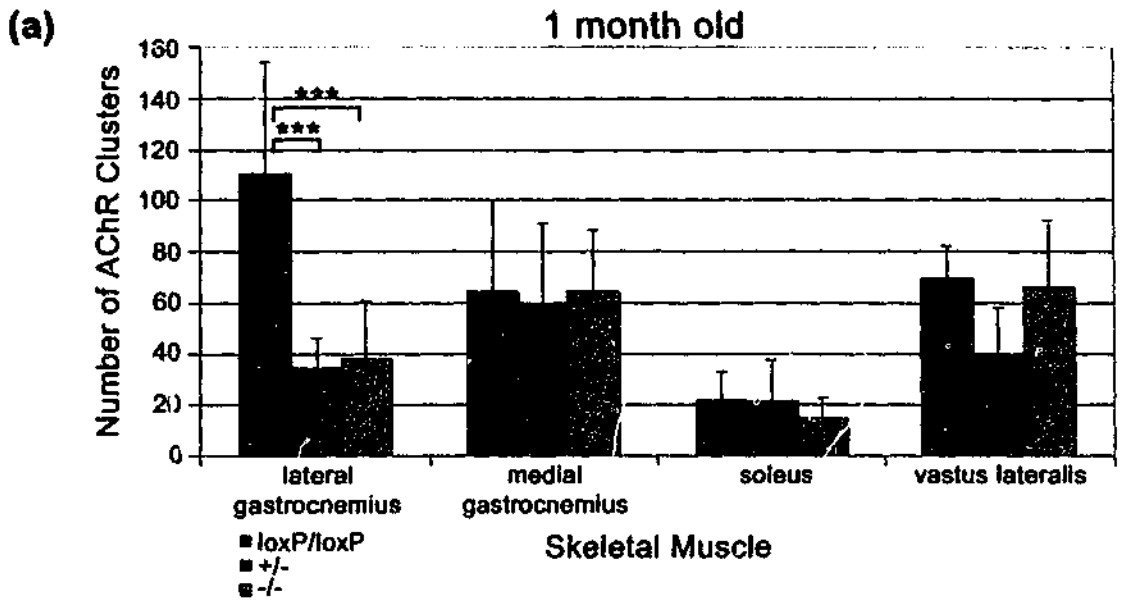
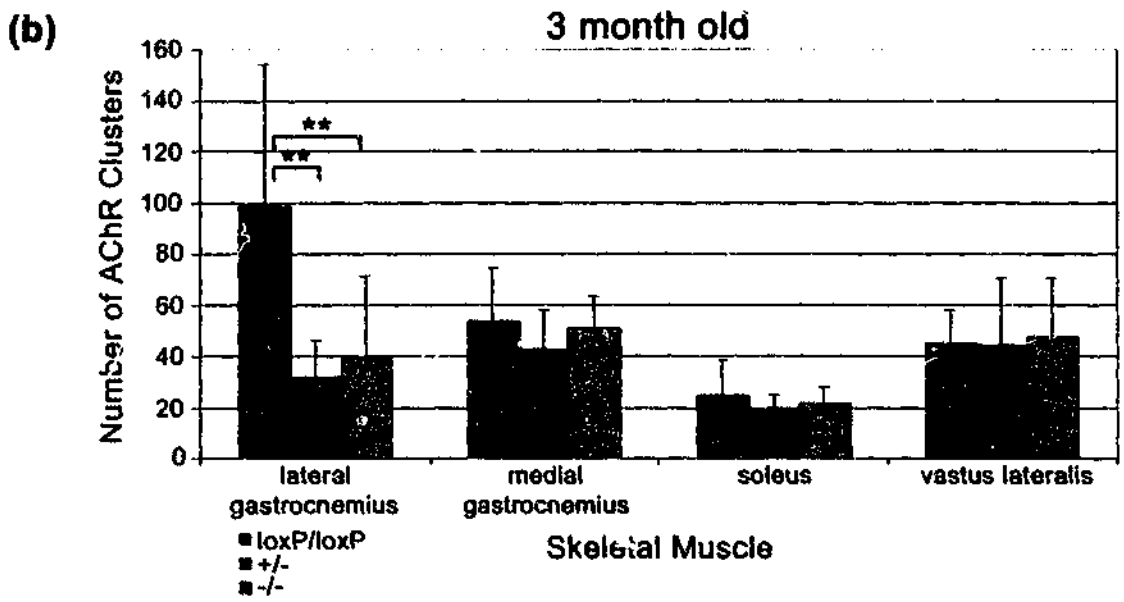


Figure 7.16 - AChR Distribution in *Gabpα* Conditional Knockout Mice.

The distribution of AChRs in longitudinal cryosections of skeletal muscle from 1 month old wildtype (loxP/loxP), heterozygous (+/-) and homozygous (-/-) *Gabpα* skeletal muscle-specific knockout mice, as shown by Texas red-conjugated α-bungarotoxin staining. Images were taken at 200x magnification.





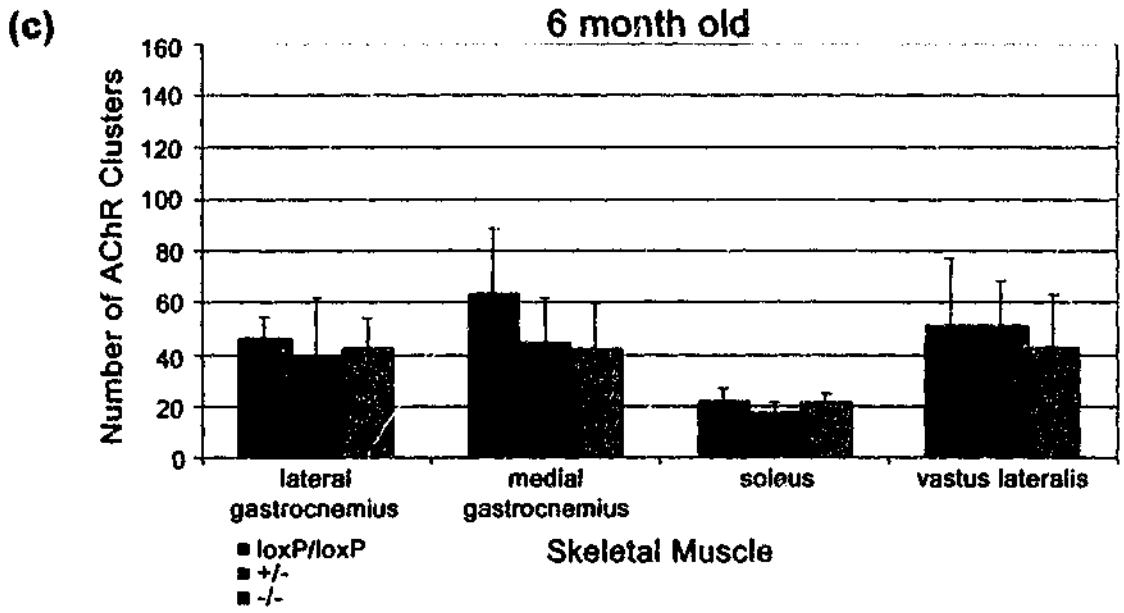


Figure 7.17 - AChR Cluster Number in *Gabpα* Conditional Knockout Mice.

The average number of AChR clusters per muscle tissue, as determined by α-bungarotoxin staining of longitudinal sections from **(a)** 1, **(b)** 3, and **(c)** 6 month old wildtype (loxP/loxP), heterozygous (+/-) and homozygous (-/-) *Gabpα* skeletal muscle-specific knockout mice. Error bars represent SD, where n=12 for 1 month old, n=10 for 3 month old and n=8 for 6 month old animal groups. ** and *** represent statistical significance, as determined by two-tailed t-tests, where p=<0.005 and <0.0005, respectively.

loss of Gabpα protein expression (see **Figure 7.12**). AChR clusters were stained using Texas red-conjugated α-bungarotoxin (Molecular Probes) at 1 μg/ml, and a mixture of rabbit sera raised against mouse 200 kDa Neurofilament (8 μg/ml, Sigma) and human Synaptophysin (1/50 neat sera, DAKO) proteins, using 7.5 μg/ml FITC-conjugated goat anti-rabbit IgG secondary antibody (Zymed). This enabled localisation of nerves and synaptic terminals at NMJs, respectively. Representative images from each muscle tissue are shown in **Figure 7.18**.

In agreement with analysis of mice expressing an ETS dominant negative protein in skeletal muscle, approximately 70 % of NMJs of homozygous *Gabpα* skeletal muscle-specific knockout mice show less branching than homozygous floxed *Gabpα* controls (type 1) and the remaining 30 % show a single ring structure (type 2) (de Kerchove d'Exaerde et al. 2002). This altered NMJ morphology is most evident in soleus. An overall reduction in NMJ size is also apparent in soleus (**Figure 7.18i**) and sternomastoid (**Figure 7.18ii**) muscle fibres of homozygous *Gabpα* skeletal muscle-specific knockout mice. However the small size of NMJs in wildtype diaphragm may mask any slight decrease in this tissue (**Figure 7.18iii**). Additional mice need to be studied in order to quantify the area occupied by the AChRs within the NMJs of *Gabpα* skeletal muscle-specific knockout mice. However, this preliminary analysis suggests a reduction in area occupied by AChRs in conditional knockout mice.

Diaphragm and soleus muscles were more closely examined by electron microscopy, to study the effect of loss of Gabpα expression upon the structure of postsynaptic junctional folds, and mitochondrial integrity (kindly performed by Lynn Tolley, Department of Physiology, University of Queensland, Queensland, Australia). Tissue from four homozygous floxed and four homozygous *Gabpα* skeletal muscle-specific knockout mice of 3 months of age was analysed. As shown in **Figure 7.19**, there is no apparent difference between the two genotypes in the two-dimensional ultrastructure and distribution of mitochondria, synaptic vesicles, postsynaptic junctional folds or muscle fibres at the NMJs of diaphragm or soleus muscles. Therefore, whether the change in fibre type proportions and three-dimensional NMJ morphology in *Gabpα* conditional knockout mice results in altered skeletal muscle function was investigated.

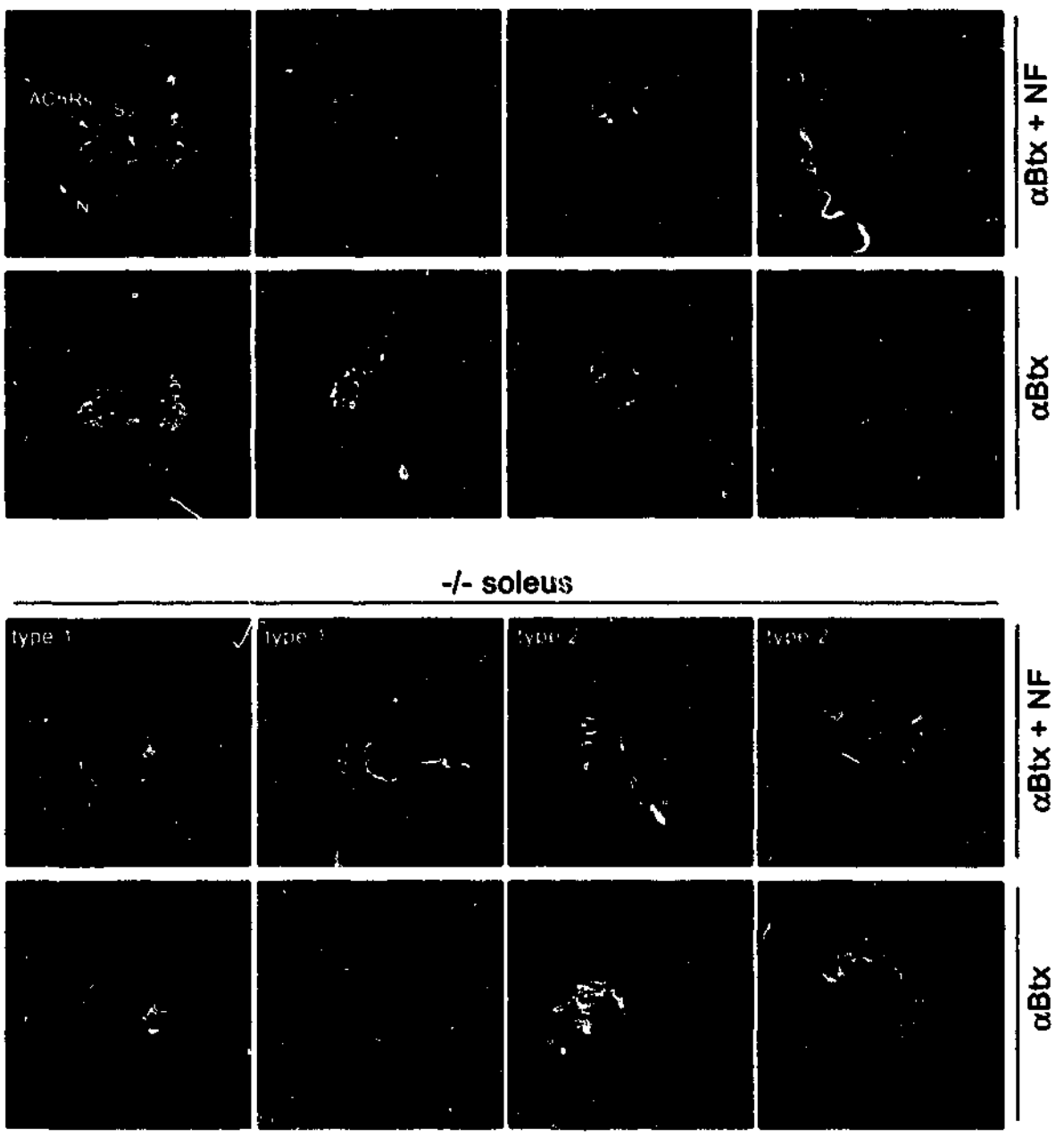


Figure 7.18 - NMJ Morphology in *Gabpα* Conditional Knockout Mice.

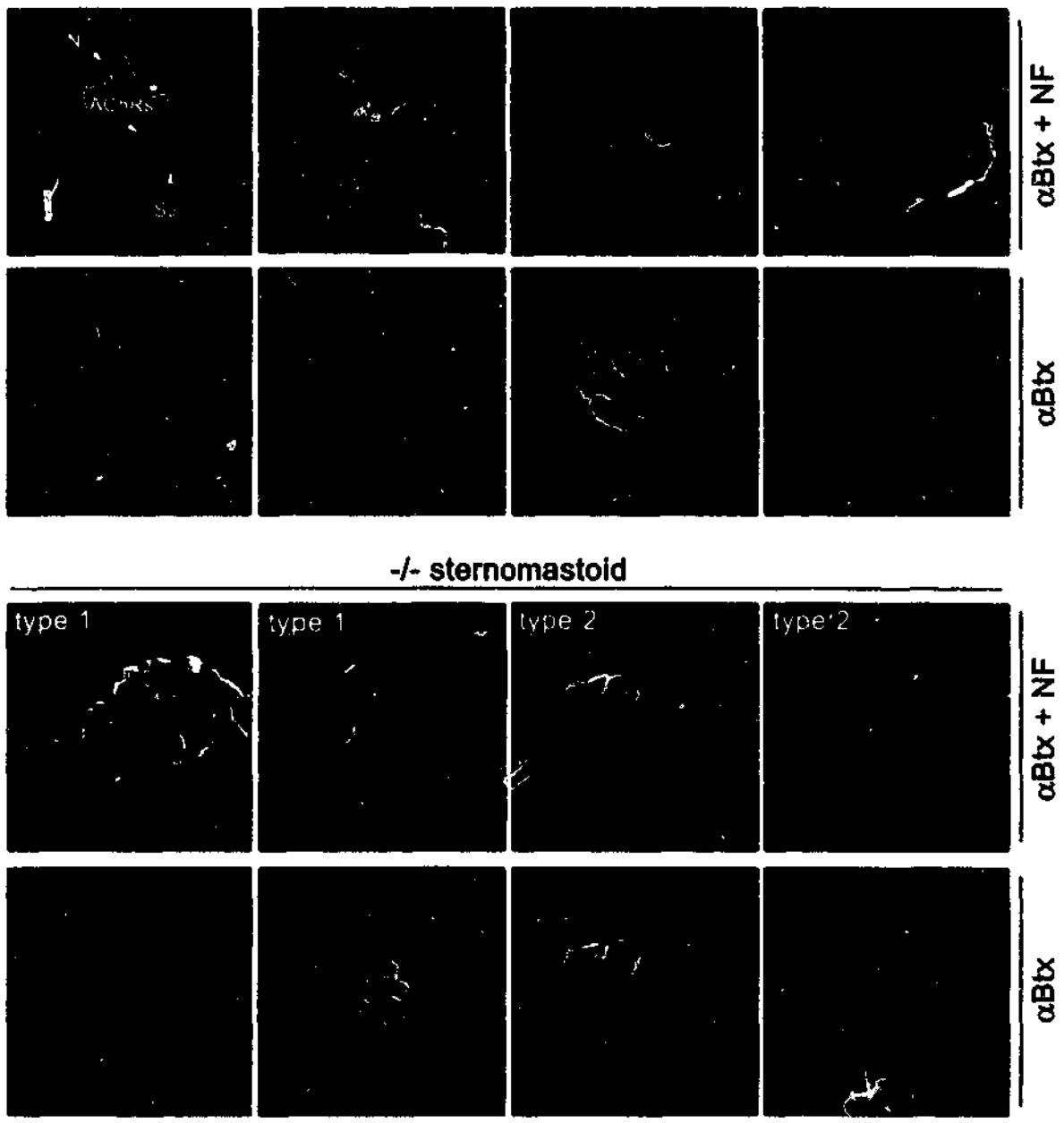


Figure 7.18 - NMJ Morphology in *Gabp*α Conditional Knockout Mice.

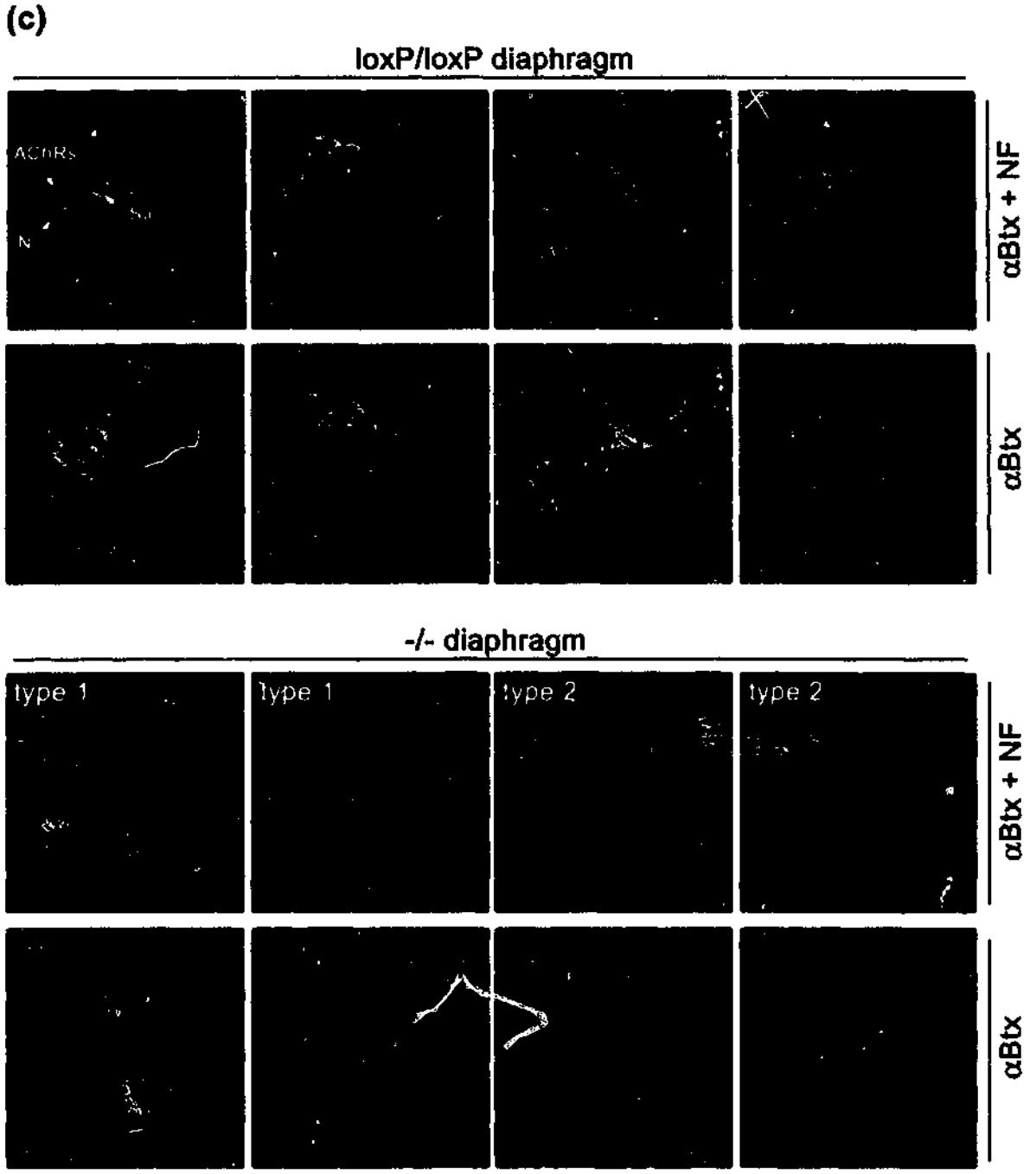


Figure 7.18 - NMJ Morphology in *Gabpα* Conditional Knockout Mice.

Wholemount immunostaining of single muscle fibres from **(a)** soleus, **(b)** sterno-mastoid and **(c)** intact diaphragm from wildtype (loxP/loxP) and homozygous (-/-) *Gabpα* skeletal muscle-specific knockout mice. NMJ shape is revealed by AChR staining with Texas red-conjugated α-bungarotoxin (αBtx), and nerves and synaptic junctions with FITC-labelled antibodies against neurofilament and synaptophysin, respectively (indicated as NF). Relative positions of nerves (N), synaptic junctions (SJ) and AChRs are indicated on the top left panel for each muscle tissue. NMJs of -/- mice are classified as showing less branching than those of loxP/loxP mice (type 1) or single ring structures (type 2). All images are at 1000x magnification.

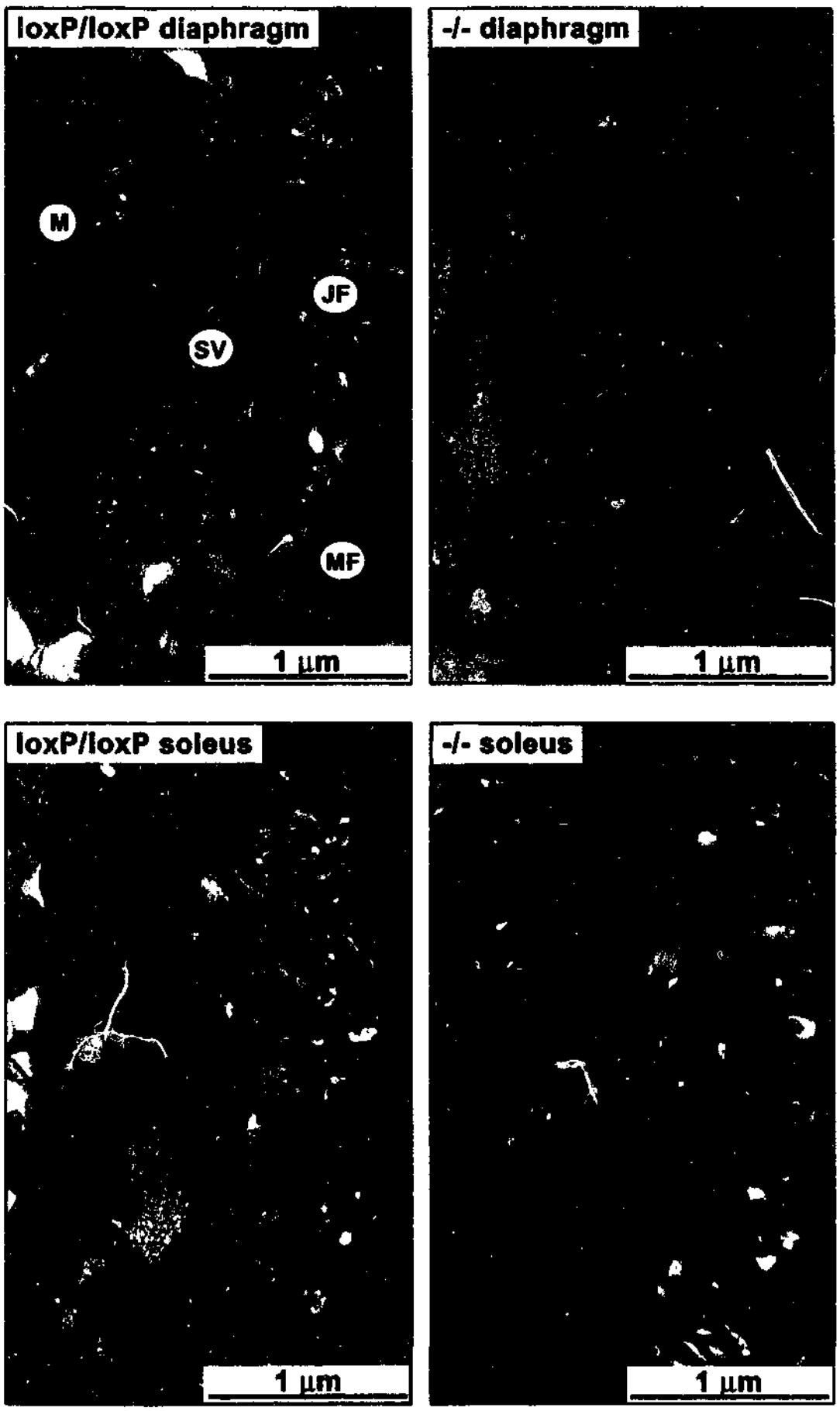


Figure 7.19 - Ultrastructure of NMJs in *Gabpα* Conditional Knockout Mice.

Electron microscopy of NMJs of diaphragm and soleus muscles from 3 month old wildtype (loxP/loxP) and *Gabpα* skeletal muscle-specific knockout mice (-/-). The relative positions of postsynaptic muscle fibres (MF), junctional folds (JF), synaptic vesicles (SV) and mitochondria (M) are indicated in the upper left panel.

7.3.6 Skeletal Muscle Function of *Gabpα* Conditional Knockout Mice

The grip strength of heterozygous and homozygous *Gabpα* skeletal muscle-specific knockout mice was measured at 1, 3 and 6 months of age, and compared to that of wildtype, floxed and *hαSA-Cre* littermates (see **Figure 7.20**). This was measured by ability to grip to an inverted wire cage lid, for a maximum test time of 60 seconds. The only significant difference seen was at 6 months of age, when heterozygous and homozygous conditional knockout mice exhibit greater grip strength than their wildtype littermates. This is due to maintenance of strength in the knockout mice, which significantly diminishes with age in wildtype mice. An explanation for this result is unknown, however it may reflect the altered fibre type proportions observed in muscles of *Gabpα* conditional knockout mice, and tests of muscle endurance such as the rotor rod may reveal deficits of mice lacking Gabpα. As the gripping time of mice varied considerably, a greater number of animals should be analysed in future studies.

The gait of homozygous floxed, heterozygous, and homozygous *Gabpα* skeletal muscle-specific knockout mice was evaluated at 1, 3 and 6 months of age. No difference in the regularity and angle of footsteps was observed between the three animal groups (see **Figure 7.21** for representative images). Therefore, loss of Gabpα expression may result in decreased mitochondrial function, a reduced number of AChR clusters, and irregular AChR cluster formation at the NMJ without resulting in any overt impairment of skeletal muscle function.

Electrophysiological properties of skeletal muscles from homozygous floxed and homozygous *Gabpα* skeletal muscle-specific knockout mice were also measured, as a means of detecting slight differences in skeletal muscle function. Intact soleus muscles of four wildtype and four *Gabpα* conditional knockout 3 month old mice were analysed extracellularly for changes in characteristics of spontaneous/miniature endplate currents (MEPCs) and evoked endplate currents (EPCs) (measurements were recorded by Dr. Nick Lavidis, Department of Physiology, University of Queensland, Queensland, Australia). As shown in **Figure 7.22a**, the average amplitude of MEPCs in *Gabpα* skeletal muscle-specific knockout mice is significantly smaller than that of wildtype littermates, as determined by a two-tailed t-test. A similar trend is also apparent for EPCs, however this is not significantly different, due to the high degree of variability between sites analysed within each muscle. As shown in **Figure 7.22b**, some sites within muscle of *Gabpα*

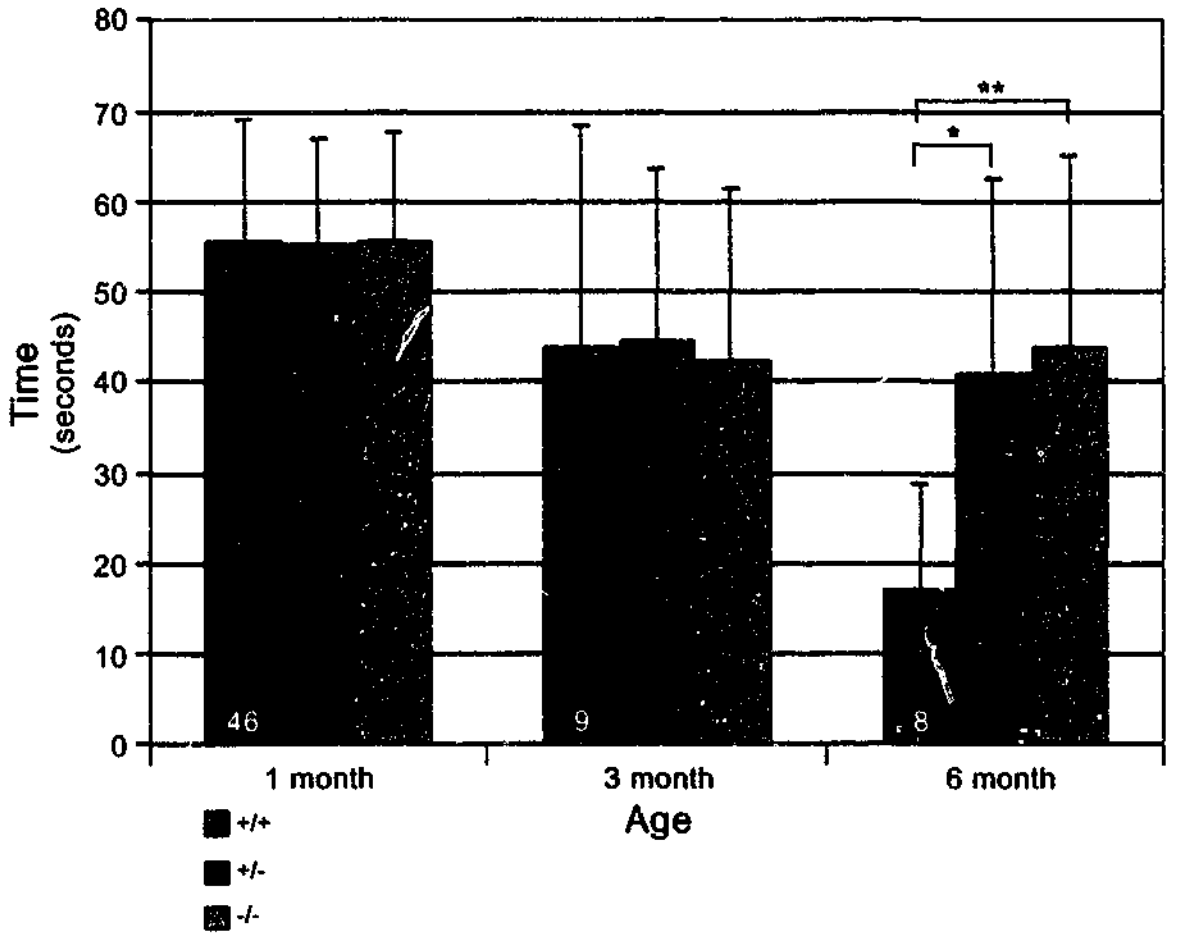


Figure 7.20 - Grip Strength of *Gabpα* Conditional Knockout Mice.
The grip strength of wildtype (+/+), heterozygous (+/-) and homozygous (-/-) *Gabpα* skeletal muscle-specific knockout mice at 1, 3 and 6 months of age, as measured by the time to fall from an inverted wire cage lid. Wildtype animal groups included mice with +/+, +/loxP, loxP/loxP and +/cre genotypes. Error bars represent SD, and n values are indicated on each column. * and ** show statistical significance, as determined by two-tailed t-tests, where p=<0.05 and <0.005, respectively.

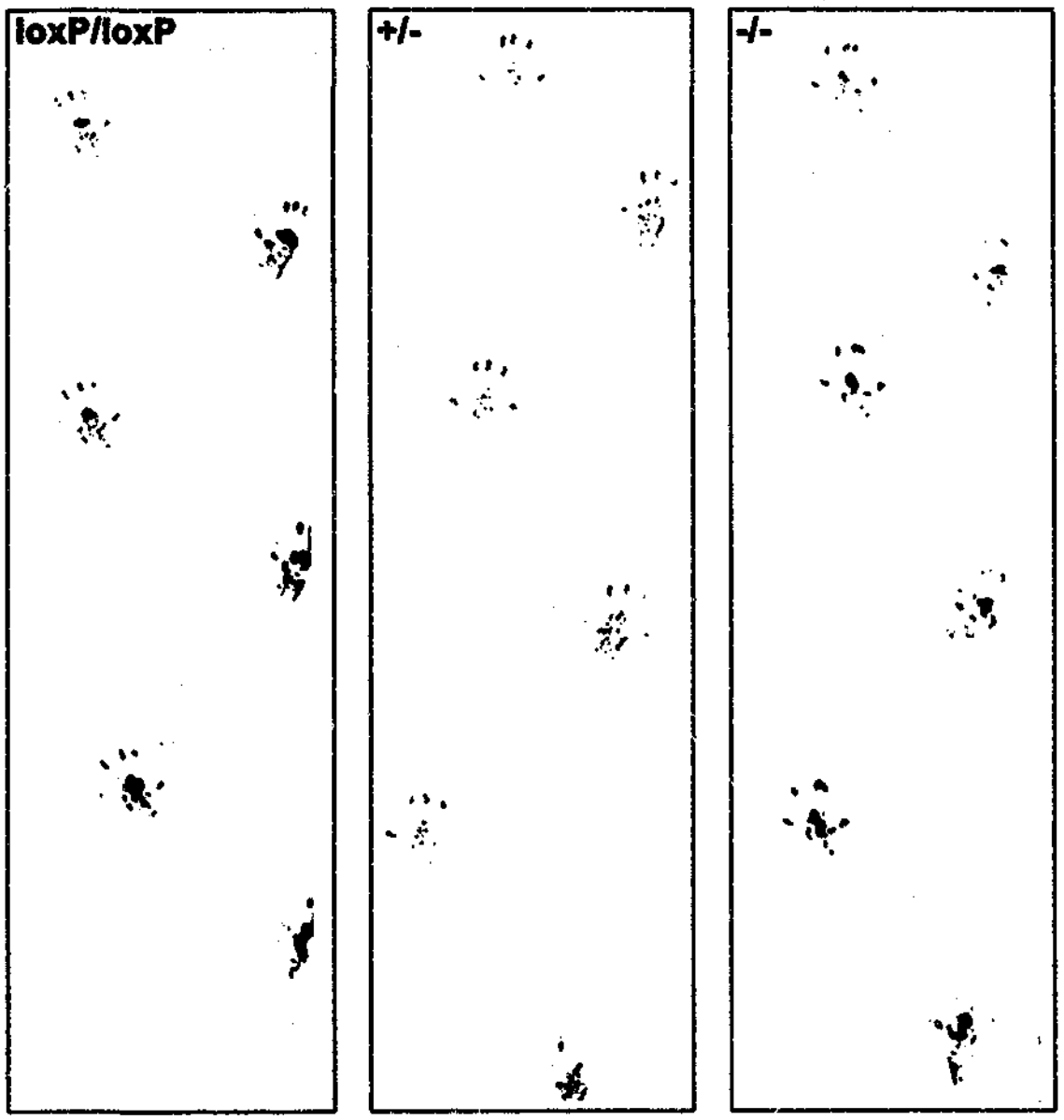


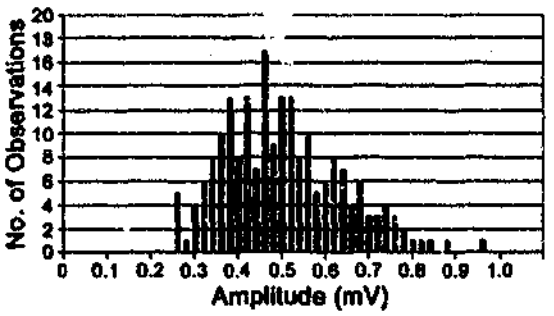
Figure 7.21 - Gait Analysis of *Gabpα* Conditional Knockout Mice.
Footprints of wildtype (loxP/loxP), heterozygous (+/-) and homozygous (-/-) *Gabpα* skeletal muscle-specific knockout mice at 3 months of age, showing balanced gait.

(a)

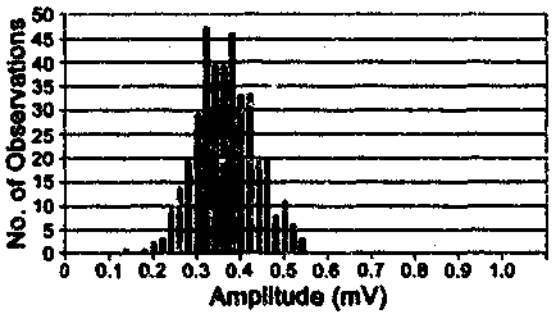
Genotype	loxP/loxP	-/-
Number of mice	4	4
Total no. of recorded sites:		
MEPC	12	12
EPC	8	8
MEPC amplitude (mV)	0.27 ± 0.03	0.17 ± 0.02*
MEPC rise time (ms)	0.64 ± 0.01	0.64 ± 0.02
MEPC decay time (ms)	1.78 ± 0.06	1.66 ± 0.15
EPC amplitude (mV)	0.29 ± 0.22	0.08 ± 0.02
EPC rise time (ms)	0.99 ± 0.11	1.08 ± 0.09
EPC decay time (ms)	2.55 ± 0.23	3.89 ± 0.82
Quantal content	0.84 ± 0.47	0.60 ± 0.20

(b)

(i)



(ii)



(iii)

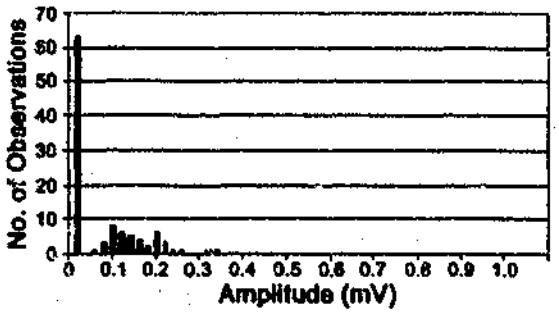


Figure 7.22 - Electrophysiology of NMJs of *Gabpα* Conditional Knockout Mice.

A summary of the biophysical properties of NMJs of soleus muscles of 3 month old wildtype (loxP/loxP) and *Gabpα* skeletal muscle-specific knockout mice (-/-), using extracellular recordings, is shown in **(a)**. The average data of spontaneous/ miniature endplate currents (MEPC) and evoked endplate currents (EPC) is shown (± SEM). * represents statistical significance, as determined by a two-tailed t-test, where p=<0.05. **(b)** Histograms of EPC recordings from single sites, showing the variability in NMJ activity in *Gabpα* skeletal muscle-specific knockout mice; (i) wildtype site, (ii) knockout site with reduced EPC amplitude, (iii) knockout site with reduced EPC amplitude and activity.

skeletal muscle-specific knockout mice display slight reductions in EPC amplitude, while others have reduced EPC amplitude and loss of activity. This may be a result of heterogeneous deletion of *Gabpα* within myonuclei of each fibre. No difference in the rise time or decay time of endplate currents was seen between the two genotypes, indicating that neurotransmitter release and channel opening are not affected by loss of Gabpα expression.

The reduced amplitude of MEPCs observed in soleus muscles of *Gabpα* skeletal muscle-specific knockout mice reflects an alteration in postsynaptic properties of the conditional knockout mice (independent of nerve activity). The most likely explanation is a reduction in AChR number, and a similar reduction in the amplitude of endplate currents is observed in *AChRε* knockout mice (Witzemann et al. 1996). Although counting of AChR cluster numbers in skeletal muscle sections did not reveal any such change in soleus of *Gabpα* skeletal muscle-specific knockout mice, future experiments will involve a more accurate measure of AChR number, by means of radioactively labelling the receptors.

7.4 Summary – Gabpα *Function in Skeletal Muscle*

Production of ubiquitous and skeletal muscle-specific knockout mouse models demonstrates that Gabpα is not essential for skeletal muscle formation and function, but does play an important role in the formation of AChR clusters at the NMJ. Specific skeletal muscle tissues, such as the gastrocnemius, are also reliant upon Gabpα expression for the establishment of normal muscle fibre type proportions and number of AChR clusters, and loss of Gabpα expression results in a significant reduction in *AChRε* and *MTFA* transcript expression levels in some skeletal muscle tissues, such as diaphragm. The effect of loss of Gabpα protein expression upon Gabp target gene expression results in changes in the electrophysiological properties of NMJs indicative of a reduced number of AChRs. However, *Gabpα* skeletal muscle-specific knockout mice do not display any overt skeletal muscle weakness, as occurs in humans suffering from Congenital Myasthenic Syndrome, lacking AChRε expression. The greater safety factor of neuromuscular transmission (ratio between the endplate potential generated under normal conditions and that required to generate an action potential) in mice and rats, compared to humans (Le Treut et al. 1990), may explain why no obvious alteration in skeletal muscle

function is seen when Gabpα is lacking. Further study of the *Gabpα* skeletal muscle-specific mouse model, by means of mitochondrial function assays, and a more comprehensive study of different skeletal muscle tissues for target gene expression levels, and AChR morphology and number, should enable a better understanding of how the Gabp complex regulates the expression of NMJ and mitochondrial proteins within particular tissues. However, taken together, this preliminary analysis demonstrates that Gabpα is the ETS transcription factor necessary for formation of intact AChR clusters at the NMJ.

Chapter 8

Discussion:

Gabpα: *A Tightly Controlled Regulator of Skeletal Muscle Transcription*

8.1 GABPα is a Highly Conserved Gene Locus

The genomic structure of 10 exons and 9 introns of *Gabpα*, and their relative sizes, are very similar to those of the human *GABPα* locus (see **Chapter 4**). High sequence similarity (≥75 %) is observed within the coding regions of the mouse and human genes, resulting in protein products that share 96 % amino acid identity. However, the alternative 5' UTRs, exons 1a and 1b, are unique to mouse, suggesting a species-specific means of regulation. This finding is not unique to *Gabpα*, as recent analysis of 9434 orthologous genes in human and mouse revealed that 28 % of exons unique to minor transcripts are conserved between the two species, compared to 98 % of exons contained in major transcripts (Modrek and Lee 2003). Therefore, this suggests a recent event of exon creation in mouse *Gabpα* or exon loss in human *GABPα*, since the genomes of the two species diverged 75-110 million years ago (Modrek and Lee 2003). Given that exon 1a and 1b are expressed in *Gabpα* transcripts in the mouse, it is likely that they are the result of exon creation, providing a species-specific means of post-transcriptional regulation. The *Gabpα* exon 1a region and beginning of exon 1b are also ≥75 % identical in mouse and rat, indicating that the *Gabpα* exon 1a transcript may also be expressed in rat.

The gene arrangement of the *GABPα* locus and flanking regions is conserved between human, mouse and rat. Located 165-240 bp upstream of the 5' UTR of mouse *Gabpα* is the 5' UTR of *Mitochondrial ATP Synthase Coupling Factor VI*, in the reverse orientation to *Gabpα*. The ATP synthase protein forms an essential component (complex V) of the mitochondrial respiratory chain, and presence of bi-directional promoter elements between the *Gabpα* and *Mitochondrial ATP Synthase Coupling Factor VI* genes (Chinenov

et al. 2000) is suggestive of coupled regulation. This bi-directional promoter region contains ETS, YY1 and Sp1 binding sites which are common to promoters of other mitochondrial protein genes, such as the bi-directional promoter regulating expression of Surf-1 and Surf-2 proteins of the inner mitochondrial membrane (Cole and Gaston 1997), and promoters of genes encoding the mitochondrial regulator MTFA (Virbasius and Scarpulla 1994) and Cytochrome-c-oxidase subunits COXIV and COXVb (Lenka et al. 1998). Presence of ETS, YY1 and Sp1 binding sites reflects the housekeeping role of mitochondrial proteins, a number of which are proposed to be regulated by the Gabp transcription factor complex. *Gabpα* expression may also be auto-regulated by a feedback loop, as several of the ETS sites within the *Gabpα* promoter are consensus Gabp binding sites. Site-specific mutagenesis would clarify whether ETS and other transcription factors positively or negatively regulate expression from the *Gabpα* promoter.

Within the non-coding regions of *GABPα* there are three regions that are ≥75 % conserved between mouse and human. These are the CpG island flanking exon 1, a 100 bp region within intron 3, and a 750 bp region within intron 9, immediately upstream of the last exon. These areas may contain regulatory sequences that have been conserved during evolution. CpG islands are associated with the 5' ends of all housekeeping genes and many widely expressed and tissue-specific genes, and are found in the same relative position in orthologous genes of different species (Gardiner-Garden and Frommer 1987). Their exact function is unknown, however it has been suggested that basal transcription factors such as Sp1 bind to elements within CpG islands, and the location of CpG islands is often associated with regions of basal promoter activity. Within the conserved *GABPα* CpG island are general transcription initiation sequences and binding sites for ETS transcription factors, Sp1, YY1, NFκB, Myc/Max and AP2, correlating with the ubiquitous expression of Gabpα. However, the presence of muscle-specific transcription initiation sequences and binding sites for the haematopoietic transcription factor Mzf1 reflect the ability of GABP to transactivate cell type-specific genes such as *AChRδ* and *ε* in skeletal muscle cells (Koike et al. 1995; Duclert et al. 1996), and CD18 in leukocytes (Rosmarin et al. 1998).

Together, this data shows that the *GABPα* coding and promoter regions of mouse and human are highly conserved, making the mouse an excellent model system for the study of GABPα protein function. However, exons 1a and 1b are unique to mouse, suggesting the existence of additional species-specific regulation of GABPα expression.

8.2 *Gabpα Expression is Regulated in a Tissue-Specific Manner*

The identification of several alternative transcript forms of *Gabpα* is consistent with the presence of multiple forms detected by Northern blot analysis (see **Chapter 4**). Exon 1a and 1b are two alternative 5' UTRs that lie within *Gabpα* intron 1, both capable of splicing into exon 2, producing identical coding regions to that of the exon 1 transcript (as the translation initiation site is located in exon 2). However, the exon 1a and 1b 5' UTRs of *Gabpα* are expressed in a limited number of tissues in contrast to the ubiquitous exon 1 transcript. Four alternative polyadenylation sites within exon 10 are also used to generate *Gabpα* transcripts differing in the length of their 3' UTRs. Therefore, the twelve possible *Gabpα* transcripts resulting from alternative 5' and 3' UTR usage all encode a single protein product, but may function to regulate tissue-specific transcription, mRNA stability, translation efficiency, and/or transcript localisation of *Gabpα*.

The alternative 5' UTRs of mouse *Gabpα* do not function as alternative promoters. However, the CpG island encompassing promoter region P2 and the upstream P1 promoter region are active individually, and in combination there is an additive rather than synergistic effect. This suggests that these promoters may function independently to regulate the expression of the three 5' UTR *Gabpα* transcripts. The use of a different transcription initiation site to generate the alternative 5' UTR transcripts of *Gabpα* could explain their tissue-specific expression patterns. Cell type-specific isoforms of TATA box-binding proteins (TBPs) and TBP-associated factors (TAFs) of the transcription initiation complex have recently been identified, and knockout mouse models lacking TBP-related factor-2 (TRF2) or TAF4b expression have shown these proteins are essential for spermatogenesis or folliculogenesis, respectively (Hochheimer and Tjian 2003). Therefore the initiation complex that binds to each transcription start site of *Gabpα* might determine the expression pattern of resulting transcripts. Site-specific mutation studies are required to understand the role of the various transcription factors and initiation complexes in the tissue-specific regulation of *Gabpα* expression.

Several enzymes of the heme biosynthetic pathway are encoded by genes that utilise alternative promoters to regulate expression of alternative 5' UTRs. The human *Uroporphyrinogen III Synthase*, *δ-Aminolevulinate Dehydratase* and *Porphobilinogen Deaminase* genes feature one promoter that controls expression of a ubiquitously

expressed transcript and another that regulates expression of an erythroid-specific transcript containing the same coding region but alternative 5' UTR (Chretien et al. 1988; Kaya et al. 1994; Aizencang et al. 2000). Transcription factors other than Gabpα also generate alternative 5' UTRs encoding the same protein product. For example, the *thyroid hormone receptor* α gene of the rat generates isoforms differing in their C-terminal end, as well as two transcripts differing only in their 5' UTR sequences (Izumo and Mahdavi 1988). The use of multiple promoters allows more flexibility in gene expression, and generation of transcripts differing only in their 5' UTRs is thought to render a gene less vulnerable to the effects of promoter mutations (Ayoubi and Van De Ven 1996).

Given that *Gabpα* exon 1a and exon 1b transcripts are expressed in a tissue-specific manner and that their predicted secondary structures and stabilities differ from those of the major exon 1 transcript, the three 5' UTR transcripts of *Gabpα* may be translated with varying efficiency in a cell type-specific manner. The low free energy of the exon 1 *Gabpα* transcript, together with the central location of the initiation codon within the predicted mRNA secondary structure, may result in decreased translation efficiency, as more free energy would be required to melt the secondary structure and provide access to the initiating AUG. Therefore, *Gabpα* transcripts containing the less structured exon 1a may be favoured for translation due to the ease of ribosomal binding, and transcripts containing exon 1 may be less efficiently translated, due to increased secondary structure. This case holds true for expression of *Gabpα* in spleen, where a high expression level of the exon 1a transcript and moderate expression level of the exon 1 transcript coincides with a high level of Gabpα protein. Whereas, the large intestine, a tissue of high exon 1 *Gabpα* transcript expression and low exon 1a transcript expression, displays low Gabpα protein expression levels. The presence of three putative ORFs within exon 1b may also negatively regulate translation of this 5' UTR *Gabpα* transcript. Upstream ORFs have been shown to interfere with ribosome scanning and lead to a reduction in translation efficiency (Kozak 1996; van der Velden and Thomas 1999). Therefore, the relative expression levels of the three 5' UTR *Gabpα* transcripts may determine the translation efficiency, and hence protein expression level, of Gabpα within a cell.

An example of translational regulation of a gene by alternative 5' UTR usage is that of the *GLI-1* oncogene. *In vitro* assessment of the translation efficiency of the three *GLI-1* 5' UTRs (α, β and γ) showed that the 5' UTRs of the larger transcripts, α and β, with more

stable predicted secondary structures and several upstream ORFs, suppressed protein production, whereas the shorter 5' UTR of the γ? transcript enabled enhanced protein expression (Wang and Rothnagel 2001). Similar assessment of the translation efficiency of the three *Gabpα* 5' UTRs is necessary before their function can be established. Differences in translation efficiency of alternative 5' UTR transcripts can have important implications for availability of the protein product. For example, the lowly abundant γ 5' UTR variant of *GLI-1* is usually absent in adult skin, but increased mRNA expression correlates with an increase in cell proliferation in cases of basal cell carcinoma (Wang and Rothnagel 2001). Concurrent analysis of *Gabpα* transcript and protein expression levels in individual cell types is necessary before the function of alternative 5' and 3' UTRs can be concluded.

Although *Gabpα* and *β* are expressed in all adult tissues, many Gabp target genes have a restricted expression pattern and specialised function, such as subunits of the AChR at the NMJ of skeletal muscle. Reporter gene expression under control of the *Gabpα* genomic regulatory sequence demonstrates that, although detectable in all tissues in the adult mouse, *Gabpα* is expressed predominately in epithelial cells. However, in skeletal muscle, reporter gene expression was observed throughout the entire tissue. *Gabpα* mRNA has previously been shown (by *in situ* hybridisation) to accumulate in sub-synaptic nuclei within skeletal muscle (Schaeffer et al. 1998). Taken together, this suggests that upstream promoter regulatory elements determine cell type-specific expression of Gabpα protein, and the choice of 5' and 3' UTRs may control the sub-cellular localisation of the *Gabpα* transcript.

The 3' UTR of *Utrophin* is known to be necessary for stabilising the transcript and localising it to the cytoskeletal-bound pool of polysomes within skeletal muscle cells (Gramolini et al. 2001a). Most regulatory functions of 3' UTRs occur through protein interactions (Sonenberg 1994). Transcript localisation can be mediated by protein interactions with microtubules or microfilaments, or proteins with specific nuclear export signals, and differences in mRNA stabilities also allows for the accumulation of a transcript at the desired site and degradation throughout most of the cytoplasm (Lipshitz and Smibert 2000). Interaction of polyadenylation factors with the C-terminal domain of RNA polymerase II is also suggested to enhance the efficiency of transcript polyadenylation (Calvo and Manley 2003). Therefore, the various combinations of *Gabpα*

5' and 3' UTRs may be processed with differing efficiency. The lack of a conserved $^{5'}$AAUAAA$^{3'}$ signal sequence in two of the four 3' UTRs of *Gabpα* suggests that these transcripts would be less efficiently cleaved and processed than those containing the consensus sequence (Wickens and Stephenson 1984).

Comparative analysis of *Gabpα* mRNA and protein levels in adult mouse tissues demonstrates that post-transcriptional regulation of *Gabpα* expression does occur in a tissue-specific manner (see **Chapter 5**). The same conclusion was reached previously, that there is no correlation between *Gabpα* mRNA and protein levels, in rat testes, liver and brain (Vallejo et al. 2000). In the present study, exogenous overexpression of *Gabpα* cDNA in NIH3T3 fibroblast cells did not result in increased Gabpα protein levels, even though the *EF-1α* promoter has previously been used to overexpress *ETS-2* mRNA, resulting in increased expression of ETS-2 protein in the same cell line (Sanij et al. 2001). It is possible that use of an artificial polyadenylation sequence within the overexpression construct may not have allowed for the production of a stable *Gabpα* transcript, as only a 3 to 8-fold increase in total *Gabpα* mRNA expression levels (above that of mock transfected cells) was observed when exogenous *Gabpα* expression was driven by the powerful mammalian *EF-1α* promoter (Mizushima and Nagata 1990). However, Down syndrome fibroblast cell lines that exhibit a 1.75-fold increase in *GABPα* mRNA expression relative to diploid fibroblasts do not show altered GABPα protein levels either. This, together with the increased expression of Gabpα protein in brain and skeletal muscle of Ts65Dn partial trisomy 16 mice, suggests that GABPα protein levels are not regulated by the same post-transcriptional mechanisms in all cell types. Production of Gabpα-specific monoclonal antibodies and ELISA analysis would be necessary to confirm unchanged Gabpα protein expression levels in other tissues.

The high mitochondrial load of brain and skeletal muscle makes these tissues highly dependent upon mitochondrial function. Therefore, perhaps a relatively high level of GABPα protein expression is needed in these tissues in the instance of Down syndrome, to compensate for the detrimental affects of pro-oxidant conditions upon mitochondrial function. However, the pro-oxidant state of Down syndrome cells would impair GABP function, by means of oxidation of two cysteine residues in the ETS and dimerisation domains of GABPα (Martin et al. 1996; Chinenov et al. 1998). Hence, reduced activity of the Gabp complex would mean that target genes may not be expressed at as high a level as

would be expected from GABPα protein levels in Down syndrome tissues. Generation and analysis of transgenic mouse lines ubiquitously overexpressing varying levels of Gabpα protein should allow us to determine whether overexpression of GABPα contributes to the tissue-specific phenotypes of muscular hypotonia and redox instability observed in Down syndrome.

Post-transcriptional regulation of *Gabpα* may result in profound effects on the availability of Gabpα protein. In addition, given that Gabpα functions in a complex with β, regulation of the expression of α is likely to affect the resulting function of the Gabp complex. Therefore, tight control of the availability of the α subunit could be an efficient mechanism for regulation of Gabp complex function in a tissue-specific manner.

8.3 Gabpβ1 Expression is Regulated in a Tissue-Specific Manner

The β subunit of Gabp facilitates complex formation, nuclear localisation and transcriptional activity. Seven alternate splice forms of the *Gabpβ1* transcript were identified in this study, potentially resulting in production of 9 isoforms of Gabpβ1 protein (see **Chapter 4**). Outside of the conserved functional domains the sequence of Gabpβ and γ subunits differ more so than the α subunits, both within and between species (de la Brousse et al. 1994). In addition, *D. melanogaster* expresses a homologue of GABPα, D-Elg, but not β (Pribyl et al. 1991). Taken together with the existence of multiple isoforms of Gabpβ, yet only one isoform of Gabpα, this suggests that α is likely to be a limiting component of the Gabp complex, and that different β subunits allow for formation of functionally distinct complexes in different cell types.

Previous experiments have identified the regions of Gabpβ necessary for nuclear localisation (PPAKR), transactivation (amino acids 153-267) (Sawa et al. 1996), and interaction with α (W35, L37, K69, V70, M102, L103, E113, K135, F136, K138) (Batchelor et al. 1998). The novel *β1* transcripts described here would give rise to proteins lacking the transactivation, nuclear localisation signal and/or α interaction domain. These isoforms fall into two functionally distinct classes, those that interact with Gabpα, and those that interact only weakly or not at all. The net effect of β isoforms that bind the α subunit, but fail to translocate and/or activate target genes is likely to be a dominant negative function.

The generation of Gabpβ1 molecules with opposing functions to the major transcript is not an uncommon outcome of alternative splicing. For instance, C-terminal truncation of the FosB component of the AP1 transcription factor complex results in an antagonistic ΔFosB protein product capable of dimerisation and DNA binding, but lacking the ability to transactivate target genes (Nakabeppu and Nathans 1991). Expression of the *ΔFosB* mRNA is induced in a similar manner to full length *FosB* transcript, and the ΔFosB protein has a dominant negative effect, inhibiting the activity of Jun or Fos/Jun by competing for AP1 binding sites (Nakabeppu and Nathans 1991). In addition, alternatively spliced DNA binding domains of the *cyclic AMP-responsive element modulator* (*CREM*) gene results in expression of transcription factors of antagonistic and activating functions during the various stages of spermatogenesis (Foulkes et al. 1992). Differential tissue expression of the alternative *Gabpβ1* transcripts allows for a mechanism whereby Gabp complex activity can be modulated depending on the β subunit. For example, the Gabp complex may be cytoplasmic or nuclear localised, activate transcription or prevent the binding of an active complex.

The Gabpα/β interaction results in a stable DNA binding complex whereas α binding to DNA alone is relatively unstable (Graves 1998) and therefore unlikely to regulate transcription. The amino acid residues of Gabpβ responsible for the interaction with α are positioned at the tip of each ankyrin repeat loop and form hydrophobic and hydrogen bonds with the carboxy terminal extension of the α ETS domain (Ely and Kodandapani 1998). The Gabpβ1 isoforms lacking these amino acids would be unlikely to form a stable complex with α and therefore may result in reduced Gabp complex activity. It is possible that these β1 isoforms may interact with other proteins.

Recently yeast-2-hybrid experiments identified Polycomb protein cofactors YAF-2 and YEAF1 as proteins that interact with amino acids 249-310 of GABPβ and γ isoforms, overlapping with the NLS (Sawa et al. 2002). Therefore Gabpβ1 isoforms lacking the NLS may not be able to bind these two proteins. GABP has also been shown to physically interact with Sp1 and Sp3 on the *Utrophin* promoter (Galvagni et al. 2001), however the interacting amino acids of GABPα and/or β have not been identified. It is interesting to speculate whether lack of the α interaction region of the Gabpβ1 ankyrin repeats affects these and other protein interactions, by means of altered protein structure. The net effect of any alternative interactions may be altered transactivation of Gabp target genes. The

identity and function of the various Gabpβ interactions remains to be determined, however it is likely that various complexes may play different functional roles within a cell. For instance, the Max basic helix-loop-helix zipper protein is capable of forming homodimeric or heterodimeric transcription factor complexes with Myc or Mad proteins, to exert effects of repression, proliferation or differentiation and quiescence, respectively (Ayer et al. 1993).

Gabp is a ubiquitously expressed transcription factor complex, and can be composed of one α and multiple β subunits that have the capacity to form various complexes. A more thorough investigation of the sub-cellular distribution and function of the various Gabpα/β complexes is necessary, in order to better understand how Gabp achieves transcriptional regulation of such a broad spectrum of target genes.

8.4 Gabpα *is Necessary for Embryonic Development*

The specific role of Gabpα in the expression and function of Gabp target genes was studied by generating mouse models lacking *Gabpα* (see **Chapter 7**). Mice carrying a homozygous deletion in the *Gabpα* locus die prior to implantation, indicating that Gabpα function is essential for early embryonic development. Gabp is known to co-operate with Nuclear respiratory factor 1 (Nrf-1) to regulate the expression of many nuclear-encoded proteins of mitochondrial function (Scarpulla 2002a). Homozygous deletion of *Nrf-1* results in a decrease in number of mitochondria in blastocysts and subsequent pre-implantation lethality (Huo and Scarpulla 2001). A high rate of mitochondrial transcription is known to occur during cleavage of the embryo, and may require the new synthesis and transport of nuclear-encoded components (Pikó and Taylor 1987). Therefore, the early embryonic lethality of mice lacking either Nrf-1 or Gabpα may be due to decreased expression of nuclear-encoded genes of mitochondrial function that are transcriptionally regulated by these two factors, such as *MTFA*. Complete loss of MTFA protein expression in the mouse results in depletion of mitochondrial DNA and death prior to E10.5 (Larsson et al. 1998). Therefore, the earlier death of mice null for Gabpα protein expression is presumed to be due to a simultaneous decrease in *MTFA* expression and that of other Gabp target genes.

Gabpα heterozygous mice are of normal body and organ weight, have normal numbers of cells of immune function, and histology of all tissues of *Gabpα* heterozygous mice appears normal. Gabpα protein levels in tissues of *Gabpα* heterozygous mice are not significantly different from those of wildtype mice, consistent with a tight post-transcriptional means of regulation of *Gabpα* expression. Given the essential role of Gabpα in embryogenesis, and the number of mitochondrial and other housekeeping genes that are transcriptional targets of the Gabp complex, it is not surprising that tight regulatory measures are in place to maintain steady Gabpα protein levels.

8.5 Gabpα *is not Essential for Development of Skeletal Muscle Tissue*

The role of Gabpα in skeletal muscle development and function was examined by the generation and analysis of mice lacking Gabpα protein specifically in skeletal muscle cells (see **Chapter 6**). Genotyping of *Gabpα* using DNA extracted from different tissues confirmed that *Gabpα* was deleted in skeletal muscle tissues only. However, the presence of blood vessels and connective tissue within skeletal muscle tissues meant that residual Gabpα protein was detected upon Western blot analysis of muscle lysates from *Gabpα* conditional knockout mice, as Gabpα protein is known to be expressed in smooth muscle, cartilage and fibroblast cells. A 65-75 % in Gabpα protein levels is observed in lateral gastrocnemius, medial gastrocnemius and soleus calf muscles, as well as vastus lateralis quadricep muscle of *Gabpα* conditional knockout mice. However, it is presumed that less efficient Cre-mediated excision of *Gabpα* or the additional smooth muscle present in diaphragm is responsible for the lesser reduction (40 %) in Gabpα protein levels observed in this tissue.

Loss of Gabpα protein expression was confirmed in ~50 % of lysates from primary skeletal muscle cell cultures from conditional knockout mice, validating generation of a skeletal muscle cell-specific *Gabpα* knockout mouse model. The variability in Gabpα expression levels between primary skeletal muscle cell cultures could be due in part to the presence of contaminating fibroblast cells within individual cultures, or may simply reflect heterogeneous Cre expression within one multinucleated muscle fibre or between individual fibres. Further analysis of Cre splicing efficiency is required by Southern blot analysis of spliced DNA and immunohistochemical analysis of Cre and Gabpα protein

expression patterns in tissue sections and single fibres of the different skeletal muscle tissues.

Gabpα protein expression levels remain unchanged in the C2C12 mouse skeletal muscle cell line pre- and post-differentiation, suggesting that increased Gabpα expression is not necessary for the increased level of transcription of Gabp target genes *Retinoblastoma protein* (*Rb*) (Okuyama et al. 1996), *Utrophin* (Gramolini and Jasmin 1999) or *AChRδ* (Buonanno and Merlie 1986) during differentiation of myoblasts into myotubes. Post-translational regulatory mechanisms such as phosphorylation may result in increased Gabp complex activity and subsequent transcriptional activation of target genes (Fromm and Burden 2001). However, any role of Gabpα during myogenesis may be compensated for by other co-expressed ETS proteins when Gabpα protein expression is lost. The expected number of homozygous *Gabpα* skeletal muscle-specific knockout mice are born from matings of heterozygous mice. In addition, conditional knockout *Gabpα* mice show no difference in body weight, weight of skeletal muscle tissues, or cellular morphology of skeletal muscle when compared to wildtype littermates.

It is possible that some redundancy exists for ETS transcription factor functions. As highlighted by the exacerbation of phenotype observed when *Pu.1* heterozygous and *Spi-B* knockout mouse models are inter-crossed (Garrett-Sinha et al. 2001), ETS transcription factors of overlapping expression pattern may regulate some of the same gene targets or genes that function in the same biological pathway, and one ETS protein may be capable of partially compensating for another in their absence. Some knockout mouse models of muscle disease do not show an overt phenotype due to the complementing role of another closely related protein resulting in complete or partial functional redundancy. For example, mice lacking the structural protein Utrophin are healthy and show no signs of muscle weakness (Deconinck et al. 1997a), and expression of Utrophin at the sarcolemma as well as at the NMJ (Jasmin et al. 1995), together with activation of muscle regeneration processes are able to successfully compensate for lack of Dystrophin in the mouse, allowing for a lifespan of up to 2 years (Deconinck et al. 1997b). However, mice lacking both Utrophin and Dystrophin proteins suffer from severe progressive muscular dystrophy resulting in premature death by 5 months of age, similar to humans suffering from Duchenne Muscular Dystrophy due to a lack of Dystrophin expression, who die in their early twenties due to respiratory or cardiac failure (Deconinck et al. 1997b). Hence, the

redundancy of ETS protein function in skeletal muscle could be investigated by breeding *Gabpα* conditional knockout mice with other ETS knockout mouse models.

Genetic complementation by highly related genes of a transcription factor family is possible even if protein products do not show complete functional redundancy when knockout mouse models are studied. For example, knock-in mouse models have demonstrated that JunB can compensate for loss of Jun function during liver and heart development, even though the two subunits of the AP1 transcription factor complex differ considerably in their ability to transactivate target genes (Passengué et al. 2002), and Fra-1 can compensate for loss of c-Fos expression during bone development but not during photoreceptor apoptosis (Fleischmann et al. 2000). In both cases, the ability of the homologous family member to compensate for loss of the other is dependent upon overlapping expression, ability to interact with the same proteins, and recognise the same binding site. Creation of knock-in mouse models where the *Gabpα* gene is replaced by that of another related ETS protein should determine if compensation for Gabpα function is possible. However redundancy of Gabpα function is unlikely, as gel shift analysis has previously shown that Gabpβ cannot interact with the highly related Ets-1, ER71 and ER81 ETS proteins (Brown and McKnight 1992), and Gabpβ is likely to be necessary to form interactions with other transcription factors binding to promoters of Gabp target genes.

8.6 Gabpα *Maintains Cellular Oxidative State in Skeletal Muscle*

The importance of Gabpα in co-ordinating the regulation of nuclear and mitochondrial genomes is highlighted by the fact that the expression level of the Gabp target gene *MTFA* is significantly reduced in the diaphragm of *Gabpα* skeletal muscle-specific knockout mice (see **Chapter 7**). A similar trend was observed in soleus, and future analysis of glycolytic skeletal muscle tissues such as gastrocnemius will allow us to see if all muscles are affected in a similar manner.

The effect of loss of Gabpα expression upon the protein expression levels of two Gabp targets of mitochondrial function, Cytochrome-c-oxidase subunits COXIV and Vb, was assessed. Unexpectedly, a significant increase in COXVb protein levels was observed in the soleus muscle of *Gabpα* conditional knockout mice. This suggests that another transcriptional regulator of nuclear-encoded mitochondrial proteins, such as Nrf-1, may be

compensating for loss of Gabpα expression, as binding sites for both Nrf-1 and Gabpα have been identified in the *COXVb* promoter, and promoters of many other *Cytochrome-c-oxidase* genes (Carter et al. 1992; Lenka et al. 1998). Mutations in the three genes of mitochondrial-encoded subunits of Cytochrome-c-oxidase have been linked with cases of human mitochondrial disease, yet no mutations have been identified in genes of the enzyme's ten nuclear-encoded subunits (Barrientos et al. 2002). This suggests that strict protective mechanisms are in place to ensure stable expression levels of nuclear-encoded proteins, and that lack of these subunits may not be compatible with cell survival. In addition, protein levels of COXVb vary between tissues in the rat, and increased COXVb levels correlate with increased Cytochrome-c-oxidase activity in the heart and kidney, as compared to the liver (Lenka et al. 1998; Vijayasarathy et al. 1998). Therefore, increased COXVb expression resulting from compensation of loss of Gabpα expression is probably most evident in the soleus, as this muscle utilises more oxygen than muscle tissues of lesser type I fibre content.

Analysis of the fibre type composition of skeletal muscle tissues showed a significant reduction in the proportion of oxidative type I fibres in the medial gastrocnemius of ubiquitous *Gabpα* heterozygous mice, compared to wildtype littermates. This is consistent with a decrease in the activity or number of mitochondria in skeletal muscle with a loss of Gabpα protein expression. The medial gastrocnemius is the only muscle affected and features a low proportion of oxidative fibres. This suggests that there may be slight loss of type I fibres in *Gabpα* heterozygous mice, and that muscles containing a high proportion of type I fibres (such as soleus) are resistant to any impairment of mitochondrial function. The effect of mutations resulting in a partial loss of mitochondrial function is thought to be more severe in tissues in which the concentration of the affected product is most limiting (Barrientos et al. 2002). Hence, the fact that the medial gastrocnemius is the only muscle significantly affected in ubiquitous *Gabpα* heterozygous mice correlates with the significant reduction in Gabpα protein levels in this muscle when one allele of *Gabpα* is lost, and the low proportion of type I oxidative fibres normally found in this tissue.

In *Gabpα* conditional knockout mice, the proportion of type I oxidative (slow) fibres is significantly reduced in lateral gastrocnemius and vastus lateralis at 3 months of age. A trend towards reduced type I fibre proportions in conditional knockout mice is also seen in

lateral and medial gastrocnemius at 1 month, medial gastrocnemius at 3 months, and vastus lateralis at 6 months of age. It would therefore be interesting to study 3 month old mice heterozygous for ubiquitous Gabpα protein to see if any differences in fibre type proportions in lateral gastrocnemius and vastus lateralis become apparent. Analysis of a greater number of mice will also ensure that fibre type proportions are not changed at 1 and 6 months of age. However, the preferential atrophy of type IIb fibres of skeletal muscle with age (Musarò et al. 1995) may have obscured the correct classification of fibres in tissue sections from 6 month old mice, so it might be better in future analyses to use myosin isoform-specific antibodies to confirm fibre type. It should then also be possible to conclude whether the decrease in proportion of type I fibres coincides with an increase in proportion of type IIa or type IIb fibres, or the rarer type IIc and IIx fibres. Future studies of mitochondrial function in *Gabpα* conditional knockout mice will include analysis of enzyme activities of Cytochrome-c-oxidase and Succinate dehydrogenase, another transcriptional target of the Gabp complex, to confirm if a muscle-specific phenotype is apparent in tissues of *Gabpα* conditional knockout mice. Comparison of the expression level of the nearby *Mitochondrial ATP Synthase Coupling Factor 6* gene in wildtype and *Gabpα* conditional knockout mice will also ensure that changes in mitochondrial function are due only to loss of Gabpα expression.

From this analysis, it appears that different skeletal muscle tissues respond to the loss of Gabpα expression in different ways, dependent upon innate properties of each muscle. A significant reduction in the proportion of oxidative fibres may be apparent in predominantly glycolytic muscles, such as the gastrocnemius, because loss of type I fibres does not effect the normal function of these muscles. Whereas, oxidative muscles, such as the soleus, may show increased COXVb expression and no apparent loss of type I fibres due to the compensatory effects of other transcription factors upon expression levels of Gabp target genes essential for oxidative cellular respiration. Further analysis of skeletal muscle tissues lacking Gabpα protein for expression levels of Nrf-1 and other ETS proteins will help establish whether the effects of loss of Gabpα in skeletal muscle are partially masked by upregulation of transcription factors that can functionally compensate for its loss.

8.7 Gabpα *is Necessary for Healthy NMJ Formation*

The role of Gabpα in the formation of functional NMJs was also assessed in mouse models of *Gabpα* deficiency (see **Chapter 7**). Expression of the Gabp target gene *AChRε* is significantly reduced in the diaphragm of skeletal muscle-specific *Gabpα* knockout mice, however no change in *AChRε* expression is apparent in soleus. A trend toward reduced mRNA levels of *AChRδ* is observed in both tissues, and no change in *Utrophin* expression levels is apparent. This indicates that other transcription factors, including other ETS proteins, expressed in skeletal muscle cells are able to compensate for loss of Gabpα expression to some degree. The fact that significantly reduced levels of *Utrophin* (as well as *AChRε* and *Acetylcholine esterase*) were observed in vastus lateralis of mice expressing a dominant negative ETS protein in skeletal muscle (de Kerchove d'Exaerde et al. 2002) suggests that it is other ETS transcription factors that maintain stable levels of *Utrophin* transcription in the absence of Gabpα. Alternatively, some Gabp gene targets are more dependent upon Gabpα expression in particular skeletal muscle tissues. Levels of *Utrophin* mRNA are significantly higher in the slow soleus muscle than the fast extensor digitorum longus, due to an increased stability of the transcript (Gramolini et al. 2001b). Therefore, perhaps a decrease in transcription of *Utrophin* is easier to detect in muscles consisting of mostly glycolytic fibres than oxidative muscles such as diaphragm and soleus. Further analysis of different muscle tissues, from mice of different ages, is necessary before a complete understanding of the consequences of loss of the Gabpα protein upon target gene expression can be gained. To decrease the variability observed between samples, future studies may also use RNA isolated from the synaptic region of skeletal muscle tissues, to increase the relative abundance and NMJ-specific transcripts in the cDNA analysed. Breeding the conditional knockout mice onto a pure genetic background should also decrease variation in mRNA expression levels between animals.

The decrease in *AChR* expression has no effect upon AChR distribution in muscle tissues of *Gabpα* skeletal muscle-specific knockout mice and mice ubiquitously heterozygous for Gabpα expression. This indicates that Gabpα is not essential for synapse-specific expression of *AChRδ* and *ε*. Given that previous studies of site-specific mutation have identified the N box (ETS site) as the element critical for synapse-specific expression of both *AChRδ* (Koike et al. 1995; Fromm and Burden 1998a) and *ε* (Duclert et al. 1996), this suggests that other ETS transcription factors expressed in skeletal muscle

cells are capable of transactivating these genes in the absence of Gabpα. A likely candidate would be Ets-2, as this ETS protein is ubiquitously expressed, and possesses a DNA binding domain of high amino acid identity with that of Gabpα. Therefore, it would be interesting to study the expression levels of other ETS proteins, such as Ets-2, at the NMJs of skeletal muscle of *Gabpα* conditional knockout mice. Partial functional redundancy of ETS transcription factors expressed within skeletal muscle may act as a protective mechanism against neuromuscular disease, and can only be tested by generating mice deficient for multiple ETS proteins.

Although no difference in the distribution of AChRs was seen in *Gabpα* conditional knockout or ubiquitous *Gabpα* heterozygous mice, the number of AChR clusters is significantly reduced in medial gastrocnemius of 1 month old mice ubiquitously heterozygous for Gabpα protein expression, with a similar trend seen in vastus lateralis. A significant reduction in AChR cluster number is also apparent in lateral gastrocnemius of heterozygous and homozygous *Gabpα* skeletal muscle-specific knockout mice at 1 and 3 months of age, with a similar trend seen in medial gastrocnemius at 6 months of age. Therefore, in both instances, fast glycolytic muscles appear to be more affected than slow oxidative muscles.

The study of a greater number of animals or use of a more quantitative technique for determining AChR number, such as labelling AChRs with radioactivity, may show a more considerable difference in AChR number in muscles of wildtype and *Gabpα* deficient mice, as a high variance in AChR cluster number was observed in both *Gabpα* conditional knockout and ubiquitous *Gabpα* heterozygous mice. However, the medial gastrocnemius shows both a reduction in AChR cluster number and a reduction in the proportion of type I oxidative fibres in ubiquitous *Gabpα* heterozygous mice, whereas the lateral gastrocnemius muscle is affected in the *Gabpα* conditional knockout. This suggests that there could be an underlying explanation for the differences seen between the two model systems, such as variable Cre splicing efficiency of *Gabpα* in skeletal muscle tissues of conditional knockout mice.

The age-related reduction in the number of AChR clusters observed specifically in the lateral gastrocnemius of wildtype 6 month old mice is consistent with data from the rat, where a decrease in NMJ density has been observed with age in bicep and gastrocnemius muscles (Ma et al. 2002). However, no change in the number of AChR clusters is seen in

lateral gastrocnemius of aging *Gabpα* conditional knockout mice. It is possible that a lower turnover rate of AChRs partially compensates for loss of Gabpα protein. Future studies will compare the AChR turnover rate in skeletal muscle tissues from wildtype, *Gabpα* conditional knockout and ubiquitously heterozygous mice to test this hypothesis.

We examined skeletal muscle of *Gabpα* conditional knockout mice by confocal microscopy, to examine whether a decrease in AChR expression results in altered NMJ morphology. In agreement with the results of phenotypic analysis of mice expressing an ETS dominant negative protein in skeletal muscle, approximately 30 % of NMJs of soleus, sternomastoid and diaphragm muscles from 3 month old *Gabpα* skeletal muscle-specific knockout mice show a single ring structure, with the remaining 70 % showing less branching than those of homozygous floxed *Gabpα* controls (de Kerchove d'Exaerde et al. 2002). Additional mice need to be studied in order to quantify the area occupied by the AChRs within the NMJ of individual muscle fibres, however, visual inspection of both soleus and sternomastoid muscle fibres suggests a reduction in area occupied by AChRs in *Gabpα* skeletal muscle-specific knockout mice. Such a reduction was similarly observed in NMJs of mice expressing the dominant negative ETS protein in skeletal muscle (de Kerchove d'Exaerde et al. 2002). This suggests that a reduced number of functional AChR complexes are formed at the NMJs of *Gabpα* conditional knockout mice and correlates with the observed reduction in *AChRδ* and *ε* expression levels in diaphragm and soleus muscles. Future studies aim to investigate whether the two morphological classes of NMJs observed in *Gabpα* conditional knockout mice correlate with differences in Gabpα and Cre protein expression levels between muscle fibres.

Despite the apparent morphological changes in NMJs of muscles from *Gabpα* skeletal muscle-specific knockout mice, electron microscopy revealed no significant alterations in structure of postsynaptic junctional folds in diaphragm and soleus muscles, when compared to wildtype littermates. Degeneration of junctional folds is often observed in muscle biopsies from individuals suffering from Congenital Myasthenic Syndrome (Engel et al. 1997). However, as *AChRε* mRNA expression is still detectable in diaphragm and soleus muscles of *Gabpα* conditional knockout mice, it is not surprising that the postsynaptic membrane remains intact. Furthermore, skeletal muscle from mice deficient for the structural proteins Utrophin (Grady et al. 1997) or Rapsyn (Gautam et al. 1995) shows no alteration in the ultrastructure of postsynaptic folds.

Taken together, these results suggest that Gabpα expression is necessary for formation of healthy AChR clusters at NMJs in skeletal muscle. A decrease in AChR density has been observed in mice lacking the Utrophin protein (Grady et al. 1997), however this does not result in an alteration in NMJ shape or size. In agreement with real-time RT-PCR analysis, this indicates that it is the decreased expression of AChR subunits, rather than structural proteins such as Utrophin, that causes altered NMJ morphology in *Gabpα* skeletal muscle-specific knockout mice. Complete loss of AChRε expression in mice results in severe impairment of neuromuscular transmission, due to maintenance of expression of the foetal AChRγ subunit, progressive muscle weakness and death at 2-3 months of age (Witzemann et al. 1996). However, *Gabpα* conditional knockout mice do not show signs of overt muscle weakness and have a normal lifespan. Therefore, whether decreased *AChR* expression and altered NMJ shape in skeletal muscle of *Gabpα* conditional knockout mice affects skeletal muscle function was assessed by biophysical measurements.

8.8 Loss of Gabpα *Expression Alters Skeletal Muscle Function*

The grip strength of heterozygous and homozygous *Gabpα* skeletal muscle-specific knockout mice is maintained with age, while that of wildtype littermates deteriorates with age (see **Chapter 7**). It is not surprising that decreased *MTFA* expression in *Gabpα* skeletal muscle-specific knockout mice does not correlate with decreased grip strength, as complete loss of MTFA expression in mouse skeletal muscle does not result in premature fatigue of EDL or soleus muscles, as determined by generation of tetanic contractions (Wredenberg et al. 2002). The maintenance of grip strength seen in aging conditional *Gabpα* knockout mice may be due to the altered fibre type ratio of skeletal muscle tissues. An increase in the proportion of glycolytic fibres may enhance performance in such a brief test of muscle strength, but could diminish the ability of conditional *Gabpα* knockout mice to sustain muscle activity over a longer period of time. The measurement time (from 1-6 minutes) of static maximal grip strength in human subjects has been reported to greatly affect the results obtained, and 1 minute was not sufficient to reflect steady state grip strength (Yamaji et al. 2002). Therefore, a longer gripping test time may reveal differences due to a decrease in type I fibres in conditional *Gabpα* knockout mice. Other tests such as the rotor rod or running wheel may help determine whether a decrease in type

I oxidative fibres and AChR clusters results in reduced endurance strength of skeletal muscles lacking Gabpα protein.

Analysis of the biophysical properties of NMJs in soleus muscles of wildtype and *Gabpα* conditional knockout mice revealed that the amplitude of spontaneous endplate currents is significantly reduced in *Gabpα* deficient mice, indicating that the number of AChRs is decreased. A similar trend was also observed for evoked endplate currents, however analysis of a larger group of animals will be necessary to counteract the high degree of variability of response seen between NMJs of the same animal. No change in rise time or decay time of currents was observed, suggesting that mechanisms of transmitter release and channel opening and closing are unaffected by loss of Gabpα expression. Upregulation of AChRγ expression is a compensatory mechanism for loss of AChRε expression, and has been observed in mice lacking AChRε (Witzemann et al. 1996) and some humans suffering from Congenital Myasthenic Syndrome (Engel et al. 1996), resulting in an increase in channel opening time (and current decay time). Therefore the unaltered AChR channel opening and closing times of *Gabpα* skeletal muscle-specific knockout mice, together with the balanced gait of both conditional knockout and ubiquitously heterozygous *Gabpα* mice, suggests that enough AChRε protein is made in the absence of Gabpα expression to allow for overtly normal skeletal muscle function.

Gabpα skeletal muscle-specific knockout mice exhibit no obvious impairment of skeletal muscle function, probably due to the combination of compensatory mechanisms of Nrf-1 and other ETS transcription factors. In addition, the higher safety factor of neuromuscular transmission in mice compared to humans (Le Treut et al. 1990) makes humans more susceptible to pathological changes resulting in myasthenic syndromes than other species (Liyanage et al. 2002). In total, loss of Gabpα expression results in a decreased oxidative capacity of skeletal muscle tissues, a reduced number of AChR clusters, irregular NMJ morphology and decreased endplate current amplitudes. This indicates that the Gabp complex plays an important role in the co-ordinate regulation of mitochondrial and nuclear genomes and in the development of functional NMJs within skeletal muscle fibres. Disruption of GABPα expression could, therefore, be an underlying cause of a subset of mitochondrial diseases and/or CMS.

8.9 *Implications for the Role of* GABP *in Down Syndrome*

Increased levels of Gabpα protein could only be detected in brain and skeletal muscle of Ts65Dn partial trisomy 16 mice, suggesting that post-transcriptional regulatory mechanisms are unable to buffer the effect of increased *Gabpα* gene dosage in these tissues. GABP is known to regulate the transcription of genes necessary for mitochondrial respiration (Scarpulla 2002b) and neuromuscular signalling (Schaeffer et al. 2001), and may also regulate the expression of neuronal nicotinic acetylcholine receptors in the brain (McDonough et al. 2000). Taken together with the tissue-specific overexpression of Gabpα in Ts65Dn mice, this implicates the GABP complex in the causation of skeletal muscle hypotonia, premature aging due to oxidative damage, and mental retardation due to defects in cholinergic neuron signalling in the brain of DS individuals.

One possible mechanism of muscular hypotonia is that increased expression of AChRδ and ε protein subunits in skeletal muscle as a result of increased GABP-mediated transcriptional upregulation may lead to the formation of AChRs with an imbalance in subunit proportions, resulting in decreased AChR function. Similarly in brain, formation of receptor complexes of inappropriate stoichiometric composition may result in impaired function of cholinergic neurons. Oxidative damage in the brain may result from enhanced mitochondrial activity resulting from increased GABPα expression, causing the generation of an increased proportion of reactive oxygen species and premature aging. In addition, increased GABPα expression in DS may enable the GABP complex to compete with other ETS proteins to transactivate promoters of genes other than the normal spectrum of GABP targets, contributing to a number of conditions associated with DS.

However, the overlapping phenotypes of muscular hypotonia and mental retardation of monosomy 21 (Chettouh et al. 1995) and trisomy 21 (Jackson et al. 1976) suggests that overexpression and loss of expression of dosage sensitive genes can lead to the same outcome. Use of knockout and transgenic mouse models has shown similar defects of axial skeleton development resulting from deficiency and overexpression of Polycomb group protein Ring1A transcription factor (del Mar Lorente et al. 2000) or the Homeobox transcription factor Hox3.1 (Le Mouellic et al. 1992; Pollock et al. 1992). Therefore, as for GABP, a functional transcription factor complex requires a specific concentration of each subunit, so absence or overabundance of any given subunit results in a non-functional complex, and ultimately the same phenotype. The decreased expression of type I oxidative

fibres in skeletal muscle of *Gabpα* conditional knockout mice, taken together with increased proportion of fast type IIa fibres in the tongue of DS individuals (Yarom et al. 1986), suggests that GABP may be a dosage sensitive protein complex, and that overexpression and loss of expression of GABPα may lead to loss of transcription factor function. Hence, the muscular hypotonia observed in DS individuals may result from decreased transcriptional activation of GABP target genes that function at the NMJ and a reduced number of functional AChRs. Similarly, decreased expression of MTFA and subunits of Cytochrome-c-Oxidase, as a result of a decreased number of functional GABP complexes, may contribute to the premature aging and mental retardation characteristic of DS, as loss of function of more than one mitochondrial gene results in increased superoxide production (Schuchmann and Heinemann 2000).

If GABPα is the limiting component of the GABP complex, increased GABPα expression may enable formation of complexes with dominant negative isoforms of GABPβ1, resulting in loss of transactivation of GABP target genes. Alternatively spliced *Gabpβ1* transcripts that encode isoforms of proposed dominant negative function are present in high abundance in mouse brain and skeletal muscle relative to the major transcript. Therefore human tissues need to be assessed for the presence of dominant negative GABPβ1 isoforms. In addition, the presence of GABP binding sites within the *GABPα* promoter is suggestive of autoregulation. Site-specific mutation will allow determination of whether *GABPα* negatively regulates its own expression. Such studies have shown that autoregulation of the Brn3a POU-domain transcription factor results in suppression of the endogenous gene upon transgenic overexpression, and that Brn3a autoregulation can compensate for loss of one allele by increasing transcription from the remaining allele in heterozygous mice (Trieu et al. 2003). Finally, the pro-oxidant cellular conditions found in DS tissues may compound the inhibition of GABP complex function, by decreasing the ability of GABPα to bind to DNA and GABPβ (Chinenov et al. 1998).

Therefore, while *in vitro* overexpression of *Gabpα* did not result in increased Gabpα protein expression, results from Gabpα protein expression analysis of Ts65Dn mouse tissues suggest that there could be tissue-specific differences in the post-transcriptional regulation of Gabpα expression. Therefore, further analysis of *Gabpα* regulation and comparative analysis of mice deficient in Gabpα or overexpressing Gabpα protein should help determine the contribution of overexpression of GABPα to conditions apparent in DS.

Chapter 9

Concluding Remarks

The GABPα/β complex is an ETS transcription factor expressed in all tissues, but not all cell types. The alternative 5' and 3' UTRs of mouse *Gabpα* and novel alternative splice forms of *Gabpβ1* identified in this study are thought to help regulate the expression and activity of the Gabp complex, allowing Gabp to up-regulate transcription of target genes of diverse function. These include *AChRδ* and *ε*, *Utrophin* and *Acetylcholine esterase* at the NMJ of skeletal muscle, and nuclear-encoded proteins of mitochondrial function such as *COXIV*, *COXVb* and *MTFA*.

Mice carrying a homozygous deletion in the *Gabpα* locus die prior to implantation, indicating that Gabpα protein is essential for early embryonic development. However, deletion of *Gabpα* specifically in skeletal muscle cells demonstrates that Gabpα is not necessary for the development of functional skeletal muscle. *Gabpα* conditional knockout mice exhibit subtle alterations in NMJ morphology, and decreased amplitude of spontaneous endplate currents. This phenotype overlaps with that of *AChRε* knockout mice, further validating *AChRε* as a Gabp target gene. In addition, the tissue-specific decrease in the number of AChR clusters and proportion of oxidative type I muscle fibres observed in *Gabpα* conditional knockout mice suggests that innate properties of different skeletal muscle tissues determine their ability to compensate for loss of Gabpα expression.

Further characterisation of the *Gabpα* skeletal muscle-specific knockout mouse model, together with mice overexpressing the Gabpα protein, will aid in our understanding of how the deregulated expression of GABP target genes contributes to the phenotypes of severe muscle weakness in Congenital Myasthenic Syndrome and muscular hypotonia, redox instability and mental retardation in Down syndrome. However, this preliminary analysis demonstrates that Gabpα is the ETS protein necessary for formation of intact AChR clusters at the NMJ.

Appendix A

Solutions

DNA Preparation and Methods

SM Buffer

100 mM	NaCl
8 mM	$MgSO_4.7H_2O$
50 mM	Tris-HCl (pH 7.5)
0.1 %	Gelatin (w/v)

- dissolved in MQ H_2O and autoclaved.

λ Diluent

10 mM	Tris-HCl (pH 7.5)
10 mM	$MgSO_4$

- dissolved in MQ H_2O and autoclaved.

TE

10 mM	Tris-HCl (pH 8.0)
1 mM	Ethylene diamine tetra-acetic acid (EDTA) (pH 8.0)

- dissolved in MQ H_2O and autoclaved.

SOC Medium

20 mg/ml	Bacto-tryptone
5 mg/ml	Bacto-yeast extract
10 mM	NaCl
10 mM	KCl
20 mM	Mg^{2+} (10 mM $MgCl_2.6H_2O$, 10 mM $MgCl_2.7H_2O$), filtered
20 mM	Glucose, filtered

- tryptone, yeast, NaCl and KCl added to 97 ml MQ H_2O and autoclaved;
- Mg^{2+} and glucose then added;
- passed through 0.2 µm filter (adjusted to pH 7.0).

Luria Bertani (LB) Broth

10 mg/ml	Bacto tryptone
5 mg/ml	Bacto yeast extract
10 mg/ml	NaCl

- dissolved in MQ H_2O;
- pH adjusted to 7.5 with NaOH, then autoclaved.

LB bottom agar

15 mg/ml agar in LB broth, autoclaved.

LB top agarose

7 mg/ml agarose in LB broth, autoclaved.

LB/amp Plates

100 µg/ml Ampicillin sodium (CSL Ltd.) in LB/agar

LB/amp/X-Gal/IPTG Plates

100 µg/ml	Ampicillin sodium (CSL Ltd.)
100 µg/ml	5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (Progen)
0.1 mM	Isopropyl-beta-D-thiogalacto-pyranoside (IPTG) (Promega)

- Dissolved in LB/agar.

LB/kan Plates

30 µg/ml Kanamycin sulphate (Roche) in LB/agar

NZY Broth

86 mM	NaCl
8 mM	$MgSO_4.7H_2O$
5 mg/ml	Bacto yeast extract
1g/ml	NZ amine (casein hydrolysate)

- dissolved in MQ H_2O and autoclaved.

Terrific Broth (TB)

12 mg/ml	Bacto tryptone
24 mg/ml	Bacto yeast extract
4 %	Glycerol (v/v)

- dissolved in MQ H_2O and autoclaved;
- 10 % (v/v) TB salts added prior to use.

TB Salts

0.17 M	KH_2PO_4
0.72 M	K_2HPO_4

Glycerol Stocks

70 %	Bacterial culture (v/v)
30 %	Glycerol (v/v)

- stored at –80°C.

Three Solution Maxiprep/Miniprep

Solution I

25 mM	Tris-HCl (pH 8.0)
10 mM	EDTA (pH 8.0)
15 %	Sucrose (w/v)

- dissolved in MQ H_2O and autoclaved.

Solution II

1 %	Sodium dodecyl sulfate (SDS) (w/v)
0.2 M	NaOH

- dissolved in MQ H_2O (made fresh).

Solution III

3 M	Potassium acetate
11.5 %	Glacial acetic acid (v/v)

- dissolved in MQ H_2O.

CsCl Maxiprep

Solution I

25 mM	Tris-HCl (pH 8.0)
10 mM	EDTA (pH 8.0)
50 mM	Glucose

- dissolved in MQ H_2O and autoclaved.

Solution II

1 %	SDS (w/v)
0.2 M	NaOH

- dissolved in MQ H_2O (made fresh).

Solution III

As per Three Solution method.

Ear Clip Lysis Buffer

10 mM	Tris-HCl (pH 8.3)
50 mM	NaCl
0.2 %	Tween 20 (v/v) (Sigma)
10 µg/ml	Proteinase K (Roche)

- dissolved in MQ H_2O;
- Proteinase K added immediately prior to use.

Tail Buffer

1 %	SDS (w/v)
0.1 M	NaCl
0.1 M	EDTA (pH 8.0)
0.05 M	Tris-HCl (pH 8.0)

- dissolved in MQ H_2O.

6 X DNA Loading Dye

40 %	Sucrose (w/v)
0.25 %	Bromophenol blue (Sigma) (w/v)
0.25 %	Xylene cyanol FF (Sigma) (w/v)

Oligonucleotide Hybridisation Mix

5 X	SSC
5 X	Denhardt's
1 %	SDS (w/v)

- dissolved in MQ H_2O and stored at –20°C.

Dextran Sulfate Hybridisation Mix

10 %	Dextran Sulfate (w/v) (Progen)
1 %	SDS (w/v)
1 M	NaCl

- dissolved in MQ H_2O and stored at –20°C.

20 X Standard Saline Citrate (SSC)

3 M	NaCl
0.3 M	tri-Sodium citrate

- dissolved in DEPC-treated MQ H_2O;
- adjusted to pH 7.2, then autoclaved.

RNA Preparation and Methods

DEPC-treated MQ H_2O

0.1 % (v/v) Diethyl pyrocarbonate (DEPC) (Sigma) added to MQ H_2O, left O/N in fume hood, then autoclaved.

Extraction Buffer

0.5 %	SDS (w/v)
0.1 M	NaCl
20 mM	Tris-HCl (pH 8.0)
1 mM	EDTA (pH 8.0)

- dissolved in DEPC-treated MQ H_2O and autoclaved (SDS added after autoclaving).

Binding Buffer

0.1 %	SDS (w/v)
0.5 M	NaCl
10 mM	Tris-HCl (pH 8.0)
1 mM	EDTA (pH 8.0)

- dissolved in DEPC-treated MQ H_2O and autoclaved (SDS added after autoclaving).

Wash Buffer

0.1 %	SDS (w/v)
0.15 M	NaCl
10 mM	Tris-HCl (pH 8.0)
1 mM	EDTA (pH 8.0)

- dissolved in DEPC-treated MQ H_2O and autoclaved (SDS added after autoclaving).

Elution Buffer

0.1 %	SDS (w/v)
10 mM	Tris-HCl (pH 8.0)
1 mM	EDTA (pH 8.0)

- dissolved in DEPC-treated MQ H_2O and autoclaved (SDS added after autoclaving).

Solution D

6 mM	Guanidinium thiocyanate (Fluke)
38 mM	Sodium citrate (pH 7.0)
0.8 %	Sarkosyl (w/v) (Sigma)
3 M	β-mercaptoethanol (Sigma)

- β-mercaptoethanol added immediately prior to use.

Deionised Formamide

Formamide stirred overnight at R/T with amberlite MB-1 monobed mixed resin (BDH), then filtered through 135 gsm blotting paper and stored at 4°C.

RNA Resuspension Buffer

50 %	Deionised formamide (v/v)
6 %	Formaldehyde (v/v)
3 %	Glycerol (v/v)

- dissolved in 1 x MOPS and stored at –20°C.

RNA Loading Dye

0.2 %	Bromophenol blue (w/v) (Sigma)
0.2 %	Xylene cyanol FF (w/v) (Sigma)
20 %	Ficoll type 400 (w/v)

- dissolved in MQ H_2O and stored at –20°C.

10X MOPS

0.2 M	4-morpholine-propane sulfonic acid (MOPS) (Roche)
10 mM	EDTA (pH 8.0)
50 mM	Sodium acetate (pH 5.2) (Sigma)

- dissolved in MQ H_2O;
- adjusted to pH 7.0 and 2-3 drops of DEPC added;
- stored at 4°C in foil.

Northern Pre-Hybridisation Mix

50 %	Deionised Formamide (v/v)
5 X	SSC
1 X	Denhardt's

- dissolved in MQ H_2O and stored at –20°C.

Northern Hybridisation Mix

50 %	Deionised Formamide (v/v)
10 %	SDS (w/v)
1 M	NaCl
10 %	Dextran sulfate (Progen) (w/v)

- dissolved in MQ H_2O and stored at –20°C.

100 X Denhardt's

2 %	BSA (w/v)
2 %	Ficoll 400 (w/v)
2 %	polyvinylpirollidone (w/v)

- dissolved in MQ and stored at –20°C.

Protein Preparation and Detection

2X EDTA Sample Buffer

125 mM	Tris-HCl (pH 6.8)
2 %	SDS (w/v)
20 %	Glycerol (v/v)
1.4 M	β-mercaptoethanol (Sigma)
0.25 %	Bromophenol blue (Sigma) (w/v)
2 mM	EDTA (pH 8.0)

- dissolved in MQ H_2O and stored at –20°C ;
- β-mercaptoethanol added immediately prior to use.

Coommassie Brilliant Blue Staining Solution

0.25 %	Coommassie Brilliant Blue R-250 (w/v) (Sigma)
50 %	Methanol (v/v)
10 %	Glacial acetic acid (v/v)

- dissolved in MQ H_2O.

Coommassie Brilliant Blue De-staining Solution

10 %	Methanol (v/v)
7.5 %	Glacial acetic acid (v/v)

- dissolved in MQ H_2O.

SDS–PAGE Running Buffer

25 mM	Tris-HCl (pH 8.0)
192 mM	Glycine
0.1 %	SDS (w/v)

- dissolved in MQ H_2O.

10X TBST

0.2 M	Tris-HCl (pH 8.0)
1.37 M	NaCl

- dissolved in MQ H_2O;
- adjusted to pH 7.6 and 5 ml Tween 20 added.

Semi-Dry Anode Buffer I

0.3 M	Tris-HCl (pH 10.4)
10 %	Methanol (v/v)

- dissolved in MQ H_2O;
- methanol added immediately prior to use.

Semi-Dry Anode Buffer II

25 mM	Tris-HCl (pH 10.4)
10 %	Methanol (v/v)

- dissolved in MQ H_2O;
- methanol added immediately prior to use.

Semi-Dry Cathode Buffer

25 mM	Tris-HCl (pH 9.4)
40 mM	Glycine
10 %	Methanol (v/v)

- dissolved in MQ H_2O;
- methanol added immediately prior to use.

Recombinant Protein Cell Lysis Buffer

6 M	Guanidine-HCl
20 mM	Tris-HCl (pH 8.0)
50 mM	NaCl

- dissolved in MQ H_2O.

Recombinant Protein Wash Buffer

8 M	Urea
20 mM	Tris-HCl (pH 8.0)
50 mM	NaCl

- dissolved in MQ H_2O.

Recombinant Protein Renaturing Buffer

20 mM	Tris-HCl (pH 8.0)
50 mM	NaCl
0.1 %	Nonidet P40 (NP40) (v/v)
0.1 %	Tween-20 (v/v) (Sigma)
0.1 %	Triton X-100 (v/v)

- dissolved in MQ H_2O.

Recombinant Protein Elution Buffer

20 mM	Tris-HCl (pH 8.0)
50 mM	NaCl
100 mM	Imidizole

- dissolved in MQ H_2O.

ELISA Carbonate Coating Buffer

15 mM	Na_2CO_3
35 mM	$NaHCO_3$
3 mM	NaN_3

- dissolved in MQ H_2O;
- adjusted to pH 9.6.

ABTS

1 mM	ABTS (2, 2' Azinobis-3-ethylbanzthiazoline sulfonic acid)
0.03 %	H_2O_2 (v/v)

- dissolved in Citrate Buffer (pH 4.2), immediately prior to use.

Tissue Culture

Dulbecco's Modification of Eagles Media (DMEM) with 25mM HEPES

1 L	DMEM + HEPES powder pack (Gibco™ Invitrogen Corporation)
44 mM	$NaHCO_3$
2 mM	NaOH

- dissolved in MQ H_2O and adjusted to pH 7.2;
- passed through a 0.2 µm filter and stored at 4°C.

Primary Skeletal Muscle Cell Undifferentiated Growth Media

84 %	DMEM with 25 mM HEPES (v/v) (Gibco™ Invitrogen Corporation)
10 %	Heat-inactivated horse serum (v/v) (JRH Biosciences)
5 %	Heat-inactivated fetal calf serum (v/v) (JRH Biosciences)
1 %	10000 U/ml Penicillin G sodium, 10000 µg/ml Streptomycin sulfate in 0.85% saline (v/v) (Gibco™ Invitrogen Corporation)

- stored at 4°C.

Primary Skeletal Muscle Cell Differentiated Growth Media

97 %	DMEM with 25 mM HEPES (v/v) (Gibco™ Invitrogen Corporation)
2 %	Heat-inactivated horse serum (v/v) (JRH Biosciences)
1 %	10000 U/ml Penicillin G sodium, 10000 µg/ml Streptomycin sulfate in 0.85% saline (v/v) (Gibco™ Invitrogen Corporation)

- stored at 4°C.

3T3 Growth Media

89 %	DMEM with 25 mM HEPES (v/v) (Gibco™ Invitrogen Corporation)
10 %	Heat-inactivated fetal calf serum (v/v) (JRH Biosciences)
1 %	10000 U/ml Penicillin G sodium, 10000 µg/ml Streptomycin sulfate in 0.85% saline (v/v) (Gibco™ Invitrogen Corporation)

- stored at 4°C.

C2C12 Undifferentiated Growth Media

78 %	DMEM with 25 mM HEPES (v/v) (Gibco™ Invitrogen Corporation)
20 %	Heat-inactivated fetal calf serum (v/v) (JRH Biosciences)
1 %	10000 U/ml Penicillin G sodium, 10000 µg/ml Streptomycin sulfate in 0.85% saline (v/v) (Gibco™ Invitrogen Corporation)
1 mM	MEM Sodium Pyruvate Solution (Gibco™ Invitrogen Corporation)

- stored at 4°C.

C2C12 Differentiation Growth Media

96 %	DMEM with 25 mM HEPES (v/v) (Gibco™ Invitrogen Corporation)
2 %	Heat-inactivated horse serum (v/v) (JRH Biosciences)
1 %	10000 U/ml Penicillin G sodium, 10000 µg/ml Streptomycin sulfate in 0.85% saline (v/v) (Gibco™ Invitrogen Corporation)
1 mM	MEM Sodium Pyruvate Solution (Gibco™ Invitrogen Corporation)

- stored at 4°C.

PC12 Growth Media

84 %	Roswell Park Memorial Institute (RPMI) media (v/v) (Gibco™ Invitrogen Corporation)
10 %	Heat-inactivated horse serum (v/v) (JRH Biosciences)
5 %	Heat-inactivated fetal calf serum (v/v) (JRH Biosciences)
1 %	10000 U/ml Penicillin G sodium, 10000 µg/ml Streptomycin sulfate in 0.85% saline (v/v) (Gibco™ Invitrogen Corporation)

- stored at 4°C.

J1 ES Cell Media

83 %	DMEM with 25 mM HEPES (v/v) (Gibco™ Invitrogen Corporation)
15 %	Heat-inactivated fetal calf serum (v/v) (JRH Biosciences)
0.5 %	10000 U/ml Penicillin G sodium, 10000 µg/ml Streptomycin sulfate in 0.85% saline (v/v) (Gibco™ Invitrogen Corporation)
0.1 mM	Non essential Amino Acids (Gibco™ Invitrogen Corporation)
0.1 mM	β-Mercaptoethanol (Gibco™ Invitrogen Corporation)
1000 U/ml	Leukemia inhibitory factor (LIF) (Chemicon)

- stored at 4°C.

Embryonic Feeder Cell Media (EFM)

89 %	DMEM without Hepes (v/v) (Gibco™ Invitrogen Corporation)
10 %	Heat-inactivated fetal calf serum (v/v) (JRH Biosciences)
1 %	10000 U/ml Penicillin G sodium, 10000 µg/ml Streptomycin sulfate in 0.85% saline (v/v) (Gibco™ Invitrogen Corporation)

- stored at 4°C.

Phosphate-Buffered Saline (PBS)

1.4 M	NaCl
27 mM	KCl
54 mM	Na2PO4
18 mM	KH2PO4

- dissolved in MQ H_2O;
- adjusted to pH 7.4, then autoclaved.

Hanks-HEPES

10 %	Hanks Buffer (v/v) (Gibco™ Invitrogen Corporation)
20 mM	HEPES (Gibco™ Invitrogen Corporation)
2.5 mM	NaOH

- dissolved in MQ H_2O and stored at 4°C.

0.25 % Trypsin-EDTA + HEPES

98 %	0.25 % Trypsin, 1 mM EDTA (v/v) (Gibco™ Invitrogen Corporation)
20 mM	HEPES (Gibco™ Invitrogen Corporation)

- stored at 4°C.

Electroporation Buffer

10 %	Hanks Buffer (v/v) (Gibco™ Invitrogen Corporation)
20 mM	Hepes (Gibco™ Invitrogen Corporation)
0.11 mM	β-Mercaptoethanol (Gibco™ Invitrogen Corporation)
1 mM	NaOH

- dissolved in MQ H_2O immediately prior to use.

2 X ES Cell Freezing Media

60 %	J1 media (v/v)
20 %	Heat-inactivated fetal calf serum (v/v) (JRH Biosciences)
20 %	Dimethylsulphoxide (v/v) (DMSO)

- stored at 4°C.

Freezing Media (general use)

90 %	Heat-inactivated fetal calf serum (v/v) (JRH Biosciences)
10 %	DMSO (v/v)

- stored at 4°C.

Cell Lysis Buffer

200 mM	NaCl
100 mM	Tris-HCl (pH 8.0)
5 mM	EDTA (pH 8.0)
0.2 %	SDS (w/v)
100 µg/ml	Proteinase K (Roche)

- dissolved in MQ H_2O and autoclaved;
- Proteinase K added immediately prior to use.

2 X β-Galactosidase Buffer

98 mM	β-Mercaptoethanol (Sigma)
2 mM	$MgCl_2$
110 mM	Na_2HPO_4
102 mM	NaH_2PO_4
4.4 mM	2-Nitrophenyl-β-D-galactopyranoside (ONPG)

- dissolved in MQ H_2O and stored at –20°C.

Histology

Acid Alcohol

0.5 % HCl (v/v) dissolved in 70 % ethanol (v/v).

X-Gal Rinse Buffer

5 mM	Ethylene Glycol-bis(beta-aminoethyl-ether)-N,N,N',N'-TetraAcetate(EGTA) (pH 7.4)
2 mM	$MgCl_2$
0.02 %	NP40 (v/v)
0.01 %	Deoxycholate (w/v)

- dissolved in PBS.

X-Gal Staining Buffer

5 mM	$K_3Fe(CN)_6$
5 mM	$K_4Fe(CN)_6$
5 mM	EGTA (pH 7.4)
2 mM	$MgCl_2$
0.02 %	Nonidet P-40 (NP40) (v/v)
0.01 %	Deoxycholate (w/v)
1.25 mg/ml	X-Gal

- dissolved in PBS immediately prior to use;

Nicotinamide adenine dinucleotide (NADH) Staining Solution

1 mg/ml	4-Nitro Blue tetrazolium chloride (NBT) (Sigma)
0.8 mg/ml	NADH (Sigma)

- dissolved in 0.2 M Tris-HCl (pH 7.4) immediately prior to use.

Phosphate-Tween-Magnesium (PTM) Buffer

2 mM	$MgCl_2$
0.1 %	Tween 20

- dissolved in PBS.

Tyrode Solution

123.4 mM	NaCl
4.7 mM	KCl
1.0 mM	$MgCl_2$
1.3 mM	NaH_2PO_4
16.3 mM	$NaHCO_3$
0.3 mM	$CaCl_2$
7.8 mM	D-glucose

- dissolved in MQ H_2O.

Appendix B

List of Suppliers

Amber Scientific	Selmont, WA, Australia
Ambion Inc.	Austin, TX, USA
Amersham Pharmacia Biotech	Buckinghamshire, UK
American Type Culture Collection (ATCC)	Manassas, VA, USA
BACPAC Resources	CA, USA
Berthold	Pforzheim, Germany
Bartelt Instruments	Heidelberg West, VIC, Australia
BDH Biochemicals	Poole, UK
Beckman Coulter	Fullerton, CA, USA
Becton Dickinson/Falcon	Lincoln Park, NJ, USA
BioRad Laboratories, Inc.	Hercules, CA, USA
Boehringer-Mannheim	Mannheim, Germany
Canberra Packard	Australia
Clontech	Palo Alto, CA, USA
Cole-Parmer	Illinios, USA
Crown Scientific	Burwood, VIC, Australia
Daido Sango Co. Ltd.	Tokyo, Japan
DAKO	Carpinteria, CA, USA
Dow Corning Australia Pty. Ltd.	North Ryde, NSW, Australia
Dynavac	Melbourne, VIC, Australia
Eberhard Faber Inc.	Lewisburg, TN, USA
Fluka	Buchs SG, Switzerland
Gibco™ Invitrogen Corporation	Paisley, UK
Grass Instruments (an Astro-Med Inc. corporation)	West Warwick, RI, USA
Heraus Instruments	Hanau, Germany
HiChem	Czech Republic
Hoefer (an Amersham Pharmacia Biotech corporation)	San Francisco, CA, USA
JRH Biosciences (a CSL corporation)	Lenexa, KS, USA
Kodak	Rochester, NY, USA

Leica Instruments	Nussloch, Germany
Menzel-Glaser	Braunschweig, Germany
Metal Mesh Pty. Ltd.	Moorabbin, VIC, Australia
Millipore Corporation	Bedford, MA, USA
Molecular Probes Inc.	Eugene, OR, USA
NEN® Life Science Products Inc.	Boston, MA, USA
New England Biolabs (NEB)	Beverly, MA, USA
Novagen Inc.	Milwaukee, WI, USA
NUNC	Naperville, IL, USA
PE Applied Biosystems	CA, USA
Pierce	Rockford, IL, USA
Progen Industries	Rockford, IL, USA
Promega	Madison, WI, USA
Qiagen	Chatsworth, CA, USA
Quantum Scientific	VIC, Australia
Ratek Instruments Pty. Ltd.	Boronia, VIC, Australia
Roche	Mannheim, Germany
Sakura Finetek USA Inc.	Torrance, CA, USA
Sanyo Australia Pty. Ltd.	Arndell Park, NSW, Victoria
Scientifix	Cheltenham VIC, Australia
Sigma-Aldrich Inc.	Saint Louis, MO, USA
Smith + Nephew	Clayton, VIC, Australia
Stratagene	La Jolla, CA, USA
Vector Laboratories	Burlingame, CA, USA
Zymed	South San Francisco, CA, USA

Appendix C

List of Equipment

Agarose gel electrophoresis:	Mini-sub Cell GT, BioRad
	GNA 200, Pharmacia Biotech
Autoclave:	TOMY ES-315, Quantum Scientific
Centrifuges:	Sigma 1-15 microfuge, Quantum Scientific
	J2-21 M/E, Beckman
	Biofuge stratos, Heraeus Instruments
	Spintron GT-175S swing-out rotor centrifuge
	TL-100 ultracentrifuge, Beckman
Cell incubator	HERA Cell, Heraeus Instruments
Convection oven	Sanyo
Cryostat	CM 3050, Leica Instruments
DNA sequencer	Model 373A, PE Applied Biosystems
Dry block heater	Xtron, Bartelt instruments
Electrical stimulator	SD48, Grass Instruments
Electrical stimulus isolator	SIU5, Grass Instruments
Electroporation apparatus	Gene Pulsar, BioRad
Film processor	X-OMAT 480 RA, Kodak
Flow cytometer	EPICS 752
Haemocytometer	Precicolor, HBG, Germany
Homogeniser	Ika Ultra Turrax T25 (Janke & Kinkel IKA®), Crown Scientific
Hybridisation ovens	Xtron HI 2002, Bartelt Instruments
Liquid scintillation counter	1900 TR Tri-Carb, Canberra Packard, Australia
Luminometer	Lumi Count™, Packard
Paraffin embedding centre	EG1160, Leica Instruments
Paraffin microtome	RM2135, Leica Instruments
Paraffin processor	TP1020, Leica Instruments
Protein gel caster	Mighty Small multiple gel caster SE200, Hoefer Scientific Instruments

Microcentrifuge	1-15 Sigma, Quantum Scientific
Microscopes :	DM IRB light microscope, Leica Instruments
	MPS60 on DMR light/fluorescence microscope, Leica Instruments
	TCS NT on DMRXE upright confocal microscope, Leica Instruments
	1010 transmission electron microscope, JEOL
Microplate reader	3550, BioRad
PCR machines	GeneAmp PCR System 2400 and 9600, Applied Biosystems
Phosphorimager	FLA-2000, Fujifilm
Power supplies:	Power Pac 300 or 3000, BioRad
	GPS 200/400 Power Pac, Pharmacia
Real-time PCR machine	Light Cycler, Roche
Rotating wheel	R.S.M.6, Ratek instruments
Scanner	N1220U, Canon
SDS-PAGE apparatus	AB MightySmall II SE250, Hoefer Scientific Instruments
Shaking incubator	Innova 4300, New Brunswick Scientific
Spectophotometer	Lambda Bio 20, Perkin Elmer
Tilting platform	Xtron SH-2004, Bartelt instruments
UV illuminator	Gel Doc 1000, BioRad
Ultracentrifuge	TL-100, Beckman
Ultrasonics homogeniser	Cole Parmer
Vacuum oven	V015/200 500 W, Dynavac
Waterbath	Ratek instruments
Western transfer apparatus	TE77 SemiPhor, Hoefer Scientific Instruments

Appendix D

Oligonucleotide Sequences

No.	Name	Position	Sequence	T^A
1	T7	pGEM-T:2984-3	5' TAATACGACTCACTATAGGG 3'	51°C
2	SP6	pGEM-T:124-143	5' ATTTAGGTGACACTATAGAA 3'	47°C
3	pGL-3f	V:4760-4779	5' CTAGCAAAATAGGCTGTCCC 3'	55°C
4	loxPf	-	5' ATATACTAGTATAACTTCGTATAGCATA 3'	65°C
6	AP-1	-	5' CCATCCTAATACGACTCACTATAGGGC 3'	75°C
51	PQE-5'	V:3371-3388	5' CCCGAAAAGTGCCACCTG 3'	53°C
52	Gabpa-5'	G:413-428	5' CGAATTCCGGAGCTGGACTG 3'	59°C
53	Gabpa-3'	G:25549-25568	5' GGTTAGAGCAAAGCTTGCTC 3'	55°C
58	GAPD-5'	cDNA:585-605	5' CTGCCACCCAGAAGACTGTGG 3'	63°C
59	GAPD-3'	cDNA:967-985	5' GTCATACCAGGAAATGAGC 3'	51°C
61	Gabpa 61r	G:4036-4055	5' GTCCCGTCGATCTCAATTTC 3'	55°C
87	Gabpb1-1 5'	cDNA:1150-1177	5' CAGCTGGATGAAGCCAAC 3'	51°C
88	Gabpb1-1 3'	cDNA:1401-1418	5' CTCATGGCTTAAGTCCCG 3'	51°C
102	Gabpa E3f	G:6254-6272	5' GCTGAATGTGTAAGCCAGG 3'	53°C
103	Gabpa E4f	G:9220-9237	5' GGACTGTACAGCTTAGTG 3'	49°C
114	Gabpb E1f	cDNA:49-69	5' GTCTGCCCTGACGGCTCCGAG 3'	67°C
117	Gabpa E2f	G:4064-4082	5' CAGAGTGCACAGAAGAAAGC 3'	55°C
118	Gabpa E3r	G:6236-6255	5' GCTGGGGTATAGGTTTGTTC 3'	55°C
119	Gabpa E4r	G:9180-9198	5' CAAACAAGCTTCGGTCTGG 3'	53°C
120	Gabpa E5r	G:11048-11065	5' CGTTTCCGCAGTCTTAAC 3'	49°C
121	Gabpa E6r	G:17429-17446	5' GTTGAGTGTGGTGAGGTC 3'	51°C
128	Gabpa INTRON1 2f	G:2745-2764	5' GCTGTGCTTTGCCAGTGTTG 3'	57°C
134	Gabpa INTRON1 3f	G:3316-3335	5' CTCCTGAGGTCCCTGCTTTG 3'	59°C
135	Gabpa E3-I2 4r	G:4484-4503	5' GACTAAGTGAGGACTATTGG 3'	53°C
140	Gabpa PROM 15f	S1:5019-5037	5' GACTCCCATTTCCGTTCGT 3'	53°C
151	Gabpa E7 FOR	G:21371-21388	5' GAGCAACAGATGAATGAG 3'	47°C
152	Gabpa E8 REV2	G:22034-22051	5' GTGAACTTCTGTCTTCTC 3'	47°C
153	Gabpa E8 FOR	G:22000-22017	5' GCAGCTAAAGTGCAACGG 3'	51°C
154	Gabpa E9 REV	G:22322-22339	5' GTCTCGAGCATCCTTGTC 3'	51°C
155	Gabpa E9 FOR	G:22316-22335	5' CTTACTGACAAGGATGCTCG 3'	55°C
160	Gabpa E10 REV	G:25372-25399	5' GACCAGACGGTTCAGTTCTG 3'	57°C
169	Gabpa KO Bcl-3'	G:4070-4089	5' CATCAACCTTTCTTCTGTGC 3'	53°C
170	Gabpa KO Bcl-5'	G:3747-3769	5' ATAACTTCGTATAATGTATGCTATACGAAGTT ATCCTGAACACCAACAAGTTCC 3'	55°C
177	Gabpa E5 FOR2	G:11068-11085	5' CGAGGTGGTCATCGATCC 3'	53°C
179	Gabpa E6 FOR2	G:17362-17378	5' GCTGGTCCACGGACCAAG 3'	55°C
180	Gabpa E7 REV2	G:21362-21379	5' CTGTTGCTCTTGGCTGGC 3'	53°C
187	Cre 5' (Myo tag)	cDNA:574-597	5' GCCTTTTGCGACCTGATGCCCAAGAAGAAGAG GAAGGTG 3'	67°C
188	Cre 3'	cDNA:1931-1950	5' GAACTAGTGGATCTCGAGCC 3'	57°C
189	Myo 3' (Cre tag)	cDNA:1595-1621	5' CTTCTTCTTGGGCATCAGGTCGCAAAAGGCTT GTTCCTG 3'	77°C
190	Myo 5'	cDNA:27-46	5' GTGATGACTCAGGCAGGAAG 3'	57°C
192	Gabpa F3 5'	G:3973-3996	5' CTTCCAGGACTGAACTTTTGAACG 3'	65°C
193	Gabpa B3 3'	G:4142-4165	5' AAAAACAAGCACACTGGCCTACTC 3'	65°C
194	Neo F8 5'	cDNA:1043-1063	5' TCTCCTGTCATCTCACCTTGC 3'	59°C
201	3' pEF-puro-BOS	V:1990-2009	5' GTCTCCCACGTGGGAGACCT 3'	61°C
206	Neo4 reverse	cDNA:572-593	5' CATTGGGTGGAAACATTCCAGG 3'	61°C
209	Gabpa prom-Age 3'	S1:8469-8490	5' CACCGGTCTCTTAGTCATGGTTGAAGAC 3'	55°C
213	Cre FORWARD	cDNA:626-647	5' TGCCTGCATTACCGGTCGATGC 3'	65°C
214	Cre REVERSE	cDNA:1020-1043	5' CCATGAGTGAACGAACCTGGTCG 3'	67°C
217	AChR delta 5'	cDNA:1021-1043	5' GGACGCCCAGCACCCATGTGCTG 3'	73°C
218	AChR delta 3'	cDNA:1808-1830	5' GTCCCAAGGTCACCTCTCTGGCC 3'	71°C
219	Utrophin 5'	cDNA:3014-3037	5' CCAGAGACTTACACTTGCTCGAGG 3'	69°C
220	Utrophin 3'	cDNA:3993-4016	5' CGGAGGACCTGGAGTTGCTTCTCC 3'	73°C
223	AChR epsilon 5'	cDNA:3-25	5' GACCTGAGGACACTGTCACCATC 3'	67°C
224	AChR epsilon 3'	cDNA:236-258	5' GCAAAGATGAACTCCACCTCCTC 3'	65°C
225	MTFA 5'	cDNA:351-373	5' CCTGTGGAGGGAGCTACCAGAAG 3'	69°C
226	MTFA 3'	cDNA:1039-1061	5' CACCACTGCATGCAGATGCATGG 3'	67°C
227	Gabpa E2r	G:4047-4073	5' GTGCACTCTGCTTTCTCAGTCCCGTCG 3'	81°C
229	Gabpa E5f	G:11179-11205	5' CGAGACCTCGGAGCAGGTGACGAGATG 3'	83°C
231	Gabpa E7f	G:21362-21388	5' GCCAGCCAAGAGCAACAGATGAATGAG 3'	77°C
232	Gabpa E9f	G:22316-22341	5' CTTACTGACAAGGATGCTCGAGACTG 3'	73°C
242	Gabpb1 5'	cDNA:244-264	5' GGAACTTCTCCACTTCATCTG 3'	57°C

243	Gabpb1-1 3'	cDNA:1177-1197	5' CAGCTGCTGTCGGTATTTCTG 3'	59°C
245	Gabpb1-2 3'	cDNA:1152-1171	5' GGATGGCTGCAGCAAAACAC 3'	57°C
246	Gabpa EXON1af2	G:1290-1309	5' GCTATGTAGATTAGACCGGC 3'	55°C
265	HSA-Cre 5'	cDNA:1808-1828	5' CCGGTCGATGCAACGAGTGAT 3'	61°C
266	HSA-Cre 3'	cDNA:2585-2605	5' ACCAGAGTCATCCTTAGCGCC 3'	61°C
267	ROSA26-lacZ 5'	cDNA:927-950	5' TCGTGCGGTGGTTGAACTGCACAC 3'	71°C
268	ROSA26-lacZ 3'	cDNA:1251-1274	5' CGATTCATTGGCACCATGCCGTGG 3'	71°C
270	pEGFP-1 polyA 3'	V:842-861	5' CTACAAATGTGGTATGGCTG 3'	53°C
273	Rapsyn 5'	cDNA:827-846	5' GCAGTGCCATGGAGTGTTGT 3'	57°C
274	Rapsyn 3'	cDNA:897-918	5' GGCAAAGCAGAGCAGACAGAGT 3'	63°C
277	pEGFP-1 reverse	V:142-163	5' CGTCGCCGTCCAGCTCGACCAG 3'	71°C
280	Beta actin 5'	cDNA:344-363	5' CCCTGTATGCCTCTGGTCGT 3'	59°C
281	Beta actin 3'	cDNA:428-446	5' ATGGCGTGAGGGAGAGCAT 3'	55°C

Oligonucleotide numbers, names, reference sequences and annealing temperatures (T^A) are shown. G= Total genomic sequence of *Gabpα* (see **Appendix E**), S1= segment 1 *Gabpα* genomic sequence (GI:27960443), *Gabpβ1-1* cDNA= GI:193384, *Gabpβ1-2* cDNA= GI:6753933, *Gapd* cDNA= GI:193423, *Cre* cDNA= *PGK-Cre* in pBS II KS, *Myogenin* cDNA= GI:200003, *Neo* cDNA= GI:1244765, *lacZ* cDNA= GI:41901, *HSA-Cre* cDNA= GI:12965137, *AChRδ* cDNA= GI:191609, *AChRε* cDNA= GI:2660742, *Utrophin* cDNA= GI:1934962, *MTFA* cDNA= GI:1575122, *Rapsyn* cDNA= GI:53804, *β-actin* cDNA= GI:49867, V= vector sequence. pEF-puro-BOS= elongation factor 1a (*EF-1a*) promoter in the pUC119 backbone (Mizushima and Nagata 1990) with a puromycin resistance gene, pGL-3 basic= GI:U47295, pEGFP-1= GI:1377908, pGEM-T sequence as per http://www.promega.com, pQE-30 sequence as per http://www.qiagen.com.

Appendix E

Gabpα Genomic Sequences

GI:27960443 Mus musculus ATP synthase coupling factor VI gene, partial cds; and GA-binding protein alpha-subunit (Gabpα) gene, exons 1-5.
Length 16236 bp

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[redacted] *ATP Synthase Coupling Factor 6* Exon
[redacted] *Gabpα* Exon
atg Start Codon
[redacted] Predicted promoter region
CpG island
[redacted] *Gabpα* Exon 1a
[redacted] *Gabpα* Exon 1b

GI:27960444 Mus musculus GA-binding protein alpha-subunit (Gabpα) gene, exon 6.
Length 1179 bp

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1141 tcctcttgtg agcttggcta gaactgaagt catgaaagt
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[redacted] *Gabpα* Exon

GI:27960445 Mus musculus GA-binding protein alpha-subunit (Gabpα) gene, exons 7 through 10.
Length 4922 bp

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 121 tcttcttgga gtcccagtct tatgagccac ccagtccgag ggtgatgacc aacaggttca
 181 gtttcaatag atagactaga tgtctgaagt gctggaaact gggatattga atatgtgaag
 241 gagttaacaa tggcatttgg tctgggaaga ccagatagat acttactgag gcttttgtag
 301 aagattcttt tcaggtctgt tagtcagggt tctctaagga acagagctaa cagaatgaac
 361 aagtctatgg ctgtagcaaa agtcttccat tgagagcagt cttttaaaga tgactaatca
 421 gtctcctagg acagctgata ttttaatttt aggagtatta tcgcactaag tttcagttgt
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4681
4741
4801
4861
4921
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Gabpα Exon

tga Stop Codon

Unpublished Mus musculus GA-binding protein alpha-subunit (Gabpα) genomic sequence, exons 1 through 10.
Length 26595 bp

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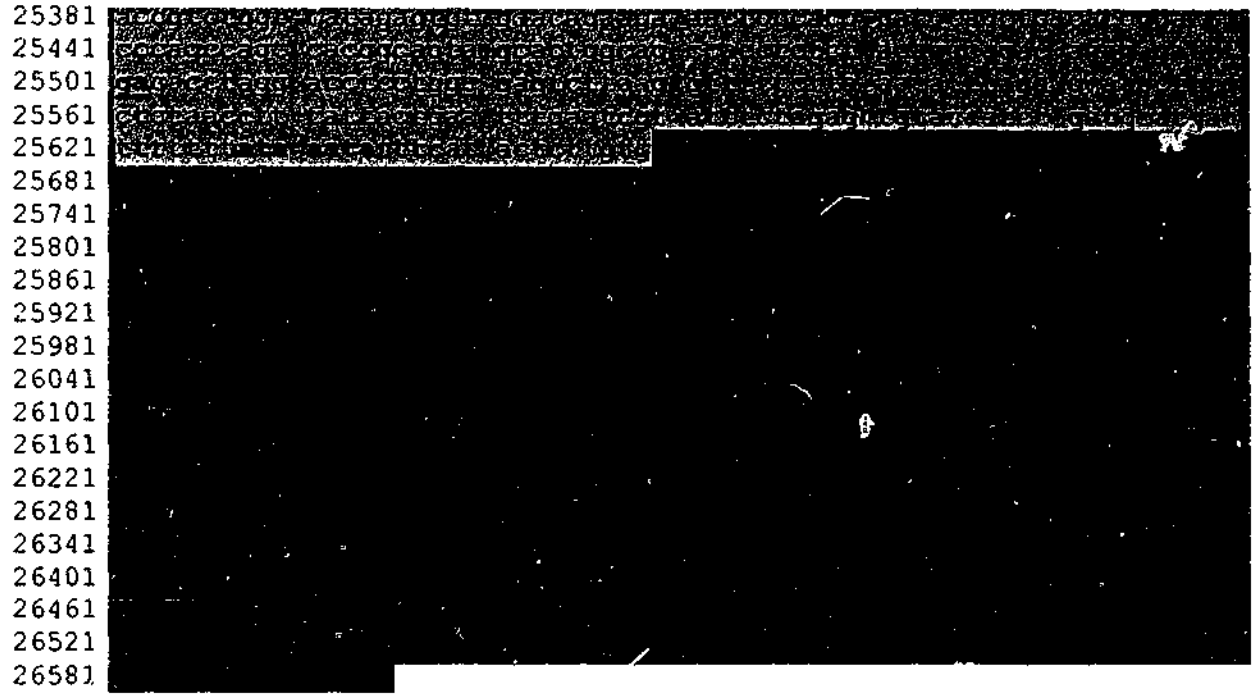
20881 gtccgagggt gatgaccaac aggttcagtt tcaatagata gactagatgt ctgaagtgct
20941 ggaaactggg atattgaata tgtgaaggag ttaacaatgg catttggtct gggaagacca
21001 gatagatact tactgaggct tttgtagaag attcttttca ggtctgttag tcagggttct
21061 ctaaggaaca gagctaacag aatgaacaag tctatggctg tagcaaaagt cttccattga
21121 gagcagtctt ttaaagatga ctaatcagtc tcttaggaca gctgatattt taattttagg
21181 agtattatcg cactaagttt cagttgttaa tatatatctt atttacttgt tttactggtt
21241 ttgtttttaa ccatggagct ttttttaaag gctgtctttg ctaaattcat ttttacagtg
21301 ttgggagattg tattttcagt ttcaaatgag aatgaatgtt ttgtgttctt cag[redacted]
21361 [redacted]gtg agtattcatt
21421 cctgtagagt gcgcccactt accaggaaga aatgtttcct tattagatag cttcaagttc
21481 aagctttcag cagaattttc tgactacaga ggacggaagc agctgaactg agtgtgttgg
21541 ctttgcttgc cctgttcctc tctcgctaaa ctgagaactg attagcctca actcttcttt
21601 cagggtttga tttctacaaa ctatgccacc ttaagtgctg cagagccatg gccatgcaca
21661 tgcttgaggc ttgctctatg aatccttgat cactcgatag acttagcttt gatgcttgca

21721 actcggtttc attcattttg actgctgagt agcatgtcta aaaatgctgc aaatttcact
21781 agatttaaga tggtattaac aatttctatt ctacccttcc atttcaaatg agctaacata
21841 atcgcaggaa cgtcagtttt attgattgca tttatttcta ttttctgtat tagaagaaaa
21901 gtatattctt ctttttcct tttag[redacted]
21961 [redacted]
22021 [redacted]gtac ttttgcatga
22081 actggtgtta gtaaacttgt cgatttagat atattctaag ttggactagc tctctggcag
22141 tgttaagcta atatttataa attgagaagt ttgtttttgc tttggttgag attggaccag
22201 agggcttgtg cactgccttt acctgtgtta gcttctaaca tgacctatgc ttattctgct
22261 tacggattta g[redacted]
22321 [redacted]
22381 [redacted]
22441 [redacted]gtaaga atttgttttt tgtcttctaa tatcatactt
22501 ctctagaaat gtagcttctt attcttgata taaacctggt agttaatgaa agtaaaatac
22561 tttaagattg tgtgcatccc gcatgcatgt gtgagcgtgt gcacgccgtg tcagtgttgg
22621 ttgctgaggt ggctcagtga acaaaggttt tcaccccaga gcctgctgcc cagctgaatc
22681 tcttctctga acccacatgg taggagacta gccggcttca ggttcacaca cacacatata
22741 tatacccaga gaaaaaacag agaacgtgca tttacaagat ttatttacaa gaatttaatt
22801 tacagttatt tatgtgagta cactgcaagc tgtgttcaga cccaggccca ggagatgcct
22861 cccatagaca tggttctgat gggacttgaa ctcaggacct ctgcgagagc cagcagccag
22921 tgctgctaac ctctgtgcca tctctccagc ccctttaaaa aatttttta agttaaatgt
22981 tttatgcatt atgttaaaaa ttacttaaga gtttttctta caattttgag gtgcatagac
23041 ctttataaaa agattgttat aagattattt atttaaagtg atttaaaagt atatcatttt
23101 tgttcaaaac caatcttctt attaatctta aatttttttt ttagtttgca ttacaaagat
23161 gctgtttaca tattgaatat atattaaatt catgaattcc tattttggca attacattta
23221 cagttttatc attctacatc tgcctaaatc tgaatatcca aaagttgaac ttggaaagat
23281 ttcccctagt tcctttggat aatcttaagt ggaaagacct ctactcctga gtgttggtga
23341 ggctatggga gtgcttttgc actgtggcca gaggtgtgcc atgcaggatt ctgtccaggg
23401 tggattgcaa tttctgccct gctaagttac caagcaactt ttcttagtag accacaagca
23461 gacatgtctg aatgtccata cacagcatcc taaatcagaa accatccctg tatccatcag
23521 cagacaaacg agtgaagcag ttgtgatgta ctcaagtatc aaagaggaac tgctgcctct
23581 gatgacagta cagagtccaa aaggggtctg cgttcaaagg acatgtgtgg tcactgtaga
23641 acatagctat agcctgagtg tattcactta ggataggagg ggtctgggtt caaaggactg
23701 taaggtaggt aactgtagga tagagcttgt gaatgtggtc acttacgata atttgataaa
23761 ctaggaaaag caaactacag catttatgta tgttccttta aggaaaaaaa atacagagaa
23821 aagcagtgga gttctgtctt gaagcaagct tgtgtttata cttttagatt aaggatgctt
23881 ggtaaaggac agagtgctca gttattggca gtctgttgtt agactgagtg agaactgaat
23941 ggtgaattgg catttctaat gttaatgtgt gtgtttcttc tatctatatg gtagagctca
24001 aaatgtcaga ggttttaaaa actagtttca caaaggtaag tgggggcatc atgtaacaac
24061 atagggggag aacgatgcgg ctvctgcttc tgtatggtat catgtgatgt gtgctccaga
24121 ctgaagaaag gaagtattag acctggtgaa aagagagaga gagagtgtgt gtgtgtgtgt
24181 gtgtgtgtgt gtgtgtgtgt gtgtgtcttt gtgtgcgcgc ggtgcatgca ccctttttc
24241 ttttaccaag aaatgacgat tacaccagtc ctaattgcat tctagaaata tgaaaccact
24301 ttttttcctt ttcccaaatg tctatttatc ataaacttat atttttaaac tgcatgacaa
24361 gtagtttagt ggttagtcaa ttcaaatttt ctgttgatgt gaagtctgat gtacaaagta
24421 gctataaact cggctgcttc tgcctccata gagagtgtag taaagtagtc tagcgtagcc
24481 tccgaggccg ctctgcagtg cgggctagac agtggtgctg cttcatgatg cctgcccttc
24541 cctcttttcc tggagcctgt ttcttctgac cttagagtta ttcttttggg aatccctcgt
24601 ggtttggaga atttctccaa aatgtggcaa gcctttatat aataaatgga agatgaagat
24661 atttctttat tgtattaaat atatcatatt agattttata taaaacaaaa gtggacttca
24721 caaccaacca tctgtaactt tttgttatct ggattcctgt taacatagct tatattttat
24781 agagagttaa ctgtggcagt cattgtaagc taagcacttt ataaagatta tccctctatt
24841 caacagttct atgagggtaa gtactgtttt aacctcattt tctagatgag gaataaagga
24901 ttaaaggaac taggatggtc ctgtggtcac agaggcagca agtgaggcct tgcggtgtat
24961 ctctgacttt ccaaggctct tttgttttgt cttctcttta gtaattctgt tggtttctaa
25021 agcaatgtta tcagagttgt taaaaatgta ttttgaaagt ttcatatata agattctgga
25081 gtagtgagga ctgaagtttt ggtgagtaat gttctagagt ttgatttgta atgtgaaata
25141 ctgtgacgtc aatgttaact gttgtagtgg attcagaact ctgctaggtc ttctgtttgc
25201 agaaaagtct aaaaagtagg gagtacaaac tgttcatctt tacgaggtcc tgatgtggtt
25261 tctcttttta aag[redacted]
25321 [redacted]



Gabpα Exon 1-10a
Gabpα Exon 1a
Gabpα Exon 1b
Gabpα Exon 10b
Gabpα Exon 10c
Gabpα Exon 10d
tga Start Codon
tag Stop Codon
aataaa Poly-A Signal

Appendix F

Gabpα Alternative Transcripts

AY282793 Mus musculus Gabp alpha mRNA, partial cds (exon 1-exon 3).
Length 675 bp

```
  1 gcatgcgtat tctggcctgt cccctagttc aagctccctt cggagtcagc gtcccgatcc
 61 ttggggttgg ggaggtggat gtagggtggg ggtacaaagg agcgcgtaaa aaccaaaacc
121 aaaacaaaac aaaaaaccaa agcggctggg gggcgtttgt gcctcgtgag ccccgtcgtc
181 gcgggcgttt cgggccgcgc agcctgctgg gagttgtagt tccaggcccg ggggtgcctg
241 ggaggcggga gaggggctgg ggttccttag cgccgattcc acgggaaggg ccccgggacc
301 ttaccctgct actcgcggga gcggcgcggc tcgcccggag aggcgctgta cctggagccc
361 gcgccgccgc cgttcccagc cggctgtgga agcgcgggct agggccacgg gacgaattcc
421 ggagctggac tgaacttttg aacgtcttca accatgacta agagagaagc agaagagctg
481 atagaaattg agatcgacgg gactgagaaa gcagagtgca cagaagaaag cattgtggaa
541 caaacctata ccccagctga atgtgtaagc caggccatag acatcaatga accaataggc
601 aatttaaaga aactactaga accaagactg cagtgttctt tggatgctca tgaaatttgc
661 ctgcaagata ttcag
```

AY282794 Mus musculus Gabp alpha mRNA, partial cds (exon 1a-exon 3).
Length 334 bp

```
  1 ctgaccttga acttgctatg tagattagac cggcctagag tttagaggcc tatctgcctc
 61 tattttctga gcgccggcat taaagggact gaacttttga acgtcttcaa ccatgactaa
121 gagagaagca gaagagctga tagaaattga gatcgacggg actgagaaag cagagtgcac
181 agaagaaagc attgtggaac aaacctatac cccagctgaa tgtgtaagcc aggccataga
241 catcaatgaa ccaataggca atttaaagaa actactagaa ccaagactgc agtgttcttt
301 ggatgctcat gaaatttgcc tgcaagatat tcag
```

AY282795 Mus musculus Gabp alpha mRNA, partial cds (exon 1b-exon 3).
Length 550 bp

```
  1 ctgaccttga acttgctatg tagattagac cggcctagag tttagaggcc tatctgcctc
 61 tattttctga gcgccggcat taaaggtgtg cactaccttg cctagcaaaa taagtgcatt
121 ttttaattgg ttatttttatt tatttacatt tcaaatgttg acccctttct tggcctcccc
181 acctcaattt ttttaaaaca ggtagattga gctaatgaca ggactgactg ctggcaagag
241 gaaaacattt atatagatcc ccctcacatt cttaggtacc ccatgccact aaacactggc
301 cagactgaac ttttgaacgt cttcaaccat gactaagaga gaagcagaag agctgataga
361 aattgagatc gacgggactg agaaagcaga gtgcacagaa gaaagcattg tggaacaaac
421 ctatacccca gctgaatgtg taagccaggc catagacatc aatgaaccaa taggcaattt
481 aaagaaacta ctagaaccaa gactgcagtg ttctttggat gctcatgaaa tttgcctgca
541 agatattcag
```

AY282796 Mus musculus Gabp alpha mRNA, partial cds (exon 8-exon 10a).
Length 710 bp

```
   1 ctgtgcagat tattccagcc tcagtgcctc ccgctacacc gactacgatt aaagttataa
  61 acagcagtgc aaaagcagct aaagtgcaac ggtccccaag gatttcagga gaagacagaa
 121 gttcaccggg gaacagaaca ggaaacaatg gtcagatcca actatggcag tttttgctag
 181 aacttcttac tgacaaggat gctcgagact gtatttcttg ggttggtgat gaaggtgaat
 241 ttaagctaaa tcagcctgag ttggttgcgc aaaaatgggg acaacgtaag aacaagccta
 301 ccatgaacta tgagaaactt agccgtgcat tacggtatta ttatgatggg gacatgattt
 361 gtaaagttca aggcaagaga tttgtgtaca aatttgtttg tgacttgaag actcttattg
 421 gatacagtgc agcagaactg aaccgtctgg tcatagagtg tgaacagaag aaactggcac
 481 ggatgcagct gcatgggatt gcccagccag tcacggcagt agcactggca gccacctctc
 541 tacaggcaga caaagagatt tgagacctag gacctcctgg ggagtcttaa ggttttttct
 601 aaatatttag agcaagcttt gctctaacct ttattactga atttgaatcg tatttctaga
 661 gtgtacaatc tgatgcatga tttttttttat aaatatttca tactcttgtg
```

AY282797 Mus musculus Gabp alpha mRNA, partial cds (exon 8-exon 10b).
Length 1273 bp

```
   1 ctgtgcagat tattccagcc tcagtgcctc ccgctacacc gactacgatt aaagttataa
  61 acagcagtgc aaaagcagct aaagtgcaac ggtccccaag gatttcagga gaagacagaa
 121 gttcaccggg gaacagaaca ggaaacaatg gtcagatcca actatggcag tttttgctag
 181 aacttcttac tgacaaggat gctcgagact gtatttcttg ggttggtgat gaaggtgaat
 241 ttaagctaaa tcagcctgag ttggttgcgc aaaaatgggg acaacgtaag aacaagccta
 301 ccatgaacta tgagaaactt agccgtgcat tacggtatta ttatgatggg gacatgattt
 361 gtaaagttca aggcaagaga tttgtgtaca aatttgtttg tgacttgaag actcttattg
 421 gatacagtgc agcagaactg aaccgtctgg tcatagagtg tgaacagaag aaactggcac
 481 ggatgcagct gcatgggatt gcccagccag tcacggcagt agcactggca gccacctctc
 541 tacaggcaga caaagagatt tgagacctag gacctcctgg ggagtcttaa ggttttttct
 601 aaatatttag agcaagcttt gctctaacct ttattactga atttgaatcg tatttctaga
 661 gtgtacaatc tgatgcatga tttttttttat aaatatttca tactcttgtg aatttggatc
 721 tttttacgtt gaacatatat tttagaatat gttaaaggat caccacgatg cctgcaatgt
 781 gagagcaggt tcatcggggt agtttgctaa cagtcaggaa agctaaactg gtcagtatta
 841 atgtctagcc ctaccaaaaa atagccagta gcgtctgaag atgaaaggag ggaagcattt
 901 ccaggaaaca gcctggtgta gccactgcgg gcgtggagct gtttcctctc cgctgcttta
 961 cacagagcct ggaaaaagga cattctcagc taaagcatgt gtgcctgttt catctaatca
1021 agcagagcta agcagtccta tcaaatacaa ttacattggt aattactaaa ctatcaggtt
1081 attaatttat aatttacaag aagagaggaa gttaactggc agaagaaagt gttgaatgag
1141 acccagattt aaagctggct taactcaaac cactattaat ggttttctgt atagattatt
1201 cattatgtca agccaaggtc ttaaattatt ttgagagagg catttaattc taataaacca
1261 gctattgcaa aaa
```

AY282798 Mus musculus Gabp alpha mRNA, partial cds (exon 8-exon 10c).
Length 1554 bp

```
   1 ctgtgcagat tattccagcc tcagtgcctc ccgctacacc gactacgatt aaagttataa
  61 acagcagtgc aaaagcagct aaagtgcaac ggtccccaag gatttcagga gaagacagaa
 121 gttcaccggg gaacagaaca ggaaacaatg gtcagatcca actatggcag tttttgctag
 181 aacttcttac tgacaaggat gctcgagact gtatttcttg ggttggtgat gaaggtgaat
 241 ttaagctaaa tcagcctgag ttggttgcgc aaaaatgggg acaacgtaag aacaagccta
 301 ccatgaacta tgagaaactt agccgtgcat tacggtatta ttatgatggg gacatgattt
 361 gtaaagttca aggcaagaga tttgtgtaca aatttgtttg tgacttgaag actcttattg
 421 gatacagtgc agcagaactg aaccgtctgg tcatagagtg tgaacagaag aaactggcac
 481 ggatgcagct gcatgggatt gcccagccag tcacggcagt agcactggca gccacctctc
 541 tacaggcaga caaagagatt tgagacctag gacctcctgg ggagtcttaa ggttttttct
 601 aaatatttag agcaagcttt gctctaacct ttattactga atttgaatcg tatttctaga
 661 gtgtacaatc tgatgcatga tttttttttat aaatatttca tactcttgtg aatttggatc
 721 tttttacgtt gaacatatat tttagaatat gttaaaggat caccacgatg cctgcaatgt
 781 gagagcaggt tcatcggggt agtttgctaa cagtcaggaa agctaaactg gtcagtatta
 841 atgtctagcc ctaccaaaaa atagccagta gcgtctgaag atgaaaggag ggaagcattt
```

```
 901 ccaggaaaca gcctggtgta gccactgcgg gcgtggagct gtttcctctc cgctgcttta
 961 cacagagcct ggaaaaagga cattctcagc taaagcatgt gtgcctgttt catctaatca
1021 agcagagcta agcagtccta tcaaatacaa ttacattggt aattactaaa ctatcaggtt
1081 attaatttat aatttacaag aagagaggaa gttaactggc agaagaaagt gttgaatgag
1141 acccagattt aaagctggct taactcaaac cactattaat ggttttctgt atagattatt
1201 cattatgtca agccaaggtc ttaaattatt ttgagagagg catttaattc taataaacca
1261 gctattgcaa aaattccaaa atgatatcat tctttctctc agattttaaa aagtatacct
1321 cagaaagaaa aaattggttt agttttgact tcctatttct ttaaaaatta atctgtagta
1381 ccagattatt atgcaagacc gtttatatat tcccttctgt gtttctgata aaatgaagac
1441 tttagatcag gcgacatgtt taacgtttga cgtaagttcc ttgtgagtag ttatgaaagg
1501 gtttgactgg gctctgtagg cgatcttctt taaataaagt ttcccacttg tttc
```

AY282799 Mus musculus Gabp alpha mRNA, partial cds (exon 8-exon 10d).
Length 1656 bp

```
   1 ctgtgcagat tattccagcc tcagtgcctc ccgctacacc gactacgatt aaagttataa
  61 acagcagtgc aaaagcagct aaagtgcaac ggtccccaag gatttcagga gaagacagaa
 121 gttcaccggg gaacagaaca ggaaacaatg gtcagatcca actatggcag tttttgctag
 181 aacttcttac tgacaaggat gctcgagact gtatttcttg ggttggtgat gaaggtgaat
 241 ttaagctaaa tcagcctgag ttggttgcgc aaaaatgggg acaacgtaag aacaagccta
 301 ccatgaacta tgagaaactt agccgtgcat tacggtatta ttatgatggg gacatgattt
 361 gtaaagttca aggcaagaga tttgtgtaca aatttgtttg tgacttgaag actcttattg
 421 gatacagtgc agcagaactg aaccgtctgg tcatagagtg tgaacagaag aaactggcac
 481 ggatgcagct gcatgggatt gcccagccag tcacggcagt agcactggca gccacctctc
 541 tacaggcaga caaagagatt tgagacctag gacctcctgg ggagtcttaa ggtttttctt
 601 aaatatttag agcaagcttt gctctaacct ttattactga atttgaatcg tatttctaga
 661 gtgtacaatc tgatgcatga ttttttttat aaatatttca tactcttgtg aatttggatc
 721 tttttacgtt gaacatatat tttagaatat gttaaaggat caccacgatg cctgcaatgt
 781 gagagcaggt tcatcggggt agtttgctaa cagtcaggaa agctaaactg gtcagtatta
 841 atgtctagcc ctaccaaaaa atagccagta gcgtctgaag atgaaaggag ggaagcattt
 901 ccaggaaaca gcctggtgta gccactgcgg gcgtggagct gtttcctctc cgctgcttta
 961 cacagagcct ggaaaaagga cattctcagc taaagcatgt gtgcctgttt catctaatca
1021 agcagagcta agcagtccta tcaaatacaa ttacattggt aattactaaa ctatcaggtt
1081 attaatttat aatttacaag aagagaggaa gttaactggc agaagaaagt gttgaatgag
1141 acccagattt aaagctggct taactcaaac cactattaat ggttttctgt atagattatt
1201 cattatgtca agccaaggtc ttaaattatt ttgagagagg catttaattc taataaacca
1261 gctattgcaa aaattccaaa atgatatcat tctttctctc agattttaaa aagtatacct
1321 cagaaagaaa aaattggttt agttttgact tcctatttct ttaaaaatta atctgtagta
1381 ccagattatt atgcaagacc gtttatatat tcccttctgt gtttctgata aaatgaagac
1441 tttagatcag gcgacatgtt taacgtttga cgtaagttcc ttgtgagtag ttatgaaagg
1501 gtttgactgg gctctgtagg cgatcttctt taaataaagt ttcccacttg tttcattttt
1561 ttctywtkww twtykywtga atattgcata yatgactctt aaaaaatact gtacacatga
1621 ctagctaacc atttctgcat atatgaagac agatat
```

Appendix G

Gabpβ1 Alternative Transcripts

AY282802 Mus musculus Gabp beta1-1 mRNA, exon 7-9 alternative splice form, partial cds (exon 3-exon 9).
Length 841 bp

```
  1 ggaacttctc cacttcatct ggccgcacag tatgggcatt tctctaccac agaggttctt
 61 ctccgagccg gtgtaagtag agatgccagg accaaagtgg accggacacc actgcacatg
121 gcggcttctg agggccatgc caacatagta gaagttttgc ttaagcatgg tgctgacgtc
181 aatgcaaagg atatgttaaa gatgacagct ctgcattggg caacagaaca taatcatcaa
241 gaggtggtgg agcttttaat caaatatggt gctgatgtac acacgcagag taaattttgt
301 aaaactgcat ttgatatttc aatagacaat ggaaatgaag atttagcaga gatattacag
361 attgctatgc agaaccaaat caacaccaac ccggagagtc ctgacactgt gacaatacac
421 gctgccacac cacagttcat cattggaccc ggaggggtgg tgaacctcac agatgaaaca
481 ggagtatctg ctgttcagtt tggaaactcc tctacgtcag tattagctac attagctgcc
541 ttagctgaag cttctgcccc attgtccaat tcttcagaaa ctccagtagt ggccacagag
601 gaagtggtta ccgcagaatc tgtggatggt gcaattcagc aagtagttag ctcagggggt
661 cagcaagtca tcacgatagt tacagatgga atccagctgg ggaatttgca ctccatacca
721 accagtggga tgggtcagcc catcatggtg acgatgcccg atggacagca aggagagaga
781 agcgcttcag aaacagctgg atgaagccaa ccgagaggcc cagaaatacc gacagcagct
841 g
```

AY282800 Mus musculus Gabp beta1-1 mRNA, exon 3-5 alternative splice form, partial cds (exon 3-exon 9).
Length 762 bp

```
  1 ggaacttctc cacttcatct ggccgcacag tatgggcatt tctctaccac agaggttctt
 61 ctccgagccg gtgtaagtag agatgccagg accaaagtgg accggacacc actgcacatg
121 gcggcttctg agggccatgc caacatagta gaagttttgc ttaagattgc tatgcagaac
181 caaatcaaca ccaacccgga gagtcctgac actgtgacaa tacacgctgc cacaccacag
241 ttcatcattg gacccggagg ggtggtgaac ctcacagatg aaacaggagt atctgctgtt
301 cagtttggaa actcctctac gtcagtatta gctacattag ctgccttagc tgaagcttct
361 gccccattgt ccaattcttc agaaactcca gtagtggcca cagaggaagt ggttaccgca
421 gaatctgtgg atggtgcaat tcagcaagta gttagctcag ggggtcagca agtcatcacg
481 atagttacag atggaatcca gctggggaat ttgcactcca taccaaccag tgggatgggt
541 cagcccatca ttgtgacgat gcccgatgga cagcaagtat tgacagtacc agcaacagac
601 attgctgaag aaactgtcat cagtgaagag ccaccagcta agagacagtg tatggaaata
661 attgagagcc gggtggaatg tgcagaaatt gaagagagag aagcgcttca gaaacagctg
721 gatgaagcca accgagaggc ccagaaatac cgacagcagc tg
```

AY282801 Mus musculus Gabp beta1-1 mRNA, exon 3-9 alternative splice form, partial cds (exon 1-exon 9).
Length 649 bp

```
  1 gtctgccctg acggctccga ggcgccgcgg tccccgcacc tcctcccgct gctcccccgc
 61 cgccgctccc gaagcttttc cagatgtccc tggtagattt ggggaagaag cttttagaag
121 cggcacgagc cggtcaagat gatgaagttc gcattttgat ggcaagtgga gctccttta
181 ctacagactg gttgggaact tctccacttc atctggccgc acagtatggg catttctcta
241 ccacagaggt tcttctccga gccggtgtaa gtagagatgc caggaccaaa gtggaccgga
301 caccactgca catggcggct tctgagggcc atgccaacat agtagaagtt ttgcttaagg
361 agagagaagc gcttcagaaa cagctggatg aagccaaccg agaggcccag aaataccgac
421 agcagctgct taagaaggag caggaggcag aggcctacag gcagaagctg gaggccatga
481 cacgcatcca gaccaacaaa gaagccgttt agctgccatg aacaccagtt tgcttttacc
541 ttttgtccag aaagaatata gtcttgaact gcacacagta aggacacagc catgggaata
601 ccgaataata gaaaatacta cagcttgata acgggactta agccatgag
```

AY282805 Mus musculus Gabp beta1-2 mRNA, exon 4-6 alternative splice form, partial cds (exon 3-exon 8).
Length 817 bp

```
  1 ggaacttctc cacttcatct ggccgcacag tatgggcatt tctctaccac agaggttctt
 61 ctccgagccg gtgtaagtag agatgccagg accaaagtgg accggacacc actgcacatg
121 gcggcttctg agggccatgc caacatagta gaagttttgc ttaagcatgg tgctgacgtc
181 aatgcaaagg atatgttaaa gatgacagct ctgcattggg caacagaaca taatcatcaa
241 gaggtggtgg agcttttaat caaatatggt gctgatgtac acacgcagag taaattttgt
301 aaaactgcat ttgatatttc aatagacaat ggaaatgaag atttagcaga gatattacag
361 atgaaacagg agtatctgct gttcagtttg gaaactcctc tacgtcagta ttagctacgt
421 tagctgcctt agccgaagct tctgccccat tgtccaattc ttcagaaact ccagtagtgg
481 ccacagagga agtggttacc gcagaatctg tggatggtgc aattcagcga gtagttagct
541 cagggggtca gcaagtcatc acgatagtta cagatggaat ccagctgggg aatttgcact
601 ccataccaac cagtgggatg ggtcagccca tcattgtgac gatgcccgat ggacagcaag
661 tattgacagt accagcaaca gacattgctg aagaaactgt catcagtgaa gagccaccag
721 ctaagagaca gtgtatggaa ataattgaga gccgggtgga atgtgcagaa attgaagtaa
781 ggagccttat acccggtgtg ttttgctgca gccatcc
```

AY282804 Mus musculus Gabp beta1-2 mRNA, exon 3-6 alternative splice form, partial cds (exon 1-exon 8).
Length 816 bp

```
  1 gtctgccctg acggctccga ggcgccgcgg tccccgcacc tcctcccgct gctccccccgc
 61 cgccgctccc gaagcttttc cagatgtccc tggtagattt ggggaagaag cttttaggag
121 cggcacgagc cggtcaagat gatgaagttc gcattttgat ggcaaatgga gctcctttta
181 ctacagactg gttgggaact tctccacttc atctggccgc acagtatggg catttctcta
241 ccacagaggt tcttctccga gccggtgtaa gtagagatgc caggaccaaa gtggaccgga
301 caccactgca catggcggct tctgagggcc atgccaacat agtagaagtt ttgcttaaga
361 tgaaacagga gtatctgctg ttcagtttgg aaactcctct acgtcagtat tagctacgtt
421 agctgcctta gctgaagctt ctgccccatt gtccaattct tcagaaactc cagtagtggc
481 cacagaggaa gtggttaccg cagaatctgt ggatggtgca attcagcaag tagttagctc
541 aggggtcag caagtcatca cgatagttac agatggaatc cagctgggga atttgcactc
601 cataccaacc agtgggatgg gtcagcccat cattgtgacg atgcccgatg gacagcaagt
661 attgacagta ccagcaacag acattgctga agaaactgtc atcagtgaag agccaccagc
721 taagagacag tgtatggaaa taattgagag ccgggtggaa tgtgcagaaa ttgaagtaag
781 gagccttata cccggtgtgt tttgctgcag ccatcc
```

AY282803 Mus musculus Gabp beta1-2 mRNA, exon 3-5 alternative splice form, partial cds (exon 3-exon 8).
Length 734 bp

```
  1 ggaacttctc cacttcatct ggccgcacag tatgggcatt tctctaccac agaggttctt
 61 ctccgagccg gtgtaagtag agatgccagg accaaagtgg accggacacc actgcacatg
121 gcggcttctg agggccatgc caacatagta gaagttttgc ttaagattgc tatgcagaac
181 caaatcaaca ccaacccgga gagtcctgac actgtgacaa tacacgctgc cacaccacag
241 ttcatcattg gacccggagg ggtggtgaac ctcacagatg aaacaggagt atctgctgtt
301 cagtttggaa actcctctac gtcagtatta gctacgttag ctgccttagc tgaagcttct
361 gccccattgt ccaattcttc agaaactcca gtagtggcca cagaggaagt ggttaccgca
421 gaatctgtgg atggtgcaat tcagcaagta gtcagctcag ggggtcagca agtcatcacg
481 atagttacag atggaatcca gctggggaat ttgcactcca taccaaccag tgggatgggt
541 cagcccatca ttgtgacgat gcccgatgga cagcaagtat cgacagtacc agcaacagac
601 attgctgaag aaactgtcat cagtgaagag ccaccagcta agagacagtg tatggaaata
661 attgagagcc gggtggaatg tgcagaaatt gaagtaagga gccttatacc cggtgtgttt
721 tgctgcagcc atcc
```

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