MULTIPLE MECHANISMS MEDIATING THE STARVATION INDUCED ACTIVATION OF RECOMBINATION AT *HIS4* IN *Saccharomyces cerevisiae*

A thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by
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ABSTRACT

Meiotic recombination occurs at relatively high levels at specific regions in the genome called hotspots. The transcription factor-dependent hotspots (α-hotspots) have been widely studied in yeast, and are beginning to be elucidated in mammals. The *HIS4* hotspot activity in *Saccharomyces cerevisiae* requires binding of Bas1p, Bas2p, Rap1p and Gcn4p. Bas1p acts in conjunction with Bas2p to regulate basal level of transcription of their target genes, and can be stimulated under conditions of adenine starvation and accumulation of metabolites AICAR and SAICAR from the purine biosynthesis pathway. Gcn4p activates transcription of yeast genes in response to starvation for amino acids and purines. This study focused on the influence of nutritional starvation on *HIS4* hotspot activity, and different mechanisms mediating this effect. Our data suggests that deletion of genes known to accumulate AICAR/SAICAR can stimulate recombination at *HIS4* in a Bas1p-dependent manner. Furthermore, intracellular and extracellular starvation for adenine and amino acids also activates recombination at *HIS4*. In addition, moderate levels of starvation only affect recombination when chromatin is already hyperacetylated, by the inactivation of the Set2p methyltransferase. Bas1p plays an essential role in mediating the effect of starvation and the *set2* mutation on recombination. We showed that Gcn4p is not required for *HIS4* hotspot activity, but plays a modest role in the effect of starvation in an adenine auxotrophic strain. Additionally, the starvation effect is also mediated by an as yet unknown factor independent from Bas1p/Bas2p and Gcn4p. This work provides additional information regarding the regulation of a transcription factor-dependent hotspot activity, and factors influencing its activation. Furthermore, data in this study indicate that *BAS1*, and not *BAS2* exhibit haploinsufficiency with respect to its function in activating meiotic recombination. This implies that Bas1p is rate-limiting for *HIS4* hotspot activity.

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ABBREVIATIONS

5′-FOA	5′-fluoroorotic acid
10-HCO-THF	10-formyl tetrahydrofolate
µM	Micromolar
AICAR	5'-phosphoribosyl-4-carboxamide-5-aminoimidazole
AIR	1-(5′-phosphoribosyl)-5-aminoimidazole
AS	adenylosuccinate
ATP	Adenine triphosphate
BIRD	Bas1p Interaction and Regulatory Domain
bp	Base pairs
CAIR	1-(5′-phosphoribosyl)-5-aminoimidazole-4-carboxylate
CHEF gels	Clamped homogenous electric field gels
cM	CentiMorgan
dHJ	Double Holliday junction
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DSB	Double-strand break
DSBR	Double-strand break repair
EDTA	Ethylenediaminetetraacetic acid
FAICAR	5-formamido-1-(5′-phosphoribosyl)-imidazole-4-carboxyamide
FGAM	5′-phosphoribosyl-*N*′-formylglycinamidine
FGAR	5′-phosphoribosyl-*N*′-formylglycinamide
GAR	5′-phosphoribosylglycinamide
HAT	Histone acetyltransferase
HCl	Hydrochloric acid
HDAC	Histone deacetylase
hDNA	Heteroduplex deoxyribonucleic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HJ	Holliday junction
KAc	Potassium acetate
kb	Kilobases
kDa	KiloDaltons
IMP	Inosine 5′-monophosphate
LB	Luria-Bertani medium
LMP	Low melting point
M	Molar
mM	Millimolar
MMS	Methyl-methanosulfonate
MRX	Mre11p, Rad50p, Xrs2p
M Wt	Molecular weight
ng	Nanogram
NMS	Non-Mendelian segregation
NPD	Non-parental ditype
OD	Optical density

ORF	Open reading frame
P	Phosphate
PCR	Polymerase chain reaction
PD	Parental ditype
PMS	Post-meiotic segregation
PMSF	Phenylmethylsulfonyl fluoride
PR	5-phosphoribosyl
PRA	5-phosphorybosylamine
PRPP	5-phosphoribosyl diphosphate
PVDF	Polyvinylidene fluoride
RPA	Replication protein A
r.p.m	Revolutions per minute
SAICAR	1-(5′-phosphoribosyl)-4-(*N*-succinocarboxyamido)-5-aminoimidazole
SC	Synaptonemal complex
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDSA	Synthesis-dependent strand annealing
SEI	Single-end invasion
SPS	Supplemented pre-sporulation medium
SSC	150 mM sodium chloride, 15 mM sodium citrate; pH7.0
TE	10 mM Tris-HCl, 1 mM EDTA; pH8.0
TBE	90 mM Tris, 90 mM Boric acid, 2 mM EDTA
THF	Tetrahydrofolate
TT	Tetratype
UV	Ultraviolet
WT	Wild type
YEPD	Yeast extract, peptone, dextrose medium
YEPEG	Yeast extract, peptone, ethanol, glycerol medium
YPER	Yeast protein extraction reagent
ZMM	Zip1, Zip2, Zip3, Zip4, Msh4, Msh5, Mer3, Spo16

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Chapter 1 : Introduction

1.1 An Overview of Meiosis

Meiosis is a unique cell division that is essential in sexually reproducing organisms to produce offspring. Products of meiosis include gametes (sperm and ovum) in mammals, pollens in higher plants and spores in fungi. Unlike mitosis that produces two identical daughter cells, a single meiotic event generates four non-identical haploid progeny with each carrying half the amount of genetic material of the diploid parents (Figure 1.1). In meiosis, pairs of sister chromatids are generated after one round of DNA replication. This is followed by two rounds of cell division, without an intervening round of DNA replication, hence reducing the chromosome number (Petronczki *et al.*, 2003). Homologous chromosomes segregate in meiosis I and the sister chromatid pairs segregate in meiosis II (Petronczki *et al.*, 2003). The chromosome number is restored to diploid when two haploid gametes fuse together. Therefore, halving of genetic material in meiosis is vital for maintaining the total number of chromosomes.

A primary feature of meiosis is inter-homolog recombination which occurs during the prophase stage in meiosis I (Figure 1.1). During prophase I, homologous chromosomes of maternal and paternal origin pair and undergo synapsis. This occurs concurrently with the polymerisation of a highly conserved proteinaceous structure, the synaptonemal complex (SC) between paired homologs. Homologous chromosomes then undergo reciprocal recombination (crossing over) that involves the physical exchange of genetic material.

Genetic recombination intermediates in meiosis I provides a mechanical attachment between homologous chromosomes, known as chiasmata, which hold homologous chromosomes together (Roeder, 1997). The success of gamete formation lies in this connection that directs coordinated segregation of homologs to the opposite spindle poles in the first meiotic division. Therefore, at least one crossover per chromosome pair is indispensable for ensuring accurate chromosome segregation into meiotic products. Absence of a crossover in a homolog pair can result in chromosome non-disjunction which leads to aneuploidy (Hassold *et al.*, 2007). Crossover failure may also be the cause of embryonic death, developmental defects and infertility in humans.

In the second meiotic division, sister chromatids are segregated in a manner similar to mitosis. Ultimately, all four haploid gametes inherit a single set of chromosomes. New combinations of DNA in daughter cells are also generated as a result of crossovers. Indeed, the shuffling of genetic information between homologous chromosomes creates genetic variation among the offspring. Additionally, non-reciprocal recombination (gene conversion) which involves the transfer of genetic information from one homologous chromosome to another, can also contribute to variation. Conversion events can result in allelic changes due to the repair of mismatches in heteroduplex DNA (Section 1.3). Hence meiosis also functions to produce genetic diversity within the populations of organisms on which evolution can act.

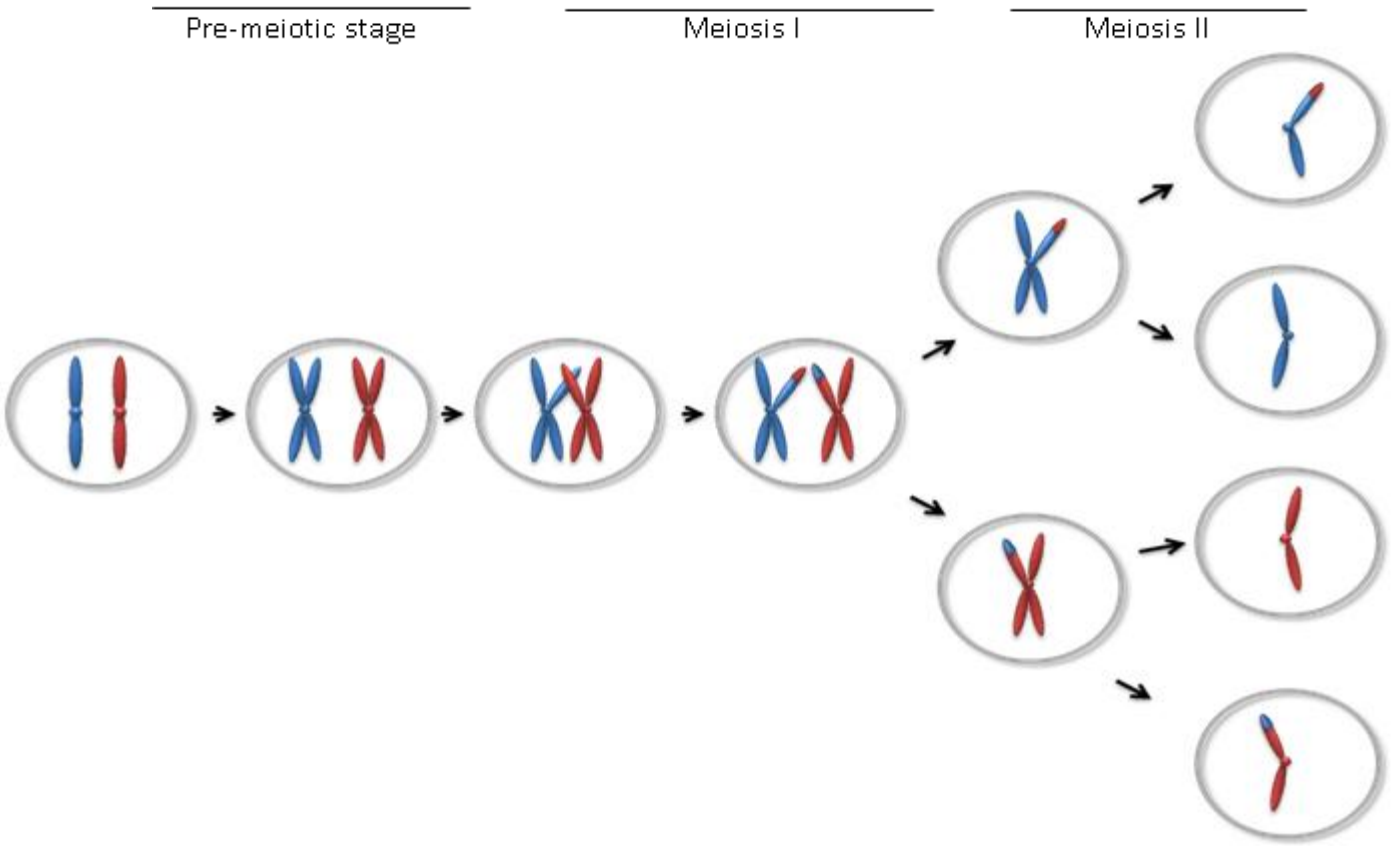


Figure 1.1 : Meiosis produces four haploid daughter cells.

Prior to meiosis, DNA replication generates pairs of sister chromatids. During the prophase stage of meiosis I, homologous chromosomes align and undergo recombination. Homologous chromosomes are segregated into two different cells at the end of meiosis I, and sister chromatids are segregated at the end of meiosis II. In meiosis, four unique daughter cells are produced from a diploid parent.

1.2 Model for Meiotic Recombination

Early models for meiotic recombination pathway, such as the Holliday model and the subsequent Meselson-Radding model, were mainly based upon initiation by nicking of the DNA, followed by the formation of a Holliday junction (Holliday, 1964, Meselson and Radding, 1975). Resolution of the Holliday junction can result in crossing over. The Double-Strand Break Repair model (DSBR) was later suggested (Resnick and Martin, 1976) and expanded (Szostak *et al.*, 1983), proposing that recombination is initiated by the formation of DNA double-strand breaks (DSB). A novel feature of the DSBR model is the formation of double Holliday junction (dHJ), whose resolution can generate either crossover or non-crossover products. In numerous later studies of meiotic recombination, several intermediates have been identified to confirm certain features of the DSBR model including the appearance of dHJ (Schwacha and Kleckner, 1995). However, other findings also indicate that crossovers and non-crossovers may not be generated from the same pathway, contradicting the DSBR model (discussed in Section 1.2.3).

1.2.1 Recombination Initiation Proteins

Formation of DSBs are catalysed by the widely conserved Spo11p, a type II topoisomerase-related enzyme (Keeney *et al.*, 1997). In *Saccharomyces cerevisiae*, Spo11p acts together with at least nine auxiliary proteins, including Mre11, Rad50p, Xrs2p, Mei4p, Mer2p, Rec102p, Rec104p, Rec114p and Ski8p (Rec103p), to generate DSBs (Hunter, 2006). Although the precise molecular roles of some of these proteins in DSB formation have yet to be determined, several of them have been well-characterised. These proteins can be divided into subgroups based on their physical interactions and subcellular localisation behaviour (Cole *et al.*, 2010). Mre11p, Rad50p

and Xrs2p (Nbs1p in higher organisms) form the evolutionarily conserved MRX complex (Borde and Cobb, 2009). This complex plays a role in the early stages of DSB initiation, as well as in the subsequent processing of DSBs. This was shown by studies comparing phenotypes of complete deletions of the MRX components with specific point mutations, such as *mre11S* or *rad50S* (Haber *et al.*, 2004). Deletion of members of the MRX complex eliminates DSB production, while these point mutations result in the accumulation of unrepaired DSBs.

The proteins Rec102p, Rec104p and Rec114p have been shown to be directly required for the association of Spo11p with the DSB site (Prieler *et al.*, 2005). The interaction of Spo11p with other recombination initiation proteins particularly Rec102p and Rec104p, is facilitated by Ski8p (Arora *et al.*, 2004). Furthermore, Ski8p was suggested to be involved in promoting the recruitment of Spo11p and other cofactors to the chromosome (Arora *et al.*, 2004). The remaining proteins Mei4p, Mer2p and Rec114p potentially interact with Rec102p/Rec104p via Rec114p (Maleki *et al.*, 2007). In addition, Rec102p/Rec104p also act as a mediator for the interaction of the Mei4p/Mer2p/Rec114p complex with Spo11p/Ski8p (Maleki *et al.*, 2007).

The function of Spo11p and some of the auxilliary proteins in the formation of meiotic DSBs are conserved in other organisms. For example, Kumar *et al.* (2010) had characterised the orthologs of Mei4p and Rec114p in mice. Deletion of *mei4* results in deficiency in the formation of meiotic DSBs, suggesting that Mei4p plays an essential role in meiotic DSB formation in mice (Kumar *et al.*, 2010). However, not all of the Spo11p-associated proteins for *S. cerevisiae* are functionally conserved in other organisms. In particular, Ski8p performs the same function in *S. cerevisiae*,

Schizosaccharomyces pombe and *Sordaria* (Tesse *et al.*, 2003), but its meiotic function has not been found in *Arabidopsis thaliana* (Jolivet *et al.*, 2006). This observation suggests that some of the specific details of DSB formation may vary between organisms.

1.2.2 Double-strand Break Repair Pathway

Spo11p catalyses the formation of DSBs by creating a dimer that cleaves the DNA duplex via a reaction similar to transesterification (Keeney, 2001). Following DSB formation, Spo11p remains covalently attached to each of the 5' termini of the DSB (Keeney and Kleckner, 1995, Keeney *et al.*, 1997). Spo11p then needs to be removed from the DNA ends for DSB processing to occur. It has been demonstrated that Spo11p is liberated through an endonucleolytic excision that also removes an oligonucleotide attached to Spo11p (Neale *et al.*, 2005). The MRX complex was suggested to be involved in this process, since Mre11p possesses a single-stranded endonuclease activity (Neale *et al.*, 2005). Furthermore, several point mutations of the MRX components (as stated above, Section 1.2.1) and a mutation that eliminates the Mre11 nuclease, results in prevention of further processing of DSBs, leaving Spo11p covalently bound to the DSB ends (Tsubouchi and Ogawa, 1998, Moreau *et al.*, 1999).

The Sae2 protein was also found to be required for the removal of the Spo11p-oligonucleotide complex (Keeney and Kleckner, 1995, Uanschou *et al.*, 2007, Manfrini *et al.*, 2010). It has been suggested that Sae2p acts in concert with the MRX complex to promote removal of Spo11p, since Sae2p was shown to cooperate with the MRX complex *in vitro* (Lengsfeld *et al.*, 2007).

The 5’ termini of the DSB then undergo nucleolytic resection by several hundred base pairs (Sun *et al.*, 1991, Neale *et al.*, 2005). A specific exonuclease, Exo1p has been suggested to be responsible for the 5’ to 3’ DSB end processing to produce 3’ single stranded overhangs (Khazanehdari and Borts, 2000, Tsubouchi and Ogawa, 2000, Keelagher *et al.*, 2010, Manfrini *et al.*, 2010, Zakharyevich *et al.*, 2010, Hodgson *et al.*, 2010). Deletion of *EXO1* results in reduction in the extent of meiotic DSB resection (Keelagher *et al.*, 2010, Zakharyevich *et al.*, 2010). In addition, Mre11p/Sae2p has also been proposed to be responsible in catalysing the limited resection of DSBs in the absence of Exo1p.

Following resection, one of the 3’ single-stranded overhangs then invades the intact homologous duplex and primes DNA synthesis using the homologous sequence as a template. Single end invasion (SEI) by the 3’ end subsequently displaces the homologous DNA and forms a D-loop structure (Hunter and Kleckner, 2001). Two eukaryotic homologues of bacterial RecA, Rad51p and Dmc1p, assemble at the 3’ tails and form helical filaments (Sun *et al.*, 1991, Keeney *et al.*, 1997, Chen *et al.*, 2008). The nucleoprotein filaments function by searching for sequence homology that generates a connection with the unbroken homologous chromosome and catalyse strand invasion (Neale and Keeney, 2006) . However, the specific mechanism of action of Rad51p and Dmc1p in meiotic DSB repair is still under investigation (Kagawa and Kurumizaka, 2010).

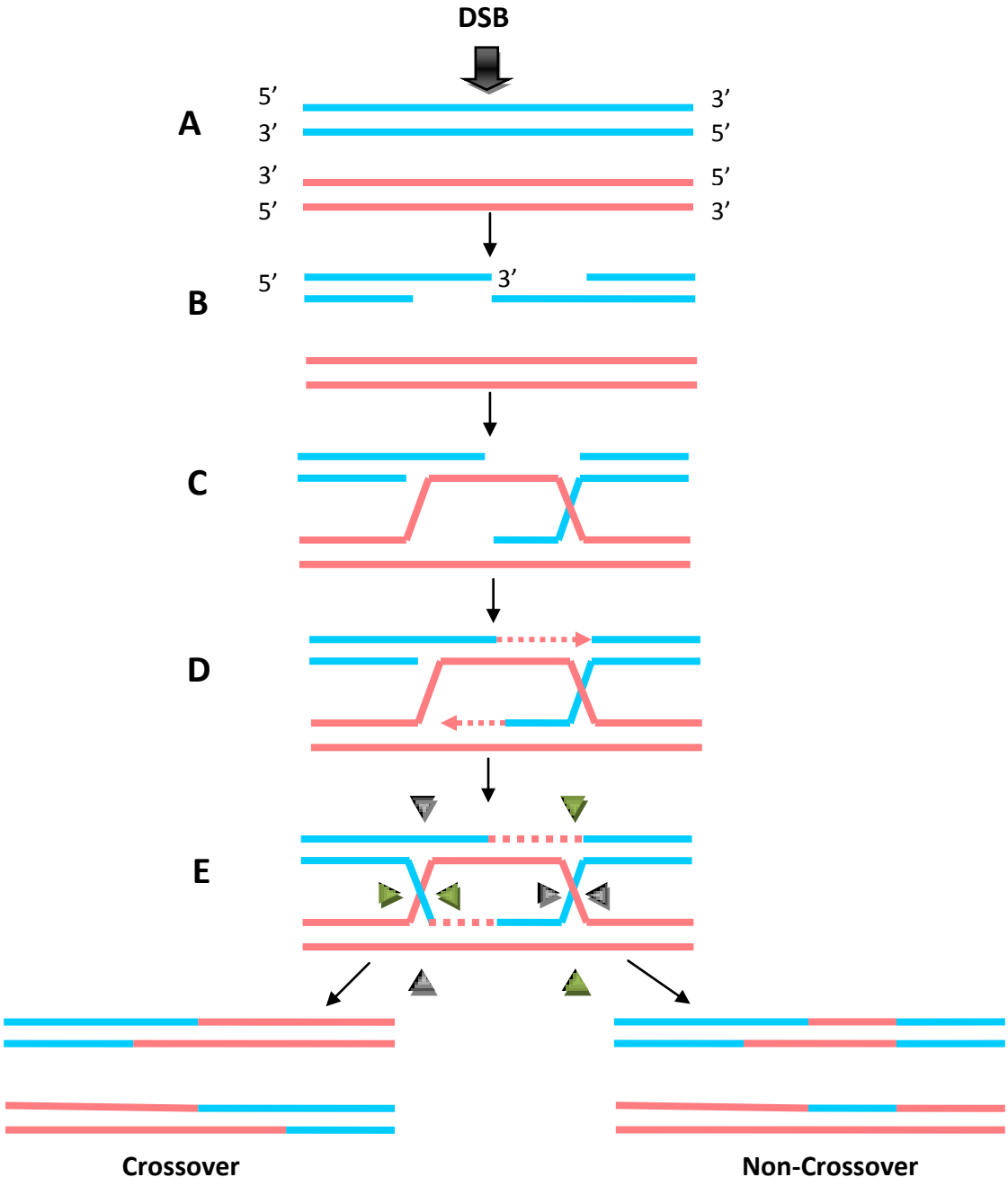


Figure 1.2 : The double-strand break repair model.
A) Formation of DSB. B) DNA is resected in a 5’ to 3’ direction to produce 3’ single-stranded overhangs. C) One of the 3’ overhangs invades the double-stranded DNA homolog forming a single end invasion (SEI) and a D-loop. D) DNA is synthesised using the homologous DNA as template (dotted lines indicates the newly synthesised DNA and arrowheads indicate direction of synthesis). E) Capture of the opposite DNA end and ligation results in the formation of a double Holliday Junction (dHJ). Resolution of the dHJ by cleaving in the opposite orientation (cutting by the same colour of arrowheads) results in a crossover. Cleaving in the same orientation (green and black arrowheads) generates non-crossover products. Adapted from Szostak *et al.* (1983) and Paques and Haber (1999).

Sheridan *et al.* (2008) demonstrated that the filament produced by Dmc1p is similar to those produced by Rad51p. This suggests that the functional difference between them is not due to the intrinsic filament structure (Sheridan *et al.*, 2008). Rather, the distinct activities of Rad51p and Dmc1p are potentially attributed to the different accessory proteins they interact with, that assist their functions (Bugreev *et al.*, 2005, Kagawa and Kurumizaka, 2010).

The major accessory factors of Rad51p include replication protein A (RPA), Rad52p, Rad55p-Rad57p, Rad54p and Tid1p (a homolog of Rad54p). RPA is a single-stranded DNA (ssDNA) binding protein that removes secondary structures to promote Rad51p filament formation (Sugiyama and Kowalczykowski, 2002). However, RPA can also inhibit the binding activity of Rad51p through direct competition for the ssDNA sites (Neale and Keeney, 2006). This inhibitory effect is overcome by the proteins Rad52 and the Rad55-Rad57 complex, thereby facilitating assembly of the Rad51p filament (Sung *et al.*, 2003). Rad54p and Tid1p are members of an ATP-dependent chromatin remodelling activity, and are partially redundant (Shinohara *et al.*, 1997). These proteins have been suggested to promote co-localisation of Rad51p and Dmc1p to the DNA, and blocking assembly of Dmc1p until Rad51p is present (Shinohara *et al.*, 2000).

Dmc1p is assisted by a heterodimeric complex, Hop2p-Mnd1p, in accurate homology searching by distinguishing between homologous and non-homologous sequences (Tsubouchi and Roeder, 2003). This has been demonstrated by deletion of *hop2* or *mnd1* that result in the formation of synaptonemal complex between the non-homologous sequences (Tsubouchi and Roeder, 2003). Mei5p and Sae3p interact specifically with Dmc1p, and mutation of these proteins eliminates the ability of

Dmc1p to bind near a DSB site (Hayase *et al.*, 2004). Furthermore, Mei5p/Sae3p is also required to overcome RPA inhibition of Dmc1p binding to the DNA (Ferrari *et al.*, 2009). These findings suggest that Mei5p and Sae3p are involved in promoting the loading of Dmc1p to the RPA-coated single stranded DNA.

Further synthesis of the invading 3' end extends the D-loop followed by 'capture' of the other DSB end. Subsequent synthesis and ligation of both ends leads to the formation of dHJ, which has been physically detected (Schwacha and Kleckner, 1995). In the original DSBR model, it was thought that either crossovers or non-crossovers are produced, depending on the direction of dHJ resolution (Figure 1.2). The eukaryotic proteins that are involved in dHJ resolution remain elusive but studies have found several proteins that function as the classical dHJ resolvases in *Escherichia coli*¸ such as the RuvC and RusA endonucleases (West, 1997, Boddy *et al.*, 2001). A potential candidate is the Yen1 protein (human orthologue Gen1) in *S. cerevisiae*, which is thought to give rise to crossovers and non-crossovers in equal efficiency (Ip *et al.*, 2008).

In *S. pombe*, it has been demonstrated that single HJs were mainly produced as meiotic recombination intermediates, which can be resolved to produce crossovers (Cromie *et al.*, 2006). The Mus81p-Eme1p complex (Mus81p-Mms4p in *S. cerevisiae*) has been suggested to function as the HJ resolvase in *S. pombe* (Boddy *et al.*, 2001). This hypothesis is supported by the observation that *mus81* mutants that generate inviable spores can be rescued by overexpression of the bacterial HJ resolvase, *rusA* (Boddy *et al.*, 2001). Therefore, it was thought that RusA can directly substitute for the Mus81p-Mms4p activity. However, only modest reduction of crossovers was seen in the *mms4*

mutant in *S. cerevisiae* (de los Santos *et al.*, 2001). It was also demonstrated that expression of the bacterial dHJ resolvase in *S. cerevisiae* did not suppress the *mus81* phenotypes (de los Santos *et al.*, 2003). Additionally, the *mms4* mutant does not accumulate dHJs as would be expected if *MMS4* is solely required for dHJ resolution (Hollingsworth and Brill, 2004). These observations suggest that Mus81p-Mms4p is not solely required for dHJ resolution in *S. cerevisiae.*

The proteins Msh4p and Msh5p, which act as a heterodimer, were also proposed to be required in the formation of crossing over in *S. cerevisiae*, by binding to and stabilising dHJ (Hollingsworth and Brill, 2004, Snowden *et al.*, 2004). Furthermore, the Mlh1p and Mlh3p heterodimer may be involved in catalysing dHJ resolution, since they were found to act at a later step in recombination (Hunter and Borts, 1997, Wang *et al.*, 1999, Cotton *et al.*, 2010). Mlh3p is also known to possess endonuclease activity, and disruption of the putative endonuclease domain of Mlh3p led to a defect in crossover formation (Nishant *et al.*, 2008). This further supports the hypothesis that Mlh3p is essential in the resolution of dHJ. Moreover, ATP binding by Mlh1p and Mlh3p were shown to be required for crossing over (Hoffmann *et al.*, 2003, Hoffmann and Borts, 2004, Cotton *et al.*, 2010). This suggests that dHJ resolution may be facilitated by conformational change of Mlh1p and Mlh3p by ATP binding.

1.2.3 Synthesis-Dependent Strand Annealing

Although many studies have confirmed the presence of DSBR pathway intermediates including the formation of DSBs and HJs, several observations conflict with some ideas of the canonical DSBR model. In particular, Schwacha and Kleckner (1995) detected the existence of HJs before the formation of heteroduplex DNA (hDNA). This is contrary to

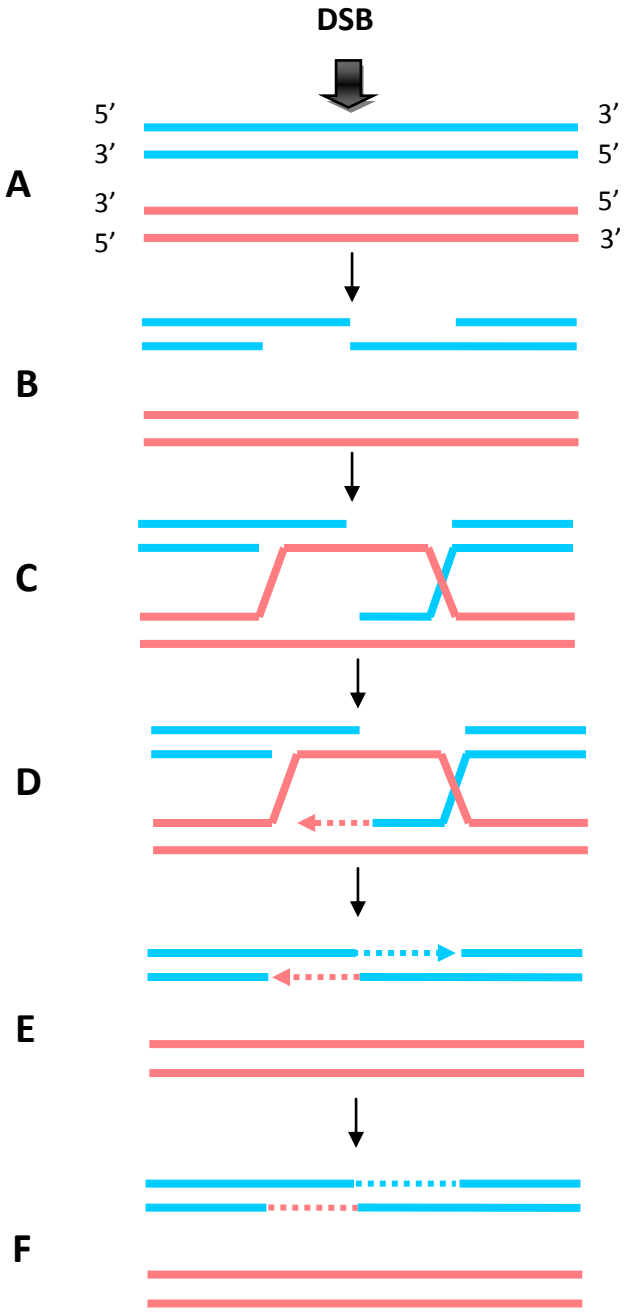


Figure 1.3 : The synthesis dependent strand annealing model
A - D) Steps similar to the DSBR model (Figure 1.2). E) Displacement of the invading strand disrupted the D-loop. F) Annealing of the displaced 3’ end with the second end of the break forming a non-crossover product. Adapted from Paques and Haber (1999).

the DSBR model that proposed earlier generation of hDNA, during the time of strand invasion. It has also been demonstrated that non-crossovers were produced at normal levels in the mutation of *ndt80*, which accumulates unresolved dHJs and is defective in crossover formation (Allers and Lichten, 2001). This implies that the dHJ is a precursor only for crossover products whereas the non-crossover products are derived from a different pathway. Therefore, an alternative model termed synthesis-dependent strand annealing (SDSA) was proposed to explain the production of non-crossover products (Figure 1.3) (Paques and Haber, 1999). In the SDSA model, DSBs are repaired by strand invasion of the 3' single stranded overhang into the unbroken homologous duplex. Similar to the DSBR model, the 3' end then acts as a primer for DNA synthesis using the invaded duplex as template (Cromie and Smith, 2007). The newly synthesised DNA is subsequently displaced from the invaded duplex and re-anneals with the other end of the break. Both ends are then ligated to conclude the repair.

1.3 Non-Mendelian Segregation in Yeast

Meiotic recombination has been most extensively studied in model organisms particularly in the budding yeast, *S. cerevisiae*. Being a single-celled organism, yeast cells are easily manipulated. Additionally, *S. cerevisiae* is stable as both a diploid and a haploid. The four meiotic products can easily be recovered and exploited for the study of meiosis. Spores are enclosed in a sac called an ascus that can be digested to release them. Thus, different classes of recombination including crossing over, gene conversion and post-meiotic segregation (PMS) can be genetically analysed by micro-dissection of yeast tetrads.

During strand exchange, mismatched DNA can be formed between two homologous chromosomes (Petes, 2001). Repair of the mismatched DNA can give rise to gene conversion or can be restored to the parental genotype. Failure to correct mismatches can result in PMS event. The level of gene conversion and PMS can be monitored by analysis of a single heterozygous marker (Figure 1.4). Normal Mendelian segregation of a given heterozygous marker results in 2:2 segregation pattern in which 2 alleles from each parents are recovered. For fungi such as *Neurospora* that contain eight spores in an ascus due to mitotic duplication of each chromosome after meiosis, normal Mendelian segregation is 4:4 (Figure 1.4). Restoration of the parental genotype following mismatch repair also gives rise to a 4:4 pattern.

Gene conversion events have 6:2 or 2:6 segregation that represent 6 alleles from one parent while 2 alleles from another. PMS give rise to 5:3 or 3:5 patterns, and are characterised by sectored spore colonies in *S. cerevisiae* tetrads. Crossing over of the chromosome can be determined using at least two heterozygous markers (Petes, 2001). Crossovers are reflected by the outcome of non-parental combinations of the linked markers flanking a given segment in the chromosome (Lichten and Goldman, 1995).

1.4 Crossover Control

Crossing over between homologous chromosomes is essential for the success of meiosis by ensuring faithful segregation in the first meiotic division. Hence, crossover events need to be strictly regulated so that crossovers are properly distributed along the chromosome. One regulatory mechanism observed in most eukaryotes for accurate chromosome disjunction is the obligate crossover (Jones and Franklin, 2006).

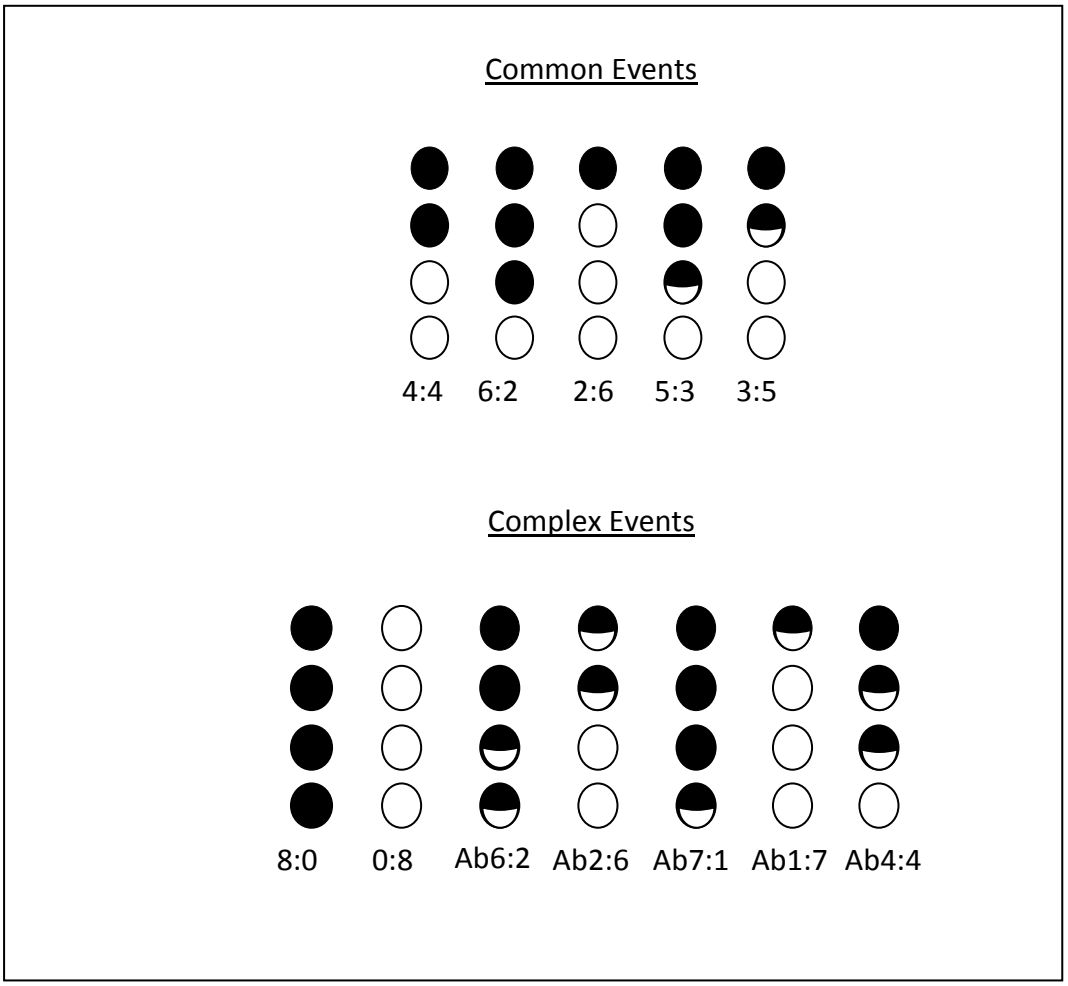


Figure 1.4 : The patterns of segregation of a heterozygous marker observed from tetrad analysis. Following meiosis, a diploid cell that is heterozygous for a single marker can give rise to a number of common segregation patterns including normal Mendelian segregation (4:4), gene conversion (6:2 or 2:6) and post-meiotic segregation (5:3 or 3:5). Restoration events can also produce 4:4 segregation and are undetectable. Occasionally some complex aberrant segregation events involving all four chromatids can occur from the repair of mismatched DNA. These include two gene conversion events (8:0 or 0:8), two independent PMS events (Ab6:2 or Ab2:6), and simultaneous gene conversion and PMS (7:1 or 1:7).

This refers to the guaranteed event that each chromosome receives at least one cross-over despite a low average crossover number per chromosome. Another manifestation of crossover control is the process called crossover interference (Hillers, 2004). Interference refers to the phenomenon in which a crossover at one chromosomal site limits the probability that another event occurs simultaneously in the immediate vicinity. The intensity of crossover interference is maximal over short distances and reduces progressively with increasing distance from the crossover site (Malkova *et al.*, 2004). Hence, interference creates regularly spaced crossovers within a chromosome.

Crossover homeostasis is another mechanism of crossover control that has been suggested to contribute to the production of an obligate crossover. It has been observed that the number of crossovers is maintained at the expense of non-crossovers even when the number of DSBs is reduced (Jones *et al.*, 2006). This was shown by analysing a series of Spo11p mutants with varying levels of activity, in which reduction in DSB production did not result in a parallel decrease in crossovers (Martini *et al.*, 2006). In addition, crossover interference was also maintained when the DSB frequency was reduced, implying an association between both mechanisms for crossover control (Martini *et al.*, 2006).

It is not yet well established how crossover control works. A 'counting' model posits that each crossover event is separated by a fixed number of non-crossover outcomes and thus exhibit interference (Foss *et al.*, 1993). However, the observation that the crossover/non-crossover ratio varied with changes in the level of DSBs appears to contradict the counting theory (Martini *et al.*, 2006). Another hypothesis to explain interference is described by the condition that chromatin is under internal compaction

stress (Borner *et al.*, 2004, Kleckner *et al.*, 2004). Crossover formation releases this stress for a certain chromosomal distance and prevents other crossovers from occurring within the area (Borner *et al.*, 2004, Kleckner *et al.*, 2004).

The mechanism of crossover control was initially thought to be a consequence of the synaptonemal complex (SC) polymerisation, which transmits an inhibitory signal preventing the occurrence of a crossover within the area (Egel, 1978). This hypothesis is supported by several observations from genetic assays that showed simultaneous impairment of SC formation and crossover interference in certain *zmm* mutants (e.g., *zip1* and *msh4*) (Sym and Roeder, 1994, Novak *et al.*, 2001). The ZMM groups (also known as the synapsis initiation complex; SIC) are the meiosis-specific proteins which consists of Zip1, Zip2, Zip3, Zip4, Msh4, Msh5, Mer3 (Lynn *et al.*, 2007) and the newly characterised Spo16 protein (Shinohara *et al.*, 2008). Mutation of these proteins are known to disrupt strand exchange structure formation and the assembly of the SC (Székvölgyi and Nicolas, 2009).

However, several other observations argue that the SC is not required for interference (Bishop and Zickler, 2004). One such observation was obtained from cytological analysis of the ZMM proteins (including the Zip2p and Zip3p), which are known to localise with crossover-designated sites (Chua and Roeder, 1998, Agarwal and Roeder, 2000). Immunostaining of Zip2 foci revealed an interference distribution in the wild type, *zip1*Δ and *msh4*Δ strains, indicating that interference can occur in the absence of the SC (Fung *et al.*, 2004). Consistent with these findings, mutants that lack the ZMM proteins at high temperature are defective in SC formation and crossover production while exhibiting normal levels of DSBs and non-crossovers (Borner *et al.*, 2004). This

further implies that the crossover control is independent of SC and suggested that crossover control is imposed at an early stage of recombination (Bishop and Zickler, 2004).

1.5 Meiotic Recombination Hotspots

Meiotic recombination is not uniform throughout the genome. There are areas in the chromosome that exhibit relatively high levels of recombination compared to other regions (Lichten and Goldman, 1995). These regions are called recombination hotspots. Several hotspots have been examined in detail in *S. cerevisiae*; *HIS4* (Detloff *et al.*, 1992, White *et al.*, 1991, White *et al.*, 1993), *ARG4* (de Massy and Nicolas, 1993, Sun *et al.*, 1991) and *HIS2* (Malone *et al.*, 1994). Other hotspots include the Tn3-derived transposable element (Stapleton and Petes, 1991) and the artificially created *HIS4-LEU2* hotspot, generated by insertion of a *LEU2* segment adjacent to the *HIS4* locus (Cao *et al.*, 1990).

The site of recombination hotspots has been shown to be strongly associated with local meiosis-specific DSBs (Sun *et al.*, 1989, Cao *et al.*, 1990). Moreover, deletions which reduce the level of DSBs (e.g., *rad50* and the promoter region of *ARG4*) also result in decreased levels of recombination (Cao *et al.*, 1990, de Massy and Nicolas, 1993, Fan *et al.*, 1995). Association between DSBs and recombination has also been suggested from genome-wide studies by mapping DSB sites across the genome. DSBs were found to be clustered at localised preferential sites where most recombination occurred within those regions (Petes, 2001, Pan *et al.*, 2011).

1.5.1 Factors Influencing Recombination Hotspots in *S. cerevisiae*

A whole-genome DSB mapping analysis has been widely used to identify DSB hotspots and to study factors influencing the pattern of DSB distribution in *S. cerevisiae* (Lichten, 2008). In early studies of genome-wide hotspots mapping, the *rad50S* mutant strain was typically used (Gerton *et al.*, 2000, Petes, 2001, Mieczkowski *et al.*, 2006). In such a mutant, the intermediate stage of meiotic prophase is blocked, and Spo11p remains covalently attached to the DSB ends (Cao *et al.*, 1990). Hence, regions with enriched Spo11p could be detected by using Spo11p-DNA complexes as hybridisation probes for yeast microarrays (Petes, 2001).

In these studies, genomic sites exhibiting low levels of recombination (coldspots) have also been reported, including the centromeric and telomeric regions (Gerton *et al.*, 2000, Mieczkowski *et al.*, 2006, Buhler *et al.*, 2007). However, some of these 'DSB coldspot' loci were observed in late replicating regions in the *rad50S* mutation, suggesting that break formation at these sites occurs later than average (Borde *et al.*, 2000). Therefore, this raised a possibility that the level of DSBs is underrepresented in maps from the *rad50S* mutant, hence might affect some of the interpretation of results in these studies.

In order to overcome limitations in the previous studies, another method for mapping DSB hotspots was then developed, using mutants lacking Dmc1p (Blitzblau *et al.*, 2007, Buhler *et al.*, 2007). Dmc1p is the protein responsible to catalyse strand invasion of the 3' single-stranded tails following DNA resection (Section 1.2.2). Therefore, the *dmc1*Δ mutant is able to remove Spo11p, but is deficient for DSB repair. Hence the

accumulated single-stranded DNA was used to prepare microarray probes instead of Spo11p.

In studies using the *dmc1*Δ mutation, high levels of DSB formation was observed at known hotspots, and substantial DSBs were also found at regions previously reported to lack DSB formation (Blitzblau *et al.*, 2007, Buhler *et al.*, 2007). This confirms that the level of DSB is underrepresented in some regions of DSB coldspots in the *rad50S* mutant. Recently, a quantitative and much higher resolution method were used for mapping recombination hotspots, by direct sequencing of purified oligonucleotides attached to Spo11p (Pan *et al.*, 2011). The study provides a more detailed compilation of DSB hotspots in *S. cerevisiae* and information underlying hotspot traits. From these genome-wide screening, together with studies of individual hotspots, various *cis*- and *trans*-acting factors influencing hotspots distribution have been suggested (discussed below).

The status of chromatin structure has been suggested to influence preferred position of DSBs and recombination hotspots in the genome. Studies in *S. cerevisiae* showed that DSB sites exhibit hypersensitivity toward nucleases, particularly DNAse I and micrococcal nuclease (MNase) (Wu and Lichten, 1994, Ohta *et al.*, 1994, Fan and Petes, 1996). Mutations that disrupt the production of nucleosomes upstream of the *PHO5* loci results in parallel increase in the level of DSBs (Wu and Lichten, 1994). Likewise, increased levels of DSB formation correlate with increased sensitivity to MNase at *ARG4* (Ohta *et al.*, 1994). Furthermore, sites with elevated DSBs at *ARG4, LEU2-CEN3* and several other loci exhibited DNase I or MNase hypersensitivity even before the onset of meiosis (Wu and Lichten, 1994, Ohta *et al.*, 1994, Mizuno *et al.*, 1997). These

observations suggest a requirement for an 'open' chromatin structure for recombination hotspot activity. Consistent with this hypothesis, comparison between the high-resolution maps of MNase-resistant nucleosomes with that of Spo11p-oligonucleotides showed that DSBs tend to occur in nucleosome-depleted regions (Pan *et al.*, 2011).

However, nuclease-sensitive chromatin alone is not sufficient to create meiotic recombination hotspot. In particular, Ohta *et al.* (1994) showed that not all of the nuclease-hypersensitive regions are sites of meiosis-specific DSBs. Furthermore, insertion of the *ARG4* promoter sequence in other chromosomal locations did not increase DSB formation even when these regions are hypersensitive to nucleases (Wu and Lichten, 1995). Moreover, mutations that abolished *HIS4* hotspot activity only eliminate some but not all of the DNase I-hypersensitive sites (Fan and Petes, 1996). These findings indicate that although an 'open' chromatin appears necessary, other factors might play a more dominant role in initiating recombination.

It has also been suggested that hotspot activity requires specific chromatin remodelling activity, particularly histone modifications (Mieczkowski *et al.*, 2007, Merker *et al.*, 2008, Borde and Cobb, 2009). For example, trimethylation of histone H3 lysine 4 was demonstrated to mark hotspot sites prior to meiosis (Borde *et al.*, 2009). Furthermore, higher levels of recombination and DSB formation at *HIS4* were found to correlate with increased acetylation of histone H3 lysine 27 (Merker *et al.*, 2008). These histone modifications facilitate DSB formation potentially by providing a favourable chromatin state, which permits access to the recombination machinery (discussed in Chapter 5).

Three major categories of hotspots have been proposed in *S. cerevisiae,* α-, β- and γ- (Kirkpatrick *et al.*, 1999a). α-hotspots are defined as hotspots that require transcription factor binding for activation (Kirkpatrick *et al.*, 1999a). A genome-wide analysis showed that Spo11p-oligonucleotide complexes were frequently mapped near binding sites of numerous transcription factors (Pan *et al.*, 2011). One example of an α-hotspot is the widely studied *HIS4*, which requires the binding of transcriptional activators, Bas1p, Bas2p, Rap1p and Gcn4p (Fan *et al.*, 1995, White *et al.*, 1993, White *et al.*, 1991, Abdullah and Borts, 2001). These transcription factors were among those whose binding sites overlapped with the Spo11p-oligonucleotide hotspots (Pan *et al.*, 2011). Interestingly, the top ranked transcription factors based on their correlation with Spo11p-oligonucleotide counts (Ino2p/Ino4p complex, Pho4p, Leu3p and Hap1p) were not previously known to influence meiotic recombination (Pan *et al.*, 2011). Therefore, this observation suggests that the presence of transcription factor-associated hotspots could be common for other regions in the genome.

The fact that most DSB sites are also located in the promoter region upstream of coding sequences further indicate the association of transcription factors with recombination hotspots (Wu and Lichten, 1994, Blitzblau *et al.*, 2007, Buhler *et al.*, 2007, Pan *et al.*, 2011). Furthermore, it has also been shown that DSBs occur preferentially in intergenic regions rather than within genes (Baudat and Nicolas, 1997, Lichten, 2008, Pan *et al.*, 2011). However, the transcription factor-dependent hotspot activity does not require high levels of transcription. This has been demonstrated by White *et al.* (1992), in which the removal of the upstream TATAA promoter element of *HIS4* only reduces the level of transcription but has no effect on the level of recombination. Instead, the binding of transcription factors is thought to maintain an

'open' state of chromatin to allow better access to the recombination machinery to form DSBs (Kirkpatrick *et al.*, 1999a). Another alternative hypothesis is that the recombination factors are directly recruited to the chromatin in a manner facilitated by the activation domain of bound transcription factors (Kirkpatrick *et al.*, 1999a).

β-hotspots are defined as hotspots that occur at specific nuclease-sensitive chromosomal regions without requiring the binding of transcription factors (Kirkpatrick *et al.*, 1999a). An example of a β-hotspot is the artificial insertion of 12 tandem repeats of the 5'-CCGNN replacing the upstream regulatory sequences of *HIS4* (Kirkpatrick *et al.*, 1999b). The $(CCGNN)_{12}$ tract is hypersensitive to DNase I and results in elevation of the recombination frequency of *HIS4*. Since no transcription factors are known to bind to the $(CCGNN)_{12}$ repeat, the hotspot is suggested to be created by the nucleosome-excluding sequences (Petes, 2001). The *HIS4-LEU2* hotspot was suggested to be another possible β-hotspot since it contains a tandem repeat of four 5'-CGGATCCG sequences (Xu and Kleckner, 1995).

Another group of hotspots, the γ-hotspots occur in regions that are associated with high GC composition (Petes, 2001). Several global analyses showed that meiotic DSBs tend to be clustered at regions with high GC content (Gerton *et al.*, 2000, Blat *et al.*, 2002, Mieczkowski *et al.*, 2006, Pan *et al.*, 2011). The mechanism by which recombination hotspots correlate with the GC-rich regions remains elusive. It has been proposed that recombination may be controlled either directly by the GC content or by a third unknown factor (e.g., transcription factors) that also affects the GC content (Marsolier-Kergoat and Yeramian, 2009). These proteins could either interact with the recombination machinery or induce chromosomal modifications to allow entry of the

recombination factors (Petes, 2001). Another alternative possibility is that the GC content is directly associated with chromatin alterations that promote recombination (Mieczkowski *et al.*, 2006).

1.5.2 Meiotic Recombination Hotspots in Other Organisms

In *Schizosaccharomyces pombe*, the widely studied *M26*-related hotspots consist of the well-characterised *ade6-M26* and the sequence-dependent $M26_{CS}$ (*M26* consensus sequence) (Pryce and McFarlane, 2009). The *ade6-M26* hotspot is a meiosis-specific hotspot generated by a G to T transversion mutation within the *ade6* gene, which produces a sequence motif of 5'-ATGACTG-3' (Gutz, 1971, Ponticelli *et al.*, 1988). This *M26* heptamer provides binding sites for the heterodimeric transcription factors, Atf1p and Pcr1p (Mts1p/Mts2p) (Wahls and Smith, 1994, Kon *et al.*, 1997). Binding of these transcription factors is required for the *ade6-M26* hotspot activity, reflecting the features of α-hotspots in *S. cerevisiae* (Wahls and Smith, 1994, Kon *et al.*, 1997).

Similarly, activation of the *ade6-M26* hotspot is not a consequence of increased levels of *ade6* transcription (Kon *et al.*, 1997). An alteration in the structure of the chromatin near the *ade6-M26* hotspot has been demonstrated upon entry into meiosis (Mizuno *et al.*, 1997). In addition, mutations that result in loss of specific chromatin modifications (including acetylation of histone H3 and H4) also reduced *ade6-M26* hotspot activity (Yamada *et al.*, 2004). These studies suggest that chromatin remodelling activity is also required in the activation of the *M26* hotspot.

A global screening of short nucleotide sequences for hotspot activity has been performed in *S. pombe*, which identified many short motifs (at around 6 bp in length) occurring multiple times in the genome (Steiner *et al.*, 2009). At least five different

DNA sequence motifs found to activate hotspots, including the motif recognised by Atf1p/Pcr1p, which appeared in at least 15% of the total pool of hotspot sequences. Wahls and Davidson (2011) hypothesised that recombination hotspots are governed by specific short DNA sequences, which can be generated in the genome by alteration of a single nucleotide. These hotspot activating motifs may be targeted by sequence-specific DNA binding proteins, which act redundantly to promote DSB formation (Wahls and Davidson, 2011). This may involve epigenetic modification of chromatin structure by the transcription factor binding, which promotes DSB formation.

Recombination hotspots in mouse were mainly identified in the major histocompatibility complex (MHC) regions, including the proteasome subunit β type-9 (*Pmsb9*) hotspot (Guillon and de Massy, 2002), the Eβ-hotspot (Shenkar *et al.*, 1991) and the *Pb* hotspot (Isobe *et al.*, 2002). Interestingly, the mouse Eβ-hotspot (located in the second intron of the class II Eβ gene) imitates certain characteristics of the α-hotspot in yeast. In particular, two DNase I hypersensitive sites were detected in the Eβ intron, with one of them located adjacent to the putative transcription factor binding sites (Shenkar *et al.*, 1991). Similar to yeast, these hypersensitive sites alone are not sufficient to produce hotspot activity, since not all DNase I hypersensitive sites are associated with recombination hotspots (Shenkar *et al.*, 1991). Additionally, nuclease hypersensitive sites were not detected at another MHC-associated hotspot, the *Psmb9* gene (Arnheim *et al.*, 2007). Therefore, the binding of specific transcription factors could be essential for hotspot activity by generating chromatin status that is more permissive to the formation of DSB.

In humans, several recombination hotspots have been identified including the MHC-associated hotspots (*DPA1*, *DNA1* and *TAP2*), the minisatellite-associated hotspot MS32 and the male-specific SHOX hotspot within the pseudoautosomal region (Arnheim *et al.*, 2007). Detailed searches for a common sequence motif for recombination hotspots in humans have result in the identification of a degenerate 13-mer sequence, which is highly associated with the presence of hotspots (Myers *et al.*, 2008). This consensus motif corresponds to the zinc-finger domains of a PR domain-containing 9 (Prdm9) protein, a transcription factor with histone H3 lysine 4 (H3K4) methyltransferase activity (Baudat *et al.*, 2010). Additionally, Grey *et al.* (2011) demonstrated that Prdm9 binding at hotspots stimulates local H3K4 trimethylation, which is correlated with the hotspot activity (discussed further in Chapter 5).

The existence of a putative α-hotspot has also been suggested in humans based on a population genetics statistical analysis (Zhang *et al.*, 2004). Using the European population samples in the Seattle SNPs database, nearly half of the detected putative hotspots were found in the promoter regions (Zhang *et al.*, 2004). In addition, the presence of transcription factor binding sites was also detected within recombination hotspots in another genome-wide computational analysis (Mani *et al.*, 2009). These observations hint at a conserved existence of transcription factor-dependent hotspot activity in humans, although genetic evidence of the α-hotspot is yet to be discovered.

Information on factors controlling the distribution of recombination hotspots can provide great benefits. Understanding the molecular mechanisms determining hotspot selection in humans could be beneficial for the study of genome evolution and the analyses of complex diseases (Jeffreys *et al.*, 2004). In specific, localisation of

recombination hotspots could provide explanations for haplotype patterns thus facilitating mapping of disease related-loci by their association with other markers (Hey, 2004). Furthermore, knowledge of factors influencing recombination hotspots in yeast and other model organisms offers a means to learn about other recombination-associated processes. The association between transcription factors with hotspot activity has also become increasingly appreciated in different organisms. However, the mechanism by which these proteins influence hotspot activity and factors controlling this interaction are yet to be fully elucidated. In this study, we focused on characterising factors influencing *HIS4* hotspot activity in *S. cerevisiae* to further understand the regulation of this transcription factor-dependent hotspot.

1.5.3 The Regulation of *HIS4* Transcription and Recombination

The recombination hotspot associated with the *HIS4* gene in *S. cerevisiae* is positioned in the region between the 5' end of *HIS4* and the 3' end of *BIK1* (Detloff *et al.*, 1992). This region displayed high levels of DSBs and recombination (White *et al.*, 1993, Detloff *et al.*, 1992, Fan *et al.*, 1995). The *HIS4* hotspot is extensively used in the study of meiotic recombination in various strain backgrounds frequently Y55 (Hoffmann and Borts, 2004, Cotton *et al.*, 2009), SK1 (Keelagher *et al.*, 2010) and S288C (White *et al.*, 1991, White *et al.*, 1992).

1.5.3.1 Transcription Factor Binding at *HIS4*

The promoter region of *HIS4* is bound by the transcription factors Bas1p/Bas2p (Basal), Gcn4p (General Control Non-derepressible) and Rap1p (Repressor Activator Protein) (White *et al.*, 1993, Arndt *et al.*, 1987). At the transcriptional level, the expression of *HIS4* is regulated by two control systems that operate independently from one another

(Tice-Baldwin *et al.*, 1989). Gcn4p activates *HIS4* via the general amino acid control (GAAC) system while the Bas1p/Bas2p (Bas1/2p) complex is responsible for the basal expression of the gene (Arndt *et al.*, 1987). Strains with mutations of *bas1, bas2* and *gcn4* require histidine for growth and only have modest level of transcription of *HIS4* (Tice-Baldwin *et al.*, 1989).

Bas1p binds to DNA sequences that include the 5'-TGACTC-3' hexanucleotide motif upstream of *HIS4* (Tice-Baldwin *et al.*, 1989, Daignan-Fornier and Fink, 1992, Høvring *et al.*, 1994). Similarly, the highly conserved 5'-TGACTC-3' binding sequence is also the core element of the recognition site for Gcn4p (Arndt and Fink, 1986). Gcn4p has five binding sites upstream of *HIS4*, sites A to E (Figure 1.5) and binds them in a selective manner (Arndt and Fink, 1986). Of these, Gcn4p binds with highest affinity to site C (Arndt and Fink, 1986, Hope and Struhl, 1985). The common binding motif for Bas2p was found at the position immediately adjacent to the Bas1p binding sites upstream of *HIS4* (Tice-Baldwin *et al.*, 1989, Rolfes *et al.*, 1997).

Rap1p is an essential DNA binding protein involved in either activating or repressing transcription of many genes, in a context-dependent manner (Piña *et al.*, 2003, Joo *et al.*, 2011). At *HIS4*, Rap1p binds adjacent to the Bas2p binding site, overlapping with the high affinity Gcn4p binding site (Figure 1.5) (Devlin *et al.*, 1991). It has been shown that Rap1p and Gcn4p compete to bind at site C *in vitro* (Arndt and Fink, 1986), but could likely bind simultaneously *in vivo* (Devlin *et al.*, 1991). The binding of Rap1p does not efficiently activate *HIS4* transcription by itself, but is required for both Gcn4p- and Bas1/2p-activation of *HIS4* (Devlin *et al.*, 1991). It has been shown that the presence of

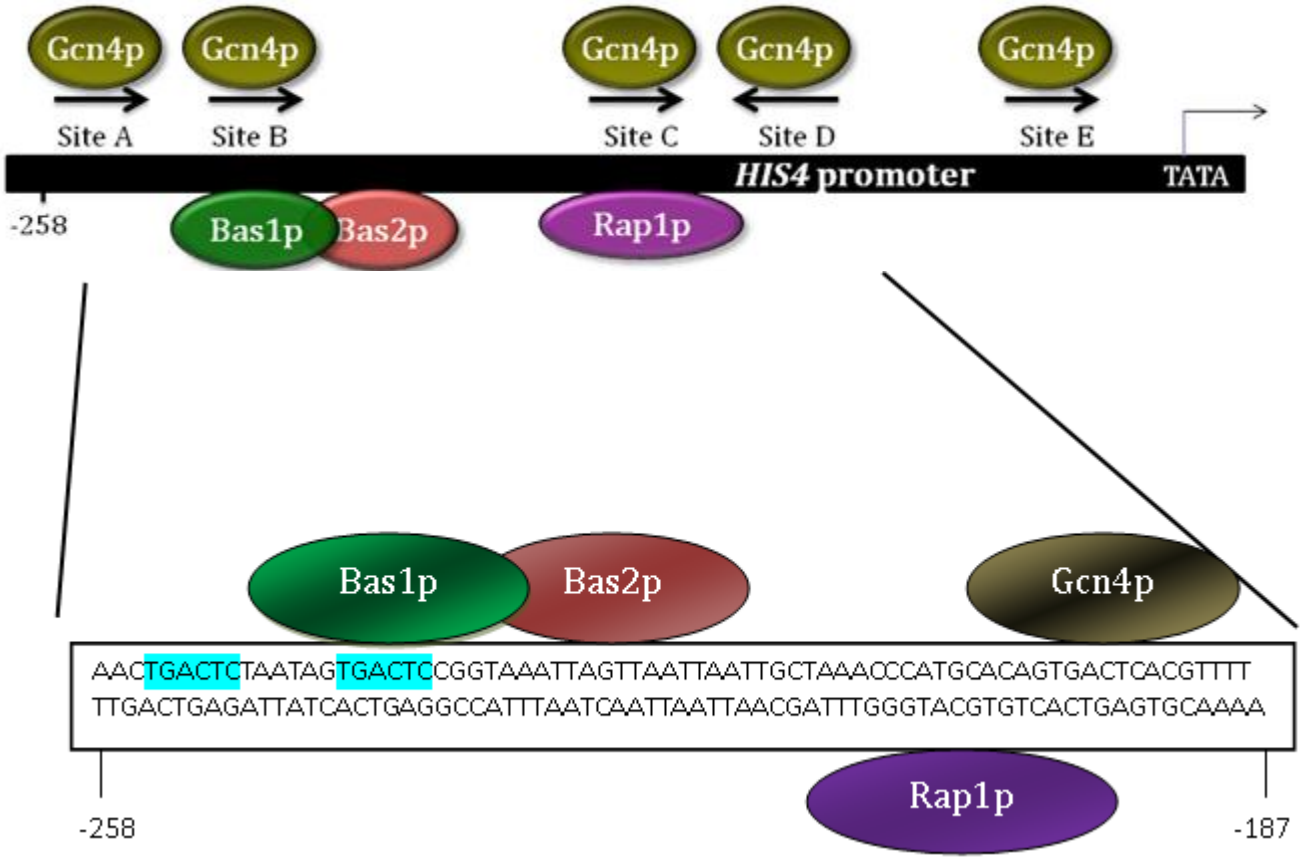


Figure 1.5 : DNA binding sites for Bas1p, Bas2p, Gcn4p and Rap1p at the promoter region of *HIS4.* Gcn4p binds at sites A – E,; Bas1p also binds at site A but is shown at site B, which it has highest affinity; Gcn4p binds with high affinity at site C. The binding site of Rap1p is overlaps with site C. Adapted from Tice-Baldwin *et al.* (1989), Devlin *et al.* (1991) and Cotton (2007).

Rap1p binding site results in increased sensitivity to nucleases at the high affinity Gcn4p binding site and the Bas1/2p binding sites (Devlin *et al.*, 1991). Therefore, this suggests that the function of Rap1p in activating the transcription of *HIS4* is by maintaining and enhancing the accessibility of binding sites for other transcription factors.

With regard to meiosis, White *et al.* (1992 and 1993) showed that removing the binding sites of Rap1p and Bas2p upstream of *HIS4* reduces the level of recombination. Mutating *bas1* or *bas2* also reduces DSB production and eliminates *HIS4* hotspot activity (White *et al.*, 1993, Fan *et al.*, 1995). Furthermore, the level of DSBs is strongly decreased when binding sites of Rap1p were removed (Fan *et al.*, 1995). These findings indicate that the binding of Bas1p, Bas2p and Rap1p are required for hotspot activity at the *HIS4* locus. White *et al.* (1993) also demonstrated that in the absence of Bas1p and Bas2p, insertion of two binding sites of Rap1p results in the wild-type level of recombination. This suggests that additional binding of Rap1p can substitute for recombination activity that depends on Bas1p and Bas2p.

Loss of Gcn4p binding activity was shown to have little effect on recombination (White *et al.*, 1992). However, Gcn4p has been demonstrated to be required and produce a positive regulatory effect on *HIS4* hotspot activity in a different strain background (Cotton *et al.*, 2009). Specifically, Abdullah and Borts (2001) demonstrated that deletion of *gcn4* reduces the level of recombination at *HIS4* while overexpression of Gcn4p results in an increased level of the NMS events. Therefore, Gcn4p could be essential in regulating recombination, particularly in response to various stress conditions known to affect Gcn4p activity.

1.5.3.2 Gene Regulation by Bas1p and Bas2p

Bas1p contains a Myb-related protein domain in its amino-terminal region that is homologous to the proto-oncogene family (Tice-Baldwin *et al.*, 1989, Høvring *et al.*, 1994). Bas2p (also known as Pho2p and Grf10p) is a DNA-binding protein that contains an amino-terminal homeobox domain (Bürglin, 1988, Tice-Baldwin *et al.*, 1989). Bas1p and Bas2p are known to cooperatively regulate several genes involved in the pathways of purine and histidine biosynthesis, and the one carbon metabolism pathway (Daignan-Fornier and Fink, 1992, Arndt *et al.*, 1987, Denis *et al.*, 1998, Springer *et al.*, 1996, Denis and Daignan-Fornier, 1998). In addition to the interaction with Bas1p, Bas2p also interacts with other partner proteins to regulate transcription of genes in other metabolic pathways. Bas2p interacts with Pho4p (a basic helix-loop-helix protein) to activate genes involved in the phosphate utilisation pathway (Barbarić *et al.*, 1996). Additionally, Bas2p interacts with Swi5p (a zinc finger protein) to regulate the expression of the *HO* genes (Brazas and Stillman, 1993a).

The cooperative activation by Bas1p and Bas2p of their target genes has been shown to be stimulated under adenine limitation (Arndt *et al.*, 1987, Daignan-Fornier and Fink, 1992, Denis *et al.*, 1998). Furthermore, the interaction between Bas1p and Bas2p is regulated by two intermediates from the purine biosynthesis pathway, 5'-phosphoribosyl-4-carboxamide-5-aminoimidazole (AICAR) and 1-(5′-phosphoribosyl)-4-(*N*-succinocarboxyamido)-5-aminoimidazole (SAICAR) (Rebora *et al.*, 2001, Rebora *et al.*, 2005, Pinson *et al.*, 2009). It has been demonstrated that mutations that accumulate AICAR/SAICAR results in increased transcription of *HIS4* through the activation of Bas1p and Bas2p interaction (Rebora *et al.*, 2005, Pinson *et al.*, 2009). A

more detailed explanation on the regulation of purine biosynthesis on *HIS4* transcription is provided in Chapter 3.

1.5.3.3 General Amino Acid Control (GAAC) by Gcn4p

Gcn4p is a leucine zipper-related activator that was shown to regulate at least 500 genes, under the general amino acid control system (Natarajan *et al.*, 2001). Gcn4p regulates genes involved in the amino acid, purines, pyrimidines and vitamin biosynthetic pathways and some genes involved in the metabolism of glycogen (Hinnebusch and Natarajan, 2002). Additionally, genes from the mitochondrial carrier family and the amino acid transporter family are also regulated by Gcn4p (Natarajan *et al.*, 2001).

The general amino acid control is a regulatory system adapted by cells in response to the deprivation of amino acids (Hinnebusch, 1988). This response operates at the translational level, increasing protein synthesis of Gcn4p when amino acids are limited. In addition, the synthesis of Gcn4p can also be induced under purine limitation, glucose deprivation and growth on a non-fermentable carbon source such as ethanol (Rolfes and Hinnebusch, 1993, Yang *et al.*, 2000). Other stimuli that were also found to activate Gcn4p include methyl-methanosulfonate (MMS) and a high salinity growth medium (Hinnebusch and Natarajan, 2002). Therefore, these findings imply that Gcn4p is an essential transcriptional regulator in yeast, having a major role in metabolic cell activities in response to various stress conditions.

1.5.4 Environmental Factors Influencing Hotspot Activity

In addition to the genetic factors, various environmental influences can also affect the level of recombination in *S. cerevisiae*. In particular, the *HIS4* hotspot was shown to be

affected by modulating temperature and the nutritional composition of sporulation media. Fan *et al.* (1995) demonstrated substantial differences in the level of recombination at *HIS4* between cells that were sporulated at two different temperatures. Specifically, the level of NMS at *HIS4* was elevated when cells were sporulated at 18°C relative to 25°C (Fan *et al.*, 1995). Furthermore, cells exhibited a 7-fold decrease in gene conversion and crossing over at *his4-ATC* (a G to C change at the third base pair of the start codon) when sporulated at 37°C compared to 23°C (Cotton *et al.*, 2009). The influence of temperature on hotspot activity was also shown to affect early steps of recombination. Specifically, the formation of crossovers in the *zmm* mutants is reduced at 33°C compared to 23°C (Borner *et al.*, 2004). These observations indicate the ability of temperature to affect meiotic recombination, although its mechanism is still poorly understood.

The effect of nutritional conditions on recombination at *HIS4* has also been studied (Cotton, 2007, Abdullah and Borts, 2001). Particularly, cells that were pre-grown and sporulated on media with limited nutrients (specifically adenine and amino acids) exhibited an approximately 1.6-fold increase in the levels of NMS and crossing over compared to sporulation on fully supplemented media (Cotton, 2007). This effect is likely associated with transcription factor binding activity since Bas1p, Bas2p and Gcn4p are controlled by exogenous starvation and stress conditions (Borts, 2009). Accordingly, these findings suggest a correlation between environmental factors with the activity of the recombination hotspot (discussed in Chapter 4).

1.6 Aims and Objectives

This project aims to further elucidate the influence of nutritional status on meiotic recombination at *HIS4*, and different mechanisms mediating this effect. More specifically, we aim to explore the influence of nutritional starvation, particularly adenine and amino acids, upon transcription factor-dependent recombination at *HIS4* in *Saccharomyces cerevisiae*. We also aim to establish the role of Gcn4p in mediating the starvation-induced activity at the *HIS4* hotspot. Given that Gcn4p regulates more than 500 genes in yeast through the general amino acid control system, the ability to manipulate recombination via Gcn4p can provide a significant contribution in the study of meiotic recombination.

The second objective is to determine the effect of AICAR and SAICAR, two intermediates from the purine biosynthesis pathway, on recombination at *HIS4.* Although AICAR/SAICAR were known to positively influence the transcription of *HIS4* through activation of Bas1/2p, the effect of these metabolites on *HIS4* recombination is yet to be investigated. Specifically, we would like to examine if the *HIS4* hotspot activity mediated by Bas1/2p can be enhanced by deleting genes in the purine biosynthesis pathway that were previously shown to increase the production of AICAR and SAICAR. This can provide further understanding on the mechanism by which Bas1p and Bas2p regulates recombination activity at *HIS4.*

Chapter 2 : Materials and Methods

2.1 Materials

2.1.1 Yeast Strains

All strains used in this study were derived from the Y55 background of *S. cerevisiae* unless otherwise stated. The list of haploid strains used in this study is presented in Table 2.1.

2.1.2 Plasmids

Plasmids used in this study are listed in Table 2.2.

2.1.3 Yeast Media

Rich growth medium, YEPD (Yeast Extract, Peptone and Dextrose) consisted of 1% (w/v) Bacto yeast extract (BD), 2% (w/v) Bacto peptone (BD), 2% (w/v) dextrose (D-glucose; Fisher Scientific) and 0.005% (w/v) adenine hemisulphate solution in 0.05 M HCl. For selection against petite mutants, YEPEG (Yeast Extract, Peptone, Ethanol and Glycerol) medium was used, consisting of 1% succinic acid (Fisher Scientific), 1% (w/v) Bacto yeast extract, 2% (w/v) Bacto peptone, 2% (v/v) glycerol and 0.5% (w/v) adenine hemisulphate in 0.05 M hydrochloric acid. The pH was adjusted to 5.5. After autoclaving, 2% ethanol was added to the medium.

Synthetic minimal medium contained 0.68% (w/v) yeast nitrogen base without amino acids (BD) and 2% (w/v) dextrose. Synthetic complete medium was prepared as the minimal medium, with the addition of 870 mg/L nutrient mixture (Table 2.3). The medium was also supplemented with 6.25 ml/L of 1% (w/v) leucine and 3 ml/L of 1% (w/v) lysine solution. Synthetic 'drop-out' media was made as the synthetic complete

Table 2.1 : List of haploid strains

Strain name	Key feature	Genotype	Source
FAH 559-4B*	Wild-type	*HIS4; LEU2; ADE1; MATα; TRP; CYH; MET; lys2-d; CANS; ura3*	Abdullah and Borts (2001)
FAH 639-14B*	Wild-type	*his4:Xho*I*; leu2-r; ADE1; MATα; TRP; cyhR; met13-4; lys2-d; CANS; ura3*	Abdullah and Borts (2001)
FAH 640-4D*	Wild-type	*HIS4; LEU2; ade1-1; MATα; trp5-1; CYH; METe; lys2-c; can1; ura3*	Abdullah and Borts (2001)
FAD 640-8C*	*gcn4*Δ	*HIS4; LEU2; ade1-1; MATα; trp5-1; CYH; METe; lys2-c; can1; ura3; gcn4::KanMX4*	Abdullah and Borts (2001)
FAH 913-6C*	*bas1*Δ	*his4: Xho*I*; leu2-r; ADE1; MATα; TRP; cyhR; met13-4; lys2-d; CANS; ura3; bas1::KanMX4*	Abdullah and Borts (2001)
FAH 913-13B*	*bas1*Δ	*his4: Xho*I*; leu2-r; ADE1; MATα; TRP; cyhR; met13-4; lys2-d; CANS; ura3; bas1::KanMX4*	Abdullah and Borts (2001)
VC 155	*ade1-1*	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; ade1-1; trp5-1; cyh2-1; MET13; lys2-c; ura3-1*	Cotton (2007)

*Derived from the H390 x H330 (Y55-like) strain backround.

Strain name	Key feature	Genotype	Source
VC 156	*ade1-1*	*HIS4-HhaI; leu2-r; MATα; ade1-1; TRP5; CYH2; met13-2; lys2-d; CANS; ura3-1*	Cotton (2007)
Y55 2830	Wild type	*HIS4-HhaI; leu2-r; MATα; TRP5; CYH2; met13-2; lys2-d; CANS; ura3-1*	This study
Y55 3569	Wild type	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; trp5-1; cyh2-1; MET13; lys2-c; ura3-1*	This study
Y55 3549	*ade1*Δ	*HIS4-HhaI; leu2-r; MATα; TRP5; CYH2; met13-2; lys2-d; CANS; ura3-1; ade1::KanMX4*	This study
Y55 3562	*ade1*Δ	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; trp5-1; cyh2-1; MET13; lys2-c; ura3-1; ade1::KanMX4*	This study
Y55 3593	*ade16*Δ	*HIS4-HhaI; leu2-r; MATα; TRP5; CYH2; met13-2; lys2-d; CANS; ura3-1; ade16::KanMX4*	This study
Y55 3594	*ade16*Δ	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; trp5-1; cyh2-1; MET13; lys2-c; ura3-1; ade16::KanMX4*	This study
Y55 3571	*ade17*Δ	*HIS4-HhaI; leu2-r; MATα; TRP5; CYH2; met13-2; lys2-d; CANS; ura3-1; ade17::KanMX4*	This study
Y55 3572	*ade17*Δ	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; trp5-1; cyh2-1; MET13; lys2-c; ura3-1; ade17::KanMX4*	This study
Y55 3602	*ade16*Δ *ade17*Δ	*HIS4-HhaI; leu2-r; MATα; TRP5; CYH2; met13-2; lys2-d; CANS; ura3-1; ade16::KanMX4; ade17::KanMX4*	This study

Strain name	Key feature	Genotype	Source
Y55 3603	*ade16*Δ *ade17*Δ	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; trp5-1; cyh2-1; MET13; lys2-c; ura3-1; ade16::KanMX4; ade17::KanMX4*	This study
Y55 3616	*his1*Δ	*HIS4-Hha*I*; leu2-r; MATα; TRP5; CYH2; met13-2; lys2-d; CANS; ura3-1; his1::KanMX4*	This study
Y55 3617	*his1*Δ	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; trp5-1; cyh2-1; MET13; lys2-c; ura3-1; his1::KanMX4*	This study
Y55 3618	*his1*Δ *ade1*Δ	*HIS4-Hha*I*; leu2-r; MATα; TRP5; CYH2; met13-2; lys2-d; CANS; ura3-1; his1::KanMX4; ade1::KanMX4*	This study
Y55 3619	*his1*Δ *ade1*Δ	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; trp5-1; cyh2-1; MET13; lys2-c; ura3-1; his1::KanMX4; ade1::KanMX4*	This study
Y55 3622	*pClb2-HA$_3$-BAS1 ade16*Δ *ade17*Δ	*HIS4-Hha*I*; leu2-r; MATα; TRP5; CYH2; met13-2; lys2-d; CANS; ura3-1; pClb2-HA$_3$-BAS1 ade16::KanMX4; ade17::KanMX4*	This study
Y55 3623	*pClb2-HA$_3$-BAS1 ade16*Δ *ade17*Δ	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; trp5-1; cyh2-1; MET13; lys2-c; ura3-1; pClb2-HA$_3$-BAS1; ade16::KanMX4; ade17::KanMX4*	This study
Y55 3595	*gcn4*Δ	*HIS4-Hha*I*; leu2-r; MATα; TRP5; CYH2; met13-2; lys2-d; CANS; ura3-1; gcn4::KanMX4*	This study

Strain name	Key feature	Genotype	Source
Y55 3596	*gcn4*Δ	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; trp5-1; cyh2-1; MET13; lys2-c; ura3-1; gcn4::KanMX4*	This study
Y55 3599	*pClb2-HA$_3$-BAS1*	*HIS4-Hha*I*; leu2-r; MATα; TRP5; CYH2; met13-2; lys2-d; CANS; ura3-1; pClb2-HA$_3$-BAS1*	This study
Y55 3600	*pClb2-HA$_3$-BAS1*	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; trp5-1; cyh2-1; MET13; lys2-c; ura3-1; pClb2-HA$_3$-BAS1*	This study
Y55 3612	*bas1*Δ	*HIS4-Hha*I*; leu2-r; MATα; TRP5; CYH2; met13-2; lys2-d; CANS; ura3-1; bas1::KanMX4*	This study
Y55 3613	*bas1*Δ	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; trp5-1; cyh2-1; MET13; lys2-c; ura3-1; bas1::KanMX4*	This study
Y55 3626	*gcn4*Δ *ade1*Δ	*HIS4-Hha*I*; leu2-r; MATα; TRP5; CYH2; met13-2; lys2-d; CANS; ura3-1; gcn4::KanMX4; ade1::KanMX4*	This study
Y55 3627	*gcn4*Δ *ade1*Δ	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; trp5-1; cyh2-1; MET13; lys2-c; ura3-1; gcn4::KanMX4; ade1::KanMX4*	This study
Y55 3620	*gcn4*Δ *bas1*Δ	*HIS4-Hha*I*; leu2-r; MATα; TRP5; CYH2; met13-2; lys2-d; CANS; ura3-1; gcn4::KanMX4; bas1::KanMX4*	This study
Y55 3621	*gcn4*Δ *bas1*Δ	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; trp5-1; cyh2-1; MET13; lys2-c; ura3-1; gcn4::KanMX4; bas1::KanMX4*	This study

Strain name	Key feature	Genotype	Source
Y55 3606	*bas2*Δ	*HIS4-Hha*I*; leu2-r; MATa; TRP5; CYH2; met13-2; lys2-d; CANS; ura3-1; bas2::KanMX4*	Williams (unpublished)
Y55 3609	*bas2*Δ	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; trp5-1; cyh2-1; MET13; lys2-c; ura3-1; bas2::KanMX4*	Williams (unpublished)
Y55 3629	*set2*Δ	*HIS4-Hha*I*; leu2-r; MATa; TRP5; CYH2; met13-2; lys2-d; CANS; ura3-1; set2::KanMX4*	This study
Y55 3630	*set2*Δ	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; trp5-1; cyh2-1; MET13; lys2-c; ura3-1; set2::KanMX4*	This study
Y55 3631	*set2*Δ *bas1*Δ	*HIS4-Hha*I*; leu2-r; MATa; TRP5; CYH2; met13-2; lys2-d; CANS; ura3-1; set2::KanMX4; bas1::KanMX4*	This study
Y55 3632	*set2*Δ *bas1*Δ	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; trp5-1; cyh2-1; MET13; lys2-c; ura3-1; set2::KanMX4; bas1::KanMX4*	This study
Y55 3634	*BAS1-myc*$_{13}$	*HIS4-Hha*I*; leu2-r; MATa; TRP5; CYH2; met13-2; lys2-d; CANS; ura3-1; BAS1-myc*$_{13}$	This study
Y55 3635	*BAS1-myc*$_{13}$	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; trp5-1; cyh2-1; MET13; lys2-c; ura3-1; BAS1-myc*$_{13}$	This study
Y55 3636	*BAS1-HA*$_3$	*HIS4-Hha*I*; leu2-r; MATa; TRP5; CYH2; met13-2; lys2-d; CANS; ura3-1; BAS1-HA*$_3$	This study
Y55 3637	*BAS1-HA*$_3$	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; trp5-1; cyh2-1; MET13; lys2-c; ura3-1; BAS1-HA*$_3$	This study

Strain name	Key feature	Genotype	Source
Y55 3638	*BAS1-HA$_3$ gcn4*Δ	*HIS4-Hha*I*; leu2-r; MATa; TRP5; CYH2; met13-2; lys2-d; CANS; ura3-1; BAS1-HA$_3$; gcn4::KanMX4*	This study
Y55 3639	*BAS1-HA$_3$ gcn4*Δ	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; trp5-1; cyh2-1; MET13; lys2-c; ura3-1; BAS1-HA$_3$; gcn4::KanMX4*	This study
Y55 3641	*pClb2-HA$_3$-BAS1 gcn4*Δ	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; trp5-1; cyh2-1; MET13; lys2-c; ura3-1; pClb2-HA$_3$-BAS1; gcn4::KanMX4*	This study
Y55 3642	*BAS1-myc$_{13}$ gcn4*Δ	*HIS4-Hha*I*; leu2-r; MATa; TRP5; CYH2; met13-2; lys2-d; CANS; ura3-1; BAS1-myc$_{13}$ gcn4::KanMX4*	This study
Y55 3643	*BAS1-myc$_{13}$ gcn4*Δ	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; trp5-1; cyh2-1; MET13; lys2-c; ura3-1; BAS1-myc$_{13}$; gcn4::KanMX4*	This study
Y55 3644	*bas1*Δ *ade1*Δ	*HIS4-Hha*I*; leu2-r; MATa; TRP5; CYH2; met13-2; lys2-d; CANS; ura3-1; bas1::KanMX4; ade1::KanMX4*	This study
Y55 3645	*bas1*Δ *ade1*Δ	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; MATα; trp5-1; cyh2-1; MET13; lys2-c; ura3-1; bas1::KanMX4; ade1::KanMX4*	This study
Y55 3646	Tester strain for *ade16ade17*	*RRP7::NAT; his4-ATC; FUS1::HYG; LEU2; HO; cyh2-1; MET13; lys2-c; ura3-1; arg4-1; CanS*	This study

Table 2.2 : List of plasmid used in this study

Plasmid	Description	Reference
pFa6-KanMX4	*KanMX4*	(Goldstein and McCusker, 1999)
pFA6a-pCLB2-HA$_3$-KanMX6	*Clb2* promoter	(Lee and Amon, 2003)
pFA6a-HA$_3$-KanMX6	3-HA tag	(Longtine *et al.*, 1998)
pFA6a-myc$_{13}$-KanMX6	13-myc tag	(Longtine *et al.*, 1998)
pWJ 716	*KlURA3*	(Erdeniz *et al.*, 1997)
PLB1 (pRED 739)	*ADE1* ORF	Aldred and Borts (unpublished)

Table 2.3 : Composition of the nutrient mixture

Amino acid	Quantity (mg)
Adenine	800
Arginine	800
Aspartic acid	4000
Histidine	800
Leucine	800
Lysine	1200
Methionine	800
Phenylalanine	2000
Threonine	8000
Tryptophan	800
Tyrosine	1200
Uracil	800

Table 2.4 : Drugs

Drug	Concentration (µg/ml)	Supplier
Ampicillin	100	Melford
Cycloheximide	10	Sigma
Geneticin (G418)	200 / 400*	Invitrogen
Hygromycin B	300	A.G. Scientific
Nourseothricin	100	Werner Bioagents
5'-FOA	1000	Apollo Scientific

* Different concentrations were used for initial selection of G418 resistant colonies (400 µg/ml), and subsequent selection to confirm G418 resistance (200 µg/ml).

medium, with omission of appropriate amino acid or nutrient from the nutrient mixture (Table 2.3). Antibiotic drugs including geneticin (G418), hygromycin B, nourseothricin and cyclohexamide were added to YEPD after autoclaving. 5'-Fluoro-orotic acid (5'-FOA) was added to uracil 'drop-out' medium, but supplemented with 50 µg/ml uracil.

Two types of sporulation media were used in this study. Complete sporulation (KAC) medium contained 2% potassium acetate (Fisher Scientific), 0.22% Bacto yeast extract, 0.05% dextrose, and 0.088% (w/v) nutrient mixture (Table 2.3). For experiments that involve starvation, minimal KAC medium containing 2% potassium acetate was used, supplemented with only the nutrients that cells were auxotrophic for (Table 2.3).

All media was prepared using distilled water, and adjusted to pH 6.0-6.5 with either 1 M hydrochloric acid or 1 M sodium hydroxide, unless otherwise stated. To make solid media, 2.5% Bacto agar (BD) was added.

2.1.4 Bacterial Media

Escherichia coli was grown in Luria-Bertani (LB) medium containing 0.5% (w/v) Bacto yeast extract, 1% (w/v) Bacto peptone and 1% (w/v) NaCl with pH adjustment to 7.0. The LB medium was supplemented with ampicillin (Table 2.4) for plasmid selection.

2.1.5 Oligonucleotides

All oligonucleotides used in this study were purchased from Fisher Scientific (Invitrogen) (Table 2.5).

2.1.6 Molecular Weight Markers

The DNA marker that was typically used for agarose gel electrophoresis was λ bacteriophage DNA digested with *Bst*EII (New England Biolabs) at a concentration of 25 ng/µl. Precision plus protein marker (Biorad) was used as a molecular weight standard for western blot analysis, at a concentration of approximately 20 µg per well.

2.2 Methods

2.2.1 Polymerase Chain Reaction (PCR)

2.2.1.1 Standard PCR Method

Taq Polymerase (KAPA Biosystems) was used for standard PCR reactions, which typically had a final volume of 25 µl or 50 µl. PCR reactions contained 0.2 µM of each primer, 1x PCR buffer from 11.1x stock (45 mM Tris-HCl pH8.8, 11 mM ammonium sulphate, 4.5 mM magnesium chloride, 6.7 mM β-mercaptoethanol, 4.4 µM EDTA pH 8, 1 mM of each deoxynucleotide triphosphate and 113 µg/ml bovine serum albumin) (Jeffreys *et al.*, 1990) and 10 to 50 ng DNA as template. Phusion Polymerase (Finnzymes) was used for PCR that required high-fidelity amplification. PCR reactions using Phusion was performed according to the manufacturer's guidelines.

Typical PCR conditions using *Taq* Polymerase involved initial denaturation at 95°C for 3 minutes followed by 35 cycles consisting of a denaturation step at 95°C for 30 seconds, an annealing step at primer-independent temperature for 30 seconds and an

Table 2.5 : List of oligonucleotides

Key feature	Sequence (5' – 3')	Purpose
ADE1 F	CATTGCTTACAAAGAATACACATACGAAATATTAACGATACGTACGCTGCAG GTCGAC	To amplify *KanMX4* from *pFa6-KanMX4* for deletion of *ADE1*
ADE1 R	GAGGAGTTACACTGGCGACTTGTAGTATATGTAAATCACGATCGATGAATTC GAGCTCG	
ADE1 A1	CACTTGCGGAAGTCTCATCA	To verify insertion of cassette into *ADE1*
ADE1 A4	TGGTTTCAGATCACGATGGA	
ADE16 F	CATTCCAAACAAAGAATCCAAATATACCATAAATAGGGACAAAAAACAAAA GAGCTAAAACGTACGCTGCAGGTCGAC	To amplify *KanMX4* from *pFa6-KanMX4* for deletion of *ADE16*
ADE16 R	ATATACACTTGTATTCAGCTATATATGTTGTTTCTGTTCTTCTCATAAAATGTA CCCTACATCGATGAATTCGAGCTCG	
ADE16 A1	AAACCCCTACCCCCTCTTCT	To verify insertion of cassette into *ADE16*
ADE16 A4	ACTGGGCTAGCGTTTTTCAA	
ADE17 F	GCAACTAATAGCCCTTGAAGTAGTTTTGCTAGCTTGGACATCAAAGCACATA TCACCATCAAATCGTACGCTGCAGGTCGAC	To amplify *KanMX4* from *pFa6-KanMX4* for deletion of *ADE17*
ADE17 R	CTATACATCGATTTGCCGTCATTTCTGTATTTATGGCATATTGACTCTGAACT CATGCCATATCGATGAATTCGAGCTCG	
ADE17 A1	CGTCGTGCTGCTAACTTGAG	To verify insertion of cassette into *ADE17*
ADE17 A4	CTGGGGGATTCTGATGAAGA	

Key feature	Sequence (5’ – 3’)	Purpose
BAS1 F	TAAAACTTTTGTTGTAGCGTTTTTGCTCTTTTTTTTTTATCGCAGAATACATTTATCGAGACGTACGCTGCAGGTCGAC	To amplify *KanMX4* from *pFa6-KanMX4* for deletion of *BAS1*
BAS1 R	ATTACAAAACTAATATGTTAAACAATTGAAAGATTTGTGTTTTTTTTCGGCCTTGCCTTCATCGATGAATTCGAGCTCG	
BAS1 A1	CACAGAATAAAGCCCCAGGA	To verify insertion of cassette into *BAS1*
BAS1 A4	AAGGAATGGATGCTGACCAC	
pClb2-Bas1 F4	GAGCAGCATGGTCTAACAATCCTCTTAAGTTCTTTAGCCGTAATTGCGAATAAAACTGTTGAATTCGAGCTCGTTTAAAC	Insertion of the *CLB2* promoter with the HA_3 tag at the N-terminal region of *BAS1*
pClb2-Bas1 R3-HA	ATCGAAGCCGGATCCTCTTTTTGGCTTACTTTTTCGTATATCTTTGGTACTTATATTCGAGCACTGAGCAGCGTAATCTG	
pClb2-Bas1 A1	ACCCATCACGTGTTCGTCTT	To verify insertion of the *CLB2* promoter at *BAS1*
pClb2-Bas1 A4	TTGGGTCCAGAGATCCTGTC	
BAS1 C-tag F2	GAGCATGATATGACGTCAGGAGGTTCTACCGATAATGGGTCAGTCCTGCCACTGAATCCTCGGATCCCCGGGTTATTAA	Insertion of the HA_3 or myc_{13} tag from *pFA6a-HA_3-KanMX6* or *pFA6a-myc_{13}-KanMX6* respectively into the C-terminal region of *BAS1*
BAS1 C-tag R1	GCTTATTACAAAACTAATATGTTAAACAATTGAAAGATTTGTGTTTTTTTTCGGCCTTGCGAATTCGAGCTCGTTTAAAC	
BAS1 C-tag A1	CGACGGCATCATATACAACG	To verify insertion of the HA_3 or myc_{13} tag into *BAS1*
BAS1 C-tag A4	TGTGTTTGTGGGCGGAAAGA	
BAS2 F	AGACATTCAACAGGGCTAGACAAGTCACGGCTTACTGCTAAATAACGTATACAATACGCTCGTACGCTGCAGGTCGAC	To amplify *KanMX4* from *pFa6-KanMX4* for deletion of *BAS2*

Key feature	Sequence (5’ – 3’)	Purpose
BAS2 F	AGACATTCAACAGGGCTAGACAAGTCACGGCTTACTGCTAAATAACGTATACAATACGCTCGTACGCTGCAGGTCGAC	To amplify *KanMX4* from *pFa6-KanMX4* for deletion of *BAS2*
BAS2 R	TAGAGTAATATTTAGAGTTGAAAATGCAATCGCAAAAAAAAAAAAACAGAATTATTTTCACATCGATGAATTCGAGCTCG	To amplify *KanMX4* from *pFa6-KanMX4* for deletion of *BAS2*
BAS2 A1	GTGTGGGATTGTTTGTCGTC	To verify insertion of cassette into *BAS2*
BAS2 A4	CGTGCTTTGTCCAGCAACTT	
CLB2 F1	TAAGGTGCCTTAGGGGAT	To verify insertion of the *CLB2* promoter at *BAS1*
CLB2 F2	GAATCTTTCTGGTATTAATTTTTC	
GCN4 F	TCGGGGAATAAAGTGCATGAGCATACATCTTGAAAAAAAAAGATGAAAAATTTCCGACTTGAATTCGAGCTCGTTTAAAC	To amplify *KanMX4* from *pFa6-KanMX4* for deletion of *GCN4*
GCN4 R	ACCATCCAATGGTGAGAAACCCATTGGATTTAAAGCAAATAAACTTGGCTGATATTCGGAGCACTGAGCAGCGTAATCTG	
GCN4 A1	GCACAAAGCACCCATACCTT	To verify insertion of cassette into *GCN4*
GCN4 A4	TCGAAGGGGTATCCTGTTTG	
HIS1 F	CGGTTTGAATCTTTGAAAATAGAAAAGAAAGGATAGGTTTCTAAAAAATTCAATAGAAAACGTACGCTGCAGGTCGAC	To amplify *KanMX4* from *pFa6-KanMX4* for deletion of *HIS1*
HIS1 R	TTATTGCCTATAAAAAATACAAAATTAATCGGGATTTGGCTTCCCTTTTTCTGTTCTATCATCGATGAATTCGAGCTCG	
HIS1 A1	ACCCGGCTAGAGAGGTTGTT	To verify insertion of cassette into *HIS1*
HIS1 A4	GCGTTACGATCCCCAATCTA	
HIS4 100R	AATCTCTTCATTACTCAGGC	To sequence the *HIS4* promoter region to check for mutations in the Gcn4p binding sites

Key feature	Sequence (5’ – 3’)	Purpose
HIS4-1277F	GGCTCACTAACCAGCTCTCTCC	To sequence the *HIS4* promoter region to check for mutations in the Gcn4p binding sites
HIS4-333F	TGCGATACGATGGGTCATAA	
HIS4+2541R	CCCACTCTTGCTACTACCTCTCTT	
K2	TTCAGAAACAACTCTGGCGCA	Internal primers to the *KanMX4* cassette to verify insertion
K3	CATCCTATGGAACTGCCTCGG	
KanMX4 F	CGTACGCTGCAGGTC	To amplify *KanMX4* DNA to be used as probe in Southern blotting to confirm double and triple gene deletions
KanMX4 R	ATCGATGAATTCGAGCTCG	
SET2 F	AGAAAACTGCATAGTCGTGCTGTCAAACCTTTCTCCTTTCCTGGTTGTTGTTTTACGTGA	To amplify *KanMX4* from *pFa6-KanMX4* for deletion of *SET2*
SET2 R	ACAAGACTTCCTTTGGGACAGAAAACGTGAAACAAGCCCCAAATATGCATGTCTGGTTAA	
SET2 A1	AACCGACGAGAAGAAGCTGA	To verify insertion of cassette into *SET2*
SET2 A4	TTCAAGGGTTCTCATCGTTC	

elongation step of 1 min/kilobase DNA at 72°C; and a final step of 10 minutes elongation at 72°C. The annealing temperature typically ranged between 51°C to 60°C and was optimised for specific primers by gradient PCR. Gradient PCR was used to determine the optimal annealing temperature that is specific for the individual primer pairs. The PCR cycling conditions were as described above, with gradual increment of the annealing temperature. The cycling conditions for Phusion PCR were based on the guidelines provided by the manufacturer. The PCR machine used was a PTC-225 Peltier Thermal Cycler (MJ Research).

2.2.1.2 Colony PCR

Colony PCR was performed mainly for quick screening of transformants by directly using yeast cells without purification of DNA. To prepare samples for colony PCR, a small scrape of a yeast colony (approximately 0.2 mm^2) was resuspended in 20 µl of 0.02 M NaOH (Fisher) and boiled at 95°C for 10 minutes. The cells were chilled on ice for 5 minutes prior to centrifugation at 13,000 r.p.m for one minute. 2 µl supernatant was added with a 25 µl PCR mixture. The PCR reaction and the PCR cycling conditions were the same as stated in the standard PCR method (Section 2.2.1.1).

2.2.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to visualise the presence of DNA and to estimate the size of PCR products. The concentration of agarose used was 1.0% to 1.5% depending on the length of the DNA fragments to be visualised. Gels were prepared by dissolving Seakem LE agarose powder (Lonza) in 1x TBE buffer (90 mM Tris-base, 90 mM Boric acid and 2mM EDTA), which was also used as the running buffer for gel electrophoresis. 0.5 µg/ml ethidium bromide was used to stain the gel. DNA samples

were loaded with 0.2x their volume of 5x loading dye consisting of 10% (w/v) Ficoll type 400, 0.1 M EDTA, 0.2% (v/v) bromophenol blue and 0.5% (w/v) SDS. Appropriate DNA molecular weight markers were used (Section 2.1.6) to determine the size of the DNA fragment. DNA was visualised under ultraviolet (UV) light using a Kodak 200L dark chamber, and the image captured using the Kodak 1D Image analysis software.

2.2.3 DNA Precipitation

DNA was precipitated by adding one tenth of 3 M sodium acetate (pH 5.2) and twice the volume of the DNA solution of 100% ice-cold ethanol. The mixture was centrifuged for 30 minutes at 13,000 r.p.m and the supernatant was discarded. 70% ice-cold ethanol was then added to the precipitated DNA followed by centrifugation for one minute to isolate the DNA from the ethanol. The pelleted DNA was left to air-dry and resuspended in the required amount of 1x TE buffer (10 mM Tris-HCl pH 8 and 1 mM EDTA) before being heated at 65°C for 20 to 30 minutes until the DNA dissolved.

2.2.4 Lithium Acetate Yeast Transformation

The yeast transformation method used was adapted from (Gietz *et al.*, 1992). Cells were cultured in 5 ml liquid YEPD with shaking at 30°C for approximately 16 hours. The cultures were diluted 1 in 10 into 5 ml fresh YEPD and allowed to grow further for 3 to 4 hours at 30°C. Cells were then harvested by centrifugation at 3,000 r.p.m for 5 minutes. 1 ml distilled water was used to wash the pellets before resuspending the pellet in 1 ml 100 mM lithium acetate. The suspension was divided into two 1.5 ml Eppendorf tubes and one was used as a negative control. These tubes were then centrifuged for one minute at 13,000 r.p.m and the supernatant was removed. After that, 240 µl (w/v) polyethylene glycol (50%, MW 3350), 36 µl 1 M lithium acetate, 50 µl

2 mg/ml salmon sperm DNA (heated at 95°C for 5 minutes and chilled on ice prior to use) and 34 µl DNA that had previously been precipitated was added to the cell. The DNA was substituted with water for the negative control. The tubes were thoroughly vortexed until the yeast pellet had completely resuspended, and then incubated at 42°C for 40 minutes. Cells were then washed with distilled water three times with gentle centrifugation at 5,000 r.p.m for 1 minute. Cells were subsequently resuspended in distilled water and spread onto the appropriate selection media. For selection of antibiotic resistance markers, the cell pellet was resuspended in 1 ml YEPD and grown further for 3 to 4 hours with shaking at 30°C before plating. The plates were incubated at 30°C for 2 to 3 days.

2.2.5 Phenol-Chloroform DNA Extraction

Yeast cells were grown overnight in 5 ml liquid YEPD, and harvested by centrifugation at 3,000 r.p.m for 5 minutes. 0.5 ml solution A (1.2 M sorbitol, 0.2 M Tris-HCl pH 8.5, 0.02 M EDTA and 0.1% β-mercaptoethanol) and 50 µl of 10 mg/ml zymolyase solution (made up in solution A without β-mercaptoethanol) was added to the cell. The cell were incubated at 37°C for 20 minutes until become spheroplasted and then harvested by centrifugation for one minute. The supernatant was then removed. Cells were gently resuspended in 50 µl 1 M sorbitol (Sigma) and 0.5 ml solution B (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 100 mM EDTA, 0.5% SDS). Solution C, containing 50 µl 1 mg/ml RNAse (Sigma) and 0.2 mg proteinase K (Roche) was also added to the mixture, and then the cells were incubated at 65°C for at least 2 hours. The cells were then chilled on ice for 5 minutes followed by addition of 0.5 ml phenol-chloroform (25 units phenol : 24 units chloroform : 1 unit isoamyl alcohol). After 10 minutes centrifugation at

13,000 r.p.m, the top aqueous layer was transferred to a new Eppendorf tube. This step was repeated twice more. DNA was precipitated using 100% ethanol and centrifuging for 10 minutes. The DNA was then washed with 70% ethanol and left to air-dry. The pellet was dissolved in 200 µl 1x TE buffer and incubated at 65°C for 30 minutes.

2.2.6 Site Directed Mutagenesis

2.2.6.1 PCR-Based Gene Disruption

Genes of interest were deleted by replacing the whole open reading frame with a selectable cassette using a PCR-based method (Wach *et al.*, 1994). Transformants were verified using PCR (Section 2.2.1) and several strains were further confirmed by DNA sequencing (Section 2.2.7). The oligonucleotides used for mutagenesis are presented in Table 2.5.

2.2.6.2 Two-Step Gene Replacement

A two-step gene replacement method was used to incorporate genes that were already present in an integrative plasmid into the yeast genome (Scherer and Davis, 1979). The plasmids contain a *URA3* selectable marker that enables selection of integrants ('pop-in') on the uracil 'drop-out' media and counter-selection of the *URA3* marker ('pop-out') on 5'-FOA media. The plasmid was digested with an enzyme that had a unique restriction site. The digested plasmid was transferred into yeast using lithium acetate transformation (Section 2.2.4). The plasmid can integrate into the yeast genes through homologous recombination. Successful integrants were first selected on uracil 'drop-out' medium before being transferred to 5'-FOA media, which is toxic to cells containing a functional *URA3* gene. Therefore, only cells that have the *URA3*

cassette (pop-out event) are able to grow. The integrants were eventually confirmed by sequencing.

2.2.7 DNA sequencing

Approximately 100 ng DNA was used as a template for sequencing reactions. The sequencing reactions also consisted of 1 µl BigDye® Terminator v3.1 (PNACL), 3 µl 5 x sequencing buffer (PNACL) and 3.2 pmol of the appropriate primer. The PCR conditions consisted of an initial denaturation at 96°C for 1 minute followed by 29 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. The reaction was purified using Performa DTR Gel Filtration Cartridges (EDGE Biosystems) according to the instructions provided, before being sent to the Protein Nucleic Acid Chemistry Laboratory (PNACL) for samples to be analysed using an Applied Biosystems 3730 sequencer.

2.2.8 Preparation of Plasmid DNA

Plasmids were extracted from *E. coli* cells that were cultured overnight in 5 ml LB media supplemented with ampicillin, using the E.Z.N.A.™ Plasmid Mini Kit I (Omega Bio-tek) according to the manufacturer's instructions.

2.2.9 Restriction Enzyme Digestion

All restriction enzymes and buffers were purchased from New England Biolabs (NEB) and were used according to the manufacturer's guidelines.

2.2.10 DNA and Protein Quantification

DNA and protein extracts were quantified using 2.0 µl of each sample on a NanoDrop™ ND-1000 spectrophotometer according to manufacturer's guidelines.

2.2.11 Genetic Procedures

2.2.11.1 Mating and Sporulation

Haploid strains of opposite mating types (*MAT*a and *MAT*α) were mated on a solid YEPD medium and incubated at 30°C overnight (approximately 17 hours). Diploid cells were induced to sporulate by starving cells of nitrogen. After mating, cells were either directly replicated to sporulation media or were selected for diploids on synthetic minimal medium prior to sporulation. Two types of KAc were used in this study; complete KAc and minimal KAc. Minimal KAc medium was used for experiments involving starvation and was supplemented with only nutrients that cells were unable to synthesise (Table 2.3). This was necessary to enable sporulation of cells. Cells were then incubated at 23°C for 3 to 5 days until tetrads were formed. Small samples of cells were scraped off from the KAc media and visualised using a Swift M400D phase contrast microscope (Swift Instruments) to confirm sporulation.

2.2.11.2 Selection for Diploids

Diploid cells were selected by replicating mated cells to a synthetic minimal medium supplemented with the appropriate nutrients that neither haploid could synthesise. Therefore, only cells that were unable to synthesise nutrients not supplemented in the medium would be unable to grow. Hence, only diploid cells were able to grow on the medium.

2.2.11.3 Tetrad Dissection

To prepare tetrads for dissection, cells were scraped from solid sporulation medium and resuspended in 100 µl cold dissecting buffer (1 M sorbitol, 10 mM EDTA, 10 mM $NaPO_4$; pH 7.2) supplemented with 5 µl 5 mg/ml zymolyase (Seikagaku Biobusiness).

Samples were then incubated at 37°C for 30 minutes. A further 400 µl dissecting buffer was added to the sample which was then stored at 4°C. Tetrads were dissected on YEPD medium using a Zeiss Axioscope microscope fitted with a micromanipulator needle (Singer Instruments). Spores that had been separated were allowed to germinate at 30°C for 2 days.

2.2.11.4 Mating Type Testing

To test the mating type of a particular strain, cells were replicated to a YEPD plate covered with either a *MATa* or *MAT*α tester strain and incubated overnight at 30°C. The following day, the YEPD plate was replicated to a synthetic minimal medium and incubated overnight at 30°C to select for diploids. The tester strains contained *ura2* and *tyr1* mutations, which are not present in any other strains used in this study. Therefore, only strains that have mated and formed diploids can grow on the minimal medium due to complementation of the auxotrophic genotypes. Thus, strains that grow on the minimal medium when mated with the *MATa* tester strain will have α mating type. Likewise, strains that were able to grow when mated with the *MAT*α tester strain were *MATa*.

2.2.11.5 Recombination Analysis

Diploid strains used in this study were heterozygous at *HIS4* and several other loci in chromosome III and VII (Table 2.1; Figure 2.1). Therefore, meiotic recombination events that include non-Mendelian segregation (NMS; Section 1.3) at these loci and crossing over in different gene intervals can be evaluated. Genetic intervals analysed in this study include the *NAT-HYG* region flanking *HIS4*, marked with a nourseothricin resistance cassette (*natMX4*; or *NAT*), 5130 bp downstream of the start codon of *HIS4*

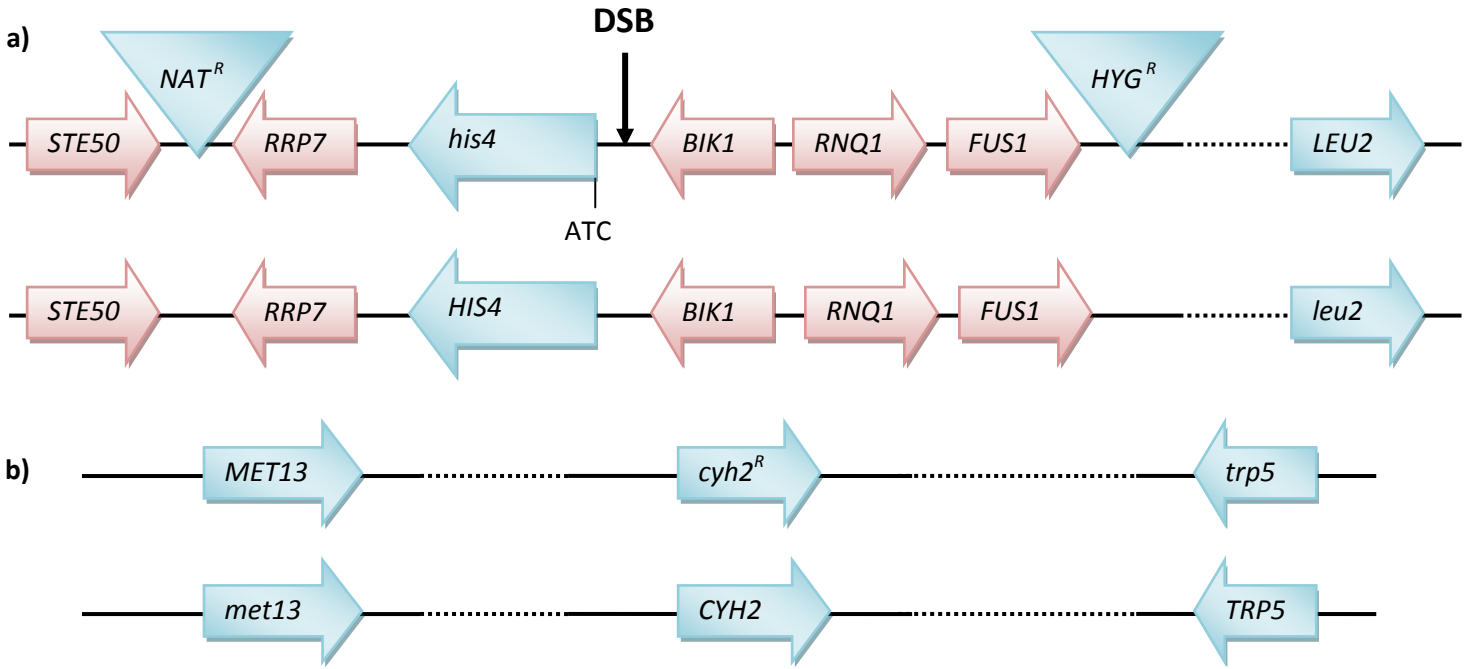


Figure 2.1 : All relevant genetic markers in both haploid wild type strains, Y55 2830 (top) and Y55 3569 (bottom) on chromosome III (a) and VII (b) are presented. Genetic markers used for recombination analysis in this study are shown in blue, and the block arrow indicates the direction of transcription. **a)** The *HIS4* gene in the Y55 3569 strain contains a silent mutation that destroys a *Hha*I restriction site (C to G change at position 1605 downstream of the start codon). The mutated *HIS4* in the Y55 2830 strain (*his4-ATC*) is a G to C change at the third base pair of the start codon. The small black arrow represents the position of the DSB at *HIS4*, approximately 300 bp downstream of the start of the ORF. The cassettes *natMX4* (*NAT*) and *hphMX4* (*HYG*) were inserted into the Y55 2830 strain, adding an interval flanking *HIS4* (Hoffmann *et al.*, 2005). The *LEU2* locus in the Y55 3569 strain contains a specific mutation at the *Eco*RI restriction site at position 637, which disrupts the gene function. **b)** The *MET13* locus in Y55 3569 has a C to A mutation at position 278. The *TRP5* gene in Y55 2830 has a single A to T change at position 2039. *cyh2* in Y55 2830 is an uncharacterised mutation. Adapted from (Hoffmann *et al.*, 2005), (Abdullah, 2002) and (Abdullah *et al.*, 2004) not drawn to scale.

and a hygromicin B resistance cassette (*hphMX4*; or *HYG*) 3804 bp downstream of *HIS4* (Hoffmann *et al.*, 2005). Other intervals included *HYG-LEU2* and *LEU2-MAT* on chromosome III and *MET13-CYH2* and *CYH2-TRP5* on chromosome VII (Figure 2.1). Dissected tetrads were replicated to various synthetic 'drop-out' or drug-containing media followed by overnight incubation at 30°C. The segregation pattern of heterozygous markers was then determined from the media, and only tetrads with four viable spores were used for analysis. The data was entered into a Microsoft Excel worksheet and analysed using MacTetrad 6.9 software (Greene, 1994). This software scored the number of normal Mendelian segregation (2:2) and non-Mendelian segregation (NMS; non-2:2) events at each genetic marker.

The MacTetrad 6.9 software was also used to calculate map distances for each genetic interval (in centiMorgans; cM) according to the formula of Perkins (1949):

$$cM = ½ (TT + 6NPD) / (TT + NPD + PD)$$

TT is the number of tetratypes, NPD is the number of non-parental ditypes and PD is the number of parental ditypes (Perkins, 1949).

2.2.12 Clamped Homogenous Electric Field (CHEF) Gel and Southern Blot Analysis

CHEF gel and Southern blot assay were used for further confirmation of double or triple gene deletions after verification by PCR (Section 2.2.1.1). Double or triple mutants were generated by crossing two single mutant strains that were previously mutated by replacing relevant genes with the *KanMX4* cassette (Section 2.2.4). These

genes were located on different chromosomes allowing the presence of individual *KanMX4* cassettes to be detected.

2.2.12.1 CHEF Gel Analysis

CHEF gels separate the large individual chromosomes by changing the orientation of the electric field during electrophoresis. For this experiment, DNA was extracted and prepared in agarose plugs. Yeast cells were cultured overnight in 3 ml YEPD medium at 30°C. Cells were then harvested by centrifugation at 3,000 r.p.m for 5 minutes. The pellet was then resuspended in 1 ml 50 mM EDTA and centrifuged at 13,000 r.p.m for 30 seconds. The supernatant was discarded before adding a further 200 µl of 50 mM EDTA. To make the CHEF plugs, 100 µl SCE solution (1 M sorbitol, 0.1 M sodium citrate, 10 mM EDTA, 10 mg/ml zymolyase and 5% (w/v) β-mercaptoethanol) was added to the cells, followed by 0.5 ml low melting point (LMP) agarose (prepared by melting 1% (w/v) Seakem LMP agarose [*Cambrex Bio Science*] in 0.125 M EDTA).

The mixture was then pipetted immediately into plug formers (Bio-Rad) that were placed on ice. Once set, the CHEF plugs were transferred into sterile Eppendorf tubes, and 0.5 ml EDTA solution (0.5 M EDTA, 1 M Tris-HCl pH 8 and 5% (w/v) β-mercaptoethanol) was then added. The CHEF plugs were subsequently incubated at 37°C for a minimum of 4 hours. The EDTA solution was then removed before adding 0.5 ml proteinase K solution (1 mg/ml proteinase K, 0.5 M EDTA, 1% (w/v) sodium sarkosyl and 0.1 mg/ml RNAse) to the plugs and incubating for a further 6 hours at 37°C. The proteinase K solution was then discarded and 1x TE buffer (0.1 M Tris-HCl pH 8 and 1 mM EDTA) was used to wash the plugs by incubating for one hour at room temperature. The plugs were stored in 1 ml Tris/EDTA solution (0.5 M EDTA and 0.1 M

Tris-HCl pH 8) at 4°C. For running the CHEF gel, approximately one third of the CHEF plug was loaded into the well of 1% agarose gel (prepared by dissolving LE agarose powder in 0.5 x TBE running buffer). The running buffer was cooled to 14°C before running the CHEF gel for 24 hours at 6 volts/cm with a reorientation angle of 120°. The program that was used to separate the chromosomes is as follows:

Stage 1: Initial Switch Time: 60 seconds
Final Switch Time: 60 seconds
Run Time: 15 hours
Stage 2: Initial Switch Time: 90 seconds
Final Switch Time: 90 seconds
Run Time: 9 hours
Pump speed: 80

For staining, the CHEF gel was submerged in 0.5x TBE running buffer with 0.5 µg/ml ethidium bromide added and gently agitated for 20 minutes. Visualisation of the gel was done under UV light as described in Section 2.2.2.

2.2.12.2 Southern Blot Hybridisation

2.2.12.2.1 DNA Transfer and Fixation

Subsequent to visualisation, the CHEF gel was then immersed in denaturing solution (0.5 M NaOH and 1 M NaCl) for 30 minutes with gentle shaking at room temperature, then washed with neutralising solution (0.5 M Tris-HCl pH 7.5 and 3 M NaCl) for 30 minutes, followed by 20 x SSC buffer (3 M NaCl, 0.3 M sodium citrate) for 10 minutes. The DNA was transferred to a positively charged nylon membrane (Roche) using an upward capillary system. The gel was placed on a wick of Whatman 3MM paper on top of a tray filled with 20 x SSC buffer, followed by the membrane, another Whatman paper, a stack of paper towels and a weight to apply pressure. DNA was left to transfer

overnight. Afterward, the DNA was fixed to the membrane by UV cross-linking at 120 mJ/cm^2.

2.2.12.2.2 DNA Probe Labelling

The DNA probe was prepared by PCR-amplifying the *KanMX4* gene from the *pFa6-KanMX4* plasmid (Table 2.2) using a DIG Probe Synthesis Kit (Roche). The PCR reaction was carried out according to the manufacturer's guidelines. Verification of the labelled probe was performed by comparing its size with the unlabelled *KanMX4* DNA using agarose gel (1% w/v) electrophoresis (Section 2.2.2).

2.2.12.2.3 Hybridisation

Following DNA fixation, the membrane was placed in a glass cylinder and incubated with 10 ml DIG Easy Hyb (Roche) at 42°C for 30 minutes with gentle agitation. Prior to membrane hybridisation, 50 µl distilled water was added to the labelled probe, and heated at 95°C for 5 minutes to denature the DNA before quickly chilling on ice. Fresh hybridisation solution (3.5 ml DIG Easy Hyb) with the 100 µl DNA probe, was then added to the membrane immediately and left to incubate overnight at 42°C with continuous rotation.

After hybridisation, the membrane was twice washed with 200 ml low stringency buffer (2x SSC, 0.1% SDS) for 5 minutes with gentle rotation at room temperature. The membrane was then washed twice with 200 ml preheated high stringency buffer (0.5x SSC, 0.1% SDS) for 15 minutes at 60°C. The membrane was then transferred into a plastic tray containing 100 ml washing buffer (0.1 M maleic acid, 0.15 M NaCl pH 7.5 and 0.3% (w/v) Tween 20) and incubated for 2 minutes at room temperature with gentle shaking. The washing buffer was then discarded, and the membrane was

incubated in 100 ml blocking solution (1 g Roche blocking reagent dissolved in 100 ml maleic acid buffer containing 0.1 M maleic acid and 0.15 M NaCl pH7.5) for 30 minutes. This was followed by incubation of the membrane in 20 ml antibody solution (75 mU/ml anti-digoxigenin-AP diluted 1:10,000 in blocking solution) at room temperature for 30 minutes. The membrane was then washed twice with 100 ml washing buffer for 15 minutes at room temperature. The washing buffer was then discarded. This was followed by adding 20 ml detection buffer (0.1 M Tris-HCl and 0.1 M NaCl pH 9.5) to the membrane for 3 minutes at room temperature. The membrane was then covered with 0.25 mM CSPD (Roche) diluted 1:100 in detection buffer and left in a cling film for 5 minutes at room temperature, followed by 10 minutes incubation at 37°C to increase the luminescence reaction. The membrane was exposed to an X-ray film (Fujifilm) for one hour before the film was being processed and visualised.

2.2.13 Statistical Analysis

The G-test of homogeneity (Sokal and Rohlf, 1969), an equivalent to the chi-squared (χ^2) test for goodness of fit was used in this study to determine any significant differences between datasets. In this study, a P-value of less than 0.05 was considered to reject the null hypothesis, hence the difference between data was said to be statistically significant. For comparison of multiple datasets, the P-value considered significant was corrected according to the number of datasets being analysed using the Benjamini-Hochberg false discovery rate (Benjamini and Hochberg, 1995). Corrections were made to minimise the chance of type I errors (false rejection of the null hypothesis even when it is true). The Benjamini-Hochberg multiple testing in a given comparison was carried out by first ranking the P-values of each sample from smallest

to largest. Except for the largest P-value, all other P-values were then corrected using the formula below (N is the total number of samples in a specific comparison and is divided by the rank of the sample to be corrected):

$$\text{Corrected P-value} = \text{P-value} \times (N / N-1)$$

Chapter 3 : Modulating Metabolic Intermediates (AICAR/SAICAR) in the Purine and Histidine Biosynthetic Pathways

3.1 Introduction

3.1.1 Regulation of the *de novo* Purine Biosynthesis Pathway

The regulation of both Bas1/2p activity and the purine biosynthesis pathway are interrelated. Bas1/2p plays a major role as a transcriptional activator of *ADE1, ADE2, ADE4, ADE5,7, ADE6, ADE8, ADE12, ADE13* and *ADE17* genes from the purine biosynthesis pathway (Figure 3.1) (Daignan-Fornier and Fink, 1992). In addition, Bas1/2p also regulate the expression of *HIS1, HIS4* and *HIS7* genes in the histidine biosynthesis pathway (Arndt *et al.*, 1987, Denis *et al.*, 1998, Springer *et al.*, 1996) and *SHM2, GLN1* and *MTD1* genes from the glycine, glutamate and 10-formyltetrahydrofolate (10-formyl THF) synthesis pathways respectively (Denis and Daignan-Fornier, 1998).

Moreover, these Bas1/2p-regulated pathways are interconnected (Figure 3.1). The production of one molecule of ATP from the purine biosynthesis pathway consumes two molecules of glutamine, two molecules of 10-formyl THF and one molecule of glycine supplied from one carbon metabolism. ATP is also utilised in the histidine biosynthesis pathway for the production of phosphoribosyl-ATP. In fact, a purine metabolite AICAR is also synthesised via the histidine biosynthesis pathway as a by-product of the metabolism of phosphoribosylformimino AICAR phosphate (Rebora *et al.*, 2005).

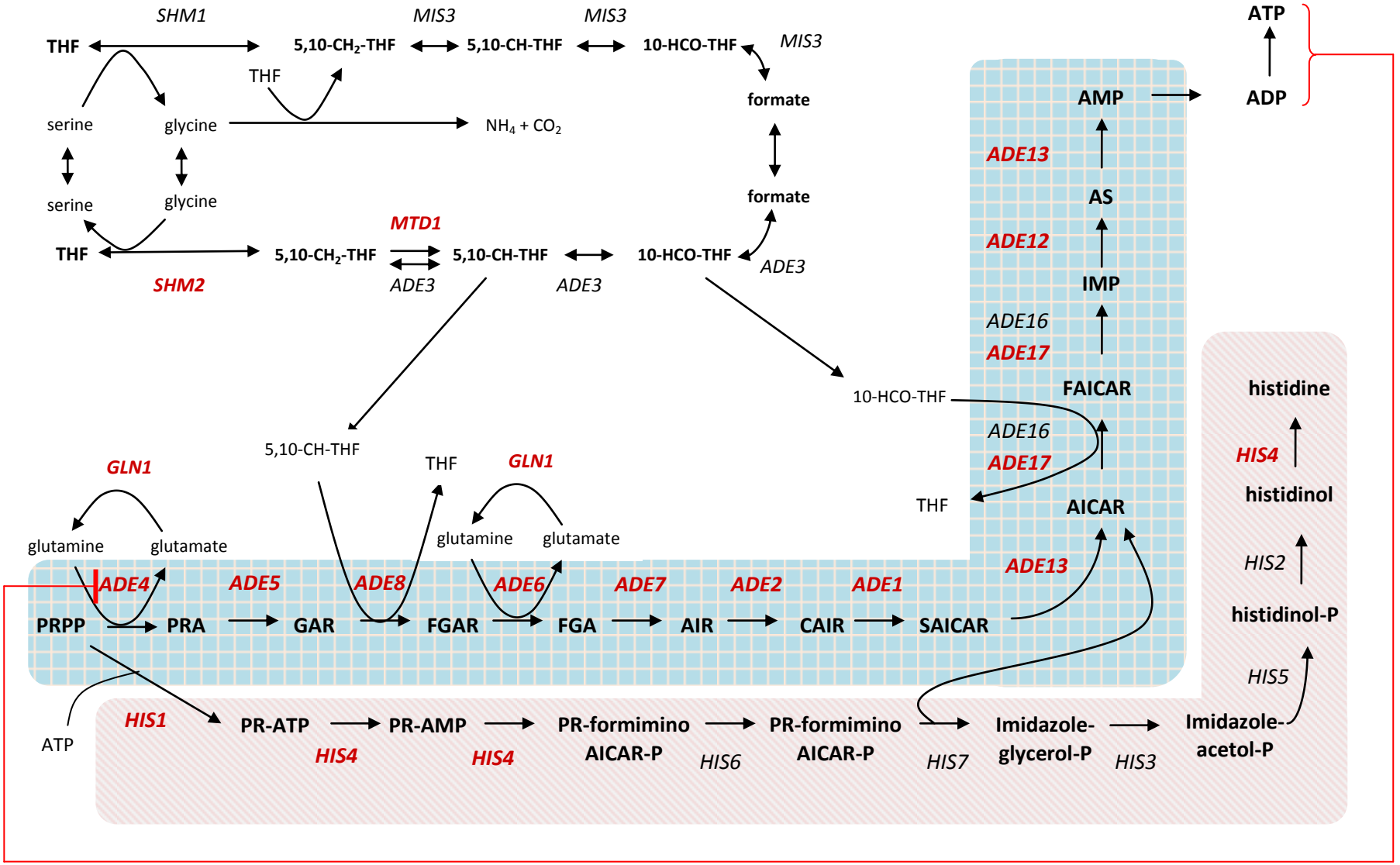

SHM1
MIS3
MIS3
MIS3
THF
5,10-CH2-THF
5,10-CH-THF
10-HCO-THF
formate
THF
serine
glycine
NH4 + CO2
serine
glycine
formate
MTD1
THF
5,10-CH2-THF
5,10-CH-THF
10-HCO-THF
ADE3
SHM2
ADE3
ADE3
ATP
ADP
AMP
ADE13
AS
ADE12
IMP
ADE16
ADE17
FAICAR
10-HCO-THF
ADE16
ADE17
AICAR
THF
ADE13
5,10-CH-THF
GLN1
THF
GLN1
glutamine
glutamate
glutamine
glutamate
ADE4
ADE5
ADE8
ADE6
ADE7
ADE2
ADE1
PRPP
PRA
GAR
FGAR
FGA
AIR
CAIR
SAICAR
histidine
HIS4
histidinol
HIS2
histidinol-P
HIS5
ATP
HIS1
PR-ATP
HIS4
PR-AMP
HIS4
PR-formimino AICAR-P
HIS6
PR-formimino AICAR-P
HIS7
Imidazole-glycerol-P
HIS3
Imidazole-acetol-P

Figure 3.1 : Schematic representation of the connection between the purine biosynthesis, histidine biosynthesis and one carbon metabolism pathways. The purine biosynthesis pathway is highlighted in blue while the histidine biosynthesis pathway is depicted in purple. The red lines represent feedback inhibition of PRPP amidotransferase, the first enzyme of the purine biosynthesis pathway encoded by *ADE4*, by ADP and ATP. Several co-substrates from one carbon metabolism are supplied to the purine pathway. Genes encoding enzymes are italicised. Genes regulated by Bas1p and Bas2p are highlighted in red.

PRPP, 5-phosphoribosyl diphosphate; PRA, 5-phosphorybosylamine; GAR, 5′-phosphoribosylglycinamide; FGAR, 5′-phosphoribosyl-*N*′-formylglycinamide; FGAM, 5′-phosphoribosyl-*N*′-formylglycinamidine; AIR, 1-(5′-phosphoribosyl)-5-aminoimidazole; CAIR, 1-(5′-phosphoribosyl)-5-aminoimidazole-4-carboxylate; SAICAR, 1-(5′-phosphoribosyl)-4-(*N*-succinocarboxyamido)-5-aminoimidazole; AICAR, 5-amino-1-(5′-phosphoribosyl)-imidazole-4-carboxyamide; FAICAR, 5-formamido-1-(5′-phosphoribosyl)-imidazole-4-carboxyamide; IMP, inosine 5′-monophosphate; AS, adenylosuccinate; THF, tetrahydrofolate; 10-HCO-THF, 10-formyl tetrahydrofolate; PR, 5-phosphoribosyl; P, Phosphate. Adapted from Mieckowski *et al*. (2006), Denis and Daignan-Fornier (1998) and Cotton (2007).

Studies to date have characterised factors controlling the ability of Bas1/2p to activate transcription of genes under their regulation. Under normal rich growth conditions, Bas1/2p were shown to regulate the basal expression of many of their target genes (Daignan-Fornier and Fink, 1992). Under starvation for adenine, expression of these genes was up-regulated in a manner dependent on Bas1/2p (Daignan-Fornier and Fink, 1992, Arndt *et al.*, 1987, Springer *et al.*, 1996, Denis *et al.*, 1998).

Two intermediates from the purine biosynthesis pathway, AICAR and SAICAR have been found to be essential to promote the expression of Bas1/2p target genes (Figure 3.1) (Rebora *et al.*, 2001, Rebora *et al.*, 2005, Pinson *et al.*, 2009). This was demonstrated by studies that monitored the expression of the *ADE1-lacZ* and *ADE17-lacZ* fusion constructs in strains bearing various mutations impairing different steps of the pathway (Rebora *et al.*, 2001, Rebora *et al.*, 2005). Mutation of any of the genes involved in the steps preceding AICAR synthesis led to the loss of expression of *ADE1-lacZ* and *ADE17-lacZ* fusions, even when the cells were starved for adenine (Figure 3.1) (Rebora *et al*., 2001, Rebora *et al*., 2005). In contrast, inactivation of genes downstream of AICAR and SAICAR production, including the functionally redundant *ADE16* and *ADE17* genes (Figure 3.1), resulted in increased expression of *ADE1-lacZ* and *ADE17-lacZ* fusions (Rebora *et al.*, 2001). Furthermore, it has been demonstrated that mutation of the *ADE16* and *ADE17* genes led to the accumulation of both AICAR and SAICAR to the concentration of 1.6 mM and 0.3 mM respectively, whilst it was below the level of detection in the wild type (< 10 µM) (Pinson *et al.*, 2009). Therefore, these findings indicate that AICAR and SAICAR are important for the activation of the purine regulon genes.

The influence of AICAR/SAICAR and extracellular adenine upon transcription of Bas1/2p-target genes is related to a feedback mechanism of the purine biosynthesis pathway. The pathway is negatively regulated as its end-products, ADP and ATP, inhibit the activity of the first enzyme, PRPP amidotransferase encoded by *ADE4* (Figure 3.1) (Rebora *et al.*, 2001). In cells supplemented with adenine, ADP and ATP are continuously present, leading to the inhibition of Ade4p activity (Rebora *et al.*, 2001). This inhibition results in reduced synthesis of AICAR/SAICAR, which leads to the lack of Bas1/2p activation. Consequently, transcription of the *ADE* regulon genes is reduced, decreasing adenine synthesis.

When cells are starved for extracellular adenine, Ade4p is activated and the production of AICAR/SAICAR increased (Rebora *et al.*, 2001). This stimulates Bas1/2p to promote transcription of their target genes, thus activating purine biosynthesis and increasing the production of adenine. Therefore, the synthesis of the purine biosynthetic intermediates is maintained by this feedback loop of the purine biosynthesis pathway.

3.1.2 AICAR/SAICAR Accumulation and Adenine Starvation Promote Transcription of *HIS4* via Bas1p and Bas2p Interaction and Binding Activity

It has been shown that increased transcription of Bas1/2p-regulated genes, in response to adenine starvation, does not require a high expression level of either *BAS1* or *BAS2* (Tice-Baldwin *et al.*, 1989, Daignan-Fornier and Fink, 1992). Instead, several studies suggested that adenine starvation and AICAR/SAICAR accumulation stimulate transcription of Bas1/2p target genes by inducing physical interaction between Bas1p and Bas2p and their binding to gene promoters (Zhang *et al.*, 1997, Pinson *et al.*, 2000, Pinson *et al.*, 2009).

This was initially suggested by Zhang *et al.* (1997) using an assay that monitored the ability of LexA-Bas1p (Bas1p fused to the DNA binding domain of the bacterial LexAp) to activate the transcription of the *lexAop-lacZ* reporter (a bacterial *lacZ* reporter containing a *lexA* operator sites upstream of the TATA box) in the presence and absence of adenine. They found that the *lexAop-lacZ* reporter was activated by LexA-Bas1p only when the native Bas2p was present, and reporter activation was inhibited when adenine was added to the medium (Zhang *et al.*, 1997). In addition, LexA-Bas1p could also promote activation of the *lexAop-lacZ* reporter in conjuction with Bas2p even when the DNA binding ability of Bas2p was abolished (Zhang *et al*., 1997). This suggests that Bas2p is recruited to the promoter by interacting with LexA-Bas1p.

Furthermore, Pinson *et al*. (2000) demonstrated that a covalent fusion between Bas1p and Bas2p led to transcriptional activation of the *ADE* regulon even in the presence of adenine, similar to the level seen under starvation for adenine. This confirmed that interaction between Bas1p and Bas2p is essential to activate expression of the *ADE* genes in response to the absence of external adenine.

In another assay based on the two-hybrid method, AICAR/SAICAR accumulation was also demonstrated to promote the interaction of Bas1p and Bas2p (Pinson *et al.*, 2009). Specifically, LexA-Bas1p strongly stimulated the transcription of the *lexAop-lacZ* reporter in strains that accumulated AICAR/SAICAR compared to wild type, in a Bas2p-dependent manner. Similarly, a LexA-Bas2p fusion also activated the *lexAop-lacZ* reporter when AICAR/SAICAR was accumulated, but only when Bas1p was present (Pinson *et al.*, 2009). These observations indicate that complex formation between the two factors is necessary

for the transcriptional regulation of their target genes in response to adenine starvation and AICAR/SAICAR accumulation.

A model for Bas1/2p-mediated activation of the *ADE* genes and *HIS4*, in response to adenine deprivation, has been proposed (Figure 3.2). This model involved unmasking of the trans-activation domain of Bas1p upon interaction with Bas2p, and is based primarily on findings from two-hybrid experiments (Zhang *et al.*, 1997, Pinson *et al.*, 2000, Som *et al.*, 2005). Truncation of the amino- and carboxy- termini flanking a central region of LexA-Bas1p led to the activation of the *lexAop-lacZ* reporter, independently of Bas2p and adenine availability (Zhang *et al.*, 1997). This suggests that the activation domain of Bas1p is located in the central region.

From the truncation experiment, another region between residues 630 and 664 in Bas1p termed BIRD (Bas1p Interaction and Regulatory Domain), was shown to inhibit the Bas1p trans-activation domain, and is critical for the interaction with Bas2p (Pinson *et al.*, 2000). When Bas2p was absent, inclusion of the BIRD region adjacent to the trans-activation domain of Bas1p led to loss of activation of the *ADE1-lacZ, ADE17-lacZ* and *HIS4-lacZ* reporters (Pinson *et al.*, 2000). These reporters were only activated under adenine deprivation and in the presence of Bas2p. However, when adenine was supplemented in the media and Bas2p was present, *HIS4-lacZ* activity was significantly elevated upon inclusion of the BIRD region, while only basal activity was seen in *ADE1-lacZ* and *ADE17-lacZ* (Pinson *et al.*, 2000). Therefore, it has been suggested that BIRD has an additional effect as the transcriptional activator at the *HIS4* promoter when adenine is present. This also suggests that the BIRD domain acts in a promoter-dependent manner.

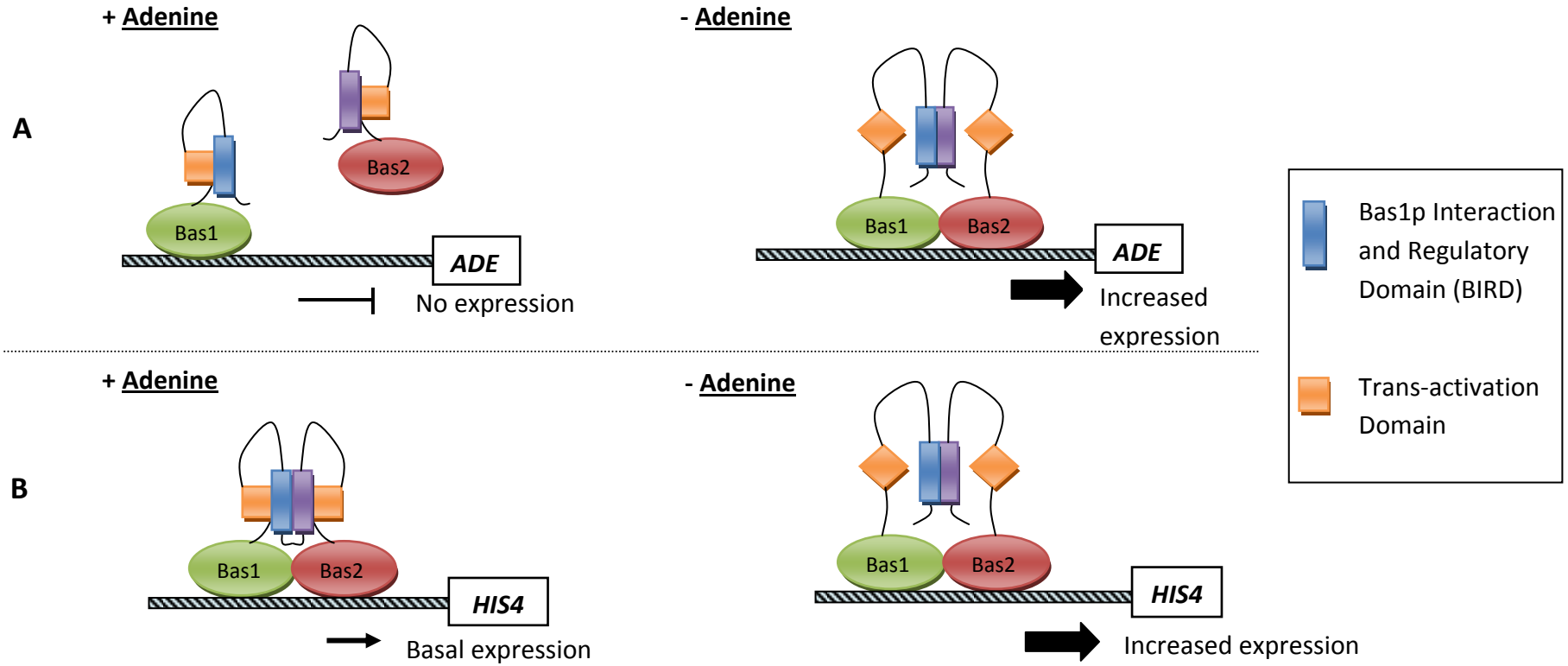


Figure 3.2 : Model of the promoter-specific regulation of Bas1p and Bas2p interaction in response to adenine. The presence and absence of adenine is indicated by + and – respectively. Adapted from Pinson *et al*., (2000), Som *et al*., (2005) and Cotton (2007).
A) *ADE1* : In the presence of adenine, only Bas1p binds to the promoter while Bas2p is not recruited. The trans-activation domains are masked, resulting in repressed activity. In the absence of adenine, interaction between Bas1p and Bas2p is stimulated. Trans-activation domain of Bas1p is exposed, inducing high expression. **B)** *HIS4* : In the presence of adenine, low level of interaction and binding of Bas1/2p to the promoter. Though the trans-activation domain of Bas1p is still masked by BIRD, the binding of both proteins resulted in high basal expression. In the absence of adenine, fuller interaction between Bas1p and Bas2p is stimulated, exposing the trans-activation domain of Bas1p, hence increasing expression.

It has been proposed that BIRD acts as an internal repressor that functionally blocks the central activation domain of Bas1p under normal conditions when adenine is present (Figure 3.2) (Pinson *et al*., 2000). At the *ADE* gene promoters, Bas1p binds constitutively in a conformation where its trans-activation domain is masked unless it interacts with Bas2p (Som *et al.*, 2005). Bas2p binds poorly to the *ADE* gene promoters in the presence of adenine, resulting in basal transcription of the *ADE* genes (Figure 3.2A) (Pinson *et al.*, 2000, Som *et al.*, 2005). In contrast, neither Bas1p nor Bas2p alone can bind efficiently upstream of *HIS4* (Figure 3.2B) (Pinson *et al.*, 2000). However, BIRD plays an additional role at the *HIS4* promoter by activating interaction and cooperative binding of Bas1/2p in the presence of adenine, promoting a high basal expression of *HIS4* (Pinson *et al.*, 2000). In the absence of adenine, interaction between Bas1p and Bas2p is promoted, leading to a conformational change of Bas1p that exposes its trans-activation domain (Zhang *et al.*, 1997). This increases the binding of Bas1/2p to the *ADE* genes and *HIS4* promoter, hence up-regulating gene expression (Som *et al.*, 2005).

As stated before, AICAR is also generated from the histidine biosynthesis pathway. It has been shown that AICAR from this pathway is insufficient to produce enough adenine required for cells to grow in the absence of extracellular adenine (Rebora *et al.*, 2005). Apart from interaction between Bas1p and Bas2p, AICAR accumulation can also promote Bas2p and Pho4p interaction, as well as activating expression of genes in the phosphate utilisation pathway (Pinson *et al.*, 2009). This suggests that the purine biosynthesis pathway and phosphate consumption are co-regulated, in addition to the histidine biosynthesis pathway and one carbon metabolism (Pinson *et al.*, 2009). Since the

regulation of the phosphate regulon by Bas2p/Pho4p is independent of Bas1p, it has been proposed that Bas2p plays the main role in mediating the AICAR response (Pinson *et al.*, 2009). This hypothesis was further confirmed by the observation that AICAR can interact directly with Bas2p and Pho4p *in vitro* (Pinson *et al.*, 2009), although how AICAR can promote interaction between Bas1p and Bas2p is not yet well understood (discussed in Section 3.4.1).

Bas1p and Bas2p are also required for recombination at *HIS4* (White *et al.*, 1993, Fan *et al.*, 1995). Mutation of *bas1* and/or *bas2*, or the DNA binding site of Bas1p and Bas2p significantly reduced recombination at *HIS4* (White *et al.*, 1993, White *et al.*, 1991). However, *HIS4* transcription and recombination activities are not directly correlated to one another (White *et al.*, 1992). A genome-wide analysis by Mieckzkowski *et al.* (2006) showed that the rate of recombination at nine of the Bas1p-bound loci was reduced by *BAS1* deletion. Only six of these genes are known to be transcriptionally regulated by Bas1p (Mieczkowski *et al.*, 2006). This indicates possible differences in the regulatory properties of Bas1/2p on the transcription and recombination activity at some loci. Although extensive research has been performed to study the influence of AICAR/SAICAR from the purine and histidine biosynthesis pathways on the transcription of *HIS4*, their effect on recombination is yet to be elucidated. In this study, we sought to determine if recombination at *HIS4* also responds to AICAR/SAICAR from the purine and histidine biosynthesis pathways as was observed for the transcription activity.

3.1.3 Modulating the Metabolic Phenotypes of the Cell Influences Recombination Frequency

Mutation of the *ADE1* gene from the purine biosynthesis pathway (Figure 3.1) has been demonstrated to elevate non-Mendelian segregation (NMS) events at *HIS4* (Abdullah and Borts, 2001). At the time of the study by Abdullah and Borts (2001), the *ade1* mutant (*ade1-1*) was presumed to be null based on the adenine auxotrophy displayed by the strain. In particular, the *ade1* mutant appeared red in colour due to the production of a pigment from the polymerisation of 1-(5′-phosphoribosyl)-5-aminoimidazole (AIR) (Myasnikov *et al.*, 1991). Cells with an *ade1* mutation would inhibit the conversion of AIR into subsequent intermediates, hence accumulate AIR and exhibit red appearance (Figure 3.1). At that time, it was also thought that adenine had a direct inhibitory effect on the interaction between Bas1p and Bas2p (Rolfes *et al.*, 1997). Therefore, it was hypothesised that the stimulation of *HIS4* recombination in the *ade1* mutant was due to the activation of Bas1/2p in the absence of intracellular adenine (Abdullah and Borts, 2001).

Furthermore, deprivation of extracellular adenine was also known to activate transcription of *HIS4* via Bas1/2p (Daignan-Fornier and Fink, 1992, Rolfes and Hinnebusch, 1993). However, as described in the previous section, subsequent studies revealed that AICAR/SAICAR is essential to induce interaction between Bas1p and Bas2p (Rebora *et al.*, 2001, Rebora *et al.*, 2005). Since *ADE1* is involved in the step prior to AICAR/SAICAR production (Figure 3.1), mutating *ADE1* should obstruct the production of these metabolites, hence recombination activity should be reduced.

3.1.4 Aims

1. To investigate the effect of an *ade1* null mutation on meiotic recombination at *HIS4* in comparison with the *ade1-1* mutant.
2. To investigate the association between the levels of AICAR and meiotic recombination at *HIS4.* This is to determine if recombination at *HIS4* is mediated by the same regulation that affects transcription.
3. To determine if the effect of AICAR and SAICAR accumulation on meiotic recombination is dependent on Bas1p.

3.2 Materials and Methods

3.2.1 Production of Double Mutants

Double or triple mutants were generated by crossing two single mutant strains together and subsequent tetrad dissection. For example, in order to produce an *ade16*Δ *ade17*Δ double mutant, *ade16::KanMX4* and *ade17::KanMX4* strains were mated on YEPD and induced to sporulate (Y55 3602 and Y55 3603; Section 2.2.11.1). Following tetrad dissection, the germinated spores were replicated to G418-200 and the segregation pattern of *KanMX4* was scored. Since *ade16::KanMX4* and *ade17::KanMX4* are located on different chromosomes (chromosome XII and XIII respectively), the deletions were either transmitted together or separately into the meiotic products. Consequently, the segregation patterns of tetrad will mainly be 3:1 and occasionally 2:2 or 4:0. The two *KanMX4* resistant spores from a tetrad segregating *KanMX4* 2:2 were selected as they should have both disrupted genes. The candidate double mutants were confirmed by PCR

or Southern blot hybridisation (Section 2.2.12) before being used in subsequent experiments. A similar technique was applied for generating other double and triple mutants in this study.

3.2.2 Assessing *HIS4* Gene Conversion in Strains Auxotrophic for Histidine

Strains with an *ade16*Δ *ade17*Δ double deletion exhibit auxotrophy for histidine (Tibbetts and Appling, 2000). Consequently, the segregation of *HIS4* (*HIS4* / *his4-ATC*) could not be evaluated using histidine omission media. To analyse the NMS events at *HIS4* in the *ade16*Δ *ade17*Δ deletion, the dissected spore colonies were crossed to a haploid 'tester strain' carrying the *his4-ATC* allele and wild type *ADE16* and *ADE17* genes (Table 2.1). The tester strain also contained a functional *HO* gene that permits self-diploidisation, and the *arg4* mutation, which is not present in other strains used in this study.

The self-diploidisation tester strain was induced to sporulate, then mated with the *ade16*Δ *ade17*Δ colonies on a YEPD plate and grown overnight. Since the sporulated cells contain both *MATa* and *MAT*α, the cells are able to mate with all spore colonies in the tetrad. The following day, these mated cells were replicated to a synthetic minimal medium supplemented with appropriate nutrients and grown overnight at 30°C. Only diploids that have a functional copy of *HIS4* were able to grow on the minimal medium while the ones carrying the *his4-ATC* allele did not grow. Therefore the segregation pattern of the *HIS4* marker in a tetrad can be observed and the frequency of NMS recorded. The same technique was used to analyse *HIS4* gene conversion in the *his1*Δ strain.

3.3 Results

3.3.1 Verification of Double and Triple Mutations

Southern blot hybridisation and CHEF gels were used to verify strains with multiple mutations including *pCLB2-bas1::KanMX6 ade16::KanMX4 ade17::KanMX4* (Y55 3622 and Y55 3623), *his1::KanMX4 ade1*::*KanMX4* (Y55 3618 and Y55 3619), *gcn4::KanMX4 ade1*::*KanMX4* (Y55 3626 and Y55 3627) and *bas1::KanMX4 gcn4*::*KanMX4* (Y55 3620 and Y55 3621). The *KanMX* gene was used to probe the blot. The strains were confirmed by analysing the size of each band related to the predicted size of the chromosomes (Figure 3.3). In addition, one of the parental haploid wild-type strains (Y55 3569) also contains *NatMX* and *HphMX* markers on chromosome III, that share some homology with *KanMX*. As expected, an extra band of the size of chromosome III was also detected for the haploid strains containing these markers (Y55 3621, Y55 3623 and Y55 3627).

3.3.2 The *ADE1* Null Mutant Exhibited Wild Type Level of *HIS4* Hotspot Activity.

As discussed in the introduction, the *ade1-1* point mutation was previously demonstrated to elevate the level of NMS at *HIS4* in the 'H390 x H330' (Y55-like) strain background, from 27% in the wild type to 40% (Abdullah and Borts, 2001). However, subsequent findings relating to the regulation of Bas1/2p suggested that the *ade1-1* mutant should not exhibit increased level of recombination at *HIS4* due to the lack of activation of Bas1/2p by AICAR/SAICAR. A similar increase in NMS was also demonstrated in the Y55 strain background, from 14.3% in the wild type to 26.7% in the *ade1-1* mutant (Table 3.1; $P = 6.7 \times 10^{-7}$, G-test) (Cotton, 2007)

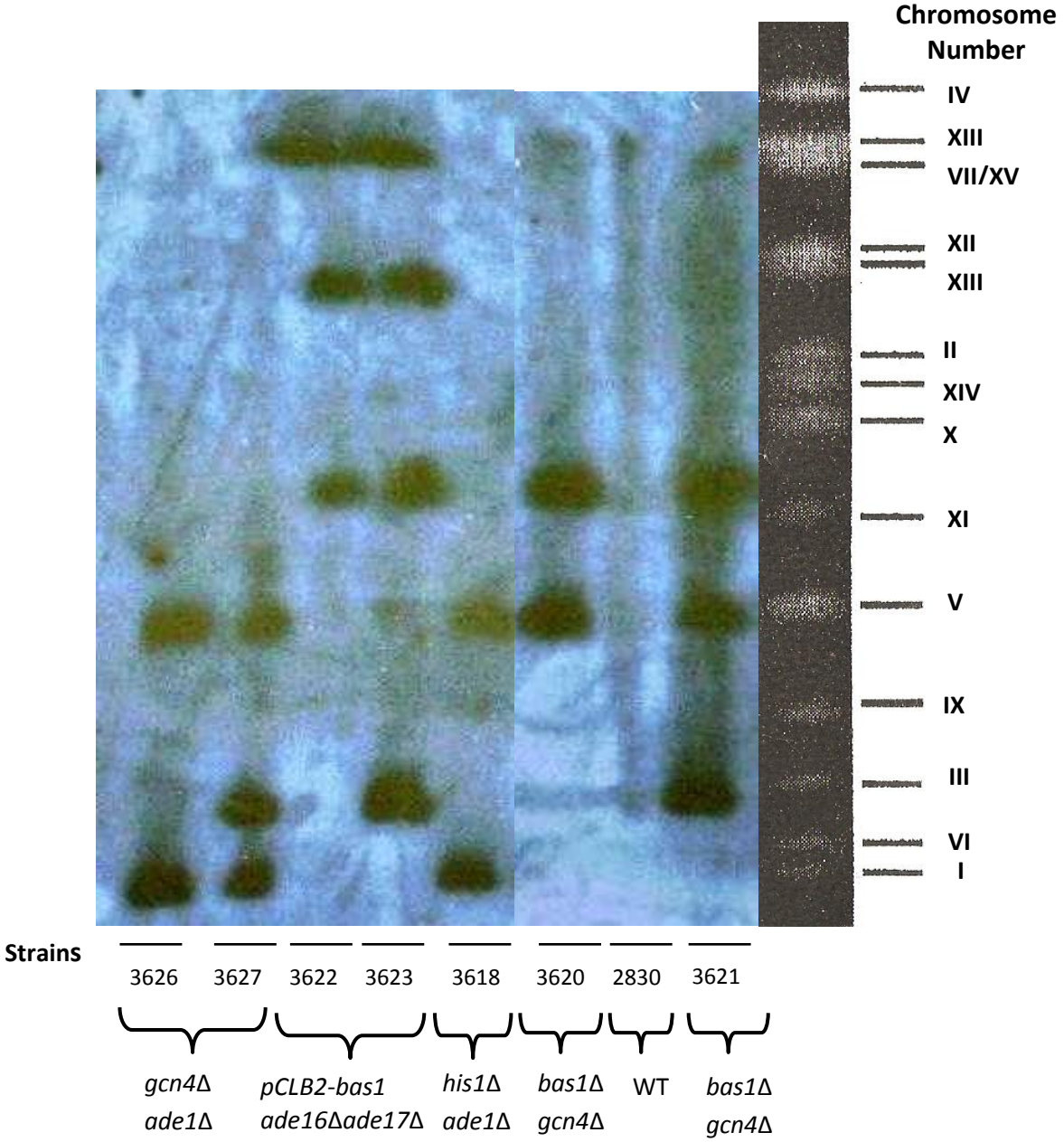


Figure 3.3 : Southern blot analysis of the *KanMX4* gene on different chromosomes. Analysis of both haploids from four different mutant strains is shown: *gcn4*Δ *ade1*Δ (Y55 3626 and Y55 3627), *pCLB2-bas1 ade16*Δ *ade17*Δ (Y55 3622 and Y55 3623), *his1*Δ *ade1*Δ (Y55 3618 and Y55 3619) and *bas1*Δ *gcn4*Δ (Y55 3620 and Y55 3621). The genomic location of each gene is as follows: *ADE1*, chromosome I; *ADE16*, chromosome XII; *ADE17*, chromosome XIII; *BAS1*, chromosome XI; *GCN4*, chromosome V; *HIS1*, chromosome V.

Therefore, in order to test the effect of an *ade1* complete deletion on recombination, the function of *ADE1* was completely obliterated by replacing the whole open reading frame of the gene with the *KanMX4* cassette (Section 2.2.4). The *ADE1* null mutant (*ade1*Δ) exhibited a 2.4-fold lower level of NMS (11.1%) as compared to the *ade1-1* point mutant (Table 3.1). Furthermore, the level of NMS in *ade1*Δ was not significantly different to the wild type (12.9%). This is more consistent with the model of how purine biosynthesis influences *HIS4* transcription than the data from the *ade1-1* mutant (discussed in Section 3.4.1).

3.3.3 Deletion of *ade16* and *ade17* Increases Recombination Activity at *HIS4*

Given that AICAR/SAICAR is predicted to affect NMS at *HIS4* via Bas1/2p, we wanted to investigate if accumulation of the metabolites could increase the level of NMS. To investigate this, we disrupted the genes involved downstream of AICAR and SAICAR production in the purine biosynthesis pathway (Figure 3.1). *ADE16* and *ADE17* genes are functionally redundant, encoding AICAR transformylase and IMP cyclohydrolase isozymes that catalyse the conversion of AICAR to IMP (Tibbetts and Appling, 1997). Deletion of both genes is needed to inhibit progression of the biosynthetic pathway and consequently accumulate AICAR and SAICAR. Therefore, we created an *ade16*Δ *ade17*Δ double mutant (Section 3.2.1) and analysed the level of NMS at *HIS4*. The data revealed a highly significant 4-fold increase of NMS events at *HIS4* in the *ade16*Δ *ade17*Δ double mutant (53%) compared to that in the wild type Ade^+ strain (12.9%; $P = 2.69 \times 10^{-35}$) (Table 3.1).

Table 3.1 : The level of NMS at *HIS4* in different *ADE* gene mutations

Strain Genotype	**NMS**[§]	**Total Tetrads**	**% NMS at *HIS4***
Wild type (*ADE*)	50	388	12.9
*ade1*Δ	28	252	11.1
*ade1-1**	113	423	26.7[‡]
*ade16*Δ *ade17*Δ	221	417	53.0[‡]

[§]NMS is non-Mendelian Segregation events that include gene conversion and post-meiotic segregation. The percentage of NMS (%) was calculated as the number of NMS events / total tetrads x 100. *The *ade1-1* data was taken from Cotton (2007). [‡]Represents data that is significantly different compared to the corresponding wild type. The G-test of homogeneity was used for the comparison.

3.3.4 Deletion of *ade16* and *ade17* Increases Crossing Over at the *NAT-HYG* Interval

We also monitored the effect of deletions of the *ADE1*Δ and *ADE16*Δ *ADE17*Δ genes from the purine biosynthesis pathways on reciprocal crossing over at five different intervals on chromosomes III and VII. This included the *NAT-HYG* interval, which flanks *HIS4* (Figure 2.1). No significant increase in crossing over was seen in the *ade1*Δ strain at *NAT-HYG* and the adjacent *HYG-LEU2* region compared to the wild type (Table 3.2, P = 0.79 and 0.95 respectively). In comparison, the *ade1-1* point mutant was shown to significantly increase crossing over at the *NAT-HYG* interval compared to the wild type ($P = 6.72 \times 10^{-7}$). These data revealed further differences between the complete *ade1*Δ and the *ade1-1* point mutant strains.

The *ade16*Δ *ade17*Δ strain showed a significant elevation in crossover events in the *NAT-HYG* interval as compared to the wild type (45.8 cM compared with 11.4 cM respectively; $P = 3.41 \times 10^{-42}$). This increase was in conjunction with the NMS events at *HIS4* in the *ade16*Δ *ade17*Δ strain which presumably accumulates AICAR/SAICAR. These data demonstrated a correlation between the levels of reciprocal crossing over at the *NAT-HYG* interval with *HIS4* gene conversion. The neighbouring interval *HYG-LEU2* showed a concomitant 2.3-fold reduction of the map distance compared to the wild-type (4.8 cM compared with 11.1 cM respectively; $P = 2.94 \times 10^{-5}$) (discussed in Section 7.8). Crossing over was also analysed at three other intervals that were not expected to be affected by the *ADE* gene mutations, *LEU2-MAT*, *MET13-CYH* and *CYH-TRP* (Table 3.3). No statistically

Table 3.2 : Map distances in different genetic intervals on chromosome III in strains with different *ADE* gene mutations

Strain Genotype	Genetic intervals											
	NAT – HYG				*HYG – LEU2*				*LEU2 – MAT*			
	PD	NPD	TT	**cM**[§]	PD	NPD	TT	**cM**	PD	NPD	TT	**cM**
Wild type (*ADE*)	307	3	68	**11.4**	305	2	72	**11.1**	162	15	205	**38.6**
*ade1*Δ	194	3	47	**13.3**	195	1	48	**11.1**	124	5	116	**29.8**
*ade1-1**	273	5	130	**19.6**[‡]	362	0	45	**5.5**[‡]	186	9	208	**32.5**
*ade16*Δ *ade17*Δ	140	21	253	**45.8**[‡]	380	1	34	**4.8**[‡]	199	10	206	**32.0**

*The *ade1-1* data was taken from Cotton (2007). [§]Map distances were calculated in centiMorgan (cM) according to Perkins (1949). PD is parental ditype, NPD is non-parental ditype and TT is tetratype. [‡]Represent values that are significantly different compared to the corresponding wild type. G-test of homogeneity was used for comparing the distributions of PD, NPD and TT between different samples.

Table 3.3 : Map distances in different genetic intervals on chromosome VII in strains with different *ADE* gene mutations

Strain Genotype	Genetic intervals							
	MET13-CYH2				*CYH2-TRP5*			
	PD	NPD	TT	**cM**[§]	PD	NPD	TT	**cM**
Wild type (*ADE*)	233	1	125	**18.2**	85	24	273	**54.6**
*ade1*Δ	146	1	83	**19.3**	70	14	166	**50.0**
*ade1-1**	300	1	118	**14.8**	84	44	289	**66.3**
*ade16*Δ *ade17*Δ	241	3	142	**20.7**	92	25	291	**54.0**

*The *ade1-1* data was taken from Cotton (2007). [§]Map distances were calculated in centiMorgan (cM) according to Perkins (1949). PD is parental ditype, NPD is non-parental ditype and TT is tetratype.

significant difference was observed between crossing over at these intervals in all strains and conditions tested in this section and in the following chapters unless otherwise stated.

3.3.5 The Effect of Deletion of *HIS1* from the Histidine Biosynthesis Pathway upon Recombination at *HIS4*

In addition to the purine biosynthetic pathway, AICAR is also generated from histidine biosynthesis as a byproduct of *HIS7* (Figure 3.1). We then deleted the *HIS1* gene, which is involved in the first step of the histidine biosynthesis pathway, to determine if the level of recombination at *HIS4* can be affected by blocking the production of AICAR from this pathway (Figure 3.1). As mutating the gene results in auxotrophy for histidine, NMS events at *HIS4* were analysed by mating dissected spores with a *his4-ATC* tester strain (Section 3.2.2). There was no significant difference in the level of NMS between *his1*Δ and the wild type strains (14.4% and 12.9% respectively; Table 3.4). Furthermore, no significant effect was detected upon crossing over at the *NAT-HYG* interval as compared to the wild type (Table 3.5). This suggests that mutating the *HIS1* gene, which should prevent AICAR production from the histidine biosynthesis pathway, did not appear to affect the level of recombination at *HIS4*.

Next, we analysed recombination at *HIS4* in a *his1*Δ *ade1*Δ double mutant to determine if the level of recombination could be further reduced by blocking AICAR synthesis from both adenine and histidine biosynthesis pathways. We discovered that the *his1*Δ *ade1*Δ double deletion did not affect the level of NMS at *HIS4* and crossing over at *NAT-HYG* more severely than either single mutant (Table 3.4 and Table 3.5).

Table 3.4 : The levels of NMS at *HIS4* in the *his1*Δ, *ade1*Δ and *his1*Δ *ade1*Δ strains

Strain Genotype	**NMS**	**Total**	**% NMS at *HIS4***
Wild type (*HIS1 ADE1*)	50	388	12.9
*his1*Δ	61	423	14.4
*his1*Δ *ade1*Δ	25	287	8.7
*ade1*Δ	28	252	11.1

*There was no statistically significant difference in the levels of NMS between all strains tested.

Table 3.5 : Map distances in different genetic intervals on chromosome III in the *his1*Δ and *his1*Δ *ade1*Δ strains

Strain Genotype	Genetic intervals											
	NAT – HYG				*HYG – LEU2*				*LEU2 – MAT*			
	PD	NPD	TT	**cM**[§]	PD	NPD	TT	**cM**	PD	NPD	TT	**cM**
Wild type (*HIS1 ADE1*)	307	3	68	**11.4**	305	2	72	**11.1**	162	15	205	**38.6**
*his1*Δ	342	2	72	**10.1**	345	1	65	**8.6**	175	11	228	**35.5**
*his1*Δ *ade1*Δ	232	0	44	**8.0**	224	0	57	**10.1**	141	8	133	**32.1**
*ade1*Δ	194	3	47	**13.3**	195	1	48	**11.1**	124	5	116	**29.8**

[§]Map distances were calculated in centiMorgan (cM) according to Perkins (1949). PD is parental ditype, NPD is non-parental ditype and TT is tetratype. There was no statistical significance difference between the map distances at *NAT-HYG, HYG-LEU2* and *LEU2-MAT* between all strains tested.

Table 3.6 : Map distances in different genetic intervals on chromosome VII in the *his1*Δ and *his1*Δ *ade1*Δ strains

Strain Genotype	Genetic intervals							
	MET13-CYH2				*CYH2-TRP5*			
	PD	NPD	TT	**cM**	PD	NPD	TT	**cM**
Wild type (*HIS1 ADE1*)	233	1	125	**18.2**	85	24	273	**54.6**
*his1*Δ	264	1	129	**17.1**	92	32	297	**58.1**
*his1*Δ *ade1*Δ	189	1	82	**16.2**	64	29	192	**64.2**
*ade1*Δ	146	1	83	**19.3**	70	14	166	**50.0**

*There was no statistically significant difference between the map distances at *MET13-CYH2* and *CYH2-TRP5* between all strains tested.

3.3.6 The Increase in Recombination at *HIS4* in *ade16*Δ *ade17*Δ is Dependent on Bas1p

The accumulation of AICAR/SAICAR was previously shown to stimulate transcription of *HIS4* through Bas1/2p activation (Pinson *et al.*, 2009). Here, we examined if the increase in the NMS events observed at *HIS4* in the *ade16*Δ *ade17*Δ mutant was also mediated by Bas1p. To test this hypothesis, we crossed a strain in which *BAS1* was under control of the meiotic-repressed *CLB2* promoter (*pCLB2-BAS1*) with an *ade16*Δ *ade17*Δ mutant. As discussed later in Chapter 5, the insertion of the *CLB2* promoter (*pCLB2-3HA*) at the N-terminus of Bas1p resulted in a non-functional *BAS1* gene. The *pCLB2-BAS1* strain exhibited similar level of NMS events as that of the *bas1*Δ strain (Table 3.7).

Our data showed that the level of NMS was reduced from 53% in the *ade16*Δ *ade17*Δ strain to 4.6% in the *pCLB2-BAS1 ade16*Δ *ade17*Δ strain (Table 3.7; $P = 4.39 \times 10^{-40}$). Furthermore, the level of NMS in the *pCLB2-BAS1 ade16*Δ *ade17*Δ strain was not different from that of *pCLB2-BAS1* or *bas1*Δ. These results indicate that the increase in the level of NMS in the *ade16*Δ *ade17*Δ strain is dependent on a functional *BAS1* gene.

Concomitantly, the occurrence of crossover events in the *NAT-HYG* region was significantly lower in the *pCLB2-BAS1 ade16*Δ *ade17*Δ strain than in the *ade16*Δ *ade17*Δ strain ($P = 4.16 \times 10^{-45}$) (Table 3.8). In addition, the level of crossing over at *NAT-HYG* was significantly reduced in the *pCLB2-BAS1 ade16*Δ *ade17*Δ strain compared to the wild type ($P = 0.005$).

Table 3.7 : Loss of Bas1p activity results in reduced level of NMS at *HIS4* in the *ade16*Δ *ade17*Δ strain

Strain Genotype	**NMS**	**Total**	**% NMS at *HIS4***
Wild type	50	388	12.9
*pCLB2-BAS1 ade16*Δ *ade17*Δ	10	219	4.6[‡†]
pCLB2-BAS1	8	201	3.9[‡†]
*ade16*Δ *ade17*Δ	221	417	53.0[‡]
*bas1*Δ	6	331	1.8[‡†]

[‡]Represent data that are significantly different compared to the corresponding wild type.
[†]Represent data that are significantly different compared to the corresponding *ade16*Δ *ade17*Δ strain.

Table 3.8 : Map distances in different genetic intervals on chromosome III in *bas1* mutant strains

Strain Genotypes	Genetic intervals											
	NAT – HYG				*HYG – LEU2*				*LEU2 – MAT*			
	PD	NPD	TT	**cM**[§]	PD	NPD	TT	**cM**	PD	NPD	TT	**cM**
Wild type	307	3	68	**11.4**	305	2	72	**11.1**	162	15	205	**38.6**
*pCLB2-bas1 ade16*Δ *ade17*Δ	193	0	21	**4.9**[‡†]	158	0	54	**12.7**[†]	87	11	118	**42.6**
pCLB2-bas1	180	0	18	**4.5**[‡†]	137	2	52	**16.8**[†]	92	2	98	**28.6**
*ade16*Δ *ade17*Δ	140	21	253	**45.8**[‡]	380	1	34	**4.8**[‡]	199	10	206	**32.0**
*bas1*Δ	297	0	28	**4.3**[‡†]	234	1	81	**13.8**[†]	179	10	130	**29.8**

[§]Map distances were calculated in centiMorgan (cM) according to Perkins (1949). PD is parental ditype, NPD is non-parental ditype and TT is tetratype. [‡]Represent data that are significantly different compared to the corresponding wild type. [†]Represent data that are significantly different compared to the corresponding *ade16*Δ *ade17*Δ strain.

Table 3.9 : Map distances at different genetic intervals on chromosome VII in *bas1* mutant strains

Strain Genotype	Genetic intervals							
	MET13-CYH2				*CYH2-TRP5*			
	PD	NPD	TT	**cM**[§]	PD	NPD	TT	**cM**
Wild type	233	1	125	**18.2**	85	24	273	**54.6**
*pCLB2-bas1 ade16*Δ *ade17*Δ	134	1	66	**17.9**	56	16	144	**55.6**
pCLB2-bas1	118	2	67	**21.1**	39	26	130	**73.3**
*ade16*Δ *ade17*Δ	241	3	142	**20.7**	92	25	291	**54.0**
*bas1*Δ	225	0	88	**14.1**	67	19	240	**54.3**

[§]Map distances were calculated in centiMorgan (cM) according to Perkins (1949). PD is parental ditype, NPD is non-parental ditype and TT is tetratype.

3.4 Discussion

3.4.1 Purine Biosynthetic Intermediate AICAR/SAICAR is Likely Involved in Activating Bas1/2p-Mediated Recombination at *HIS4*

We have investigated the effect of mutating *ADE16* and *ADE17*, the purine biosynthetic genes involved in steps downstream of AICAR and SAICAR production, on recombination at *HIS4*. Both gene conversion and crossover events were strongly up-regulated in the *ade16*Δ *ade17*Δ strain (Tables 3.7 and 3.8). Since AICAR and SAICAR have been shown to accumulate in the deletion of *ade16*Δ *ade17*Δ (Pinson *et al*., 2009), we hypothesised that increased activity of recombination in the *ade16*Δ *ade17*Δ strain is caused by high levels of AICAR/SAICAR.

The effect of deleting *ADE16* and *ADE17* genes on recombination correlates with the transcriptional response of *HIS4* to the level of AICAR/SAICAR (Rebora *et al*., 2001, Rebora *et al*., 2005, Pinson *et al*., 2009). Together with our data, this suggests that the amount of AICAR and SAICAR could be an important regulator for both transcription and recombination activity of *HIS4*. We also demonstrated that loss of Bas1p leads to a reduction in the level of recombination at *HIS4* in the *ade16*Δ *ade17*Δ strain (11.5-fold and 9.3-fold reduction respectively). This indicates that Bas1p is necessary for the effects of *ade16 ade17* deletion on recombination at *HIS4*. Since AICAR/SAICAR has been suggested to stimulate interaction and binding of Bas1/2p to gene promoters (Section 3.1.2), we propose that AICAR/SAICAR positively activate recombination at *HIS4* by mediating Bas1/2p activity.

Interestingly, the study by Pinson *et al*. (2009) suggested a difference in the influence of SAICAR between the transcription of the *ADE* genes and *HIS4* in the *ade13-42* mutant. The gene *ADE13* encodes enzyme adenylosuccinate lyase that converts SAICAR to AICAR in the purine biosynthesis pathway (Figure 3.1). SAICAR was shown to accumulate in the *ade13-42* mutant, while the amount of AICAR was undetectable, presumably due to the inhibition of AICAR synthesis (Pinson *et al.*, 2009). Although SAICAR accumulation was shown to induce expression of the *ADE* genes, the transcript levels of *HIS4* appeared to be reduced in the *ade13-42* mutant compared to the wild type (Pinson *et al.*, 2009). Furthermore, the abundance of *HIS4* transcripts in the *ade13-42* mutant was comparable to that seen in a *bas1* or *bas2* deletion. This suggests that AICAR may be solely responsible for the induced transcription of *HIS4*.

Moreover, the binding ability of Bas1/2p also differs at the promoters of the *ADE* genes and *HIS4* (Section 3.3.2) (Pinson *et al.*, 2000). In particular, Bas1p alone can bind to the *ADE* gene promoters, while the interaction between Bas1p and Bas2p is required for binding upstream of *HIS4* (Pinson *et al.*, 2000, Som *et al.*, 2005). Therefore, one possible model to explain these observations is that the SAICAR-induced interaction of Bas1p and Bas2p is insufficient to promote the conformation required for Bas1/2p to be recruited to the *HIS4* promoter (Figure 3.4B). In contrast, AICAR may have the ability to promote this conformational change of Bas1/2p.

Consistent with this, Pinson *et al.* (2009) demonstrated that the accumulation of AICAR alone is sufficient to activate the purine regulon. However, in promoters of the *ADE* gene, SAICAR alone can promote the Bas1/2p-mediated activation of the genes, perhaps due to

the ability of Bas1p to bind constitutively to the DNA (Pinson *et al*., 2000). If that is the case, in the *ade13-42* mutant, it is possible that Bas2p has better access to promoters of the *ADE* gene by interacting with the already bound Bas1p in a manner facilitated by SAICAR (Figure 3.4A). Based on this model, it is possible that the increased level of transcription and recombination at *HIS4* in the *ade16*Δ *ade17*Δ strain is mediated only by AICAR, and not SAICAR.

What is the mechanism by which AICAR stimulates Bas1/2p-mediated transcription and recombination at *HIS4*? Bas2p has been proposed to play a major role in the transcriptional activation of the purine and phosphate regulons in response to AICAR (Pinson *et al.*, 2009). This hypothesis is based on findings that AICAR binds Bas2p *in vitro* and that the interaction of Bas2p with another partner protein, Pho4p was also stimulated in response to a high level of AICAR (Section 3.1.2) (Pinson *et al.*, 2009).

Furthermore, accumulation of AICAR has been shown to enhance the occupancy of Bas1p and Bas2p at the promoter region of the *ADE* genes (Pinson *et al.*, 2009). This suggests a direct role of AICAR in promoting the interaction of Bas2p with Bas1p, and therefore could also facilitate their binding to the *HIS4* promoter. Moreover, it is possible that the AICAR-mediated interaction between Bas1p and Bas2p occurs in the BIRD region of Bas1p (Section 3.1.2). Consequently, the conformation of Bas1p may be altered to expose its latent trans-activation domain, leading to the activation of *HIS4* transcription (Figure 3.4). In terms of recombination, accumulation of AICAR leads to an increased binding of Bas1/2p to the *HIS4* promoter, hence stimulating DSB formation in an unknown manner. Several possibilities are discussed in Chapters 5 and 7.

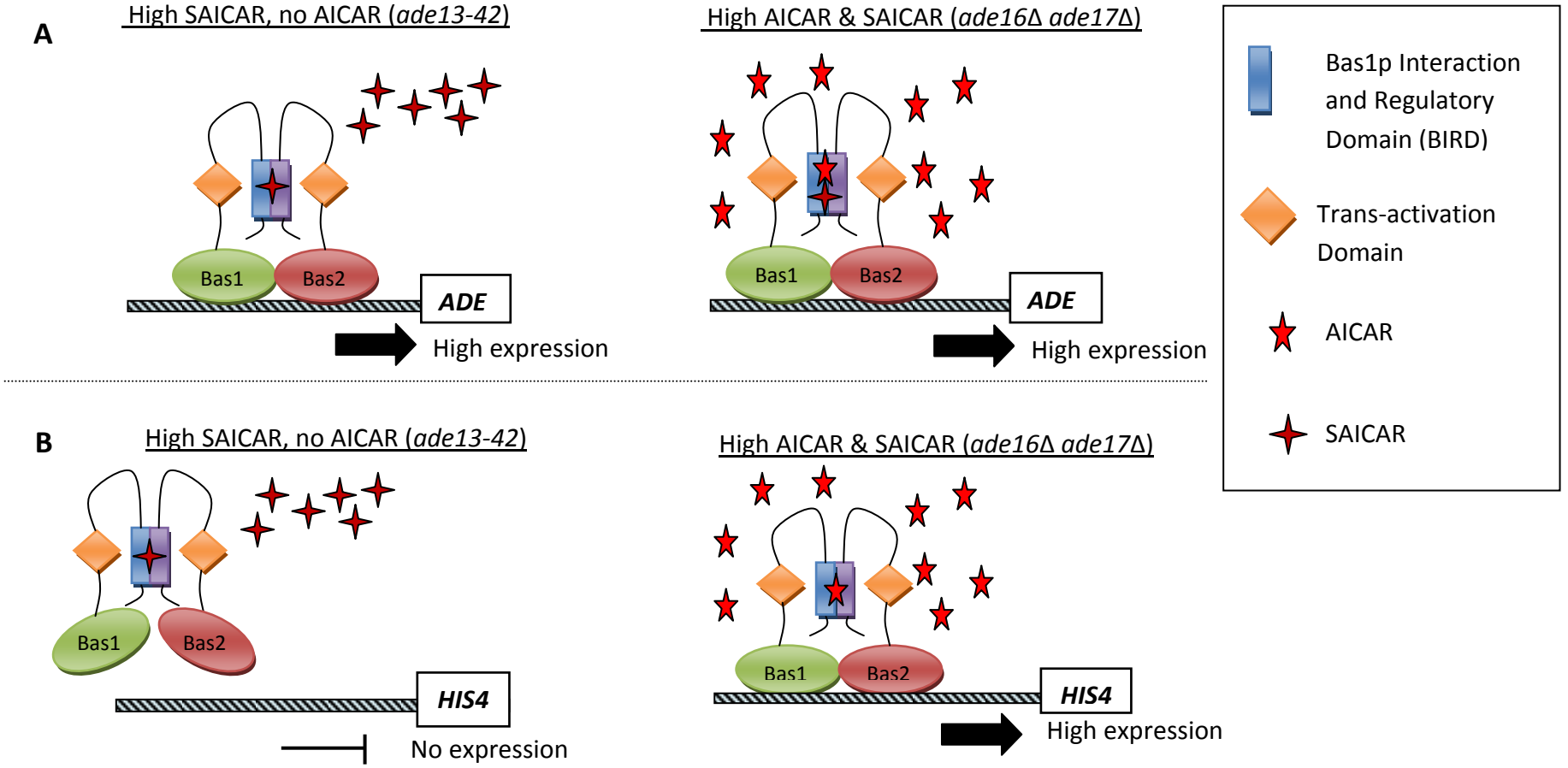


Figure 3.4 : A model for the cooperative activation of Bas1p and Bas2p at the promoter of *HIS4* and *ADE1* in response to AICAR and/or SAICAR accumulation. See text for details.

3.4.2 AICAR from the Histidine Biosynthesis Pathway may be Insufficient to Activate Recombination

In this study, we also investigated the contribution of AICAR synthesised from the histidine biosynthesis pathway upon recombination activity at *HIS4*. Our data suggest that AICAR produced from this pathway may be insufficient to activate the *HIS4* hotspot activity. This was indicated by the unchanged level of recombination observed in a *his1*Δ strain compared to the wild type and *ade1*Δ strains. The data is consistent with the transcriptional study by Rebora *et al*. (2005), which showed that AICAR produced from the histidine biosynthesis pathway is insufficient to allow cells to grow in the absence of adenine. Furthermore, double deletion of *his1* and *ade1* also caused no significant effect on recombination as compared to the wild type and *ade1*Δ strains. Since a *his1*Δ *ade1*Δ deletion should block the production of AICAR from both pathways, this further indicates that AICAR from the histidine biosynthesis pathway is not enough to promote recombination.

Interestingly, the levels of recombination in the absence of AICAR from both biosynthesis pathways (*his1*Δ *ade1*Δ strain) are significantly higher than that of the *bas1*Δ or *bas2*Δ strains (*bas1*Δ in tables 3.8 and 3.9; *bas2*Δ in tables 6.3 and 6.4). This suggests that Bas1p and Bas2p are able to interact without AICAR at a certain level that might not be sufficient to stimulate transcription but enough to stimulate recombination activity. Therefore, an AICAR-independent mechanism may be present to promote the Bas1/2p-mediated recombination activity at *HIS4*.

3.4.2 Why does *ade1-1* Increases Recombination?

This study demonstrates that the effect of *ADE16 ADE17* deletion on *HIS4* recombination is consistent with the current model for AICAR-mediated activation of Bas1/2p. Specifically, accumulation of AICAR in the *ade16*Δ *ade17*Δ strain led to an up-regulation of *HIS4* recombination, in a manner mediated by Bas1/2p. Deletion of *ADE1*, which should block the production of AICAR/SAICAR from the purine biosynthesis pathway, has no effect on the level of recombination at *HIS4* compared to the wild type. This may be due to the low level of recombination in the wild type strain as a result of low amount of AICAR. Consistent with this, Pinson *et al*., (2009) demonstrated that the amount of AICAR in the wild type strain is below the level of detection (< 10 µM). This may be caused by feedback inhibition of the purine biosynthesis pathway, or that AICAR produced in the wild type strain may be immediately processed hence not sufficient to promote Bas1/2p-mediated recombination. Therefore, it remains a possibility that the synthesis of AICAR from the purine biosynthesis pathway is inhibited in the *ade1*Δ strain.

The significantly higher level of recombination in the *ade1-1* point mutant (Cotton, 2007) compared to the wild type does not fit with the hypothesis that AICAR positively regulates Bas1/2p-mediated recombination. The fact that this increased level of recombination at *HIS4* was observed in three different strain backgrounds indicates that this effect is not strain-specific (Abdullah and Borts, 2001, Keelagher *et al.,* 2010, Cotton, 2007). It was previously thought that *ade1-1* is a leaky mutation and might produce a certain amount of AICAR that can stimulate Bas1/2p activity (Cotton, 2007). This is because the point mutation is located towards the end of the *ADE1* gene and might not interfere with the

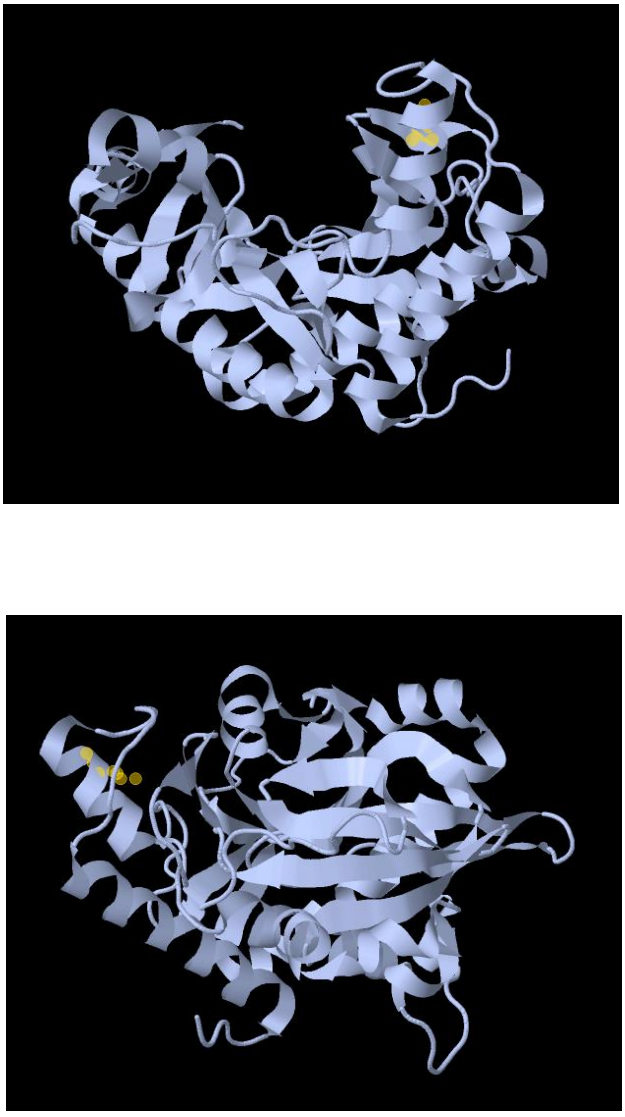


Figure 3.6 : Structure of the Ade1 protein (SAICAR synthetase) from two different angles. The position of the T to C missense mutation in the *ade1-1* strain is in the middle of an α-helix (indicated in small yellow circles), adjacent to putative protein active site. The figure is a computer-modelled structure using the FirstGlance software at http://molvis.sdsc.edu/fgij/ and is adapted from the study by Aronson *et al.* (2009).

active site of Ade1p (SAICAR synthetase). Specifically, *ade1-1* is a T to C missense mutation (changes the amino acid leucine to proline) at position 799 of the 921 base pairs of the *ADE1* gene (Abdullah *et al*., 2004).

However, an analysis of the Ade1 protein structure showed that the point mutation is located in the middle of an α-helix, adjacent to the putative active site of the protein (Figure 3.6) (Aronson and Silveira, 2009). Moreover, the amino acid proline contains a side chain that typically interferes with the conformation of the α-helix, and usually creates breaks or kinks in the helical structure (Richardson, 1981). Therefore, it is likely that the point mutant *ade1-1* disrupts the active site of Ade1p, hence making the protein non-functional. Furthermore, the growth phenotypic behaviour shown by *ade1-1* also disagrees with it being a leaky mutation. In particular, both the *ade1-1* and *ade1* deletion shared similar adenine auxotrophic characteristics that indicate a disrupted function of the *ADE1* gene (Section 3.1.3). Therefore, this suggests that both the *ade1-1* and *ade1*Δ null mutants should block the production of AICAR from the purine biosynthesis pathway.

Nevertheless, it is also possible that the *ade1-1* mutant can produce a different form of AICAR than the one normally synthesised in the wild type. Perhaps the type of AICAR produced in the *ade1-1* strain is unable to be metabolised but function normally to promote interaction between Bas1p and Bas2p. This may result in accumulation of the different form of AICAR in the *ade1-1* strain, consequently activating recombination at *HIS4*.

Apart from that, we cannot rule out the possibility that the Bas1/2p-mediated recombination activities at *HIS4* in the *ade16*Δ *ade17*Δ and *ade1*Δ strains were not

mediated by AICAR. It is possible that the physical presence of the Ade1 protein affects interaction between Bas1p and Bas2p via an unknown response. Alternatively, the physical alteration of Ade1p that was induced by the *ade1-1* point mutation might lead to a protein conformation that enables interaction with other factors. This could be other transcription factors such as Gcn4p or Rap1p, which may be able to promote recombination initiation at *HIS4*. Another possible explanation is that both the *ade1-1* and *ade16*Δ *ade17*Δ mutations have something in common that directly or indirectly stimulates DSB formation, and this feature might not be acquired by the *ade1* null mutant. This could be some unique properties that have the ability to stimulate Bas1/2p and/or other transcription factors (e.g., Gcn4p and Rap1p).

It would be of interest to determine if the frequency of recombination in the *ade1-1* mutant strain would be reduced in the absence of Bas1p and/or Gcn4p. This could be achieved by analysing recombination in a double mutant of *bas1*Δ *ade1-1* and *gcn4*Δ *ade1-1*. The outcome from this experiment would provide an indication whether Bas1/2p and Gcn4p are responsible for the increase in recombination at *HIS4* in the *ade1-1* strain.

Chapter 4 : The Effect of Nutritional Starvation upon Meiotic Recombination at *HIS4*

4.1 Introduction

4.1.1 Nutritional Conditions Affecting Meiotic Recombination at *HIS4*

The influence of the intracellular nutritional environment upon recombination hotspot activity has been demonstrated in a study by Abdullah and Borts (2001). Recombination rates were shown to be significantly affected by metabolically altering the ability of the cell to synthesise adenine and different amino acids; specifically lysine and leucine. Cells that were unable to synthesise lysine (*lys2* genotype) exhibited the highest level of NMS at the *his4-xho* allele (96 base pairs from the start codon) when combined with prototrophy for leucine and adenine (Lys^- Leu^+ Ade^+). In contrast, cells prototrophic for lysine and an auxotrophic for leucine (*leu2* genotype; Lys^+ Leu^- Ade^+) displayed the lowest level of NMS events.

Furthermore, Abdullah and Borts (2001) demonstrated that an auxotrophy for adenine results in stimulation of the *HIS4* hotspot. This hypothesis is based on the observation that an *ade1-1* mutant allele strongly increases the level of recombination regardless of the cell's ability to synthesise leucine or lysine (Abdullah and Borts, 2001). A similarly increased recombination level at *HIS4* in an *ade1-1* strain was also reported in another strain background (Cotton, 2007). The *ade1-1* allele exhibits a similar degree of adenine auxotrophy as the complete *ade1* deletion (discussed in Chapter 3) (Cotton, 2007). Since this deletion did not increase the level of *HIS4* recombination (Chapter 3), this suggests that auxotrophy for adenine alone is not sufficient to stimulate recombination.

Abdullah and Borts (2001) also showed that the influence of these metabolic phenotypes on recombination was mediated by the transcription factor Gcn4p (Abdullah and Borts, 2001). In particular, deletion of *GCN4* in all cells studied reduces the NMS events to the level of the Lys^+ Leu^- Ade^+ cells, suggesting that this represents the basal level of recombination that takes place without Gcn4p activation (Abdullah and Borts, 2001). In contrast, overproduction of Gcn4p results in an approximately 3.7-fold increase in the level of NMS at *HIS4* (Abdullah and Borts, 2001). These intriguing observations indicate that Gcn4p may be required to activate recombination at *HIS4*, a suggestion that contradicts an earlier study by White and colleagues (1992). In the study by White *et al.* (1992), it was demonstrated that the level of NMS at *HIS4* was not significantly affected by deletion of *GCN4*. Since these two experiments were performed using different strain backgrounds, it is possible that the extent of a Gcn4p effect is strain-dependent (discussed in Section 4.4.2).

More direct evidence of the nutritional influence on recombination was provided by Cotton (2007) in which the level of recombination at *HIS4* was influenced by altering the composition of extracellular nutrients, specifically adenine and amino acids. The continuous deprivation of these nutrients before and during sporulation elevated the recombination level, compared to cells that were pre-grown and sporulated in nutrient-supplemented conditions (Cotton, 2007). In particular, the influence of starvation on recombination was analysed using diploid cells that were freshly mated and selected on a nutrient-limited growth medium. These diploid cells were then sporulated on a potassium acetate (KAc) medium that was only supplemented with nutrients that cells were unable to synthesise ('minimal' KAc) (Figure 4.1B). Therefore,

these cells were continuously starved for adenine and amino acids even before sporulation.

Recombination levels were subsequently compared with cells that were sporulated with complete nutrient supplements ('complete' KAc), using diploid cells that were pre-grown on rich medium (YEPD; Section 2.1.3; Figure 4.1A). Hence, recombination events were compared between two extreme cases, the continuously starved cells and the constantly supplemented cells. In this study, we further investigated *HIS4* hotspot activity by using constant pre-meiotic cell preparation techniques, and only varied the nutrient composition during sporulation. This is to investigate the possibility that only the different cell conditions prior to meiosis contributed to the apparent effect of starvation on recombination.

Complete KAc medium differs from minimal KAc by the presence of a nutrient mixture containing 0.003% (w/v) adenine and 0.008% (w/v) amino acids (Table 2.4, Figure 4.1B). The rich vegetative growth medium, YEPD, differs from synthetic minimal medium by containing 0.005% (w/v) adenine in addition to yeast extract and peptone (Section 2.1.3). Since Gcn4p is a transcriptional up-regulator of *HIS4* that acts in response to amino acid and purine starvation, it was hypothesised that the starvation induced recombination was being promoted via Gcn4p (Cotton, 2007). Gcn4p is also an essential regulator of various genes involved in overcoming other stress stimuli such as glucose deprivation and a high salinity growth medium (Hinnebusch and Natarajan, 2002). When triggered by amino acid limitation, Gcn4p is up-regulated via the general amino acid control system, hence activating transcription of its target genes including *HIS4*.

4.1.2 Aims

1. To confirm the effect of nutritional starvation on recombination at *HIS4.*
2. To investigate the role of Gcn4p and Bas1p/Bas2p in the starvation influenced level of meiotic recombination.

4.2 Materials

4.2.1 Sporulation Media

Cells were sporulated on solid KAc media, which include the complete nutrient-supplemented KAc and minimal KAc (Section 2.1.3). In addition, a 'half complete' KAc medium was produced with ingredients similar to complete KAc except for the addition of only half amounts of the nutrient mixture (0.044% (w/v); Table 2.4). For sporulation on minimal KAc, two populations of cells were used; either 'selected' diploids (Section 2.2.11.2) or 'unselected' diploids (Figure 4.1). Since diploids that were pre-selected on minimal medium ('selected') were unable to sporulate on minimal KAc, relevant nutrients were supplemented to the medium. These nutrients include amino acid and nucleotide that cells were auxotrophic for (0.003% (w/v) uracil and 0.004% (w/v) lysine for the wild type cells).

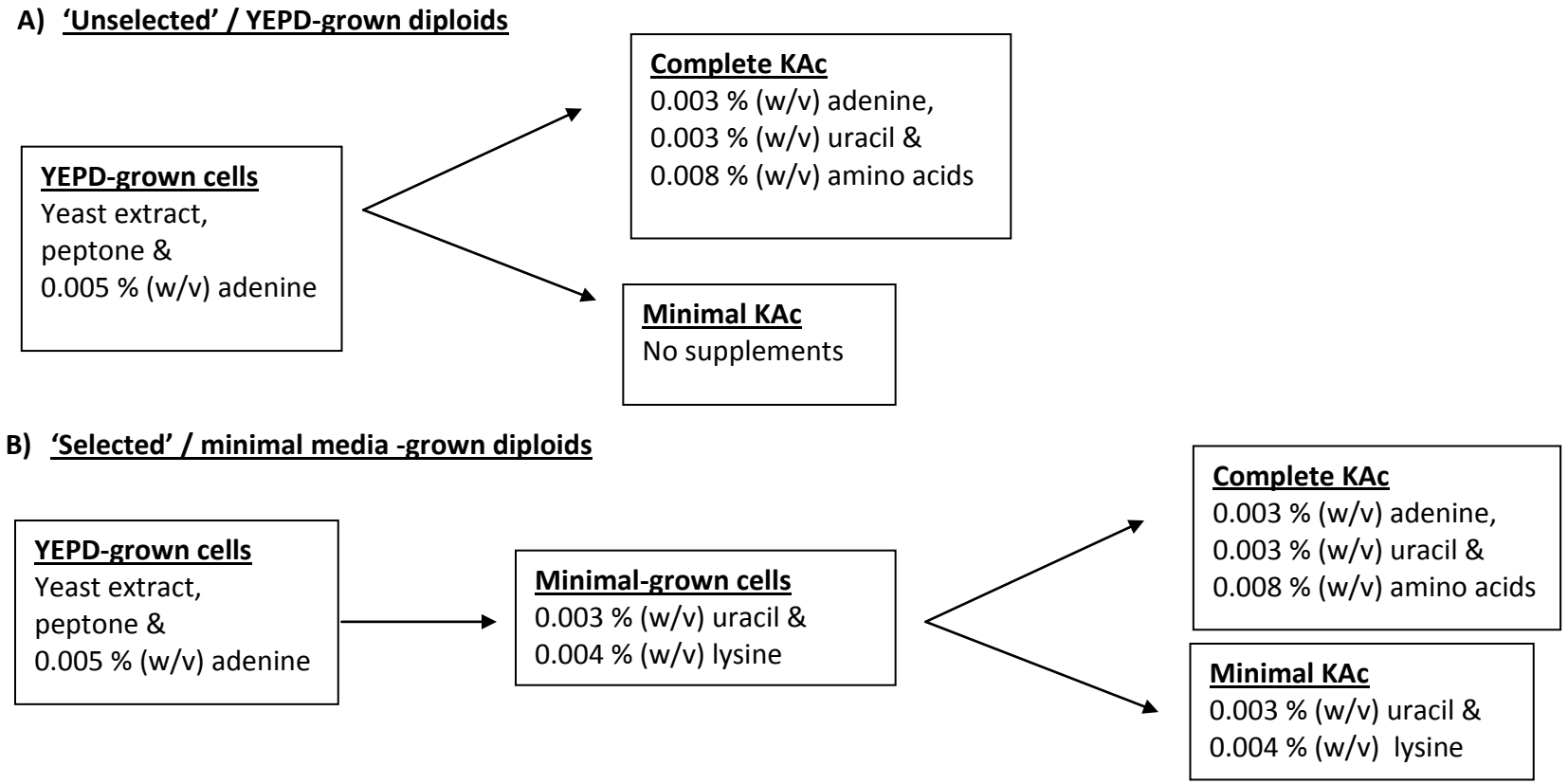


Figure 4.1 : Different methods of sporulation and pre-sporulation used in this study for the wild type strain. The amount of specific nutrients supplemented in each medium is indicated. A) Sporulation of unselected diploid cells that were vegetatively grown on rich medium (YEPD). The unselected diploids were able to sporulate on minimal KAc without nutrient supplements. B) Sporulation of diploid cells that were selected on partially supplemented synthetic minimal medium. Cells selected this way were unable to sporulate on minimal KAc without added supplements as indicated.

4.3 Results

4.3.1 Nutritional Starvation Increases the Level of NMS at *HIS4* in the Wild Type (Ade$^+$) Strain only in Pre-selected Diploid Cells

As stated in the introduction, it has been demonstrated that recombination activity at *HIS4* was elevated when the wild type cells were sporulated in nutrient-limiting conditions (Cotton, 2007). In the study by Cotton (2007), recombination events at *HIS4* were monitored in cells sporulated on two different media, complete KAc and minimal KAc (Section 4.2.1). Prior to sporulation on minimal KAc, diploids were selected on synthetic minimal medium that was supplemented with nutrients that both haploids were auxotrophic for (0.003% (w/v) uracil and 0.004% (w/v) lysine; Figure 4.1B) (Cotton, 2007). In addition, 0.003% (w/v) uracil and 0.004% (w/v) lysine were further added onto minimal KAc medium to enable sporulation of cells. This is due to the inability of selected diploid cells to sporulate without supplementation of the required nutrients.

In this study, we used two different experimental approaches to evaluate the effect of nutritional starvation on recombination at *HIS4* in the wild type (Ade$^+$) strain (Figure 4.1). The first method involved selecting diploid cells on a partially supplemented growth medium prior to sporulation on either complete KAc or minimal KAc (supplemented with uracil and lysine), as described above. The second method used another slightly modified version of this technique that eliminated the diploid selection step (Figure 4.1A). This was carried out by direct replication of mated cells from YEPD medium onto the sporulation media. Since diploids that were pre-grown on YEPD were able to sporulate on minimal KAc even without supplements, no additional nutrients

were added to the medium. Cells that were subjected to these techniques are going to be termed 'selected' (pre-grown on minimal medium plus supplements; Figure 4.1B) and 'unselected' (pre-grown on YEPD; Figure 4.1A) hereafter.

Using diploids that were selected on minimal medium (Figure 4.1B), sporulation of the wild type (Ade^+) cells on minimal KAc increased the level of NMS up to 2-fold compared to sporulation on complete KAc (P = 0.003; Table 4.1). Comparing between diploids selected on minimal medium with non-selected (YEPD-grown) diploid cells, sporulation of these cells with complete nutrients did not result in significantly different levels of NMS (Table 4.1). However, when sporulated on minimal KAc, diploids pre-selected on minimal media exhibited significantly higher levels of NMS than that of the non-selected cells (P = 0.025). Additionally, when the non-selected diploids were sporulated on complete and minimal KAc, no significant difference was observed in the level of NMS. These data suggest that the nutritional starvation only affects the NMS events when diploid cells were continuously starved before and during sporulation.

4.3.2 Nutritional Starvation during Sporulation Increases Crossing Over in the *NAT-HYG* Interval in the Selected, but not Unselected Wild Type (Ade^+) Diploid Cells

We also monitored the influence of nutritional starvation on crossing over in different regions on chromosomes III and VII. Crossing over in the *NAT-HYG* interval was increased in the selected diploids sporulated on minimal KAc compared to the YEPD-grown diploids sporulated on complete KAc ($P = 1.22 \times 10^{-5}$; Table 4.2). This elevated level of crossing over in the region flanking *HIS4* correlates with the increased level of NMS observed at the *HIS4* hotspot site (Table 4.2). In other intervals examined,

crossing over was unaffected in response to nutritional starvation in both selected and unselected cells (Tables 4.2 and 4.3).

Interestingly, sporulation of selected cells on minimal KAc did not result in reduction in the level of crossing over at *HYG-LEU2* compared to the YEPD-grown cells sporulated on minimal KAc. This observation did not correlate with the increased level of crossing over at *NAT-HYG* between selected cells sporulated on minimal KAc compared to non-selected cells, inconsistent with the hypothesis of crossover interference. However, since map distance between these two conditions are low (5.8 cM and 5.6 cM), it is possible that the amount of samples collected (202 and 218 tetrads) may not be enough to detect a significant difference between these conditions. Therefore, it is possible that crossover interference could be observed between these conditions if more tetrads were collected.

Table 4.1 : The level of NMS at *HIS4* for different sporulation conditions using the wild type (Ade^+) selected and unselected diploid cells

Strain Genotype	**Sporulation Condition**	**Pre-growth Media** [$]	**NMS / Total Tetrads**	**% NMS at *HIS4***
Wild type	Complete KAc	YEPD	50 / 388	12.9
Wild type	Complete KAc	Minimal (+ura, lys)	24 / 193	12.4
Wild type	Minimal KAc	YEPD	30 / 202	14.9
Wild type	Minimal KAc (+ura, lys)	Minimal (+ura, lys)	51 / 218	23.4[‡]

[$]YEPD –Unselected diploid cells that were pre-grown on YEPD medium; Minimal – Diploids that were pre-selected on minimal medium added with relevant nutrients. [‡]Significantly different compared to data from other conditions studied. The G-test of homogeneity was used for comparison.

Table 4.2 : Map distances in different genetic intervals on chromosome III for different sporulation conditions using the wild type (Ade^{+}) selected and unselected diploid cells

Sporulation Condition	Pre-growth Media	Genetic intervals											
		NAT – HYG				*HYG – LEU2*				*LEU2 – MAT*			
		PD	NPD	TT	**cM**[§]	PD	NPD	TT	**cM**	PD	NPD	TT	**cM**
Complete KAc	YEPD	307	3	68	**11.4**	305	2	72	**11.1**	162	15	205	**38.6**
Complete KAc	Minimal (+ura, lys)	155	1	32	**10.1**	153	0	36	**9.5**	93	4	93	**38.6**
Minimal KAc	YEPD	161	2	34	**11.7**	176	0	23	**5.8**	96	4	101	**31.1**
Minimal KAc (+ura, lys)	Minimal (+ura, lys)	131	3	80	**22.9**[‡]	192	0	24	**5.6**	94	2	120	**30.6**

[§]Map distances were calculated in centiMorgan (cM) according to Perkins (1949). PD is parental ditype, NPD is non-parental ditype and TT is tetratype. [‡]Represent values that are statistically significant compared to other conditions tested. G-test of homogeneity was used to compare the distributions of PD, NPD and TT between different samples.

Table 4.3 : Map distances in different genetic intervals on chromosome VII for different sporulation conditions using the wild type selected and unselected diploid cells

Sporulation Condition	Pre-growth Media	Genetic intervals							
		MET13 - CYH2				*CYH2 – TRP5*			
		PD	NPD	TT	**cM**[§]	PD	NPD	TT	**cM**
Complete KAc	YEPD	233	1	125	**18.2**	85	24	273	**54.6**
Complete KAc	Minimal (+ura, lys)	129	1	56	**16.7**	42	12	136	**54.7**
Minimal KAc	YEPD	123	0	57	**15.8**	46	11	140	**52.3**
Minimal KAc (+ura, lys)	Minimal (+ura, lys)	131	3	63	**20.6**	41	17	157	**60.2**

[§]Map distances were calculated in centiMorgan (cM) according to Perkins (1949). PD is parental ditype, NPD is non-parental ditype and TT is tetratype.

4.3.3 The Effect of Starvation on Recombination in the Selected Wild Type (Ade^+) Cells is Dependent on Bas1p but not Gcn4p

Gcn4p is the transcriptional activator known to be required for overcoming amino acid limiting conditions in yeast. Both a previous study (Cotton, 2007) and our data (Table 4.1 and 4.2) showed that starvation before and during sporulation for adenine and amino acids activates *HIS4* hotspot activity. Since the absence of these same nutrients stimulates Gcn4p activity (Section 1.5.3.3), this prompted us to investigate if the effect of starvation is facilitated by Gcn4p. Therefore, we analysed the levels of NMS and crossing over under two different conditions in the presence and absence of Gcn4p.

The data obtained demonstrated that even in the absence of Gcn4p (*gcn4*Δ), the level of NMS was increases when pre-selected diploids were sporulated with limited nutrients, compared to that of non-selected *gcn4*Δ diploid cells sporulated with complete nutrients (Table 4.4; $P = 6.9 \times 10^{-7}$). Similarly, crossover events increases at *NAT-HYG* in *gcn4*Δ cells that were starved both during and prior to sporulation, compared to the *gcn4*Δ cells fully supplemented with nutrients (P = 0.003; Table 4.5). As the level of NMS and crossing over still increase in the absence of Gcn4p, this suggests that the effect is not dependent on Gcn4p.

Since most of the recombination activity at *HIS4* is dependent on Bas1p (Chapter 3), we then asked if the *HIS4* hotspot can be activated by starvation in the absence of Bas1p. When the selected diploids of *bas1*Δ were sporulated on minimal KAc, the strain exhibited 5.4% NMS, which is significantly higher than that of the non-selected *bas1*Δ cells sporulated on complete KAc (1.8% NMS; P = 0.02). This data suggests that

although the majority of NMS events at *HIS4* are dependent on Bas1p, the level of NMS can still be induced by continuous starvation in the absence of Bas1p.

In the *NAT-HYG* region spanning *HIS4*, we found a subtle increase in the level of crossing over when *bas1*Δ cells were continuously starved as opposed to the supplemented cells. However, the difference is not statistically significant ($P > 0.05$). The observation did not correlate with the significantly increased level of NMS at *HIS4* when *bas1*Δ cells were starved during sporulation. This is inconsistent with other observations in our study that showed concomitant changes of the NMS events at *HIS4* with crossing over of flanking markers under various conditions (discussed in Section 7.8, Chapter 7).

However, the sample size tested in this experiment (467 and 212 tetrads for complete and minimal sporulation conditions respectively) may be too small to detect a significant difference and therefore may be subjected to a type II error (failure to reject null hypothesis even when it is false). Therefore, we used a method (inference for proportion, http://www.stat.ubc.ca/~rollin/stats/ssize/b2.html) to statistically estimate the sample size necessary to provide a significant difference between these populations. Approximately 650 tetrads for each sample would be sufficient to give an 80% chance that the data would be significantly different. Hence, we speculate that crossing over events at *NAT-HYG* in *bas1*Δ may also be elevated under starvation before and during sporulation, as was observed for the level of NMS at *HIS4.*

Table 4.4 : The level of NMS at *HIS4* in the *gcn4*Δ and *bas1*Δ strains under different sporulation and pre-sporulation conditions

Strain Genotype	**Sporulation Condition**	**Pre-growth Media**	**NMS / Total Tetrads**	**% NMS at *HIS4***
Wild type	Complete KAc	YEPD	50 / 388	12.9
Wild type	Minimal KAc (+ura, lys)	Minimal (+ura, lys)	51 / 218	23.4‡
*gcn4*Δ	Complete KAc	YEPD	45 / 467	9.6
*gcn4*Δ	Minimal KAc (+ura, lys)	Minimal (+ura, lys)	52 / 212	24.5‡
*bas1*Δ	Complete KAc	YEPD	6 / 331	1.8
*bas1*Δ	Minimal KAc (+ura, lys)	Minimal (+ura, lys)	11 / 203	5.4‡

‡Represent data that are significantly different compared to the same strain that were sporulated on complete KAc. The G-test of homogeneity was used for the comparison.

Table 4.5 : Map distances in different genetic intervals on chromosome III in the *gcn4*Δ and *bas1*Δ strains under different sporulation and pre-sporulation conditions

Strain Genotype	Sporulation Condition	Pre-growth Media	Genetic intervals											
			NAT – HYG				*HYG – LEU2*				*LEU2 – MAT*			
			PD	NPD	TT	**cM**[§]	PD	NPD	TT	**cM**	PD	NPD	TT	**cM**
Wild type	Complete KAc	YEPD	307	3	68	**11.4**	305	2	72	**11.1**	162	15	205	**38.6**
Wild type	Minimal KAc (+ura, lys)	Minimal (+ura, lys)	131	3	80	**22.9***	192	0	24	**5.6***	94	2	120	**30.6**
*gcn4*Δ	Complete KAc	YEPD	382	0	76	**8.3**	362	0	88	**9.8**	219	13	220	**33.0**
*gcn4*Δ	Minimal KAc (+ura, lys)	Minimal (+ura, lys)	159	3	50	**16.0***	191	0	19	**4.5***	85	2	123	**32.1**
*bas1*Δ	Complete KAc	YEPD	297	0	28	**4.3**	234	1	81	**13.8**	179	10	130	**29.8**
*bas1*Δ	Minimal KAc (+ura, lys)	Minimal (+ura, lys)	175	0	27	**6.7**	162	1	34	**10.2**	106	9	82	**34.5**

[§]Map distances were calculated in centiMorgan (cM) according to Perkins (1949). PD is parental ditype, NPD is non-parental ditype and TT is tetratype. *Significantly different compared to the same strain sporulated on the complete KAc. G-test of homogeneity was used to compare the distributions of PD, NPD and TT between different samples.

Table 4.6 : Map distances in different genetic intervals on chromosome VII in the *gcn4*Δ and *bas1*Δ strains under different sporulation and pre-sporulation conditions

Strain Genotype	Sporulation Condition	Pre-growth Media	Genetic intervals							
			MET13 - CYH2				*CYH2 – TRP5*			
			PD	NPD	TT	**cM**[§]	PD	NPD	TT	**cM**
Wild type	Complete KAc	YEPD	233	1	125	**18.2**	85	24	273	**54.6**
Wild type	Minimal KAc (+ura, lys)	Minimal (+ura, lys)	131	3	63	**20.6**	41	17	157	**60.2**
*gcn4*Δ	Complete KAc	YEPD	271	5	151	**21.2**	105	39	313	**59.8**
*gcn4*Δ	Minimal KAc (+ura, lys)	Minimal (+ura, lys)	130	2	66	**19.7**	48	22	141	**64.7**
*bas1*Δ	Complete KAc	YEPD	225	0	88	**14.1**	67	19	240	**54.3**
*bas1*Δ	Minimal KAc (+ura, lys)	Minimal (+ura, lys)	134	1	59	**16.8**	43	17	142	**60.4**

4.3.4 Starvation during Sporulation Increases Recombination in the *ade1*Δ Strain

Previous studies have reported that a point mutation of *ADE1*, *ade1-1*, increases the level of recombination at *HIS4* (Chapter 3) (Abdullah and Borts, 2001, Cotton, 2007). Cotton (2007) demonstrated an elevated level of recombination in the *ade1-1* strain, when selected diploids were used for sporulation on minimal KAc (supplemented with adenine, uracil and amino acids) compared to unselected diploids sporulated on complete KAc. We extended the study by analysing the effect of full starvation during sporulation upon recombination in the *ade1* null mutant (*ade1*Δ) strain, using non-selected (YEPD-grown) diploids for sporulation on complete and minimal KAc.

Surprisingly, without prior selection, the *ade1*Δ cells that were starved during sporulation exhibited a 3.8-fold increased level of NMS (41.9%; Table 4.7) relative to the cells sporulated on complete KAc (11.1%; $P = 6.3 \times 10^{-16}$). These data indicate that complete starvation for adenine, uracil and amino acids during sporulation increases recombination levels in the *ade1* deletion strain, even when the cells were pre-grown on rich medium. Similarly, a concomitant increase in crossing over at *NAT-HYG* was observed in the *ade1*Δ cells when sporulated on minimal KAc, compared to sporulation on complete KAc ($P = 9.6 \times 10^{-14}$; Table 4.8). Crossing over in other intervals remained unaffected under nutrient limiting condition.

Table 4.7 : The level of NMS at *HIS4* in the *ade1*Δ strain under different sporulation conditions using YEPD-grown diploids

Strain Genotype	**Sporulation Condition**	**NMS / Total Tetrads**	**% NMS at *HIS4***
Wild type	Complete KAc	50 / 388	12.9
Wild type	Minimal KAc	30 / 202	14.9
*ade1*Δ	Complete KAc	28 / 252	11.1
*ade1*Δ	Minimal KAc	109 / 260	41.9‡*

‡Significantly different compared to the corresponding wild type. *Significantly different compared to the same strain sporulated on the complete KAc

Table 4.8 : Map distances in different genetic intervals on chromosome III in the *ade1*Δ strain under different sporulation conditions using the non-selected (YEPD-grown) diploids

Strain Genotype	Sporulation Condition	Genetic intervals											
		NAT – HYG				*HYG – LEU2*				*LEU2 – MAT*			
		PD	NPD	TT	**cM**[§]	PD	NPD	TT	**cM**	PD	NPD	TT	**cM**
Wild type	Complete KAc	307	3	68	**11.4**	305	2	72	**11.1**	162	15	205	**38.6**
Wild type	Minimal KAc	161	2	34	**11.7**	176	0	23	**5.8**	94	6	101	**31.1**
*ade1*Δ	Complete KAc	194	3	47	**13.3**	195	1	48	**11.1**	124	5	116	**29.8**
*ade1*Δ	Minimal KAc	119	8	129	**34.6**[‡#]	237	2	21	**6.3**[#]	132	5	123	**29.4**

[§]Map distances were calculated in centiMorgan (cM) according to Perkins (1949). PD is parental ditype, NPD is non-parental ditype and TT is tetratype. [‡]Significantly different compared to the corresponding wild type. [#]Significantly different compared to the same strain sporulated on complete KAc.

Table 4.9 : Map distances in different genetic intervals on chromosome VII in the wild type and *ade1*Δ strains under different sporulation conditions using the YEPD-grown diploids

Strain Genotype	Sporulation Condition	Genetic intervals							
		MET13 - CYH2				*CYH2 – TRP5*			
		PD	NPD	TT	**cM**[§]	PD	NPD	TT	**cM**
Wild type	Complete KAc	233	1	125	**18.2**	85	24	273	**54.6**
Wild type	Minimal KAc	123	0	57	**15.8**	46	11	140	**52.3**
*ade1*Δ	Complete KAc	146	1	83	**19.6**	70	14	166	**50.0**
*ade1*Δ	Minimal KAc	166	1	70	**16.0**	62	22	171	**59.6**

[§]Map distances were calculated in centiMorgan (cM) according to Perkins (1949).

4.3.5 Modulating the Amount of Nutrients during Sporulation Affects Recombination in the *ade1*Δ Strain

We further evaluated the nutritional effect on recombination at *HIS4* in the *ade1*Δ strain, by varying the amount of nutritional supplements (containing adenine, uracil and amino acids) in the sporulation media. To achieve this, the level of recombination was compared between cells sporulated on different KAc media containing different amounts of the nutrient mixture (complete KAc, half complete KAc and minimal KAc). Our data demonstrated a correlation between the quantity of the nutrient mixture and the level of NMS (Figure 4.2). By supplying half the amount of nutrients during sporulation, the level of NMS was elevated (25.6%) compared to complete supplementation (11.1%; $P = 5.3 \times 10^{-5}$). Additionally, this level of NMS in cells sporulated on the partially starved KAc, is significantly lower compared to the cells that were completely starved of the nutrient mixture (41.9%; $P = 2.0 \times 10^{-4}$).

Cells that were sporulated on 'half-complete' KAc also exhibited an increased level of crossing over in the *NAT-HYG* interval compared to sporulation with complete nutrients ($P = 0.008$). This reduction is concomitant with the elevated level of NMS events at *HIS4.* Similarly, crossing over was decreased in cells sporulated on 'half-complete' KAc relative to the total starvation condition ($P = 1.5 \times 10^{-5}$). This further confirms previous data that adenine, uracil and amino acids in the nutrient mixture (Table 2.3) are the determining factors whose absence influences the enrichment of *HIS4* recombination events in minimal KAc medium.

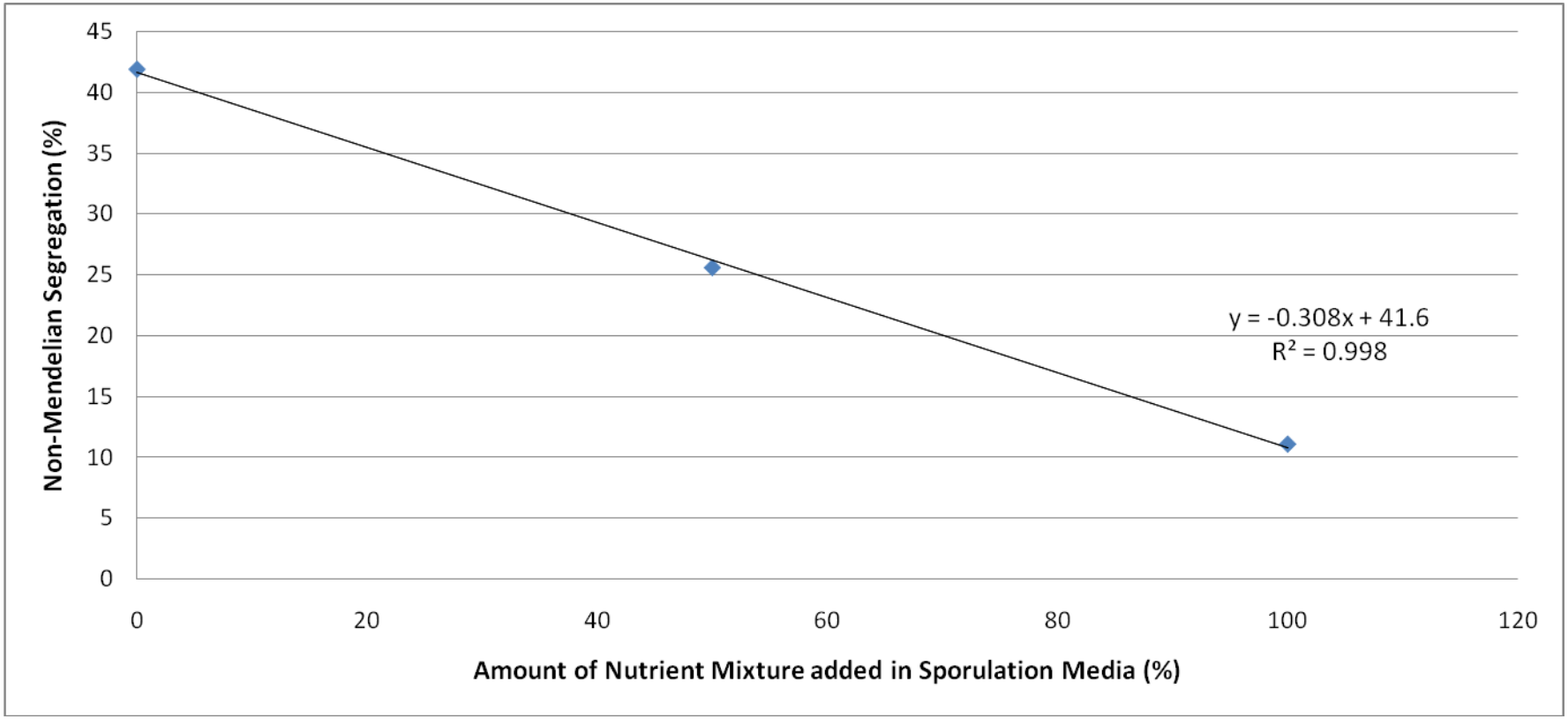


Figure 4.2 : A negative correlation between the level of NMS at *HIS4* in the *ade1*Δ strain with the amount of nutrients supplemented in sporulation media; 'minimal' KAc (0% of the nutrient mixture), 'half complete' KAc (50% of the nutrient mixture) and 'complete' KAc (100% of the nutrient mixture). Linear regression curves are represented, with R^2 corresponding to the square of Pearson's product moment correlation coefficient and was conducted using Excel (Microsoft Corporation).

Table 4.10 : Map distances in different genetic intervals on chromosome III for different sporulation conditions in the *ade1*Δ strains

Sporulation Condition	Genetic intervals											
	NAT – HYG				*HYG – LEU2*				*LEU2 – MAT*			
	PD	NPD	TT	**cM**[§]	PD	NPD	TT	**cM**	PD	NPD	TT	**cM**
Complete KAc	194	3	47	**13.3**	195	1	48	**11.1**	124	5	116	**29.8**
Half complete KAc	131	1	62	**17.5**[‡#]	164	0	35	**8.8**	113	5	84	**28.2**
Minimal KAc	119	8	129	**34.6**[‡]	237	2	21	**6.3**[‡]	132	5	123	**29.4**

[§]Map distances were calculated in centiMorgan (cM) [‡]Represent values that are significantly different to the sporulation on complete KAc. [#]Significantly different than sporulation on minimal KAc.

4.3.6 Gcn4p is a Minor Contributor to the Starvation Effect on Recombination in a Strain Auxotrophic for Adenine (*ade1*Δ)

The interesting observation in which depletion of nutrient mixture stimulates recombination activity in the non-selected *ade1*Δ cells, led us to investigate if this response is mediated by Gcn4p. Hence, we analysed recombination activity at *HIS4* in the *gcn4*Δ *ade1*Δ double deletion strain under starvation conditions. Our data demonstrated that the level of NMS in the *gcn4*Δ *ade1*Δ strain is slightly but significantly reduced under starvation, compared to the *ade1*Δ strain (P = 0.03; Figure 4.3). Consistent with this, a significant reduction was also observed for crossing over in *NAT-HYG* in the *gcn4*Δ *ade1*Δ strain compared to the *ade1*Δ strain, in cells that were sporulated on minimal KAc (P = 0.043). These observations suggest that Gcn4p plays a role in activating recombination in the *ade1*Δ strain under starvation (during sporulation).

However, we also found a 2-fold increase in the level of NMS in the *gcn4*Δ *ade1*Δ strain for cells sporulated on minimal KAc medium (32.7%) relative to sporulation on complete KAc (16.4%; P = 1.4×10^{-5}) (Figure 4.3). Correspondingly, similar increased of crossing over was also observed in the *NAT-HYG* interval in the *gcn4*Δ *ade1*Δ strain under starvation during sporulation, compared to the non-starved sporulation condition (P = 9×10^{-4}; Table 4.11). This suggests that even in the absence of Gcn4p, the level of recombination at *HIS4* was still stimulated in response to the nutritional starvation in the *ade1*Δ strain. Based on these data, the Gcn4p-dependent NMS activity in response to starvation is only approximately 22%, which is much lower than the starvation induced activity of NMS that is independent of Gcn4p (78%; Figure 4.3).

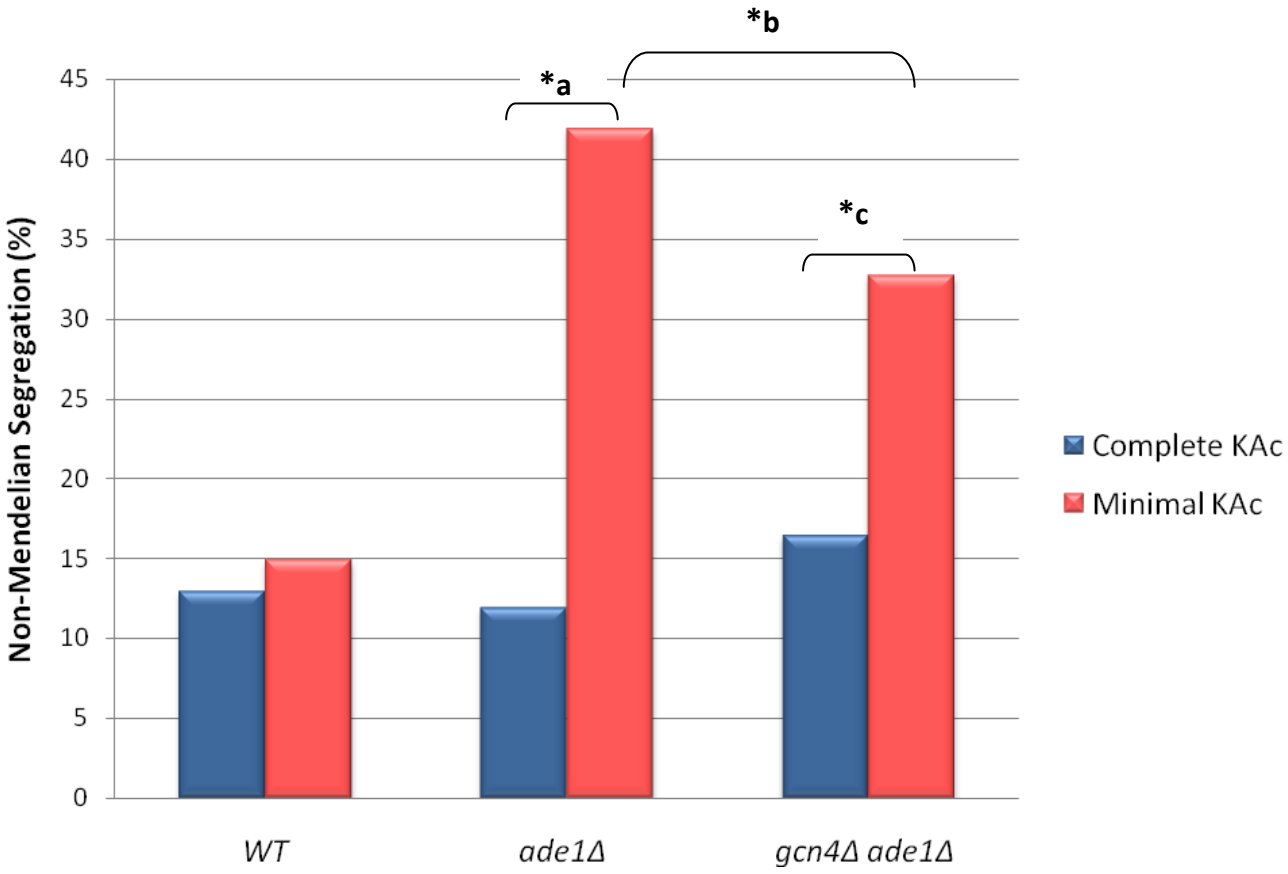


Figure 4.3 : The level of NMS at *HIS4* under different sporulation conditions in the *ade1*Δ and *gcn4*Δ *ade1*Δ strains using non-selected (YEPD-grown) diploids. *Pair wise comparisons showing data that are significantly different from each other; a) P value = 6.3×10^{-16}; b) P value = 0.03; c) P value = 9×10^{-4}

Table 4.11: Map distances in different genetic intervals on chromosome III for different sporulation conditions in the *ade1*Δ and *gcn4*Δ *ade1*Δ strains using YEPD-grown diploids

Strain genotype	Sporulation Condition	Genetic intervals											
		NAT – HYG				*HYG – LEU2*				*LEU2 – MAT*			
		PD	NPD	TT	**cM**[§]	PD	NPD	TT	**cM**	PD	NPD	TT	**cM**
*ade1*Δ	Complete KAc	194	3	47	**13.3**	195	1	48	**11.1**	124	5	116	**29.8**
*ade1*Δ	Minimal KAc	119	8	129	**34.6**[#]	237	2	21	**6.3**[#]	132	5	123	**29.4**
*gcn4*Δ *ade1*Δ	Complete KAc	200	3	69	**16.0**	225	0	48	**8.8**	138	5	131	**29.4**
*gcn4*Δ *ade1*Δ	Minimal KAc	139	5	96	**26.3**[‡#]	201	0	42	**8.6**	121	4	119	**29.3**

[§]Map distances were calculated in centiMorgan (cM) according to Perkins (1949). PD is parental ditype, NPD is non-parental ditype and TT is tetratype. [‡]Represent values that are significantly different compared to the corresponding wild type. [#]Significantly different compared to the same strain sporulated on the complete KAc.

Taken together, these observations suggest that Gcn4p makes a modest contribution towards mediating the effect of starvation (during sporulation) on recombination in the *ade1*Δ strain, while most of the activity is mediated by a Gcn4p-independent factor.

4.3.7 Bas1p is Required for the Starvation Induced Recombination at *HIS4* in the *ade1*Δ Strain

Since a significant proportion of the starvation effect on recombination at *HIS4* in the *ade1*Δ strain is independent of Gcn4p, we next looked at the involvement of Bas1p in the starvation induced recombination. Our data demonstrated that in the absence of Bas1p, the level of NMS in the *ade1*Δ strain was strongly reduced under starvation conditions (12-fold; $P = 3.62 \times 10^{-27}$; Table 4.12). Similarly, crossing over in the *NAT-HYG* interval was decreased in the *bas1*Δ *ade1*Δ strain compared to the *ade1*Δ strain ($P = 5.33 \times 10^{-28}$; Table 4.13). This implies a major requirement for Bas1p in mediating the increased levels of recombination at *HIS4* in the *ade1*Δ strain in response to starvation during sporulation.

4.3.8 AICAR from the Histidine Biosynthesis Pathway Partly Contributes to the Starvation Induced Recombination in the *ade1*Δ Strain

As stated above, Bas1p is required for the elevated levels of recombination at *HIS4* in the *ade1*Δ strain when cells were sporulated under starvation conditions. Bas1p likely mediates this starvation effect in conjunction with Bas2p, since interaction of both proteins is needed for binding to the *HIS4* promoter (Chapter 3). The purine metabolic intermediate, AICAR, is known to be important in activating Bas1/2p interaction, and is also produced via the histidine biosynthesis pathway (Figure 3.1; Chapter 3).

Table 4.12 : The level of NMS at *HIS4* in the *ade1*Δ, *bas1*Δ *ade1*Δ and *bas1*Δ strains sporulated on minimal KAc using non-selected (YEPD-grown) diploids

Strain Genotype	**Sporulation Condition**	**NMS / Total Tetrads**	**% NMS at *HIS4***
*ade1*Δ	Minimal KAc	109 / 260	41.9
*bas1*Δ *ade1*Δ	Minimal KAc	8 / 232	3.45‡
*bas1*Δ	Minimal KAc	12 / 354	3.39‡

‡Represent data that are significantly different compared to the corresponding *ade1*Δ cells.

Table 4.13 : Map distances in different genetic intervals on chromosome III in the *ade1*Δ, *bas1*Δ *ade1*Δ and *bas1*Δ strains sporulated on minimal KAc using YEPD-grown diploids

Strain Genotype	Sporulation Condition	Genetic intervals											
		NAT – HYG				*HYG – LEU2*				*LEU2 – MAT*			
		PD	NPD	TT	**cM**[§]	PD	NPD	TT	**cM**	PD	NPD	TT	**cM**
*ade1*Δ	Minimal KAc	119	8	129	**34.6**	237	2	21	**6.3**	132	5	123	**29.4**
*bas1*Δ *ade1*Δ	Minimal KAc	208	0	19	**4.2**[‡]	191	0	36	**7.9**[‡]	128	3	99	**25.4**
*bas1*Δ	Minimal KAc	313	1	33	**5.6**[‡]	282	0	61	**8.9**[‡]	190	8	149	**28.4**

[§]Map distances were calculated in centiMorgan (cM) according to Perkins (1949). PD is parental ditype, NPD is non-parental ditype and TT is tetratype. [‡]Represent values that are significantly different compared to the corresponding *ade1*Δ cells.

Since Bas1/2p-activated recombination in the *ade1*Δ strain in response to starvation is not stimulated by AICAR from the purine biosynthesis pathway, we asked if AICAR produced from the histidine biosynthesis pathway is responsible for this activation. To address this, recombination activity was analysed in a *his1*Δ *ade1*Δ strain where the production of AICAR from both adenine and histidine biosynthesis pathways is blocked (Figure 3.1; Chapter 3).

The levels of NMS and crossing over were significantly decreased in the *his1*Δ *ade1*Δ strain compared to *ade1*Δ, when both strains were sporulated under starvation conditions ($P = 8.3 \times 10^{-6}$ and $P = 5.1 \times 10^{-7}$; Table 4.14 and 4.15 respectively). These data may suggest that the recombination increase in the *ade1*Δ strain in response to starvation is partially reliant upon AICAR produced via the histidine biosynthesis pathway. However, significantly higher levels of NMS and crossing over were observed when the *his1*Δ *ade1*Δ cells were sporulated on minimal KAc as opposed to complete KAc ($P = 1.7 \times 10^{-5}$ and $P = 0.001$; Table 4.14 and 4.15 respectively). These data suggest that the starvation induced recombination at *HIS4* occurs even when AICAR synthesis is completely blocked. Thus, although AICAR from the histidine biosynthesis pathway is partly responsible for mediating recombination in the *ade1*Δ strain in response to starvation, there are factors other than AICAR stimulation on recombination yet to be identified.

Table 4.14 : The level of NMS at *HIS4* in the *ade1*Δ and *his1*Δ *ade1*Δ strains under different sporulation conditions using YEPD-grown diploids

Strain Genotype	**Sporulation Condition**	**NMS / Total Tetrads**	**% NMS at *HIS4***
*ade1*Δ	Complete KAc	28 / 252	11.1
*ade1*Δ	Minimal KAc	109 / 260	41.9[#]
*his1*Δ *ade1*Δ	Complete KAc	25 / 287	8.7
*his1*Δ *ade1*Δ	Minimal KAc	47 / 208	22.6[‡#]

[‡]Significantly different compared to the *ade1*Δ cells sporulated under starvation condition. [#]Significantly different compared to the same strain sporulated on the complete KAc.

Table 4.15 : Map distances in different genetic intervals on chromosome III in the *ade1*Δ and *his1*Δ *ade1*Δ strains under different sporulation conditions using YEPD-grown diploids

Strain Genotype	Sporulation Condition	Genetic intervals											
		NAT – HYG				*HYG – LEU2*				*LEU2 – MAT*			
		PD	NPD	TT	**cM**[§]	PD	NPD	TT	**cM**	PD	NPD	TT	**cM**
*ade1*Δ	Complete KAc	194	3	47	**13.3**	195	1	48	**11.1**	124	5	116	**29.8**
*ade1*Δ	Minimal KAc	119	8	129	**34.6**[#]	237	2	21	**6.3**[#]	132	5	123	**29.4**
*his1*Δ *ade1*Δ	Complete KAc	232	0	44	**8.0**	224	0	57	**10.1**	141	8	133	**32.1**
*his1*Δ *ade1*Δ	Minimal KAc	143	1	59	**16.0**[‡#]	183	0	22	**5.4**[#]	102	4	102	**30.3**

[§]Map distances were calculated in centiMorgan (cM) according to Perkins (1949). PD is parental ditype, NPD is non-parental ditype and TT is tetratype. [‡] Significantly different compared to the *ade1*Δ cells sporulated under starvation condition. [#] Significantly different compared to the same strain sporulated on the complete KAc.

4.3.9 *HIS4* Recombination Cannot be Further Increased in the *ade16 ade17* Double Deletion

Even when nutrients were plentiful during sporulation, the *ade16*Δ *ade17*Δ cells exhibited very high levels of recombination (53% NMS events; 45.8 cM; Chapter 3). We hypothesised that this effect is due to the accumulation of AICAR which activates interaction between Bas1p and Bas2p. We wished to determine if the *HIS4* hotspot activity could be further increased in the *ade16*Δ *ade17*Δ strain by subjecting it to starvation of adenine, uracil and amino acids during sporulation. We found unchanged levels of both the NMS at *HIS4* and crossing over in the *NAT-HYG* interval in both sporulation conditions (Table 4.16). Furthermore, no effect of starvation was detected at other intervals in the *ade16*Δ *ade17*Δ strain.

Table 4.16 : The level of NMS at *HIS4* in the *ade1*Δ and *ade16*Δ *ade17*Δ strains under different sporulation conditions using YEPD-grown diploids

Strain Genotype	**Sporulation Condition**	**NMS / Total Tetrads**	**% NMS at *HIS4***
*ade16*Δ *ade17*Δ	Complete KAc	221 / 417	53.0
*ade16*Δ *ade17*Δ	Minimal KAc	233 / 259	47.4

There was no statistical significance in the frequency of NMS between the conditions tested.

Table 4.17 : Map distances in different genetic intervals on chromosome III in the *ade1*Δ and *ade16*Δ *ade17*Δ strains under different sporulation conditions using YEPD-grown diploids

Strain Genotype	Sporulation Condition	Genetic intervals											
		NAT – HYG				*HYG – LEU2*				*LEU2 – MAT*			
		PD	NPD	TT	**cM**[§]	PD	NPD	TT	**cM**	PD	NPD	TT	**cM**
*ade16*Δ *ade17*Δ	Complete KAc	140	21	253	**45.8**	380	1	34	**4.8**	199	10	206	**32.0**
*ade16*Δ *ade17*Δ	Minimal KAc	177	28	282	**46.8**	439	8	43	**9.3**	231	22	239	**37.7**

[§]Map distances were calculated in centiMorgan (cM) according to Perkins (1949). PD is parental ditype, NPD is non-parental ditype and TT is tetratype. There was no statistical significance in the map distances at all intervals between different sporulating conditions.

4.4 Discussion

4.4.1 Starvation Before and During Sporulation for Adenine and Amino Acids Activates Recombination at *HIS4* in the Wild Type (Ade$^+$) Strain

In this study, we found no effect of adenine and amino acid starvation (during sporulation) on *HIS4* hotspot activity when cells were pre-grown on a rich medium (YEPD) prior to sporulation ('unselected diploids'; Figure 4.1). In contrast, by selecting diploid populations on a synthetic minimal medium prior to meiosis, sporulation on minimal KAc led to a two-fold increase in the level of recombination compared to sporulation on complete KAc (Table 4.1; Figure 4.1). These data are consistent with the previously reported observation, in which nutritional starvation during and prior to sporulation influence the recombination level at *HIS4* (Cotton, 2007).

We hypothesised that the differences in the effect of starvation on *HIS4* hotspot activity between selected and unselected diploids is the result of different degrees of starvation for adenine and amino acids. Particularly, diploid cells were already deprived of adenine and amino acids when selected on the synthetic minimal medium prior to sporulation (Figure 4.1). Therefore, further starvation throughout meiosis might maintain or enhance the activity of regulators responsible for inducing recombination in response to nutritional limitations (discussed in Section 4.4.3).

Furthermore, the activity of these starvation-induced factors may return to the non-activated state when sufficient nutrients are supplemented in the sporulation media. This could explain the lower level of recombination obtained when cells were starved during vegetative growth but sporulated with complete nutrients (Table 4.1 and 4.2). In cells that were supplemented with complete nutrients prior to sporulation, the

regulators that respond to starvation may be in an inactivated state. Therefore, starvation during sporulation may not provide adequate time for these regulators to be activated in a manner that can stimulate DSB formation. These results indicate that being continuously starved for adenine and amino acids (pre-sporulation and during sporulation) is necessary to increase the level of recombination at *HIS4*, likely by activating as yet unknown factors to induce DSB production.

4.4.2 Differences of *GCN4* Deletion between Different Strain Backgrounds

This study showed that deletion of *GCN4* did not affect the levels of NMS and crossing over at *HIS4* compared to the wild type, under both starvation and non-starvation conditions (Table 4.4 and 4.5). These data suggested that the transcription factor Gcn4p is not required for recombination at *HIS4* in the Y55 strain, consistent with a previous observation by White *et al.* (1992) in the S288C strain background. However, these data contrast with another previous observation in which recombination events at *HIS4* were reduced when *GCN4* is deleted (Abdullah and Borts, 2001).

The experiment by Abdullah and Borts (2001) was performed in the H390 x H330 strain background, which is the Y55-related strain. One possibility could have been that the difference between these strains is due to a possible mutation of Gcn4p binding sites at the *HIS4* promoter in one of the strains. However, DNA sequencing of the promoter regions of *HIS4* in the Y55 and H390 x H330 strains ruled out this possibility. Therefore, it is possible that the requirement of Gcn4p for recombination at *HIS4* is strain-specific for unknown reasons.

Furthermore, the haploid *gcn4*Δ cells of H390 x H330 have aberrant growth phenotypes that include a slower growth on synthetic omission media, and aberrant

germination of some of its spores following tetrad dissection (M.F.F. Abdullah, personal communication). However, no aberrant growth phenotype for the *gcn4*Δ cells was observed in the Y55 strain background used in this study (Appendix I). This observation supports the suggestion that the Y55 and H390 x H330 strains respond differently in the absence of Gcn4p.

4.4.3 Elevation of *HIS4* Recombination in the Absence of Gcn4p or Bas1p in Response to Starvation, in the Wild Type Strain Prototrophic for Adenine (Ade^+)

We have demonstrated that both levels of gene conversion and crossing over in the *gcn4*Δ strain were increased in response to starvation during and prior to sporulation for adenine and amino acids (2.6- and 1.9-fold respectively; Table 4.4 and 4.5). This observation suggests that Gcn4p is not required for the effect of starvation upon recombination in adenine prototrophic cells.

Cells also exhibited low levels of recombination (5.4% NMS, 6.7 cM) in response to starvation before and during sporulation for adenine and amino acids, when *BAS1* was deleted (Table 4.4 and 4.5). However, the level of NMS in the *bas1*Δ strain was significantly elevated under starvation before and during sporulation, as opposed to that pre-grown and sporulated with complete nutrient supplements (Table 4.4 and 4.5). These data argue that although the vast majority of gene conversion events at *HIS4* are dependent on Bas1p, nutrient starvation can still stimulate the NMS events independently of Bas1p. This also suggests that there may be an unknown component that can activate gene conversion at *HIS4* in response to continuous starvation.

One possibility is that Rap1, a protein that also binds to the *HIS4* promoter, is involved in this recombination activation in response to starvation. Rap1p is a context-

dependent transcriptional activator and repressor, which is involved in various cellular processes including telomere lengthening and shortening, as well as controlling transcription of genes involved in the glycolytic pathway and ribosomal proteins (Sussel and Shore, 1991, Piña *et al.*, 2003, Joo *et al.*, 2011). At *HIS4,* Rap1p functions as a 'chromatin opener' by preventing the formation of nearby nucleosomes specifically at regions containing Bas1/2p binding sites and the high affinity Gcn4p binding site (Section 1.5.3.1) (Devlin *et al.*, 1991). Therefore, it has been suggested that Rap1p facilitates binding of Bas1/2p and Gcn4p to the promoter region, and the chromatin status is maintained by the transcription factors (Devlin *et al.*, 1991, Kirkpatrick *et al.*, 1999a).

The presence of Rap1p binding site has also been reported to be required for *HIS4* recombination (White *et al.*, 1993, Fan *et al.*, 1995). Therefore, it is possible that the starvation induced recombination activity at *HIS4*, which is independent of Bas1p and Gcn4p, is mediated by Rap1p. This could involve a Rap1p-mediated chromatin organisation that directly promotes access to recombination machinery to generate DSBs under starvation condition. It is also possible that nutrient starvation affect specific alterations in the chromatin structure by stimulating factors other than Rap1p, such as a chromatin remodelling protein that responds to starvation. This then creates an optimal environment for recombination initiating factors to form DSBs.

4.4.4 Starvation only during Sporulation Affects Recombination in Cells Auxotrophic for Adenine (*ade1*Δ)

As mentioned above, no effect on recombination was detected in the wild type Ade^{+} strain when the non-selected (YEPD-grown) diploid cells were starved for adenine,

amino acids and uracil only during sporulation. However, we found an intriguing observation in which the *ade1*Δ strain exhibited an elevated level of recombination when non-selected diploids were sporulated on minimal KAc (Table 4.7 and 4.8). In addition, this starvation effect in the *ade1*Δ strain requires Bas1p with little contribution of Gcn4p (Section 4.3.6 and 4.3.7). This suggests that although Gcn4p is not required for the effect of nutrient starvation before and during sporulation on recombination in the wild type Ade^+ strain, the effect of starvation (during sporulation) in the *ade1*Δ strain is partly dependent on Gcn4p. These data also suggest that Bas1p plays a major role in the effect of starvation during sporulation in cells auxotrophic for adenine (*ade1*Δ). Furthermore, this activation of Bas1/2p is independent of AICAR from the purine biosynthesis pathway, since complete deletion of *ADE1* should inhibit the synthesis of AICAR from the pathway (Figure 3.1).

We also demonstrated a significant reduction in the level of recombination in the *his1*Δ *ade1*Δ strain relative to the *ade1*Δ strain (Table 4.14 and 4.15). This result suggests that AICAR from the histidine biosynthesis pathway is partially involved in activating recombination in the *ade1*Δ strain in response to starvation. Gcn4p may be involved in this activation of the histidine biosynthesis pathway in response to nutrient starvation during sporulation, in cells auxotrophic for adenine. This is because Gcn4p is known to activate transcription of genes in the histidine biosynthesis pathways including *HIS4* and *HIS7* (Arndt *et al.*, 1987, Denis *et al.*, 1998, Springer *et al.*, 1996). Thus, when AICAR production was blocked from the purine biosynthesis pathway (in the *ade1*Δ strain), starvation for adenine and amino acids might activate the histidine biosynthesis pathway via Gcn4p for unknown reasons. Therefore, it would be of interest to determine if Gcn4p is involved in activating recombination in the *his1*Δ

*ade1*Δ strain under nutrient starvation during sporulation. To do this, the level of recombination in a triple deletion of *his1*, *ade1* and *gcn4* would need to be compared with the *his1*Δ *ade1*Δ strain.

We also found a significant increase in the level of recombination when the *his1*Δ *ade1*Δ cells were starved during sporulation compared to sporulation on complete KAc (Table 4.14 and 4.15). These data suggest that nutrient starvation could increase recombination at *HIS4* even in the absence of AICAR from both the purine and histidine biosynthesis pathways. This indicates that an AICAR-independent factor is also responsible for activating recombination in response to extracellular and intracellular nutrient starvation. One possible mechanism is that starvation potentially promotes phosphorylation of Bas1p, which leads to either increased binding of Bas1/2p to the *HIS4* promoter or direct recruitment of recombination initiating factors (discussed in Section 7.6). Alternatively, unknown chromatin remodelling protein could also respond to the starvation signals and thus increase binding of Bas1/2p to the promoter.

4.4.5 No Effect of Nutritional Starvation upon Accumulation of AICAR

In Chapter 3, we demonstrated that the accumulation of AICAR in the *ade16*Δ *ade17*Δ strain elevates the *HIS4* hotspot activity in a manner mediated by Bas1/2p. We initially expected that by starving the cells during sporulation, the level of recombination could be further increased due to an up-regulation of Bas1/2p and/or other factors. However, the unchanged level of recombination in the *ade16*Δ *ade17*Δ strain in response to starvation does not support this hypothesis. One possibility is that the level of recombination in the *ade16*Δ *ade17*Δ strain was at a very high level even under

non-starvation conditions (Table 4.16 and 4.17). Therefore the activity of Bas1/2p could already be at the maximum level (40% to 54% NMS), in which starvation does not further activate Bas1/2p. In addition, it is also possible that the formation of DSBs is already at the highest possible level, so that even if Bas1/2p were further activated, there would be no further production of DSBs.

4.4.6 Future Work

Since the activation of recombination at *HIS4* in response to starvation can be mediated by an AICAR-independent factor(s) that may stimulate Bas1p and Bas2p interaction, it is of interest to determine what these other mechanisms are. We could do this by testing if the effect of Bas1/2p on the *HIS4* hotspot activity involves specific chromatin alterations and/or post-translational modifications of Bas1p. Therefore, in Chapter 5, we attempted to investigate the influence of starvation in meiosis on specific histone modification activities using genetic analysis. In addition, we also attempted to evaluate the phosphorylation status of Bas1p in both starvation and non-starvation conditions using phospho-labelling (discussed in Chapter 5). In addition, it would also be of interest to distinguish between the components in the nutrient mixture (adenine, uracil and amino acids) to determine the effect of starvation for individual nutrient on recombination at *HIS4.*

Chapter 5 : The Influence of Nutrient Starvation on the Phosphorylation of Bas1p, and the Set2p-Mediated *HIS4* Hotspot Activity

5.1 Introduction

5.1.1 Chromatin Modification Activities Associated with Meiotic Recombination

In yeast and higher eukaryotes, the position and activity of many meiotic recombination hotspots have been demonstrated to be influenced, at least partly, by the status of local chromatin structure. It has been shown that some hotspots are associated with an 'open' chromatin configuration, which presumably makes the DNA more accessible to recombination initiating factors. In addition, local and global genomic studies have also characterised several histone post-translational modification activities that correspond with recombination hotspots.

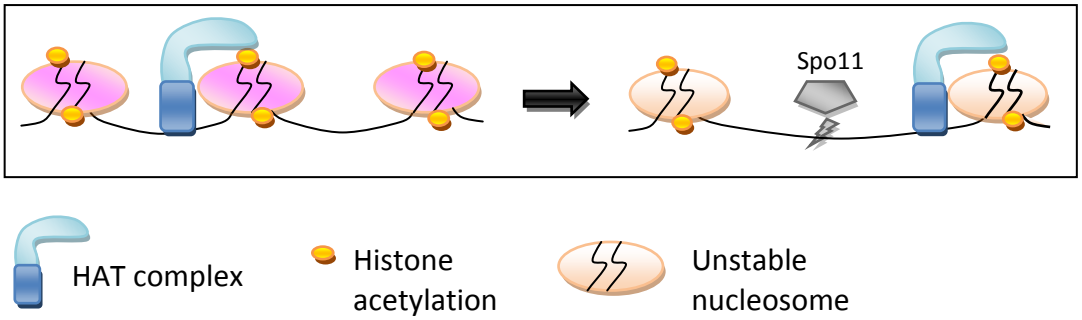
Trimethylation of lysine 4 of histone 3 (H3K4me3) has been reported to be associated with recombination hotspots in several organisms. In mice, a high level of H3K4me3 is correlated with elevated levels of recombination activity at *Psmb9* and *Hlx1* hotspots (Buard *et al.*, 2009). It has also been reported that a H3K4 trimethylase, Prdm9, is responsible for the distribution of a subset of recombination hotspots in mice and humans (Baudat *et al.*, 2010). Prdm9 contains a zinc-finger domain that binds to a degenerate 13-mer motif which is associated with a substantial group of recombination hotspots in humans (Section 1.5.2) (Myers *et al.*, 2010 268, Hochwagen and Marais, 2010 265). Therefore, it has been suggested that the enrichment of H3K4me3 at hotspot regions results from Prdm9 activity (Muers, 2010). Furthermore, a modification of the Prdm9 zinc-finger domain of transgenic mice was shown to affect

the activity of the *Psmb9* and *Hlx1* hotspots, as well as the level of H3K4me3 at these hotspot regions (Grey *et al.*, 2011). This further indicates that the Prdm9-catalysed H3K4me3 is important in regulating the distribution of hotspots in mammals. The binding of Prdm9 may promote recruitment of other proteins or chromatin remodelling factors, which could lead to chromatin alterations that favour DSB formation (Figure 5.1c.i) (Grey *et al.*, 2011).

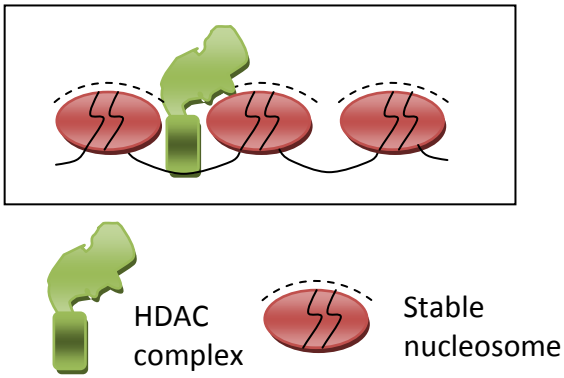
The link between H3K4me3 and recombination hotspots has also been reported in *S. cerevisiae*. Deletion of *SET1*, which encodes the only H3K4 methyltransferase in yeast, severely reduces DSB formation at the *CYS3* and *YCR047C* loci (Sollier *et al.*, 2004). Similarly, Borde *et al.* (2009) demonstrated that inactivation of Set1p methyltransferase reduces the rate of DSBs at 84% of *S. cerevisiae* hotspots genome-wide, including the *HIS4* hotspot. A constitutively higher level of H3K4me3 was also found near DSB regions (Borde *et al.*, 2009).

Furthermore, it has been shown that H3K4me3 already exists in vegetative cells at higher levels at the DSB-preferred sites relative to regions with few or no DSBs (Borde *et al.*, 2009). This implies that H3K4me3 serves to mark the chromatin sites for future recombination initiation (Székvölgyi and Nicolas, 2009). However, whilst the Set1p-mediated H3K4me3 was enriched at the majority of recombination hotspots in yeast, the formation of DSBs at some other loci was strongly stimulated in the absence of Set1p (Borde *et al.*, 2009). This suggests that recombination initiation is not completely dependent on H3K4me3, and could imply a requirement for other factors (e.g., other chromatin features). Moreover, not all regions that contains elevated levels of H3K4me3 are recombination hotspots, suggesting that additional factors may also be

a) Acetylation

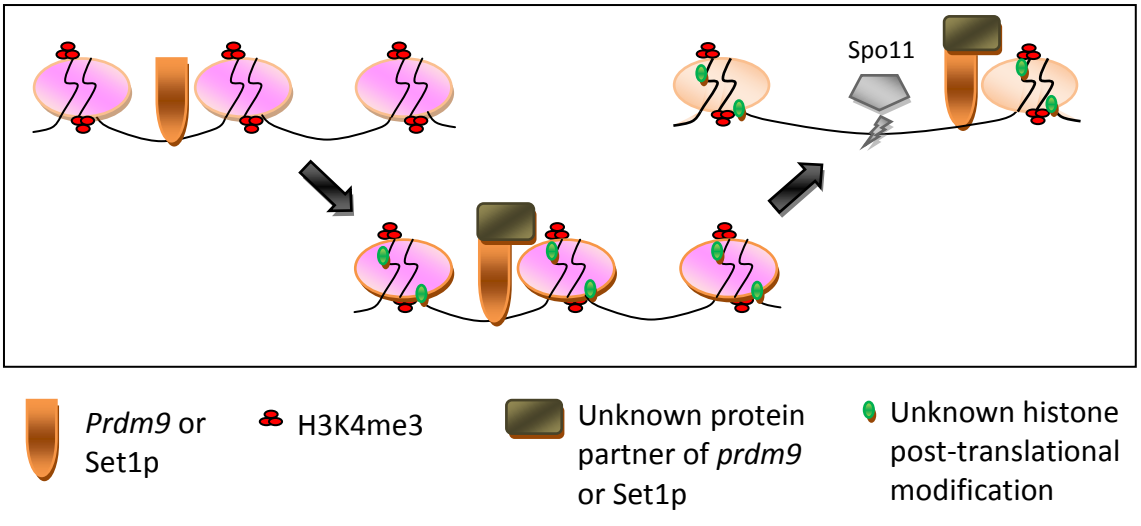


b) Deacetylation



c) Methylation

i. H3K4me3



ii. H3K36me3

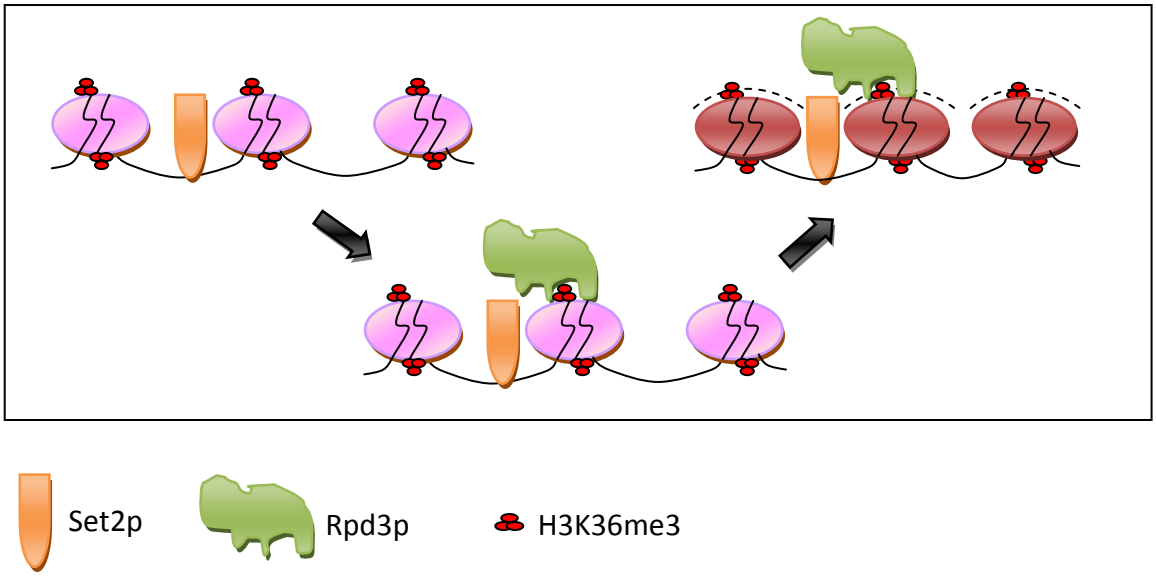


Figure 5.1 : Models for the association of several histone post-translational modifications with meiotic recombination hotspots in *S. cerevisiae* and other organisms.

a) Histone acetylation (e.g., H3K27ac at *HIS4*) is mediated by HATs (e.g., Gcn5p and Ada2p), and provides a chromatin structure that favours access of recombination factors.

b) Histone deacetylation (e.g., H4K16 near telomeres), mediated by HDACs (e.g., Hda1p, Rpd3p and Sir2p), stabilises nucleosomes hence 'closing-up' the chromatin.

c) Trimethylation of histones:

i) H3K4me3, mediated by Set1p in *S. cerevisiae* and Prdm9 in humans and mice. Prdm9 recognise its target DNA motif (the 13-mer motif), and binds the DNA through the zinc finger domain. Histone trimethylation mediated by Prdm9 or Set1p may promote other proteins that can modify the chromatin structure to favour access of Spo11p, or proteins that can directly recruit recombination machinery.

ii) The Set2p-mediated H3K36me3 at the *HIS4* locus in *S. cerevisiae*. The Rpd3 HDAC binds to the H3K36-methylated nucleosomes via its Eaf3p chromodomain subunit, and stabilises the nucleosome.

involved in hotspot specification (Hochwagen and Marais, 2010).

Several other histone post-translational modification activities have also been reported to influence recombination in yeast. These suggestions came primarily from studies that manipulate enzymes involved in modifications of histones and chromatin remodelling. In another genome-wide analysis of *S. cerevisiae*, deletion of *SIR2*, which encodes a histone deacetylase (HDAC), elevates the DSB formation in 5% of the genes (most of which are located near telomeres and the rRNA genes) and reduces them in 7% of the genes (Mieczkowski *et al.*, 2007). Moreover, the increased level of DSB formation near telomeres was suggested to be the result of hyperacetylation of histones, since Sir2p deacetylates H4K16 near telomeres (Robyr *et al.*, 2002).

Histone acetylation has often been associated with transcriptionally active regions potentially by mediating an 'unravelling' of chromatin, hence making it more accessible for proteins that mediate transcription (Kouzarides, 2007). Consistent with this, histone deacetylation by HDAC has the opposite effect 'closing-up' the chromatin, hence repressing transcription (Kouzarides, 2007). Therefore, the effect of histone acetylation on meiotic recombination may be regulated via a similar mechanism as transcription, by promoting an accessible state of chromatin for recombination machinery (Figure 5.1a).

A correlation between DSB formation and histone acetylation has also been demonstrated in *S. pombe*, in which hyperacetylation of histones H3 and H4 were observed near the region of the *ade6-M26* hotspot (Yamada *et al.*, 2004). This hyperacetylation at H3 and H4 is also dependent on transcription factors Atf1/Pcr1 and

the '*M26* sequence' (a heptameric DNA sequence created by the *M26* mutation), suggesting a site-specific chromatin effect (Yamada *et al.*, 2004). Furthermore, deletion of both *gcn5* and *ada2*, members of the SAGA family of histone acetyltransferases (HAT) in *S. pombe*, significantly reduces *M26* hotspot activity and histone H3 acetylation (Pryce and McFarlane, 2009). A more severe effect was observed on DSB formation and chromatin remodelling in the *ade6-M26* hotspot as a result of inactivation of Snf22p (a putative Swi2/Snf2 ATP-dependent Chromatin Remodelling Factors; ADCR) (Yamada *et al.*, 2004). These findings suggest that both the ADCR-mediated chromatin remodelling and histone acetylation influence *ade6-M26* hotspot activity, perhaps by providing preferential loading sites for DSB machinery (Yamada *et al.*, 2004).

At *HIS4* in *S. cerevisiae*, Merker *et al.* (2008) demonstrated that loss of Set2p histone methyltransferase (HMT) and Rpd3 deacetylase activities strongly stimulate recombination and DSB formation. Set2p mediates trimethylation of histone H3 lysine 36 (H3K36), which is known to recruit a repressive Rpd3 complex, Rpd3C(S), via its chromodomain subunit Eaf3p (Figure 5.1c.ii) (Keogh *et al.*, 2005). Therefore, the effect of *SET2* deletion on the *HIS4* hotspot activity was suggested to be due to the loss of Set2p-dependent recruitment of Rpd3 deacetylase, leading to a hyperacetylation of local chromatin (Merker *et al.*, 2008). Indeed, inactivation of Rpd3p has been shown to increase acetylation at many lysine residues of histone H3 and H4 at several loci including *INO1* and *IME2* (Rundlett *et al.*, 1996, Suka *et al.*, 2001, Robyr *et al.*, 2002).

Similarly, mutation of *SET2* was demonstrated to induce histone H3 and H4 acetylation including H3K9, H3K14 and H4K5 at many different locations in the genome (Carrozza

et al., 2005, Keogh *et al.*, 2005, Lin *et al.*, 2010). Therefore, it has been hypothesised that the recombination increase at *HIS4* in the absence of Set2p and Rpd3p is a consequence of increased acetylation of histones.

Consistent with this hypothesis, Merker *et al.* (2008) also demonstrated an elevated acetylation level of histone H3 lysine 27 (H3K27) in the *set2*Δ and *rpd3*Δ strains adjacent to the meiosis-specific DSB site of *HIS4*. Specifically, the levels of H3K27 acetylation were analysed at four regions flanking the DSB site, within approximately 200 bp and 1 kb of both upstream and downstream of the DSB site (Merker *et al.*, 2008). All four regions were hyperacetylated at the H3K27 sites in *set2*Δ and *rpd3*Δ, and stronger enrichments were observed at regions nearer to the DSB sites (Merker *et al.*, 2008). These observations suggest a link between increased levels of histone acetylation with the *HIS4* hotspot activity.

The regulation of Bas1/2p on the transcription of *HIS4* and other Bas1/2p-target genes has also been demonstrated to be associated with histone acetylation and the presence of HAT activity. Valerius *et al.* (2003) demonstrated that the activation of *HIS7* transcription by Bas1/2p, under adenine starvation condition, requires the Gcn5p-mediated acetylation of histones. *GCN5* encodes a HAT protein that targets lysine residues of H2B and H3 including H3K27, and exists predominantly as a subunit of chromatin-modifying complexes including SAGA and ADA (Lee and Young, 2000). Yu *et al.* (2006) demonstrated that the transcription of *HIS4* is dependent on Gcn5p since deleting *GCN5* results in a significant reduction of *HIS4* expression (Yu *et al.*, 2006). Furthermore, point mutations of H3K14 and H3K18, which are the important targets of Gcn5p, also led to a strong reduction of *HIS4* transcription activity (Yu *et al.*, 2006). In

the *ADE5,7* promoter, it has been reported that an increased occupancy of Bas1/2p in response to adenine deprivation requires chromatin remodelling complexes SAGA and SWI/SNF (Koehler *et al.*, 2007). Taken together, these observations may suggest that Bas1/2p regulate transcription of their target genes through the activity of specific HAT complexes.

As with other hotspots, it has been hypothesised that histone hyperacetylation (resulted from loss of Set2p and Rpd3p) stimulates recombination at *HIS4* by promoting an accessibility of chromatin to recombination machinery (Merker *et al.*, 2008). It has also been suggested that the transcription factors Bas1/2p and/or Rap1p could be involved in recruiting histone modifying proteins to the hotspot site (Merker *et al.*, 2008). In this chapter, we aimed to investigate if Bas1p is involved in the activation of the *HIS4* hotspot in the absence of Set2p. The effect of adenine and amino acid starvation upon recombination at *HIS4* in the *set2*Δ strain was also investigated. This is to determine if the *HIS4* hotspot activity can be stimulated further by modulating extracellular nutrient composition during sporulation when the Set2p methyltransferase is inactivated.

5.1.2 Post-Translational Modification Associated with Bas1p and Bas2p

5.1.2.1 Phosphorylation of Swi5p and Pho4p (Bas2p Partner Proteins) Activates Transcription of the *HO* and *PHO* Genes Respectively

Bas2p regulates transcription of several groups of genes in conjunction with at least three different co-activators, Bas1p, Pho4p and Swi5p (Section 1.5.3.2). Bas2p/Pho4p activates the expression of *PHO5* gene encoding a secreted acid phosphatase in response to deprivation for inorganic phosphate (Barbarić *et al.*, 1996). Bas2p/Swi5p

mediates the expression of the *HO* endonuclease, which initiates mating type switching, in response to cell cycle signals (Brazas and Stillman, 1993a).

The regulation of Pho4p and Swi5p in activating their target genes upon interaction with Bas2p involved phosphorylation (Nasmyth *et al.*, 1990, O'Neill *et al.*, 1996). The mechanisms by which phosphorylation regulates the activity of several transcription factors in response to internal or external signals includes conformational changes, altering their subcellular localisation, DNA binding activity or interaction with co-regulators (Locker, 2000). Swi5p and Pho4p are examples of transcription factors that are regulated through subcellular localisation by phosphorylation.

The activation of *PHO5* by Bas2p/Pho4p in response to deprivation of inorganic phosphate is regulated by dephosphorylation of Pho4p (Komeili and O'Shea, 1999, Liu *et al.*, 2000). In high phosphate conditions, Pho80p/Pho85p (a cyclin/cyclin-dependent kinase complex) mediates phosphorylation of Pho4p which inhibits its nuclear entry, promotes its nuclear export, and prevents interaction with Bas2p (Schneider *et al.*, 1994, Komeili and O'Shea, 1999). Under starvation for inorganic phosphate, Pho80/Pho85 are inactivated, leading to an increased interaction of Pho4p and Bas2p, hence stimulates the expression of *PHO5* (Schneider *et al.*, 1994, Kaffman *et al.*, 1994). The activation of *PHO5* transcription by Bas2p/Pho4p also requires phosphorylation of Bas2p, possibly by Cdc28 kinase (Liu *et al.*, 2000).

The transcriptional regulation of the *HO* gene by Swi5p is more complex, and is known to require phosphorylation of Swi5p by the Cdc28 kinase (Moll *et al.*, 1991). Phosphorylation of Swi5p inhibits its nuclear entry at the G_2 phase of the cell cycle, while inactivation of the Cdc28 kinase facilitates entry into nucleus on the start of the

G_1 phase and activates *HO* gene expression (Moll *et al.*, 1991). In addition, Swi5p needs to interact with Bas2p to facilitate binding specificity of Swi5p and stimulate high level of *HO* transcription (Brazas and Stillman, 1993a, Brazas and Stillman, 1993b). Since both Pho4p and Swi5p are co-partners with Bas2p, it has been thought that the regulation of Bas1/2p on the expression of their target genes also involved subcellular localisation of Bas1p. However, Som *et al.* (2005) showed that Bas1p does not exhibit changes in subcellular localisation even when its interaction with Bas2p is stimulated by adenine deprivation (Som *et al.*, 2005). Nevertheless, whether the interaction between Bas1p and Bas2p can be regulated by phosphorylation of Bas1p is yet to be determined.

5.1.2.2 Phosphorylation Associated with Bas1p

In Chapter 4, we have demonstrated that starvation for adenine and amino acids during sporulation elevates *HIS4* recombination even when the production of AICAR was completely blocked from both the purine and histidine biosynthesis pathways. The observation suggests that some of the starvation-induced recombination activity that was mediated by Bas1/2p can occur in an AICAR-independent manner. This raises the question of what promotes interaction between Bas1p and Bas2p without AICAR.

One of the possibilities considered is that the formation of Bas1/2p complex is partially mediated via phosphorylation of Bas1p which alters its conformation, hence facilitates its interaction with Bas2p (Section 4.4.4). Furthermore, a system-wide analysis of phosphorylation in *S. cerevisiae* had identified a phosphorylation site at the amino acid 653 of Bas1 protein when cells were grown in a complete-supplemented growth

condition (Bodenmiller *et al.*, 2010). This suggests that Bas1p can be phosphorylated under a normal growth condition.

Bas1p has also been suggested to be a substrate of the Snf1 protein kinase based on a regulatory network of Snf1p (Usaite *et al.*, 2009). The interaction map was constructed by measuring the abundance of genes, proteins and metabolites in the absence of *SNF1*, followed by integration with previously compiled data (Usaite *et al.*, 2009). The study suggested that Bas1p can be positively regulated by Snf1 kinase, since deleting *SNF1* results in significantly lower abundance of *BAS1* expression relative to the wild type (Usaite *et al.*, 2009). Snf1 kinase plays essential functions in metabolic control in response to various nutrients and environmental stress conditions including glucose and nitrogen deprivation, and oxidative stress (Orlova *et al.*, 2006, Hong and Carlson, 2007). In addition, Snf1p also plays an important role in regulating entry into meiosis, presumably by controlling cell adaptation for growth on media with acetate as the carbon source (Honigberg and Lee, 1998).

Interestingly, the mammalian homolog of *SNF1* is the AMP-activated kinase (AMPK), which is known to be activated by AICAR (Stefanelli *et al.*, 1998). However, a genome-wide study of gene transcription by Pinson *et al.* (2009) suggested that AICAR has no effect on Snf1 kinase activity in *S. cerevisiae*. This was based on the observation that modulating the amount of AICAR did not appear to affect expression of genes that were known to be strongly regulated by Snf1p. Nevertheless, an AICAR-independent activation of Snf1 kinase on Bas1p may be possible. In this chapter, we attempted to investigate the phosphorylation status of Bas1p under different nutritional conditions, including intracellular and extracellular starvation for adenine.

5.1.3 Meiotic-Null Allele of Bas1p

In Chapter 4, we showed that the *HIS4* hotspot activity was elevated in response to constant starvation before and during sporulation for adenine and amino acids. This effect is mediated by Bas1p. Here, we wished to determine if the Bas1p-mediated changes leading to elevated recombination would persist in meiosis, in the absence of further expression of Bas1p. In order to test this, we wanted to generate a meiotic-null allele of *BAS1*.

The promoter of the *CLB2* gene is often used to generate meiotic-null alleles of various genes, including *SGS1* (Lee and Amon, 2003, Amin *et al.*, 2010) and *SCC3* (Lin *et al.*, 2011). This is because the *CLB2* promoter does not facilitate gene expression in meiosis but functions only during vegetative growth (Grandin and Reed, 1993). The expression of *SGS1* during meiosis has been shown to be switched off when *CLB2* was used to replace the endogenous promoter of *SGS1* (Lee and Amon, 2003). Therefore, we sought to create a meiotic-null allele of *BAS1* by controlling its expression using the *CLB2* promoter. We would then determine the effect of pre-meiotic growth conditions on recombination at *HIS4*. These include nutritional starvation conditions, the Set2p-mediated histone hyperacetylation, and other modifications of the chromatin structure.

5.1.4 Aims

1. To determine the effect of adenine and amino acid starvation during sporulation on recombination at *HIS4* in the absence of the Set2p methyltransferase.

2. To investigate the influence of nutritional starvation on phosphorylation state of Bas1p.
3. To create and analyse a meiotic-null mutant of *BAS1*.

5.2 Materials and Methods

5.2.1 Materials

5.2.1.1 Media

Low phosphate growth medium (LP-YEPD) was prepared by mixing 1% (w/v) Bacto yeast extracts, 2% (w/v) Bacto peptone, 0.24% (w/v) magnesium sulphate (BDH) and 1 in 100^{th} volume of ammonium hydroxide (Fisher Scientific). The mixture was then incubated at room temperature to allow precipitation of phosphate. The mixture was twice vacuum-filtered using a 0.22 µm filter circle (Milipore) before adding 2% (w/v) glucose. The medium was then adjusted to pH 6.5 to 7.0 with 6 M HCl before autoclaving, and stored at 4°C.

5.2.1.2 Antibodies

For immunoblotting experiments, a mouse anti-HA monoclonal antibody (Santa Cruz) or mouse anti-Myc monoclonal antibody (Santa Cruz) were used as primary antibodies. A rabbit anti-mouse antibody (Santa Cruz) was used as the secondary antibody. For immunoprecipitation of Myc-tagged proteins, immobilised Myc-Tag mouse monoclonal antibody (Cell Signaling Technology) was used following manufacturer's guidelines. For the loading control, rat monoclonal α-tubulin antibody (Santa Cruz) was used as the primary antibody, and a goat anti-rabbit antibody conjugated to horseradish peroxidase (Santa Cruz) was used as the secondary antibody.

5.2.2 Methods

5.2.2.1 PCR-Based Chromosomal Gene Modification

The PCR-mediated technique for chromosomal gene modification was used to generate a meiotic-null allele of *BAS1*, as well as for epitope tagging of endogenous *BAS1* (Longtine *et al.*, 1998). To generate the meiotic-null allele, a *pFA6a-pCLB2-HA$_3$-KanMX6* plasmid was used as the PCR template for insertion of the *CLB2* promoter into *BAS1*. PCR primers were designed to contain 60 bp sequence complementary to regions immediately upstream and downstream of the start codon of *BAS1*, and 20 bp sequence homologous to the plasmid (Table 2.2). Subsequent transformation of the PCR product into genomic DNA will replace the native ATG start codon with the *pCLB2-HA$_3$-KanMX6* construct. Transformants were selected on a G418-400 medium to select for cell colonies that contains the *KanMX6* marker (Table 2.4; Section 2.1.3).

A similar method was used for epitope tagging of *BAS1*. In particular, the carboxyl terminal region of Bas1p was tagged with either thirteen copies of a Myc epitope or three copies of an HA epitope using the PCR-mediated approach. As above, PCR primers were designed with additional sequences that were homologous to the sequence immediately upstream and downstream of the stop codon of *BAS1*. The *pFA6a-HA$_3$-KanMX6* and *pFA6a-myc$_{13}$-KanMX6* plasmids were used as PCR templates for generating *BAS1-HA$_3$* and *BAS1-myc$_{13}$* strains respectively. These tags were transformed into yeast chromosomal locations by homologous recombination and selected on the G418-400 medium. Successful integrants were confirmed by PCR and DNA sequencing using primers listed in Table 2.5 (Section 2.2.7).

5.2.2.2 Preparation of Protein Extracts

The following method was used for all protein experiments except for the [^{32}P]-orthophosphate labelling (Section 5.2.2.5). For protein expression analysis of the vegetatively grown cells, diploids were streaked as single colonies on a solid YEPD medium and incubated overnight at 30°C. A single colony was then inoculated in 5 ml liquid growth media, either YEPD or synthetic minimal medium, and incubated overnight at 30°C with shaking. The following day, the culture was diluted 1:10 by transferring into a 250 ml conical flask containing 45 ml of the same media and further grown at 30°C with shaking.

Cells that were grown in minimal medium were left to grow overnight, while cells grown in YEPD medium were cultured for five hours until the culture reached an $OD_{\lambda 600}$ of 1.0 to 2.0. Phenylmethylsulfonyl fluoride (PMSF [Sigma]; dissolved in isopropanol) was added to cultures at a final concentration of 1 mM and incubated for 30 seconds with shaking at 30°C. Cells were then harvested in a 50 ml Falcon tube by centrifugation at 3500 r.p.m for 5 minutes at 4°C. 40 ml ice-cold Tris-buffered saline (TBS) solution (20 mM Tris-HCl pH 7.5 and 150 mM NaCl) was used to wash the cells before pelleted again by centrifugation at 3500 r.p.m for 5 minutes at 4°C. Prior to cell lysis, the residual solution was completely removed from the pellet.

For lysis, the pellet was resuspended in 400 µl cold B60 buffer (50 mM Hepes-KOH pH 7.3, 60 mM K-Acetate, 5 mM Mg-Acetate, 0.1% (w/v) Triton-X 100, 10% (w/v) glycerol, 1 mM sodium fluoride, 20 mM glycerophosphate, 1 mM DTT, a capsule of proteinase inhibitor [Roche] and 1 mM PMSF). Cells were then transferred into a fresh tube containing approximately 250 µl of glass beads (Sigma). A Bead Beater was used at

maximum speed for 1 minute to shear cells before pelleted by centrifugation for 5 minutes at 4°C (13,000 r.p.m). The supernatant was transferred to a fresh tube and centrifuged again for 20 minutes at 4°C to remove any remaining cell debris.

Approximately 300 µl (50 mg/ml) of the cleared protein lysate in the supernatant were transferred to a new tube. Each protein samples were quantified using 2.0 µl of each sample on a Nanodrop^{TM} ND-1000 spectrophotometer according to manufacturer's instructions (Section 2.2.10). The protein extracts were then incubated with 0.2 volumes 5x Laemmli-buffer (60 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.1% (w/v) bromophenol blue) at 95°C for 5 minutes prior to run on SDS-PAGE.

5.2.2.3 Western Blot Analysis

Proteins were separated on the Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). A standard 8% (w/v) separating gel (0.38 M Tris (pH 8.8), 8% (w/v) acrylamide mix (Protogel), 0.1% (w/v) SDS (Fisher Scientific), 0.1% (w/v) ammonium persulfate and 9 µl TEMED) was used unless otherwise stated. A thin layer of approximately 200 µl isopropanol was used to level the gel. After the gel set and isopropanol removed, approximately 2 ml stacking gel (0.125 M Tris-HCl pH 6.8, 0.1% (w/v) SDS, 6% (w/v) acrylamide mix, 0.2% ammonium persulfate and 5 µl TEMED) was poured and left to polymerise. Protein samples (normalised to contain the same amount of proteins) were loaded in each lane, together with an appropriate marker (Section 2.1.6). Electrophoresis was carried out for 45 minutes at 200 volts in running buffer (25 mM Tris-HCl, 0.2 M glycine (Fisher Scientific) and 0.1% (w/v) SDS, pH 8) until sufficient separation was obtained.

The proteins were then transferred to a Polyvinylidene fluoride (PVDF) membrane (Millipore) treated with 100% methanol using wet or semi-dry electrophoresis transfer methods. Typical wet transfer was mediated by electrophoresis at 100 volts for one hour and buffered by cold blotting buffer A (25 mM Tris-HCl, 0.2 M glycine, 0.1% (w/v) SDS and 2% (v/v) methanol). Semi-dry transfer was carried out at room temperature using blotting buffer B (192 mM glycine, 0.2 (v/v) methanol, 0.1% (w/v) SDS and 0.3% (w/v) Tris-HCl) mediated by electrophoresis at 25 volts for one hour. The PVDF membrane was then incubated in blocking solution (5% (w/v) milk solution in 1x TBST) for 30 minutes.

Following blocking, the membrane was incubated in primary antibody diluted 1 in 1000 in blocking solution and incubated on rocker for 2 hours at room temperature. The primary antibody was washed off from the membrane three times in 1x TBST. The membrane was then incubated in secondary antibody diluted 1 in 1000 in blocking solution and incubated on rocker for 2 hours at room temperature. The membrane was washed three times in 1x TBST. The band was visualised with ECL reagent (Biological industries) and X-ray film (Fujifilm).

5.2.2.4 Protein Immunoprecipitation

Protein extracts (2 mg/ml) were immunoprecipitated with 10 µl immobilised Myc-Tag mouse monoclonal antibody at 4°C with rotation overnight. The following day, beads were pelleted by centrifugation at 5,000 r.p.m for one minute. Immunoprecipitates were then washed with 1 ml TBS buffer (20 mM Tris pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.5% Nonidet P-40) and harvested again by gentle centrifugation as above. The washing step was repeated twice more. 0.2 volumes 5x Laemmli buffer (section 5.2.2.2)

was mixed with the remaining beads and subsequently boiled at 95°C for 5 minutes. Approximately 2 mg of the immunoprecipitated protein sample was separated on 8% (w/v) SDS gel, and western blot was performed as described above (Section 5.2.2.3).

5.2.2.5 [^{32}P]-orthophosphate Labelling

[^{32}P]-orthophosphate labelling is a radioactive method to detect protein phosphorylation. Yeast cells were grown in 5 ml YEPD overnight at 30°C with shaking. The next day, the culture was diluted 1:10 by transferring into a flask containing 45 ml YEPD and further grown for 4 to 5 hours. 5 ml of the culture were transferred to a Falcon tube and cells were harvested. Cells were then resuspended in 2 ml low phosphate growth medium (Section 5.2.1.1). The culture was added with 100 µCi/ml [^{32}P]-orthophosphate (GE Healthcare) and incubated for 2 hours on a rotating wheel at room temperature. Reaction mixtures were then lysed with an YPER lysis solution (Yeast Protein Extraction Reagent [Thermo Fisher Scientific]) and treated according to the manufacturer's guidelines. Proteins were quantified by Bradford assay (Bradford reagent, BioRad), and immunoprecipitated with 10 µl immobilised Myc-Tag mouse monoclonal antibody.

The immunoprecipitated proteins were then added with 0.2 volumes 5x Laemmli buffer and heating at 95°C for 5 minutes before resolved by 8% SDS-PAGE gel for 45 minutes. Proteins were then transferred to a nitrocellulose membrane (Milipore) using a semi-dry electrophoresis transfer method for one hour (Section 5.2.2.3). The presence of protein phosphorylation was first determined by visualising the signal of radioactive phosphate using a STORM phosphor-imager (GE Healthcare). Once phosphorylation

signal was detected, the membrane was subsequently exposed to autoradiographic film (Fujifilm) for two to seven days at -80°C.

Following exposure to autoradiography, the membrane was subsequently incubated in blocking solution (Section 5.2.2.3) for 30 minutes followed by incubation with rabbit anti-Myc polyclonal antibody (1:1000 dilution in blocking solution) for 2 hours. The membrane was then washed three times with 1x TBST, and incubated in secondary antibody (1:1000 dilution in blocking solution) for 2 hours. The membrane was then washed three times with 1x TBST. The band was visualised with ECL reagent (Biological industries) and X-ray film (Fujifilm).

5.3 Results

5.3.1 Recombination Frequencies at *HIS4* were Elevated in the *set2*Δ Strain

It has previously been reported that the *HIS4* hotspot activity was increased by the deletion of *SET2* in the S288C strain background (Merker *et al.*, 2008). This is presumably due to the loss of deacetylation by Rpd3p, which results in hyperacetylation of histone H3 lysine 27 (H3K27). Here, we asked if the effect of *SET2* inactivation on recombination is similar in the Y55 strain background, and whether nutritional starvation can further activate recombination in the *set2*Δ strain.

As expected, the level of NMS increased in the *set2*Δ strain (28.6%) as opposed to the wild type under non-starvation condition (12.9%; P = 2.4 x 10^{-6}; Figure 5.2). Under starvation for adenine, uracil and amino acids during sporulation, the level of NMS was further elevated (41.4%) compared to the non-starvation condition (P = 0.002; Figure 5.1 and 5.2). Correspondingly, crossovers at *NAT-HYG* flanking *HIS4* were also elevated in the *set2*Δ strain relative to the wild type, and further increased in response to starvation (P = 9.5 x 10-7 and 0.02 respectively; Table 5.1). These data suggest that starvation for adenine, uracil and amino acids can further increase recombination at *HIS4* in the absence of Set2p methyltransferase activity.

Interestingly, we found that crossing over in the *HYG-LEU2* interval were unaffected in *set2*Δ cells sporulated on complete KAc compared to the wild type strain. Similarly, no effect was observed in crossing over when the *set2*Δ cells were starved during sporulation as opposed to that sporulated with complete nutrients. These observations did not correspond to significant changes of crossing over in the adjacent

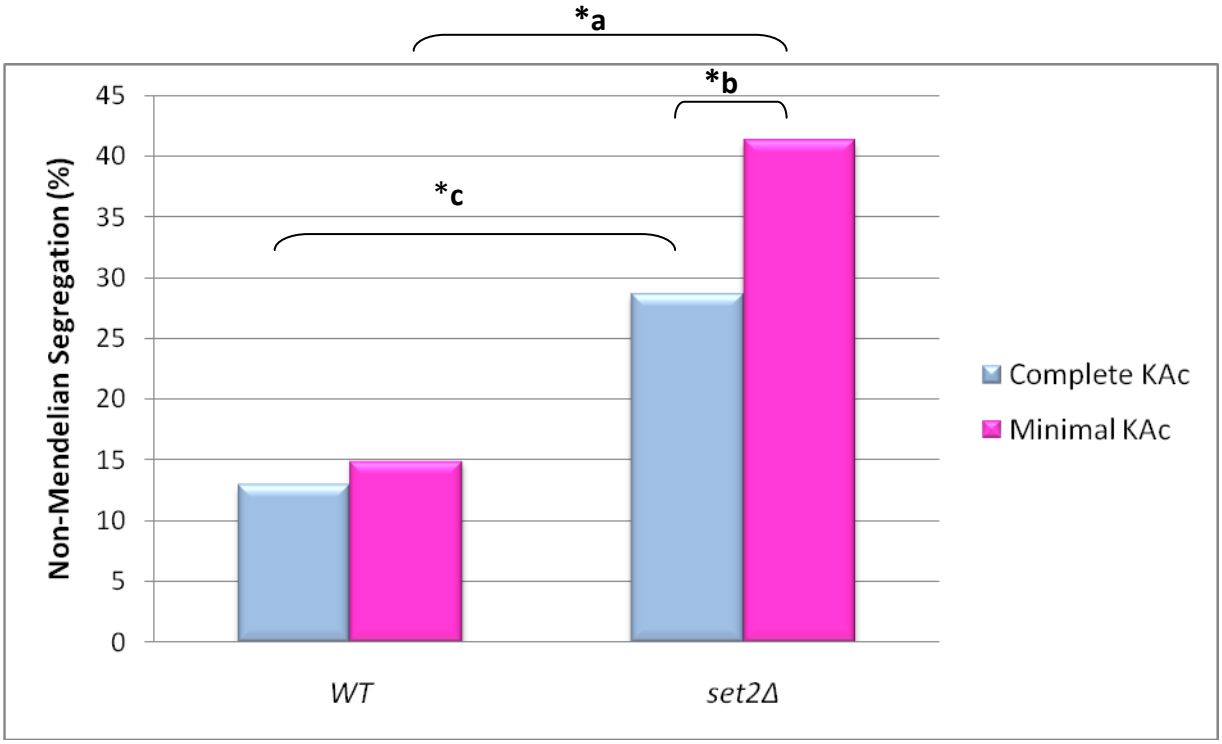


Figure 5.2 : The level of NMS at *HIS4* in the wild type and *set2*Δ strains sporulated with complete and minimal (without adenine, uracil and amino acid) nutritional supplements. *P-value of pair wise comparisons of data that are significantly different from each other; **a)** P = 5 x 10^{-4}, **b)** P = 0.002 and **c)** P = 2.4 x 10^{-6}.

Table 5.1 : Map distances in different genetic intervals on chromosome III in the *set2*Δ strain under different sporulation conditions

Strains	Sporulation Condition	Genetic intervals											
		NAT – HYG				*HYG – LEU2*				*LEU2 – MAT*			
		PD	NPD	TT	**cM**[§]	PD	NPD	TT	**cM**	PD	NPD	TT	**cM**
Wild type	Complete KAc	307	3	68	**11.4**	305	2	72	**11.1**	162	15	205	**38.6**
Wild type	Minimal KAc	161	2	34	**11.7**	176	0	23	**5.8**	94	6	101	**31.1**
*set2*Δ	Complete KAc	133	4	80	**24.0**[‡]	172	0	44	**10.2**	102	3	112	**30.0**
*set2*Δ	Minimal KAc	155	7	152	**30.9**[#‡]	281	0	40	**6.2**	147	12	163	**36.5**

[§]Map distances were calculated in centiMorgan (cM) according to Perkins (1949). PD is parental ditype, NPD is non-parental ditype and TT is tetratype. [‡]Represent values that are significantly different compared to the wild type sporulated on complete KAc. [#]Significantly different compared to the *set2*Δ strain sporulated on complete KAc.

NAT-HYG interval under similar conditions. These data may suggest that crossover interference does not occur when the *SET2* gene is inactivated.

5.3.2 Bas1p is Required for the Increased Level of *HIS4* Recombination in the *set2*Δ Strain

As shown above, our data supports the previous observation by Merker *et al.* (2008) in which the *HIS4* hotspot activity is stimulated by deleting the *SET2* gene. In addition, the level of recombination at *HIS4* in the absence of Set2p was further increased in response to starvation during sporulation. We then asked if the starvation effect on recombination in the *set2*Δ strain is mediated by Bas1p.

Our data demonstrated that the level of NMS in the *set2*Δ *bas1*Δ strain was significantly reduced compared to the single *set2*Δ strain, both under non-starvation and starvation conditions ($P = 1.3 \times 10^{-12}$ and 6.7×10^{-31}; Figure 5.3). Similarly, crossovers in the *NAT-HYG* region were reduced in *set2*Δ *bas1*Δ as compared to *set2*Δ under both non-starvation and starvation conditions ($P = 5.4 \times 10^{-11}$ and $P = 3.8 \times 10^{-18}$ respectively; Table 5.2). These data suggested that Bas1p is involved in the activation of recombination at *HIS4* in the absence of *SET2*.

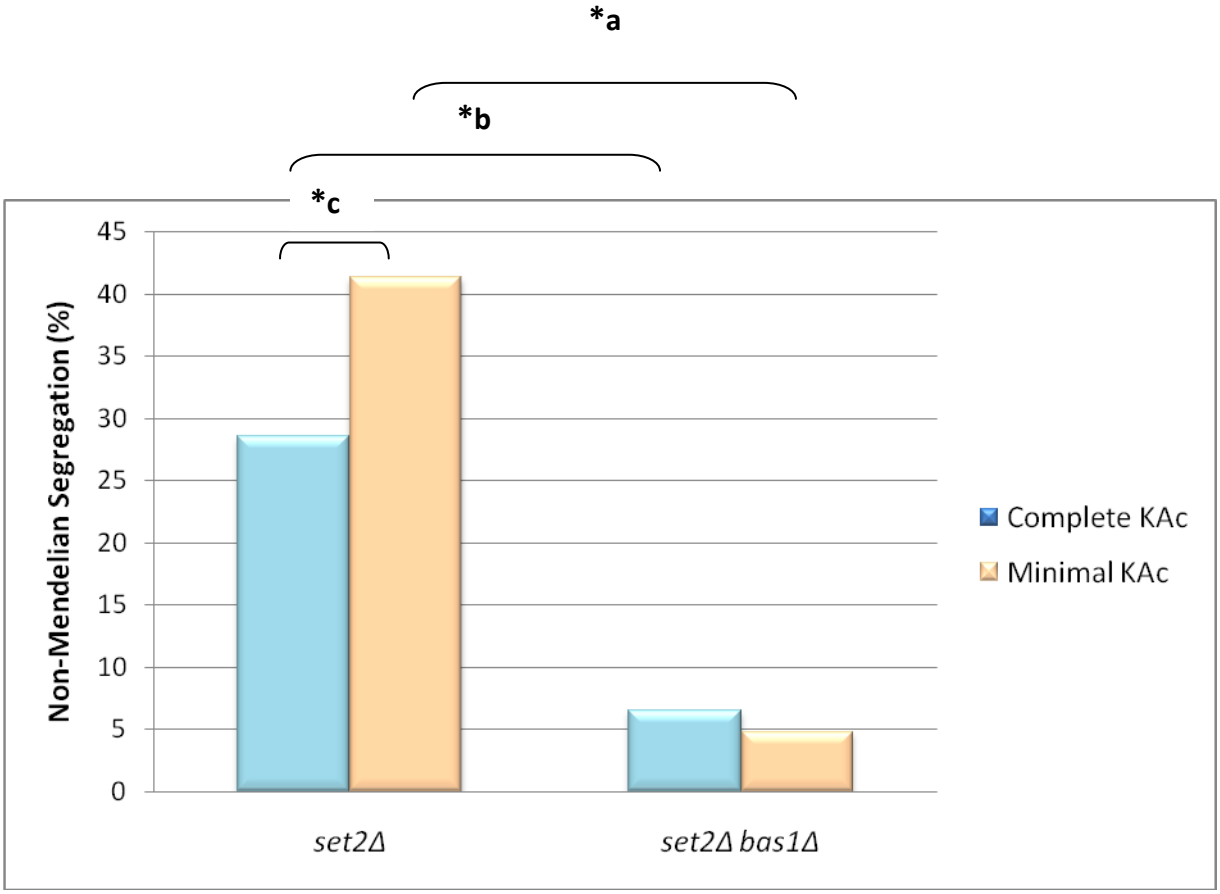


Figure 5.3 : The level of NMS at *HIS4* in the *set2*Δ and *set2*Δ *bas1*Δ strains sporulated with complete and minimal nutritional supplements. *P-value of pair wise comparisons of data that are significantly different from each other; **a)** P = 6.7 x 10^{-31}, **b)** P = 1.3 x 10^{-12} and **c)** P = 0.002.

Table 5.2 : Map distances in different genetic intervals on chromosome III in the *set2*Δ, *set2*Δ *bas1*Δ and *bas1*Δ strains under different sporulation conditions

Strains	Sporulation Condition	Genetic intervals											
		NAT – HYG				*HYG – LEU2*				*LEU2 – MAT*			
		PD	NPD	TT	**cM**	PD	NPD	TT	**cM**	PD	NPD	TT	**cM**
*set2*Δ	Complete KAc	133	4	80	**24.0**	172	0	44	**10.2**	102	3	112	**30.0**
*set2*Δ	Minimal KAc	155	7	152	**30.9**[#]	281	0	40	**6.2**	147	12	163	**36.5**
*set2*Δ *bas1*Δ	Complete KAc	280	0	45	**6.9**[‡]	246	0	82	**12.5**	148	16	165	**39.7**
*set2*Δ *bas1*Δ	Minimal KAc	252	0	55	**9.0**[‡]	238	0	68	**11.1**[‡]	153	5	150	**29.2**

[‡]Represent values that are significantly different compared to the corresponding *set2*Δ. [#]Significantly different compared to the same strain sporulated on complete KAc.

5.3.3 Insertion of the *HA$_3$* and *CLB2-HA$_3$* Constructs Inactivates Bas1p

In this study, we generated two different constructs of the HA-tagged Bas1p; one was tagged at the carboxyl terminus (*BAS1-HA$_3$*), whilst another construct contains a *CLB2* promoter that was inserted in the amino terminus of Bas1p (*pCLB2-HA$_3$-BAS1*). The *pCLB2-HA$_3$-BAS1* strain was generated with the aim to generate a meiotic-null allele of Bas1p, using the *CLB2* promoter to lead expression only during mitosis. In contrast, the *BAS1-HA$_3$* strain was generated for experiments that involved analysing Bas1p expression (e.g., radioactive labelling of Bas1p, Section 5.3.5).

Following addition of the epitopes into *BAS1*, it is important to determine whether the altered Bas1p retains its function as in the wild type strain. We first investigated if Bas1p is functional during mitotic growth in the *pCLB2-HA$_3$-BAS1* and *BAS1-HA$_3$* strains by determining if they could support growth in the absence of Gcn4p. It has been shown that cells wild type for histidine biosynthesis (His$^+$) were unable to grow in the absence of histidine, if they lack both Bas1p and Gcn4p (Section 1.5.3.1) (Arndt *et al.*, 1989). Therefore, we analysed the growth of *pCLB2-HA$_3$-BAS1* and *BAS1-HA$_3$* cells with a wild type His$^+$ phenotype and mutated *gcn4*, on media lacking histidine (Figure 5.4).

The *pCLB2-HA$_3$-BAS1 gcn4*Δ and the *BAS1-HA$_3$ gcn4*Δ cell colonies did not grow on the histidine omission medium after two days of incubation, similar to the phenotype of a *bas1*Δ *gcn4*Δ double deletion (Figure 5.4). This result indicates that the *pCLB2-HA$_3$-BAS1* strain is inactivated in mitotic growth. Together with the observation of lack of growth of *BAS1-HA$_3$ gcn4*Δ, this may suggest that insertion of the *HA$_3$* construct can disrupt normal Bas1p function.

Genetic analysis of the homozygous *pCLB2-HA$_3$-BAS1* and *BAS1-HA$_3$* strains demonstrated significantly lower levels of NMS as compared to the wild type (P = 2.3 x 10^{-4} and 9 x 10^{-4} respectively). Furthermore, the levels of NMS in these strains were not significantly different compared to *bas1*Δ (Table 5.3). Therefore, this suggests that Bas1p in the *pCLB2-HA$_3$-BAS1* and *BAS1-HA$_3$* strains is not functional in meiosis.

Interestingly, heterozygosity of *BAS1-HA$_3$* (*BAS1-HA$_3$* / wild type) results in reduced level of NMS compared to the homozygous wild type (*BAS1*) strain (P = 2 x 10^{-4}; Table 5.3). In addition, this level of NMS in the heterozygous *BAS1-HA$_3$* strain was not significantly different than the homozygous *bas1*Δ strain. These observations raised the possibilities that *BAS1-HA$_3$* may be a dominant negative mutant or that the *BAS1* gene is haploinsufficient. Therefore, in Chapter 6, we tested the possibility that *BAS1* may exhibit haploinsufficiency.

5.3.4 *BAS1-myc$_{13}$* Exhibited Normal Vegetative Growth and Expression

It is important to generate a functional epitope-tagged Bas1p for experiments involving Bas1p phospho-labelling. Since the HA-tagged Bas1p strains were not functional, a *BAS1-myc$_{13}$* strain construct was generated and the functionality of Bas1p analysed. We first evaluated the growth phenotype of *BAS1-myc$_{13}$* strain in the absence of Gcn4p on histidine omission medium (Figure 5.4B.i). The *BAS1-myc$_{13}$ gcn4*Δ strain was able to grow on the medium after two days of incubation at 30°C, indicating that Bas1p is functional in the *BAS1-myc$_{13}$* strain construct. A single band of approximately 120 kDa was also detected from immunoblot analysis which correlates to the size of *BAS1-myc$_{13}$* protein, confirming that Bas1p is present (Figure 5.6).

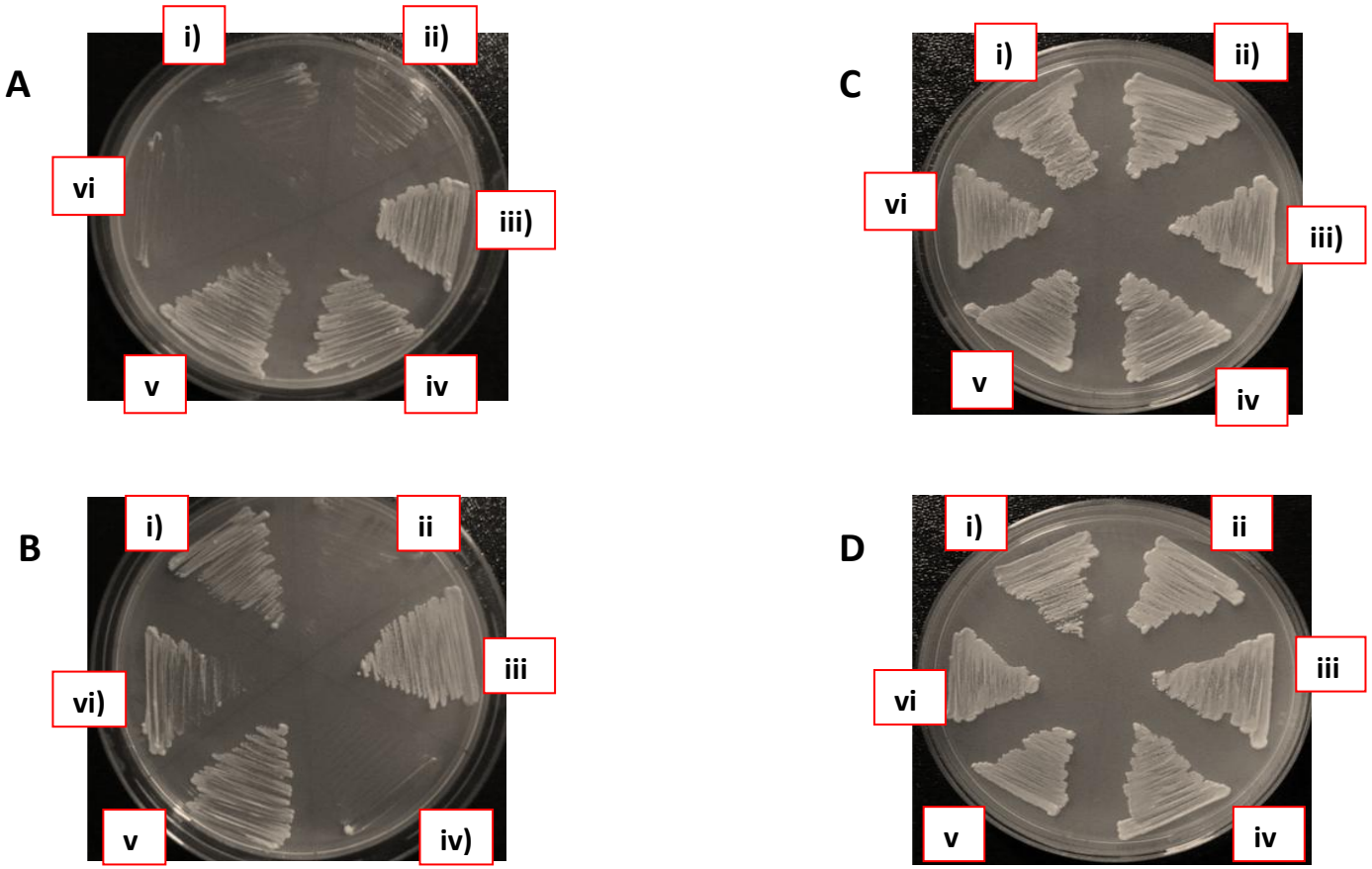


Figure 5.4 : Growth phenotype of various strains (all containing a His^+ phenotype except the wild type Y55 3569 strain which contains a His^- phenotype) on the histidine omission medium (A and B); and on the synthetic complete medium (C and D) after two days of incubation at 30°C. Strains shown in the figure are as follows:

A and C: **i)** *pCLB2-HA3-BAS1 gcn4Δ* **ii)** *bas1*Δ *gcn4*Δ **iii)** *gcn4*Δ **iv)** *bas1*Δ **v)** wild type (Y55 2830) **vi)** wild type (Y55 3569)
B and D: **i)** *BAS1-myc13 gcn4*Δ **ii)** *bas1*Δ *gcn4*Δ **iii)** wild type (Y55 2830) **iv)** *BAS1-HA3 gcn4Δ* **v)** *gcn4*Δ **vi)** *bas1*Δ

Table 5.3 : Reduction in the level of NMS at *HIS4* in the *pCLB2-HA$_3$-BAS1* and *BAS1-HA$_3$* strains

Strain Genotype	**NMS**	**Total**	**% NMS at *HIS4***
BAS1 / *BAS1*	50	388	12.9
pCLB2-HA$_3$-BAS1 / *pCLB2-HA$_3$-BAS1*	8	201	3.9‡
BAS1-HA$_3$ / *BAS1-HA$_3$*	9	195	4.6‡
BAS1-HA$_3$ / *BAS1*	9	218	4.2‡
*bas1*Δ / *bas1*Δ	6	331	1.8‡

‡Represent data that are significantly different compared to the corresponding wild type. The G-test of homogeneity was used for the comparison.

To determine if Bas1p remains active for recombination in meiosis, the level of NMS at *HIS4* in the *BAS1-myc$_{13}$* strain was also examined (Table 5.4). For the homozygous *BAS1-myc$_{13}$* diploid, the level of NMS remained unchanged compared to the wild type strain. Similarly, no significant difference in the level of NMS was observed for strains that are heterozygous for *BAS1-myc$_{13}$*, as opposed to the homozygous wild type diploid. These data indicate that Bas1p retains its meiotic function in the *BAS1-myc$_{13}$* construct, and that no dominant negative effect of *BAS1-myc$_{13}$* was observed on gene conversion at *HIS4*.

Table 5.4 : Comparison between the levels of NMS at *HIS4* in the homozygous *BAS1-myc$_{13}$*, heterozygous *BAS1-myc$_{13}$* and the homozygous *bas1*Δ Strains

Strain Crosses (Y55)	**Relevant Genotype**	**NMS / Total Tetrads**	**% NMS**
2830 x 3569	*BAS1* / *BAS1*	50 / 388	12.9
3634 x 3635	*BAS1-myc$_{13}$* / *BAS1-myc$_{13}$*	31 / 216	14.4
3635 x 2830	*BAS1-myc$_{13}$* / *BAS1*	28 / 222	12.6
3634 x 3569	*BAS1-myc$_{13}$* / *BAS1*	24 / 224	10.7
3612 x 3613	*bas1*Δ / *bas1*Δ	6 / 331	1.8‡

‡Represent data that are significantly different compared to the corresponding wild type. The G-test of homogeneity was used for the comparison.

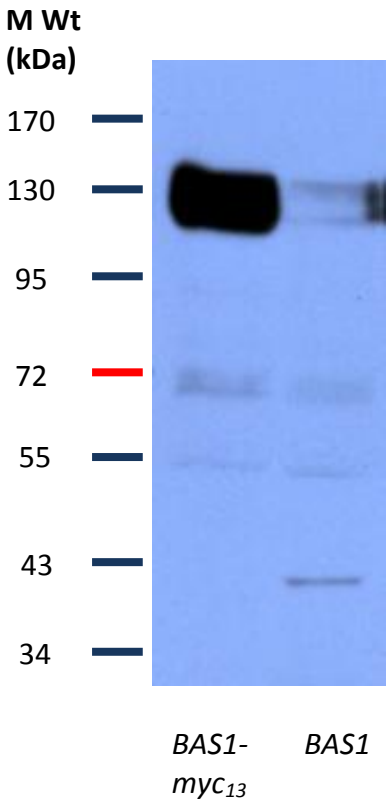


Figure 5.6 : Western blot analysis of mitotic expression of *BAS1-myc*$_{13}$ (first lane) showed a single band of approximately 120 kDa, the expected size of *BAS1-myc*$_{13.}$ No band was detected at the corresponding position in the second lane of the untagged wild type strain.

5.3.5 Potential Phosphorylation of Bas1p under Starvation and Non-starvation Conditions

In this study, we attempted to investigate if *BAS1-myc$_{13}$* is phosphorylated during vegetative growth under different nutritional conditions. A preliminary test included the wild type and *BAS1-myc$_{13}$* strains grown in liquid YEPD medium, as well as the *BAS1-myc$_{13}$* and *BAS1-myc$_{13}$ ade1*Δ grown in synthetic minimal medium. The study aimed to determine if there is any alteration in the level of expression and phosphorylation of *BAS1-myc$_{13}$* when cells were grown in rich and minimal nutritional conditions (deprived of adenine and amino acids), and when auxotrophic for adenine (*ade1*Δ).

We initially carried out western blotting of proteins prepared from *BAS1-myc$_{13}$*, *BAS1-myc$_{13}$ ade1*Δ and wild type strains that were sheared using the bead beater and B60 buffer (Section 5.2.2.2) to see if *BAS1-myc$_{13}$* were present in these strains. Bands of approximately 120 kDa were observed for *BAS1-myc$_{13}$* and *BAS1-myc$_{13}$ ade1*Δ samples under both starvation and non-starvation conditions, which correlate with the expected size of *BAS1-myc$_{13}$* (Figure 5.7A). No band was observed for the untagged wild type strain that was used as a negative control. This suggests that *BAS1-myc$_{13}$* is normally expressed under rich and minimal vegetative growth conditions.

Since the protocols involving the bead beater cannot be used for shearing radioactively-labelled cells, another method needs to be used. Therefore, a YPER lysis buffer (Yeast Protein Extraction Reagent [Thermo Fisher Scientific]) was used to extract

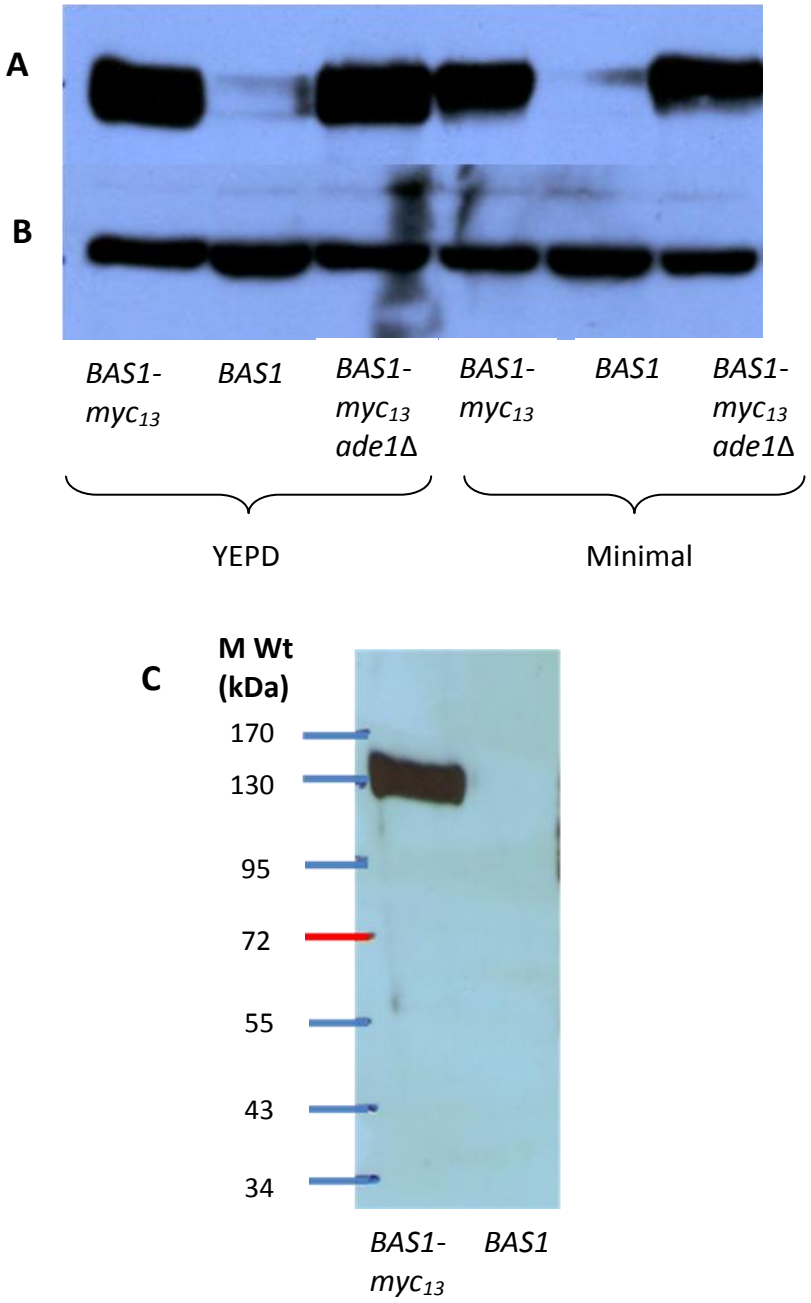


Figure 5.7 : **A.** Western blot showing expressions of *BAS1-myc*$_{13}$ and *BAS1-myc*$_{13}$ *ade1*Δ vegetatively grown in YEPD and synthetic minimal media. No bands were observed for untagged wild type strain under both conditions. **B.** α-tubulin antibody was used as the loading control. **C.** Western blotting of immunoprecipitated *BAS1-myc*$_{13}$ that were prepared using YPER lysis method. Precision plus protein marker (Biorad) was used as the molecular weight standard in this experiment.

labelled proteins (Section 5.2.2.5). To determine if this is sufficient for the phosphorylation experiment, we first performed immunoprecipitation and subsequent western blotting of *BAS1-myc*$_{13}$ prepared from unlabelled cells that were purified using YPER. A band of the expected size of *BAS1-myc*$_{13}$ was observed, suggesting that lysis was successful (Figure 5.7C).

To determine the phosphorylation status of Bas1 protein, a radioactive labelling was obtained by incorporating [^{32}P]-orthophosphate. The labelled proteins were immunoprecipitated with the immobilised Myc-Tag mouse monoclonal antibody to pull-down Bas1p, before being separated by SDS-PAGE, and transfer to nitrocellulose membrane and visualised by a STORM phosphor-imager and autoradiography. A preliminary experiment showed a phosphorylated band with the size of approximately 120 kDa for all samples with *BAS1-myc*$_{13}$, grown with complete nutrients and under starvation conditions (Figure 5.8A). Approximately three to four other bands of varying sizes were also observed, possibly the product of protein degradation.

Subsequent immunoblotting of the same membrane with rabbit polyclonal anti-Myc antibody recognised faint bands at approximately the size that also correlates with *BAS1-myc*$_{13}$ (Figure 5.8B). Furthermore, no band was detected for the negative control in both the western blot and autoradiography when the Myc-tag was absent (Figure 5.8A). Since no band was also observed in the western blot for the expected heavy chain of immunoglobulin (approximately 50 kDa) at the second lane of the negative control, this suggests that no protein extract is present in the second lane.

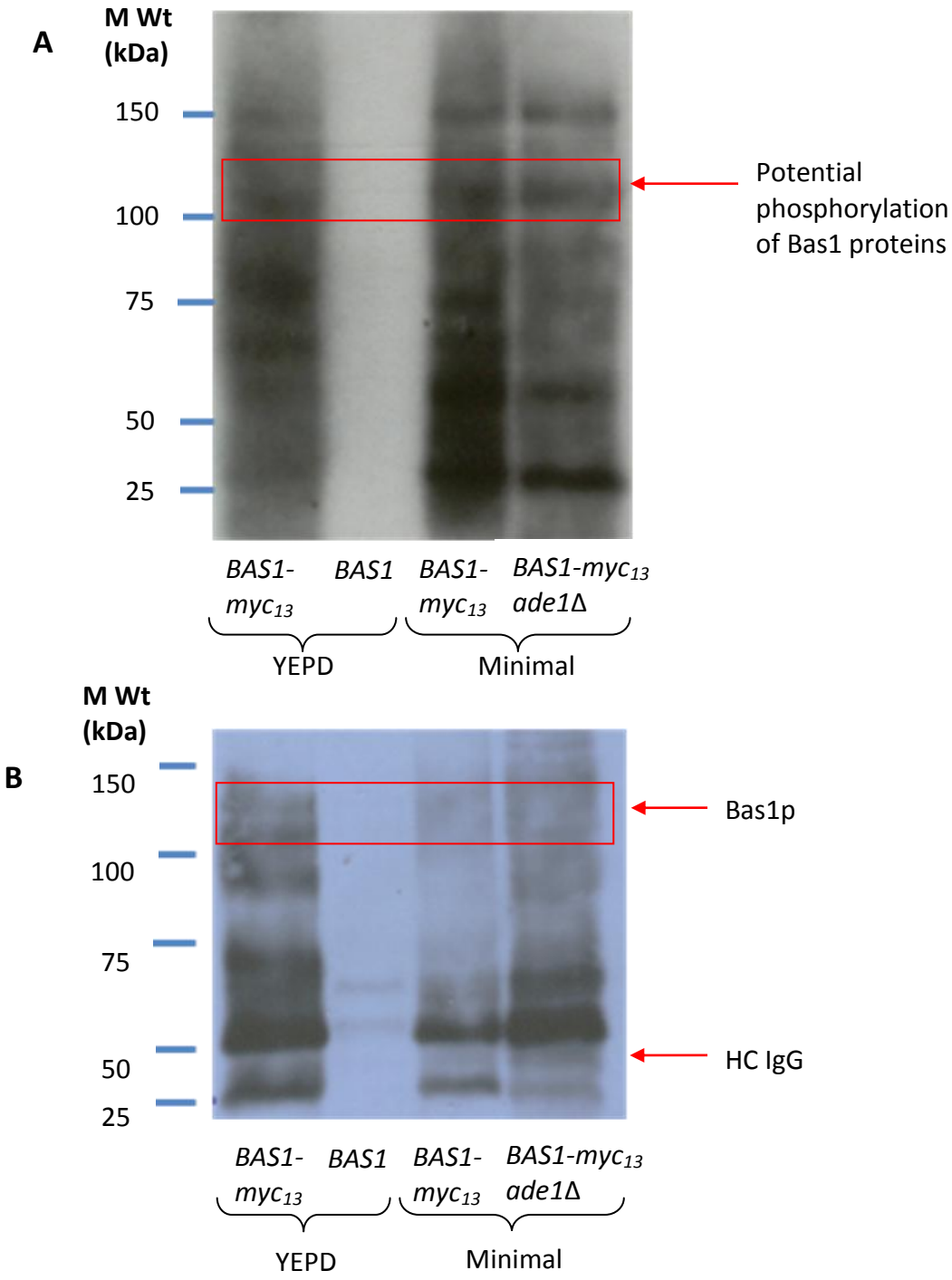


Figure 5.8: **A.** Potential phosphorylation of Bas1 protein. Autoradiograph of samples immunoprecipitated from [^{32}P]-orthophosphate labelled cells prepared from strains grown in liquid YEPD medium (wild type and *BAS1-myc$_{13}$*) and synthetic minimal medium (*BAS1-myc$_{13}$* and *BAS1-myc$_{13}$ ade1*Δ). Bands of approximately 120 kDa were recognised, correlated with the size of *BAS1-myc$_{13}$*. **B.** Western blot analysis of the same membrane showed bands with size that correlates with *BAS1-myc$_{13}$* and the phosphorylated bands. No bands were detected on the second lane of untagged wild type which serves as negative control. Bands of the size of heavy chain (HC) IgG were only detected in lane 1, 3 and 4 suggesting that proteins may not be present on lane 2 of the negative control.

5.4 Discussion

5.4.1 Bas1p is Required to Stimulate *HIS4* Hotspot Activity on the Loss of Set2p Methyltransferase under Starvation and Non-Starvation Conditions

In this study, we confirmed previous observation by Merker *et al.* (2005) that deletion of Set2p methyltransferase increases the level of recombination (Figure 5.2 and Table 5.1). Loss of Set2p and Rpd3p deacetylase has also been shown to elevate the formation of DSBs and hyperacetylates H3K27 at regions near the DSB site (Merker *et al.*, 2008). Therefore, it has been proposed that the increased recombination activity in *set2*Δ is the result of histone acetylation, which provides a favourable chromatin condition to recombination machinery.

A strong reduction in the level of recombination at *HIS4* in the *set2*Δ *bas1*Δ strain was also observed compared to the *set2*Δ strain (Figure 5.3 and Table 5.2). These data suggest that Bas1p is required in the stimulation of *HIS4* hotspot activity even when Set2p is inactivated. This also implies that the potential 'open' chromatin structure, mediated by inactivating Set2p, is not enough to activate recombination without the presence of Bas1p. Moreover, we also found that by starving the *set2*Δ cells for adenine and amino acids during sporulation, a further elevation of recombination activity was observed (Figure 5.2 and Table 5.1). Similar experiments have not been performed on *RPD3* because mutation of the gene results in sporulation defect of cells. However, it is possible that inactivating Rpd3p will have similar effect as the *set2*Δ strain.

Note that the effect of extracellular starvation in *set2*Δ occurs in a strain prototrophic for adenine (Ade^+), which was unaffected by starvation during sporulation when Set2p

is active (Table 4.1 and 4.2; Chapter 4). This may suggest that nutrient starvation can stimulate recombination activity in the wild type (Ade^+) strain when chromatin is in a highly 'opened' state. Furthermore, this effect also requires Bas1p, since the highly activated recombination in *set2*Δ under nutrient starvation was repressed in the *set2*Δ *bas1*Δ strain.

The mechanism by which Bas1p affects recombination in the absence of Set2p is unclear. We speculate that the hyperacetylation of histones, resulting from loss of Set2p, may lead to the unravelling of nucleosomes at the *HIS4* promoter, particularly at the binding sites of Bas1/2p (Figure 5.8). This leads to an increased occupancy of Bas1/2p to their binding sites, which then promotes DSB formation in an as yet unknown manner. One possibility is that Bas1p or Bas2p can recruit other histone modifying proteins, which can further remodel the chromatin organisation into a structure that favour better access for recombination factors (Figure 5.8). Alternatively, Bas1p or Bas2p may have the ability to directly interact with components of the recombination initiating factors (Section 1.2.1), hence recruiting the complex to the DSB site at *HIS4* (discussed further in Section 7.6).

It is also unclear how extracellular starvation for adenine and amino acids during sporulation further activates the level of recombination in the *set2*Δ strain. It is possible that nutrient starvation promotes an unknown chromatin modification other than the H3K27 acetylation, which increases Bas1/2p binding activity. Another possibility is that nutrient starvation can induce phosphorylation of Bas1p via an as yet unknown protein kinase, which may affect conformational alteration of Bas1p. This may lead to an increased interaction between Bas1p and Bas2p, and stimulate their

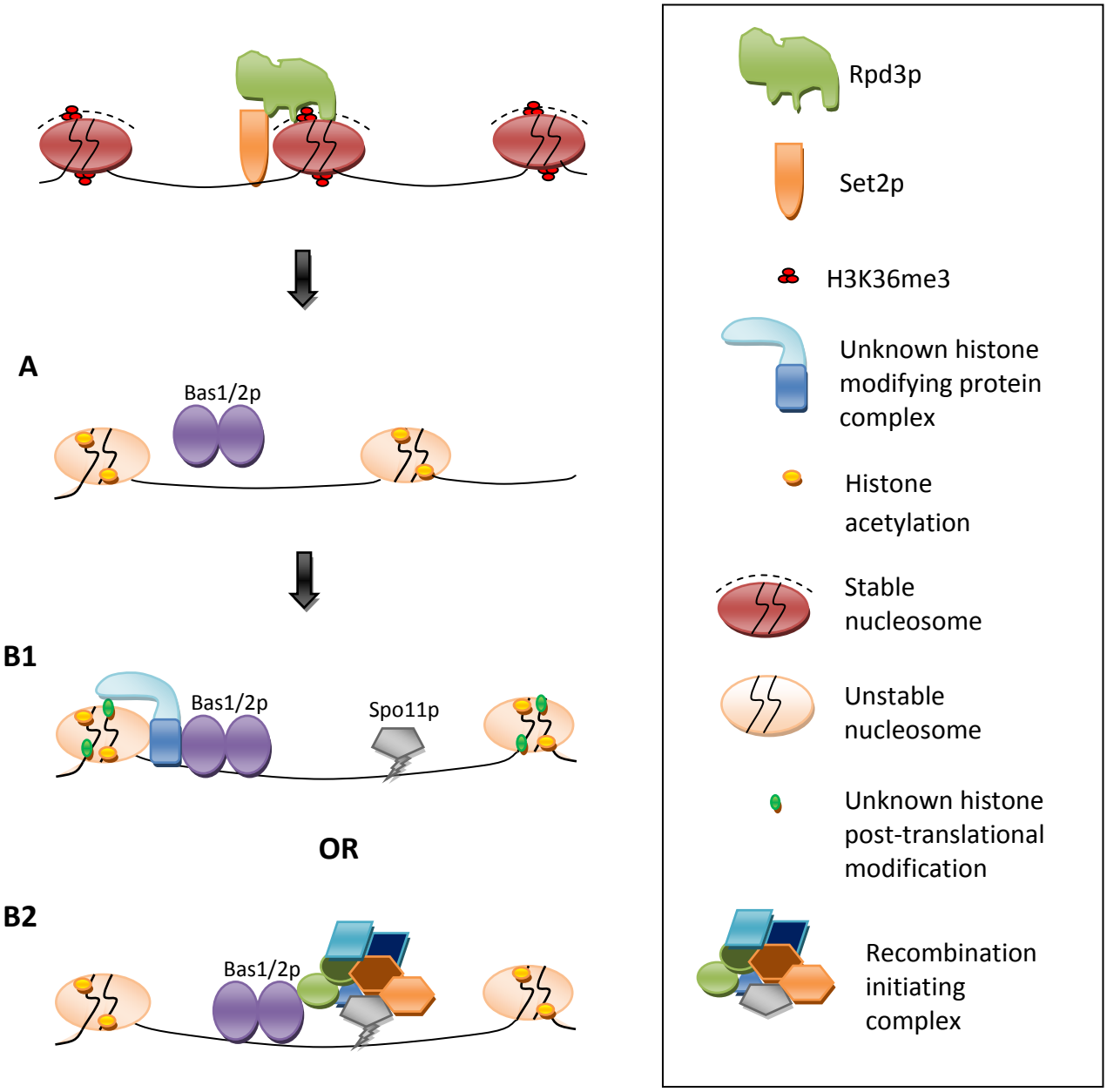


Figure 5.8 : A model for the influence of Set2p inactivation on recombination activity at *HIS4*.

A) Loss of the Set2p-dependent H3K36 trimethylation and Rpd3 deacetylation lead to hyperacetylation of H3K27. Nucleosomes become unstable, and promote better access of Bas1/2p to their binding sites.

B1) Bas1p or Bas2p may recruit unknown histone modifying enzymes that catalyse another modification of histone, which promote a favourable condition for DSB formation.

B2) Alternatively, recombination initiation factors (Spo11p complex) are recruited by either Bas1p or Bas2p to the DSB site.

binding at *HIS4*. Alternatively, if the hypothesis that Bas1p or Bas2p can interact with the recombination machinery is true, perhaps this direct contact can be further stimulated by the starvation signal.

5.4.2 Insertion of an HA_3 Tag Inactivates the Function of Bas1p

This study showed that insertion of HA_3 and *pCLB2-*HA_3 constructs at the carboxyl- and amino- termini of Bas1p respectively, led to a reduction in the level of NMS at *HIS4* to the level of the *bas1* deletion strain (Table 5.3). Furthermore, the His^+ strains homozygous for *pCLB2-*HA_3*-BAS1* and *BAS1-*HA_3 were unable to grow normally on the histidine omission media in the absence of Gcn4p (Figure 5.4). These observations suggest that Bas1p is defective in the *pCLB2-*HA_3*-BAS1* and *BAS1-*HA_3 strains.

Since the *pCLB2-*HA_3 construct inactivates Bas1p function in mitosis, it was not possible to generate a conditional meiotic-null allele of Bas1p. It would be of interest to use a Myc_{13} epitope with the *pCLB2-BAS1* construct in the future, since insertion of the Myc_{13} epitope did not appear to affect Bas1p function. This construct would then be used to confirm the effect of Bas1p-mediated starvation conditions prior to meiosis on recombination (Chapter 4).

Furthermore, we have shown that in the *set2* deletion strain, in which histones were already hyperacetylated, recombination can be further elevated by starvation for nutrients during sporulation. This effect is mediated by Bas1p. Therefore, using the *pCLB2-BAS1* construct, we would be able to support the hypothesis that Bas1p is required throughout meiosis even when the chromatin configurations were already fixed prior to meiosis.

5.4.3 Phosphorylation State of Bas1p

We used phospho-labelling method to investigate the phosphorylation state of Bas1p under different nutrient conditions in terms of the composition of growth media, as well as adenine auxotrophy. Vegetatively grown cells of the wild type, *BAS1-myc*$_{13}$ and *BAS1-myc*$_{13}$ *ade1*Δ strains were first labelled, immunoprecipitated with anti-Myc antibody, and visualised by autoradiography.

From the phospho-labelling experiment, some phosphorylated bands were observed including the one that corresponds to the expected size of *BAS1-myc*$_{13}$, which was present in all strains tested except the negative control (Figure 5.8A). Similar bands with the size of *BAS1-myc*$_{13}$ were also observed in all strains containing *BAS1-myc*$_{13}$ in a subsequent western blot analysis of the same immunoprecipitated protein samples (Figure 5.8B). However, in the negative control lane, no bands were detected at all including the band of the expected heavy chain immunoglobulin, suggesting that the negative control was invalid. This negative control is a sample prepared from the wild type strain which does not contain a Myc-tagged protein, and subjected to immunoprecipitation as in other samples. Therefore, it is not to be ruled out the possibility that the phosphorylated bands were non-specific.

However, similar bands of *BAS1-myc*$_{13}$ were also observed in a repeated experiment in all lanes of proteins that contain *BAS1-myc*$_{13}$ (data not shown), suggesting that these phosphorylated bands may be real. By taking these observations into consideration, one explanation is that *BAS1-myc*$_{13}$ is phosphorylated under both rich and deprived nutrient conditions, and in the auxotrophy for adenine. However, given that the resolution of these bands was very low, the relative amounts of phosphorylation

between these conditions could not be evaluated. Therefore, it remains a possibility that the levels of phosphorylation between these samples were different.

Our result of the presence of Bas1p phosphorylation is consistent with the previously found phosphorylated peptide of Bas1 when cells were grown with complete nutrients (Bodenmiller *et al.*, 2010). Potential kinases involved in this phosphorylation activity include Snf1p, although the amino acid sequence of the phosphopeptide found by Bodenmiller *et al.* (2010) does not correlate with Snf1p binding site. However, Bas1p has at least three potential binding sites of Snf1 kinase, and phosphorylation activities of these sites have not yet been documented.

While the preliminary observations might provide some hints on the phosphorylation activity of Bas1p prior to meiosis, more experiments are needed to confirm this hypothesis. Additionally, an investigation of Bas1p phosphorylation in meiotic cells could also provide a clue whether Bas1p is dephosphorylated during meiosis under adenine deprivation. A specific antibody targeted against Bas1p or specific to the known phosphorylation site in Bas1p can be used in future experiment for detecting Bas1p phosphorylation in response to different nutrient conditions. This would be useful to get a higher sensitivity of the western blot, and higher specificity for Bas1p, or the particular phosphorylation site in Bas1p.

5.4.4 Future work

Acetylation of histones H3 and H4 potentially plays an important role in regulating the activation of recombination at the *HIS4* hotspot in response to nutrient stress conditions. A chromatin immunoprecipitation (ChIP) technique using antibodies directed against specific histone modifications could provide a more detailed

explanation on the position of histones in which acetylation occurs and specific modifications associated with the *HIS4* hotspot. The enrichment of these modifications for meiotic cells that were subjected to different nutritional conditions would also be measured.

It would be of interest to use a ChIP-sequencing (ChIP-seq) method (Johnson *et al.*, 2007) to analyse the enrichment of different histone modifications at *HIS4* in response to various conditions. The ability of the ChIP-seq technique to sequence millions of short DNA fragments in a single run would provide more information of other genomic locations that were also affected under different nutrient conditions. Therefore, we would be able to determine if the same modification of histones occurred at other Bas1p-regulated genes in these conditions, as was observed at *HIS4*. Comparison of enrichments in histone modifications with the level of Bas1p activity can also provide indications if the changes of the specific histone modifications at *HIS4* is associated with the changes in Bas1p.

Chapter 6 : Analysis of Haploinsufficiency of *BAS1* and *BAS2*

6.1 Introduction

Haploinsufficiency refers to a detectable phenotype observed in diploid organisms that results from inactivation of a single allele at a given locus. Haploinsufficiency can also be defined as a phenomenon in which one copy of a gene in a diploid organism is not sufficient to maintain a wild type function. In Chapter 5 (Table 5.3), we have shown that strains containing one copy of the HA-tagged *BAS1* (*BAS1-HA$_3$* / *BAS1*) exhibit a reduced level of NMS compared to the homozygous wild type strain (*BAS1* / *BAS1*). The level of NMS in the *BAS1-HA$_3$* / *BAS1* strain was not significantly different to that of the homozygous *bas1* deletion strain (*bas1*Δ / *bas1*Δ; 4.2% and 1.8% respectively). One explanation for this observation is that *BAS1* is haploinsufficient. Therefore, these interesting data prompted us to investigate if the reduced level of recombination in the *BAS1-HA$_3$* / *BAS1* strain is due to haploinsufficiency of *BAS1*.

In humans, haploinsufficiency has been suggested to be associated with tumour-suppressor genes including *BRCA1* and *PTEN* (Santarosa and Ashworth, 2004). Staff *et al.* (2003) demonstrated that loss of one copy of the *BRCA1* gene in sporadic breast tumours is highly correlated with reduced expression of the mRNA. This suggests that *BRCA1* exhibits haploinsufficiency in sporadic breast cancer (Staff *et al.*, 2003). It has also been reported that mice that are heterozygous for the *BRCA1* gene (*BRCA1*$^{+/-}$) have shortened life span with 70% tumour incidence (Jeng *et al.*, 2007). These mice also had a 3- to 5-fold increased rate of ovarian tumour when exposed to ionising radiation, compared to the *BRCA1*$^{+/+}$ mice (Jeng *et al.*, 2007). This indicates that mice with haploinsufficiency of *BRCA1* gene have an increased risk of cancer.

Haploinsufficiency in tumour-suppressor genes has also been suggested to facilitate tumour progression (Quon and Berns, 2001). One example was shown by Kwabi-Addo *et al.* (2001) in which one copy of the *PTEN* gene was unable to prevent the progression of prostate cancer. In particular, a significantly higher rate of tumorigenesis was detected in mice containing a single *PTEN* allele compared to mice that retained two wild type copies of *PTEN* (Kwabi-Addo *et al.*, 2001).

In *S. pombe*, it has been demonstrated that the *ATF1* gene, which encodes the transcription factor Atf1p, is haploinsufficient with respect to its function in meiotic recombination at the *ade6-M26* hotspot (Gao *et al.*, 2009). Specifically, recombination at the *ade6-M26* hotspot in strains that were heterozygous for *ATF1* (*ATF1* / *atf1*Δ) had an intermediate frequency between the homozygous wild type and homozygous deletion strains (Gao *et al.*, 2009). This indicates that genes which encode transcription factors required for a specific recombination hotspot can exhibit haploinsufficiency in meiotic diploid cells.

In *S. cerevisiae*, a genome-wide profiling study has previously been carried out to determine genes that exhibit haploinsufficiency in terms of growth in YEPD medium (Deutschbauer *et al.*, 2005). Specifically, parallel fitness profiling (Giaever *et al.*, 2002) was used to quantitatively assess the relative growth of all the heterozygous and homozygous deletion strains in YEPD medium (Deutschbauer *et al.*, 2005). *BAS1* and *BAS2* were not included among the 184 heterozygous strains that were detected to exhibit haploinsufficient growth defects. However, the study by Deutschbauer *et al.* (2005) focused on their fitness profiling in terms of vegetative growth activity. Therefore, *BAS1* and *BAS2* may exhibit haploinsufficiency with respect to meiotic

recombination. Furthermore, it is also possible that this assay was not sensitive enough to detect the fitness defect of *BAS1* and *BAS2*, and that heterozygosity of these genes may still exhibit a subtle reduction in growth activity.

In meiotic recombination studies which require analysis of point mutants that are difficult to construct, experiments often involve modifications of only one copy of the gene to determine the effect of these mutations on recombination activity. Specifically, experiments were typically carried out on diploids generated by crossing a haploid strain containing the modified gene, with another haploid strain containing the gene deletion ('point mutant / Δ'). The activity of recombination in this heterozygous point mutant strain was then compared with the homozygous wild type ('WT / WT') and homozygous deletion ('Δ / Δ') strains.

One potential problem that can arise from such an experiment is if the tested gene exhibits haploinsufficiency. Since haploinsufficient genes might generate only partial activity compared to the wild type or be similar to that of gene deletion, this can interfere with interpretation of results. For example, if the level of recombination in the 'point mutant / Δ' strain is significantly reduced compared to that of the 'WT / WT', one explanation is that this reduction may be the consequence of the point mutation. However, it is also possible that the point mutant has no effect on recombination activity, but one copy of the gene in a diploid is not enough to exhibit a wild type level of recombination activity. Therefore, in such experiment, it is important to analyse a control strain of heterozygous wild type ('WT / Δ') to ensure that these genes do not exhibit haploinsufficiency. The activity of recombination in the 'WT / Δ' strain should be equal to that of the homozygous wild type (WT / WT), before the reduced activity of

recombination in the 'point mutant / Δ' strain can be considered as likely to be the effect of the mutation.

Examples of this type of experiment in previous studies include recombination analysis in point mutant of the mismatch repair genes, *MLH1* and *MLH3* (Hoffmann *et al.*, 2003, Cotton, 2007). These point mutants disrupted the ATP binding or ATP hydrolysis activities of Mlh1p and Mlh3p, and were used to investigate the functional requirements of the Mlh1p and Mlh3p ATPase domains in meiosis (Cotton *et al.*, 2010). Crossing over and gene conversion activities were analysed in strains heterozygous for the point mutants (e.g., *mlh3-N35A* / *MLH3*; Cotton, 2007), and compared with the homozygous wild type and homozygous deletion strains. In these studies, recombination analyses were also carried out in heterozygous wild type strains (e.g., *MLH3* / *mlh3*Δ), which were found to exhibit similar level of activities as the homozygous wild type strain (e.g., *MLH3* / *MLH3*). Therefore, these observations confirmed that the *MLH1* and *MLH3* genes were not haploinsufficient, hence the heterozygous point mutant diploids were used for recombination analyses (Hoffmann *et al.*, 2003, Cotton *et al.*, 2010).

An example of such experiment designed for our future studies is the analysis of recombination activities in the *pCLB2-BAS1* strain, a meiotic-null allele of *BAS1* (Chapter 5). Due to the difficulty of creating the mutant strain, recombination activity may have to be analysed in a strain generated by crossing between the *pCLB2-BAS1* and *bas1*Δ strains.

Another example is a study aimed to verify the hypothesis that interaction of Bas1p and Bas2p is the essential mechanism involved in promoting recombination under

various stimulatory factors. This would be tested by evaluating recombination activity at *HIS4* in a strain with a disrupted domain in Bas2p for interaction with Bas1p. Therefore, the effect of starvation on recombination at *HIS4*, in the absence of Bas1p and Bas2p interaction, could be determined. The experiment was designed using a point mutation of the specific domain in Bas2p that has been proposed to be essential for interaction with Bas1p (Bhoite *et al.*, 2002). Subsequently, the *HIS4* hotspot activity in a diploid strain containing the *BAS2* point mutant over *bas2*Δ would be evaluated under different nutrient conditions during sporulation.

6.1.1 Aims

This project aimed to determine if *BAS1* and *BAS2* exhibit haploinsufficiency with respect to vegetative growth on media lacking histidine and meiotic recombination at *HIS4*.

6.2 Results

6.2.1 Heterozygousity for *BAS1* is Insufficient to Promote Normal Vegetative Growth on Media Lacking Histidine in the *gcn4*Δ Strain

Cells wild type for histidine (His^+), and lacking both Bas1p and Gcn4p, lost their ability to grow in the absence of extracellular histidine (Section 1.5.3.1). To test if *BAS1* exhibited haploinsufficiency with respect to its function in promoting vegetative growth, we analysed the growth phenotype of a diploid His^+ strain, which was homozygous for *gcn4*Δ and heterozygous for *BAS1* (*BAS1* / *bas1*Δ), on a histidine 'drop-out' medium. This was then compared to the growth phenotype of the homozygous wild type strain (*BAS1* / *BAS1*, *GCN4* / *GCN4*) and homozygous deletion strain (*bas1*Δ / *bas1*Δ, *gcn4*Δ / *gcn4*Δ).

The *BAS1* / *bas1*Δ strain appeared to grow much slower on the histidine omission medium compared to strains with homozygous *BAS1,* when *gcn4* was deleted (Figure 6.1). This observation suggests that the presence of only one copy of *BAS1* in a diploid is not sufficient to promote normal vegetative growth on media lacking histidine in the absent of Gcn4p. This also implies that *BAS1* is haploinsufficient with respect to its function in facilitating transcription of *HIS4*.

6.2.2 *BAS1* Exhibits Haploinsufficiency in Activating Recombination at *HIS4*

In order to determine if *BAS1* exhibits haploinsufficiency with respect to *HIS4* hotspot activity, the level of recombination in strains containing a single chromosomal copy of *BAS1* were analysed (Table 6.1). Our data showed that heterozygosity for *bas1*Δ correlates with reduced levels of NMS and crossing over compared to the homozygous wild type (Tables 6.1 and 6.3; P values between 3×10^{-4} and 0.02). In addition, the levels of NMS and crossing over were not significantly different as compared to the homozygous *bas1*Δ. These data indicate that a single chromosomal copy of *BAS1* is not sufficient to generate normal levels of recombination at *HIS4*, suggesting that *BAS1* is haploinsufficient. We also analysed the NMS events at *HIS4* in strains heterozygous for *BAS1* in the H390 x H330 (Y55-like) strain background (Table 6.2). Strains with the *BAS1* / *bas1*Δ genotype exhibited significantly reduced levels of NMS compared to the wild type strain (Table 6.2; P = 0.0097 and 3×10^{-4}). This observation in the H390 x H330 strain background is consistent with that in Y55, suggesting that this experiment is reproducible.

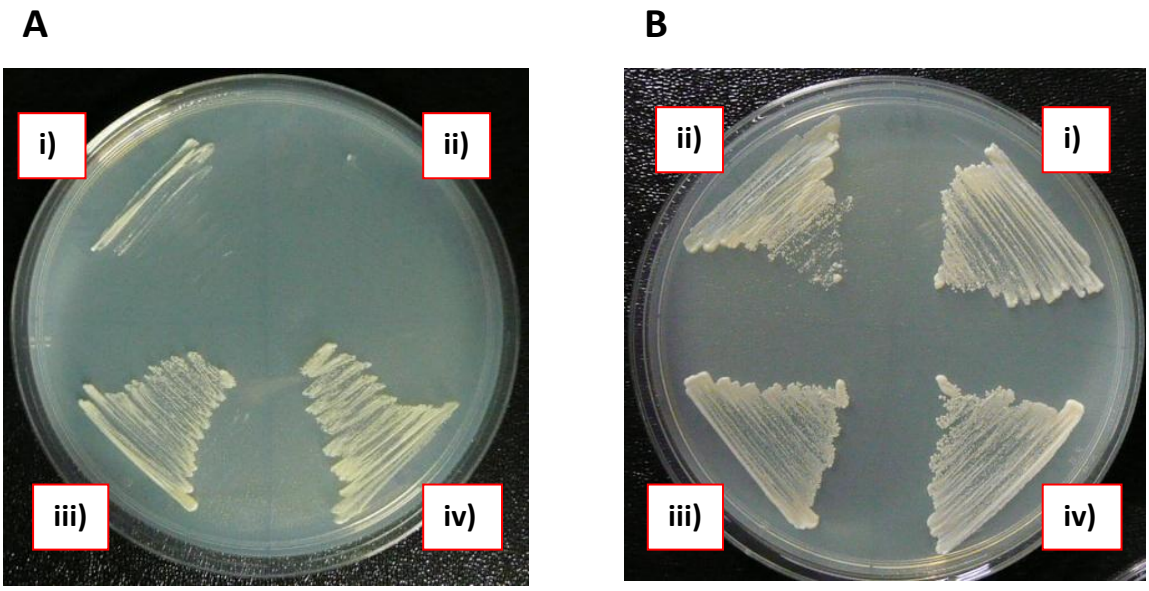


Figure 6.1 : Growth phenotype of strains wild type for histidine (His^+) on (A) the histidine omission medium and (B) the synthetic complete medium after two days of incubation at 30°C. The genotype of strains shown in the figure is as follows:

i) *BAS1* / *bas1*Δ, *gcn4*Δ / *gcn4*Δ ; **ii)** *bas1*Δ / *bas1*Δ, *gcn4*Δ / *gcn4*Δ;

iii) *BAS1* / *bas1*Δ, *GCN4* / *gcn4*Δ; **iv)** *BAS1* / *BAS1*, *GCN4* / *GCN4*.

Table 6.1 : The level of NMS at *HIS4* in different crosses of *BAS1* and *bas1*Δ strains in the Y55 strain background

Strain Crosses (Y55)	**Relevant Genotype**	**NMS[§] / Total Tetrads**	**% NMS**
2830 x 3569	*BAS1* / *BAS1*	50 / 388	12.9
3613 x 2830	*bas1*Δ / *BAS1*	9 / 214	4.9[‡]
3612 x 3569	*bas1*Δ / *BAS1*	10 / 219	4.6[‡]
3612 x 3613	*bas1*Δ / *bas1*Δ	6 / 331	1.8[‡]

[§]NMS is the non-Mendelian segregation events that include meiotic gene conversion and post-meiotic segregation activity. [‡]Represent data that are significantly different compared to the corresponding wild type. The G-test of homogeneity was used for the comparison.

Table 6.2 : The level of NMS at *HIS4* in different crosses of *BAS1* and *bas1*Δ strains in the H390 x H330 strain background

Strain Crosses (H390 x H330)	**Relevant Genotype**	**NMS / Total Tetrads**	**% NMS**
640-4D x 639-14B	*BAS1* / *BAS1*	33 / 167	19.8
913-6C x 640-4D	*bas1*Δ / *BAS1*	10 / 154	6.5[‡]
639-14B x 559-4B	*bas1*Δ / *BAS1*	21 / 205	10.2[‡]

[‡]Represent data that are significantly different compared to the corresponding wild type.

Table 6.3 : Map distances in different genetic intervals on chromosome III in different crosses of *BAS1* and *bas1*Δ strain in the Y55 strain background

Genotype	Strain Crosses (Y55)	Genetic intervals											
		NAT – HYG				*HYG – LEU2*				*LEU2 – MAT*			
		PD	NPD	TT	**cM**[§]	PD	NPD	TT	**cM**	PD	NPD	TT	**cM**
BAS1 / *BAS1*	2830 x 3569	307	3	68	**11.4**	305	2	72	**11.1**	162	15	205	**38.6**
*bas1*Δ / *BAS1*	3613 x 2830	186	0	20	**4.9**[‡]	154	5	49	**19.0**	93	9	110	**38.7**
*bas1*Δ / *BAS1*	3612 x 3569	185	0	24	**5.7**[‡]	162	1	49	**13.0**	96	7	111	**35.7**
*bas1*Δ / *bas1*Δ	3612 x 3613	297	0	28	**4.3**[‡]	234	1	81	**13.8**	179	10	130	**29.8**

[§]Map distances were calculated in centiMorgan (cM) according to Perkins (1949). PD is parental ditype, NPD is non-parental ditype and TT is tetratype. [‡]Represent values that are significantly different compared to the corresponding wild type.

Table 6.4 : Map distances in different genetic intervals on chromosome VII for different crosses of *BAS1* and *bas1*Δ strain he Y55 strain background.

Genotype	Strain Crosses (Y55)	Genetic intervals							
		MET13 - CYH2				*CYH2 – TRP5*			
		PD	NPD	TT	**cM**[§]	PD	NPD	TT	**cM**
BAS1 / *BAS1*	2830 x 3569	233	1	125	**18.2**	85	24	273	**54.6**
*bas1*Δ / *BAS1*	3613 x 2830	139	0	64	**15.8**	52	21	140	**62.4**
*bas1*Δ / *BAS1*	3612 x 3569	145	1	61	**16.2**	53	16	148	**56.2**
*bas1*Δ / *bas1*Δ	3612 x 3613	225	0	88	**14.1**	67	19	240	**54.3**

[§]Map distances were calculated in centiMorgan (cM) according to Perkins (1949). PD is parental ditype, NPD is non-parental ditype and TT is tetratype. There was no statistical significance between the map distances at *MET13-CYH2* and *CYH2-TRP5* between all strains tested.

6.2.3 *BAS2* is not Haploinsufficient in Activating Recombination at *HIS4*

To determine if *BAS2* exhibits haploinsufficiency, similar experiments were carried out as performed in *BAS1* (Section 6.2.2). No significant difference was observed in the level of NMS at *HIS4* and crossovers at *NAT-HYG* for all strains containing only one functional copy of *BAS2* compared to the homozygous *BAS2* strain (Tables 6.5 and 6.6). These observations suggest that *BAS2* does not exhibit haploinsufficiency for its function in recombination at *HIS4*, in contrast to what was observed in *BAS1*.

Table 6.5 : The level of NMS at *HIS4* in different crosses of *BAS2* and *bas2*Δ strains

Strain Crosses (Y55)	**Relevant Genotype**	**NMS / Total Tetrads**	**% NMS**
2830 x 3569	*BAS2* / *BAS2*	50 / 388	12.9
3609 x 2830	*bas2*Δ / *BAS2*	22 / 206	10.7
3606 x 3569	*bas2*Δ / *BAS2*	15 / 208	7.2
3606 x 3609	*bas2*Δ / *bas2*Δ	8 / 209	3.8[‡]

[‡]Significantly different compared to the corresponding homozygous and heterozygous *BAS2* crosses.

Table 6.6 : Map distances at different genetic intervals in chromosome III in different strain crosses of *BAS2* and *bas2*Δ strain

Genotype	Strain Crosses (Y55)	Genetic intervals											
		NAT – HYG				*HYG – LEU2*				*LEU2 – MAT*			
		PD	NPD	TT	**cM**[§]	PD	NPD	TT	**cM**	PD	NPD	TT	**cM**
BAS2 / *BAS2*	2830 x 3569	307	3	68	**11.4**	305	2	72	**11.1**	162	15	205	**38.6**
*bas2*Δ / *BAS2*	3609 x 2830	174	0	27	**6.7**	160	1	42	**11.8**	104	5	96	**30.7**
*bas2*Δ / *BAS2*	3606 x 3569	170	1	29	**8.8**	164	0	36	**9.0**	100	1	100	**26.4**
*bas2*Δ / *bas2*Δ	3606 x 3609	184	0	22	**5.3**[‡]	165	0	33	**8.3**	95	2	101	**28.5**

[§]Map distances were calculated in centiMorgan (cM) according to Perkins (1949). PD is parental ditype, NPD is non-parental ditype and TT is tetratype. [‡]Represent value that is significantly different compared to the corresponding homozygous and heterozygous *BAS2* crosses.

Table 6.7 : Map distances in different genetic Intervals on chromosome VII in different crosses of *BAS2* and *bas2*Δ strain.

Genotype	Strain Crosses (Y55)	Genetic intervals							
		MET13 - CYH2				*CYH2 – TRP5*			
		PD	NPD	TT	**cM**[§]	PD	NPD	TT	**cM**
BAS2 / *BAS2*	2830 x 3569	233	1	125	**18.2**	85	24	273	**54.6**
*bas2*Δ / *BAS2*	3609 x 2830	135	0	59	**15.2**	51	15	135	**56.0**
*bas2*Δ / *BAS2*	3606 x 3569	123	2	67	**20.6**	49	14	140	**55.2**
*bas2*Δ / *bas2*Δ	3606 x 3609	126	2	65	**19.9**	45	13	144	**55.0**

[§]Map distances were calculated in centiMorgan (cM) according to Perkins (1949). PD is parental ditype, NPD is non-parental ditype and TT is tetratype. There was no statistical significance between the map distances at *MET13-CYH2* and *CYH2-TRP5* between all strains tested.

6.3 Discussion

In this study, we have shown that strains which contain only one functional copy of *BAS1* (*BAS1* / *bas1*Δ) exhibited significantly lower levels of recombination at *HIS4* compared to strains containing two copies of *BAS1* (Section 6.2.2). This indicates that *BAS1* is haploinsufficient with respect to its function in promoting the *HIS4* hotspot activity. Therefore, this observation suggests that the low level of NMS observed in the *BAS1-HA$_3$* / *BAS1* strain (Chapter 5) is caused by haploinsufficiency of *BAS1*. Furthermore, we showed that a diploid strain heterozygous for *BAS1* had a slower growth rate on histidine omission media in the absence of Gcn4p, compared to the homozygous *bas1* deletion strain (Figure 6.1). This suggests that *BAS1* also exhibits haploinsufficiency in promoting *HIS4* transcription.

However, these findings contradict with a genome-wide study conducted by Deutschbauer *et al.* (2005), which demonstrated that *BAS1* did not exhibit haploinsufficiency in terms of growth in YEPD medium. Specifically, heterozygous deletion of *BAS1* was not found among the strains that exhibited a fitness defect, while the homozygous deletion of *BAS1* was detected among the slow growing strains (Deutschbauer *et al.*, 2005). One possible explanation for the differences is that *BAS1* haploinsufficiency could be context-dependent. Specifically, *BAS1* may exhibit haploinsufficiency in activating transcription and recombination at *HIS4*¸ but not in promoting cell growth in complete media.

What is the underlying cause of *BAS1* haploinsufficiency? It has been shown that proteins encoded by genes that exhibit haploinsufficiency tend to be involved in protein complexes (Papp *et al.*, 2003). Therefore one theory for gene

haploinsufficiency, the 'balance hypothesis' proposed by Papp *et al.* (2003), argues that the relative amount of subcomponents in a protein complex is in a stoichiometric balance, and deviations of the normal balance can be deleterious. Hence, underexpression and overexpression of a protein subunit complex could exhibit a functional defect. Since overexpression of Bas1p is toxic for cell viability (Valerius *et al.*, 2003), our result of *BAS1* haploinsufficiency appears to support the balance hypothesis. Furthermore, the current model of Bas1p regulation on the *HIS4* hotspot activity includes the requirement for Bas1p to form a complex with Bas2p, and their cooperative binding at the *HIS4* promoter (Chapter 3). Therefore, the production of Bas1p may be in a tight regulation in order to get the right balance of Bas1p molecules to form a complex with Bas2p.

However, the 'balance hypothesis' may not be applied to all genes exhibiting haploinsufficiency. Deutschbauer *et al.* (2005) showed that overexpression of haploinsufficient genes, even those whose products are members of protein complexes, did not exhibit a similar fitness reduction as seen in the heterozygous deletion. Hence, an opposing theory has been proposed, stating that a haploinsufficient phenotype is simply the result of reduced levels of protein produced in the heterozygous state, and overexpression is not expected to be deleterious (Deutschbauer *et al.*, 2005). Therefore, it is possible that haploinsufficiency of *BAS1* in recombination at *HIS4* is not the consequence of a tight requirement for balance in the amount of Bas1p to interact with Bas2p, but simply due to the low number of Bas1p molecules available for interaction.

We speculate that in a heterozygous state of *BAS1*, the production of Bas1p molecules was reduced to the level that is not sufficient to promote wild type activity of *HIS4* recombination. It is possible that even the normal expression of Bas1p is low and the amount of Bas1p produced in the homozygous wild type (*BAS1* / *BAS1*) strain is limited, most of which become active and interacts with Bas2p. These Bas1/2p complexes then bind to the *HIS4* promoter and stimulate the wild type level of recombination. In the heterozygous deletion (*BAS1* / *bas1*Δ) strain, even less Bas1p molecules are produced, therefore only a few active Bas1p molecules are available and form complexes with Bas2p. Hence, recombination activity at *HIS4* is reduced.

Interestingly, our data showed that unlike *BAS1, BAS2* did not exhibit haploinsufficiency in its function in meiosis. This suggests that Bas2p may be expressed at a high enough level in the heterozygous strain (*BAS2* / *bas2*Δ), and interaction with Bas1p can occur at a normal level to promote recombination. Since Bas2p is also involved in the regulation of other genes by partnering with other transcription factors, we would predict that the production of Bas2p should be high. Consistent with this, there are approximately 6420 Bas2p molecules per cell during log-phase growth in rich media, while only 861 molecules of Bas1p are present per cell (Ghaemmaghami *et al.*, 2003). This suggests that Bas2p molecules are produced far in excess compared to Bas1p, consistent with our observation that *BAS2* is not haploinsufficient. From these haploinsufficiency data, we conclude that Bas1p is the rate-limiting factor for recombination activity at the *HIS4* hotspot.

There have been no previous reports of *BAS1* haploinsufficiency with respect to meiotic recombination activity at *HIS4*. Previous studies on the effect of Bas1p on *HIS4*

hotspot activity were carried out using strains homozygous for mutations of *BAS1* (White *et al.*, 1993, Fan *et al.*, 1995). Therefore, the knowledge of *BAS1* haploinsufficiency in this study could provide a significant contribution for future experiments on meiotic recombination at *HIS4* and other hotspots, especially for experiments that involved crossing between modified genes with gene deletions.

Chapter 7 : Discussion

7.1 The Purine Biosynthetic Intermediate AICAR Positively Regulates Recombination at *HIS4* by Promoting Bas1/2p Activity

The data presented in Chapter 3 suggest a positive influence of AICAR on gene conversion and crossing over frequencies at the *HIS4* hotspot. This finding is consistent with the model of Bas1/2p-mediated transcription of *HIS4*, in which transcription was activated in response to AICAR accumulation (Rebora *et al.*, 2001, Rebora *et al.*, 2005, Pinson *et al.*, 2009). Our data showed that deletion of the functionally redundant *ADE16 ADE17* genes, which accumulate AICAR (Pinson *et al.*, 2009), strongly up-regulates the level of recombination. We also found that this effect is dependent on Bas1p. Furthermore, both levels of NMS and crossing over observed in the *ADE16 ADE17* deletion strain were among the highest obtained compared to other strains and conditions tested in this study. This suggests that AICAR plays an essential role as an internal activator for the *HIS4* hotspot activity in a manner mediated by Bas1p.

The influence of AICAR on transcription and recombination at *HIS4* is likely attributed to its role in stimulating interaction between Bas1p and Bas2p, and their binding ability to the *HIS4* promoter (Section 3.4.1) (Pinson *et al.*, 2009). The mechanism by which AICAR potentiates interaction of Bas1p and Bas2p is not completely understood. Since AICAR is known to bind Bas2p *in vitro* (Pinson *et al.*, 2009), this direct contact with AICAR might induce conformational change of Bas2p that promotes its interaction with Bas1p. This AICAR-mediated interaction between Bas1p and Bas2p then facilitates their binding to the DNA (model in Figure 3.4). Furthermore, this interaction may also lead to subsequent alteration of Bas1p conformation, which results in unmasking of its

latent trans-activation domain (Section 3.4.1). Hence, this domain may interact with transcriptional machinery and activates transcription of *HIS4*. With respect to recombination, increased occupancy of Bas1/2p at the promoter region may create a chromatin state that favours access for recombination initiating complex to form DSB. Another possible mechanism is that the recombination complex is directly recruited by Bas1p or Bas2p, hence increasing DSB formation (discussed in Section 7.6).

Does AICAR activate transcription and recombination activities genome-wide? A microarray study by Pinson *et al.* (2009) demonstrated that transcription of a subset of yeast genes were activated in strains that accumulate AICAR. The vast majority of these genes were known to be transcriptionally regulated by Bas1/2p and Bas2p/Pho4p (phosphate regulon). Since Bas1/2p has varying binding ability between the *HIS4* promoter and the *ADE* gene promoters (Section 3.1.2) (Pinson *et al.*, 2000), it is also possible that the degree by which AICAR affects transcription varies between different loci.

In terms of recombination activity, it is as yet unknown whether accumulation of AICAR can activate recombination at genes other than *HIS4*. A genome-wide analysis has been performed by Mieczkowski *et al.* (2006) to map all genomic Bas1p binding sites and to determine the effect of *bas1* deletion on the level of DSBs globally. The genome-wide mapping of Bas1p binding sites was performed using chromatin immunoprecipitation for isolating Bas1p-associated DNA, followed by DNA microarray analysis (ChIP-chip method). In samples derived from sporulating cells, 56 binding sites of Bas1p were detected, while only nine sites were identified in vegetative cells grown in rich media. They proposed that the higher number of Bas1p binding sites detected

in sporulating cells may be the consequence of adenine deprivation in the sporulation media (Mieczkowski *et al.*, 2006). This may lead to an increase production of AICAR, which promotes interaction between Bas1p and Bas2p, hence stimulating occupancy of Bas1p at the promoter. Therefore, this observation implies that adenine starvation and increased level of AICAR could stimulate Bas1p binding activity to its target gene promoters globally.

Furthermore, the genome-wide analysis by Mieczkowski *et al.* (2006) also showed that the formation of DSBs at some regions with Bas1p binding sites was strongly reduced by *BAS1* deletion, while DSB formation at other genes was increased or unaffected. Most of the genes that require Bas1p for DSB formation were known to be transcriptionally regulated by Bas1/2p. Therefore, it is possible that recombination activity at these loci also responds to a high level of AICAR, as was observed at *HIS4*. The finding by Mieczkowski *et al.* (2006) also suggested that Bas1p is not required for recombination activity, indeed even inhibits DSB formation, at several genes containing Bas1p binding sites, some of which were known to be transcriptionally dependent on Bas1/2p. This suggests that the effect of Bas1p on recombination activity is context-dependent. However, AICAR can activate transcription of other genes unrelated to Bas1p (Pinson *et al.*, 2009) as discussed above. Therefore, it remains a possibility that recombination activity at these loci and other genes without Bas1p binding sites can be activated in response to AICAR accumulation.

7.2 Interaction between Bas1p and Bas2p can be Mediated by an AICAR-Independent Mechanism that is Sufficient for Recombination Activity

AICAR is produced from the purine and histidine biosynthesis pathways. The data in Chapter 3 suggested that a certain level of recombination can be stimulated even when AICAR synthesis was completely inhibited from both pathways, in the *his1*Δ *ade1*Δ deletion. The level of recombination from this double deletion strain was significantly higher compared to that found in the absence of Bas1p or Bas2p (up to 4-fold in NMS and 2-fold for crossovers).

Therefore, one possible explanation is that there is an AICAR-independent mechanism that can promote interaction of Bas1p and Bas2p, and is sufficient to activate recombination at *HIS4*. Given that sporulating cells were also subjected to various nutrient starvation including glucose and nitrogen, it is possible that these nutrient signals modestly contribute to the cooperative binding of Bas1/2p to the DNA by an as yet unknown factor. Perhaps other metabolic molecules can promote interaction between Bas1p and Bas2p, or that conformation of either protein can be altered by post-translational modification such as phosphorylation. This conformational change might then induce interaction and binding of Bas1/2p to the *HIS4* promoter.

7.3 Adenine and Amino Acid Starvation Increases Recombination at *HIS4*

This study provides further evidence supporting the hypothesis that constant deprivation for adenine and amino acids stimulates meiotic recombination activity at *HIS4*. We have demonstrated that by modulating the nutrient composition both in the growth and sporulation media, an elevated level of meiotic recombination can be obtained. Wild-type cells prototrophic for adenine (Ade^+) exhibit approximately 1.8-

fold increase in recombination level when subjected to starvation for adenine and amino acids during and prior to sporulation (Tables 4.1 and 4.2). This suggests that continuous nutrient starvation can increase recombination activity even in the wild type Ade^+ strain.

When cells were starved only during sporulation, the recombination level was only activated in cells that were auxotrophic for adenine (*ade1*Δ). This elevated level of recombination was up to 3.7-fold higher than in cells sporulated with complete nutrient supplements, and 1.8-fold higher than cells prototrophic for adenine that were continuously starved (Tables 4.7 and 4.8). We hypothesised that the greater degree of starvation, which include extracellular nutrient limitation and the inability of cells to synthesise adenine, contributes to this induced recombination activity.

The increased level of recombination in response to the high nutrient stress conditions may be contributed by a combination of different factors and mechanisms. Deletion analysis showed that most of the starvation response on recombination is highly dependent on Bas1p (Tables 4.4, 4.5, 4.12 and 4.13). Furthermore, AICAR from the histidine biosynthesis pathway also plays a role to activate recombination in the adenine auxotrophic strain, in response to starvation. This is based on the data that showed significant reduction in the level of recombination in the *his1*Δ *ade1*Δ strain relative to the *ade1*Δ strain under starvation during sporulation (Tables 4.14 and 4.15). In addition, the *his1*Δ *ade1*Δ strain also exhibited a 2.5-fold increase in recombination when cells were subjected to starvation during sporulation, as opposed to cells sporulated with complete nutrients. This suggests that starvation can also promote Bas1p to activate recombination independently of AICAR. This data supports the

hypothesis that interaction between Bas1p and Bas2p can be stimulated by factors other than AICAR, and these factors respond to starvation.

Gcn4p is also required for the effect of starvation during sporulation in cells auxotrophic for adenine, but not for the effect of continuous starvation in cells containing the Ade^+ phenotype (discussed below in Section 7.4). Furthermore, in the absence of Bas1p, a significant increase in the level of NMS was also observed in the Ade^+ strain in response to continuous starvation (Table 4.4). These data indicate that there may be factors other than Bas1p and Gcn4p, which can activate recombination under starvation for adenine and amino acids before and during sporulation. This may be proteins involved in chromatin modifications or other transcription factors such as Rap1p (discussed in Section 4.4.3 and 7.5).

Alternatively, the nutrient stress signals may induce a yet unknown protein kinase to phosphorylate Bas1p, and changed its conformation which promotes interaction with Bas2p and binding to the *HIS4* promoter. Our preliminary experiment to determine phosphorylation of Bas1p under starvation and non-starvation conditions suggests that Bas1p may be phosphorylated in all conditions. However, differences in the level of phosphorylation between these conditions have not yet been studied. Therefore, it remains a possibility that Bas1p is phosphorylated or dephosphorylated under different nutrient conditions.

7.4 Gcn4p is not Required for *HIS4* Hotspot Activity but Contributes to Activate Recombination in the *ade1*Δ Strain when Starved during Sporulation

Our initial expectation in this study was that Gcn4p is required for the *HIS4* hotspot activity particularly in response to starvation. This is because Gcn4p was shown to be

necessary for meiotic recombination at *HIS4*, and was required to activate recombination in response to intracellular starvation for adenine and amino acids (Abdullah and Borts, 2001). In *S. cerevisiae,* Gcn4p is involved in the general amino acid control system that activates transcription of its target genes in response to various stress conditions including purine and amino acid starvation. This system acts by increasing Gcn4p production when cells were subjected to a stress condition, which subsequently activates transcription of genes under its regulation.

The data in Chapter 4 demonstrated that Gcn4p is not essential for recombination activity at *HIS4*. As described above (Section 7.3), Gcn4p also appeared to have no role on recombination activity in response to starvation for adenine and amino acids before and during sporulation, in cells prototrophic for adenine (Ade^+). However, in cells auxotrophic for adenine (*ade1*Δ), Gcn4p has a small contribution for activating recombination in response to starvation (during sporulation). This suggests that Gcn4p can influence recombination activity at *HIS4* when cells were subjected to both extracellular and intracellular nutrient starvation.

It is not clear why continuous nutrient starvation did not stimulate Gcn4p for activating recombination in the wild type Ade^+ strain. Perhaps the amount of Gcn4p produced in response to the external nutrient starvation signals was not sufficient to provide conditions that can activate recombination, but enough to stimulate transcription. This could be due to the chromatin factors recruited by Gcn4p, which favour more access of transcription machinery than recombination initiating factors.

Furthermore, Gcn4p may also act in conjunction with Bas1/2p to activate recombination under starvation condition in the adenine auxotrophic cells. This

combinatorial effect may explain the greater activation of recombination activity observed under starvation condition in cells unable to produce adenine, compared to the Ade^+ strain. Moreover, no effect of starvation in the adenine auxotrophic cells was observed in other control genes (e.g., *MET13* and *TRP5*) that were expected to respond to Gcn4p but have no binding sites of Bas1/2p. This further suggests that the activity of Gcn4p and Bas1/2p in activating recombination at *HIS4* under starvation could be combinatorial.

7.5 The Bas1p-Mediated *HIS4* Hotspot Activity is Associated with Removal of Histone Deacetylation

The data presented in Chapter 5 is consistent with previous observations by Merker *et al.* (2008) in which removal of Set2p-dependent histone deacetylation increases the level of recombination at *HIS4.* Furthermore, inactivation of Set2p methyltransferase and Rpd3p deacetylase also stimulated the formation of DSBs and elevated the level of H3K27 acetylation (Merker *et al.*, 2008). Therefore, it has been hypothesised that removal of histone deacetylase Rpd3p and subsequent acetylation of H3K27 in the *set2*Δ strain provides a chromatin configuration that favours recombination initiation at *HIS4*.

The inactivation of Set2p may also stimulate recombination activity through hyperacetylation of histone H3 and H4 at sites other than H3K27. This is because deletions of *SET2* or *RPD3* were found to acetylate many lysine residues at histone H3 and H4 including H3K9, H3K14, H4K5 and H4K8 (Lin *et al.*, 2010). Furthermore, loss of Hda1p, a deacetylase of histone H3 and H2B also showed a small stimulatory effect on the *HIS4* hotspot activity (Merker *et al.*, 2008). This suggests that histone deacetylase

complexes (HDAC) other than Rpd3p might also have a negative effect on recombination at *HIS4*.

We have also established that Bas1p is required for the effect of hyperacetylation on recombination activity in the *set2*Δ strain (Figure 5.3 and Table 5.2). This finding indicates that the highly opened chromatin structure resulting from histone hyperacetylation has no effect on recombination activity without the activity of Bas1p. In addition, our data demonstrated a further up-regulation of recombination level in the hyperacetylated cells when subjected to starvation for adenine and amino acids during sporulation. Bas1p is also required for this effect on recombination. Furthermore, the effect of starvation during sporulation, in cells prototrophic for adenine, was only observed when the chromatin was already hyperacetylated. As discussed above (Section 7.3), no effect of starvation during sporulation was detected in the wild type (Ade^+) strain in the presence of Set2p, presumably when the chromatin was not in a highly opened state. Perhaps this chromatin structure provides better condition for starvation-induced Bas1p and/or other yet unidentified factors to activate recombination initiation.

7.6 How do Bas1p and Bas2p Induce Meiotic Recombination at *HIS4*?

This study showed that Bas1p is an essential factor for the effect of modulating various environmental conditions including nutrient starvation and removal of Set2p-mediated histone deacetylation. In previous studies of the *HIS4* hotspot, two models have been proposed to explain the mechanism by which the binding of transcription factors Bas1p, Bas2p and Rap1p stimulate recombination activity (White *et al.*, 1993, Kirkpatrick *et al.*, 1999a, Petes, 2001). First, the binding of Bas1/2p and Rap1p

upstream of *HIS4* may promote alteration of chromatin configuration into an 'open structure', which promotes entry of recombination machinery. Second, the transcription factors may have the ability to interact with recombination initiating proteins, hence tethering the recombination complex to the DSB site at *HIS4*.

As stated above (Section 7.5), we have shown that the elevated level of recombination in the *set2*Δ strain, presumably caused by an 'open' chromatin structure via hyperacetylation of histones, was repressed when *BAS1* was deleted. Therefore, this raised a possibility supporting the second model, in which Bas1/2p may have a direct effect in activating recombination initiation. This also suggests that the influence of chromatin on recombination was mediated by modulating Bas1/2p occupancy at *HIS4*. Given that various proteins are involved in the formation of DSB, one of these proteins may be able to interact with Bas1/2p or Rap1p and be recruited to the DSB site. These recombination proteins include Mre11p, Rad50p, Xrs2p, Mei4p, Mer2p, Rec102p, Rec104p, Rec114p and Ski8p (Section 1.2.1), which act together with Spo11p to initiate recombination.

However, if Bas1p has the ability to recruit recombination proteins, it would be predicted that the level of DSBs at all of Bas1p-regulated loci globally would be significantly reduced in the absence of Bas1p. The genome-wide study of *BAS1* deletion by Mieczkowski *et al.* (2006) demonstrated that this was not the case. One explanation for this observation is that Bas1/2p and Rap1p were acting together in mediating the recombination initiation. Thus, chromosomal regions which contain both Bas1/2p and Rap1p binding sites will probably have the same effect as was observed at *HIS4.* Possibly Rap1p has the ability to recruit the recombination complex,

and this effect may be strengthened by Bas1/2p binding in a yet unknown manner. This hypothesis seems to be consistent with a previous finding in which insertion of two Rap1p binding sites could retain the wild type level of recombination in the absence of Bas1p and Bas2p (White *et al.*, 1993). This suggests that Rap1p may be the main player responsible for recruiting recombination proteins, and requires Bas1/2p to stimulate its activity.

For future experiments, it would be of interest to determine if Bas1p, Bas2p or Rap1p can interact with any members of the recombination initiation complex. One way to test this would be using yeast two-hybrid analysis by fusing the transcription factor (e.g., Bas1p) to the DNA binding domain of *E. coli* LexAp, while its activation domain would be fused onto a library of genes encoding recombination initiating proteins. Potential interaction between these proteins will be analysed by testing for reporter gene activity. Additionally, co-immunoprecipitation would also be used for validating the result of possible interaction between transcription factors and recombination proteins in meiosis. This will be carried out by immunoprecipitation of Bas1p at different stages of meiosis, and analysing the presence of other proteins (a recombination initiating protein) using western blot. Therefore, potential interaction between Bas1p and the protein members of recombination complex can be detected.

7.7 Bas1p is Rate-Limiting for the *HIS4* Hotspot Activity

The data in Chapter 6 suggests that *BAS1* is haploinsufficient in terms of its function in meiotic recombination at *HIS4* (Tables 6.1, 6.2 and 6.3). We hypothesised that under normal conditions (in a strain with two copies of *BAS1*), a limited number of Bas1p molecules are produced, and not all of them are able to interact with Bas2p. This

results in a wild type basal level of recombination. In the heterozygous *BAS1* strain (containing only one copy of the gene), the amount of Bas1p is reduced, and even fewer molecules are able to interact with Bas2p, hence reducing the level of recombination. This implies that Bas1p is the rate-limiting factor for recombination activity at *HIS4.*

This observation of *BAS1* haploinsufficiency in meiosis provides additional explanation to other findings in this study on how Bas1/2p regulates *HIS4* hotspot activity. In this study, strains that were used for testing the effect of other gene deletions and conditions on recombination activity were mostly homozygous for *BAS1*. In the model of *HIS4* recombination (Figure 3.4), AICAR is mainly responsible to mediate interaction between Bas1p and Bas2p, presumably by direct binding to Bas2p, followed by activation of Bas1p in a yet unknown manner (Section 3.4.1 and 7.1). Therefore, under normal conditions when the synthesis of AICAR is low (due to feedback inhibition of the purine biosynthesis pathway; Chapter 3), the amount of AICAR that was available to bind to Bas2p may not be sufficient to mediate interaction with the limited number of Bas1p molecules. In conditions where AICAR is highly synthesised (in the *ade16*Δ *ade17*Δ strain and nutrient starvation), it is possible that maximum amount of Bas1p molecules, produced in the state of homozygous *BAS1*, becomes activated. Specifically, when the quantity of AICAR is high, all of Bas1p molecules may become activated by the AICAR-Bas2p complex. This leads to an increased interaction and occupancy of Bas1/2p at *HIS4*.

The data of *BAS1* haploinsufficiency in this study may also indicate an additional characteristic for the transcription factor-dependent hotspot (α-hotspot) activity. It is

possible that a specific transcription factor required for the activity of a given meiotic recombination hotspot can be rate-limiting for recombination. Consistent with the hypothesis, genes encoding the transcription factor Atf1p in *S. pombe*, which is required for the *ade6-M26* hotspot, also exhibit haploinsufficiency (Gao *et al.*, 2009). Therefore, this may be a conserved characteristic of the α-hotspot.

Haploinsufficiency is also becoming an important phenomenon especially in humans, since haploinsufficient genes are increasingly implicated with cancer (Section 6.1). Therefore, identification of haploinsufficient genes in model organisms and further understanding of genetic mechanisms underlying this phenotype could provide important insights into the association between haploinsufficiency and human diseases.

7.8 NMS and Crossover Events at *HIS4* are Correlated

In all experiments, our data consistently exhibit a correlation between the level of NMS at *HIS4* and crossover events of markers flanking *HIS4* (Figure 7.1). The same correlation between both events was also reported in previous studies for various mutations affecting Bas1/2p-mediated *HIS4* hotspot (Fan *et al.*, 1995, Cotton, 2007, White *et al.*, 1993). In our analysis, strains that elevate or reduced the level of NMS at the *HIS4* hotspot (e.g., *ade16*Δ *ade17*Δ) also showed a concomitant increase or reduction of crossing over in the region flanking *HIS4* (*NAT-HYG* interval).

The reason for this correlation between NMS at *HIS4* and crossing over of flanking markers is likely due to the initiation of both events from the same DSBs. For example, mutations that were expected to reduce interaction of Bas1p and Bas2p, as well as *bas1* deletion repressed both levels of NMS and crossing over, since both events were

potentially initiated by the same Bas1/2p-influenced DSB. It has also been shown that deletion of *BAS1* strongly reduces both the levels of DSBs and recombination at *HIS4* (M.F.F. Abdullah, personal communication). This further confirms the hypothesis that the effect of modulating Bas1/2p activity on *HIS4* recombination was a consequence of alteration in the rate of DSBs.

However, the linear correlation between crossovers and NMS (and likely DSBs; Figure 7.1) in our data does not fit with the principal of crossover homeostasis. This aspect of crossover control proposed by Martini *et al.* (2006) demonstrated that the number of crossovers was maintained at the expense of non-crossovers regardless of reduced level of DSBs. However, they monitored global rates of DSB initiation by directly altering Spo11p activity, while we studied a locus specific event by modulating DSBs under different stimulatory conditions. Moreover, Martini *et al.* (2006) also reported that the artificial interval *HIS4-LEU2* exhibited little or no evidence of crossover homeostasis at the strong DSB site even when surrounding regions displayed the phenomenon. Therefore, factors that influence crossover homeostasis globally might not apply regionally as was shown in the regions flanking *HIS4*.

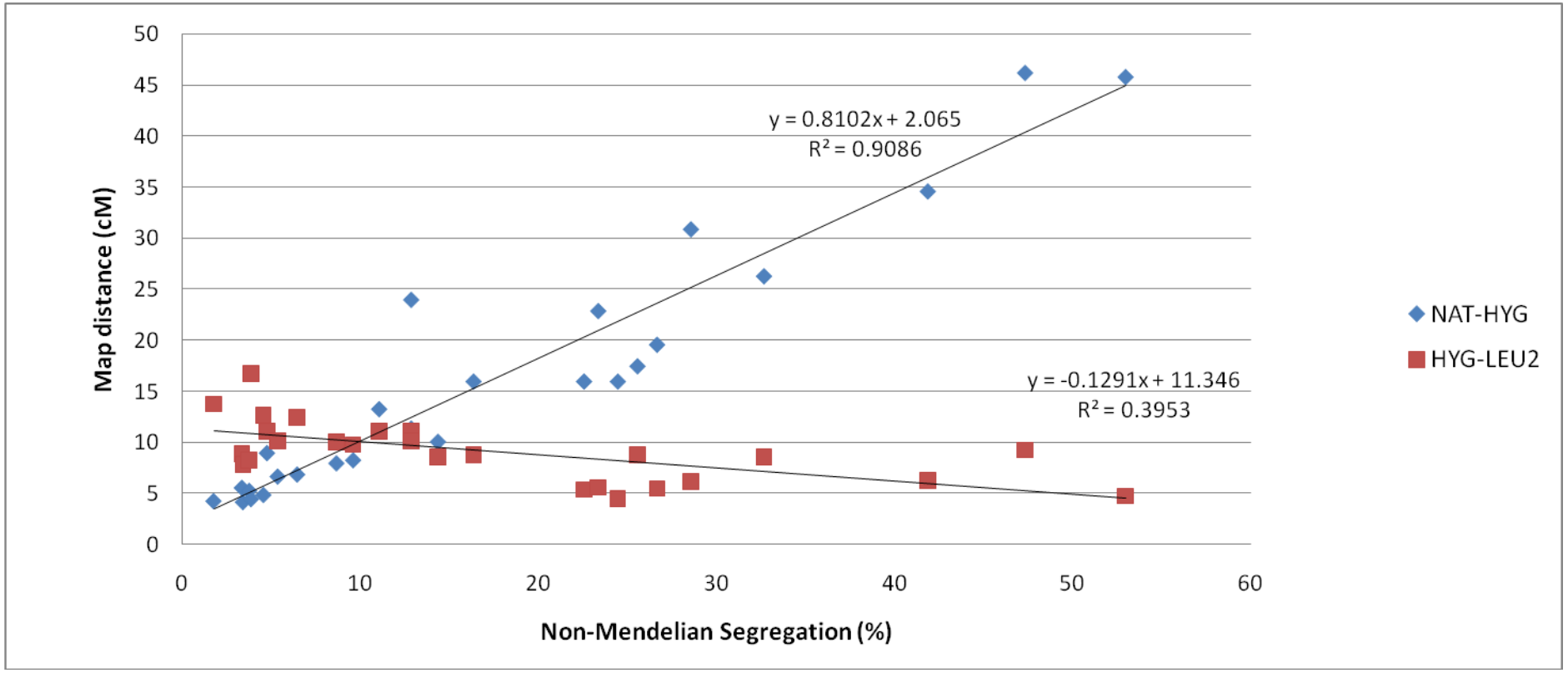


Figure 7.1 : Correlation between crossovers at *NAT-HYG* (blue) and *HYG-LEU2* (red) with NMS at *HIS4*. The map distance data for *NAT-HYG* and *HYG-LEU2* in all strains studied were plotted against the corresponding NMS at *HIS4*. Linear regression curves were represented, and the data of crossing over at *NAT-HYG* and *HYG-LEU2* were fit to each line by least squares (R^2 = 0.9086 and 0.3953 respectively). R^2 corresponds to the square of Pearson's product moment correlation coefficient and was conducted using Excel (Microsoft Corporation).

In this study, we also observed concomitant changes in the level of crossing over at *HYG-LEU2* in response to the changes in crossover rates in the adjacent *NAT-HYG* interval (Figure 7.1; Tables 3.3, 3.6 and 3.9). For example, a 4-fold increase in crossovers at *NAT-HYG* in the *ade16*Δ *ade17*Δ deletion under a normal condition resulted in a 2.3-fold decrease in crossing over at *HYG-LEU2* (Table 3.3). This observation can be explained by the phenomenon of crossover interference in which the occurrence of one crossover limits another event in the immediate vicinity. Another explanation is that the presence of DSBs in one region suppresses another DSB in the nearby region (Wu and Lichten, 1995). Therefore, increasing DSBs in the *HIS4* region could reduce the presence of DSBs at the adjacent interval *HYG-LEU2*.

7.9 Conclusion

In *S. cerevisiae*, the knowledge of how cells adapt to environmental stress conditions in the regulation of cellular processes such as gene expression has long been studied (Gasch and Werner-Washburne, 2002). The influence of environmental factors, specifically temperature and nutritional starvation, on meiotic recombination has only beginning to be elucidated. Our study provides further evidence on the effect of nutritional environment upon recombination hotspot at *HIS4*, which is mediated by a variety of different mechanisms (Table 7.1).

We showed that the level of gene conversion at *HIS4* and crossing over of flanking markers can be manipulated by modulating intracellular and extracellular nutrients, specifically adenine and amino acids. Bas1/2p plays an essential role in regulating the effect of starvation and chromatin alteration on recombination, while Gcn4p also partly contributes for starvation-induced activation of recombination. Furthermore, we

suggest that the level of the purine biosynthetic intermediate AICAR is the major factor influencing Bas1/2p-dependent activity of the *HIS4* hotspot.

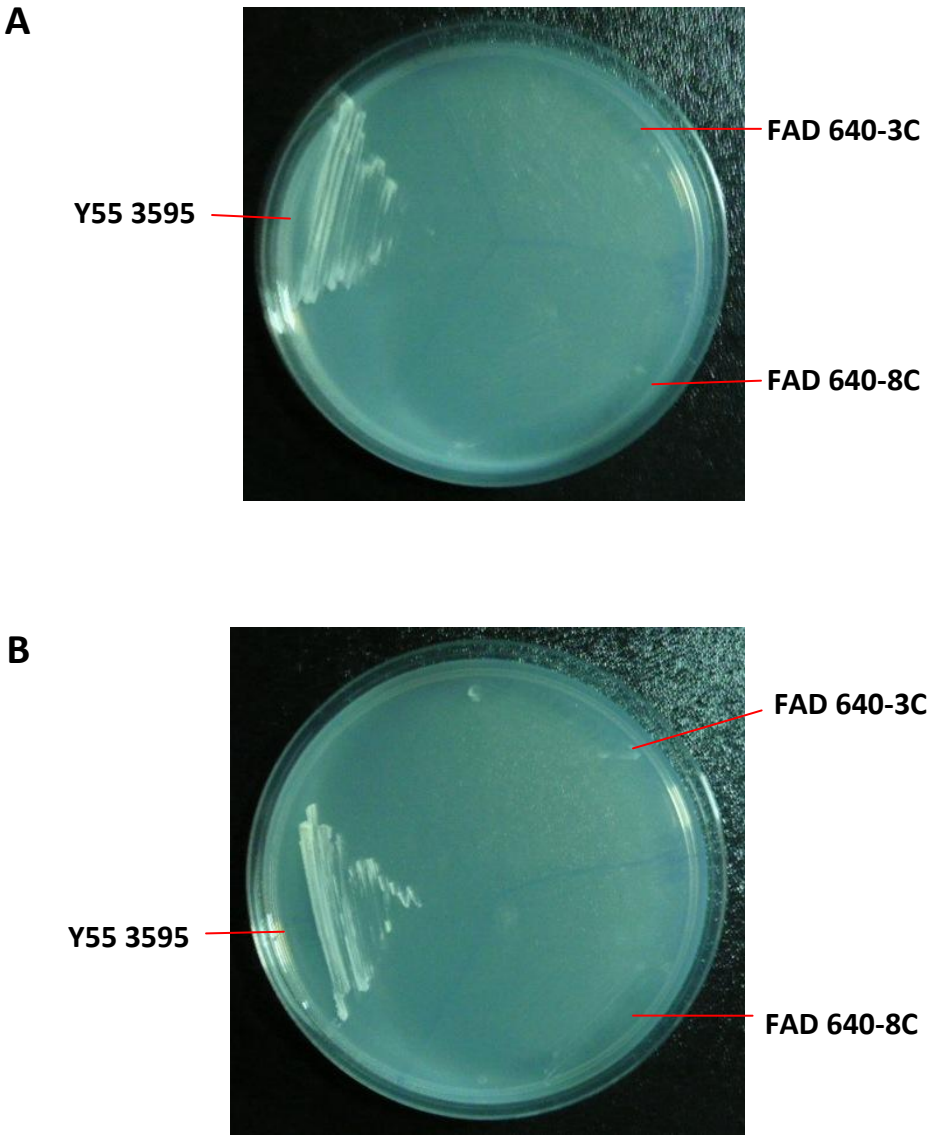
Information regarding environmental modulation upon the level of recombination is useful for meiotic recombination studies for generating a high starting level of recombination. Substantial comparison between different tested conditions or gene modifications can be obtained, when a 'wild type' condition is at a relatively high level. For example, experiments on the effect of a specific gene mutation on meiotic recombination can be compared to the wild type, by consistently sporulated under starvation conditions.

Observations obtained in this study can also provide significant implication in understanding different aspect of meiotic recombination. If the environmental influences on recombination activity can be seen at other Bas1/2p and Gcn4p target genes genome-wide, this can provide information of the controlling factors mediating global distribution of recombination hotspots in yeast. Furthermore, the data in this study also demonstrated that an unknown factor(s) also plays a role in activating recombination in response to starvation, independently of Bas1/2p and Gcn4p. If genes other than *HIS4* can be found to respond to this same effect, analysis of these loci may provide clues to predict this unknown factor. The data from this study can also contribute for the understanding of the regulation of transcription factor-dependent hotspot activity, which is conserved in higher eukaryotes.

APPENDICES

Appendix I: Factors affecting Meiotic Recombination Activity at the *HIS4* Hotspot in *S. cerevisiae*

1. AICAR, an intermediate from the purine and histidine biosynthesis pathways.
2. Continuous starvation for adenine and amino acids before and during sporulation.
3. Starvation for adenine and amino acids during sporulation in cells auxotrophic for adenine.
4. Inactivation of the Set2 methyltransferase activity, presumably due to hyperacetylation of histones near the region of DSB at *HIS4.*



Appendix II: Growth phenotype of *gcn4*Δ cells (Met^+ and Leu^+ phenotypes) in the Y55 and the H390 x H330 strain background (FAD 640 strains) after one day of incubation at 30°C. **A:** Growth on the methionine 'drop-out' medium. **B:** Growth on the leucine 'drop-out' medium.

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