PML-Retinoic Acid Receptor-α

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Novel insight into the function of promyelocytic leukaemia (PML) and PML-retinoic acid receptor alpha

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Abstract

The promyelocytic leukaemia protein (PML) is a tumour suppressor initially identified in acute promyelocytic leukaemia (APL). In APL, *PML* and the retinoic acid receptor α *(RARα)* genes are fused as a consequence of the translocation t(15;17). The product of the chimeric gene is the oncogenic PML-RARα protein, which inhibits the functions of PML and RARα, thus promoting the block of myeloid differentiation and the survival of malignant blasts. The *PML* gene encodes multiple nuclear and cytoplasmic isoforms. PML nuclear isoforms (nPML) are the main components of the PML nuclear bodies (PML-NBs), sub-nuclear structures involved in the modulation of essential cellular players including the tumour suppressor p53. Nuclear PML has been intensively studied, while, the role of cytoplasmic PML remains poorly understood. Increasing evidence indicates that PML could bear cytoplasmic functions in both physiological and pathological settings. This study aims to gain more insights into the function of PML and PML-RARα cytoplasmic pool of proteins. Recently, two missense mutations resulting in truncated PML cytoplasmic protein (Mut PML) have been identified in aggressive APL cases. We found that Mut PML alters the structure and the function of the PML-NB mainly through the cytoplasmic relocation of nPML. Remarkably, Mut PML inhibits p53 transcriptional, growth suppressive and apoptotic functions. In the cytoplasm, Mut PML interacts and stabilizes PML-RARα, thus potentiating its block of RA-induced transcription and differentiation. A mutant of PML-RARα (Δ2) accumulating in the cytoplasm is able to inhibit RA-dependent transcription and differentiation, suggesting that cytoplasmic localization of PML-RARα may contribute to transformation. Finally, we found that Δ2 expression blocks G-CSF-dependent myeloid differentiation and causes partial transformation of primary haematopoietic progenitor cells. Taken together these findings reveal novel insights into the cytoplasmic functions of PML and PML-RARα and shed new light on the molecular mechanisms underlying the pathogenesis of APL.

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This thesis is dedicated to my parents.

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Chapter 1

Introduction

1.1 The Acute Promyelocytic Leukaemia (APL)

Leukaemias are cancers of the bone marrow and the other blood forming organs leading to defective production of leukocytes. These diseases are recurrent with genomic instability including chromosomal deletions, inversions and translocations (Rabbitts, 2001). This genomic instability results in the abnormal production of mis-functioning blood cellular elements, referred to as leukaemic blasts. Blasts remain for the most part phenotypically immature, so that, they continue to proliferate, thus invading initially the bone marrow and the blood to then, eventually infiltrate other organs such as the spleen, lymphnodes and liver. Leukaemias can be classified in two main subgroups: acute leukaemias and chronic leukaemias based on how fast the disease develops. Acute promyelocytic leukaemia (APL) is a distinct subtype of acute myelogenous leukaemia (AML) that accounts for about 10% of all AMLs. APL patients are characterized by the clonal expansion of malignant blasts blocked at the promyelocytic stage. In the early nineties, it was reported for the first time that a reciprocal and balanced chromosomal translocation involving chromosome 15 and 17, t(15;17), was the genomic lesion consistently found in the vast majority of APL patients (more than 90% of the cases) (de The et al., 1990; Rowley et al., 1977). As a consequence of the translocation t(15;17) two recombinant chromosomes are formed: 15q+ and 17q-. Further studies identified the break sites on the two chromosomes. On the one hand, the breakpoint on chromosome 15 is mapped within a previously unknown gene, originally named *myl* and subsequently renamed *PML*, for promyelocytes. On the other hand, the breakpoint on chromosome 17 is located within the locus encoding for the retinoic acid receptor alpha (*RARα*). Therefore, the translocation yields two chimeric genes: *PML-RARα* and *RARα-PML*. The former retains most of the functional domains of PML and RARα and is believed to impair the physiological functions of both proteins and to be the oncogene in APL. Indeed, it has

been shown that PML-RARα functions at the level of the DNA inducing chromatin remodelling and transcriptional repression, thus contributing to the development of the disease (Salomoni and Pandolfi, 2002). Importantly, while the RARα region involved in the translocation is invariant, heterogeneous breakpoints within the PML gene lead to the generation of diverse isoforms of the fusion protein (Chang et al., 1992a; Chang et al., 1992b; Dong et al., 1993; Huang et al., 1993; Tong et al., 1992).

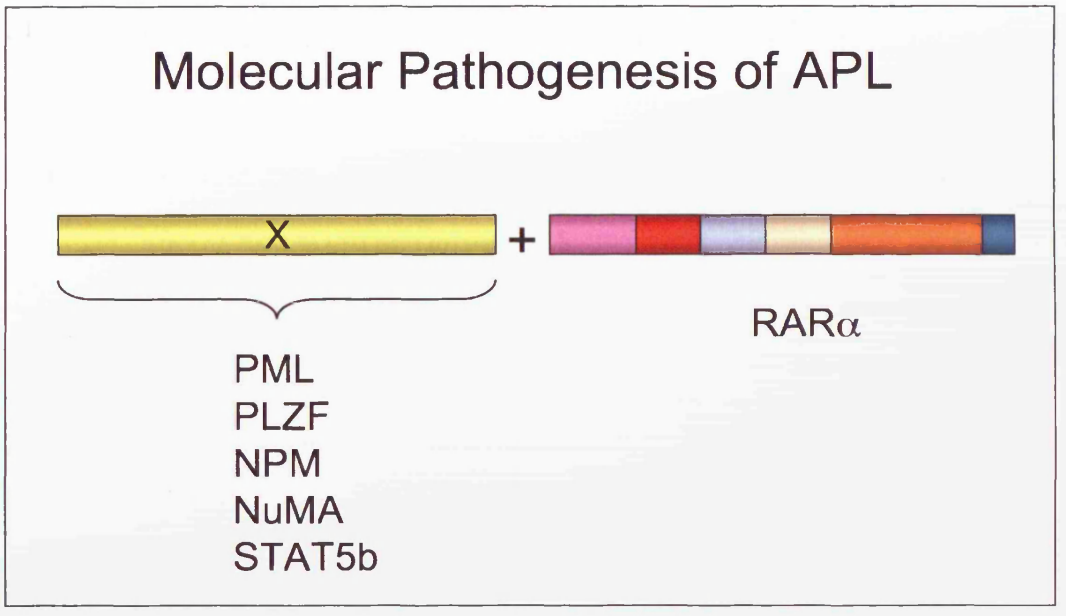

Figure 1.1 Chromosomal translocation described in APL. Molecular pathogenesis of APL accounts for different chromosomal translocations. These rearrangements always involve the RARα gene, which fuses to five distinct genes: promyelocytic gene (*PML*), promyelocytic zinc finger gene (*PLZF*), nucleophosmin gene (*NPM*), nuclear matrix associate protein (*NuMA*) or the signal transducer and activator of transcription 5B (*STAT5B*) gene.

Specifically, two major PML-RARα isotypes are found in patients: the most 5' breakpoint in PML generates *bcr3* (S or short), whereas the most 3' yields to *bcr1* (L or long). In adults the long form is found in about 55% and the short in 35% of APL patients (Huang et al., 1993). An additional breakpoint *bcr2*, that accounts for less than

10% of the APL patients, has been described and involves sites in and around exon 6 of PML that generate a fusion protein with an intermediate length as compare to the other two variants. Interestingly, some studies indicate that patients carrying the *bcr3* translocation experience a worse prognosis as compared to those having the *bcr1*, however, this remains a controversial matter in the field, as other studies did not find such an association (Huang et al., 1993; Jurcic et al., 2001; Vahdat et al., 1994). This aspect will be discussed more in detail below. Besides the t(15;7) other rare variants of translocations have been described in the remaining APL cases (Figure 1.1). These rearrangements always involve the RARα gene, which fuses to three distinct genes: the promyelocytic zinc finger gene (PLZF), nucleophosmin gene (NPM) and the nuclear matrix associate protein (NuMA) gene to produce the translocations t(11q23;17), t(5;17) and t(11q13;17) (Melnick and Licht, 1999). More recently a fifth gene, the signal transducer and activator of transcription 5B (*STAT5B)* gene has been found fused to *RARα* due to an interstitial deletion on chromosome 17 (Arnould et al., 1999). These observations imply that the disruption of the RARα functions is a critical prerequisite in the pathogenesis of this subtype of leukaemia. However, it is becoming evident that the molecular mechanisms leading to APL pathogenesis are more complex as suggested by a number of recent studies that will be discussed more in detail later in this thesis. In conclusion, APL has become a paradigm in the field of leukaemia research as pharmacological doses of all-*trans* retinoic acid (ATRA) induce the differentiation of the malignant blasts and remission of the disease in the patients. Thus, this disorder represents a sort of "Rosetta Stone" to gain more understanding on the molecular basis of leukaemogenesis in order to develop of new therapeutic agents and successfully cure also other AMLs.

1.2 The Retinoic acid receptor

1.2.1 RARα structure

Retinoids including vitamin A and its biological derivates retinal and retinoic acid (RA) are essential regulators of development in both embryonic and adult tissue and are implicated in the proliferation and differentiation of a variety of cell types. A role of these molecules in myeloid differentiation was inferred from clinical observations that retinoic acid could induce remission of the disease in APL patients. Indeed, in APL RA induces the terminal differentiation of the leukaemic cells, in which maturation is blocked at the promyelocytic stage. As a result, the function of ATRA in regulating haematopoiesis has been extensively studied. Retinoids exert their functions by binding to specific members of a superfamily of nuclear receptors governing gene expression in a ligand-dependent manner (Glass and Rosenfeld, 2000). This family includes retinoic acid (RAR), thyroid (TR), estrogens (ER) and glucorticoid (GR) receptors. Three RAR genes have been cloned α, β, γ; however, only RARα is preferentially expressed in the haematopoietic compartment and has its peak of expression in myeloid cells (Chambon, 1996; de The et al., 1989). Remarkably, the different nuclear receptors recapitulate in a similar modular structure consisting of 6 evolutionary conserved domains (A to F, Figure 1.2.1). Three main functional motifs are encoded by different regions moving from the 5' to 3' ends of the gene: a DNA binding domain (DBD), a ligand binding domain (LBD) and activation function 2 domain (AF2). The C domain encodes for the DBD motif responsible for the recognition of specific responsive elements (RE) on the DNA. The DBD is linked through a hinge region to a high affinity LBD retained in the E, which dictates the ligand specificity. Moreover, the E domain also includes the AF2 motif required for ligand-dependent activation of transcription.

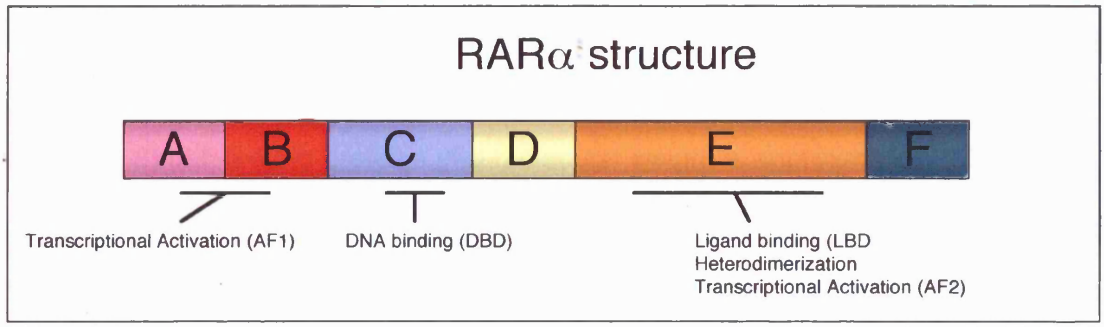

Figure 1.2.1 The structure of RARα. RARs have a modular structure consisting of 6 domains (A-F). Region A and B retain the transcriptional activation domain 1 (AF1) mediating promoter recognition. A DNA-binding domain (DBD) is present in the C region. E region includes the transcriptional activation domain 2 (AF2) that mediates ligand binding, hetero-dimerization and interaction with the transcriptional co-regulators.

RARα recognizes consensus retinoic acid responsive elements (RARE) within the promoters of RA target genes that consist of a direct repeat (A/G)G(G/T)TCA that are separated by two (DR2) or five (DR5) nucleotides. RARα binds to RAREs as a heterodimer along with the retinoic X receptor protein (RXR). RXRs also belong to the superfamily of the nuclear receptor and exist in three different isoforms (α, β, γ), which are similar to RARs but they only bind the 9-*cis* isomer of the retinoic acid (9-*cis* RA) (Glass and Rosenfeld, 2000). Remarkably, RXRs heterodimerize with other nuclear receptors including TR, vitamin D3 (VDR), peroxisome proliferator activator receptor (PPAR) and several orphan receptors (Khorasanizadeh and Rastinejad, 2001; Rastinejad, 2001). RARα dimerizes primarily with the alpha isoform of RXR (RXRα) through the AF2 domain that is also responsible for the recruitment of the different transcriptional co-regulators. In fact, the heterodimerization is of critical importance for the activity of the nuclear receptor, as RARα/RXRα recognize RARE with higher affinity as opposed to RARα/RARα homodimers *in vitro* and *in vivo* (Mascrez et al., 1998). Remarkably, RARα modulates transcription in a biphasic manner depending on its interactions with the transcriptional co-factors. Specifically, in the absence of ligand

the heterodimer RARα/RXRα is constitutively bound to co-repressor proteins, such as the nuclear receptor co-repressor N-CoR and the silencing mediator for retinoic acid and thyroid receptors (SMRT), that inhibit transcription (Chen and Evans, 1995). N-CoR and SMRT are big proteins (the molecular weight is approximately 270 KDa) that possess in their C-terminal portion two interacting domains (referred to as ID1 and ID2), which mediate binding to the nuclear receptors. Notably, N-CoR and SMRT are part of multiprotein repressor complexes including histone deacetylases (HDAC1, HDAC2 and HDAC3) and histone methyltransferases (HMTs). It has been shown that the interaction between N-CoR, SMRT and HDAC/HMT is mediated by the mammalian homologous of the yeast Sin3 protein (Khan et al., 2001a; Lin et al., 1998). In this respect, the aberrant recruitment of co-repressors mediated by PML-RARα has been implicated in the pathogenesis of APL. Accordingly, the use of HDAC inhibitors [i.e. trichostatin A (TSA)] or specific peptides decreasing the interaction between HDACs and PML-RARα, relieves the transcriptional block (Nervi et al., 2001; Racanicchi et al., 2005). Conversely, in the presence of ATRA the AF2 domain undergoes conformational changes that displace the co-repressors from the nuclear receptor complex allowing the binding of co-activators and favouring transcription of the different target genes. Nuclear co-activators include members mediating ATP-dependent remodelling of nucleosome, for instance SWI/SNF, and modifiers capable of promoting the acetylation of chromatin (HAT), such as P/CAF, p300 and CBP. These proteins possess the intrinsic capacity to acetylate histones, thus promoting a relaxed conformation of the chromatin that stimulates transcription.

1.2.2 RAR-mediated transcription and myeloid differentiation

The expression of a number of genes has been shown to vary in response to ATRA treatment of myeloid cells. Indeed, ATRA induces changes in the gene expression

profile, which are accompanied by either inhibition of cell growth or induction of terminal differentiation both contributing to the production of mature cells. Inhibition of RA-induced transcription leads to abnormal haematopoiesis, as in the case of vitamin A deficiency or in conditions disrupting the normal functions of RARα as in APL. RARα can modulate the expression of a wide variety of genes encoding for proteins in many aspects of cell homeostasis such as transcription factors (i.e. STAT1, Hox), cell cycle inhibitors (p21), modulators of myeloid differentiation (i.e. G-CSF, G-CSFR), regulators of cell death (i.e. bcl2) and cell surface molecules (i.e. CD11b, CD18). Surprisingly, *RARα* carries RAREs within its promoter, so that, the receptor itself is upregulated by ATRA. In addition, ATRA regulates the expression of the homeobox family of transcription factors (*HOX* genes), which are essential for the normal development of myeloid cells. In fact, *HOXs* are downregulated by PML-RARα in APL (Thompson et al., 2003). RARα has also been reported to activate STAT1α and is therefore involved in the transcription of interferon (IFN)-modulated genes bearing interferon responsive elements (IRE) on their promoter (Gianni et al., 1997). Moreover, the CCAAT enhancer binding protein epsilon (C-EBPε) is induced following ATRA stimulation of myeloid cells. C-EBPε expression is restricted to the haematopoietic compartment and is required for the activation of genes regulating myeloid differentiation. As a matter of fact, C-EBPε expression is often deregulated in myeloid disorders (Lee et al., 2006; Truong et al., 2003) and C-EBPε null animals have defective granulopoiesis (Yamanaka et al., 1997). Other subclasses of RARα target genes are involved in the regulation of the cell cycle such as inhibitors of cyclins and cyclin-dependent kinases (CDKs) complexes. Accordingly, the tumour suppressor protein $p21^{WAF/CIP}$ is induced by ATRA, thus restricting cell proliferation (Wang et al., 1998a). Interestingly, RAREs have been mapped with the promoter of the cytosolic retinoic acid binding protein II

(CRABPII), a protein that directly binds ATRA and promotes its nuclear import (Astrom et al., 1994).

1.2.3 *RAR* genes knockout and myeloid development

Disruption of the *RARα* gene by chromosomal translocation strongly suggests that *RARα* plays an active role in myeloid differentiation. Moreover, several *in vitro* studies have demonstrated the importance of RARα expression in myeloid differentiation (Collins et al., 1990; Damm et al., 1993; Robertson et al., 1992). Nevertheless, a direct evidence for a role of RARα-mediated pathway in neutrophil development *in vivo* remains elusive. Several laboratories have tried to investigate the *in vivo* functions of RARα by generating "knockout" animals deficient for all the three RARs (α, β and γ) (Kastner et al., 2001). Animals bearing single isoform depletion are viable and demonstrate a normal haematopoiesis. However, $RAR\alpha^{-/-}$ and $RAR\gamma^{-/-}$ double knockout mutants ($RAR\alpha^{-/-}/RAR\gamma^{-/-}$ DKO) die during foetal development or shortly after birth. Nevertheless, the analysis of the foetal bone marrow has revealed that the myeloid differentiation of $RAR\alpha^{-/-}/RAR\gamma^{-/-}$ DKO mice was substantially unaffected (Kastner et al., 2001), thus it is possible that redundant functions carried by other member of the nuclear receptor family may compensate the absence of these two proteins at least in the context of the developing haematopoietic system.

1.3 The Promyelocytic leukaemia protein (PML)

The promyelocytic leukaemia protein (PML) has been subjected to intense research because of its involvement in the translocation t(15;17). In fact, the *PML* gene, originally named *Myl*, was discovered in patients suffering APL. Strikingly, the protein appears to play a role in many important cellular processes. However, despite the substantial progress that has been made, the biological functions of PML remain only partially elucidated. Further complexity is added by the fact that a number of nuclear and cytoplasmic isoforms of PML have been identified (Jensen et al., 2001) (Table 1.3). In particular, old and new evidence suggests that the role of PML cytoplasmic isoforms seems to be overlooked (Lin et al., 2004; Seo et al., 2006). The wide implication of PML in different cellular aspects derives from the fact that PML is part of the nuclear multi-protein complexes, referred to as PML nuclear bodies (PML-NBs), nuclear domain 10 (ND10) or PML oncogenic domain (POD) (Jensen et al., 2001). PML-NBs are present in most mammalian cell nuclei and their number can vary depending on cellular status and environmental stresses. Importantly, an increasing number of proteins has been shown to stably or transiently interact with PML in the nuclear bodies (Figure 1.3). The nature and dynamics of these interactions are essential for the ability of PML to modulate various important cellular pathways (Bernardi and Pandolfi, 2007; Salomoni and Pandolfi, 2002). Yet, in APL PML-RARα disrupts the PML-NBs. Studies conducted using PML null animals suggest an essential function of PML and PML-NB in tumour suppression (Salomoni and Pandolfi, 2002).

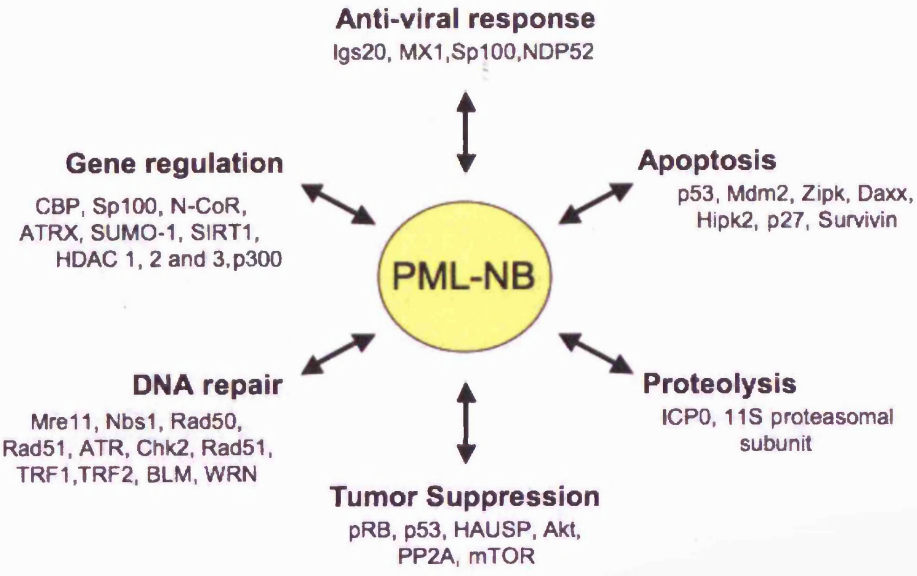

Figure 1.3 Scheme of PML-NB functions.

1.3.1 The *PML* gene and its isoforms

The *PML* gene is located on chromosome 15 and consists of nine exons spread along a locus of 35 Kb in length. A number of nuclear and cytoplasmic isoforms are originated by alternative splicing of the primary transcript (Fagioli et al., 1992) (Jensen et al., 2001) (table 1.3A). To date at least fourteen PML transcripts encoding for distinct proteins have been described (Fagioli et al., 1992); (Jensen et al., 2001). The majority of PML variants are nuclear proteins, however, three variants referred to as PML 3-4-7a-8a, 3-7a-8a and 3-4-7b predominantly accumulate in the cytoplasm. Notably, all isoforms share the N-terminal region (exon 1-3), which encodes several important domains a RING (R) zinc binding motif, one or two cysteine/histidine-rich B-boxes B-boxes (B) and an α-helical coiled-coil region (CC) that collectively form the RBCC domain. This domain is a distinctive element shared by members belonging to the tripartite motive family of proteins (TRIM). This sub-class of proteins include PML together with more than 40 other members (Condemine et al., 2006; Reymond et al.,

2001) (see table 1.3B). Conversely, the C-terminal portion of PML is very variable amongst the PML isoforms, so that, isoform-specific functions may rely on the interaction mediated by this region, i.e. p53 and PML4 (table 1.3A).

Isoforms	Exons	References	Localization
PML1	1-2-3-4-5-6-7a-8a-9	PML4 (Fagioli et al., 1992) PML-1 (Goddard et al., 1991) TRIM 19 alpha (Reymond et al., 2001)	Nucleus/Cytoplasm
PML II	1-2-3-4-5-6-7a-7b	PML-2 (Fagioli et al., 1992) PML-3 (Goddard et al., 1991) TRIM 19 gamma (Reymond et al., 2001) TRIM 19 delta (Reymond et al., 2001) TRIM 19 kappa (Reymond et al., 2001)	Nucleus
PML III	1-2-3-4-5-6-7a-7ab retained intron -7b	PML-L (de The et al., 1991)	Nucleus
PML IV	1-2-3-4-5-6-7a-8a-8b	PML-3 (Fagioli et al., 1992) Myl (Kastner et al., 1992)	Nucleus
PMLV	1-2-3-4-5-6-7a-7ab retained intron	PML1 (Fagioli et al., 1992) PML-2 (Goddard et al., 1991) TRIM 19 beta (Reymond et al., 2001)	Nucleus
PMLVI	1-2-3-4-5-6-intron sequence-7a	PML-1 (Kakizuka et al., 1991) PML-3b (Goddard et al., 1991) TRIM 19 epsilon (Reymond et al., 2001)	Nucleus
PML VIIb	1-2-3-4-7b	TRIM 19 theta (Reymond et al., 2001)	Cytoplasm
PML VIIa	1-2-3-4-7a-8a	(Fagioli et al., 1992)	Cytoplasm
PML VIIa (splice variant)	1-2-3-7a-8a	cPML (Fagioli et al., 1992; Lin et al., 2004)	Cytoplasm

Adapted from Jensen et al. Oncogene 2001

Table1.3A Summary of exon assembly of the different PML isoforms and their cellular localization.

1.3.2 PML Structure

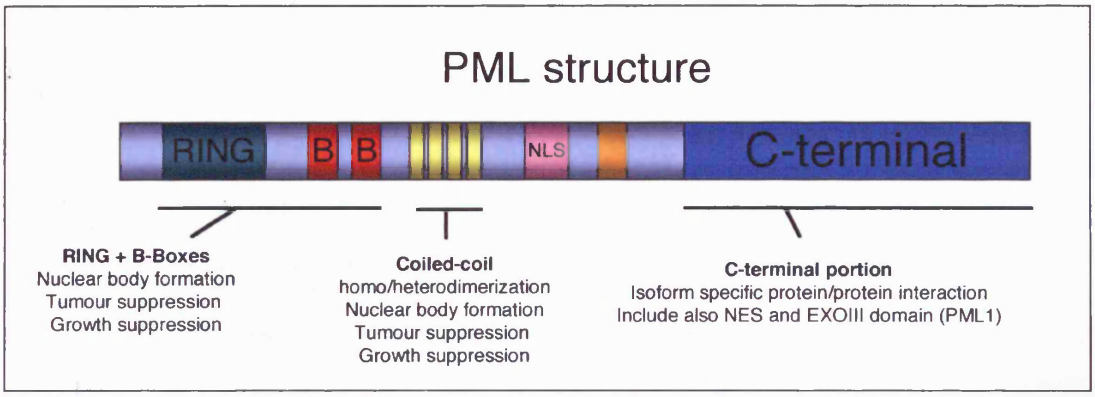

Figure 1.3.2 The structure of PML.

1.3.2.1 The RBCC domain of PML and TRIM family of protein

As previously mentioned all members of the TRIM family of protein possess a RBCC domain. The importance of this region is determined by the fact that each motif contributes the biological function of PML. Scanning the RBCC from the N-terminal to the C-terminal, the first distinctive region to be encountered is the **RING** motif. The RING domain is characterized by the presence of cysteins and histidines arranged in order to coordinate two atoms of zinc in a "cross brace" structure. This domain regulates protein-protein interactions but also possesses an intrinsic E3 ligase activity thereby promoting the attachment of the conserved polypeptide ubiquitin to lysine residues on target proteins (Meroni and Diez-Roux, 2005). Ubiquitylation is a multistep process that involves three type of enzyme: E1, E 2 and E3. These enzymes cooperate together to catalyze the transfer of multi-ubiquitin chains on the specific substrate, which is then target for proteasome-mediated degradation. In this regard, an increasing number of TRIM family members have been implicated in proteolysis (Gack et al., 2007; Kudryashova et al., 2005; Lerner et al., 2007; Meroni and Diez-Roux, 2005). Nevertheless, it is unclear whether PML retains this ability (Boddy et al., 1997; Borden et al., 1995). The RING domain is present in several proteins implicated in

cellular transformation. Mutations of the critical cysteins required for binding of the zinc cause the disruption of PML-NBs and loss of tumour suppressor activity *in vivo* (Borden et al., 1995; Fagioli et al., 1998; Kastner et al., 1992). Similarly, deletion of the RING finger in the tumour suppressor protein BRCA-1 increases the predisposition to cancer development (Saurin et al., 1996). The **B-boxes** (referred to as B1 and B2) are small zinc-binding cysteine/histidine rich modular units that upon binding of the metal modulate the secondary structure of the protein. To date a specific function has not been ascribed to this domain function, however, it appears to coordinate, together with the RING finger, protein-subcellular distribution and is required for the growth suppressive function of PML (Meroni and Diez-Roux, 2005; Reymond et al., 2001; Fagioli et al., 1998). Importantly, the first of the B-Boxes (B1) retains a critical SUMOylation site important for PML turnover. Adjacent to the two B-boxes resides the **α-Coiled-Coil** (CC) region, which consists of α-helices convoluted in a rod-like structure. This domain is of crucial importance in homo-dimerization as well as in hetero-dimerization but also in promoting the formation of multimeric complexes, for instance trimers (Jensen et al., 2001). This region is also essential for PML multimerization as well as for PML/PML-RARα hetero-dimerization in APL context (Grignani et al., 1996; Le et al., 1996). Yet, the spacing between the three regions within the RBCC is maintained amongst the TRIM family members and the architecture conserved in mammals and lower organisms, suggesting its importance in the modulation of protein function (Nisole et al., 2005). Overall these findings suggest that the integrity of the RBCC is an indispensable prerequisite for the PML-NB assembly and PML tumour suppressive functions. A study conducted using a yeast model demonstrated that heterodimerization between different TRIM members is rare (Reymond et al., 2001). Indeed, TRIM proteins tend to homodimerize to form nuclear and cytoplasmic aggregates that function as scaffold for higher-order protein complexes (Reymond et al., 2001) (Table 1.3B). Nevertheless, PML has also been

shown to heterodimerize with the Ret Finger Protein (RFP or TRIM27) in mammalian cells (Cao et al., 1998; Morris-Desbois et al., 1999). Therefore, it is conceivable that other TRIMs could heterodimerize in higher organisms, thus affecting localization and/or function. This is definitely an area of research worth investigating in the near future.

Cytoplasmic TRIM proteins		
Filaments	*Ribbon-like*	*Bodies*
TRIM1/MID2	TRIM29/ATDC	TRIM4
TRIM2/NARF		TRIM5
TRIM3/BERP		TRIM6
TRIM18/MID1		TRIM9/SPRING
		TRIM10/HERF1
		TRIM12
		TRIM14/Pub
		TRIM19/PML
		TRIM21/RO52
		TRIM22/STAF-50
		TRIM23/ARD1
		TRIM26
		TRIM27/Rfp
		TRIM30/RPT-1
		TRIM32/HT2A

Table 1.3B Human cytoplasmic TRIMs divided based on their ability to form cytoplasmic structures: filaments, ribbon-like or bodies. In bold are the cytoplasmic TRIMs potentially implicated in cancer (Horn et al., 2004; Klugbauer and Rabes, 1999; Lin et al., 2004; Salomoni and Pandolfi, 2002).

TRIM proteins are involved in physiological and pathological conditions (Meroni and Diez-Roux, 2005) (Table 1.3B). Specifically, some TRIM family members are mutated in hereditary disorders (Meroni and Diez-Roux, 2005) and the mutant proteins acquire aberrant localization (i.e. mutant MID1 in Opitz Syndrome) (Cainarca et al., 1999; Quaderi et al., 1997; Schweiger et al., 1999). In addition to PML other TRIM proteins,

such as TRIM27/RFP and TRIM24/TIF1-α, have been shown to acquire oncogenic activity when involved in chromosomal translocations (Klugbauer and Rabes, 1999; Le Douarin et al., 1995; Takahashi et al., 1988). TRIM24 regulates the activity of nuclear receptor in a ligand-specific manner (Zhong et al., 1999a). Interestingly, *TRIM24*-null mice develop more hepatocellular carcinomas (HCC) as compared to control littermates (Khetchoumian et al., 2007). Strikingly, deletion of one copy of the *RARα* gene blocks tumour formation, thus suggesting that *RARα* expression is instrumental for the development of HCC. This is the first evidence that *RARα* may act as an oncogene *in vivo* (Khetchoumian et al., 2007). Intriguingly, PML has been found delocalized to the cytoplasm in human hepatocellular carcinoma specimens, thus it would be interesting to study the role of PML in *TRIM24*-null animals.

1.3.2.2 Regulation of PML subcellular distribution: the Nuclear Localization (NLS) and Nuclear Export (NES) Sequences

The cellular distribution of PML is regulated by specific motifs sequences in the gene: a nuclear localization sequence (NLS) and a nuclear export sequence (NES). The NLS is encoded by exon 6 drives PML to the nucleus, in fact, the deletion of this motif results in a cytoplasmic and perinuclear PML localization (Le et al., 1996). Notably, exon 6 is spliced-out from transcripts encoding cytoplasmic isoforms. The nuclear export sequence (NES), present in exon 9, is uniquely retained in the C-terminal portion of PML1 allowing this particular isoform to shuttle between the nucleus and the cytoplasm (Condemine et al., 2006). It is conceivable that PML1 could have different functions depending on its cellular distribution and protein interactions. Despite initial studies suggesting that all isoforms were expressed at comparable levels (Fagioli et al., 1992); (Jensen et al., 2001), a recent analysis performed on a number of different primary and immortalized cell types revealed that PML isoforms are differentially expressed (Condemine et al., 2006). PML1 is the isoform expressed at the highest levels and,

according to its DNA sequence, the one that displays the highest homology between humans and mice (Condemine et al., 2006). Interestingly, the C-terminal portion of this isoform contains a predicted exonuclease III (EXOIII) domain that may function in chromatin remodelling mediating the interaction with DNA (Block et al., 2006; Luciani et al., 2006). Indeed, the EXOIII domain has been recently shown to be essential for the nucleolar redistribution of PML upon exposure to cellular stressors (Condemine et al., 2007). Furthermore, the expression analysis of different PML isoforms in *Pml*$^{-/-}$ cells by using isoform-specific antibodies revealed that PML1 accumulated in both nucleus and cytoplasm [(Condemine et al., 2006) and our unpublished observation]. Interestingly, PML1 mRNA levels were high in primary cells but rather low in transformed cells, thus suggesting that PML1 expression may inversely correlate with the transformation status of the cell (Condemine et al., 2006). Notably, some primary tumour samples displayed a PML cytoplasmic staining (Condemine et al., 2006). This could be due either to cytoplasmic sequestration of nuclear PML or to increased nuclear export of PML1 and/or induction of PML cytoplasmic isoforms. It remains to be established what are the consequences of PML cytoplasmic localization on transformation and tumorigenesis (discussed below). Answering this question will be critical in understanding the role of different PML isoforms in transformation and cancer. There is the possibility that the variability observed in the C-terminal portion of the different PML isoforms could reflect isoforms-specific protein/protein interactions. Indeed, some specific interactions have already been reported, for instance, the interaction between PML2 and the adenoviral protein E4orf3 leads to redistribution of PML and inhibition of the antiviral immune response (Condemine et al., 2007; Ullman et al., 2007). Another example of isoforms-specific interaction is PML4, which specifically interacts with p53, pRB and HDACs, thus participating in the modulation of tumour suppression and senescence (Alcalay et al., 1998; Guo et al., 2000; Lin et al., 1998). For these reasons, this variant of PML has been so far the most studied and

best characterized amid all other PML isoforms. In addition, in a recent study a specific PML cytoplasmic isoform has been implicated in the modulation of the TGF-β signalling through specific interactions with factors modulating the pathway: SMAD2 and SARA (Lin et al., 2004); this will be discussed in more detail below.

PML isoform	***PML interacting protein***
PML1	DAXX
PML2	CBP, E4orf3, TIF1α
PML3	Aurora A
PML4	p53, pRB, CBP, Pin1, Mdm2, HDAC, DAXX, SUMO, Survivin, UBLE1, eIF4E, Chk2, PP2A, Akt, mTOR
PML5	SUMO, Ubc9, Z-protein
PML7a (cPML)	SARA, TGIF

Adapted from Jensen et al. Oncogene 2001

Table 1.3.2.2 PML isoform-specific interactions.
Summary of some PML isoform-specific interactions.

1.3.4 PML post-translational modifications/regulations

Beside transcriptional regulation, a major role in the modulation of PML activity is due to post-translational modifications. In general, modifications of the polypeptide chain include the attachment of different groups such as phosphates (phosphorylation), acetyl groups (acetylation) or small peptides such as ubiquitin (ubiquitylation) and SUMO (SUMOylation). PML has been reported to be SUMOylated as well as phosphorylated at different residues (Figure 1.3.4A).

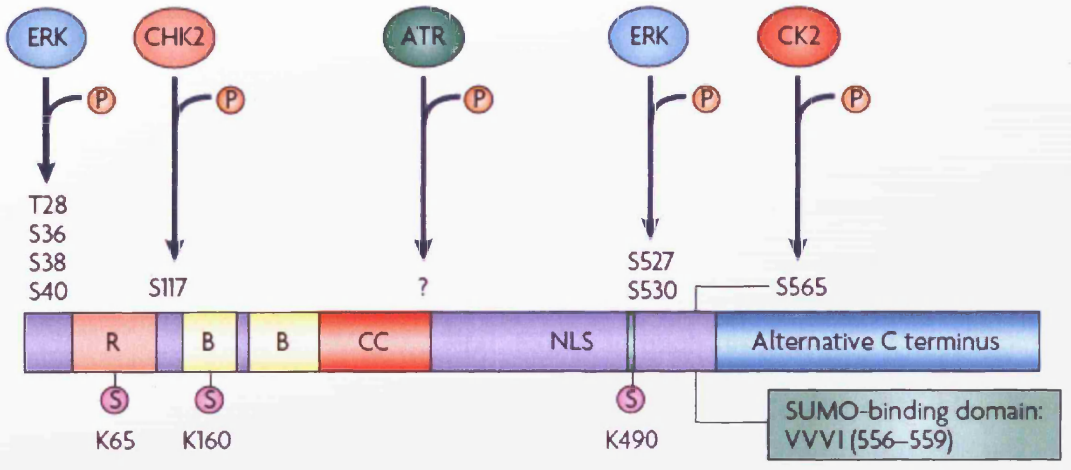

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Figure 1.3.4A Post-translational modifications of PML. Scheme showing post-translational modifications in PML. PML can be phosphorylated at different position by serine-threonine kinases such as ERK, CHK2, ATR and CK2. PML can also be SUMOylated at three different lysines. In addition, a SUMO-binding domain, referred to as SUMO interacting motif (SIM), is also present in the C-terminal portion of the protein.

1.3.4.1 PML SUMOylation

PML is covalently modified by SUMO-1, an 11 KDa ubiquitin-like polypeptide, formerly referred to as senstrin-1, UBL-1, or PIC-1. Similarly, several other proteins are SUMOylated including p53, Sp100, IκB and RanGAP1. Together a SUMO E2-

conjugating and a specific E3-conjugating enzyme catalyze SUMO-1 binding on lysine residues within the substrate. Interestingly, several members of the TRIM family of proteins function as SUMO E3 ligase, including PML, which has been shown to promote its own SUMOylation (Quimby et al., 2006). PML is SUMO-modified on three lysine (K) residues: K65 in the Ring finger, K160 in the B1-box and K490 in the NLS (Kamitani et al., 1998a; Kamitani et al., 1998b; Shen et al., 2006). Interestingly, Shen and collaborators showed that a SIM (SUMO Interacting Motif) sequence in PML is required, together with the RING domain, for the PML-NB formation (Shen et al., 2006). PML SUMOylation is also strongly and rapidly enhanced by arsenic trioxide (As_2O_3) [(Lallemand-Breitenbach et al., 2001) and references cited therein]. It has been shown that SUMOylation stabilizes IkB, a known regulator of the NFκB pathway, and Ran-Gap1, a GTPase involved in different cellular processes (Azuma and Dasso, 2002), blocking inhibitory signals (Desterro et al., 1998; Mahajan et al., 1997). Conversely, As_2O_3-mediate SUMOylation of the K160 triggers the proteasome-dependent degradation of PML and PML-RARα through mediated by the recruitment of the 11S proteasomal subunit. Accordingly, the mutagenesis of K160 abrogates of As_2O_3-mediated degradation of PML (Lallemand-Breitenbach et al., 2001). PML can be de-SUMOylated by a specific SUMO protease, referred to as SuPr-1. It has also been demonstrated that this modification causes the disassembly of the PML-NB (Best et al., 2002). Moreover, it has been shown that PML can be modified by other SUMO isoforms: SUMO-2 and -3. Interestingly, SUMO2/3 conjugation is a dynamic event regulated by environmental stresses and it appears that also PML and PML-NB may be subjected to such regulation (Saitoh and Hinchey, 2000). During the preparation of this thesis two independent groups showed that in response to As_2O_3 treatment the RING-domain containing ubiquitin E3 ligase, RNF4 (also known as SNURF), through its SIM domain recognizes poly-SUMO2/3 chains on K160 and mono-SUMOylation of K490 in PML. Notably, RNF4 triggers the poly-ubiquitylation and proteasomal

degradation of PML (Lallemand-Breitenbach et al., 2008; Tatham et al., 2008). This is the first piece of evidence describing a specific E3 ligase that directly acts on PML. Remarkably, RNF4 also mediates the catabolism of PML-RARα (*bcr-1*), which also has the essential lysines involved in RNF4 association/ubiquitylation. Nevertheless, there is the possibility that additional modifications such as phosphorylation of residues in the N-terminal or C-terminal portion of PML may also contribute to PML and PML-RARα degradation. More future efforts are required to elucidate this important aspect.

1.3.4.2 PML phosphorylation

Phosphorylation of PML occurs on both threonine and serine residues *in vivo* and appears to modulate the pro-apoptotic activity of the protein [(Bernardi and Pandolfi, 2007) and references cited within]. First of all, DNA damage induces hCds1/Chk2-mediated phosphorylation of serine 117 in PML and this results in p53 stabilization (Louria-Hayon et al., 2003; Yang et al., 2002). The extracellular signal-regulated kinase (ERK) has been shown to phosphorylate PML at several residues, thus favouring PML SUMOylation and augmenting PML-dependent apoptosis in response to As_2O_3 (Hayakawa and Privalsky, 2004). Notably, CK2-dependent phosphorylation of PML induces its catabolism (Scaglioni et al., 2006). CK2 is a nuclear–matrix-associated serine/threonine kinase that phosphorylates PML at serine 517, thus triggering the proteasomal degradation. Indeed, inhibition of CK2 activity enhances PML tumour suppressive properties. Interestingly, CK2 is frequently upregulated in many human tumours and this seems to correlate with the low levels of PML expression reported in another study by Gurrieri and collaborators (Gurrieri et al., 2004a). Interestingly, Pin1, a member of the pavrulin of peptidyl-prolyl *cis-trans* isomerases (PPIase), binds PML and promotes its degradation (Reineke et al., 2008). Yet, Pin1 has been shown to be upregulated in human breast cancers and this would suggests that Pin1 deregulation is important for tumorigenesis.

1.3.5 PML and the biogenesis of the PML-Nuclear Body (PML-NB)

Several sub-nuclear compartments can be distinguished in the nucleus of mammalian cells, which are generally referred to as "nuclear bodies" or "nuclear domains". In the late 1980's, one of such structures was shown to be reactive to anti-sera for PML, at that time a newly identified gene (de The et al., 1990; Pandolfi et al., 1991). Indeed, PML accumulates in distinct nuclear domains, referred to as PML-NB that can be classified as a specific sub-class of nuclear bodies. At the ultra-structural level, the nuclear body appears as ring-like (doughnut-shape) electron dense protein structure with a diameter ranging from 0.2 to 1 μm (Figure 1.3.5A).

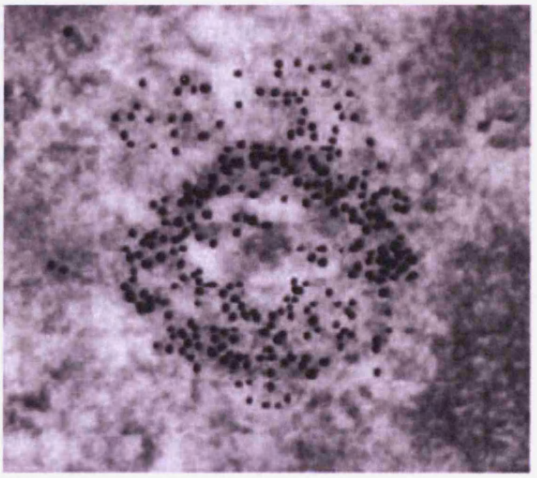


Figure 1.3.5A The PML nuclear body. Electron microscopy image of a PML-NB , black dots represent gold nanoparticles conjugated to anti-PML antibody. PML is localized to the outer edge of the nuclear bodies, which appears as a doughnut-shape structure with a diameter of 0.2 to 1 μm. This image has been kindly provided by Dr. Dinsdale (MRC Toxicology Unit).

No appreciable levels of nucleic acids were noticed within the bodies (Dellaire and Bazett-Jones, 2004). However, PML-NBs are able to contact chromatin at their periphery (Eskiw et al., 2004), thus maintaining integrity and localization (Eskiw et al., 2004). Indeed, PML-NB composition is altered in conditions that perturb chromatin

condensation such as stress, transcriptional repression and apoptosis (Ching et al., 2005). *In situ* hybridization experiments revealed that PML-NBs associate with regions rich in genes (Wang et al., 2004) such as the cluster region encoding the major histocompatibility complex (*MHC*) (Bruno et al., 2003; Zheng et al., 1998). Nevertheless, a direct role of PML transcriptional activity is still missing. It has been proposed that PML along with the Special AT-rich sequence Binding protein 1 (SATB1) modifies the chromatin architecture thus favouring the transcription of this specific genomic region (Kumar et al., 2007b). As previously mentioned, PML-NBs are heterogeneous and dynamic structures. There are 10 to 30 PML-NBs per nucleus but their number together with the size can vary for example during the cell cycle (Chelbi-Alix et al., 1995; Lavau et al., 1995) and in response to antiviral interferon (IFN) treatment (Chelbi-Alix et al., 1995; Lavau et al., 1995). Interestingly, PML-NBs, like Cajal bodies are motile structures (Lamond and Sleeman, 2003; Platani et al., 2000). Studies of PML-NB dynamics in living cells have been conducted tracking the nuclear body component Sp100 fused to the yellow fluorescent protein (YFP) (Muratani et al., 2002). Interestingly, a small portion of PML-NBs display rapid movements up to a speed of 0.4 μm/s, the fastest moving nuclear structures recorded (Muratani et al., 2002). Authors speculated that this particular class of bodies might act as "nuclear sensors" travelling within nuclear regions that are associated with "anomalous" structures, i.e. viral proteins or protein aggregates (Muratani et al., 2002). These studies, however, might underscore major caveats as authors used Sp100 to monitor the dynamics of PML-NBs. Indeed, it has been shown that Sp100 is not always associated with PML in the nucleus (Wiesmeijer et al., 2002). Moreover, the presence of these moving bodies seem to restricted only to certain type of cells (Muratani et al., 2002), thus further experiments are needed to clarify these controversies.

Deregulation of the PML-NB architecture often occurs in response to a number of cellular insults such as viral infection, heat shock, heavy metal exposure (i.e. Cadmium) and/or pathological conditions (Everett and Murray, 2005; Maul et al., 1995; Nefkens et al., 2003), thus suggesting that the integrity of the bodies is important for cellular homeostasis. Indeed, a number of crucial cellular players stably or transiently associate with the PML-NB, however, only a few directly interact with PML, including p53 (Guo et al., 2000), the retinoblastoma protein (pRB) (Alcalay et al., 1998), the death domain associated protein (DAXX) (Ishov et al., 1999; Salomoni and Khelifi, 2006; Torii et al., 1999), the acetyltransferase CBP (cAMP-response element (CREB)-binding protein) and eIF4E (eukaryotic initiation factor 4E) (Cohen et al., 2001). Studies conducted using *PML* null animals revealed that in the absence of PML all the other known components acquire aberrant diffuse nuclear localization.

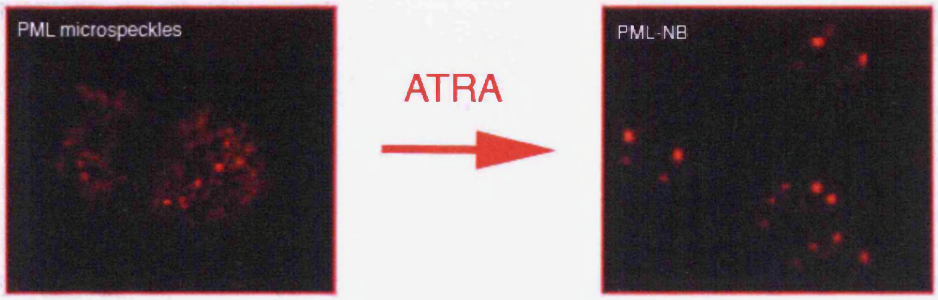


Figure 1.3.5B PML-NBs are disrupted in APL.
Confocal image of NB4 cells before and after treatment with pharmacological concentration of ATRA (10^{-7} μM). In APL, PML-RARα disrupts the PML-NB. As a matter of fact, PML is delocalized in hundreds of tiny nuclear microspeckles. PML-NB architecture is restored upon treatment with ATRA.

Accordingly, re-introduction of PML in *PML*-null cells restores the assembly and the localization of the PML-NB components (Maul et al., 2000; Zhong et al., 2000a). In APL, PML-RARα affects the architecture of PML-NBs by relocating PML into tiny nuclear and cytoplasmic speckles (Figure 1.3.5B) (Dyck et al., 1994; Kastner et al., 1992; Koken et al., 1994; Weis et al., 1994). Yet, treatment with ATRA induces the

degradation of the fusion protein and restores the PML-NBs (Weis et al., 1994). As previously mentioned, PML is covalently modified by SUMO-1 at three specific lysine residues and this post-transcriptional modification of PML is indispensable for the formation of the nuclear bodies (Shen et al., 2006; Zhong et al., 2000a). It has been demonstrated that DAXX, a transcriptional co-repressor, binds SUMOylated-PML and this results in the modulation of its transcriptional functions (Li et al., 2000). Furthermore, SUMOylation of other PML-NB-associated proteins such as p53, CBP and Sp100 has been described (Shen et al., 2006) and seems to be required for the assembly of PML-NBs (Salomoni and Pandolfi, 2002). Thus, it is conceivable that changes in the PML-NB composition could mirror the balance between SUMOylation and de-SUMOylation of PML and other nuclear body components. This aspect awaits further investigation.

1.4 Biological functions of PML

1.4.1 PML functions in tumour suppression

Several important observations suggest that PML possesses a tumour suppressor role. *PML*$^{-/-}$ mice are viable and the incidence of spontaneous tumours is not increased, but display increase susceptibility to develop infections and to die as opposed to *PML*$^{+/+}$ and *PML*$^{+/-}$ animals, thus making difficult the assessment of tumorigenesis late in life (Wang et al., 1998a). Thus, this aspect was studied in experimental models designed to accelerate tumour formation. *PML*$^{-/-}$ animals were subjected to higher rate of and to a different spectrum of tumours as compared to control littermates when injected with tumour promoting agents such as dimethylbenzanthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) (Wang et al., 1998b). These findings suggested that PML may antagonize the initiation and the progression of malignancies of different histological origin (Wang et al., 1998b). Notably, *PML*$^{-/-}$ cells were also resistant to the lethal effects of γ-irradiation, Fas, TNFα and IFNs suggesting a crucial role of PML in modulating the response to different type of stress (Wang et al., 1998b). Thus, PML tumour suppressive functions were tested in transgenic animal of leukaemia and breast cancer (Rego et al., 2001). Strikingly, the genetic reduction of *PML* dramatically increased the incidence and accelerated the onset of PML-RARα-induced leukaemia in mice. Conversely, neu-induced breast tumorigenesis was not enhanced in the absence of PML and latency, size of the tumours and rate of metastasis formations were comparable in *PML*$^{-/-}$ and *PML*$^{+/+}$ animals (Rego et al., 2001). Accordingly, PML inhibited transformation of primary mouse cells induced by expression of oncogenic Ras, mutant p53 or H-Ras along with c-myc (Mu et al., 1994). Moreover, PML inhibited the ability of prostate cancer cells to engraft tumours in transplanted sub-lethally irradiated recipient mice (He et al., 1997). Interestingly, PML regulates the proto-oncogene *AKT* (Trotman et al., 2006). Notably, Akt is aberrantly

activated in many human cancers often associated with the loss of the its negative regulator PTEN (Ruggero and Sonenberg, 2005). In human, the majority of prostate carcinomas display either heterozygosity or complete depletion of the *PTEN* gene (Whang et al., 1998; Wu et al., 1998). Trotman and co-workers studied the effect of *PML* inactivation on *PTEN*$^{+/-}$ animals and found that *PML* loss markedly increased prostate and colon carcinogenesis (Trotman et al., 2003). It has been proposed that PML may control protein phospatase 2A (PP2A)-dependent dephosphorylation and inactivation of Akt (Trotman et al., 2003). Altogether, these findings indicate that PTEN and PML cooperate to a tumour suppressor network that controls the activation of the PI3K/AKT pathway (Trotman et al., 2006). Another study employed tumour tissue microarrays (TTMs) to analyze the expression of PML in specimens of human tumours of different histological origins (Gurrieri et al., 2004a). The results showed that PML expression is lost or severely reduced in many tumours including prostate adenocarcinomas, breast and lung carcinomas, lymphomas, central nervous system (CNS) tumours, and germ line tumours (Gurrieri et al., 2004a). Nevertheless, PML mRNA levels were comparable to those measured in control specimens and the gene was rarely mutated and not subjected to loss of heterozygosity (LOH) (Gurrieri et al., 2004a). In line with this, normal expression of PML can be rescued in PML-negative colon carcinoma and gastric cancer cell lines by using proteasome inhibitors, thus suggesting that PML stability is of paramount importance in cancer (Gurrieri et al., 2004a). Nevertheless, it is unclear whether the loss of PML expression is a primary event in carcinogenesis. De The and collaborators studied the expression of PML in different tissues including human tumour specimens. This study demonstrated that in tumours the loss of PML is often accompanied by reduction of p53 levels. Thus, it is possible that decreased levels of PML may contribute to p53 inactivation. Recently, it has been shown that PML degradation in cancer cells is promoted by casein kinase 2 (CK2)-dependent phosphorylation (Scaglioni et al., 2006). Interestingly, CK2

expression is increased in many tumours correlating with the reduction of PML levels. From another standing point, it also is plausible that the activity of a PML-specific E3 ubiquitin ligase(s) may be increased in tumours. Nevertheless, the enzyme(s) responsible for the ubiquitylation of PML has not yet been identified, thus more effort is needed in the future to elucidate this aspect of PML regulation. Finally, it has been suggested that PML inhibits angiogenesis in both ischemic and neoplastic conditions (Bernardi et al., 2006). Indeed, tumours arising from *PML*$^{-/-}$ animals displayed greater microvessel density, thus suggesting that PML negatively affects the expression of the hypoxia inducible factor-1α (HIF-1α), a transcription factor essential for neoangiogenesis (Bernardi et al., 2006). This would be the direct consequence of PML-dependent inhibition of mTOR, which regulates the expression of HIF-1α. Indeed, PML sequesters mTOR in the nucleus and blocks its phosphorylation/activation (Bernardi et al., 2006). Indeed, these findings appear to correlate with data showing increase angiogenesis and the severe disease progression in cancer lacking PML (Bernardi et al., 2006; Trotman et al., 2006).

1.4.2 PML and the regulation of cell death

The evidence that PML has a critical role in the regulation of cell death has been described in a number of important studies conducted over the last decade. First and foremost, PML is important for the proper execution of apoptosis in response to a number of stresses including DNA-damage (Wang et al., 1998b). In fact that *PML-/-* mice are resistant to the lethal effect of ionizing radiation, to apoptosis induced by the pathway regulated by FasL/Fas (also referred to as CD178 and CD95, respectively) and by other molecules such as tumour necrosis factor alpha (TNFα), ceramide and interferons (type I and II IFNs) (Wang et al., 1998b). Wang and colleagues showed that in a *PML*$^{-/-}$ background the activation of the executor caspases 1 and 3 is impaired. In

keeping with this, *PML*$^{-/-}$ mice develop more B and T cell lymphomas following carcinogenic treatment, which mirrors the sensitivity of *PML*$^{-/-}$ cells to pro-apoptotic signals mediated by FasL, TNFα and IFNs (Wang et al., 1998b). Similar results were obtained using myeloid progenitors isolated from *PML-RARα* transgenic animals (Wang et al., 1998b). Strikingly, the resistance to apoptosis was further increased by reducing *PML* gene dosage to hemizygosity, obtained by crossing *PML-RARα* animals with mice having *PML-/-* background (Rego and Pandolfi, 2001). These findings outline the importance of the PML pro-apoptotic activity *in vivo* and in the context of PML-RARα-mediated leukaemogenesis. Another study showed that overexpression of PML in different cell types led to rapid death but without the typical hallmarks of apoptosis such as activation of caspases and DNA fragmentation (Quignon et al., 1998). Indeed, the main effector caspase, caspase 3, was not activated in PML-induced death and the presence of the *pan*-caspase inhibitor z-VAD-fmk, paradoxically enhanced the cell death. Authors concluded that the overexpression of PML causes a caspase-independent apoptosis, which apparently contrasts the data by Wang and co-workers obtained by using *PML*$^{-/-}$ cells and mice. This controversy may be explained envisioning a model in which low levels of PML activate "canonical" apoptosis supported by *de novo* transcription, whereas, at high levels of expression PML triggers death independently of caspases activation. Notably, many other conditions have been shown to induce caspase-independent death including oncogene overexpression, DNA damage and starvation (Rathmell and Thompson, 1999). This is possibly mediated through the recruitment of pro-apoptotic members (e.g. Bax) or the hijacking of survival factors (e.g. p27KIP1) to the PML-NB (Quignon et al., 1998), Nevertheless, it has to be taken into account that authors reported cross-reactivity of the anti-Bax antibody with Sp100. Thus, it is not clear whether PML play a role in the regulation of caspase-independent cell death. Furthermore, evidence demonstrating colocalization/interaction between PML-NB and anti/pro-apoptotic BCL-2 family proteins *in vivo* is still missing or

limited to some tumour cell lines (Hoetelmans, 2004). From another standing point, it would be worth investigating whether PML modulates autophagy, another alternative form of programmed cell death that is also important in a number of pathological conditions including cancer. Interestingly, p53 has been shown to control autophagy at different levels (Tasdemir et al., 2008). Thus, it can be hypothesized that PML and p53 could function together in regulating the expression and/or the function of some critical regulators of the autophagic pathway such as the atg proteins.

1.4.2.1 PML and p53-dependent apoptosis

PML is a central regulator of p53-dependent apoptosis and DNA-damage response. This was suggested by the observation that PML deficiency results in the protection of cells from the lethal effects of ionizing radiation (γ radiation) (Guo et al., 2000; Wang et al., 1998b). Guo and colleagues analyzed the effect of ionizing radiation in thymocytes for *wild type* (WT), *p53*$^{-/-}$ and *PML*$^{-/-}$ animals. Interestingly, *PML*$^{-/-}$ cells were resistant to γ radiation-induced apoptosis, although to a lesser extent as compared to *p53*$^{-/-}$ thymocytes. These findings suggest that PML is at least in part required for the correct p53 pro-apoptotic functions (Guo et al., 2000). Accordingly, in *PML*$^{-/-}$ cells the ionizing radiation induced transactivation of p53 target genes such as *p21*, *GADD45* and *BAX* was defective (Guo et al., 2000). Indeed, upon γ-radiation a specific PML nuclear isoforms, PML4 herein after referred to as **nPML**, has been shown to interact and activate with p53. Indeed, the recruitment of p53 to the PML-NB results in its stabilization and transcriptional activation (Guo et al., 2000). Specifically, the acetyltransferase CBP cooperates with PML in promoting the acetylation of lysine 382 on p53 (Guo et al., 2000; Pearson et al., 2000). The effects of UV-radiation were also studied in respect to the ability of PML to modulate p53 pro-apoptotic functions. Interestingly, in cells exposed to high doses of UV the homeodomain-interacting protein kinase-2 (HIPK2), PML, CBP and p53 colocalized within PML-NBs. In this

context, HIPK2 directly interacts with and phosphorylates p53 at serine 46 (S46), another critical residue for its transcriptional activation (D'Orazi et al., 2002; Hofmann et al., 2002). Furthermore, Bernardi and co-workers demonstrated that PML enhances p53 stability by sequestering Mdm2 to the nucleolus (Bernardi et al., 2004). As a result, *PML* depletion in primary mouse embryo fibroblast leads to increased p53 ubiquitylation and degradation. Similarly, DNA damage induces PML phosphorylation by the checkpoint ataxia telangectasia mutated Rad-3 related kinase (ATR) and PML accumulation along with Mdm2 in the nucleolus (Bernardi et al., 2004; Louria-Hayon et al., 2003; Wei et al., 2003; Zhu et al., 2003). Notably, the interaction between PML and Mdm2 is through the coiled-coil domain of PML (Bernardi et al., 2004; Wei et al., 2003), which is retained by all PML isoforms. Thus, it is possible that depending on the type of cellular stress different PML isoforms may interact with Mdm2. PML has also been shown to translocate to the nucleolus in cells treated with proteasome inhibitors, suggesting that nucleoli may function as alternative centres for degradation of PML-NB components (Mattsson et al., 2001). It has also been demonstrated that the nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase SIRT1, the human homologue of yeast SIRT1 (Imai et al., 2000; Landry et al., 2000) is able to interact with PML and this leads to p53 inhibition (Langley et al., 2002). Notably, *SIRT1* belongs to the silence information regulators 2 family of genes (SIR2), which are involved in diverse processes ranging from the regulation of genes silencing to DNA repair and aging (Gasser and Cockell, 2001). Moreover, SIRT1 binds and deacetylates p53 in response to oxidative stress and DNA damage, thus promoting survival (Luo et al., 2001; Vaziri et al., 2001). These findings suggest that PML may exert a pivotal role in controlling the balance between acetylation and deacetylation of p53, for instance combining the function of CBP or SIRT1 in relation to the nature of the cellular stress as proposed by Hoffmann and colleagues (Hofmann and Will, 2003).

1.4.3 Additional mechanisms

The death domain-associated protein (DAXX) is another important protein found within the PML-NB (Torii et al., 1999; Zhong et al., 2000b). Originally, DAXX has been shown to bind to the Fas death domain (DD) thereby promoting Fas and TGF-β-induced apoptosis (Perlman et al., 2001; Yang et al., 1997). In this regard, it has been reported that PML and DAXX interaction increases Fas-induced apoptosis (Torii et al., 1999). Interestingly, in the absence of PML, DAXX accumulates in dense heterochromatic nuclear regions where it colocalizes with a component of centromeric heterochromatin, the SWI/SNF protein α-thalassemia/mental retardation syndrome (ATRX) (Ishov et al., 2004). The interaction between DAXX and ATRX has been shown to play an important role in controlling the chromatin changes during the S phase of the cell cycle (Xue et al., 2003). SUMOylation of PML is required for the recruitment of DAXX to the PML-NB. Specifically, the levels of SUMO-modified PML positively correlated with the amount of DAXX recruited to these nuclear structures (Kamitani et al., 1998b; Maul et al., 2000; Zhong et al., 2000b). A number of studies suggest that DAXX is able to shuttle between the nucleus and the cytoplasm upon specific stimuli. Accordingly, it has been shown that DAXX relocates from the nucleus to the cytoplasm in response to Fas, oxidative stress and glucose deprivation (Song and Lee, 2003; Song and Lee, 2004). Studies conducted using a yeast model showed that DAXX was able to bind the cytoplasmic tail of the type II transforming growth factor beta (TGFβ) receptor and to inhibit TGFβ-induced apoptosis (Ecsedy et al., 2003; Perlman et al., 2001; Song and Lee, 2003). Interestingly, the nuclear kinase HIPK2, which also associates with PML-NB, appears to mediate TGFβ-induced phosphorylation of DAXX and to promote JNK activation (Hofmann et al., 2003). RNAi-mediated downmodulation of DAXX rendered cells resistant to apoptosis induced by IFN-γ and As_2O_3 (Kawai et al., 2003) and we demonstrated that DAXX depletion renders primary human fibroblasts more resistant

to UV- and oxidative-induced cell death (Khelifi et al., 2005). Overall these findings suggest that DAXX localization to PML-NB is required for its pro-apoptotic activity. Nevertheless, the role of DAXX remains controversial as it can have both pro- and anti-apoptotic functions, depending on different factors (Chen and Chen, 2003; Michaelson et al., 1999; Michaelson and Leder, 2003; Salomoni and Khelifi, 2006). In a recent study DAXX has been found to simultaneously associate with Mdm2 and the deubiquitinase HAUSP, thus favouring the degradation of p53 (Tang et al., 2006). It is conceivable PML interferes with DAXX-mediated p53 degradation by sequestering DAXX in the PML-NB. Moreover, DAXX also associates with proteins critical for transcriptional repression including HDAC1, HDAC2 and ATRX, implying a role for DAXX in chromatin modification and in the control of epigenetic mechanisms (Hollenbach et al., 2002; Li et al., 2000; Xue et al., 2003). In this respect, DAXX was also shown to interact with SUMO-modified CBP and to inhibit its transcriptional activity recruiting HDAC2 (Kuo et al., 2005). In APL, PML-RARα delocalizes DAXX from the nuclear bodies and exacerbates its transcriptional repressive functions (Li et al., 2000). Recently, the SUMOylation of PML-RARα has been shown to mediate DAXX recruitment, a critical event required for transcriptional repression and cellular transformation (Zhu et al., 2005). Furthermore, Xu and co-workers found that nPML modulates Survivin, a member of the inhibitor of apoptosis (IAP) family of proteins. Survivin is often overexpressed in cancer cells whereas is barely detectable in most normal adult tissues (Xu et al., 2004). Interestingly, high levels of Survivin have been found in NB4 and primary APL cells (Xu et al., 2004). Nevertheless, the function of Survivin is still a matter of debate. Indeed, data suggest that Survivin play an important role in controlling chromosomes segregation and cytokinesis rather than functioning as a survival factor (Stauber et al., 2007; Vader et al., 2006 {Verdecia, 2000 #653) (Connell et al., 2008). As abovementioned, PML is critical for p53-dependent induced cell death in response to γ-irradiation. Previous studies demonstrated that in mouse

primary fibroblasts the apoptotic response to different DNA-damaging agents and short wavelength ultraviolet (UV) light does not rely on the activity of p53 and p21 (Bissonnette and Hunting, 1998; Brugarolas et al., 1995; Smith and Fornace, 1997), but it depends on the function of the c-Jun N-terminal kinases (JNKs) (Behrens et al., 1999; Tournier et al., 2000). Salomoni and colleagues found that UV treatment redistributes PML into multiple microspeckled structures, referred to as UV-NBs, where phosphorylated c-Jun is also found (Salomoni et al., 2005). Recently, Khelifi and co-workers found that DAXX is upregulated in primary cells exposed to UV and hydrogen peroxide (H_2O_2) treatment (Khelifi et al., 2005). DAXX and PML colocalize in PML-NBs in both unstressed and stressed cells, and DAXX down-modulation inhibits JNK activation (Khelifi et al., 2005). These finding demonstrate the importance of DAXX and PML in the modulation of the JNK pathway in physiological settings. Interestingly, c-Jun has been also recently shown to modulate the function of a specific cytoplasmic isoform of PML in the context of the TGF-β signalling (Seo et al., 2006). This aspect is further discussed in section 1.5. Thus nuclear dynamics of PML may change in response to different apoptotic stimuli and activating transcription factors that controls key pro-apoptotic pathways.

1.4.4 PML and cellular senescence

Normal cells are unable to replicate indefinitely and after serial cultivation *in vitro* undergo a permanent exclusion from the cell cycle, referred to as cellular senescence. Cellular senescence has been proposed to represent a critical mechanism to limit proliferation and block tumorigenesis (Bartkova et al., 2006; Campisi and d'Adda di Fagagna, 2007; Mallette et al., 2007). Senescence was described in the early sixties by Hayflick and Moorhead (Hayflick and Moorhead, 1961) and has since then been challenging researchers. Increasing evidence indicate that senescence is accompanied by DNA damage and requires the activation of p53 and/or pRB depending on the cell type (Campisi and d'Adda di Fagagna, 2007; Mallette et al., 2004; Serrano et al., 1997). Senescence can also intercede to limit the aberrant proliferation promoted by the expression of oncogenes. Indeed, expression of oncogenes such as an active form of RAS (RAS^{G12V}) triggers cellular senescence by inducing the expression of the cyclin-dependent kinase (CDK) inhibitor $p16^{INK4a}$ and *p53* (Pearson et al., 2000; Serrano et al., 1997). Notably, like p53, $p16^{INK4a}$ (p16) is mutated in a wide range of cancers (Schmitt et al., 2002; Sherr, 2004). In fact, p16 is a tumour suppressor that binds and inhibits the activity of the CDK4/6 (Kim and Sharpless, 2006). p16 maintains pRB in a hypophosphorylated state, blocking cell cycle progression. PML role in oncogene-induced senescence was determined in primary MEFs (de Stanchina et al., 2004; Ferbeyre et al., 2000). Pearson and colleagues found that upon expression of an active form of RAS, PML is upregulated at both the transcriptional and protein levels. Furthermore, RAS triggers the formation of a ternary complex between p53, CBP and PML. This event promotes p53 stabilization and activation (Pearson et al., 2000). In keeping with this, the ability of RAS to induce *p21* is impaired in $PML^{-/-}$ cells and over-expression of PML led to induction of p53-dependent senescence (Ferbeyre et al., 2000; Pearson et al., 2000).

Altogether, these results indicate that in mouse cells senescence is regulated by the interplay between p53 and PML (Ferbeyre et al., 2000; Pearson et al., 2000). As opposed to mouse cells, in human cells induction of senescence relies more on the activation pf pRB (Bischof et al., 2005; Ferbeyre, 2002; Mallette et al., 2004). Specifically, it has been demonstrated that nPML modulates pRB phosphorylation and potentiates pRB transcriptional repression (Alcalay et al., 1998; Bischof et al., 2005; Khan et al., 2001a; Khan et al., 2001b). Overall these findings propose a critical role for PML in the regulation of cellular players, such as p53 and pRB involved in the control of cell proliferation and the induction of cellular senescence. In APL, these functions can be altered as a consequence of the dominant-negative action of PML-RARα on PML.

1.4.5 PML and translation control

Increasing evidence suggests that cellular transformation and translational control are intimately intertwined (Rosenwald, 1996). Mutations affecting ribosomal proteins are associated with increased cancer susceptibility such as in the Diamond-Blackfan anaemia and in Dyskeratosis Congenita (DC) (Ruggero and Pandolfi, 2003; Yoon et al., 2006). Protein biosynthesis consists of three major phases: initiation, elongation and termination. Initiation of translation is a critical step and its deregulation has been implicated in cellular transformation (Ruggero and Pandolfi, 2003). The eukaryotic initiation factor 4E (eIF4E) acts as both a key initiator factor and a promoter of nucleus/cytoplasmic transport of specific transcripts, such as cyclin D1. eIF4E binds the 5' m^7G cap promoting the nuclear export and transcription of capped-mRNAs. Transgenic animals expressing sustained levels of eIF4E develop tumours of various histological origins and eIF4E cooperates with c-Myc in accelerating B-cell lymphomagenesis *in vivo* (Ruggero et al., 2004). PML has been shown to co-localize

and interact with eIF4E in the PML-NB. In addition, Lai and co-workers showed that PML, through the nuclear retention of eIF4E, negatively regulates the cytoplasmic export of a subset of transcripts including the cyclin D1 messenger. PML function leads to a reduction of the cyclin D1 protein levels and inhibition of cyclin D1 transforming activity (Lai and Borden, 2000). In line with this, eIF4E-dependent cyclin D1 mRNA transport is upregulated in human specimens of acute and chronic myelogenous leukaemia (Topisirovic et al., 2003).

1.4.6 PML and genomic stability

The stability of the genome represents a major barrier against cellular transformation and PML participate to this fundamental process by modulating important proteins involved in DNA-damage response. Indeed, a number of factors involved in DNA recombination and repair have been shown to colocalize and interact with PML in the PML-NB at specific stages of the cell cycle or upon treatment with DNA-damaging agents. For example PML regulates the function of Bloom (BLM) a DNA helicase controlling non-homologous recombination events (Hanada et al., 1997; Harmon and Kowalczykowski, 1998). Mutations of BLM have been associated with the Bloom Syndrome (BS), characterized by excessive sister chromosome exchange (SCE) and chromosome breakage leading to high predisposition for cancer development in the patients (Ellis et al., 1995). Interestingly, PML and BLM colocalize in primary cells derived from BS cells (Zhong et al., 1999b) and BLM is delocalized from the PML-NB in *PML*$^{-/-}$ and APL cells. Accordingly, primary *PML*$^{-/-}$ fibroblasts display a higher rate of spontaneous SCE as compared to control cells (Zhong et al., 1999b). PML has also been shown to colocalize and directly interact with the meiotic recombination 11 (MRE11), a protein involved in the regulation of the DNA damage response. Upon DNA damage, MRE11 shuttles between PML-NB and associates with DNA damage

foci (Carbone et al., 2002). Nevertheless, it is still unclear what is the effect of PML inactivation on MRE11 functions. Interestingly, PML and MRE11 colocalize in cancer cells maintaining telomeres by a telomerase-independent alternative (ALT) mechanism. In these malignancies, PML accumulates in novel bodies, referred to as ALT associated PML bodies (APBs), which are characteristic of ALT cells and qualitatively different from the PML-NBs, where also telomeric repeat DNA sequences (TTAGGG), telomere binding proteins TRF1 and TRF2 and several proteins involved in DNA repair accumulate (Reddel, 2007). Furthermore, APBs share some components of the PML-NBs such as Sp100 and BLM (Reddel, 2007). Our laboratory recently investigated the role of PML and TRF2 in the formation of APBs in ALT cells by using RNAi-mediated silencing (Stagno D'Alcontres et al., 2007). In this study, D'Alcontres and colleagues showed that in ALT cells lacking TRF2 there is a loss of telomeric DNA and an induction of cellular senescence, rather than apoptosis, that requires intact p53 and PML (Stagno D'Alcontres et al., 2007). Interestingly, other DNA helicases have been found in the PML-NBs including Werner (WRN) and RecQL4, which are associated with human syndromes that display increased genomic instability (Blander et al., 2002; Petkovic et al., 2005). Nevertheless, the exact roles of PML and PML-NB in processes regulated by these two helicases remain unknown. Genomic instability also associates with gain or loss of chromosome (aneuploidy) and centrosome duplication (Lingle et al., 2002) (Ghadimi et al., 2000). Centrosomes have a critical role during mitosis and many proteins including cell cycle regulators, phosphatases and kinases involved in cell signalling and tumour suppressors associate with these structures (Nigg, 2002). Using isoform-specific anti-PML antibodies, Xu and co-workers demonstrated that PMLIII associated with the centrosome *in vivo* (Xu et al., 2005) and that the percentage of $PML^{-/-}$ fibroblasts with centrosome amplifications was higher as compared to $PML^{+/+}$ cells (Xu et al., 2005). The same authors showed that PMLIII physically interacts with Aurora A, a kinase regulating centrosome assembly (Hannak

2001). Interestingly, deregulation of Aurora A is implicated in centrosome amplification (Zhou et al., 1998). Nevertheless, in a recent study Condemine and colleagues investigate the expression levels and the localization of the different PML isoforms by using isoform-specific antibodies could not find any association between the centromeres and PMLIII. Thus, this remains a very controversial matter that requires further analysis. In this regard, the generation of reliable and specific tools to investigate the function of the different PML isotypes could provide a major advance in the understanding of PML's biological functions.

1.5 PML cytoplasmic functions

1.5.1 Initial observations

Despite the existence of a number of PML cytoplasmic isoforms was reported many years ago (Fagioli et al., 1992; Jensen et al., 2001), their functional characterization has been somehow limited until recently. Initial observations conducted by using a mutant of PML4 lacking the nuclear localization signal (ΔNLS), which resulted in cytoplasmic accumulation of the protein, revealed that the tumour suppressive functions were severely impaired (Le et al., 1996). ΔNLS PML expression also led to a significant reduction of PML-NBs, thus suggesting that this mutant could function as a dominant negative of wild-type nPML (Le et al., 1996). Subsequently, Fagioli and colleagues analyzed the effect of different PML splice variants on cell proliferation and found that a cytoplasmic isoform, referred to as PML 3-4-7, failed to induce growth suppression (Fagioli et al., 1998). Hence, they concluded that the presence of NLS was indispensable for the growth suppressive function of PML (Fagioli et al., 1998). Because of the greater interest on nuclear PML and its growth suppressive and pro-apoptotic functions, the role of cytoplasmic isoforms has been overlooked for a long

time. Nevertheless, there is the possibility that also cytoplasmic PML could have an important role in physiological and pathological conditions (Lin et al., 2004; Seo et al., 2006).

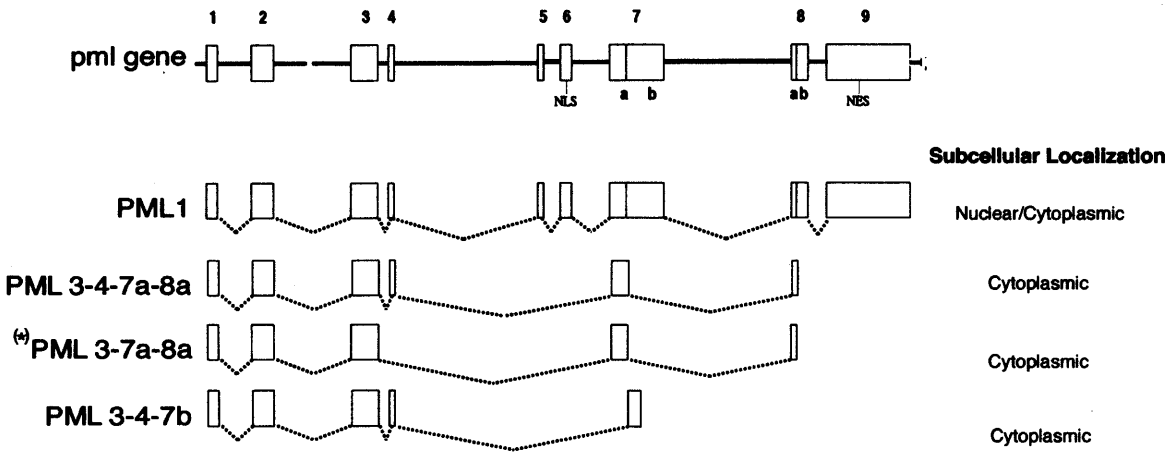


Figure 1.5.1A *PML* gene and cytoplasmic isoforms. Cellular localization is governed by the presence or absence of a nuclear localization sequence (NLS) and nuclear export sequence (NES) encoded by exon 6 and 9, respectively. Remarkably, PML1 possesses both elements so that displays nuclear and cytoplasmic distribution. The (*) indicates the PML cytoplasmic isoform involved in the modulation of TGF-β signalling (Lin et al., 2004).

1.5.2 Role of cytoplasmic PML in the TGF-β pathway

TGF-β is a pleiotropic cytokine that is crucially implicated in a variety of cellular processes such as proliferation, differentiation and apoptosis (Siegel and Massague, 2003). This pathway is tightly regulated in physiological conditions (Siegel and Massague, 2003). By contrast, alterations of the TGF-β pathway occur in cancer and has been strongly linked to the pathogenesis of several human malignancies, encompassing solid as well as haematopoietic tumours (Derynck et al., 2001; Lin et al., 2005; Shi and Massague, 2003; Siegel and Massague, 2003). The TGF-β pathway and its alterations in cancer have been extensively studied and well characterized (Siegel and Massague, 2003). The signal is originated at the level of the cell membrane by two

serine-threonine kinase receptors: TGF-β receptor I and II (TβRI and TβRII). TGF-β binds to the TβRII that associate to and activates the TβRI. Subsequently, the receptor complex is internalized through the chlatrin/early endosome pathway, and the signal is propagated to the nucleus through TβRI mediated-phosphorylation of the transcriptional factors Smad2 and Smad3. In this context, SARA (Smad Anchor for Receptor Activator) promotes the internalization of the receptors into early endosomes, thus facilitating Smad2/3 activation (Shi and Massague, 2003; Tsukazaki et al., 1998). Once activated, Smad2/3 associate with Smad4 and the complex translocates to the nucleus where it coordinates the expression of TGF-β-responsive genes (Inman and Hill, 2002; Wu et al., 2001). Furthermore, DAXX has been implicated in the modulation of TGF-β pro-apoptotic signalling. Perlman and colleagues demonstrate that upon TGF-β treatment DAXX relocates to the plasma membrane and physically associates with the TβRII, thus promoting ASK1-mediated activation of JNK (Perlman et al., 2001). In this respect, it can be hypothesized that specific PML cytoplasmic isoforms can modulate DAXX activity outside the nucleus, for instance facilitating the activation of the downstream components of the TGF-β signalling pathway (Lin et al., 2004).

1.5.2.1 Cytoplasmic PML and modulation of TGF-β signalling

In a recent work, Lin et al. (Lin et al., 2004) implicated the tumour suppressor PML in the modulation of TGF-β signalling (Figure 1.5.2.1). Remarkably, primary *Pml*$^{-/-}$ mouse embryo fibroblasts (MEFs) appeared to be insensitive to TGF-β-induced growth suppression and apoptosis (Lin et al., 2004). Surprisingly, reintroduction of the nuclear isoform PML4 failed to rescue these defects. In contrast, full restoration of TGF-β responsiveness was achieved by expressing a PML cytoplasmic isoform (cPML; figure 1.5.1A), thus suggesting a pivotal role of cPML in this pathway (Lin et al., 2004). It is presently unclear whether other PML nuclear isoforms could play any role in rescuing

TGF-β-dependent senescence and cell death. Interestingly, mRNA levels of cPML were induced by TGF-β in different cell types, thus suggesting that TGF-b may control PML expression and/or splicing (Lin et al., 2004). Importantly, *Pml*$^{-/-}$ MEFs are defective in TGF-β-mediated phosphorylation and nuclear translocation of Smad2/3, and these defects could be fully rescued by cPML expression (Lin et al., 2004). Nevertheless, it remains to be established whether depletion of endogenous cPML only in *Pml*$^{+/+}$ MEFs would result in impaired TGF-β signalling. Interestingly, cPML physically associates with Smad2/3 and with SARA (Lin et al., 2004). Furthermore, immunofluorescence and sucrose gradient-mediated fractionation revealed that cPML localized to early endosomes and that the TβRI/II-SARA localization to this subcellular compartment was compromised in *Pml*$^{-/-}$ cells. Similarly, PML-RARα is able to disrupt this complex, thus interfering with TGF-β tumour suppressive signalling (Lin et al., 2004). Based on these observations, the authors concluded that cPML is essential for the efficient recruitment and assembly of the TGF-β receptors/SARA/Smads complex. Altogether, this work has revealed an unexpected role for cPML in the modulation of the TGF-β signalling. Nevertheless, more efforts are needed to answer several outstanding questions. For example, the status of the TGF-β/cPML pathway in non-haematopoietic cancers, and in particular in metastatic solid tumours is still unknown. Finally, the absence of phenotypic overlapping between animal models lacking different components of the TGF-β pathway and *Pml*$^{-/-}$ mice suggests that the role of cPML could be confined to specific tissues or pathological conditions (Kulkarni et al., 1993; Nomura and Li, 1998; Shull et al., 1992; Yang et al., 1999; Yang et al., 1998).

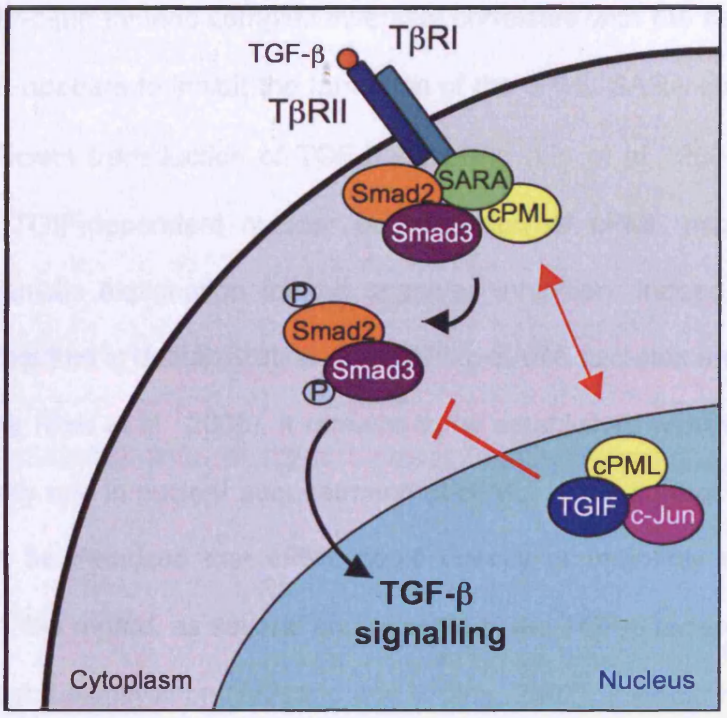


Figure 1.5.2.1 Cytoplasmic PML modulates TGF-β signalling. Upon TGF-b treatment cPML promotes the assembly of the complex TβRI/II-SARA-Smad2/3. This event induces the phosphorylation of Smad2/3 and transduction of the TGF-β pathway (Lin et al., 2004). In this context, TGIF along with c-Jun sequesters cPML in the nucleus and inhibits its function (Seo et al., 2006).

Another study has demonstrated that the TG-interacting factor (TGIF), a negative regulator of the TGF-β pathway, blocks cPML function and this results in the inhibition of Smad2 phosphorylation and activation (Seo et al., 2006) (Figure1.5.2.1). Consistent with previous reports showing that the interaction between c-jun and TGIF is essential to inhibit TGF-β-activated pathways (Pessah et al., 2001), *c-jun*$^{-/-}$ fibroblasts are impaired in TGIF-dependent inhibition of Smad2 phosphorylation. The authors also demonstrate that TGIF-dependent effects do not rely on its binding to Smad2 or on c-jun transcriptional activation. As PML physically interacts with c-jun (Salomoni et al., 2005) and modulates the TGF-β pathway (Lin et al., 2004), the authors hypothesized that PML was involved in TGIF inhibitory activity. Indeed, cPML and TGIF interact in the nucleus, and this interaction is favoured by c-jun (Seo et al., 2006). The presence

of a cPML-TGIF-c-jun trimeric complex inversely correlates with the sensitivity of cells to TGF-β. TGIF appears to inhibit the formation of the cPML-SARA complex, which is required for efficient transduction of TGF-β signalling (Lin et al., 2004). The authors conclude that TGIF-dependent nuclear sequestration of cPML might represent a possible mechanistic explanation for the observed inhibition. Indeed, cPML nuclear sequestration resulted in destabilization of the cPML-SARA complex and impairment of TGF-β signalling (Seo et al., 2006). It remains to be established whether nuclear PML isoforms play any role in nuclear sequestration of cPML. In the light of these important findings, it can be theorized that cPML could directly or indirectly modulate signal transduction. In this regard, as several components of the TGF-β receptor complex are regulated through ubiquitylation (Attisano and Wrana, 2002), it would be interesting to determine whether cPML through its RING domain could function as specific E3 ubiquitin ligase. Furthermore, nuclear sequestration of cytoplasmic PML is emerging as an important regulatory mechanism (Seo et al., 2006). *Vice versa*, it would be of extreme interest to determine whether nuclear isoforms can be regulated through nuclear exclusion and if cytoplasmic localization of nuclear isoforms could affect TGF-β-dependent signalling as well. Albeit important results have been achieved, more effort is needed in the future to gain more insights into this fascinating area of PML biology.

1.5.3 Redistribution of PML to the cytoplasm during the cell cycle

PML-NBs undergo to a dramatic re-organization during the cell cycle: the number, the shape and the composition of nuclear bodies are profoundly altered during S and M phases (Dellaire et al., 2006a; Everett et al., 1999; Koken et al., 1995; Terris et al., 1995). A recent study reported that during mitosis PML redistributes to cytoplasmic domains called mitotic accumulation of PML proteins (MAPPs), which diverge for structure and composition from PML-NBs (Dellaire et al., 2006b). Notably, even in the early G1 phase of cell cycle a large portion of PML is found to reside in cytoplasmic MAPPs. This phenomenon is very likely due to relocalization of nuclear isoforms to the cytoplasm. Nevertheless, it is still unclear whether the NES-containing isoform, PML1, or cytoplasmic isoforms are functionally involved in this process. Interestingly, the co-localization between PML and Daxx, a well-known interphase PML-NB component, is lost during cell cycle. Indeed, the exact function of cytoplasmic MAPPs and their contribution to cell cycle progression has still to be determined. Specifically, it is presently unclear whether MAPPs can bear cytoplasmic functions, for instance regulating translation (Cohen et al., 2001) or modulating TGF-β signalling (Lin et al., 2004), or whether they simply represent a transient depot for the recycling of PML proteins until the mid-G1 reorganization of the PML-NB is completed.

1.5.4 Role of cytoplasmic PML in the cellular defence against viral infection

Pioneering studies conducted by different groups showed that PML levels were induced in response to antiviral interferon induction (IFN; (Chelbi-Alix et al., 1995; Lavau et al., 1995), revealing that PML was a primary target of IFN (Stadler et al., 1995). IFN treatment caused the increase in both size and number of PML-NBs

(Chelbi-Alix et al., 1995; Lavau et al., 1995; Stadler et al., 1995). The importance of PML in the viral response is outlined by the fact that many viruses have evolved different strategies in order to disrupt the PML-NB. Interestingly, arenaviruses encode a RING protein, Z protein, which binds PML and promotes its cytoplasmic redistribution. Once in the cytoplasm, PML and protein Z interfere with the function of eIF4E by reducing its affinity for the CAP structure thereby hampering translation initiation (Kentsis et al., 2001). Accordingly, nPML was previously shown to inhibit eIF4E by targeting to the PML-NB, thus suggesting that different PML isoforms could interfere with mRNA transport and translation (Cohen et al., 2001). An elegant work by Turelli et al. demonstrated that cytoplasmic PML is part of the anti-viral cellular response during the early events of the retroviral life cycle, which spans from the cellular entry of the viral particles to the integration of the viral genome into the host genome. This part of the infection cycle is usually inefficient, as only a small portion of the viral particles entering the cells are able to successfully integrate. The integrase interactor 1 (INI-1) interacts with the HIV-1 integrase and is an essential subunit of the human SWI/SNF chromatin-remodelling complex (Turelli et al., 2001). At steady state, INI-1 presents a nuclear diffuse localization, while PML lies in punctuated PML-NBs. Subsequently, PML and INI-1 undergo a rapid but transient cytoplasmic relocation and accumulation in dense cytoplasmic bodies. The nucleus-cytoplasmic export was demonstrated to be exportin-dependent (Turelli et al., 2001). Importantly, it was found that PML/INI-1 colocalize in the cytoplasm with the incoming retroviral pre-integration complex. This event appeared to be crucial for the anti-viral response mediated by PML. Indeed, nuclear sequestration of PML induced by using leptomycin B or arsenic trioxide, As_2O_3, greatly increased viral transduction efficiency (Turelli et al., 2001). Altogether these lines of evidence suggest that PML is implicated in the cellular defence against viral infections. Interestingly, this is feature shared by several TRIM proteins such as TRIM1, TRIM5 and TRIM22, thus suggesting that PML could interplay

with other TRIMs during viral infection (Nisole et al., 2005). Alternatively, there could be a degree of redundancy between different TRIMs, and this could explain the contradictory results obtained by testing viral infection efficiency in PML-deficient cells (Everett et al., 2006; Nisole et al., 2005). Finally, what remains unclear is how PML interferes with viruses that possess different replicative strategies. This aspect awaits further investigation.

1.5.5 Cytoplasmic PML in tumours

1.5.5.1 Cytoplasmic PML in solid tumours

Loss of PML expression has been reported in tumours of both haematopoietic and epithelial origin (Gurrieri et al., 2004a), in addition, old and new evidence indicates that PML could also acquire cytoplasmic localization in some human cancers. Nevertheless, it remains to be determined whether cytoplasmic PML has a role in tumorigenesis, for example deregulating the function of essential tumour suppressor proteins. This remains an outstanding and intriguing question in PML field. Two independent laboratories have reported that PML relocates to the cytoplasm in the majority of human hepatocellular and skin cell carcinoma samples (Condemine et al., 2006; Terris et al., 1995). In these types of malignancies, PML accumulates in cytoplasmic granules however, it is still unclear whether this is due to increase expression of PML cytoplasmic isoforms or whether nuclear isoforms are aberrantly relocalized outiside the nucleus. Indeed, mutations in *PML* are extremely rare in human cancer, thus it is possible that an unbalanced production of shorter cytoplasmic isoforms occurs in these malignancies. Nevertheless this aspect has not been investigated yet. In this regard, the generation of isoform-specific antibodies recognizing PML cytoplasmic isoforms will represent an invaluable functional and prognostic tool.

1.5.5.2 Cytoplasmic mutants of PML in APL

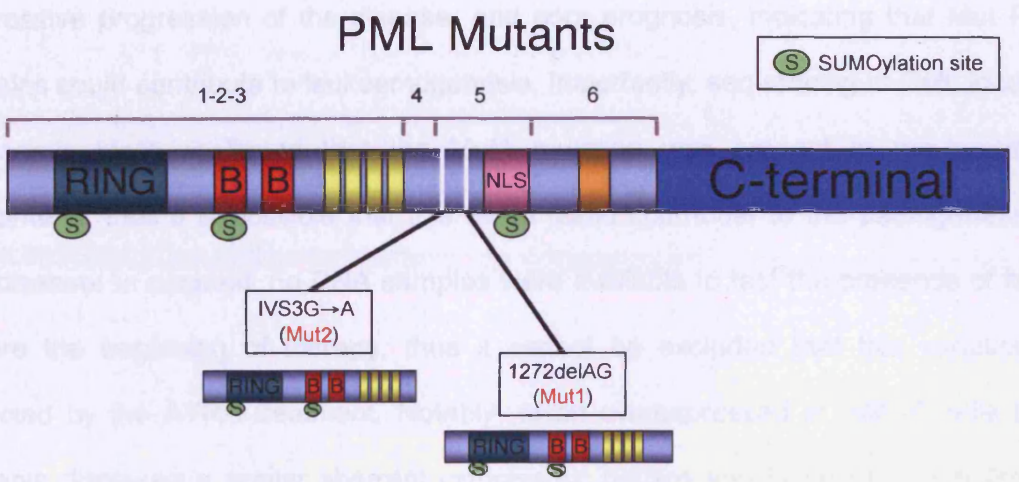


Figure 1.5.5.2A APL-associated PML mutants. Scheme of PML structure with the two missense mutations identified in APL. The mutations are a deletion 1272delAG (Mut1), and a splice site mutation IVSG→A (Mut2). Remarkably, both mutations introduce a premature stop codon upstream of the nuclear localization signal (NLS), leading to generation of cytoplasmic mutant PML proteins.

PML was found mutated in a plasmacytoma cell line, namely J558, to generate a truncated protein, which accumulates in the cytoplasm and is able to delocalize nuclear PML (Zhen et al., 1998). More precisely, the mutation occurs within exon 3 of *PML* leading to the generation of a premature stop codon and to the accumulation of PML cytoplasmic proteins with dominant negative properties (Bruno et al., 2003). Interestingly, a recent study conducted on a cohort of seventeen RA-resistant APL cases, two missense mutations were identified in the remaining *PML* allele (Gurrieri et al., 2004b). The first DNA variation, Mut1, 1272delAG, in exon 5, identified in a 9-year-old female was a splice site mutation IVSG→A identified in a 19-year-old male that causes a frameshift in the coding frame and splices out exon 4 from the mature transcript. The second mutation, Mut2, was a splice site mutation IVSG→A identified in a 19-year-old male. Interestingly, both mutations introduce a premature stop codon

upstream the nuclear localization signal (NLS) sequence present in exon 6, so that, the resulting mutant PML proteins (Mut PML) accumulate in the cytoplasm (Gurrieri et al., 2004b) (Figure 1.5.5.2A). Notably, these mutations are associated with a very aggressive progression of the disease, and poor prognosis, indicating that Mut PML proteins could contribute to leukaemogenesis. Importantly, sequencing of *PML* locus in leukaemic blast confirmed that the Mut1 mutation was present in pre-treatment specimens, thus it is possible that this event could contribute to the pathogenesis of the disease. In contrast, no DNA samples were available to test the presence of Mut2 before the beginning of therapy, thus it cannot be excluded that this variation is selected by the ATRA treatment. Notably, when overexpressed in *PML-/-* cells both mutants displayed a similar aberrant cytoplasmic pattern and failed to accumulate in either the PML-NBs or the nucleus, however, no functional analysis were performed. Thus, it would be extremely important to determine whether PML mutants cooperate with PML-RARα in promoting leukaemogenesis. This crucial aspect will be analyzed more in detail in chapter 3 and 4.

1.6 PML-RARα function in APL

The majority of of APL patients (more than 90%) is characterized by a specific genetic abnormality: the reciprocal and balanced translocation t(15;17). As a results two chimeric proteins are generated: PML-RARα and RARα-PML (Salomoni and Pandolfi, 2002; Huang et al., 1993; Jurcic et al., 2001; Vahdat et al., 1994). According to the current model, PML-RARα functions in the nucleus at the chromatin level by forming abnormal macro-molecular nuclear receptor complexes that bind to retinoic acid responsive elements (RARE) and block the transcription of essential RA-target genes (Lin and Evans, 2000; Salomoni and Pandolfi, 2002). Indeed, it has been shown that PML-RARα aberrantly recruits HDACs and hystone methyltransferases (HMTs) through the RARα moiety of the fusion protein (Di Croce et al., 2002; Grignani et al., 1998; Lin et al., 1998). Furthermore, PML-RARα has been shown to form heterodimers or multimers with RXRα. These macromolecular complexes display a relaxed DNA-binding specificity binding core motifs in any orientation, even if widely spaced on the DNA (Jansen et al., 1995; Perez et al., 1993; Zhou et al., 2006). Moreover, PML-RARα has been shown to repress *de novo* target genes such as type II transglutaminase and or CCAAT/enhancer binding proteins alpha, beta and epsilon (C/EBPα–β–ε) (Benedetti et al., 1996; Duprez et al., 2003; Truong et al., 2003). Notably, the effects of PML-RARα on C/EBP proteins may influence disease progression by diminishing sensitivity of leukaemic cells to RA treatment (Truong et al., 2003). A recent study shows that during myeloid differentiation C/EBPε expression is reduced in *PML*$^{-/-}$ background. Importantly, C/EBPε expression relies on the activity of the transcription factor PU.1, which also regulates many myeloid genes including cytokines receptors involved in granulocyte and monocyte-macrophage maturation. It has been demonstrated that

PU.1 is co-activated in the PML-NB specifically by PML4 (in this thesis referred to as nPML) (Yoshida et al., 2007).

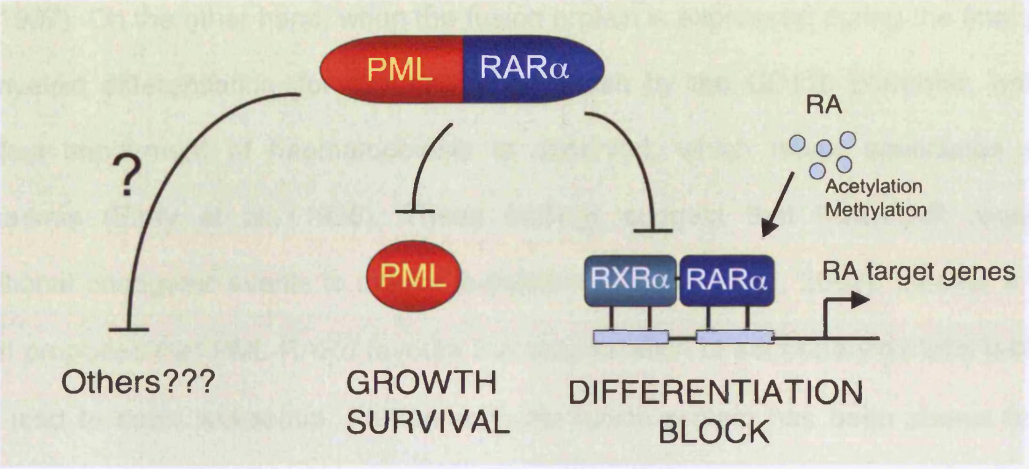


Figure 1.6A PML-RARα leukaemogenesis. The model represents the molecular mechanisms possibly involved in PML-RARα-mediated leukaemogenesis. The fusion protein displays a dominant negative activity over the physiological functions of PML and the nuclear receptor RARα/RXRα. Furthermore, it is also possible that additional inhibitory mechanisms mediated by PML-RARα can contribute to the pathogenesis of APL. Our hypothesis is that that PML-RARα also controls/disrupts cytoplasmic pathways, this will demonstrate that additional levels of complexity exist.

As aforementioned, PML-RARα inhibition of nPML is considered a critical event in APL leukaemogenesis (Salomoni and Pandolfi, 2002). Indeed, it has been reported that PML is essential for the tumour and growth suppressive activity of RA, a function exerted through the *trans*-activation of *p21* (Wang et al., 1998a). Several groups have studied the *in vivo* functions of PML-RARα by generating transgenic animals and/or testing the oncogenic potential of the fusion in *ex vivo* transformation assays of primary progenitor cells isolated from the bone marrow of mice. Controversial results have been obtained by using transgenic animals expressing PML-RARα under the control of different myeloid-specific promoters. On the one hand, the expression of PML-RARα under the control of promoters of genes express during the early phases of myeloid differentiation, such as cathepsin G (CG) and myeloid related protein 8 (MRP8), leads to mild alteration of myelopoiesis. These mice develop a myeloproliferative syndrome,

a "preleukaemic state" characterized by low penetrance of leukaemia (15-20 % of the transgenic animals) and long latency (6-18 months) (Brown et al., 1997; Grisolano et al., 1997). On the other hand, when the fusion protein is expressed during the final part of myeloid differentiation, for example when driven by the *CD11b* promoter, only a modest impairment of haematopoiesis is observed, which never associates with leukaemia (Early et al., 1996). These findings suggest that PML-RAR requires additional oncogenic events to induce leukaemia (Minucci et al., 2002). Indeed, it has been proposed that PML-RARα favours the accumulation of secondary genetic lesions that lead to overt leukaemia. Remarkably, the fusion protein has been shown to be toxic for the cells and this would explain why its levels of expression are maintained very low in the bone marrow and in the blasts (Ferrucci et al., 1997). Nevertheless, a 'knock-in' PML-RARα animal model using the entire 5' UTR of the *CG* gene lead to high-penetrance of the disease (Westervelt et al., 2003). Interestingly, also in this mouse model the fusion protein is expressed at low levels. Thus, it is possible that low levels of expression of PML-RARα in early myeloid cells are required for efficient transformation (Westervelt et al., 2003). Furthermore, experiments using primary haematopoietic progenitors have been used to dissect the functions of the fusion protein. For example, it has been shown that targeting the interaction surface between PML-RARα and HDAC has been shown to relieve in part PML-RARα-mediated gene repression and reduce cell transformation (He et al., 2001; Racanicchi et al., 2005). Another study demonstrated that forced homodimerization of RARα, induced by replacing the PML portion with the dimerization domain of p50NFκB, results in an efficient recruitment of corepressors such as SMRT, which is believed to play a central role in APL development (Sternsdorf et al., 2006). Nevertheless, RARα homodimers fail to immortalize primary progenitors *ex vivo*. Indeed, RARα forced homodimers are poor inducers of leukaemia *in vivo* (Sternsdorf et al., 2006). Interestingly, *p50-*

RARα/PML-/- transgenic animals did not show increased incidence of leukaemia, thus suggesting that additional repressive mechanism could be involved (Sternsdorf et al., 2006).

1.6.1 PML-RARα catabolism

The sensitivity to treatments with high concentrations of RA is probably the most striking feature of APL that made this disease an interesting paradigm to use in order to understand the molecular mechanism underling leukaemogenesis (Zhu et al., 1999; Zhu et al., 2001). Indeed, pharmacological concentrations of ATRA, (10^{-7} and 10^{-6} M), induce the release of HDAC and HMT from PML-RARα macromolecular complexes thus releasing the block of transcription. Nevertheless, point mutations in the RA-binding domain of PML-RARα have been associated with resistance to chemotherapy and lead to the relapse of the leukaemia in the patients. For these reasons, understanding the catabolism of the fusion protein has become of paramount therapeutical importance (Lallemand-Breitenbach et al., 1999; Zhu et al., 1999; Zhu et al., 2001). This aspect ramained poorly understood until recently. The mechanism by which ATRA induces the down-modulation of PML-RARα levels has been associated to the activity of the proteasome. It has been suggested that RA-induced degradation is dependent on the proteasome. Specifically, it has been shown that RA induces the activation of the AF2 domain in the C-terminal region of RARα, which promotes the recruitment of the SUG-1 components of the 19S proteasome (Nervi et al., 1998; Zhu et al., 2001). Similarly, arsenic, a natural poison initially used as a remedy in Chinese medicine, was shown to possess a clinical efficacy in APL (Zhu et al., 2002). Arsenic acts on the PML counterpart of PML-RARα promoting the SUMOylation of lysine K160, which promotes the recruitment of the 11S proteasome regulatory complex and the degradation of the fusion protein (Lallemand-Breitenbach et al., 2001). The role of

SUMOylation has recently emerged as an important post-translational event in the regulation of PML-RARα functions. In addition to the effects on protein stability, SUMOylation has been shown to modulate the activity of several transcriptional factors and cofactors including the glucocorticoid receptor, the mineralcorticoid receptor, the progesterone receptor, ETS, c-EBP α and c-EBPε, c-Myb and many others (Gill, 2005; Hay, 2005; Iniguez-Lluhi and Pearce, 2000). Specifically, SUMOylation favours the interaction between transcription factors and HDACs and, in turn, silencing transcription. Importantly, the transcriptional outcome is regulated through the balance between acetylation and SUMOylation (Gill, 2005; Hay, 2005). Furthermore DAXX has been shown to bind to SUMOylated transcription factors through its SUMO-interacting motif and to repress their function. Accordingly, SUMOylation of PML is required for DAXX localizion to the PML-NB and for the modulation of PML pro-apoptotic functions (Zhong et al., 2000a; Zhong et al., 2000b). In APL, PML-RARα disrupts PML-NB and abrogates the interaction between PML and DAXX, thus conferring a survival advantage to the leukaemic cells (Zhong et al., 2000a; Zhong et al., 2000b). Recently, it has been demonstrated that SUMOylation of the lysine 160 (K160) in PML-RARα is of critical importance to induce immortalization and transformation of primary haematopoietic progenitor *ex vivo* (Zhu et al., 2005). Indeed, cells expressing a PML-RARα mutant carrying the substitution lysine (K) to arginine (R) at position 160 (referred to as PML-RARα*K160R*) do not undergo transformation and transgenic animals expressing the PML-RARα*K160R* mutant under the control of the MRP8 promoter develop a myeloid hyperplasia but never APL. Importantly, SUMOylated PML-RARα recruits DAXX along with HDACs on specific DNA sequences, thus repressing transcription (Zhu et al., 2005).

1.6.2 Possible additional PML-RARα leukaemogenic functions

As previously mentioned, PML-RARα homo-oligomerization in the nucleus leads to the formation of abnormal macro-molecular nuclear receptor complexes, in which HDACs and HMTs accumulate; an event that is required for transformation *in vitro* and *in vivo* (Di Croce et al., 2002; Grignani et al., 1998; Lin and Evans, 2000; Lin et al., 1998; Minucci et al., 2000; Sternsdorf et al., 2006). Moreover, PML-RARα interacts and inhibits endogenous PML. Consistently, the loss of PML in an APL animal model resulted in acceleration and increased incidence of the disease (Rego et al., 2001; Salomoni and Pandolfi, 2002). Other studies showed that chimeric proteins lacking the PML portion maintained *in vitro,* but not *in vivo,* transforming potential (Lin and Evans, 2000; Minucci et al., 2000; Sternsdorf et al., 2006). Nevertheless, forced RARα dimerization accompanied by *PML* inactivation did not phenocopy the activity of the *wild type* PML-RARα, thus suggesting that the fusion protein may also represent a gain-of-function mutant (Sternsdorf et al., 2006). Thus, it is plausible that beside PML and RARα, PML-RARα could also interfere with the activity of other proteins (Zhu et al., 2005). In this regard, it has been suggested that distinct PML-RARα isoforms might bear distinct leukaemogenic properties that would result in a different progression of the disease. For example, a number of studies claim that *bcr3* variant of PML-RARα leads to a poorer prognosis as compare to the *bcr1* (Huang et al., 1993; Jurcic et al., 2001; Vahdat et al., 1994). Interestingly, *bcr3* PML-RARα lacks the PML NLS and this possibly correlates with a more pronounced cytoplasmic distribution (Figure 1.6.2A). Thus it can be hypothesized that cytoplasmic localization of this isoform might account for the increased aggressiveness of the leukaemia. It has been shown that when overexpressed in COS cells, *bcr1* PML-RARα also accumulated in the cytoplasm along with RXRα, the nuclear receptor partner of RARα. Thus, titration of key nuclear factors

implicated in myeloid differentiation has been proposed as one of the possible additional functions of PML-RARα (Perez et al., 1993).

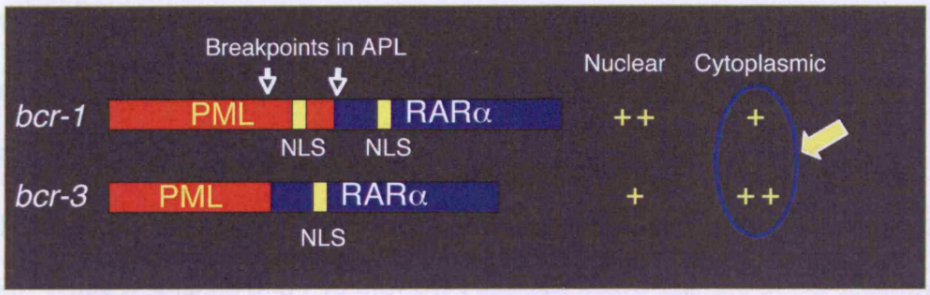


Figure 1.6.2A Schematic diagram of the 2 major PML-RARα isotypes in APL: *bcr-1* and *bcr-3*. Remarkably, *bcr-3* lacking PML NLS is predicted to have a more pronounced cytoplasmic distribution as compared to *bcr-1*.

Nevertheless, it has been demonstrated that when expressed at physiological levels, *bcr1* is mainly nuclear (Huang et al., 1993). Furthermore, direct evidence that clearly demonstrate cytoplasmic accumulation of PML-RARα *in vivo* is currently lacking. De The and colleagues showed that the majority of primary APL blasts displayed a PML cytoplasmic staining (Daniel et al., 1993). However, in this study the co-staining for RARα was not successful because of the low sensitivity of the anti-RARα antibody used. Therefore, it is still unclear whether the whole fusion protein localizes in the cytoplasmic of primary leukaemic cells. Interestingly, it has been shown that PML-RARα mRNA is subjected to alternative splicing resulting in short PML transcripts predicted to encode for cytoplasmic proteins (Pandolfi et al., 1992). Thus, it conceivable that multiple PML and PML-RARα isoforms could co-exist in the tumour cells and that these proteins have a cooperative effect in promoting leukaemogenesis. In this regard, it has been recently shown that PML-RARα is cleaved by neutrophil elastase (NE), an enzyme expressed at very high levels in promyelocytes. Specifically, NE proteolytic activity results in the cytoplasmic accumulation of the PML portion of the fusion protein. Intriguingly, the NE activity seems to be required for leukaemogenesis

(Lane and Ley, 2003). Nevertheless, it is not clear whether the cleavage products play a role in the progression of the disease. Notably, other protease expressed along with NE during myeloid differentiation in azurophil granules such as cathespin G (CG) and prolattin 3 (PR3) have been tested for the ability to cleave PML-RARα. Nonetheless, NE seems to be the main enzymatic activity responsible for PML-RARα cleavage in myeloid cells (Lane and Ley, 2003). In fact, despite the CG and PR3 were able to cleave PML-RARα they did not generate the same the cleavage pattern observed in APL cells. Thus, it is likely that CG and PR3 are not major players in the degradation of the fusion protein *in vivo.*

1.7 Aims of the project

Several lines of evidence suggest that PML could bear cytoplasmic functions beside to its nuclear ones. However, this intriguing possibility has remained mostly unexplored. Thus, the aim of this PhD project is to investigate the function/s of PML and PML-RARα cytoplasmic proteins.

To this end:

i- Cytoplasmic mutants of PML (Mut PML) (Gurrieri et al., 2004b) identified in RA-resistant APL patients, will be employed as a molecular tool to study the whether abnormal PML cytoplasmic accumulation results in deregulation of the cellular homeostasis, Thus, the effect of Mut PML expression on nuclear PML and PML-NB components will be tested. In this regard, p53 activity will be analyzed in the presence of Mut PML.

ii- Mut PML will be expressed in APL cells in order to assess whether it can contribute to PML-RARα repressive functions. To this purpose, the activity and the regulation of the fusion protein will be analyzed in the presence of Mut PML.

iii- To gain more insights into the cytoplasmic function of PML-RARα, the two major PML-RARα isotypes (*bcr1* and *bcr3*) will be analyzed *in vitro* and *in vivo*. Furthermore, a cytoplasmic PML-RARα mutant will be employed to study RA-mediated transcription and differentiation. Finally, the transforming potential of cytoplasmic PML-RARα will be tested in mouse primary haematopoietic

progenitor cells in order to gain a better view of its functions in more physiological settings.

Addressing these outstanding questions could contribute to clarify some of the controversies currently existing in the APL field and encourage new avenues of research for the development of new prognostic and therapeutic tools.

Chapter 2

Materials and methods

2.1 Cell culture

Cell culture procedures were carried out aseptically in a class II laminar flow cabinet. Cells were maintained in a Heraeus CO_2 Auto-Zero incubator at 37°C with 5% CO_2.

2.1.1 Suspension cell

Haematopoietic cell lines HL60, NB4 and U937 were cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 4.5 mg/ml glucose and L-glutamine. 32D cells were cultured in IMDM containing 10% foetal bovine serum 100 U/ml penicillin, 100 mg/ml streptomycin, 4.5 mg/ml glucose and L-glutamine. Cells were cultured at a density of 200 - 250 × 103 cells/ml and routinely passaged before confluence.

2.1.2 Adherent cells

Primary fibroblasts and established cell lines were cultured in D-MEM supplemented with 20 or 10% foetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin, 4.5 mg/ml glucose and L-glutamine. Cells were replated before they reached confluence, to maintain a logarithmic growth and routinely frozen in liquid nitrogen during culture to ensure stocks of all passages. Cells were washed with sterile phosphate buffer saline (PBS, Sigma) and detached with a solution of 10X trypsin/EDTA (Sigma). The activity of trypsin was stopped adding a double amount of culture medium. After centrifugation at 1200 rpm for 5 minutes, cells were resuspended in the appropriate medium, counted with a haemocytometer and seeded at the desired density. All reagents were purchased from GIBCO (Invitrogen) unless otherwise stated.

Cells	Derivation
BJ	Human primary fibroblasts
293T	Human embryonic kidney cells
MEFs	Mouse primary embryo fibroblasts
SAOS2	Human osteoblast-derived osteosarcoma (*p53*$^{-/-}$)
U2OS	Human osteosarcoma
H1299	Human lung carcinoma (*p53*$^{-/-}$)
HL60	Human promyelocytic leukaemia (*PML-RARα*$^{-}$ and *p53*$^{-/-}$)
U937	Human leukaemic monocyte lymphoma
NB4	Human promyelocytic leukaemia (*PML-RARα*$^{+}$)
32D	Mouse myeloid cells

Table 2.1 List of the primary and immortalized cell lines and their histological origins.

2.2 Cloning and plasmid generation

PML mutants (Gurrieri et al., 2004b) as well as ΔRING-Mut PML were generated by using PCR-based strategies, tagged at the amino-terminal with HA or Myc epitope and then subcloned into pCDNA3.0 (Invitrogen) and pBABE PURO (Morgenstern and Land, 1990). Briefly, a two step PCR was performed by using 2 external primers F1 and R2 and two internal primers R1 and F1 designed to partially overlap and to introduce the desired mutations in the target sequence (Figure 2.2). The first round of PCR was performed using the primer pairs F1-R1 and F2-R2 to generate amplicon 1 (A1) and 2 (A2). Amplicons were then run on a 1.5% agarose gel and purified by using QIAprep gel extraction kit (Qiagen) following the manufacturers instructions. The second PCR was performed using external primers F1-R2 to amplify the template DNA

consisting of a mixture of A1 and A2. The full cDNA encoding for the mutated protein was purified as described above and subcloned into the relevant expression vectors.

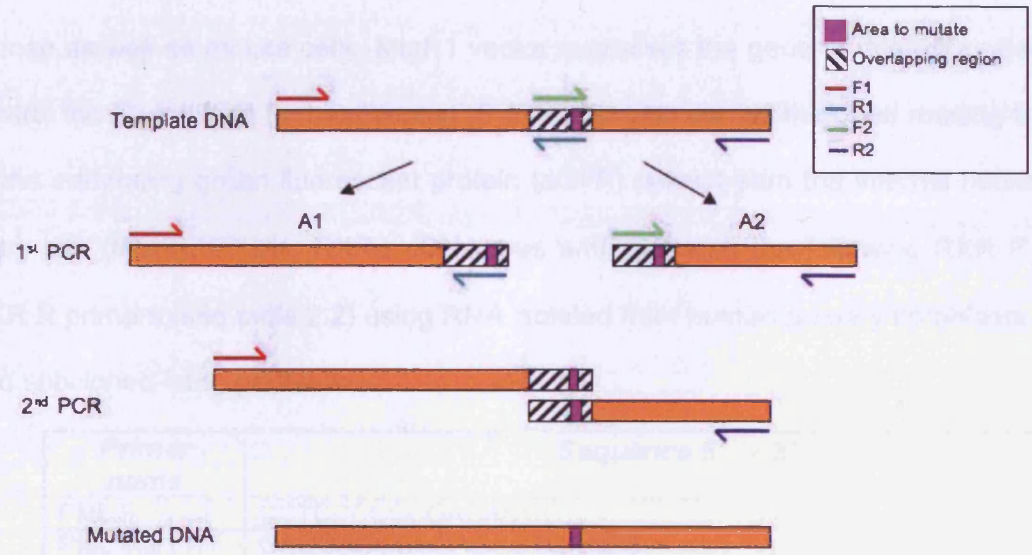


Figure 2.2 Schematic of the PCR strategy used to generate mutated versions of PML and PML-RARα.

PML-RARα *bcr1* (Alcalay et al., 1992) was cut from PINCO-PML-RARα (a kind gift of Dr. Saverio Minucci) as EcoRI fragment of approximately 3Kb and subcloned into pSG5, pcDNA3.0 and the retroviral vector pBABE PURO (Morgenstern and Land, 1990). When not specified, the PML-RARα form used in this study is *bcr1*. PML-RARα *bcr3* was generated by using PCR-based strategies as previously described (Sambrook and Russell, 2001), tagged at the amino terminus with HA or Myc epitopes, and sub-cloned into pcDNA3.0 (Invitrogen) and pBABE. QuikChange® site-directed mutagenesis kit (Stratagene) was used according to the manufacturers instructions to delete PML or both PML and RARα nuclear localization signals (NLS) of PML-RARα in order to generate PML-RARα- ΔNLS1 and - ΔNLS2 (thereafter referred to as Δ1 and Δ2). The Δ2 mutant (Δ2 M883R/T886R) lacking RXRα-binding domain described elsewhere (Zhu et al., 2005) was generated using standard PCR-based strategies

(Sambrook and Russell, 2001) and sub-cloned in pcDNA3.0 and pBABE PURO. Furthermore, cDNAs corresponding to Δ2 and PML-RARα were subcloned as EcoRI fragments into the retroviral expression vector MigR1, a mouse stem cells viral vector (MSCV), in order to produce highly efficient amphotropic retroviruses able to infect human as well as mouse cells. MigR1 vector expresses the gene of interest under the control the 5' viral long terminal repeat (5' LTR) but also carries the open reading frame of the enhancing green fluorescent protein (eGFP) downstream the internal ribosomal entry site (IRES). Finally, RXRα cDNA was amplified with the following RXR F and RXR R primers (see table 2.2) using RNA isolated from human primary fibroblasts (BJ) and subcloned into pcDNA3.0.

Primer name	***Sequence 5' → 3'***
PML F	CCTCCCCCCGAGACCC
PML Mut1 R1	GAGGAGGCAGAGAGTGAAGG
PML Mut1 F2	CCTTCACTCTCTGCCTCCTC
PML Mut1 R2	TGGAGAAGGCGTACACTGGCAC
PML Mut2 R1	TCTCTGCCTCCTCGGGCTTTCCCCTGGGTGATG
PML Mut2 F2	CATCACCCAGGGGAAAGCCCGAGGAGGCAGAGA
PML Mut2 R2	TTGATGGAGAAGGCGTACACTG
ΔRING R1	CGAAAAAGACGTTATCCGAAGCGGGGGCTC
ΔRING F2	CCCCCGCTTCGGATAACGTCTTTTTCGAGAGTC
ΔRING R2	TGGAGAAGGCGTACACTGGCAC
Δ2 ΔNLS PML F	ACAACGACAGCCCAGATGGAGTCTGAGGAG
Δ2ΔPML R	CTCCTCAGACTCCATCTGGGCTGTCGTTGT
Δ2 ΔNLS RAR F	GTGAGAAACGACCGAAACAGAAAGAGAAAGGAGGTGCCCAAGC
Δ2 ΔNLS RAR R	GCTTGGGCACCTCCTTTCTCTTTCTGTTTCGGTCGTTTCTCAC
Δ2 M873R/T873R Forward	CATGTTCCCCAAGATGCTAAGGAAGATTAGGGACCTGCGAAGCAT
Δ2 M873R/T873R Reverse	GCGCTGATGCTTCGCAGGTCCCTAATCTTCCTTAGCATCTTGGGG
RXR F	ATGGACACCAAACATTTCC
RXR R	CTAAGTCATTTGGTGCGGC

Table 2.2 List of the primers used for the cloning and preparation of the plasmids.

2.2.1 Preparation of Plasmid DNA from bacteria by miniprep and maxiprep

Chemically competent bacterial cells (*E. Coli* strain JM109) were transformed with approximately 50 ng of plasmid DNA by heat pulsing the cells for 45 seconds at 42°C. Cells were grew in an orbital shaker for 1 hour at 37°C and plated in Luria broth (LB) agar plates supplemented with the relevant antibiotic (ampicillin or kamamycin). Plates were incubated overnight at 37°C and the bacterial colonies screened by PCR in order to select only clones carrying the plasmid. Then, small culture of bacterial cells was grown in LB media overnight at 37°C in an orbital shaker. Plasmid DNA was purified from bacterial suspension using the miniprep system (Qiagen). The procedure, starting from 2 ml of bacterial suspension, was exactly as described by the manufacturer. The DNA was subjected to restriction digestion with appropriate restriction enzymes to verify that the presence of the right insert. Positive colonies were in turn used to inoculate larger bacterial preparations. Maxipreps were carried out culturing bacteria in 250ml of liquid LB supplemented with 50 mg/ml ampicillin (contained in a 500 ml conical glass tube). Cells were grown at 37°C in a shaking incubator at 220 rpm over night and plasmid DNA was purified with the Qiagen Plasmid Maxi Kit (Qiagen) according to the manufacturer's instructions. The DNA recovered from the maxi preparation was quantified using a spectrophotometer, by reading the absorbance at 260 nm and 280 nm. The quality of the DNA prepared was determined by the ratio between 260 nm and 280 nm absorbancies. Ratios between 1.8 – 1.9 are indicative of a highly pure preparations of double strand plasmid DNA.

2.3 Real-time quantitative PCR

Quantitative PCR was used to measure the transcriptional levels of mRNA expressed during myeloid differentiation. Total mRNA from 32D cells cultured in the presence or absence of 25 ng/ml G-CSF was prepared using the RNAeasy kit® (Qiagen) according to the manufacturers instructions. The concentration of RNA was determined using a spectrophotometer (Eppendorf) by reading the absorbance at 260 nm. For each sample, 2 μg of total RNA were retro-transcribed using the Retro-script™ (RT) kit (Ambion) according to the manufacturers instructions. Briefly, RNA was denatured at 95 °C for 5 minutes in the presence of random decamers and immediately incubated on ice. Subsequently, retro-transcriptase, RNAase inhibitors and dNTPs were added to the reaction mix. Retro-transcription was carried out at 23°C for 10 minutes followed by 42°C for 1 hour. Then, 1 μl of cDNA was used for the PCR. Quantitative real-time PCR (qPCR) was performed using Sybr GreenER™ qPCR master mix (Invitrogen) supplemented with 200 nM of forward and reverse primers (see table 2.3). qPCR reaction components are listed below:

1X Retrotranscription mix

RNA	2 μg
Decamers	200 nM
Retro transcriptase	5 units (1 μl)
RNAase inhibitor	10 units (1 μl)
dNTPs	200 mM

1X Real-time PCR reaction mix

Sybr Green qPCR mix	10 μl

Forward primer	200 nM
Reverse primer	200 nM
cDNA	1 μl
H_2O	up to 25 μl

In addition to the specific messengers, the levels of the GAPDH housekeeping gene, which is constitutively expressed in all samples, were analyzed in order to normalize the levels of mRNA expression in each sample. The efficiency of target and GAPDH PCRs was determined according to the Applied Biosystems User bulletin No.2 P/N 4303859 instructions, carrying out PCR amplifications on cDNA prepared from HeLa cells subjected to serial dilutions. In this regard, all transcripts analyzed displayed a similarly high efficiency (approximately 95%). The relative expression was determined using the $2^{-(\Delta\Delta Ct)}$ method as described elsewhere [(Livak and Schmittgen, 2001; Schmittgen, 2001) and Applied Biosystems User bulletin No.2 P/N 4303859]. Each reaction was carried out in triplicate and experiments were performed in triplicate.

Primer name	***Sequence 5' → 3'***
Lactoferrin Forward	CTATGCGGTAGCAGTCGTGA
Lactoferrin Reverse	CCAGGTGGCACTCCTTGTAT
ID1 Forward	GAGTCTGAAGTCGGGACCAC
ID1 Reverse	GAGAGGGTGAGGCTCTGTTG
ID2 Forward	CTCTTGGACGACATGAACCA
ID2 Reverse	ACAAGACACCTGGGCAAGAC

Table 2.3 List of the primers used for real-time qPCR

2.4 Protein Electrophoresis and Western Blotting

2.4.1 Principle of Protein Electrophoresis

Electrophoresis is the name given to the movement of charged particles in solution by applying an electrical field across the mixture. The speed at which the molecules move is dependant on their size, shape and charge making this a useful tool for the separation of large molecules such as proteins. Most proteins are separated using polyacrylamide gels. These cross-linked sponge-like structures act as size selective sieves such that molecules smaller than the gel pore size move more freely through the gel than those that are larger. Gel pore size is regulated by the concentration of polyacrylamide used where the higher the concentration the smaller the pore size. SDS polyacrylamide gel electrophoresis (SDS-PAGE) is the technique we chose to use as this allowed us to separate proteins on the basis of their molecular weight. Sodium dodecylsulfate (SDS) is an anionic detergent that us used to denature proteins. It does this by wrapping around the polypeptide backbone and conferring a net negative charge proportionally to the length.

2.4.2 Principle of Western Blotting

The transfer of proteins separated by SDS-PAGE from a gel to a membrane by electrophoretic elution is known as Western blotting. Nitrocellulose membranes are the more commonly used material. For a wet transfer the membrane is soaked in water and 2 pieces of 3MM paper are soaked in transfer buffer and then assembled. Following transfer the membrane is then incubated in blocking solution to block all non-specific binding sites and thus reduce any background. The blot is then probed with a protein-specific primary antibody followed by a secondary antibody specific for the general class of primary antibodies. Secondary antibodies are generally tagged with

either a peroxidase or alkaline phosphatase thus allowing immunoreactive bands to be detected by either colour development of enhanced chemiluminescecne (ECL) upon application of the appropriate enzyme substrates.

2.4.3 Principle of Enhanced Chemiluminescence (ECL) Detection

Chemiluminescence is the emission of light without heat as a result of a chemical reaction. One of most well characterised systems is the horseradish peroxidase (HRP)/hydrogen peroxide catalysed oxidation of luminol in alkaline conditions. The emission of light is due to the decay of excitation of the luminol. To enhance the light emission the oxidation reaction can be done in the presence of chemical enhancers such as phenols. This process is known as enhanced chemiluminescence (ECL).

2.4.4 Buffers and reagents

10X Buffer stock solution

TriZma Base (Sigma)	30g
Glycine (Sigma)	140g

Made up to 1L with distilled water.

1X Running buffer

Buffer stock solution	100ml
SDS 20%	5ml

Made up to 1L with distilled water

1X Transfer buffer

Buffer stock solution	100ml
Methanol (Sigma) 100%	200ml

Made up to 1L with distilled water

20% SDS

Powdered SDS (Sigma)	40gr
Distilled water	200ml

Phosphate Buffered Saline

1 tablet/100ml distilled water (Gibco)

PBS-Tween (0.1%) (PBS-T)

PBS	1 L
Tween-20 solution (Sigma) 20%	5ml

Membrane Blocking solution

PBS	
Tween-20	0.1%
Dry-powdered milk (Marvel)	5%

Primary/Secondary antibody solution

PBS	
Tween-20	0.1%
Dry-powdered milk (Marvel)	3%
Primary/ secondary antibody	

2.4.4 Protein Extraction

Total proteins were extracted from cells growing in culture by using the Ripa lysis buffer (see below). Cells were washed once with PBS, excess liquid was aspirated and pellets frozen in dry ice and stored at -80°C until needed. Frozen pellets were left to thaw on ice in lysis buffer for 10 minutes before mixing with a pipette. Non-frozen samples were mixed directly with the lysis buffer and then left on ice for 10 minutes before centrifuging at 13000 rpm for 5 minutes at +4°C. Total supernatant containing proteins was recovered and placed in a fresh Eppendorf tube (1.5 ml). An aliquot (1 μl) was used for determination of protein concentration as described in section 2.4.6 (see below).

Lysis Buffer	*Final Concentrations*
Triton X-100	0.5%
Tris 1M, pH 7.6	50 mM
NaCl	150 mM
Potease inhibitor cocktail 100X (Sigma)	1X
Sodium Fluoride	2.5 mM
Sodium Ortovenadate	10 mM

Make up to the desire final volume with distilled water.

2.4.6 Protein Concentration Measurement

Protein concentration in the cellular extract was determined according to the Bradford protocol. Firstly a stock solution of bovine serum albumin (BSA) of 2 mg/ml and dilutions ranging from 1 to 20 μg/ml) were prepared by adding the appropriate amount of stock solution directly to 1 ml aliquots of Bradford reagent (Sigma). The blank sample was Bradford reagent alone. The absorbance at 595 nm was measured in the

spectrophotometer and the calibration curve stored. The concentration of proteins in the samples was determined by adding 1 µl of protein extract to 1 ml of Bradford reagent and the absorbance read on a spectrophotometer (Eppendorf). Samples were processed as for the calibration curve and the protein concentration calculated.

2.4.7 SDS-PAGE

One fifth of the volume of sample buffer was added to the protein lysates and they were boiled at 95°C for 5 minutes.

Laemli 5X Sample buffer	*Final concentration*
SDS	10%
Tris pH6.8	312.5 mM
Glycerol (Sigma)	50%
β-mercaptoethanol (Sigma)	25%
Bromophenol-blue (Sigma)	0.025%

After a brief spin 60 µg of lysates were loaded in wells of a 5% stacking gel on an 8 or 12% polyacrylamide gel along with BenchMark™ Prestained Marker (Invitrogen) or precision plus dual colour protein standard (Bio-Rad).

Stacking gel	*Final concentration*
Acrylamide mix (Protogel, Gene Flow National Diagnostic)	5%
Tris pH6.8	0.13M
SDS	0.01%
APS	0.01%
TEMED	1000X

Distilled Water

8%Running Gel	*Final Concentration*
Acrylamide mix (Protogel, Gene Flow National Diagnostic)	8%
Tris pH8.8	0.4M
SDS	0.01%
APS	0.01%
TEMED	1600X
Distilled Water	

12%Running Gel	*Final Concentration*
Acrylamide mix (Protogel, Gene Flow National Diagnostic)	12%
Tris pH8.8	0.4M
SDS	0.01%
APS	0.01%
TEMED	2500X
Distilled Water	

Stacking and separating gels were prepared shortly before pouring. Ammonium persulphate (APS) catalyses polymerisation and TEMED accelerates the reaction and therefore these reagents are added last. Proteins were resolved on a minigel apparatus (Bio-Rad) and run at 40 V until the dye front entered the separating gel and then at 80 V until the dye reached the bottom of the gel.

2.4.8 Protein Transfer and Detection

The separated proteins were transferred onto nitro-cellulose (at 65 V for 2.5 hours) using Bio-Rad Mini transfer blot equipment in 1X transfer buffer. Blots were placed in a box with 5% non-fat dried milk dissolved in PBS-T 0.1% and shaken for 30 minutes at room temperature to block non-specific binding. Blots were exposed for either 1 hour at room temperature or over night at 4°C to the desired antibodies diluted to the optimal working solution. After incubation, the blots were washed three times for 5 minutes in PBS-T 0.1% and incubated with the appropriate horseradish-peroxidase linked anti-mouse or anti-rabbit IgG secondary antibody (Amersham) for 1 hour using appropriate dilutions. Blots were washed as previously described, and detection was performed with an enhanced chemiluminescent detection system (ECL, Amersham-Life Science). The blots were exposed to film for different times ranging from 10 seconds to 20 minutes and developed.

2.4.9 Antibodies used for western blotting

2.4.9.1 Primary Antibodies

Antibody	Host	Source	Concentration
PML	Rabbit	Chemicon	1:1000
RARα	Rabbit	Santa Cruz	1:400
RXRα	Rabbit	Santa Cruz	1:500
HA	Mouse	Sigma	1:5000
Myc tag	Mouse	Cell Signaling	1:200
Actin	Mouse	Sigma	1:5000
Tubulin	Mouse	Sigma	1:1000
Lamin A/C	Rabbit	Santa Cruz	1:1000
Ras	Rabbit	Cell Signaling	1:1000

2.4.9.2 Secondary Antibodies conjugated to HRP

Antibody	Host	Source	Concentration
Anti-mouse	Sheep	Amersham Bioscience	1:10,000
Anti-rabbit	Donkey	Amersham Bioscience	1:10,000

2.5 Immunoprecipitation

2.5.1 Principle of immunoprecipitation

Immunoprecipitation is one of the most used techniques to study specific protein-protein interactions. This method is based on the high affinity of the antibodies for their antigens. This feature is exploited to bind and isolate target proteins in solution. Once the antibody-antigen complexes are formed, agarose beads covalently coated with protein A and G that interacts with the constant portion of the antibodies are used to isolate them by centrifugation. Non-specific interactions can be reduced by serial washes of the bead. Finally, immunoprecipitates can be released from the antibody-beads complexes and resolved by SDS-PAGE.

2.5.2 Procedure

Cells were lysed in immunoprecipitation (IP) buffer (see the composition below). Briefly, for each IP half a milligram of lysates was used. Lysates were initially incubated with sepharose G and A beads (Amersham) coated with control mouse and rabbit IgG for 1 hr at 4°C in order to eliminate non-specific interactions. Then, pre-cleared protein

extracts were subjected to immunoprecipitation using beads coated with 1μg of anti-HA (IgG1 mouse monoclonal, Sigma) and anti-RARα (IgG rabbit polyclonal, SCBT) antibodies, respectively. The incubation was carried out for 3 hours at 4°C on a rotary shaker. Immunocomplexes were centrifuged at 3000 rpm for 5 min washed 5 times with 500 μl of IP buffer and re-suspended in SDS-sample buffer. Specific protein-protein interactions were analyzed by SDS-PAGE and western blotting as previously described.

IP buffer

Tris-Cl pH 7.6	50 mM,
NaCl	150 mM
Na_2VO_3,	2 mM
NaF	2.5 mM
Triton X-100	0.5%

Protease-inhibitor cocktail (Sigma) 100X

2.6 Immunocytochemistry

Immunocytochemistry is a method that has been developed to allow the identification of specific antigen in cells previously fixed on glass or plastic supports. The protein of interest is visualized using a combination of antibodies raised in different species. Primaries are generated against the protein of interest injecting mouse or rabbit whereas secondary are made in goat using the constant region of mouse (anti-mouse) or rabbit (anti-rabbit) primary antibodies. Thus, the primary antibody recognizes the epitote on the target protein and the secondary antibody binds the primary antibody and is conjugated with a fluorophore, thereby, the target protein can be visualized using a fluorescence microscope.

2.6.1 Buffers and Reagents

PBS

10% Goat Serum

Goat Serum (Sigma) 1ml

Made to 10ml with 1X PBS

4% Paraformaldehyde (PFA)

1X PBS 100ml

Paraformaldehyde 4g (Sigma)

2.6.2 Cell Preparation

Immunocytochemistry was performed on cells fixed with 4% PFA on glass coverslips. Briefly, cells were seeded on 22 mm x 22 mm glass coverslips that had been dipped in methanol and allowed to dry in the laminar flow cabinet, to ensure sterility. The dry coverslips were placed in 6 well dishes (NUNC) and covered with the appropriate medium. Cells were seeded at the appropriate density directly on the coverslips and allowed to attach for at least 24 hours.

2.6.2.1 Cytospin preparations of suspension cells

Immunofluorescence of suspension cells was carried using 50 to 80 $\times 10^3$ cells. Cells were resuspended in 150 μl of culture media supplemented with serum and loaded on a slide deposition chamber (see below) and immediately spun on a glass slide by using a Cytospin 4 (Thermo Shandon) centrifuge. Centrifugation was carried out at 350 rpm for 5 minutes. Then, slides were left to air-dry at room temperature for at least 30 minutes and subsequently stained using exactly the same protocol as for adherent cells (see below).

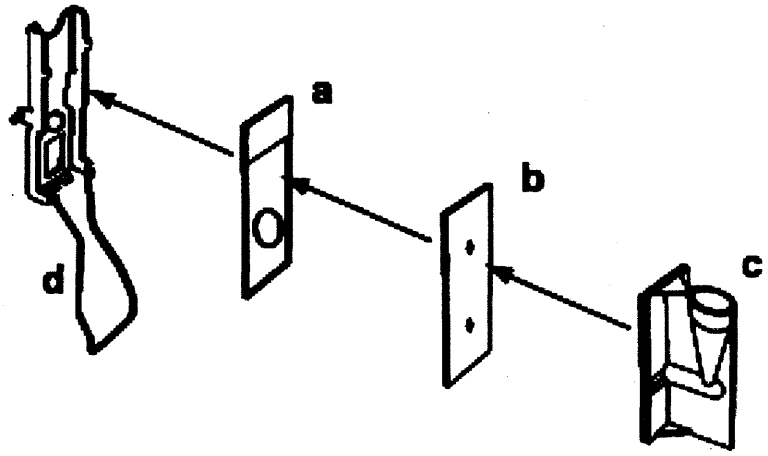


Figure 2.6.2A Assembly of the slide deposition chamber. Schematic diagram of the slide deposition chamber used to cytospin suspension cells on glass coverslips. In the figure: (a) glass slide, (b) filter card, (c) reusable sample chamber and (d) cytoclip side clip.

2.6.3 Procedure

Cells were fixed in 4% PFA for 10 minutes at room temperature and then permeabilised with ice-cold 0.1% Triton-X100 for 3 minutes. Once fixed and permeabilized, cells were blocked with a 10% goat serum-PBS solution and incubated with primary antibody for 1 hour and with secondary antibody for 1 hour at room temperature. All antibodies were diluted in 10% goat serum–PBS. Cells were then washed, counterstained with 49, 69-diamidino-2-phenylindole (DAPI), and mounted as using the ProLong™ Anti-fade kit (Molecular Probes). Primary antibody dilutions were as follows: Anti-human PML PG-M3 (Santa Cruz) 1:400, anti-human PML (Chemicon) 1:200, anti-p53 (DO-1) (Santa Cruz) 1:200, anti Daxx (Upstate) 1:400, anti-HA (Sigma) 1:1000, anti-Myc tag (Cell Signaling) 1:400, anti-FLAG (Sigma) 1:400, anti-CBP A22 (Santa Cruz) 1:200, anti-Sp100 (a kind gift of Dr. Thomas Hofmann), anti-RARα C20 (Santa Cruz) 1:400, anti-RXRα (Santa Cruz) 1:200, respectively. Alexa Fluor 488 and 568 conjugated secondary antibodies were purchased from Molecular Probes and

used at 1:1000. Slides were analyzed by confocal microscopy.

2.7 Immunohistochemistry (May-Grünwald/Giemsa)

Morphological analysis of haematopoietic cells was performed by standard May-Grunwal/Giemsa staining procedure as described by Keeshan and colleagues (Keeshan et al., 2003). In brief, cells were cytospun on poly-lysine coated microscope slides (Menzel) at 200 rpm for 5 minutes by using a cytocentrifuge (Shandon). Slides were left to air-dry for 30 minutes at room temperature, stained for 3 minutes with a May-Grunwal solution (Sigma) and rinsed with plenty of distilled water for 1 minute. Then, nuclei were counterstained for 30 minutes by using a Giemsa solution (Sigma) and rinsed exactly as abovementioned. Slides were analyzed using an Axiostar direct light microscope (Zeiss) and pictures of cells were acquired using a ProgRes C-14 RGB camera.

2.8 Determination of Senescence

2.8.1 Principle of determination of senescent cells

Normal cells possess a limited capacity to replicate and undergo to senescence, a state in which the cells are arrested and viable but display altered patterns of gene and protein expression. Senescent is a stable and metabolically active state accompanied by some characteristic morphological features. These include a flattened and enlarged appearance, expression of senescence-associated β-galactosidase (SA-βgal), and the appearance of senescence-associated heterochromatic foci. The activity of the SA-βgal can be histochemically detected and used a marker to monitor senescent cells.

2.8.2 Determination of oncogene-induced senescence in MEFs

Cellular senescence was studied as described (Serrano et al., 1997) with some modifications. In summary, early passage mouse embryo fibroblasts were plated on 6-well plate at 150 × 10^3 the day prior infection. Cells were subjected to 3 rounds of infections using high titer viral supernatants for pBABE, pBABE-H-Ras^{V12} and pBABE-HA-Mut PML. Double infections were carried out infecting cells firstly with H-Ras^{V12} and secondly with Mut PML. After that, cells were selected for 3-4 days with 2.5 μg/ml of puromycin (Sigma) and 200 × 10^3 cells were re-plated in 6-well plate. Cellular senescence was assayed one and two days later using the senescence β-galactosidase staining kit (Cell Signaling) to measure senescence associated (SA)-β-galactosidase activity according to the manufacturers instructions.

2.9 Transcriptional assays

2.9.1 Principle of transcriptional measurements

Reporter systems are used to measure the transcriptional activity, in particular to study the ability of *trans*-acting proteins to regulate promoters and enhancers in response to environmental changes. In these assays the target regulatory sequence, containing specific responsive elements (RE) motifs, is inserted upstream of a reporter gene (i.e. luciferase, CAT, β-galactosidase). As a matter of fact, the expression of the reporter directly mirrors the activity of the regulatory sequence of interest. The activity of luciferase, an enzyme absent in mammalian cells, which is encoded by the *luc* gene of the firefly (commonly derived from the North American firefly *Photonius pyralys*), represents an excellent reporter gene to use in promoter analysis. Firefly luciferase

catalyzes the oxidative decarboxylation of luciferin in the presence of ATP and magnesium (Mg^{2+}) to generate oxyluciferin and light:

Luciferin + ATP-Mg^{2+} + luciferase $\rightarrow$ luciferase:luciferyl-AMP + PP_i

luciferase:luciferyl-AMP + O_2 $\rightarrow$ luciferase + oxyluciferin + AMP + light (λ = 560 nm)

Luciferin is a generic term for substrates that generates light during oxidation catalyzed by luciferases. Pure luciferin was firstly isolated from fireflies, however, nowadays, is chemically synthesized. Light generated by the activity of luciferase can be captured, amplified and measured using a luminometer. The luminometer is an extremely sensitive instrument that can detect light produced by tiny amount of luciferase (up to 10^{-20} moles). The addition of ATP and luciferin to the extracts containing the firefly luciferase produces a flash of light that peaks 0.3 second later and lasts for a few seconds. Within one minute after mixing substrate and enzyme, the intensity of the emitted light falls by 10% and slowly decays over a period of several minutes (generally 5 – 10 minutes). Transfection controls are required when measuring the activity of specific regulatory sequences in order to compare different samples. More precisely, an internal control is needed in each sample to distinguish differences in levels of transcription from differences caused by the variability in the transfection efficiency or in the preparation of the extracts. To this purpose, cells are usually co-transfected with two reporter plasmids: one plasmid carries the regulatory sequence under investigation upstream of the *luc* gene and another plasmid that constitutively expresses a control activity that can be used to normalize the transcriptional levels amongst the samples. Control genes employed for dual reporter assay systems include the *E. coli* β-galactosidase and the luciferase from the sea pansy (*Renilla reniformis*), which utilizes a substrate different from the firefly and has different

biochemical properties. The expression of the β-galactosidase or the sea pansy luciferase is driven by constitutive eukaryotic promoters and can be assayed by measuring the light emission within the same aliquot of cell lysate initially used to assess the firefly luciferase activity.

2.9.2 Procedure

Generally cells were seeded 24 hours prior transfection in 12-well plates and cultured in DMEM supplemented with 10% FBS, Penicillin/Streptomycin and Glutamax. The transient transfection of the different expression vectors was carried out using the Calcium Phosphate transfection Kit (Invitrogen) according to the manufacturers instructions. Then, fresh medium containing different concentrations of ligand (All-Trans-Retinoic Acid, ATRA) was added to the cells 24 hours post-transfection. From 6 to 12 hours later, cells were harvested and 5 μl of protein extracts were assayed by using Dual Light System kit (Applied Biosystems) according to the manufacturers instructions. Slightly different protocols were used for each transcriptional assay as described below. For endogenous nuclear receptor reporter assays 9-10 $\times$ 10^4 COS-1 cells/well were seeded 24 hours prior to transfection in 12-well plates. Transient transfection was carried out as previously mentioned and the following expression vectors were used (100 ng unless otherwise stated): pCH110, 500 ng of luciferase reporter pRep$_4$-RARE-Luc, 1000 ng of pcDNA-HA-Mut PML along with empty vector. The effects of PML-RARα in combination with Mut PML were tested in COS-1 cells. For this purpose, cells were transfected with the following plasmids: pCH110, 300 ng of pRep$_4$-RARE-Luc and pcDNA-PML-RARα alone or in combination with 100 ng of pcDNA-HA-Mut PML. For p53 reporter assays, 8-10 $\times$ 10^4 SAOS-2 cells/well were seeded 24 hours prior to transfection in 12-well plates and transiently transfected with the following plasmids: pCH110, pcDNA3-p53, 500 ng pGADD45-Luc (p53 responsive

luciferase reporter), 500-1000 ng of pcDNA-HA-Mut PML along with empty vectors pcDNA3.0 and pUC19. The effects of nPML (nuclear isoforms 4) (kind gift of Dr. Keith Leppard) on p53-Mut PML transfected SAOS-2 cells were assayed by transfecting the above mentioned reporter plasmids in conjunction with 500 ng pcDNA-HA-Mut PML and different doses of pcDNA-FLAG-PML4 (500 ng). Finally, cells were harvested 36 hours after transfection and assayed for luciferase activity as described above. Furthermore, p53 activity was tested in HL60 cells infected with pBABE-HA-Mut PML or empty vector, respectively. After selection with puromycin, one pBABE and two Mut PML clones were isolated and used for transcriptional assays. Briefly, 1×10^6 cells were transfected using the Amaxa Nucleofector system (amaxa GmbH) according to the manufacturers instructions. The following amounts of vectors were used: 500 ng pcDNA3-p53, 2500 ng pGADD45-Luc along with 100 ng of the TK-Renilla plasmid (Promega). Luciferase activity was measured by using the dual luciferase assay system (Promega). To measure the transcriptional activity of PML-RARα NLS mutants (Δ1 and Δ2) the following expression vectors were used (100 ng unless otherwise stated): pCH110, 300 ng of pRep4-RARE-Luc together with 1 μg each of pcDNA-PML-RARα *bcr1*, Δ1 or Δ2. Fresh medium containing 10-7 M ligand (ATRA) was added 24 hours post-transfection. Luciferase activity was assessed 12 hours later using the Dual Light System kit (Applied Biosystems). Effects of Δ2 on dihydroxyvitamin D3 (VD3)-dependent transcription were investigated in transiently transduced COS1 cells by using 300 ng of vitamin D3 luciferase reporter (consisting of four DR3-type VD3-responsive elements inserted upstream of the tk-luciferase) (kind gift of Professor Carsten Carlberg), 600 ng of PML-RARα (*bcr-1*), Δ2 or empty vectors along with 100 ng of pCH110. Cells were cultured for 12 hours in presence or absence of 10^{-6} M of dihydroxyvitamin D3 (Calbiochem) and subsequently assayed for luciferase and galactosidase activities as previously mentioned.

2.10 Clonogenic assays (Colony Forming Assays)

Colony-forming assays are an insightful tool to test the long-term effect of a target gene on cell death and proliferation.

2.10.1 Procedure

Cells were seeded at clonal density in 10 cm dishes and transfected with the plasmid encoding the gene of interest using a calcium phosphate solution (Introgen) following the manufacturers instructions. Forty-eight hours after transfection the medium was replaced and cells were cultured for one week. The number of colonies formed was determined by using crystal violet staining. Crystal violet is a cationic dye that binds DNA and is soluble in ethanol. This dye allows visualization of cells that remain attached to the plate and therefore enables colony counting and cell viability to be determined. Plates were placed on ice and washed twice with ice-cold 1X PBS. Cells were then fixed with ice-cold methanol for 10 minutes. Methanol was aspirated from the plates, and 0.5% crystal violet solution (made in 25% methanol) was added to cover the bottom of the plate and incubated at room temperature for 10 minutes. The crystal violet solution was poured off and the plates were carefully rinsed in ddH_20 until no color came off in the rinse. Plates were left to air-dry at room temperature and colony formation was determined by eye. Images of the plates were acquired by using 8-bit CCD camera Syngene Bio Imaging System™.

2.10.2 p53 colony-forming assays

For p53 colony-forming assays, H1299 cells were plated at 300×10^5 in 6 cm dishes and transfected using Lipofectamine 2000 (Invitrogen) with pBABE PURO, pCDNA3.0 or pcDNA-Mdm2 or pCDNA-Mut PML1 in the presence and absence of pcDNA-p53. Cells were cultured for two weeks in the presence of puromycin, stained with crystal

violet and counted. Cell viability was evaluated using Trypan blue exclusion assay by using light microscopy.

2.11 Differentiation analysis of U937 cells

U937 cells were infected as previously described (Salomoni et al., 2005) using empty and Δ2 retroviral constructs, respectively. Infection was carried out using spinoculation (30 minutes at 2500 rpm). Cells were selected with 2.5 μg/ml puromycin and used either as clones or mixed populations. Differentiation was induced as described by Lin and colleagues (Lin and Evans, 2000), by culturing cells with 10^{-7} M ATRA. After four days, differentiation was assayed by flow cytometry analysis of differentiation-associated surface marker CD11b, using an anti-CD11b-FITC conjugated antibody (Caltag Laboratories). Flow cytometry analysis was performed with the help of my supervisor, Paolo Salomoni. Specifically, the percentage of CD11b positive cells was determined in samples from cells cultured in the presence or absence of RA green measuring the number of events on the side scatter (SSC) over the intensity of the green fluorescence (anti-CD11b antibody) acquired on the FL1 channel. Furthermore, differentiation was also evaluated by morphological analysis of U937 cells following haematoxylin-eosin and May-Grünwald/Giemnsa staining as described in section 2.7.

2.12 Transformation assay of primary haematopoietic progenitor cells

2.12.1 Principle of transformation assays

Primary haematopoietic progenitor cells possess the ability to originate different types of committed progenitors that give rise to all the subsets of blood cell lineages. Progenitors cells can be plated in semisolid media supplemented with growth factors promoting proliferation and, at the same time, inducing terminal differentiation.

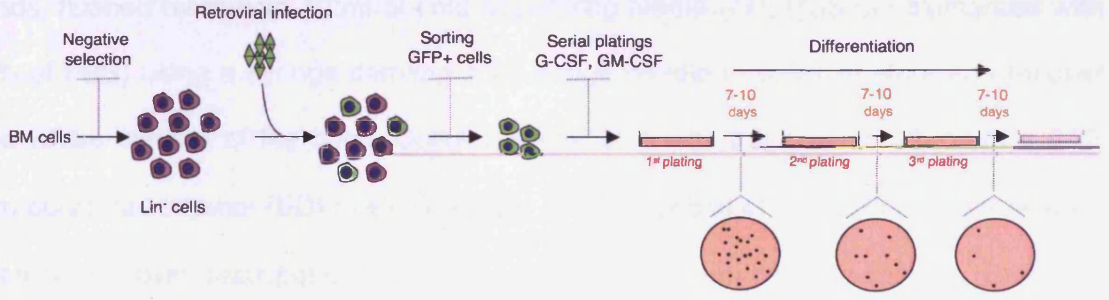


Figure 2.12A Overview of the transformation assay used. Briefly, bone marrow cells were isolated from the femurs of 6-8 wks old mice. Lin- cells were enriched by negative selection of differentiated cells. Subsequently, cells were infected with retroviruses encoding for empty vector, Δ2 or *bcr1* PML-RARα and sorted based on their ability to express the GFP. Sorted cells were used for the assay, plated in methylcellulose media in the presence of high concentration of G-CSF and GM-CSF to induced differentiation. Colonies generated were counted from 7-10 days later and cells were subjected to serial replating until no colonies were formed in control cells due to terminal differentiation.

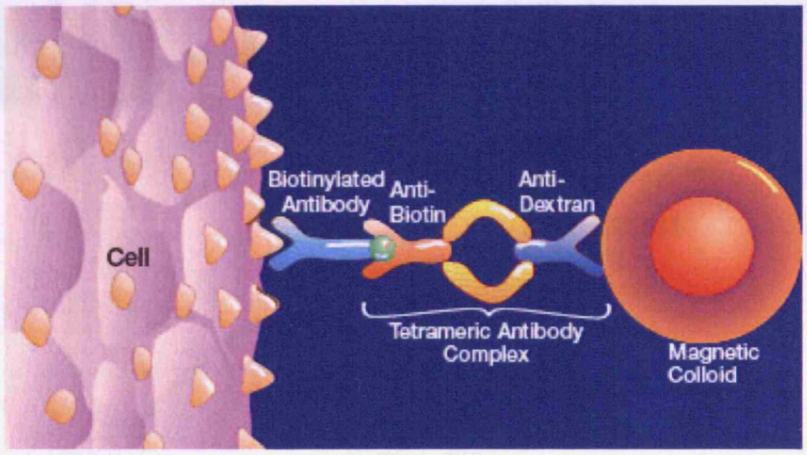
Then, cells plated in methylcellulose give rise to colonies consisting of a specific type of differentiated cells including granulocytes, monocytes, macrophages and erythrocytes. Cells can then be re-plated until no colonies can be originated due to terminal differentiation. The number of colonies at each plating directly mirrors the capability of the cells to proliferate and to differentiate. This assay represents an ideal *ex vivo* model to assess whether the expression of a certain protein in the progenitor

lineage alters normal haematopoiesis. However, transduction of myeloid progenitors is a complex process that requires the use of a sophisticated gene delivery system. In this regard, efficient transfection of myeloid progenitor can be achieved by using high-titer retroviruses followed by selection of transduced cells that can then be plated for the assay (Figure 2.12A).

2.12.2 Isolation of lineage minus (Lin^-) cells

Bone marrow was prepared from the tibias and femurs of 8 to 10 weeks old C57BL6 mice. Briefly, animals were sacrificed and rear limbs were surgically removed and bones isolated. Femurs and tibias were punctured with a 25-gauge needle at both ends, flushed twice with 10 ml of cold separating media (SP, PBS supplemented with 2% of FBS) using a syringe carrying a 27-gauge needle in order to elute and recover the whole content of the bone. Cellular suspension was then passed through a 0.22 μm pores cell strainer (BD) to eliminate the residual debris of bone. Bone marrow cells were spun down, resuspended in 2 ml of a cold ammonium chloride solution (StemCell Technologies) to lyse the erythrocytes, washed twice with cold SP buffer and counted using a haemocytometer chamber. An average of 40 - 50 million bone marrow cells were recovered from each animal. Isolation of lin^- cells was performed by using the StemSep mouse haematopoietic progenitor cell enrichment kit™ (StemCell Technologies) following the manufacturers instructions. Briefly, cells were resuspended in 500 μl of cold SP supplemented with 1/20 of rat serum to prevent non-specific binding of rat antibodies to mouse cells and incubated for 10 minutes at +4 °C. Then, cell suspension was labelled with a cocktail containing a combination of biotinylated monoclonal antibodies purified from rat ascites fluid or hybridoma culture supernatant. Antibodies are directed against differentiation-induced cell surface antigens on mouse haematopoietic cells (CD5 (Ly-1), CD11b (Mac-1), CD45R/B220, Ly-6G/C (Gr-1), Neutrophils (4-7), TER119). Subsequently, cells were incubated exactly as described

above and resuspended in 500 μl of cold SP containing 50 μl of a bispecific tetrameric complex (TAC) solution consisting of anti-biotin and anti-dextran antibodies linked together. Then, cells were transferred at +4 ºC for 15 minutes. Lastly cells were linked to magnetic dextran iron particles, which are recognized by the TAC, adding 30 μl of a solution of magnetic colloid and incubated for 15 minutes as described above (Figure 2.12.2A).

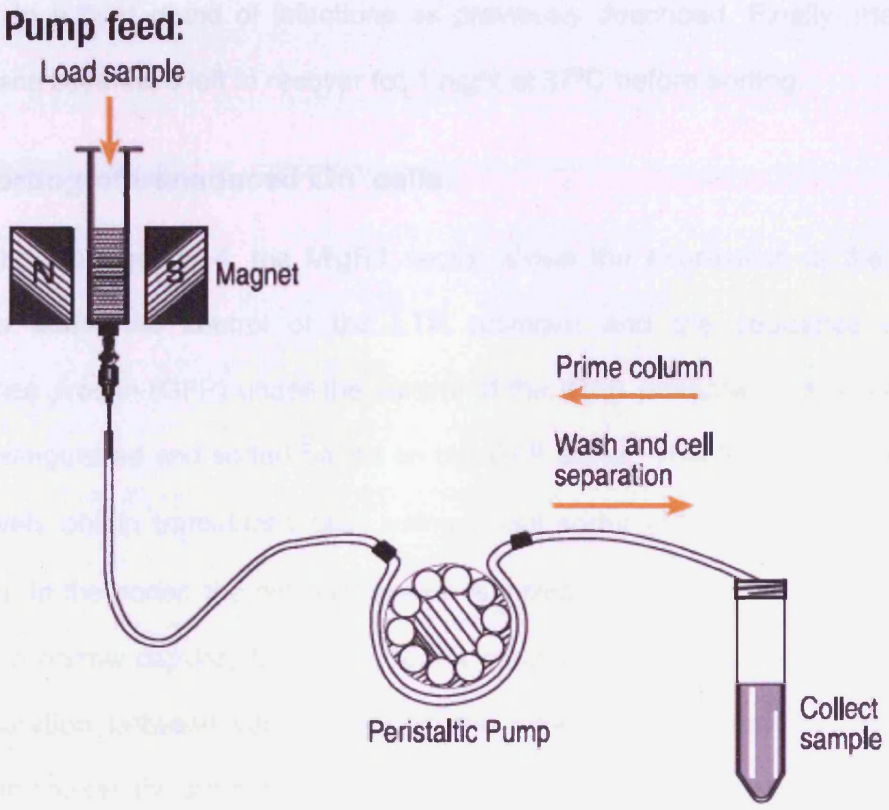


From StemSep product information sheet

Figure 2.12.2A Schematic drawing of the StemSep TAC magnetic labelling of mouse cells.

Negative selection of lineage minus cells was performed passing the cellular suspension through a 0.3" StemSep negative selection column placed on a StemSep® Magnet™ with the strength of 0.5 Tesla and fed by a peristaltic pump MINIPULS 3™ (Gilson) (Figure 2.12.2B). Columns were initially primed from the bottom with 1 ml of PBS and washed with 8 ml of cold SP. Cell suspension was loaded on the column washed from the top down 8 ml of SP. The flowthrough including the sample volume and the washes was collected in 15 ml tubes. Cells were counted and then resuspended in StemSpan SFEM media™ (StemCell Technologies). An average of 700×10^3 up to 1×10^6 lin^- cells were obtained from each mouse. Finally, cells were

cultured overnight in StemSpan SFEM media supplemented with 0.6 ng/ml interleukin-3 (IL-3), 0.4 ng/ml IL-6, 2.5 ng/ml Stem cells factor (SCF) and 5 ng/ml of Flt3 ligand (Flt3L). All cytokines were purchased from Peproteck.



From StemSep product information sheet

Figure 2.12.2B Schematic drawing of Lin$^-$ cells negative selection by using through magnetic column attached to a peristaltic pump

2.12.3 Retroviral infection of lin$^-$ cells

Isolated lin$^-$ cells were transduced by retroviral infections as aforementioned with some modifications. In brief, 500 x 10^3 cells were used, resuspended in a solution containing a mixture of 500 μl of retroviral supernatant (empty vector, PMLRARα or Δ2) and 500 ml of Stem Span Sfem media (StemCell Technologies) supplemented with 0.6 ng/ml IL-3, 0.4 ng/ml IL-6, 2.5 ng/ml Stem cells factor (SCF), 5 ng/ml Flt3L and 2 mg/ml of

polybrene. Cells were transferred to a 6-well plate, spun at 2000 rpm for 1 hour at 42°C and maintained for 3 hours in cell incubator at 42°C. Then, infections were repeated exactly as described above and cells were cultured overnight in Stem Span Sfem media containing a full cocktail of cytokines (see above). The following day, cells were subjected to a third round of infections as previously described. Finally, media was replaced and cells were left to recover for 1 night at 37°C before sorting.

2.12.4 Sorting of transduced Lin$^-$ cells

As described in Chapter 4, the MigR1 vector allows the expression of the gene of interest is under the control of the LTR promoter and the sequence of green fluorescence protein (GFP) under the control of the IRES promoter. Transduced cells can be distinguished and sorted based on the GFP signal. This feature was exploited to selectively obtain transduced cells using a cell sorter (FACS Vantage™, Becton Dickinson). In the sorter, the cell suspension is mixed with a rapidly flowing stream of liquid into a narrow capillary tube. The flow is controlled in such a way that there is a large separation between cells based on their diameter. Furthermore, a vibrating mechanism causes the stream of cells to break into individual droplets so that one cell is confined in each droplet. Just before the stream breaks into droplets the flow passes through a fluorescence measuring station where the fluorescent of each cell is acquired. A charged ring is placed just at the point where the stream breaks into droplets and an electrostatic charge is applied to each droplet based on the fluorescent intensity. Then, charged droplets pass through an electrostatic deflection system that diverts droplets into containers depending on their charge. Finally, sorted cells were incubated at + 4 °C for 30 minutes, centrifuged, resuspended in 1 ml of StemSpan SFEM media and counted.

2.12.5 Plating methylcellulose and count of colonies

A total of 30×10^3 cells was used to perform serial replating assays in methylcellulose as described elsewhere (Minucci et al., 2002). Briefly, cells were resuspended in 600 µl of StemSpan SFEM media containing 10 ng/ml IL-3, 10 ng/ml IL-6, 10 ng/ml granulocytic-macrophage colony stimulating factor (GM-CSF), 100 ng/ml SCF and 30 ng/ml granulocytic colony stimulating factor (G-CSF). Sample volume was brought to 3 ml by adding 2.4 ml of methylcellulose Methocult™ H4233 media (StemCell technologies). A homogenous suspension was achieved by mixing the cells with the methylcellulose avoiding the formation of air bubbles. Then 1 ml of the cells/methylcellulose mixture was distributed on a 3 cm tissue culture plate (Grainer) and evenly dispersed throughout the plate surface. Subsequently, dishes were incubated for 8-10 days at 37 ºC in a tissue culture incubator. Finally, the number of colonies formed in each plate was assessed using an inverted light microscope (Zeiss). After that, the methylcellulose was dissolved using warm IMDM media. Cells were counted and replated in fresh methylcellulose exactly as previously described. Serial replating of the cells was performed until no colonies were formed in the vector (control)-plates due to terminal differentiation and exhaustion of progenitor cells.

2.13 Stastical analysis

Statistical analysis was performed using Prism 5 for Macintosh (Graph Pad Software Inc, San Diego USA). Unless otherwise stated, experiments were carried out in triplicate and mean ± standard deviation was calculated. Statistical analysis was calculated using t-test and P values <0.05 were considered statistically significant. Unless otherwise stated values reported on graphs are mean ± standard error of the mean (SEM).

Chapter 3

An APL-associated mutant of PML inhibits p53 functions

3.1 Introduction

Reduction of *PML* gene dosage in an animal model of APL causes an increase in the incidence and shortening of the disease onset, thus supporting the hypothesis that PML acts as a tumour suppressor. In some human cancers such as in hepatocellular and skin carcinomas, PML is aberrantly found in the cytoplasm. Interestingly, it has been proposed that the relocalization of PML outside the nucleus could be accompanied by the gain of abnormal oncogenic functions (Condemine et al., 2006; Terris et al., 1995). Furthermore, PML mutations leading to an aberrant short cytoplasmic proteins have been described in murine plasmacytoma cells and in APL (Bruno et al., 2003; Gurrieri et al., 2004b). Specifically, two mismatch mutations in the remaining *PML* allele have been reported in RA-resistant APL patients that experienced a very aggressive form of the disease (Gurrieri et al., 2004b). The mutations are a deletion 1272delAG (Mut1), and a splice site mutation IVSG→A (Mut2) both leading to short PML proteins that accumulate in the cytoplasm (Gurrieri et al., 2004b). As role(s) of these APL-associated cytoplasmic mutants of PML has not been investigated, in this chapter we will analyze the function of Mut1 (**Mut PML**) and Mut2. Remarkably, the majority of the experiment will be performed using Mut1, herein after referred to as Mut PML for the following two reasons: i- Mut1 shows a more pronounced cytoplasmic distribution as compared to Mut2 (see below) and, ii- the original genomic lesion generating Mut1 was shown to be present in the patient before starting the therapy whereas no such information are available for Mut2. In particular, Mut PML will be employed as a tool to investigate the relationship between cytoplasmic PML, nuclear PML (PML4, in this thesis referred to as **nPML**) and p53. The modulation of p53 activity mediated by nPML within the PML-NB has been shown to have a crucial role in protecting the cells from neoplastic transformation. Thus, it is important to understand whether PML cytoplasmic proteins could promote tumorigenesis through

the inhibition of fundamental nuclear and/or cytoplasmic cellular tumour suppressor pathways. Remarkably, we found that Mut PML is able to inhibit the transcriptional, growth suppressive, and apoptotic functions of p53, through a mechanism that involves the cytoplasmic relocation of nuclear PML, an event that also lead to disruption of the PML-NB structure. Taken together, these findings shed new light on the possible functions of cytoplasmic PML proteins in disease.

3.2 Results

3.2.1 Mutant PML proteins accumulates in cytoplasmic bodies

We initially analyzed the cellular distribution of Mut1 and Mut2 in human fibroblasts. In line with previous data, confocal analysis confirmed that both mutants accumulated in discrete cytoplasmic foci, which we referred to as PML cytoplasmic bodies (PML-CB) (Gurrieri et al., 2004b).

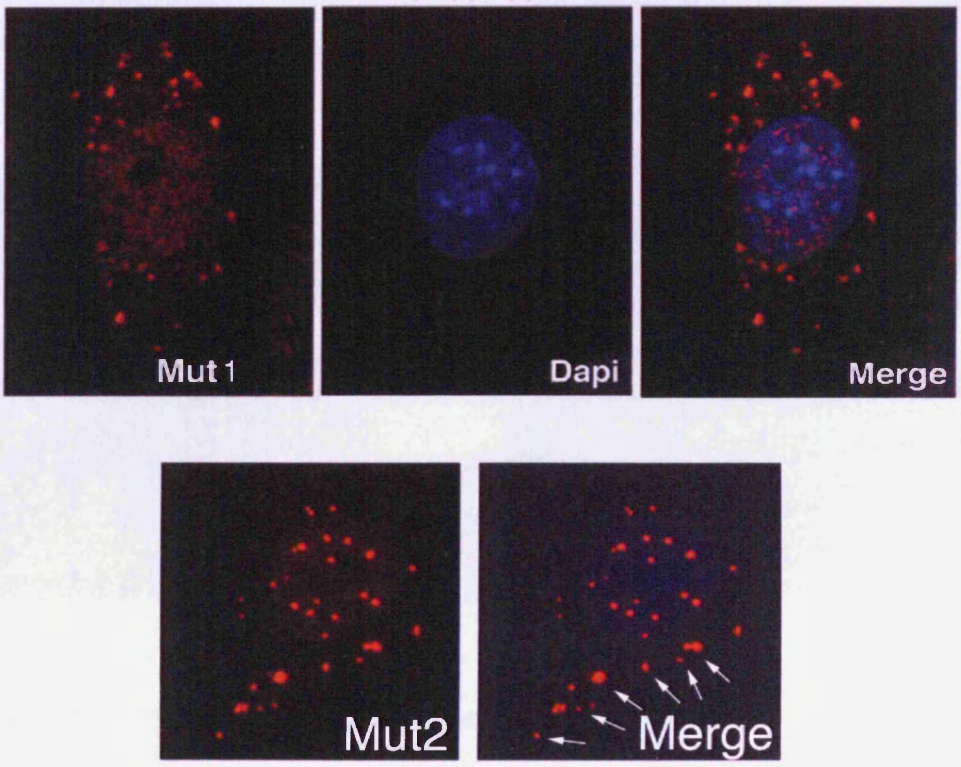


Figure 3.2.1A Mut PML forms PML-CB. Human primary fibroblasts (BJ) were infected with HA-tagged Mut1 or Myc-tagged Mut2 retroviruses. Cells were stained with anti-HA (Mut1, red top panel) and anti-Myc tag (Mut2, red lower panel) antibody. Nuclei were counterstained with DAPI (blue).

Immunofluorescence also revealed that Mut2 clearly accumulated also in PML-NB (Figure 3.2.1A top Mut1 and bottom Mut2) possibly as a consequence of homo-

oligomerization with nPML. Thus, we decided to use Mut1, **Mut PML**, to use a tool to study the cytoplasmic function of PML. As stated in the introduction, Mut1 has a more pronounced cytoplasmic localization than Mut2 and Mut1 mutation was present in the leukaemic blasts during the pre-treatment phase, thus excluding the possibility that it is a secondary event selected by RA treatment (Gurrieri et al., 2004b). Conversely, no pre-treatment sample was available for the mutation corresponding to Mut2 (Gurrieri et al., 2004b). Electron microscopy studies were conducted to better assess the structure of PML-CB using antibodies against PML or hemagglutinin (HA). Interestingly, at the ultrastructural level mutant PML formed doughnut-shaped structures, which were reminiscent of PML-NB (Figure 3.2.1B).

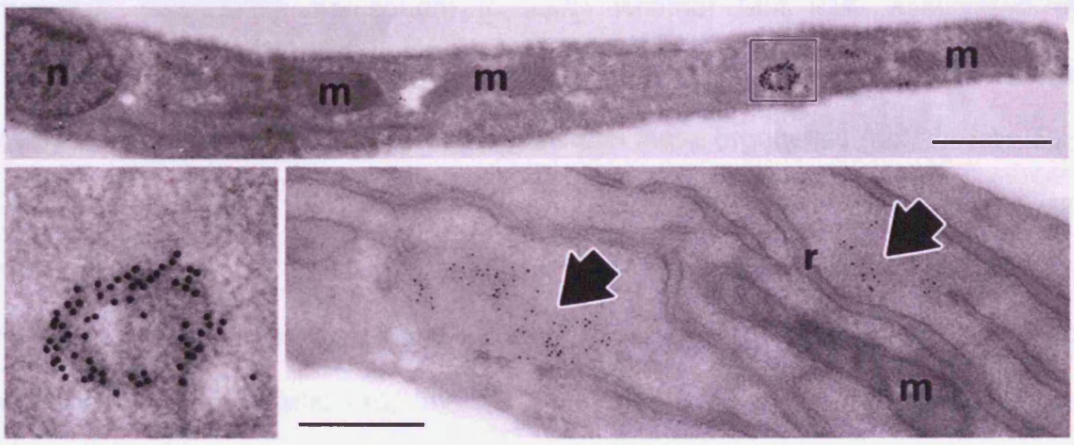


Figure 3.2.1B Mut PML forms doughnut-shaped PML-CB. Immunogold labelling of PML in a PML-CB within the cytoplasm of a cell transduced with Mut PML (top panel bar = 1 μm, right lower panel bar = 10 nm). Detail of a PML-CB within the cytoplasm (bottom left). *m* indicates mitochondria; *r* is endoplasmic reticulum and *n* is nucleus. Black arrows (bottom right) image indicate Mut PML distribution in the cytoplasm. These images were kindly acquired by Dr. Dinsdale, MRC Toxicology Unit.

Furthermore, sub-cellular fractionations showed that a portion of Mut PML accumulated in the insoluble part of the cytoplasm (P100), suggesting a possible association with intracellular membranes (Figure 3.2.1C).

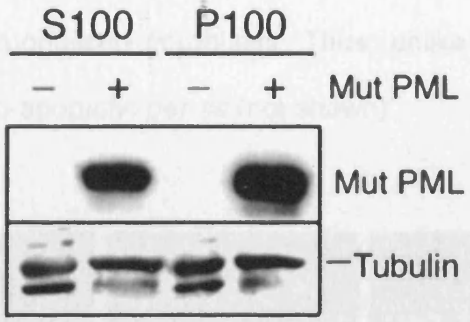


Figure 3.2.1C Mut PML accumulates in both the soluble and insoluble fraction of the cytoplasm. The pellet P100 and supernatant S100 obtained from control and Mut PML infected fibroblasts. Mut PML was detected by using anti-HA antibody (upper panel). Lower panel: tubulin was used as a loading control for the different fractions.

We next studied Mut PML localization in relation to the different cytoplasmic organelles. Specifically, we sought to study whether Mut PML associated with mitochondria, lysosomes or endoplasmic reticulum using appropriate markers. However, we could not find any colocalization with these organelles (not shown). Since it has been reported that a PML cytoplasmic isoform can associate with early endosomes (Lin et al., 2004), we tested whether this also applied to Mut PML. To prove this, confocal analysis was performed on both fibroblasts and Mut PML-expressing haematopoietic cells by using a specific marker for early endosomes (EEA1). Nevertheless, we were unable to demonstrate any significant colocalization. Accordingly, a detailed analysis of the ultra-structural images confirmed that Mut PML did not associate with any cytoplasmic organelles (3.1B bottom left panel).

3.2.2 Mutant PML alters PML nuclear body composition

To date it remains to be clarified whether Mut PML contribute to tumorigenesis. For this reason, we decided to use Mut PML to study PML cytoplasmic functions in both physiological and pathological contexts. Mut PML expression did not cause any

substantial alteration of cell death or proliferation at steady state in haematopoietic cell lines or primary and immortalized fibroblasts. Thus, unlike nPML, Mut PML is not growth-suppressive or pro-apoptotic *per se* (not shown).

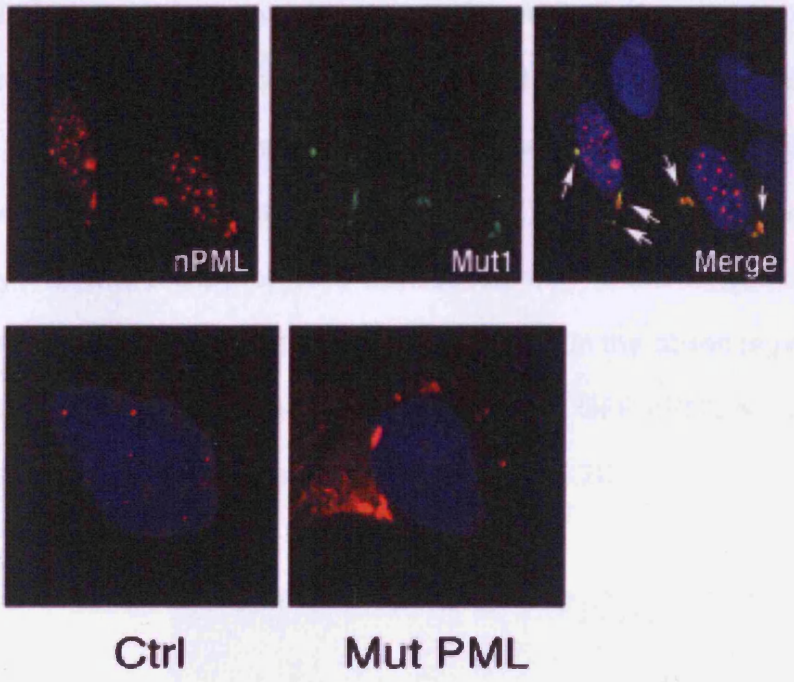


Figure 3.2.2A Mut PML colocalized with nuclear PML in PML-CB. Upper panels show exogenous nPML and Mut PML: SAOS-2 cells were transduced with FLAG-tagged nPML (PML4) and HA-tagged Mut PML. Cells were stained with anti-FLAG (red) and anti-HA (green) antibodies, respectively. Lower panels show endogenous PML: cells were transfected with empty or Mut PML expression vectors and stained with anti-PML antibody (red), which specifically recognizes the C-terminal portion of PML not included in Mut PML. Nuclei were counterstained with DAPI.

Thus, we set out to determine whether Mut PML expression had any effects on PML-NBs and the distribution of nuclear bodies components. In this regard, electron microscopy demonstrated a close structural homology between PML nuclear and cytoplasmic bodies, thus suggesting that at least some components could be shared between the two structures. Interestingly, fluorescence microscopy performed on primary fibroblasts (BJ) stably transduced with Mut PML, showed that the number of PML-NBs was reduced (not shown). Accordingly, a similar effect was reported in cells

expressing a nPML cytoplasmic mutant lacking the NLS (Le et al., 1996). As a matter of fact, it is conceivable that the localization of nuclear body components might be altered in the presence of Mut PML. The distribution of different nuclear body constituents was monitored in Mut PML-expressing cells by confocal microscopy. The first PML-NB component tested was nuclear PML, which is essential for the assembly and function of PML nuclear bodies (Salomoni and Pandolfi, 2002). Remarkably, in transduced SAOS-2 cells a fraction of both endogenous and exogenous nPML colocalized with Mut PML in PML-CB (Figure 3.2.2A). These initial observations were confirmed by live microscopy of cells transduced with nPML tagged at its amino-terminal with the green fluorescent protein (GFP-nPML) in the absence or presence of Mut PML. Remarkably, these experiments revealed that GFP-nPML accumulated only in the cytoplasm of Mut PML-expressing cells (Figure 3.2.2B).

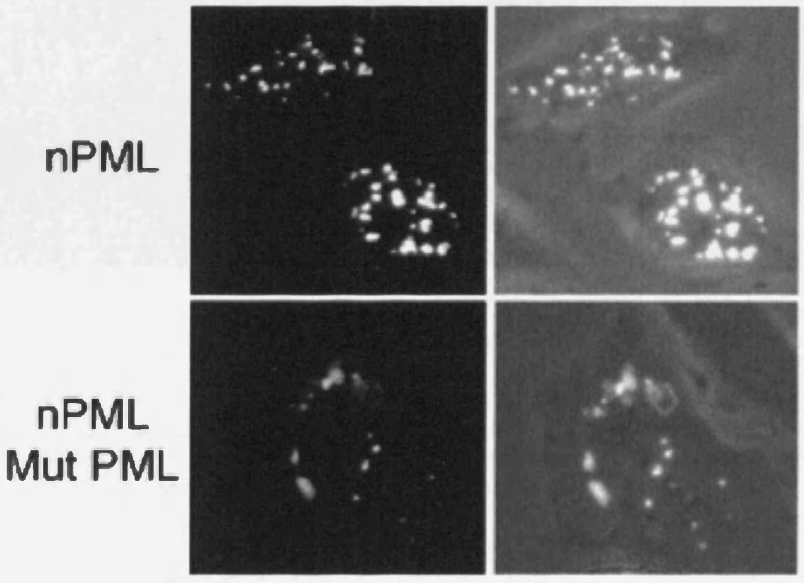


Figure 3.2.2B Mut PML induces cytoplasmic relocation of nPML in live cells. SAOS2 cells were transfected with GFP-nPML alone or in combination with Mut PML. Cells were analyzed by live microscopy.

Furthermore, in haematopoietic HL60 cells, Mut PML caused an even more drastic reduction in the number of PML-NB compared to SAOS-2 cells (Figure 3.2.2C). Notably, in a small portion of Mut PML-expressing cells, nPML was completely relocated to the cytoplasm (Figure 3.2.2C). The acetyltransferase CBP, another important nuclear body component, which was localized almost exclusively to the nucleus in control cells, was clearly found also in PML-CBs in Mut PML-expressing fibroblasts (not shown) and haematopoietic HL60 and U937 cells (Figure 3.2.2C).

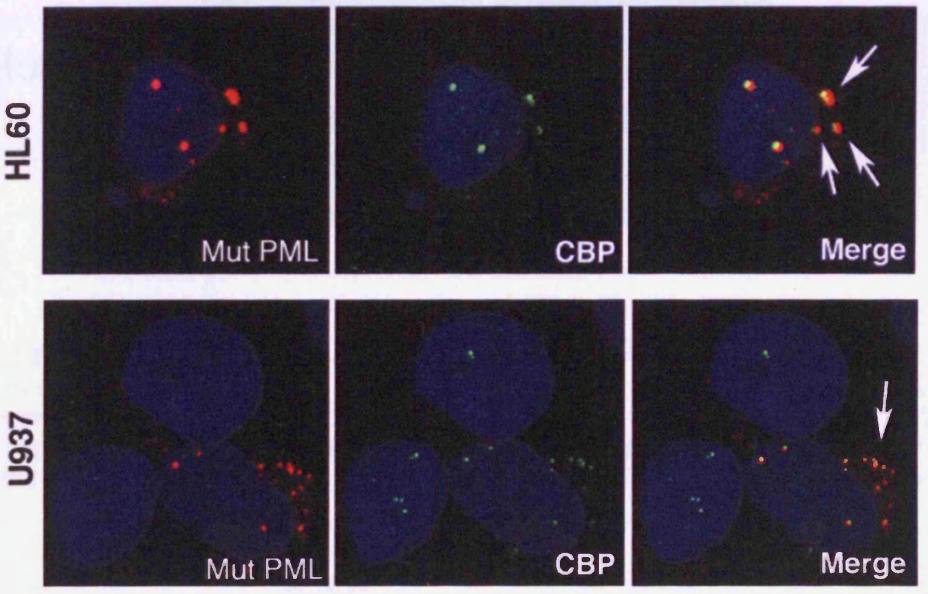


Figure 3.2.2C Mut PML induces relocation of the acetyltransferase CBP in to PML-CB. Hematopoietic HL60 and U937 cells were infected with Mut PML retroviruses and stained with anti-HA (red) and anti-CBP (green) antibodies. Nuclei were counterstained with DAPI. White arrows indicate Mut PML and CBP cytoplasmic colocalization (yellow).

Accordingly, the number of PML-NB displaying CBP was significantly reduced in Mut PML compared to vector cells. In order to prove the specificity of CBP staining, immunofluorescence analysis of cells expressing Mut PML was carried out after pre-incubating the anti-CBP antibody with either specific or unrelated CBP peptides (3.2.2D).

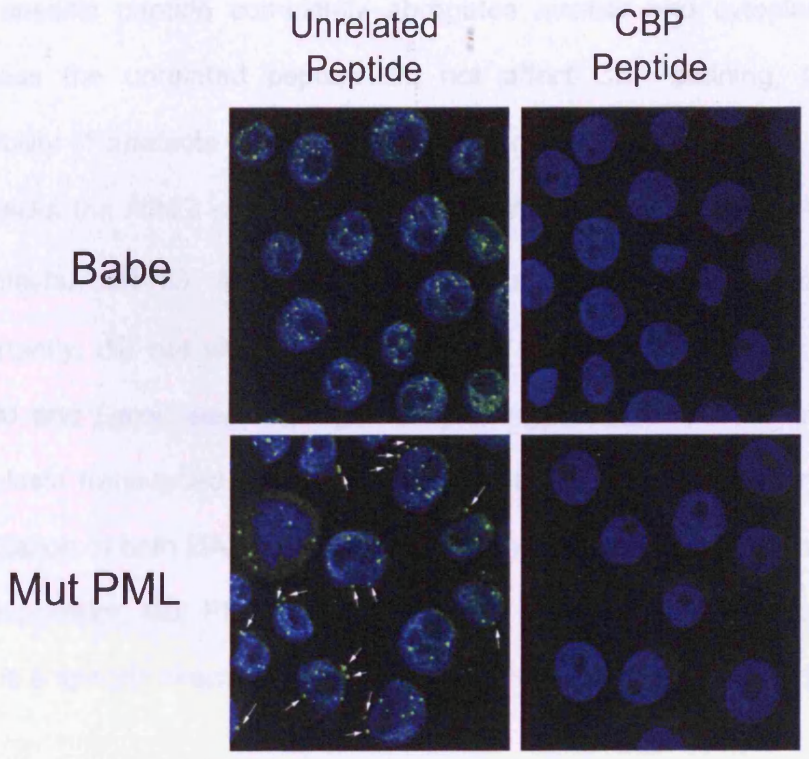


Figure 3.2.2D Specificity of Mut PML-dependent cytoplasmic relocation of CBP Haematopoietic HL60 were infected with control or Mut PML retroviruses were stained with an anti-CBP antibody (green) pre-incubated with or without an excess of unrelated (left panels) and CBP-specific (right panels) peptides. Nuclei were counterstained with DAPI. White arrows indicate the presence of cytoplasmic CBP localization in Mut PML expressing cells only.

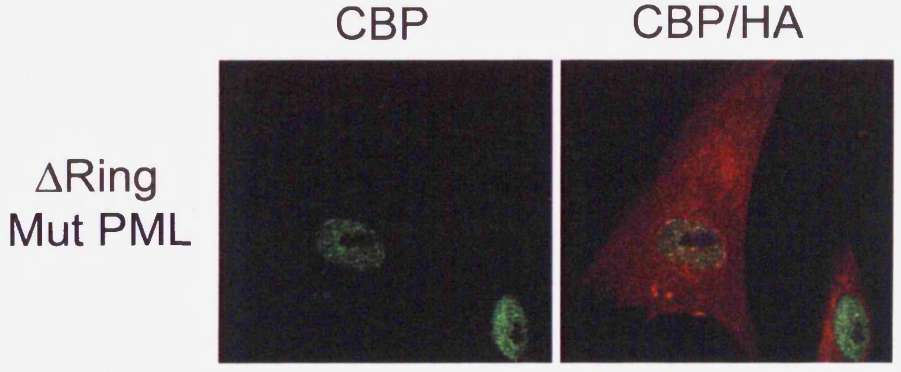


Figure 3.2.2E A ΔRING deletion mutant of Mut PML does not form PML-CB and fails to relocate CBP. Human primary fibroblasts were infected with ΔRING-Mut PML retroviruses and stained with anti-CBP (green) and anti-HA (red) antibodies.

The specific peptide completely abrogates nuclear and cytoplasmic CBP signals, whereas the unrelated peptide did not affect CBP staining, thus excluding the possibility of artefacts due to non-specific binding. Furthermore, a variant of Mut PML that lacks the RING domain (ΔRING Mut PML) was generated. When transduced in fibroblasts, ΔRING Mut PML showed a diffuse cytoplasmic distribution and, importantly, did not alter the localization of CBP (3.2.2E). Finally, the localization of Sp100 and Daxx, was investigated by using relevant specific antibodies in human fibroblasts transduced with Mut PML. Nonetheless, immunostaining showed that the localization of both DAXX and Sp100, known markers of PML-NB, was not affected by the expression Mut PML, thus proving that the cytoplasmic relocation of nPML and CBP is a specific event mediated by the mutant protein (Figure 3.2.2F).

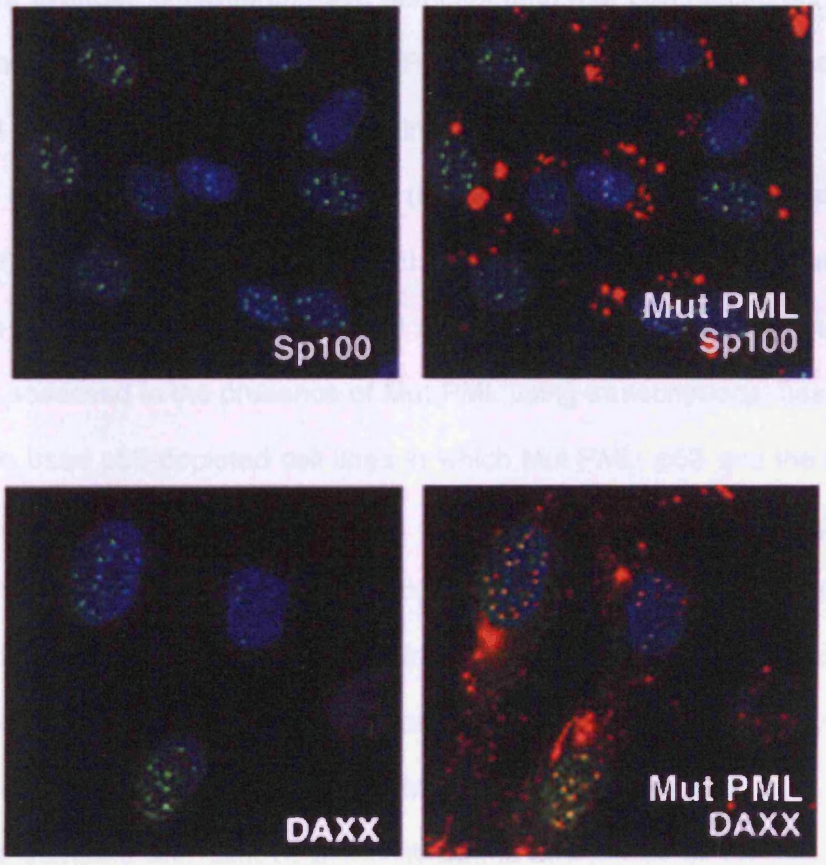


Figure 3.2.2F Mut PML does not relocate Sp100 and DAXX to PML-CB. Confocal images of human primary fibroblasts infected with HA-Mut PML and stained with anti-HA (red), anti-Sp100 (green, top panels) or anti-DAXX (green, bottom panels) antibodies. Nuclei were counterstained using DAPI.

3.2.3 Mut PML inhibits p53-dependent transcription

Independent avenues of research have demonstrated that p53 function is regulated in part by its association with nPML and the PML-NB, therefore, it is conceivable that Mut PML could inhibit p53 activity by affecting nPML and other PML-NB components involved in p53 regulation, such as CBP (Insinga et al., 2004; Pearson et al., 2000). Since nPML is a strong p53 transcriptional co-activator (Salomoni and Pandolfi, 2002) through the modulation of CBP-dependent acetylation. Thus, the transcriptional activity of p53 was assessed in the presence of Mut PML using transcriptional assays. For this purpose, we used p53-depleted cell lines in which Mut PML, p53 and the Luc-reporter vector where cotransfected. Exogenous expression of p53 allows high luciferase readouts allowing a direct, although artificial, measure of the protein's transcriptional activity. Indeed, a direct measure of endogenous p53 with this approach would be more difficult as the p53 is tightly regulated and normally kept at very low levels. Furthermore, the levels of overexpressed Mut PML and endogenous p53 would be too dissimilar to compare and data obtained difficult to analyze as all possible differences could be simply due to artefacts related to the expression levels. For these reasons, Mut PML was transduced along with p53 in SAOS-2 cells, which are depleted of endogenous p53 and represent an ideal tool to perform transcriptional assays. Interestingly, we found that the activity of a *GADD45* reporter was clearly inhibited in the presence of Mut PML (Figure 3.2.3A).

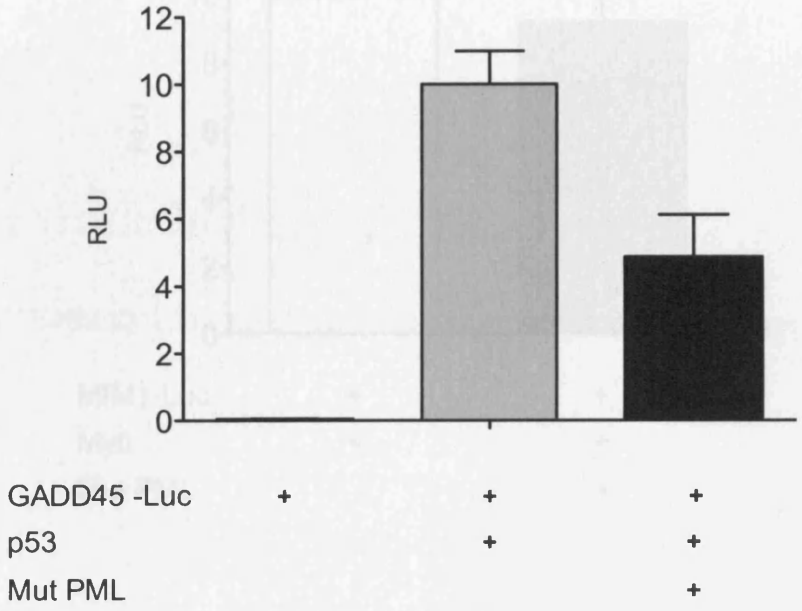


Figure 3.2.3A p53 transcriptional activity is inhibited by Mut PML in SAOS-2 cells. The activity of a GADD45-Luc reporter vector was assessed in SAOS-2 cells transiently transduced with combinations of expression vectors encoding for a p53 and Mut PML as indicated below the graph. β-galactosidase activity was used to normalize transfection efficiency. Luciferase and β-gal activity were measured 36 hours after transfection as relative luminescence units (RLU). Transcriptional assay values are means ± SD of three independent experiments performed in triplicate.

In contrast, Mut PML did not alter the activity of an unrelated *Myb* reporter, MIM1 (Figure 3.2.3B), thus confirming the specificity of the effects observed.

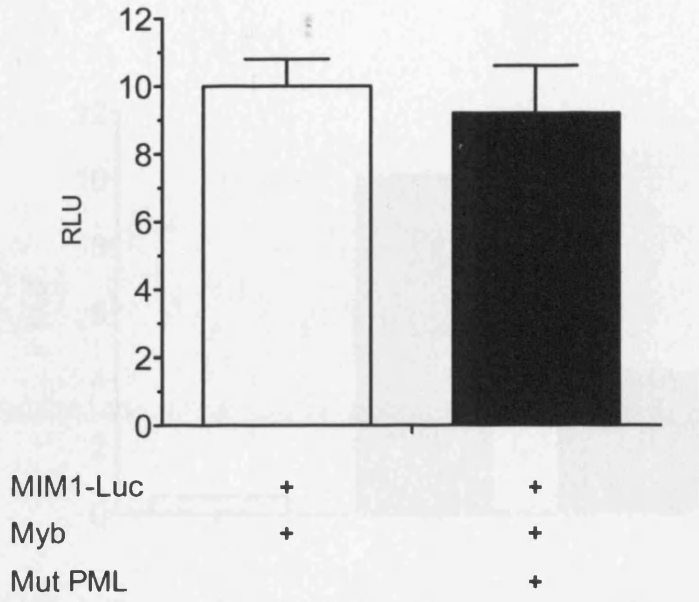


Figure 3.2.2B Mut PML does not alter tbe activity of Myb reporter in SAOS-2 cells. The activity of a Myb-Luc reporter (MIM1) was assessed in SAOS-2 cells transiently transduced with the reporter vector along with Myb in the presence or absence of Mut PML as indicated below the graph. Luciferase and β-gal activity were measured as previously described. Values are means ± SD of three independent experiments performed in triplicate.

In order to assess whether this also applied to haematopoietic cells, p53-dependent transcription was analyzed in HL60 cells (which have a major mutation in the *P53* gene leading to the depletion of the protein). Cells were infected with Mut PML or vector (pBABE) retroviruses. Similarly to the results obtained using SAOS-2 cells, p53-dependent activation of *GADD45* reporter was impaired in Mut PML-infected but not in control cells (Figure 3.2.3C).

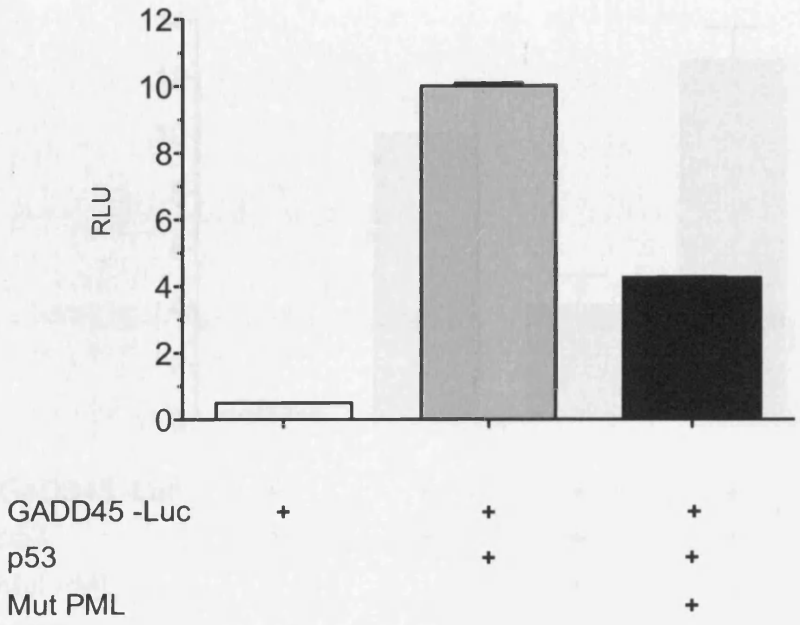


Figure 3.2.3C Mut PML inhibits p53 in hematopoietic cells. HL60 cells were infected with Mut PML or control (pBABE) viral particles. After selection with puromycin pBABE and Mut PML expressing clones were transfected with p53, *GADD45*-Luc along with the TK-Renilla plasmid using Amaxa nucleofection. Luciferase activity was assayed 8 hours after transfection. Data represent means ± SD of three independent experiments.

Interestingly, the over-expression of nPML in SAOS-2 cells completely rescues the inhibitory effects of Mut PML on p53-dependent transcription, indicating that Mut PML and nPML could possibly counteracts each other functions (Figure 3.2.3D).

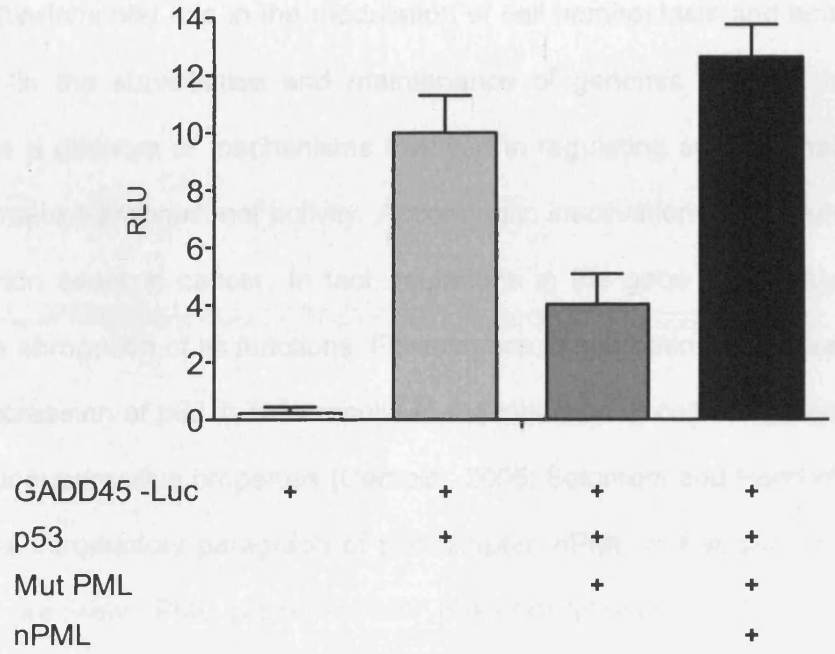


Figure 3.2.3D Over-expression of nPML (PML4) rescues Mut PML inhibition of p53 transcriptional activity. SAOS-2 cells were transduced with a *GADD45*-Luc reporter vector alone or in combination with p53, nPML and Mut PML. Luciferase activity was evaluated exactly as described in figure 3.2.3A. Data represent means ± SD of three independent experiments.

Then, p53 cellular localization was studied in immortalized fibroblasts co-transduced with Mut PML and nPML. As previously described (Doucas et al., 1999), coexpression of nPML and p53 in fibroblasts resulted in a partial colocalization into PML-NB (not shown). In contrast, the expression of Mut PML did not result in p53 relocation to the cytoplasm (not shown). Indeed, Mut PML lacks the carboxy-terminal portion involved in the interaction with p53, thus suggesting that Mut PML inhibition of p53-transcription is a consequence of an indirect inhibitory mechanism.

3.2.4 Mut PML inhibits the growth suppressive functions of p53

p53 has a fundamental role in the modulation of cell homeostasis and acts as a main gatekeeper in the surveillance and maintenance of genomic stability. Indeed, p53 orchestrates a plethora of mechanisms involved in regulating survival that extend far beyond its mere transcriptional activity. Accordingly, inactivation of p53 function is the most common event in cancer. In fact, mutations in the gene commonly occur and result in the abrogation of its functions. For instance, it has been well documented that the over-expression of p53 in cells results in the inhibition of cellular growth according to its tumour suppressive properties (Campisi, 2005; Salomoni and Pandolfi, 2002). As stated in the introductory paragraph of this chapter, nPML is a strong co-activator of p53. More precisely, PML promotes p53 post-translational modifications, such as acetylation and phosphorylation, which result in the stabilization and ultimately activation of the protein (Pearson et al., 2000). Thus, it could be theorized that Mut PML could affect p53 growth suppressive functions. To test this, colony-forming assays were performed using p53-deficient H1299 cells, which were transfected with p53 alone or in combination with Mut PML and nPML, and as a control, HDM2, which is one of the major negative regulators of p53 (Kubbutat et al., 1997). Interestingly, the experiments showed that cells over-expressing p53 and Mut PML formed a significantly higher number of colonies compared to p53 only- and p53/nPML co-transduced cells (Figure 3.2.4A), thus indicating that Mut PML inhibited p53 activity. Interestingly, Mut PML impaired cell growth to an extent similar to HDM2, as expression of both proteins lead to a comparable repression of the growth suppressive functions of p53 that ultimately resulted in a significant increase of clonogenic capability (Figure 3.2.4A).

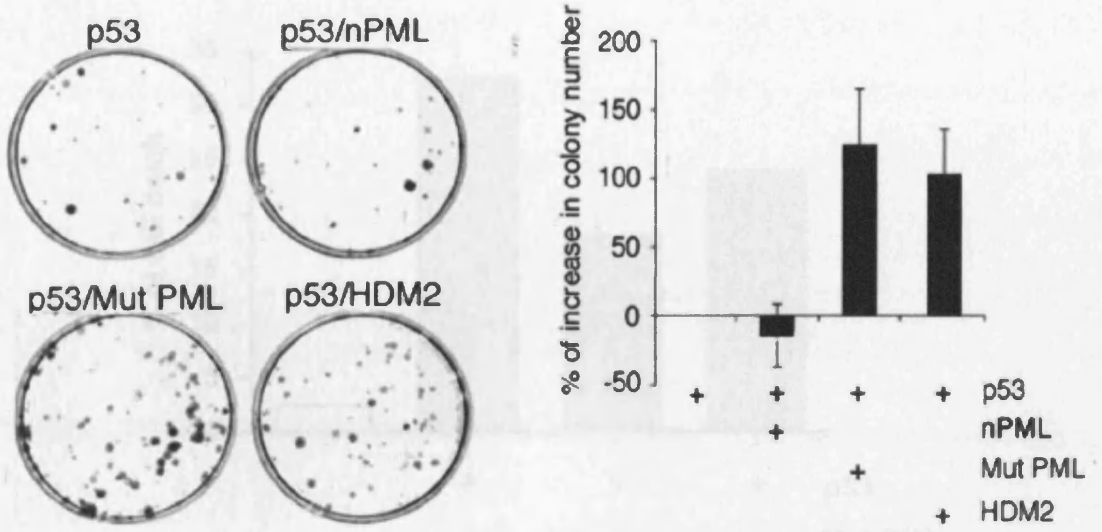


Figure 3.2.4A Mut PML inhibits the growth-suppressive functions of p53. Mut PML inhibits p53 functions in colony forming assays. H1299 cells (p53 $^{-/-}$) were transfected with a combination of different p53, Mut PML, nPML and HDM2 expression vectors. Colonies were stained with crystal violet and counted 15 days after transfection. In the left panel shows representative images of the tissue culture plates after the staining. In the right panel are means of three independent experiments. Transfected plasmids are reported below and data are expressed as mean percentage of increase in colony formation over the control (p53-only transduced cells) ± standards deviations.

These findings indicate that Mut PML interferes with the long-term effect of p53 expression resulting in a diminished proliferative potential of the cells, however, it remains to be established whether other effects, such as the induction of cell death are also altered. To assess this, cell death was measured in H1299 cells transduced in the presence or absence of p53 in combination with Mut PML and HDM2. As expected, twenty-four hours after transduction with the different expression vectors, the percentage of dead, trypan blue-positive cells was clearly induced by p53 (Figure 3.2.4B).

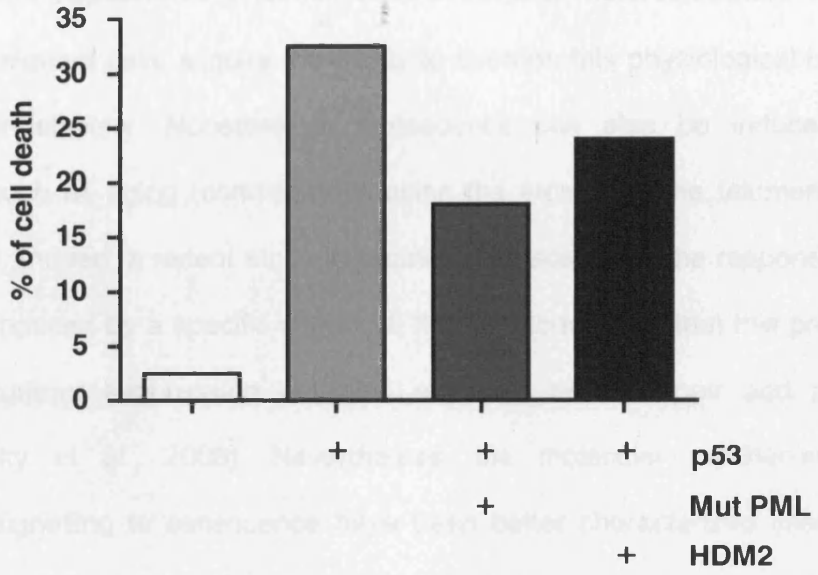


Figure 3.2.4B Mut PML inhibits p53-dependent cell death. H1299 cells were transfected with a combination of different p53, Mut PML, and HDM2 expression vectors as indicated below the graph. Cell death was evaluated 24 hours after transfection by using the trypan blue exclusion assay. Data are average of one experiment performed in duplicate.

Remarkably, expression of either Mut PML or HDM2 led to a similar and substantial reduction of p53-dependent cell death (Figure 3.2.4B, left). Overall, these observations are in accordance with the results obtained in the transcriptional assays and reinforce the hypothesis that Mut PML could impair the tumour suppressive functions of p53 both in short and long-term settings.

3.2.5 Mut PML inhibits H-Ras^{V12}- induced cellular senescence

Deregulation of cellular growth is undoubtedly a hallmark of malignant cells. Normal cells are unable to replicate indefinitely, in fact, after a serial passaging *in vitro* they enter a state of irreversible growth arrest defined as cellular senescence. The properties defining senescent cells are a permanent growth arrest, even at sub-confluent densities in the presence of serum, and a stable and metabolically active state accompanied by some characteristic features. These include a flattened and enlarged appearance, expression of senescence-associated β-galactosidase (SA-

βgal), and the appearance of senescence-associated heterochromatic foci. On the contrary, malignant cells acquire the ability to override this physiological brake and to proliferate indefinitely. Nonetheless, senescence can also be induced by other conditions such as aging (conditions inducing the erosion of the telomeres) but also tissue injury. Indeed, a recent study implicated senescence in the response to fibrosis of the liver induced by a specific chemical, thus demonstrating that this process in not limited to tumour suppression but also regulates tissue repair and regeneration (Krizhanovsky et al., 2008). Nevertheless, the molecular mechanisms relating oncogenic signalling to senescence have been better characterized (Serrano et al., 1997). As already discussed in the introduction of this thesis, ectopic expression of an oncogenic active form of RAS, H-Ras^{V12}, was shown to induce a permanent cell cycle arrest accompanied by the accumulation of p16^{INK4} (p16) and p53 in mouse embryo fibroblasts. In this regard, PML has been shown to have a critical role in the modulation of senescence in mice and humans (Bischof et al., 2002; Ferbeyre et al., 2000; Pearson et al., 2000). Indeed, the expression of H-Ras^{V12} promotes the PML-dependent recruitment of p53 and CBP in the PML-NB, thus favouring p53 stabilization and activation (Pearson et al., 2000). Taking into consideration the results obtained so far, it can be theorized that Mut PML may inhibit stress-induced senescence by disrupting the functions of p53 and nPML, which are the critical players in the regulation of this essential process. To prove this hypothesis, the effects of Mut PML were tested on oncogene-induced senescence using mouse embryonic fibroblasts (MEFs). As aforementioned, senescence is entirely controlled through the p53 pathway in primary MEFs (Pearson et al., 2000). Thus, these cells were infected with an oncogenic form of H-Ras (H-RasV^{12}) along with empty vector (pBABE) and Mut PML viral particles. Upon the completion of the infections, cells were cultured for two days in the presence of puromycin (PURO) in order to select transduced cells. After selection, cells were plated at subconfluent density and senescence was evaluated on different

days by measuring the activity of the senescence associated β-galactosidase (SA-βgal), which is optimally active at (a slightly acidic pH) pH6, and it is therefore considered a well defined marker to identify senescent cells [(Goldstein, 1990) and figure 3.2.5A]. In fact, SA-βgal positive cells become green/light blue when stained with a solution that contains X-gal.

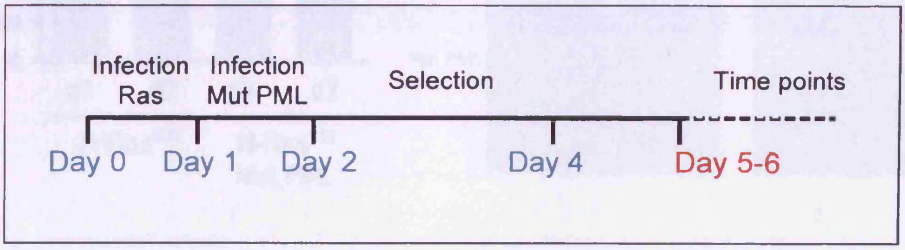


Figure 3.2.5A Overview of the experimental settings. Primary MEFs at early passages were transduced with empty vector (pBABE-PURO), H-Ras^{V12} alone or in combination with Mut PML viral particles. Note that Ras and Mut PML infections were carried out one after the other. Cells were selected with puromycin for two days and replated at low density. Senescence was assessed measuring the number of cells positive to the SA-βgal at day 5 and 6, (day 1 and day 2 post-selection) respectively.

As previously reported, after an initial burst of proliferation H-RasV^{12}-only expressing cells rapidly ceased to grow and acquired a typical senescence-like phenotype (Bischof et al., 2002; Di Micco et al., 2006). Remarkably, by day 1 the number of SA-βgal positive cells was considerably lower in cells co-transduced with H-RasV^{12} and Mut PML as compared to H-RasV^{12} expressing cells. The difference was maintained also at day 2 when almost the majority of cells of H-RasV^{12} infected cells were irreversibly arrested (Figure 3.2.5B). In contrast, control cells infected with empty vector (pBABE) appeared normal, grew nicely, did not change morphology and readily reached a confluent cellular density after 3 days (not shown).

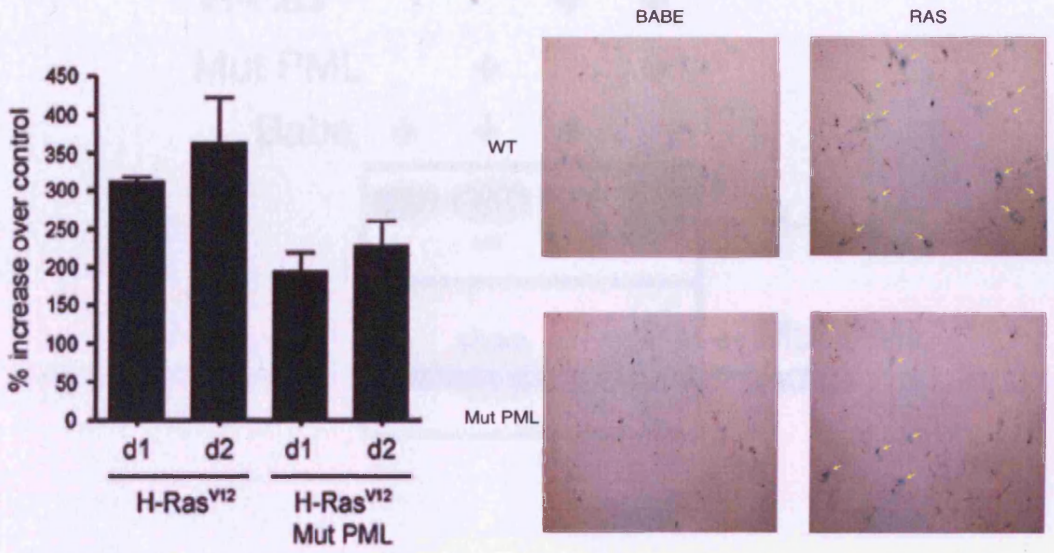


Figure 3.2.5B Mut PML inhibits H-Ras^{V12}-induced senescence. MEFs were infected with control or H-Ras^{V12} alone or in combination with Mut PML viral particles (see figure 3.2.4A). Right panel: show a representative image of infected cells. Senescent cells appear in green as a consequence of the catabolism of X-Gal by the SA-βgalactosidase activity, a well defined marker of senescence (Goldstein, 1990). Left panel: the percentage of senescent cells was measured in cells infected with empty vector (Babe), H-Ras^{V12} and Mut PML after 1 or 2 days post-infection (referred to as d1 and d2) from the end of antibiotic selection. Data are means of fold induction over empty vector infected cells ± standard deviations (SD) of three independent experiments.

To exclude that the observed effects were not simply due to differences in Ras protein levels within the different cell populations analyzed, cellular extracts were prepared from a portion of cells isolated immediately after the puromycin selection and protein analysis was performed (Figure 3.2.5C).

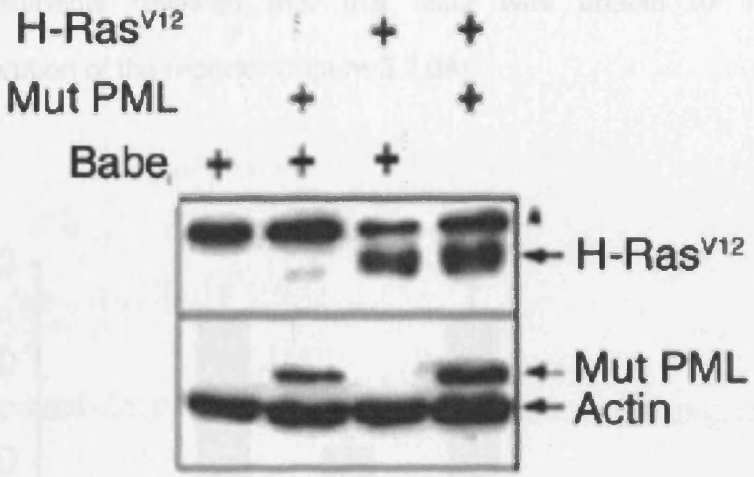


Figure 3.2.5C Mut PML and H-Ras^{V12} are equally expressed in primary MEFs. MEFs were infected with H-RasV12 alone or in combination with Mut PML viral particles. Protein extracts prepare from empty vector (Babe), single or double H-Ras/Mut PML infected cells, were analyzed using anti-Ras (upper panel) and anti-HA antibodies (lower panel). β-actin was used as loading control.

Notably, H-RasV^{12} was expressed at comparable levels in H-RasV^{12}-only and H-RasV^{12}/Mut PML transduced cells, demonstrating that Mut PML does not affect Ras protein levels. Taken together, these findings demonstrated that in response to oncogenic stress the induction of senescence is impaired by Mut PML, thus suggesting that Mut PML activity phenocopy the inactivation of nPML.

3.2.6 Mut2 impairs p53 growth suppressive function in a transcriptional-independent manner

To further strengthen the relevance of the data obtained using Mut PML (Mut1), I decided to test whether also the other mutant identified in APL, Mut2, was able to inhibit p53 transcription and growth suppressive functions. Firstly, we studied the effect of Mut2 on p53 transcriptional activity by overexpressing the protein along with p53 and a GADD45 reported in SAOS-2 cells as previously described in section 3.2.2.

Surprisingly, experiments revealed that this Mut2 was unable to inhibit p53 transcriptional activation of the reporter (Figure 3.2.6A).

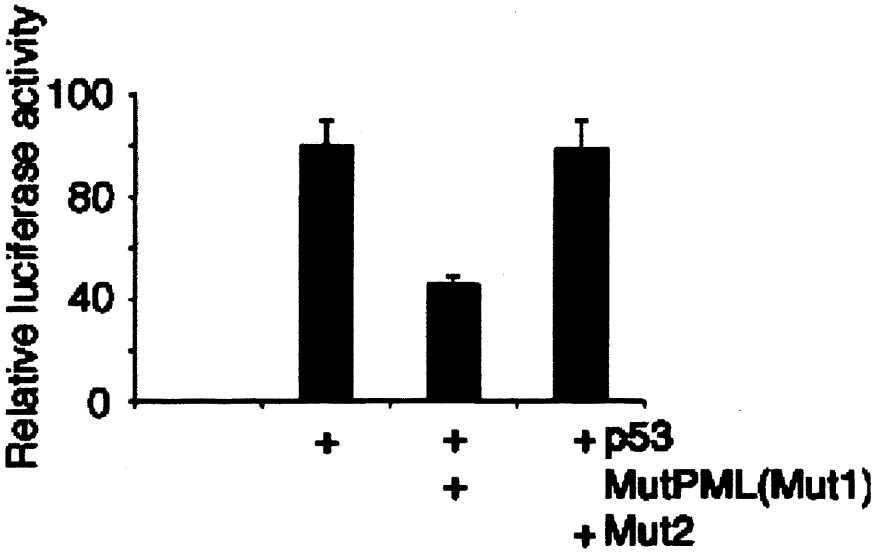


Figure 3.2.6A Mut 2 does not block p53 transcriptional activity in SAOS-2 cells. The activity of a GADD45-Luc reporter vector was assessed in SAOS-2 cells transiently transduced with combinations of expression vectors encoding for a p53 and Mut PML 1 and Mut PML 2 as indicated below the graph. β-galactosidase activity was used to normalize transfection efficiency. Luciferase and β-gal activity were measured 36 hours after transfection as relative luminescence units (RLU). Transcriptional assay values are means ± SD of three independent experiments performed in triplicate.

Notably, confocal/immunofluorescence of Mut2 transduced fibroblasts indicated that as opposed to Mut1, this mutant also accumulated in PML-NBs. This suggests that Mut2 does not have the capabilities to perturb the structure of the PML-NB thereby it might not completely phenocopy Mut 1 in blocking nPML/CBP-mediated activation of p53. However, it is possible that this effect is cell-type specific and that different nuclear PML isoforms could instead be more sensitive to the expression of Mut2. Thus, future experiments are needed to study Mut 2 activity in different cellular settings, for example in hematopoietic cells. Secondly, we tested whether Mut2 alters the growth suppressive functions of p53 in H1299 cells (Figure 3.2.6B).

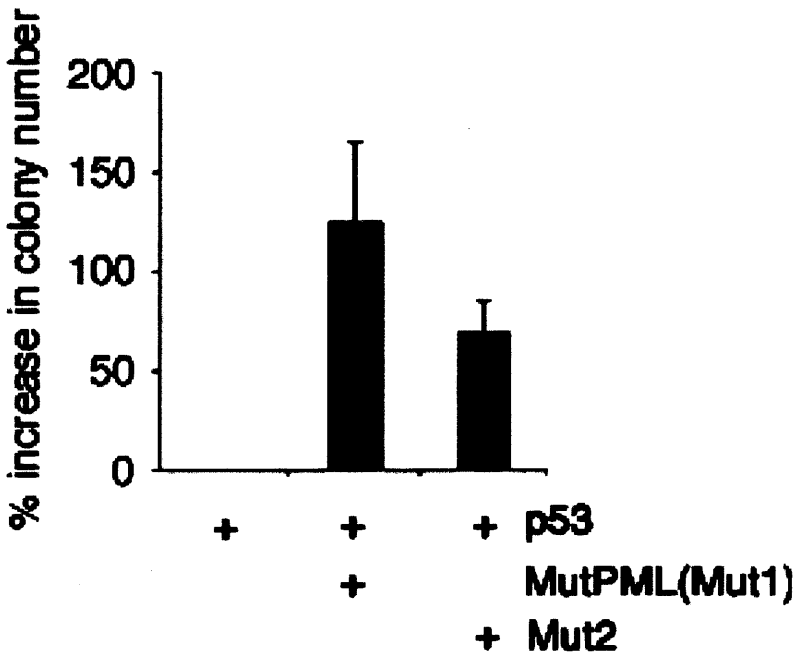


Figure 3.2.6B Mut2 inhibits the growth-suppressive functions of p53. Mut2 inhibits p53 functions in colony forming assays. H1299 cells (p53 $^{-/-}$) were transfected with p53 alone or in combination with Mut PML (Mut1) and Mut2. Colonies were stained with crystal violet and counted 15 days after transfection. Data in the graph are expressed as mean percentage of increase in colony formation over the control (p53-only transduced cells) ± standards deviations of three independent experiments.

Interestingly, experiment indicted that Mut2 inhibits p53 growth suppressive function promoting the formation of a significantly higher number of colonies as compare to cells transduced with p53 alone. Thus, Mut2 inhibits p53 function, albeit it is not as potent as Mut1, probably in a transcription-independent manner. These intriguing findings support the hypothesis that Mut PML inhibits p53 at different levels, thus suggesting that additional levels of complexity exist.

3.3 Discussion

When taken together, data presented in this chapter demonstrate that: (i) Mut PML affects the localization of PML-NB components, (ii) impairs p53-dependent transcription and cell death and (iii) alters the induction of oncogene-induced senescence in mouse primary cells, thus providing the first evidence of a potential transforming potential of PML cytoplasmic proteins.

Intriguingly, data presented in this chapter indicate that cytoplasmic Mut PML can counteract p53 tumour suppressive functions in different experimental settings, thus suggesting that aberrant PML cytoplasmic proteins may function in promoting rather than blocking tumour formation. In line with this, PML cytoplasmic delocalization was observed in some human cancers (Chan et al., 1998; Terris et al., 1995). The absence of a direct interaction between p53 and Mut PML implies that an indirect mechanism accounts for the observed effects. As a matter of fact, a possible explanation for the repressive functions of Mut PML could be ascribed to the cytoplasmic hijacking of p53 nuclear co-activators. Confocal analysis revealed that Mut PML was able to relocate specific PML-NB components to the cytoplasm both in fibroblasts and haematopoietic cells. Specifically, exogenous and endogenous PML were sequestered in the cytoplasm in the presence of Mut PML. In accordance with this, the total number of PML-NB was reduced in Mut PML-expressing cells. Further analysis identified the acetyltransferase CBP, another PML-NB component, together with Mut PML in PML-CBs. In light of the importance of both PML and CBP in controlling p53 activity, it is conceivable that Mut PML may have a profound impact on some of the tumour suppressive pathways regulated by the PML-NB. In this regard, it has recently been shown that PML-NBs undergo physiological re-organization during the cell cycle including changing in number, shape and composition (Dellaire et al., 2006a; Dellaire

et al., 2006b). In fact, a large portion of PML is found organized in cytoplasmic bodies, referred to as mitotic accumulation of PML protein (MAPP), in the early G1 phase of the cell cycle. Notably, MAPP domains are qualitatively divergent from PML-NB. Nevertheless, it is still unclear what is the exact role of MAPPs, whether they simply represent a transient depot for the recycling of PML proteins until the mid-G1 phase when reorganization of the PML-NB is completed, or whether they also bare cytoplasmic functions. To test whether Mut PML interferes with p53 functions, the transcriptional activity of p53 was assayed in the presence of Mut PML. Transcriptional assays using a *GADD45* reporter construct suggested that the ability of p53 to activate the transcription of target genes was severely reduced in the presence of Mut PML. Importantly, the over-expression of nPML could rescue the inhibitory effect of Mut PML on p53, thus suggesting that the two proteins could counteract each other's function. However, a more detailed analysis of p53 transcriptional activity is needed. For instance, it will be important to assess whether Mut PML has a general effect on p53 transcription or selectively inhibits only some p53 target genes. This will definitely corroborate the data obtained by using the artificial *GADD45* reporter assay. In line with its tumour suppressive properties, the main p53 functions are indeed as a negative regulator of the cell cycle, *via* p21 and GADD45 or pro-apoptotic factor, for instance inducing Bax levels. Furthermore, PML is itself a target gene of p53, adding another level of complexity to the whole process. The effects of Mut PML expression on the growth suppressive functions of p53 were then tested. Remarkably, Mut PML was able to significantly diminish the growth suppressive functions of p53 in colony forming assays, to an extent similar to HDM2, which is one of the major negative regulators of p53 (Kubbutat et al., 1997). The absence of a direct interaction between p53 and Mut PML implies that an indirect mechanism accounts for the observed effects. As a matter of fact, it is reasonable to believe that Mut PML may negatively influence p53 activity possibly through cytoplasmic hijacking of p53 nuclear co-

activators. Nevertheless, it cannot be ruled out that Mut PML may also affect p53 transcriptional-independent functions. This possibility is further supported by interesting data obtained using Mut2, which inhibits p53 growth suppressive functions in a transcriptional-independent manner, thus suggesting that different levels of complexity exist. Interestingly, it has been shown that the cytoplasmic portion of p53 possesses an important role in triggering apoptosis in response to stress stimuli. In this regard, it can be hypothesized that Mut PML alters the function of PUMA (p53-up-regulated modifier of apoptosis) a specific p53 transcriptional target gene, in response to cellular stimuli. Notably, PUMA has been shown to couple the nuclear and cytoplasmic pro-apoptotic functions of p53. In this regard, the analysis of PUMA transcriptional rate and protein levels would certainly provide more insights into this aspect. Finally, it is also evident that Mut PML affects p53 functions in physiological settings. Indeed, a significant defect in the activation of oncogene-induced senescence was observed in mouse fibroblasts expressing an oncogenic form of RAS together with Mut PML as compared to RAS-only infected cells. The ability of Mut PML to relocate important p53 co-activators to the cytoplasm may represent an important inhibitory mechanism. In fact, the inhibition of post-translational p53 modification may result in a profound defect of p53 activity. From another standing point, it could be also envisaged that the expression of Mut PML could alter the apparatus responsible for the maintenance of the genomic integrity. In this respect, a connection between DNA damage and senescence following oncogenic stress has recently been described (Di Micco et al., 2006). Indeed, experimental evidence indicates that oncogenic activation triggers a transient hyper-proliferative phase that in turn results in generation of stalled replication forks. These stalled forks activate a DNA damage response resulting in the induction of senescence. These findings suggested that senescence cells may also be arrested in S-phase, thus it would be interesting to analyze whether during RAS-induced senescence Mut PML preferentially alter the G1 or the S-phase checkpoints. In this

regard, it has been reported that cells challenged with oncogenes (for instance Ras) accumulated partly replicated DNA, thus indicating a critical role of the S-phase checkpoint in the induction of senescence (Di Micco et al., 2006). Evidence coming from another study suggest that the number of PML-NBs can change during the cell cycle: more PML-NBs are found in S phase and in response to cellular stress, such as DNA damage (Dellaire et al., 2006b). Albeit a direct connection has not yet been established, it is plausible that oncogene-induced senescence and S-phase control of PML-NB are co-regulated. In accordance, some essential molecular players, such as CHK2 and ATM are involved in both processes. Yet, it would be intriguing to investigate the role of PML-NB in senescence induced by DNA-damage and whether Mut PML can impair the function of the S-phase checkpoint. It would be also interesting to check whether in Mut PML expressing cells the phosphorylation status of H2AX, a checkpoint marker, is affected (Hovest et al., 2006). Another intriguing aspect to consider is how Mut PML expression can affect the regulation of the tumour suppressor protein pRB. pRB acts as a main gatekeeper of the cell cycle, colocalizes with and is in part regulated by nPML (Alcalay et al., 1998; Bischof et al., 2002; Labbaye et al., 1999). PML contributes to maintain pRB in its hypophoshorylated state, so that, it can inhibit E2F function and block the G1/S transition. In this regard, it is conceivable that in addition to p53, Mut PML may also directly or indirectly impair the function of pRB, for instance by modifying its phosphorylation status. Importantly, it has been shown that the adenoviral oncoprotein E1A can cooperate with activated Ras to bypass the induction of senescence, thus promoting transformation (Deng et al., 2005). Specifically, E1A overrides *RAS*-induced senescence by interfering with the activities of both pRB/p16 and CBP/p300 pathways. Analysis of the effect of Mut PML on oncogene-induced senescence in human cells will also be very important. As a human cell model of oncogene-induced senescence has been described, namely IDH4 cells (Jiang and Ringertz, 1997), it will be worth exploring the function of Mut PML in this

system to gain more functional insights. Furthermore, our laboratory has recently demonstrated that PML regulation of pRB function is of crucial importance for the general homeostasis of the progenitor/stem cell niche in the nervous system (Regad, T, Bellodi, C, Nicotera, P, and P Salomoni, submitted manuscript). In neuronal progenitors/stem cells in the developing neocortex, in progenitors of the gut and bone marrow, PML loss results in profound deregulation of cellular proliferation (Regad, T, Bellodi, C, Nicotera, P, and P Salomoni, submitted manuscript). At least in neural progenitor/stem cells this event appears to depend on pRB deregulation. Indeed, the lack of PML is accompanied by hyperphosphorylation and subsequent inactivation of pRB, thus leading to unrestricted proliferation of the progenitor/stem cells. In haematopoietic tissue, this may lead to the exhaustion of the pluripotent stem cell population, thus altering the ability to produce the different blood cellular elements.

Taken together, data presented in this chapter demonstrate that Mut PML: (i) affects the localization of PML-NB components, (ii) impairs p53-dependent transcription and cell death and (iii) alters the induction of the oncogene-induced senescence in mouse primary cells, thus providing the first evidence showing that PML cytoplasmic proteins favour cellular transformation. However, future investigations are needed in order to explore this possibility and gain more molecular mechanistic insights into the functions of cytoplasmic PML proteins.

Chapter 4

"Mutant PML and PML-RARα cytoplasmic functions in APL"

4.1 Introduction

In the previous chapter the effects of Mut PML have been tested in respect to the activity of nPML and p53. Collectively data demonstrated that Mut PML inhibits the growth suppressive functions of p53 through the cytoplasmic sequestration of nPML. Nevertheless, it remains unclear whether Mut PML possesses additional function in APL cells. In this chapter we will try to address this outstanding question primarily trying to investigate whether Mut PML and PML-RARα cooperate in promoting leukaemogenesis. Specifically, we will study the effect of Mut PML on the localization, the regulation and the activity of PML-RARα by co-expressing the two proteins in both fibroblasts and haematopoietic cells. These experiments will possibly provide a mechanistic explanation for the very aggressive form of leukaemia described in the APL patients carrying PML missense mutations (Gurrieri et al., 2004b). In the second part of this chapter, we will try to understand whether PML-RARα bears cytoplasmic functions and how these contribute to leukaemogenesis. To date, there is no clear evidence suggesting that PML-RARα localizes to the cytoplasm in leukaemic cells. Indeed, the only data describing PML-RARα in the cytoplasm have been obtained overexpressing the fusion protein in cell lines (Kastner et al., 1992; Perez et al., 1993). Furthermore, the majority of the functional studies on PML-RARα both *in vitro* and *in vivo* have been conducted using the *bcr1* isoform, which is for the most part nuclear. In contrast, the functions of the short form, *bcr3*, have not been investigated in depth. Remarkably, this isoform lacks the NLS of PML and is predicted to acquire a more pronounced cytoplasmic localization as compared to *bcr-1*. A number of studies have suggested that *bcr3* expression is associated with poorer prognosis as opposed to the *bcr-1* isoform (Huang et al., 1993; Jurcic et al., 2001; Vahdat et al., 1994). This is still a very controversial matter in the field of APL. To address these important questions, we

will take advantage of a cytoplasmic mutant of PML-RARα to investigate the role of the fusion protein outside the nucleus in the absence of chromatin remodelling events. Altogether, data presented in this chapter will possibly clarify some of the dogmas in the APL field and reveal new fascinating insights into the molecular mechanisms contributing to leukaemogenesis.

4.2 Results

4.2.1 Mut PML colocalizes and interacts with PML-RARα

As described in the previous chapter, two missense mutations in the *pml* allele not involved in the specific translocation t(15;17) have been identified in APL patients. Patients carrying the mutations experienced a very aggressive disease (Gurrieri et al., 2004b). It is conceivable that the presence of Mut PML may somehow modulate the functions of PML-RARα. Notably, PML-RARα is a strong inhibitor of the RARα/RXRα nuclear receptor complex, thus impairing the transcription of RA-responsive gene and the RA-dependent differentiation. Thus, it is possible that Mut PML may contribute to PML-RARα-dependent inhibition of RA activities within the leukaemic cells. In addition, as both Mut PML and PML-RARα retain the RBCC domain of PML at their N-terminal portion, which mediates proteins interactions, it is likely that the two proteins interact and form heterodimers. To test this, the cellular localization of Mut PML and PML-RARα was studied in primary fibroblasts (BJ) co-infected with retroviruses encoding for the two proteins (Figure 4.2.1A). Interestingly, confocal analysis indicated that a portion of cytoplasmic PML-RARα colocalized with Mut PML (Figure 4.2.1A).

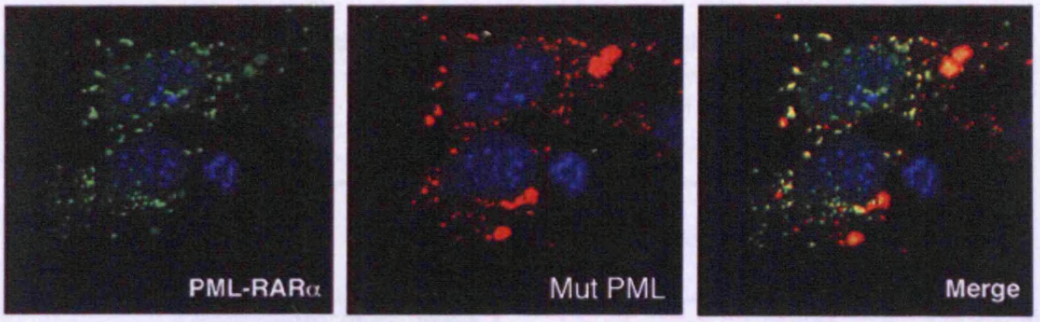


Figure 4.2.1A Mut PML colocalizes with PML-RARα in PML-CB. Human primary fibroblasts were co-infected with PML-RARα and HA-tagged Mut PML retroviruses. Cells were stained with anti-RARα (green) and anti-HA (red) antibodies. Nuclei were counterstained with DAPI (blue). Yellow speckles in the merge image (right) represent colocalization between the two proteins.

Furthermore, immunoprecipitation experiments were conducted in 293T and BJ cells by co-transfecting PML-RARα and a HA-tagged version of Mut PML in order to prove the interaction between the two proteins. In both cell lines a band corresponding to PML-RARα appeared in the anti-HA immunoprecipitates from cotransfected cells, although this interaction was less pronounced in BJ cells. These differences probably reflect the method used to transduce the proteins. Indeed, while 293T cells were transiently transfected, BJ cells were stably transduced via retroviral infection. Nevertheless, these finding convincingly demonstrated that Mut PML and PML-RARα directly interact. It is conceivable that the two proteins homodimerize through their RING and coiled-coil domains as reported for many TRIM family members (Meroni and Diez-Roux, 2005; Reymond et al., 2001) (Figure 4.2.1B).

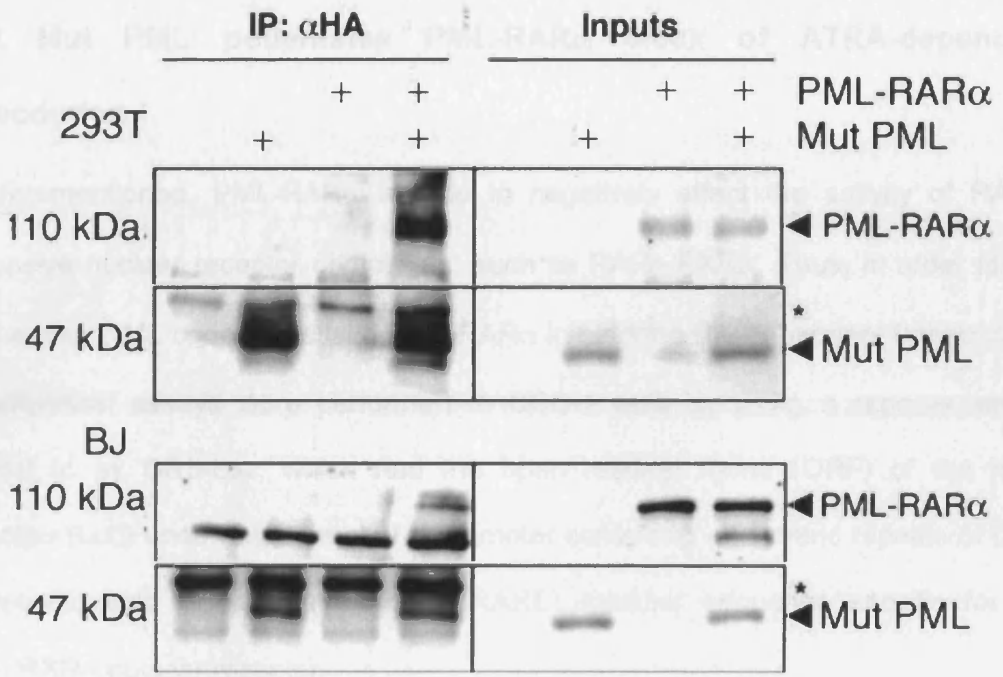


Figure 4.2.1B Mut PML interacts with PML-RARα. Interaction between Mut PML and PML-RARα was tested in co-immunoprecipitation experiments by transducing 293T and BJ cells with combinations of expressing vectors encoding for the two proteins, as indicated above in the panels. Right panels, anti-HA immunoprecipitates (IP) from 293T (top) and BJ (bottom) cells were probed using an anti-RARα antibody. 1/10 of the total lysate was loaded as a control for protein expression (left panels). Mut PML and PML-RARα bands are pointed by black arrows, whereas, asterisk indicates non-specific bands. Molecular weight are reported on the left

4.2.2 Mut PML potientates PML-RARα block of ATRA-dependent transcription

As aforementioned, PML-RARα is able to negatively affect the activity of RA on responsive nuclear receptor complexes, such as RARα/RXRα. Thus, in order to test whether Mut PML cooperates with PML-RARα in blocking RA-dependent transcription, transcriptional assays were performed in COS-1 cells by using a reporter vector, referred to as DR5-Luc, which had the open reading frame (ORF) of the firefly luciferase (Luc) under the control of a promoter containing multimeric repeats of DR5, the retinoic acid responsive element (RARE) modular sequence specific for the RARα/RXRα nuclear receptor.

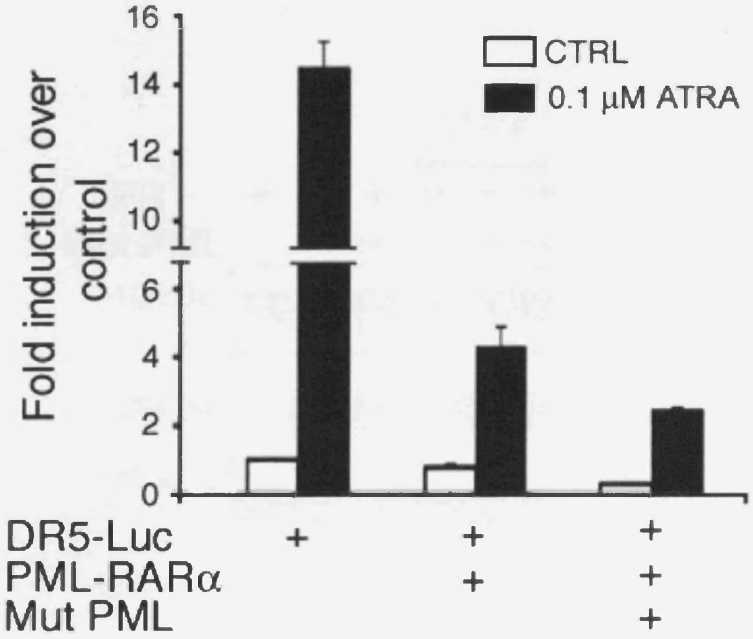


Figure 4.2.1A Mut PML potentiates PML-RARα inhibition of RA-dependent transcription. To assess the effect of the coexpression of Mut PML and PML-RARα on RA-induced transcription, three independent transcriptional assay experiments were performed in COS-1 cells. Cells were transduced with a combination of a DR5-luciferase reporter along with PML-RARα and Mut PML as indicated below the graph, and cultured for 24 hours in the absence (white) or presence of 0.1 μM of ATRA. β-galactosidase (β-gal) activity of a β-gal control vector was used to normalize the luciferase intensity in each sample. Data are mean ± SEM of three independent experiments expressed as fold induction over the value of the untreated control sample.

As expected the activity of DR5-Luc reporter was nicely induced by ATRA in control cells (10.4 ± 0,6 fold) and strongly inhibited in the presence of PML-RARα (3,1 ± 0,3). Interestingly, the expression of Mut PML potentiates the inhibitory activity of PML-RARα (1,9 ± 0,1) (Figure 4.2.2A).

4.2.3 Mut PML inhibits ATRA-dependent down-modulation of PML-RARα

To uncover the mechanisms by which Mut PML exacerbates the inhibitory functions of PML-RARα, the effect of Mut PML expression on PML-RARα stability was analyzed in the presence of ATRA and As_2O_3, two chemotherapeutic agents that, at pharmacological doses (μM concentrations), can induce the degradation of the fusion protein and are used for treating APL patients.

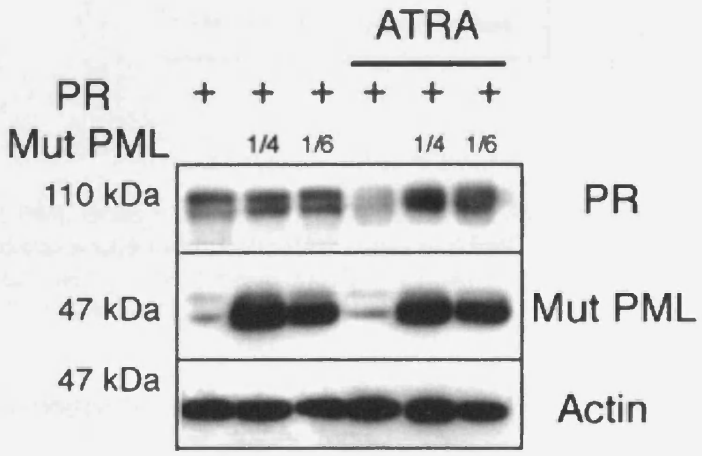


Figure 4.2.3A Mut PML inhibits ATRA-mediated downmodulation of PML-RARα.
Cos-1 cells were co-transduced with HA-tagged PML-RARα (PR, 1.6 μg) and two different amounts of Myc-tagged Mut PML: 1/4 and 1/6, respectively. Cells were cultured for 24 hours with or without 0.1 μM ATRA and protein levels were assessed using anti-HA (top) and anti-Myc (middle). Actin was used as loading control (bottom).

COS-1 cells were co-transduced with PML-RARα along with different amounts 1/4 or 1/6 of Mut PML. Cells were cultured in the presence of pharmacological concentrations

of ATRA and As_2O_3 and the levels of the proteins were measured. Surprisingly, expression of Mut PML resulted in impaired PML-RARα down-modulation in the presence of ATRA at both 1 μM and 0.1 μM (Figure 4.2.3A and not shown). In contrast, Mut PML did not influence the As_2O_3-mediated degradation of PML-RARα (Figure 4.2.3B).

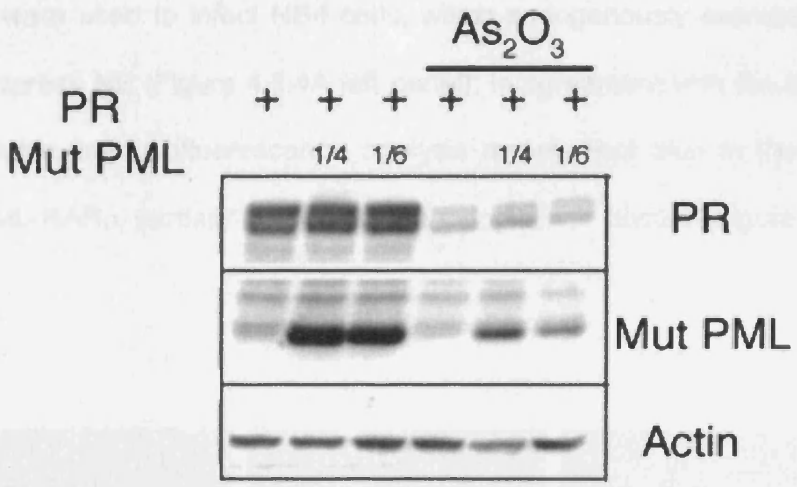


Figure 4.2.3B Mut PML does not inhibit As_2O_3-mediated downmodulation of PML-RARα. Cos-1 cells were co-transduced with PML-RARα and Mut PML as described above. Cells were cultured for 24 hours with 1 μM arsenic trioxide. Protein levels were analyzed probing the membranes with the relevant antibodies as aforementioned.

As_2O_3 is known to induce SUMOylation of the lysine K160 in the PML portion of PML-RARα, and this event is thought to mediate the recruitment of the 11S subunit of the proteasome, thus causing the proteasomal-mediated degradation of the protein (Lallemand-Breitenbach et al., 2001). In line with this, As_2O_3 also caused the down-regulation of Mut PML levels, thus explaining its lack of protection (Figure 4.2.3B). Notably, As_2O_3-induced degradation of Mut PML is PML-RARα-independent as the protein is degraded also in the absence of the fusion protein (not shown). In fact, Mut PML retains the critical lysine, K160 required for SUMOylation and proteasome-

dependent degradation (Kamitani et al., 1998b; Lallemand-Breitenbach et al., 2001). This also suggests that the interaction between Mut PML and PML-RARα does not affect the binding of the specific E3 ubiquitin ligase in response to As_2O_3.

4.2.4 Mut PML inhibits ATRA-dependent down-modulation of PML-RARα

To determine the effect of Mut PML on RA-dependent differentiation, Mut PML retroviruses were used to infect NB4 cells, which endogenously express PML-RARα but do not express NE (Figure 4.2.4A left panel). In agreement with the data obtained using fibroblasts, immunofluorescence analysis reveals that also in these cells Mut PML and PML-RARα partially colocalized in cytoplasmic bodies (Figure 4.2.4A right panel).

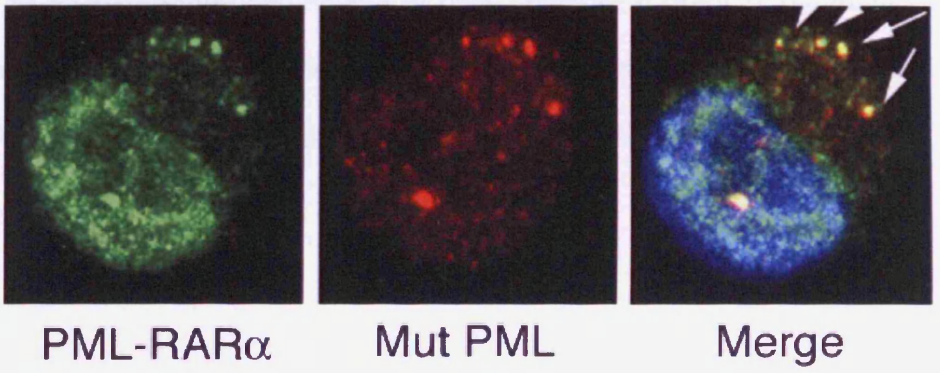


Figure 4.2.4A Mut PML colocalizes with PML-RARα in NB4 cells. NB4 cells were infected with HA-tagged Mut PML retroviruses, cytospun and stained with anti-RARα (green) anti-HA (red) antibodies. Nuclei were counterstained with DAPI. The cytoplasmic colocalization between the two proteins is indicated with white arrows.

Accordingly, subcellular fractionation studies indicated that PML-RARα equally distributed between the nuclear and cytoplasmic fractions, whereas, as expected, Mut PML accumulated exclusively in the latter (Figure 4.2.4B).

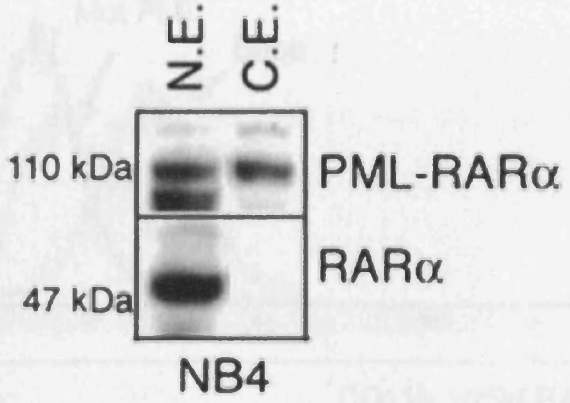


Figure 4.2.4B PML-RARα is equally distributed in the nucleus and cytoplasm of NB4 cells. NB4 cells were infected with Mut PML as previously described and protein levels were analyzed in the nuclear and cytoplasmic fraction by western blot. Membranes were probed with an anti-PML antibody and tubulin was used as loading control.

Thus, vector and Mut PML-expressing NB4 cells were cultured in the presence of 0.1 and 1 μM ATRA and the percentage of cells undergoing granulocytic maturation was assessed by measuring the levels of the myelomonocytic marker CD11b (Figure 4.2.4C).

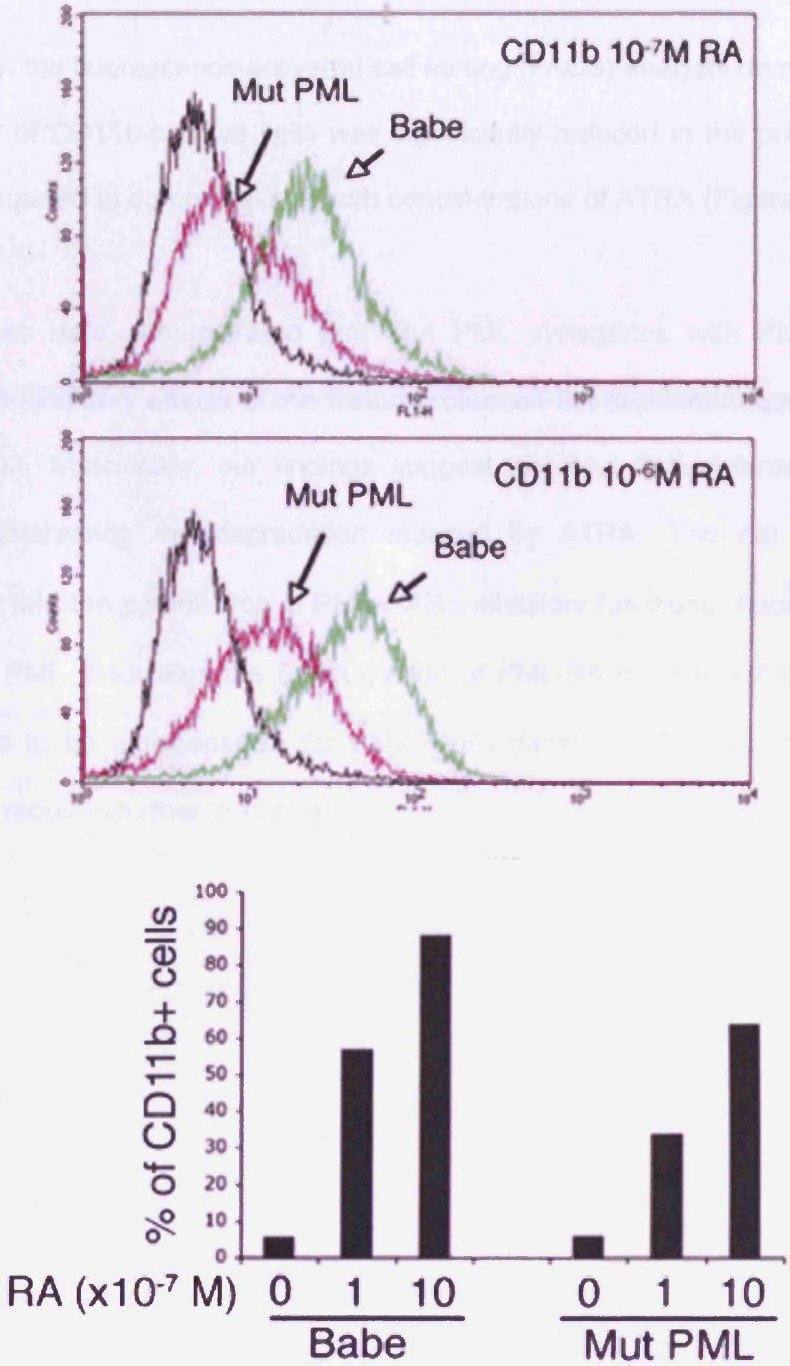


Figure 4.2.4C Mut PML inhibits RA-dependent differentiation in transduced NB4 cells. **Top:** NB4 cells were infected with Mut PML and culture in the presence or absence of either vehicle or 0.1 (top) /1 (bottom) μM ATRA. Differentiation was measured by using fluorescent activated cell sorter (FACS), staining the cells with FITC conjugated anti-CD11b antibody, which targets the granulocytic-monocytic maturation marker CD11b. Black trace represents untreated control cells. **Bottom:** histogram showing the percentage of cells undergoing terminal differentiation in NB4 cells infected with vector (Babe) or Mut PML retroviruses cultured for 4 days in the presence of 0.1 and 1 μM ATRA. Percentages were calculated as number of CD11b positive cells over the total number of cells in each sample. Measurements were performed plotting the side scatter (SSC) signal over the intensity of the fluorescence 1 channel (FL1). Data is from one experiment representative of two repeats.

Remarkably, the fluorescence-activated cell sorting (FACS) analysis demonstrated that the number of CD11b-positive cells was significantly reduced in the presence of Mut PML as compared to control cells at both concentrations of ATRA (Figure 4.2.4D).

Overall these data demonstrated that Mut PML synergizes with PML-RARα and worsens the inhibitory effects of the fusion protein on RA-dependent transcription and differentiation. Specifically, our findings suggest that Mut PML interacts with PML-RARα, counteracting the degradation induced by ATRA. The net result is the stabilization and the potentiation of PML-RARα inhibitory functions. Another possibility is that Mut PML modulates the SUMOylation of PML-RARα, a modification that has been shown to be indispensable for PML-RARα-transforming capability (Zhu et al., 2005). This requires further investigation.

4.2.5 Cytoplasmic PML-RARα inhibits the response to RA

Previous studies showed that PML accumulated in the cytoplasm in the majority of primary leukaemic cells derived from APL patients that were not yet treated with chemotherapeutic agents (Daniel et al., 1993). Furthermore, PML-RARα over-expression was also reported to accumulate in the cytoplasm (Figure 4.2.1A) (Kastner et al., 1992; Perez et al., 1993). Our confocal and subcellular fractionation studies confirmed that PML-RARα evenly accumulated both in the nucleus and in the cytoplasm of NB4 cells, which endogenously express the fusion protein (Figure 4.2.4A-B). Nevertheless, to date, very little is known on the consequences of cytoplasmic localization of PML-RARα. Yet, it is still unclear whether PML-RARα accumulates in the cytoplasm *in vivo* and whether this event contributes to transformation and leukaemogenesis. To answer these questions, two mutants of PML-RARα, isotype *bcr1*, were generated by site-direct mutagenesis of the nuclear localization sequences (NLS). Indeed, it has been proposed that PML-RARα cellular localization relies on the presence of two NLS, of which one is found in the PML portion and the other in the RARα portion (Figure 4.2.5A).

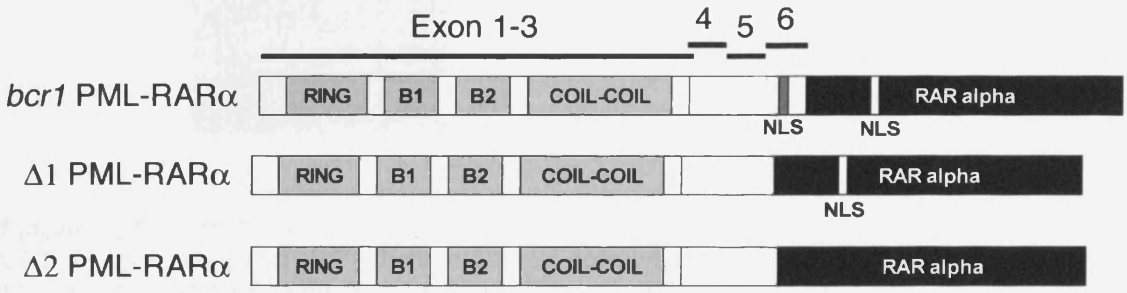


Figure 4.2.5A Generation of PML-RARα cytoplasmic mutants.
Scheme depicting PML-RARα and its NLS-depleted mutants. Top the structure of *bcr1* PML-RARα (top). Note that the bcr1 isoform retains two NLS. The PML NLS or both PML and RARα NLS were mutated to generate the Δ1 and Δ2 PML-RARα mutants, respectively.

A single (Δ1PR or Δ1) and a double (Δ2PR or Δ2) of PML-RARα were generated and their subcellular distribution was analyzed in retrovirally infected COS-1 and U937 cells by immunofluorescence (Figure 4.2.5B).

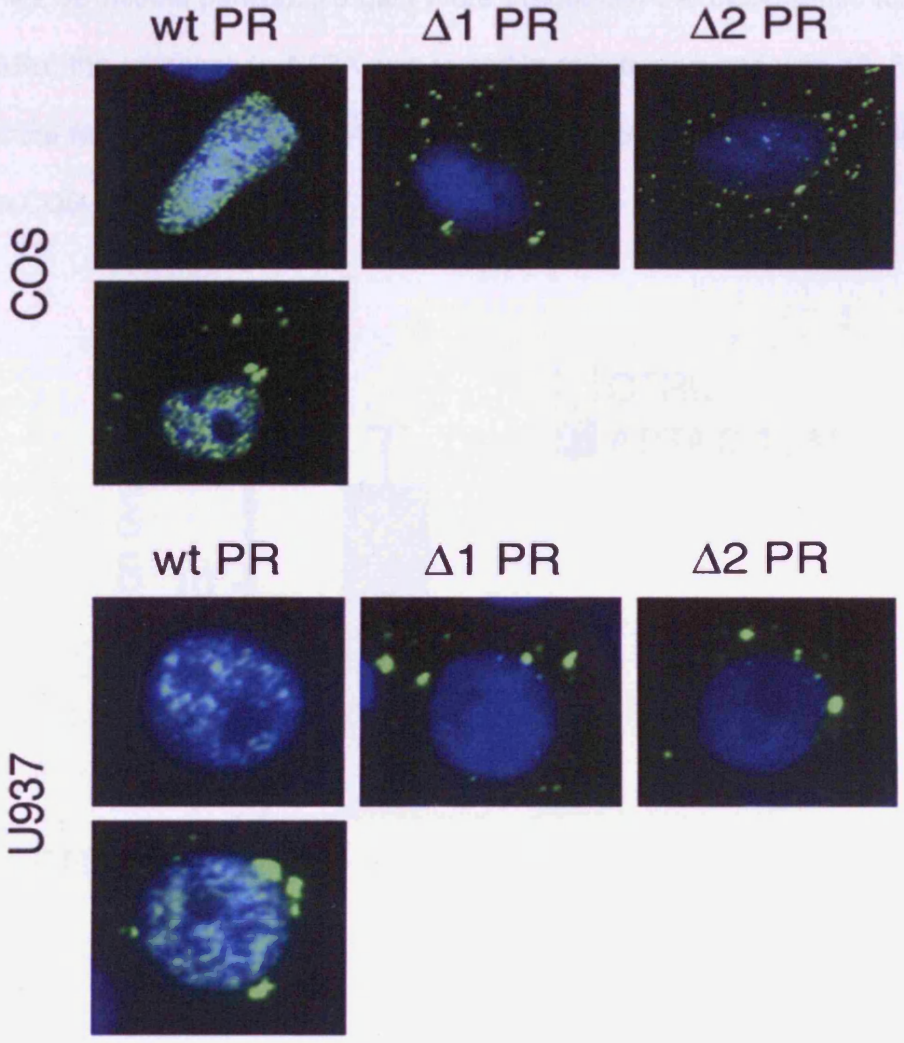


Figure 4.2.5B PML-RARα NLS mutants localize to the cytosol in PML-CB.
COS-1 (top panels) and U937 (bottom panels) cells were transduced with retroviruses for the *bcr1* and the two NLS mutants, Δ1 and Δ2, isotypes of PML-RARα. The cellular distribution of the proteins was studied by confocal microscopy. Cells were fixed and stained with anti RARα (green) antibody. Nuclei were counterstained with DAPI.

Immunofluorescence analysis revealed that Δ2 was exclusively localized to the cytoplasm (Figure 4.2.5B left panels). In contrast, the wild type PML-RARα

accumulated in the nucleus only or in the nucleus as well as in the cytoplasm, as previously described (Alcalay et al., 1992)(Figure 4.2.5B right panels). Intriguingly, the single NLS mutant, Δ1, predominantly accumulated in the cytoplasm, thus suggesting that the NLS retained in the PML portion controls the nuclear distribution of PML-RARα (Figure 4.2.5B middle panels). To gain more insight into the cytoplasmic functions of PML-RARα, the response to ATRA was tested in cells transduced with Δ2. Firstly, the effect of the two cytoplasmic PML-RARα mutants on RA-dependent transcription was tested in COS-1 cells.

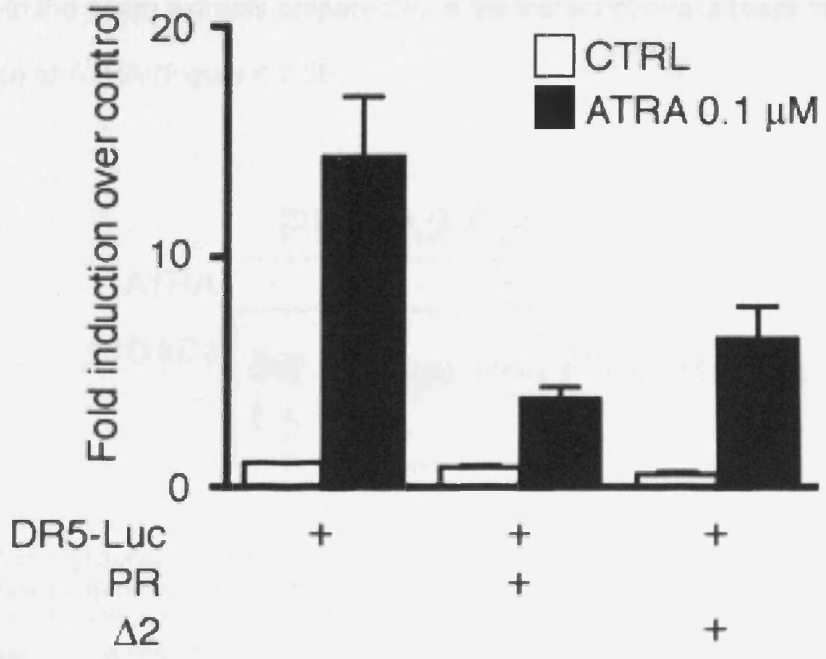


Figure 4.2.5D Cytoplasmic mutant of PML-RARα inhibits ATRA-dependent transcription. COS-1 cells were transiently transduced with a DR5-Luc reporter vector along with *bcr1* PML-RARα (PR) or its double NLS mutant Δ2. Cells were cultured in the absence or presence of 0.1 μM ATRA for 24 hours. β-galactosidase (β-gal) activity of a β-gal control vector was used to normalize the luciferase intensity in each sample. Data show mean ± SEM of three independent experiments expressed as fold induction over the value of the untreated control sample.

As expected wild type *bcr1* PML-RARα strongly blocked (3.8 ± 0.1) the activity of a DR5 luciferase reporter vector (14.2 ± 0.9) in response to 0.1 μM RA (Figure 4.2.5C). Surprisingly, ATRA-dependent transcriptional activation was also clearly inhibited in

the presence of Δ2, although to a lesser extent (6.4 ± 0.9) than *bcr1* (Figure 4.2.5C). Accordingly, Δ1 displayed a comparable inhibition of the DR5-Luc reporter activity in response to ATRA (not shown). These findings support the intriguing possibility of the presence of an additional inhibitory circuitry governed by PML-RARα. These alternative pathways may act in parallel to the nuclear chromatin remodelling activity thus complementing the oncogenic potential of the fusion protein. Furthermore, the mechanism underlying the inhibitory activity of Δ2 was studied. As suggested by the data described earlier in this chapter, cytoplasmic localization may result in a diminished RA-mediated degradation. Thus, the levels PML-RARα and Δ2 were analyzed in the same extracts prepared from the transcriptional assays in the presence or absence of ATRA (Figure 4.2.5E).

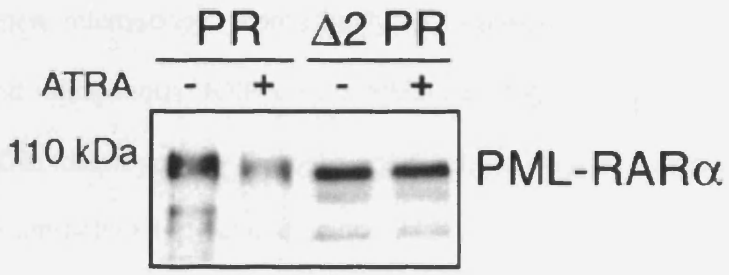


Figure 4.2.5E Cytoplasmic PML-RARα is more resistant to RA-induced degradation.
Cellular extracts from the transcriptional assays described above (Figure 4.2.5D) were analyzed by Western blot. Protein levels of PML-RARα and Δ2 (Δ2 PR) were measured in transduced cells cultured in the presence or absence of 0.1 μM ATRA by using an anti-RARα antibody. Extracts were normalized according to the β-galactosidase activity measured in each sample.

Interestingly, Δ2 was not downmodulated in the presence of ATRA. By contrast, the levels of wild type PML-RARα were substantially reduced (Figure 4.2.5E). This data implies that cytoplasmic localization may alter the degradation of PML-RARα. Alternatively, Δ2 inhibitory activity could possibly rely on the protein's ability to relocate essential nuclear co-activators of RA-functions to the cytoplasm. Notably, a similar functional mechanism has already been described for the APL-associated cytoplasmic

mutant of PML, Mut PML (discussed in chapter 3). Importantly, previous studies showed that overexpression of PML-RARα led to the sequestration of the nuclear receptor component RXRα, the transcriptional partner of RARα (Mangelsdorf and Evans, 1995), into aberrant nuclear and cytoplasmic foci (Perez et al., 1993). Specifically, PML-RARα mediated sequestration of RXRα also impairs the activity of many nuclear receptor complexes. Indeed, it has been shown that beside RARα, PML-RARα also affects the functions of the vitamin D3 receptor (VDR) and the thyroid hormone receptor (TR) (Perez et al., 1993). These findings suggested that the inhibition mediated by PML-RARα of the nuclear receptors could possibly account for a combination of nuclear and cytoplasmic repressive events. However, the function of cytoplasmic PML-RARα was still unclear. Thus, to clarify this point, exogenous and endogenous RXRα cellular distribution was analyzed in the presence or absence of Δ2 using fluorescence microscopy. Remarkably, Δ2 caused a clear relocation of both endogenous and exogenous RXRα into PML-CB (Figure 4.2.5F). Conversely, in control cells RXRα displayed an almost exclusively nuclear diffused distribution and did not show accumulation in cytoplasmic speckles (Figure 4.2.5F and not shown).

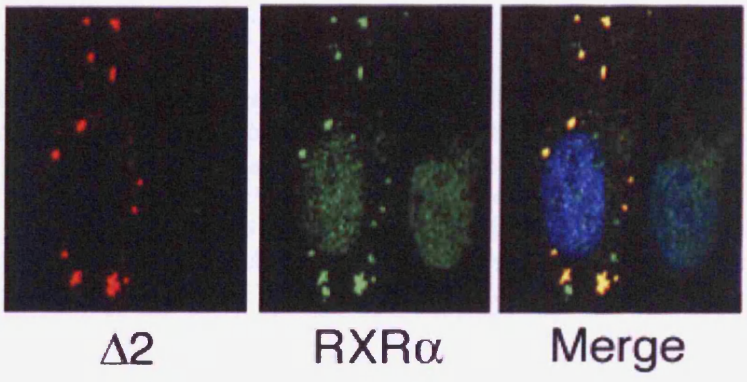


Figure 4.2.5F RXRα is delocalized to PML-CB by cytoplasmic Δ2 PML-RARα.

Cytoplasmic PML-RARα causes the RXRα relocation to PML-CB. COS-1 cells were transduced with HA-tagged Δ2, fixed and stained with anti-HA (red) and anti-RXRα (green) antibodies. Nuclei were counterstained with DAPI. Slides were analyzed by using a confocal microscope.

To determine whether the cytoplasmic delocalization RXRα is required for the transcriptional inhibition mediated by Δ2, a mutated version of Δ2 lacking the ability to interact with RXRα was generated. More precisely, the essential residues involved in RXRα binding (Zhu et al., 2005), the methionine (M) at position 883 and the threonine (T) at position 886 were mutated into arginine (R) to generate the Δ2 M883R/T886R mutant unable to bind RXRα.

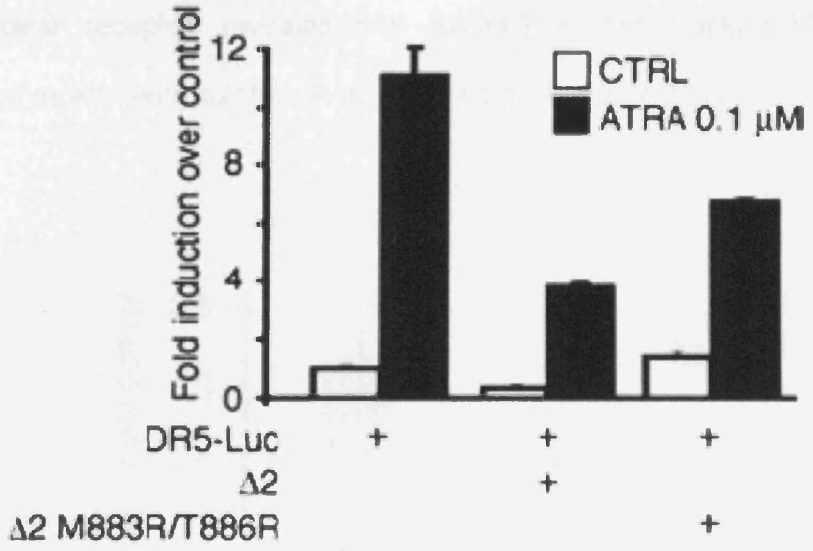


Figure 4.2.5G RXRα binding is important for Δ2-mediated RA-dependent transcription. RXRα is important for Δ2 inhibition of RA transcription. Mutation of M883R and T886R impair the RXRα binding ability of Δ2. The activity of a DR5-Luc reporter was assessed along with Δ2 or Δ2 M883R/T886R (mutant unable to bind RXRα) in transduced cells cultured in the absence or presence of 0.1 μM ATRA for 24 hours. β-galactosidase (β-gal) activity of a β-gal control vector was used to normalize the luciferase intensity in each sample. Data are mean ± SEM of three independent experiments expressed as fold induction over the value of the untreated control sample.

Subsequently, the transcriptional properties of Δ2 M883R/T886R were tested in the presence or absence of ATRA in COS-1 cells. Interestingly, the Δ2 M883R/T886R was less potent in inhibiting RA-dependent transcription (6.8 ± 0.1) as compared to the

original Δ2 (3.8 ± 0.1), thus suggesting that recruitment of RXRα is at least in part required for transcriptional repression (Figure 4.2.5G). Indeed, as the rescue of RA-dependent transcription is incomplete there is the possibility that additional alternative mechanisms might be involved. To further corroborate these findings, the activity of vitamin D_3 (VD_3), which relies on the function of RXRα (Mangelsdorf and Evans, 1995), was analyzed. Notably, it has been shown that VD_3-dependent transcription is inhibited by PML-RARα (Grignani et al., 1993b), thus it is plausible that also Δ2 may have a similar effect. Indeed, transcriptional assays carried out in the presence or absence of 1 μM dihydroxyvitamin D_3 using a VDR luciferase reporter vector, specific for the VDR-RXRα nuclear receptor, revealed that Δ2 (1.2 ± 0.4) blocked VD_3-dependent transcription as efficiently as PML-RARα (1.1 ± 0.1) (Figure 4.2.5H).

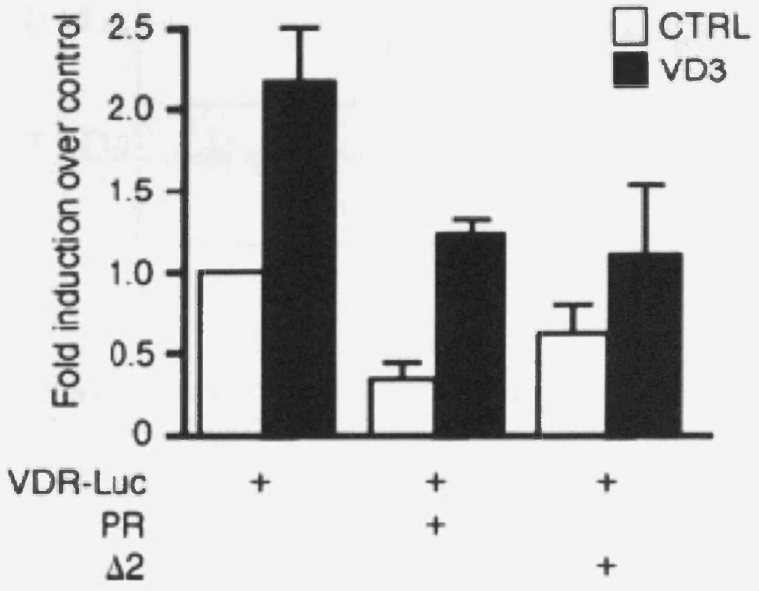


Figure 4.2.5H Δ2 inhibits vitamin D3 (VD3)-induced transcription. The activity of a VD3 responsive (VDR)-luciferase reporter was tested in COS-1 transduced cells alone or in combination with *bcr1* PML-RARα or Δ2. Cells were cultured with 1 μM VD3 for 24 hours and luciferase was measured in the extracts. β-galactosidase (β-gal) activity of a β-gal control vector was used to normalize the luciferase intensity in each sample. Data show are mean ± SEM of three independent experiment expressed as fold induction over the value of the untreated control sample.

4.2.6 Cytosolic PML-RARα inhibits differentiation of haematopoietic cells

Retinoids are potent inducers of differentiation, an effect that is associated with the RA-dependent inhibition of cellular proliferation. Therefore, we theorized that in addition to transcription, Δ2 could also alter RA-induced differentiation. To test this, U937 cells, a promyelocytic tumour cell line that does not express PML-RARα, were transduced with control and Δ2 retroviruses. Subsequently, single cellular clones that expressed high levels of Δ2 were selected by clonal dilution of the total population of infected cells (Figure 4.2.6A).

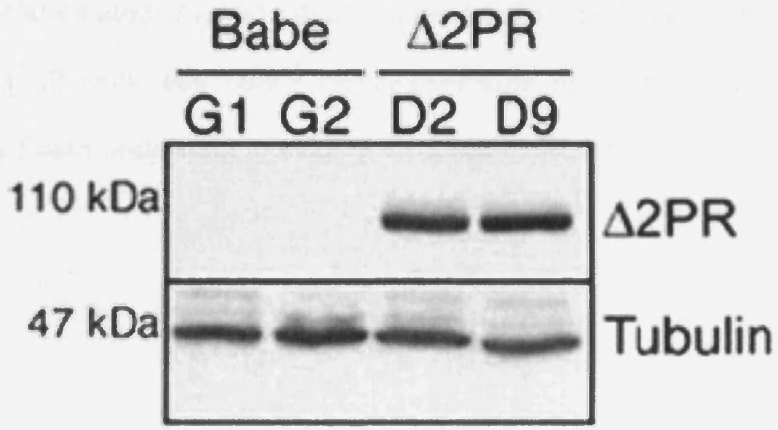


Figure 4.2.6A Cytoplasmic PML-RARα expression in retrovirally-transduced U937 cells. U937 cells were infected with control (Babe) or HA-tagged Δ2 retroviruses. Cells were selected with puromycin and two clones for Babe (G1 and G2) and Δ2 (D2 and D9) were isolated by clonal dilution of the cells. Protein levels were measured in the extracts probing the membrane with anti-HA antibody.

Specifically, two clones for control (referred to as G1 and G2) and Δ2 (referred to as D2 and D9) were analyzed for their sensitivity to ATRA-induced differentiation (Figure 4.2.6A-B).

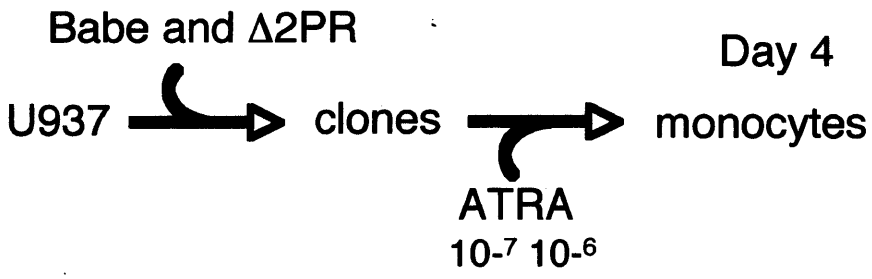


Figure 4.2.6B Overview experimental settings to assess the effect of cytoplasmic PML-RARα on RA-induced differentiation.

To this end, cells were cultured in the presence or absence of ATRA for 4 days and differentiation was studied by morphological analysis of the cells and by measuring the levels of the myelomocytic marker CD11b, which is highly expressed on the cell surface of differentiated myeloid cells (Figure 4.2.6B). Strikingly, the morphology of ATRA-treated Δ2 cells was nearly indistinguishable from untreated cells, whereas vector-infected cells underwent to evident morphological changes (Figure 4.2.6C).

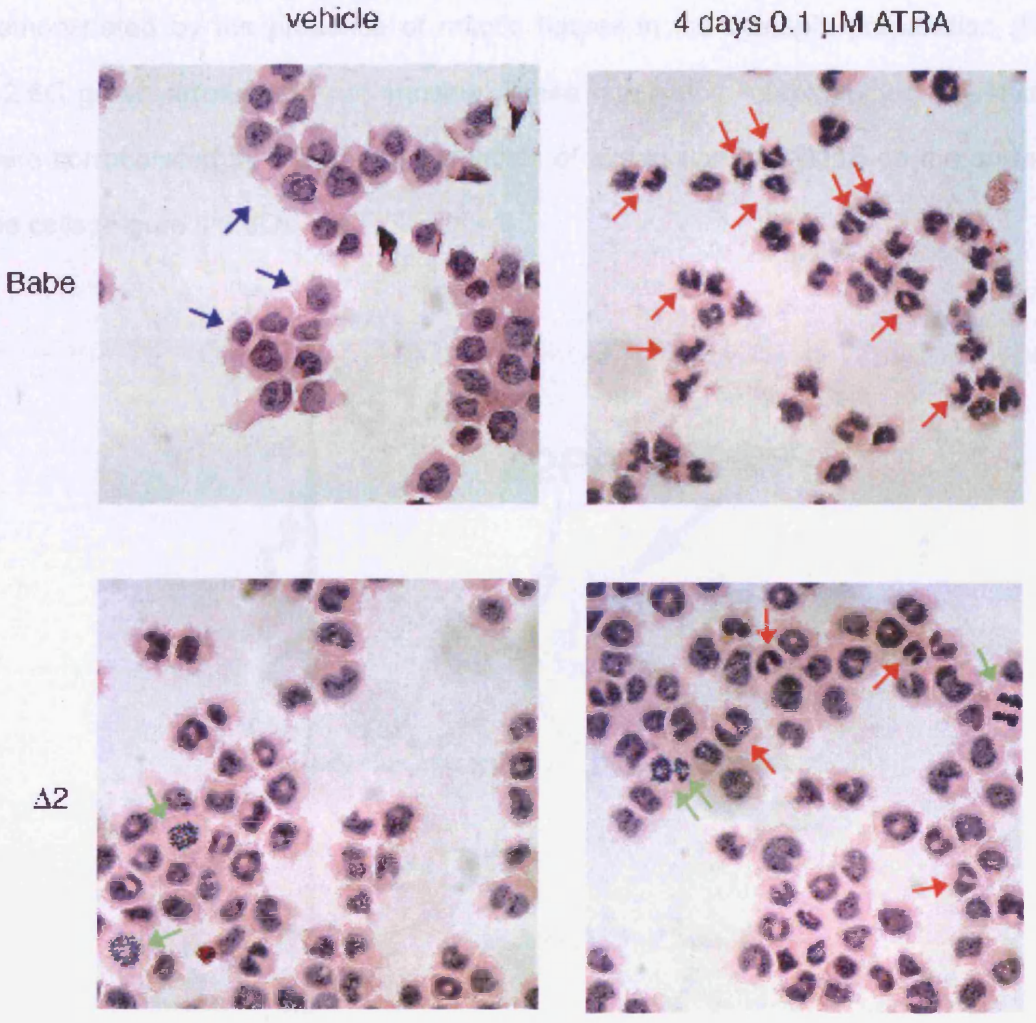


Figure 4.2.6C Cytoplasmic PML-RARα impairs RA-induced differentiation. Morphological analysis of U937 cells transduced with empty vector (Babe) or Δ2 retroviruses. Cells were treated with vehicle or 0.1 μM ATRA for 4 days, cytospun and stained with hematoxylin-eosin (H/E). Arrows indicate: undifferentiated cells (blue), differentiated cells (red) and mitotic figures (green).

Indeed, empty vector (Babe) cells in the presence of ATRA became smaller than untreated cells, which is a direct effect of the differentiation. More precisely, the nucleus, big and round in promyelocytes (blue arrows), became indented with an evident bean-shape, that is typical of mature monocytes (Figure 4.2.6C, red arrows). Strikingly, proliferation was not substantially blocked by ATRA in Δ2 cells, which

continue to proliferate even when treated with high concentrations of ATRA as demonstrated by the presence of mitotic figures in the cytospin preparation (Figure 4.2.6C green arrows and not shown). These interesting morphological observations were corroborated by measuring the levels of expression of CD11b on the surface of the cells (Figure 4.2.6D).

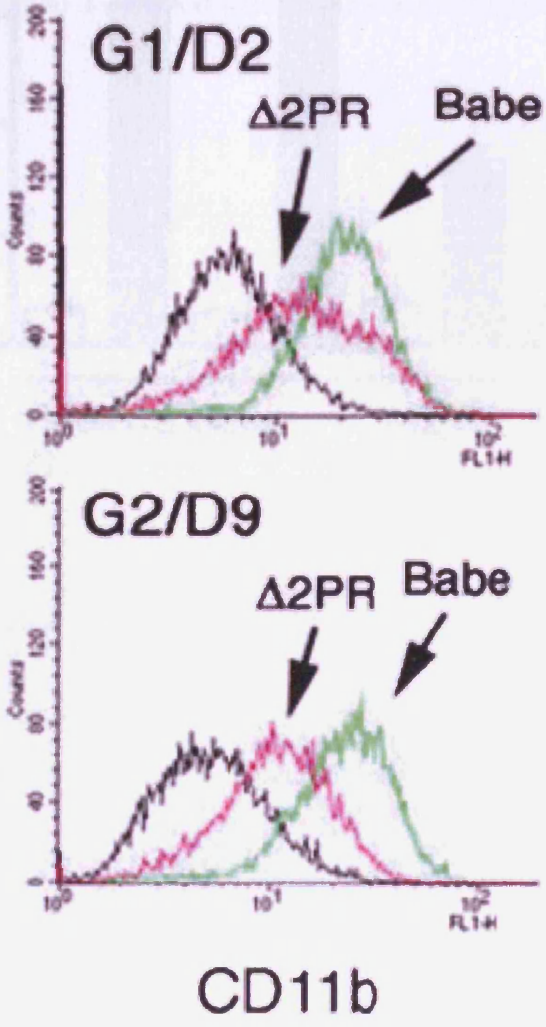


Figure 4.2.6D Cytoplasmic PML-RARα inhibits RA-dependent differentiation. Δ2-expressing cells (D2 and D9) and control (G1 and G2) clones were treated with 0.1 μM ATRA and analyzed for the expression of the differentiation marker CD11b. Flow cytometry traces of untreated (black), Babe control (green) and Δ2 (purple) cells stained with a FITC-conjugated anti-CD11b antibody.

Accordingly, a significant reduction, approximately 40%, of the percentage of mature cells (CD11b positive) was evident in both Δ2 cellular clones as compared to control cells that instead nicely expressed the marker on their surface (Figure 4.2.6E).

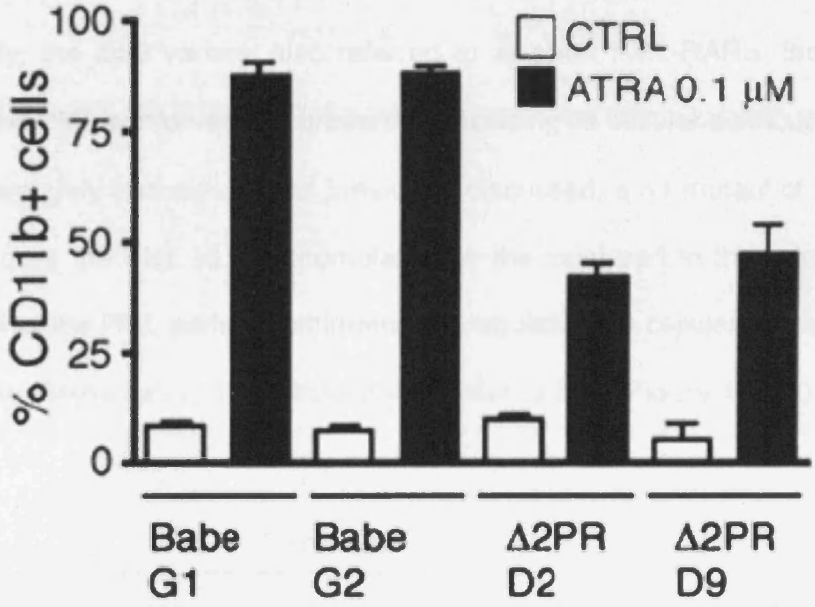


Figure 4.2.6E Cytoplasmic PML-RARα inhibits RA-dependent differentiation. Histogram showing the percentage of CD11b-positive cells in control and D2 clones cultured in the presence or absence of 0.1 μM ATRA for 4 days as described above (Figure 4.2.6D). Percentages were calculated as number of CD11b positive cells over the total number of cells in each sample. Measurements were performed plotting side scatter (SSC) over the intensity of the fluorescence 1 channel (FL1). Data are average ± range of two independent experiments.

Altogether, these results suggest that cytoplasmic PML-RARα can interfere with the physiological function mediated by ATRA on transcription and development.

4.2.7 *bcr3* PML-RARα largely localizes to the cytoplasm and colocalizes with RXRα

As mentioned in the introduction, depending on the breakpoint in the *PML* gene two predominant variants of PML-RARα are found in APL patients, referred to as *bcr1* and *bcr3.* Notably, the *bcr3* variant, also referred to as short PML-RARα, lacks the NLS retained in the PML portion and its properties, including its cellular distribution have not yet been thoroughly investigated. As previously discussed, a Δ1 mutant of PML-RARα, which also lacks the PML NLS, accumulated for the most part in the cytoplasm, thus suggesting that the PML portion dominates and regulates the cellular distribution of the fusion protein. Remarkably, Δ1 is structurally similar to *bcr3* (Figure 4.2.7A).

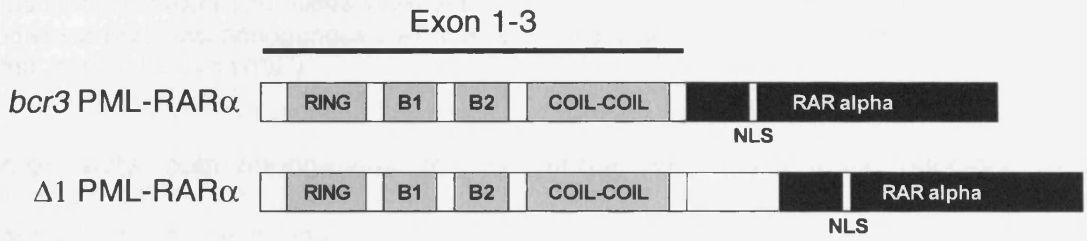


Figure 4.2.7A Schematic of *bcr3* PML-RARα and the single NLS mutant Δ1. *bcr3* is the short isoform of PML-RARα which is expressed in patients carrying a translocation involving a breakpoint in the intron 3 of PML (top). Therefore in the resulting fusion protein PML exon 3 is translocated in frame with the RARα portion. Notably, *bcr3* lacks the NLS signal present in the exon 6 of PML. *bcr-3* structure shows similarity with Δ1 mutant of *bcr-1* PML-RARα in which the PML NLS sequence has been mutated. Interestingly, Δ1 expression results in a pronounced cytoplasmic distribution of the protein (as described in figure 4.2.5B).

Thus, it is plausible that as for Δ1, *bcr3* could also be predominantly found in the cytoplasm. To test this, *bcr3* was transduced in COS-1 cells and the distribution of the protein was analyzed by confocal microscopy (Figure 4.2.7B). Indeed, the analysis of *bcr3*-expressing cells revealed that this isoform of PML-RARα accumulates mainly in cytoplasmic bodies (Figure 4.2.7B).

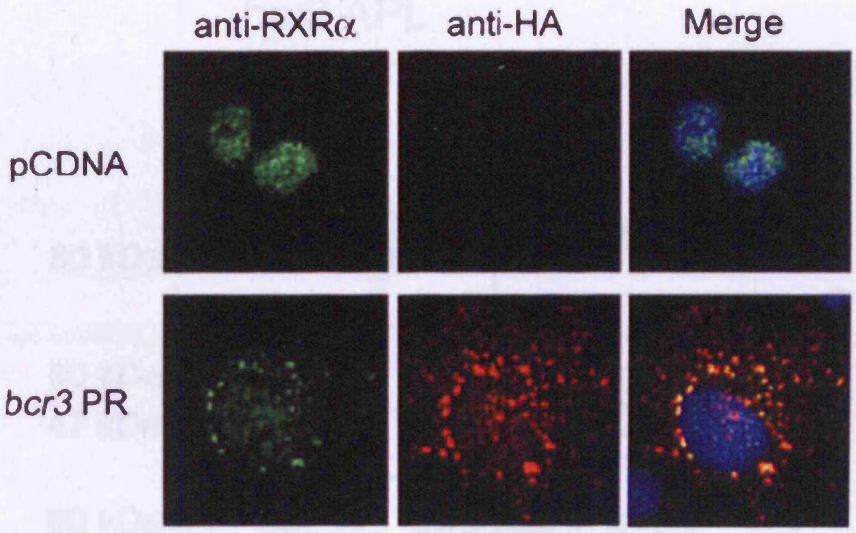


Figure 4.2.7B *bcr3* PML-RARα accumulates in the cytoplasm and delocalizes RXRα. COS-1 cells were transduced with empty or HA-*bcr3* PML-RARα expression vectors. Cells were fixed and probed with anti-RXRα (green) and anti-HA (red) antibodies. DAPI were used to counterstain nuclei and slides were analyzed by using a confocal microscope. Colocalization between *bcr3* and endogenous RXRα is represented by the yellow-orange dots in the merge image (right bottom panel).

Importantly, both endogenous and exogenous RXRα was found relocated to the cytosol only in the presence of *bcr3*. Similarly, the co-expression of RXRα and *bcr3* confirmed that the two proteins colocalized in the cytoplasm (Figure 4.2.7B and not shown). In keeping with these finding, it can be hypothesized that *bcr3* and Δ2 may share common inhibitory pathways. Thus, PML-RARα sub-cellular distribution was investigated in primary APL cells isolated from a patient carrying the translocation t(15;17), which was carrying the *bcr3* breakpoint. The type of breakpoint was assessed by RT-PCR as described elsewhere by Huang and collaborators (Huang et al., 1993) (not shown). Remarkably, sub-cellular fractionation of primary blasts clearly demonstrated that the majority of PML-RARα accumulated in the cytoplasmic fractions (Figure 4.2.7C). In addition, the analysis confirmed that also RXRα was distributed in the cytosolic fractions of primary APL cells (Figure 4.2.7C).

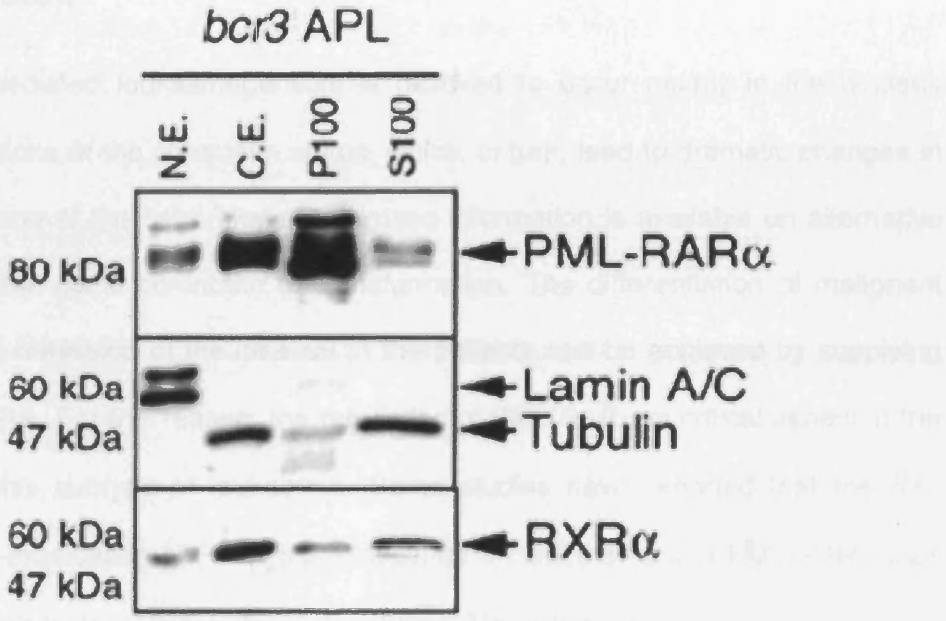


Figure 4.2.7C *bcr3* PML-RARα accumulates in the cytosolic fraction of primary APL cells. APL cells isolated from a patient carrying the *bcr3* translocation were fractionated into nuclear extracts (N.E.) and cytosolic fractions (total cytoplasmic extracts (C.E.)), pellet (P100) and supernatant (S100). Filters were probed with anti-RARα, anti-RXRα and lamin A/C anti-tubulin antibodies.

This is the very first evidence demonstrating that PML-RARα accumulates in the cytoplasm *in vivo*. For this reason, these data represent a key finding of this work and strongly support our hypothesis that cytoplasmic PML-RARα may alter transcription and differentiation independently from chromatin-remodelling phenomena.

4.3 Discussion

PML-RARα mediated leukaemogenesis is believed to occur mainly in the nucleus through alterations of the chromatin status, which, in turn, lead to dramatic changes in the transcriptome of the cells. However, limited information is available on alternative mechanisms that could contribute to transformation. The differentiation of malignant blasts and the remission of the disease in the patients can be achieved by supplying high levels of RA. For this reason, the regulation of PML-RARα is critical aspect in the treatment of this subtype of leukaemia. Some studies have reported that the RA-induced down-modulation of PML-RARα depends on whether or not PML-RARα was efficiently bound to the DNA (Zhu et al., 2005). Nevertheless, it is currently unclear whether the stability of the protein relies on its association with different cellular compartments. In the previous section the function of Mut PML, recently identified in a RA-resistant APL patient, was analyzed in respect to the PML-NB tumour suppressive functions. Nevertheless, it is not known whether Mut PML could function in the context of APL. Data presented here suggest that Mut PML could modulate the repressive functions of PML-RARα. Specifically, Mut PML and PML-RARα colocalize and interact in both adherent cells and malignant blasts. Surprisingly, Mut PML potentiates PML-RARα-mediated repression of RA-dependent transcription, thus suggesting that cytoplasmic localization of PML-RARα may function in the absence of a direct effect on chromatin. Furthermore, Mut PML augments the differentiation block exerted by PML-RARα in response to pharmacological concentrations of retinoic acid. In this regard, Mut PML hampers RA-mediated degradation of PML-RARα, thus providing a mechanistic explanation for the increased refractoriness to RA. Based on these findings, it can be theorized that Mut PML interferes with the proteasome-mediated degradation of PML-RARα, a phenomenon that is still poorly understood. Nevertheless, it cannot be ruled out that Mut PML may also affect other modifications

of the fusion protein. In this regard, it has been recently demonstrated that sumoylation of PML-RARα is required for its transforming activity (Zhu et al., 2005). It would be therefore interesting to test whether Mut PML affects the SUMOylation status of PML-RARα. Furthermore, it has been shown that NE cleaves PML-RARα leading to the cytoplasmic accumulation of the PML portion. Interestingly, this proteolytic event appears to be required for leukaemogenesis (Lane and Ley, 2003). Thus, it would be important to test whether Mut PML is able to interfere with the function of NE or other proteases such as CG and PR3, thus preventing the cleavage and stabilizing PML-RARα. This aspect warrants more future investigations. As previously showed (Kastner et al., 1992; Khan et al., 2004; Koken et al., 1994; Perez et al., 1993), over-expression of PML-RARα results in both nuclear and cytoplasmic localization. One of the outstanding questions in the APL field is whether or not PML-RARα bears alternative functions. To address this question we generated a cytoplasmic mutant of the fusion protein, Δ2, by site direct mutagenesis of the nuclear localization sequences present in PML and RARα portions. Albeit, Δ2 almost exclusively localizes to the cytoplasm, still retains the ability to inhibit RA-dependent transactivation of a DR5-luciferase reporter. Indeed, Δ2 and the full-length PML-RARα display a comparable repressive activity of RA-induced transcription. Interestingly, Δ2 appears to be less sensitive to RA-dependent degradation than its *bcr1* counterpart, thus suggesting that cytoplasmic localization may hamper the RA-mediated proteasomal degradation of the fusion protein. However, more investigations are needed to clarify this aspect. Furthermore, we also found that RXRα, the nuclear receptor partner of RARα, is relocated and colocalizes with Δ2 in discrete cytoplasmic foci. Accordingly, previous studies proposed that cytoplasmic titration of RXRα results in its transcriptional inactivation (Perez et al., 1993). Interestingly, we found that Δ2-mediated sequestration of RXRα accounts for part of the repressive activities of this cytoplasmic isoform.

Notably, a mutant of Δ2 unable to bind RXRα is less potent in repressing RA-mediated transcription. However, as the transcriptional rescue was not complete, there is the possibility that additional inhibitory mechanisms might be involved. Furthermore, we also found that ATRA-induced differentiation of U937 cells is impaired in the presence of Δ2. Indeed, Δ2 decreases the percentage of CD11b-positive differentiated cells by approximately 40% in the presence of pharmacological concentrations of RA. In addition, Δ2 also abrogates the growth suppressive functions of RA. In fact, Δ2 expressing cells continue to proliferate even at very high concentrations of ATRA (1 μM). Remarkably, we had the possibility to analyzed primary blasts derived from an APL patient carrying the *bcr3* breakpoint. Biochemical analysis of these cells provided the first *in vivo* evidence showing that a large portion of PML-RARα is found in the cytoplasm. Notably, we were able to demonstrate that *bcr3* PML-RARα and RXRα accumulates in the same cytoplasmic fractions in primary leukaemic cells. These findings are of paramount importance for our hypothesis and contribute to clarify the mechanisms underlying PML-RARα-dependent leukemogenesis.

Chapter 5

"PML-RARα cytoplasmic functions in myeloid progenitor and primary haematopoietic stem cells"

5.1 Introduction

In the previous chapter two major findings have been achieved: i- the first evidence of cytoplasmic accumulation of PML-RARα *in vivo* and ii- the demonstration that PML-RARα is also able to inhibit RA-dependent transcription and differentiation from the cytoplasm. Altogether these data strongly support our hypothesis. As the majority of the data has been so far obtained by using cell lines, it would be extremely important to assess whether these observations can be confirmed in physiological settings. Therefore, in this chapter we will test the function of Δ2 in i- a non-tumorigenic murine cellular model, 32Dcl3 myeloid precursor cell line (32D) (Valtieri et al., 1987) and ii- in primary haematopoietic progenitors cells. In particular, proliferation and differentiation can be modulated in 32D cells, by culturing the cells in the presence of two different cytokines: interleukin-3 (IL-3) and the granulocytic-colony stimulating factor (G-CSF), respectively. IL-3 induces the cells to proliferate and its removal from the culture media triggers caspase-dependent apoptosis (Hamilton et al., 2001; Valtieri et al., 1987). Alternatively, cells can be differentiated into mature granulocytes within 5 to 10 days in the presence of G-CSF, which is indeed a potent inducer of granulocytic differentiation in both human and murine bone marrow cells (Figure 5.1A).

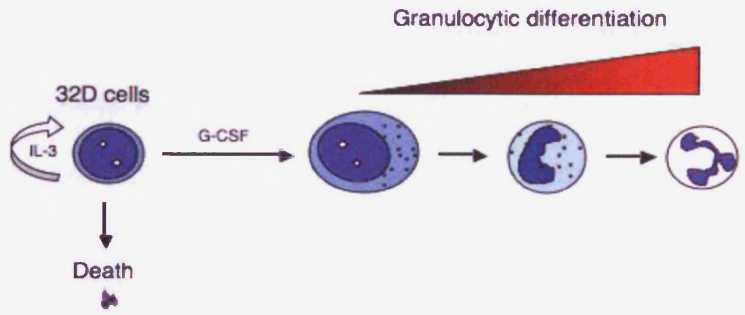


Figure 5.1A 32D cellular model.
32D cells consist of mouse myeloid precursor cells that grow in IL-3 dependent manner. Cells cultured in the presence of G-CSF differentiate in mature granulocytes within 5 to 10 days. Alternatively, if IL-3 is removed from the culture, cells undergo apoptosis.

Notably, the kinetics of differentiation of these cells mimics the physiological process occurring in the normal marrow. For this reason, we decided to use 32D cells as a model to study the effects of Δ2 on myeloid differentiation. As previously mentioned, no information is available on the leukaemogenic potential of *bcr3* PML-RARα *in vivo*. Indeed, all animal models of APL currently available have been generated using *bcr1* isoform (Rego and Pandolfi, 2001; Westervelt et al., 2003). Thus, in the second part of this chapter, we will analyze the effect of Δ2 on primary haematopoietic murine progenitors. In adult mammals, all the cellular elements of the blood arise from somatic haematopoietic stem cells (HSCs) residing in the bone marrow. HSCs possess the unique property of self-renewal and also, through cell division and differentiation give rise to progenitor cells committed toward all the main haematopoietic lineages. Importantly, progenitors cells are referred to lineage minus (Lin^-) cells as they do not expressed any of the lineage-specific surface markers (Terskikh et al., 2003; Weissman, 2002). The direct descendents of HSC are the common lymphoid progenitor (CLP) and myeloid progenitor (CMP), respectively. The former give rise to lymphoid lineage-committed cells that, in turn, lead to mature B and T lymphocytes, whereas, the latter is the progenitor of all the different types of leukocytes (white blood cells), erythrocytes and megakaryocytes. Interestingly, a number of culture systems have been developed to study the proliferation of the committed progenitors toward the major marrow lineages. In this chapter, long-term colony forming assay of primary progenitor cells transduced with *bcr1* and Δ2 will be performed to compare their ability to grow in methylcellulose containing growth factors that promote myeloid differentiation. These data will possibly provide important information on the role of cytoplasmic PML-RARα *in vivo*.

5.2 Results

5.2.1 Generation of 32D clones stably expressing *bcr1* PML-RARα and its cytoplasmic mutant Δ2.

32D cells were transduced with PML-RARα and Δ2 using retroviral-based techniques. Initially, cDNAs corresponding to Δ2 and PML-RARα were subcloned into the retroviral expression vector MigR1, a mouse stem cells viral vector (MSCV), in order to produce highly efficient amphotropic retroviruses able to infect human as well as mouse cells. This transduction system allows the expression of both: the gene of interest, under the control the 5' viral long terminal repeat (5' LTR) and the enhancing green fluorescent protein (eGFP) downstream the internal ribosomal entry site (IRES) (Figure 5.2.1A).

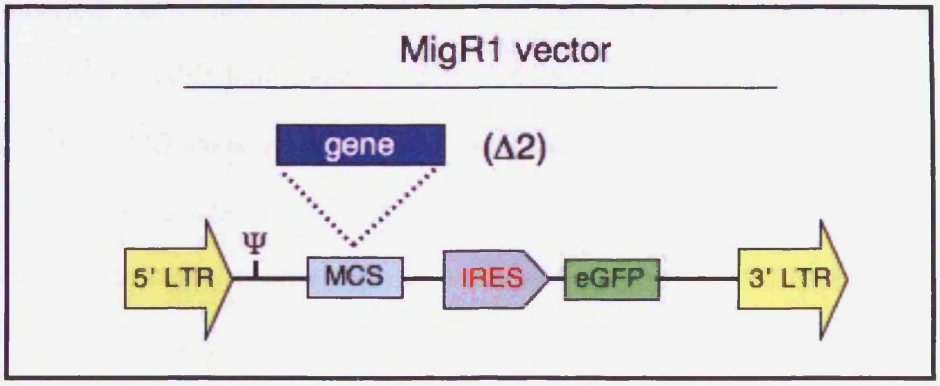


Figure 5.2.1A MigR1 backbone schematic structure. Scheme summarizing the structure of MigR1 expression cassette. cDNA encoding for the gene of interest (i.e. Δ2) is inserted upstream an IRES-GFP cassette, so that transduced cells co-express the transgene along with the GFP. MCS, multiple cloning sites, IRES, internal ribosomal sequence, eGFP, enhanced green fluorescence protein, 5'/3' LTR, long terminal repeat, Ψ, packaging sequence.

Infected cells can be monitored and sorted based on their ability to express the GFP, and finally plated for the relevant assays (Figure 5.2.1A). Notably, the different retroviral preparations were first tested in 293T cells before being used for the infection

of 32D cells (Figure 5.2.1B). Preliminary experiments confirmed that infected 293T cells expressed both transgenes: *bcr1* PML-RARα and Δ2 (Figure 5.2.1B).

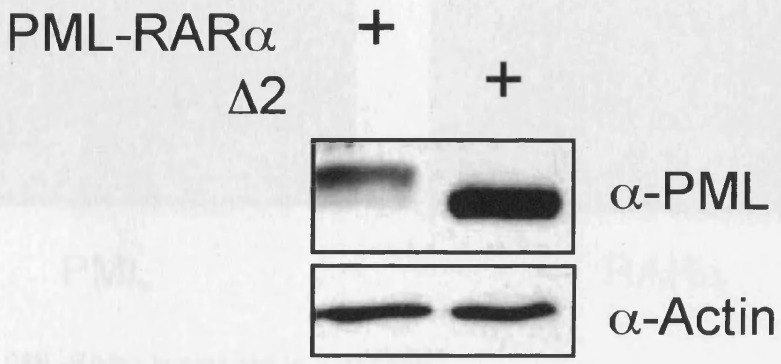


Figure 5.2.1B Retroviral transduction of cytoplasmic PML-RARα.
Retroviruses encoding for PML-RARα and Δ2 were tested in 293T cells before being used to infect haematopoietic cells. Protein levels were assessed using an anti-PML antibody. Actin was used as loading control.

In all infected cells, the expression of the transgene was accompanied by the expression of the GFP that could be detected using a fluorescence microscope (not shown). Thus, 32D cells were infected with control, PML-RARα and Δ2 retroviral particles and GFP-positive cells, sorted and plated at clonal density in order to isolate single cell clones highly expressing the transgene. A number of different clones from vector, PML-RARα (*bcr1*) and Δ2 were screened by immunostaining and western blot and two Δ2 clones (referred as C3 and C6) were identified. Conversely, it was no possible to generate any PML-RARα clone. Indeed, out of 96 clones, only a few showed a PML and/or a RARα nuclear and/or cytoplasmic staining that, however, never colocalized (i.e. clone B23, Figure 5.2.1C). This suggests that the *bcr1* is extremely unstable in these cells.

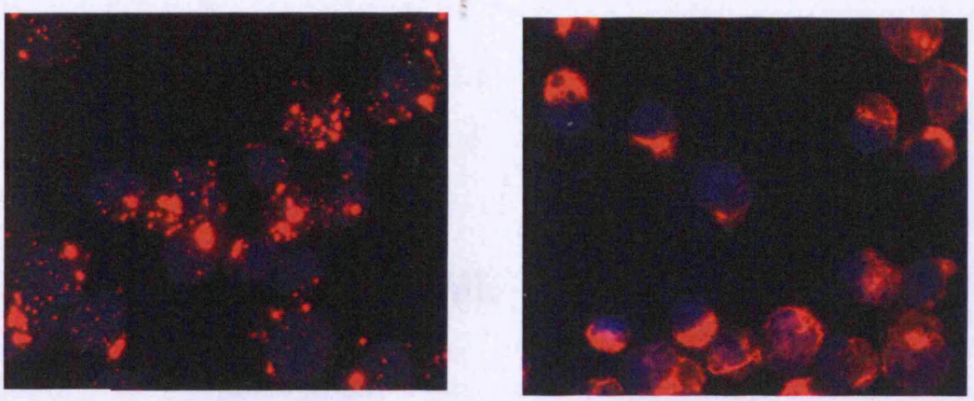


Figure 5.2.1C PML-RARα is cleaved in 32D cells.
Expression of PML-RARa in myeloid precursor cells give rise to different PML and RARa localization patterns. Immunofluorescence analysis of 32D cells (clone B23) infected with PML-RARα retroviral particles, cytospun and stained with anti-PML (left) and anti-RARα (right) antibodies. Nuclei were counterstained with DAPI (blue).

Accordingly, western blot analysis of one clone transduced with *bcr1* retroviruses, referred to as B23, confirmed that *bcr1* is cleaved to produce two faster migrating PML and RARα fragments (Figure 5.2.1D). Another clone, B29, carried only a RARα fragment (not shown), thus suggesting that in these cells different enzymatic activity/ies might cleave the fusion protein. Indeed, promyelocytes are characterized by high levels of neutrophil elastase (NE), a serine protease that is expressed during the early stages of myeloid-differentiation. Importantly, NE has been shown to cleave PML-RARα and that this event may have a role in leukaemogenesis (Lane and Ley, 2003). Lane and collaborators demonstrated that NE recognizes a specific cleavage consensus motif located before the NLS in PML portion also recognizes alternative motifs that are spread along the aminoacidic sequence of the fusion protein (Lane and Ley, 2003). This would explain the presence of multiple cleavage fragments in the extracts of 32D clones transduced with PML-RARα. However, there is the possibility that NE may not be the only protease involved in PML-RARα proteolysis.

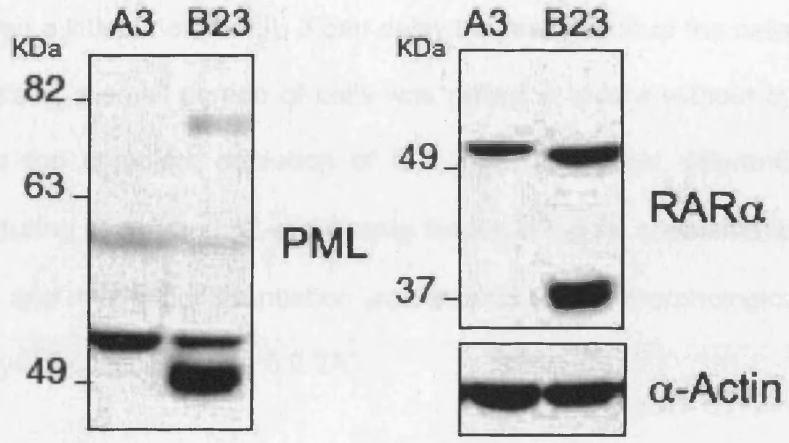


Figure 5.2.1D PML-RARα cleavage results in the generation of PML and RARα fragments. Analysis of protein extracts prepared from a cells retrovirally transduced with PML-RARα (clone B23). The membrane was probed using anti-PML (left) and anti-RARα (right up). Actin was used to normalize the protein levels.

It can be theorized that during differentiation PML-RARα might be cleaved by additional proteases that somehow cooperate with NE to the catabolism of the fusion protein (Lane and Ley, 2003). This aspect warrants further investigations. Furthermore, it would be extremely important to establish whether the resulting PML and RARα fragments, some of which display cytoplasmic localization (Figure 5.2.1C), contribute to the development of the leukaemia.

5.2.2 Cytoplasmic PML-RARα inhibits G-CSF-induced differentiation of transduced myeloid precursor cells (32D cells)

To study the effect of cytoplasmic PML-RARα expression on myeloid differentiation induced by G-CSF, two cellular clones for vector (A2 and A3) and Δ2 (C3 and C6) were selected (Figure 5.2.2A). Initially, cells were carefully washed out from all possible residuals of IL-3 and, subsequently cultured in the presence of G-CSF. The

removal of IL-3 is critical for the proper assessment of differentiation using this cellular model in fact even a little amount of IL-3 can delay the maturation of the cells. To avoid experimental pitfalls, a small portion of cells was plated in media without cytokines in order to assess the complete depletion of IL-3. Then, terminal differentiation was assessed by culturing vector and Δ2-expressing clones in media supplemented with 25 ng/ml of G-CSF and myeloid differentiation was determined by morphological analysis after 5 and 7 days of culture (Figure 5.2.2A).

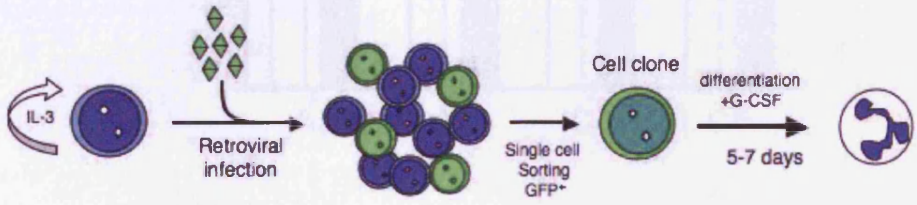


Figure 5.2.2A Schematic overview of the experimental settings.
32D cells were infected with control and Δ2 retroviral particles. GFP-positive cells were isolated using a cell sorter and 2 clones for control and Δ2 were used to study G-CSF induced myeloid differentiation. Specifically, the morphology of the cells was assessed after 5 or 7 days of culture by counting the number of terminally differentiated cells in the May-Grünwald preparations.

The expressions of Δ2 did not affect survival/proliferation of the cells during the differentiation. Indeed, no substantial differences in proliferation (not shown) or survival (Figure 5.2.2B) were found between G-CSF culture of control and Δ2-expressing cells.

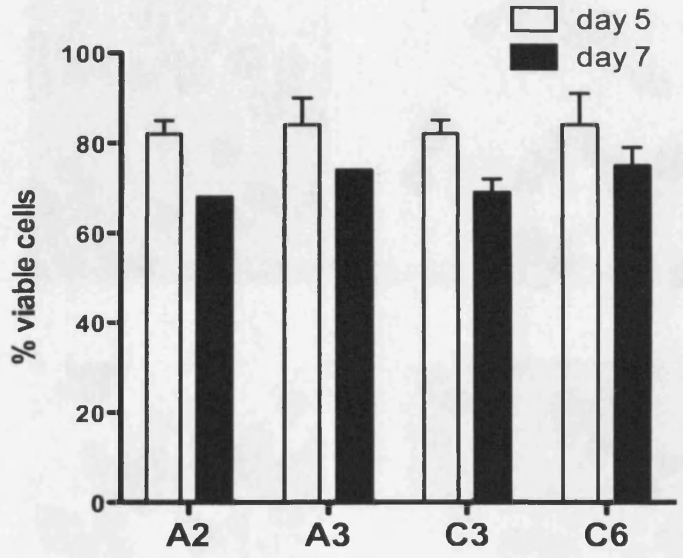


Figure 5.2.2B Cytoplasmic PML-RARα does not affect cell viability.
Control (A1 and A2) and Δ2 (C3 and C6) clones were culture in the presence of 25 ng/ml G-CSF and the percentage of viable cells was assessed after 5 and 7 days of culture. Viability was measured using trypan blue-exclusion assay. Data are mean ± SEM of three independent experiments.

May-Grünwal preparations revealed that a large portion of vector cells clearly underwent differentiation after 7 days of culture in the presence of G-CSF. In fact, vector-transduced myelocytes, which were reminiscent of large lymphocytes (rounded-shaped with an big ovoid nucleus), differentiated to neutrophils (smaller cells easily distinguishable for their multi-lobulate nucleus and clear cytoplasm) (Figure 5.2.2C). Conversely, Δ2 expressing cells appeared significantly less sensitive to G-CSF treatment. In fact, only a few mature neutrophils were found in the morphological preparations of these cells at day 7 (Figure 5.2.2C).

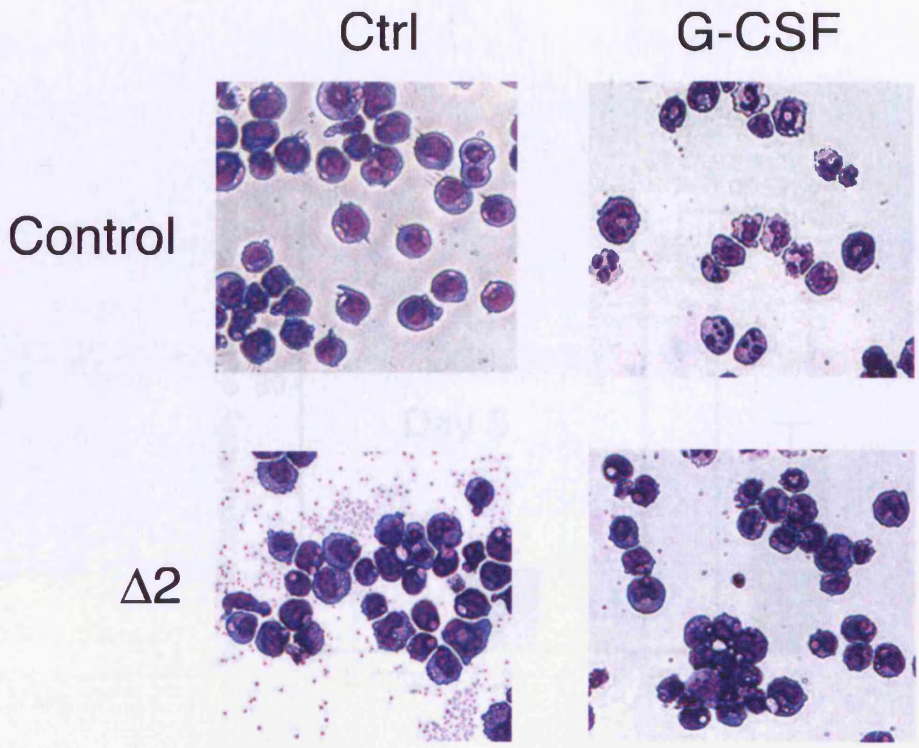


Figure 5.2.2C Cytoplasmic PML-RARα affects G-CSF-induced differentiation. Empty vector and Δ2 expressing cells were culture in the presence of IL-3 (left panels) or G-CSF (right panels). Cells were cytospun after 5 and 7 days and morphology was analyzed by using the May-Grunwald/Giemsa staining protocol.

These observations were corroborated by counting the number of terminally differentiated cells in control and Δ2 clones in the cytological preparations. More than 200 cells were counted for each clone at the different time-points (day 5 and 7 of differentiation) and experiments were performed in triplicate. The result of the cellular counts confirmed that the number of mature neutrophils was decreased in Δ2 compared to control cells as soon as after 5 days. Importantly, this negative trend became even more pronounced and statistically significative (P=0.0052) with days in culture. Indeed, at day 7 of differentiation, the number of differentiated cells was diminished of approximately 40% in Δ2 as compare to control cells (Figure 5.2.2D).

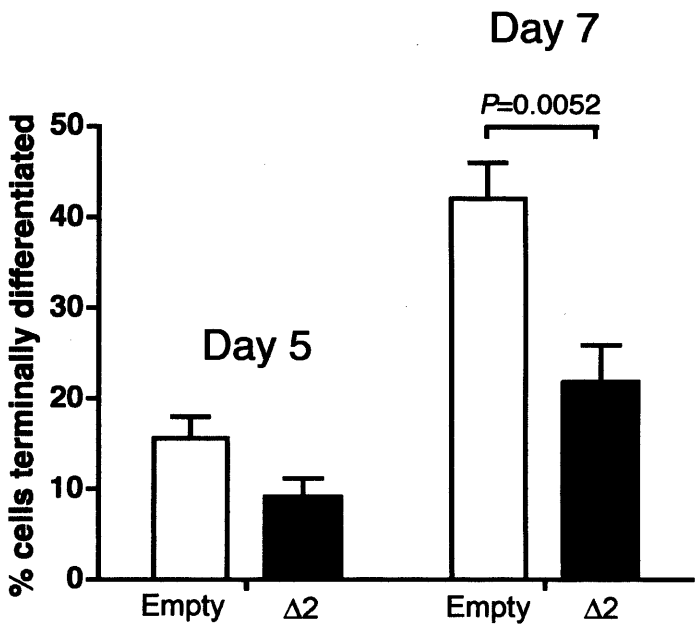


Figure 5.2.2D Cytoplasmic PML-RARα inhibits G-CSF-dependent differentiation of 32D cells. Cytoplasmic PML-RARα expression reduces number of mature neutrophils as compare to control cells. The number of terminally differentiated cells was determined in cytological preparations (Figure 5.2.2C) from empty vector and Δ2 infected cells, cultured in the presence of G-CSF (25 ng/ml). Cells were cytospun and stained by using May Grunwald/Giemsa protocol after 5 and 7 days. Data are mean ± SEM of three independent experiments. Statistical analysis was carried out using the un-paired *t*-test.

To further support our findings, the expression levels of three genes known to be upregulated during granulocytic maturation were analyzed using reverse transcription (RT) followed by quantitative real time PCR (qPCR). The first two genes analyzed were both belonging to the inhibitor of DNA binding (*ID1* and *ID2*) family of proteins, which were reported to heterodimerize with helix-loop-helix (HLH) transcription factors such as the stem-cell leukaemia/T-cell acute lymphoblastic leukaemia-1 (SCL/Tal-1) and lymphoblastic leukaemia derive sequence 1 (Lyl-1). It has been reported that IDs inhibit HLH-dependent transcriptional activation through the sequestration of HLH members into non-transcriptional active complexes. Indeed, HLH-ID dimers contained

an incomplete and therefore inefficient DNA binding region (Buitenhuis et al., 2005). *IDs* genes appeared to be widely expressed in both lymphoid as well as myeloid cells lines, however, their levels are differentially modulated throughout the haematopoietic development (Cooper and Newburger, 1998). Moreover, a study reported that ID1 mRNA was absent or expressed at very low levels in HSC and developing lymphoid and erythroid cells (Cooper et al., 1997). In contrast, ID1 expression increased in cells undergoing myeloid maturation, thus implying a role in controlling the specific commitment of myeloid cells (Leeanansaksiri et al., 2005). Another study indicated that ID1 was induced during early granulopoiesis but that the levels diminished in more terminally differentiated cells (Buitenhuis et al., 2005). ID2 mRNA levels were reported to be markedly increased during granulocytic differentiation of both primary and established human myeloid cells (Ishiguro et al., 1995; Ishiguro et al., 1996). A recent study conducted using APL cells demonstrated that both *ID1* and *ID2* were induced following ATRA treatment, thus suggesting a role for these proteins in the RA-dependent differentiation (Nigten et al., 2005). The third messenger analyzed was the lactoferrin (LF) a protein involved in the innate immune defence. Importantly, LF associates with the late secondary granules within the cytoplasm of granulocytes, and it is expression is consider one of the hallmarks of terminal neutrophil differentiation (Gupta et al., 2003). Furthermore, LF expression is controlled by the CCAAT-enhancer binding protein alpha and epsilon (C-EBPα and C-EBPε) and, is reduced during ATRA-induced differentiation of NB4 cells (Gupta et al., 2003). Thus, total RNA was prepared from vector and Δ2 cells at day 7, retro-transcribed into cDNA and used to perform a gene specific quantitative real-time PCRs (qPCR) (Figure 5.2.2E). Importantly, the results obtained using the qPCR, were in line with the morphological study previously described. Indeed, the levels of transcription of the three genes, *LF, ID1* and *ID2* were significantly diminished in cells expressing Δ2 as compared to control cells. Specifically, among the three messengers analyzed LF showed the most important

reduction in Δ2-expressing cells, almost a 50% reduction in Δ2 cells. Notably, LF expression is restricted to the very final steps of differentiation (Valtieri et al., 1987), thus it is conceivable that Δ2 might exerts its maximal inhibitory activity during the final stage of the myeloid maturation (Figure 5.2.2E).

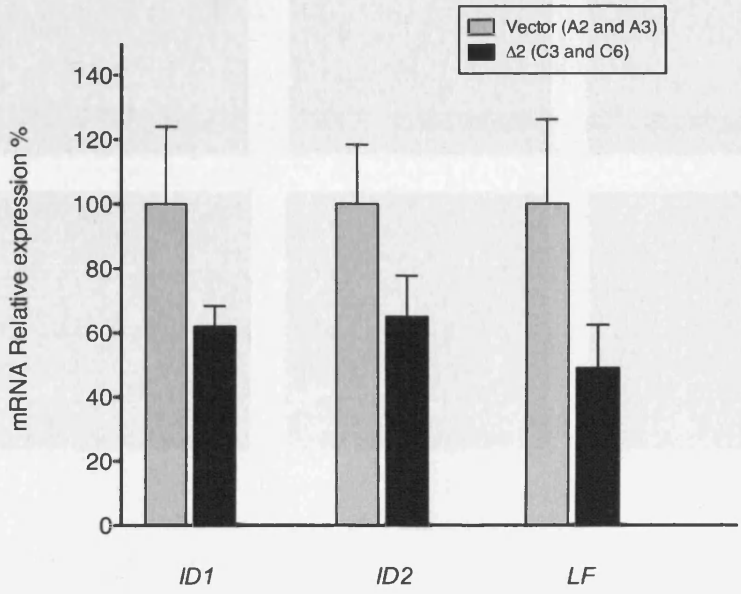


Figure 5.2.2E Cytoplasmic PML-RARα inhibits the expression of genes induced during myeloid differentiation. Total RNA was prepared from control and Δ2 clones cultured for 7 days in the presence or absence of G-SCF at concentration 25 ng/ml. The levels of inhibitor of differentiation ID1, ID2 and lactoferrin (LF) were measured by using quantitative real-time PCR (qPCR). Mouse GAPDH was used as internal mRNA housekeeping control. Each sample was analyzed in triplicate and values represent the means ± SD of two independent experiments.

The levels of myeloperoxidase (MPO), another gene up-regulated during differentiation (Valtieri et al., 1987), were also assessed and found decreased in the presence of Δ2 (not shown). Furthermore, immunofluorescence was performed to analyze the expression of Δ2 in the cells during differentiation. Confocal/immunofluorescence analysis of Δ2-expressing clones was performed using anti-human PML and anti-RARα antibodies (Figure 5.2.2F).

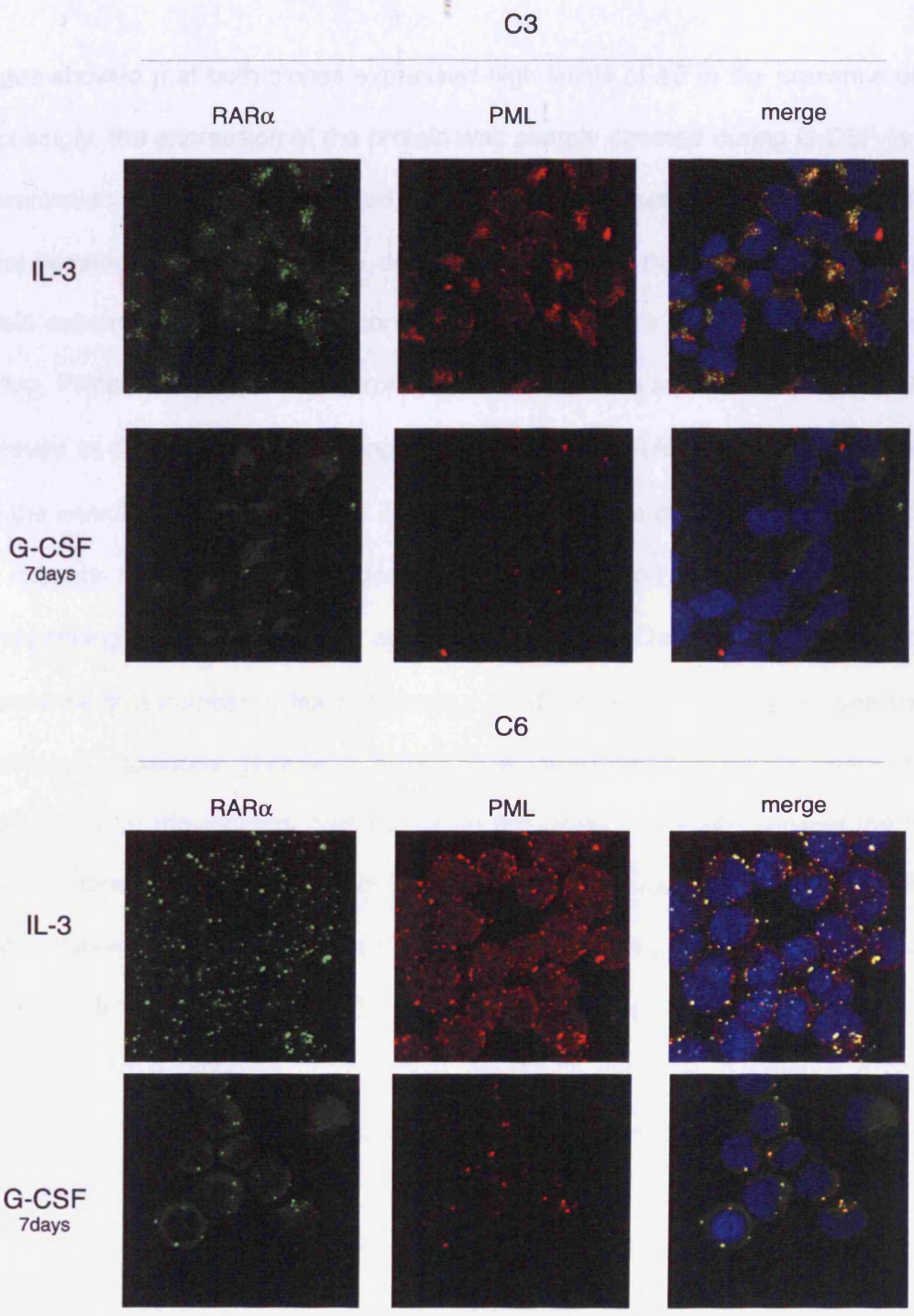


Figure 5.2.2F Δ2 is downmodulated during myeloid differentiation. The expression of Δ2 during G-CSF-induced granulocytic differentiation of 32D cells was analyzed by confocal/immunofluorescence. Cells from two different Δ2-expressing clones (C3 and C6) were cultured in the presence of IL-3 or G-CSF for 7 days. Cells were cytospun and stained by using anti-PML (red) and anti-RARα (green) antibodies. Nuclei were counterstained with DAPI (blue). Colocalization between PML and RARα signals is represented by yellow dots in the cytoplasm of the cells in the merge images (right column).

Images showed that both clones expressed high levels of Δ2 in the presence of IL-3. Surprisingly, the expression of the protein was sharply dimmed during G-CSF-induced differentiation (Figure 5.2.2F). Indeed, by using the same settings both PML and RARα signal became diffused and barely detectable, indicating that down-modulation of the protein occurred during differentiation. These findings were confirmed also by western blotting. Protein extracts from control and Δ2 clones were analyzed during the G-CSF treatment at day 0, 3, 5 and 7 using anti-PML and anti-RARα antibodies. In keeping with the immunofluorescence data, Δ2 protein levels were diminishing upon G-CSF in the extracts from C3 and C6 clones. Interestingly, the decrement of the band corresponding to the full-length Δ2, approximately 80-90 kDa, was accompanied by the appearance of a number of faster migrating bands, suggesting that a Δ2 was cleaved at different positions (Figure 5.2.2G). The degradation products were clearly accumulating in the extracts prepared from the clone C6, which showed the higher levels of expression of the cytoplasmic fusion protein (Figure 5.2.2G). Interestingly, the proteolytical-activity responsible for the G-CSF-dependent down-modulation of Δ2 had its maximal activity after 3 and 5 days of culture. This implied that the cleavage could be ascribed to a protease, for example neutrophil elastase, transiently expressed during myeloid differentiation. Despite a degree of Δ2 downmodulation during myeloid differentiation, these data indicate that cytoplasmic localization results in a significant increase in the stabilization of the fusion protein, This aspect warrants further investigation, and for instance inhibitors of different proteolytical activities could be exploited to understand the mechanism underlying Δ2 degradation during G-CSF-induced differentiation.

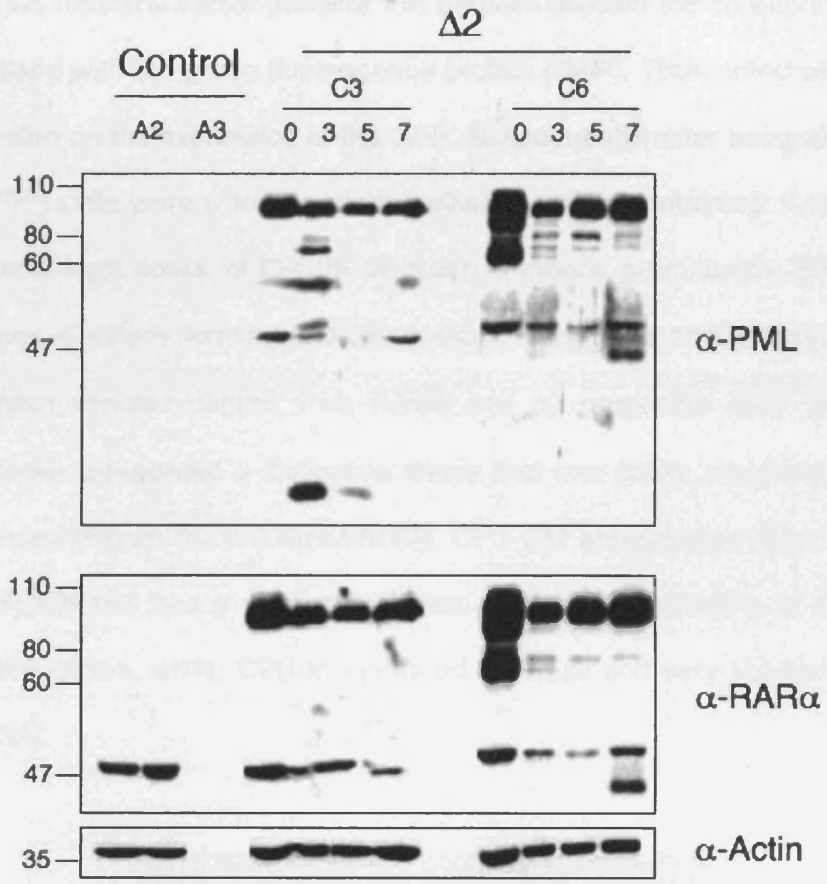


Figure 5.2.2G Δ2 is downregulated during myelopoiesis. Levels of expression of Δ2 were analyzed during G-CSF-induce myeloid differentiation. Protein extracts prepared from control (A1 and A2) and Δ2-expressing (C3 and C6) clones were analyzed using anti-PML (upper panel) and anti-RARα (middle panel) antibodies. Actin was used as loading control (lower panel).

5.2.3 Functions of cytoplasmic PML-RARα in primary haematopoietic progenitor cells

To test the effect of cytoplasmic PML-RARα on differentiation of primary haematopoietic progenitors, lineage negative (Lin-) cells were isolated from 6-8 weeks old mice, cultured and transduced with vector, PML-RARα (*bcr1*) and Δ2 retroviral particles. The infections were carried out in the presence of a cocktail of cytokines to maintain viabilitity and at the same time preserve the primitive state. As previously

mentioned, the retroviral vector used for this purpose allowed the co-expression of the transgene along with the green fluorescence protein (GFP). Thus, infected cells could be sorted based on the expression of the GFP. Subsequently, after being sorted, GFP-positive (GFP^+) cells were plated in methylcellulose media containing: IL-3, IL-6, GM-CSF, SCF and high doses of G-CSF in order to induce granulocytic differentiation. Different types of colony forming unit (granulocyte, monocyte and macrophage) were generated from infected control, PML-RARα and Δ2 progenitor cells. Notably, the different colonies possessed a distinctive shape that was easily recognizable using a light microscope (Figure 5.2.3A). Specifically, CFU-GM appeared as large and sparse colonies, CFU-GEMM had a very dense core surrounded by a ring of cells spread away from the centre, while, CFU-M appeared as small and very condense colonies (Figure 5.2.3A).

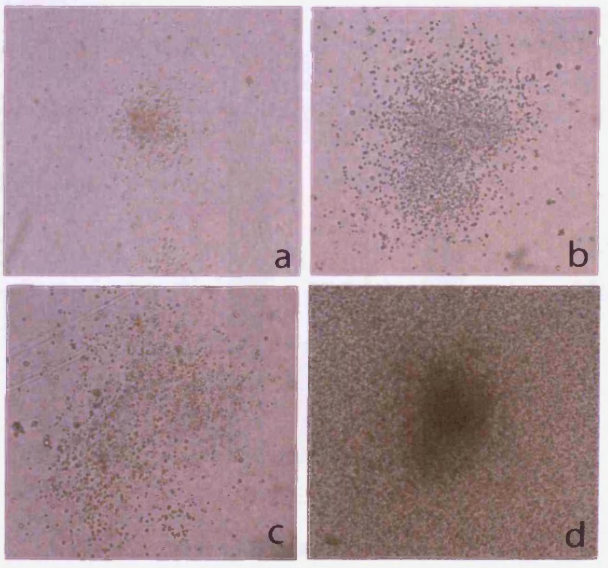


Figure 5.2.3A Morphological differences between the colony forming units (CFU) generated in methylcellulose. Representative images of colonies generated by lineage minus cells plated in methylcellulose in the presence of the following cytokines: 60 ng/ml G-CSF, 100 ng/ml SCF, 20 ng/ml GM-CSF, IL-3 and IL-6, respectively. Colonies were analyzed under a light microscope 8-10 days after plating. From the top: (a) CFU-M (monocyte), (b and c) CFU-GM (grunulocyte-macrophage) and (d) CFU-G (grunulocyte).

Importantly, the number of colonies originated directly mirrors the clonogenic potential, a feature related to the proliferative properties of the cells. Serial replatings of the cells is therefore of extreme importance to test whether a protein interferes with the maturation process leading to transformation of the progenitors cells. Thus, vector-, PML-RARα and Δ2-infected cells were plated on methylcellulose and colonies formed were counted 8 to 10 days later. Cells were recovered from methylcellulose and replated until no colonies were formed in vector-transduced cells, and this normally occurred after the third plating. Three independent experiments were performed and, as expected, in the presence of high concentrations of G-CSF control cells terminally differentiated as soon as after the first plating (Figure 5.2.3B).

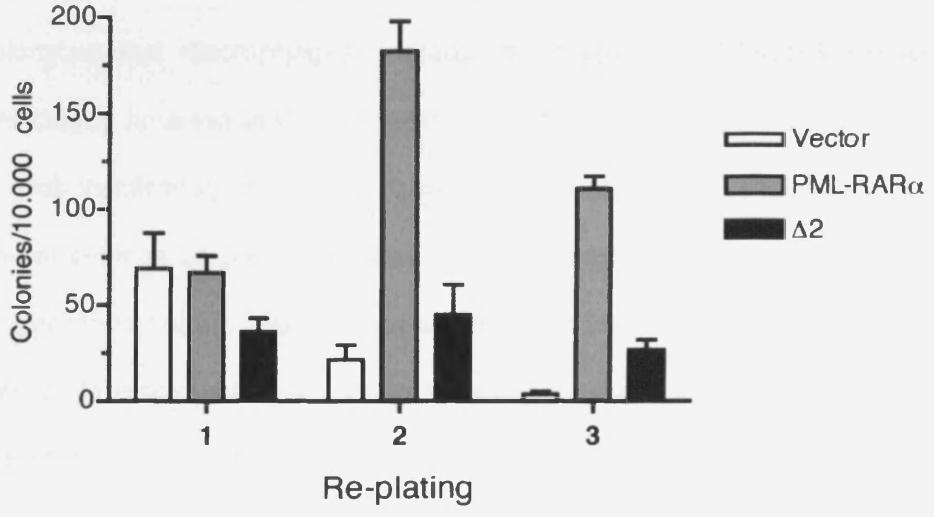


Figure 5.2.3B Cytoplasmic PML-RARα induces a partial transformation of primary haematopoietic progenitors. The number of colonies formed in vector, PML-RARα and Δ2 lineage minus cells was assessed after individual plating (approximately every 10 days). Data represent mean ± SEM of three independent experiments.

Conversely, the number of colonies arising from cells transduced with PML-RARα augmented over the passages indicating that these cells remained fully capable of generating colonies regardless to the presence of differentiating factors (Figure 5.2.3B). Expression of Δ2 led to different results (Figure 5.2.3B). Although the number of colonies formed throughout the serial passaging was substantially diminished, a residual clonogenic activity of Δ2-transduced cells was still noticeable at the third replating resulting in a number significantly higher than the control (P=0.043, t-test). However, this effect appears of a limited extent as compared to the strong transforming activity of the *bcr1* PML-RARα. Taken together, these data suggest that cytoplasmic PML-RARα may have a transforming capacity albeit limited when compared to *bcr1* PML-RARα. Primary progenitor cells were isolated from the methylcellulose at each replating in order to assess cellular morphology and to measure the expression levels of the fusion proteins. Remarkably, the cytological analysis of the cells after the first passage in methylcellulose revealed that the control cells readily differentiated mainly to granulocytes and macrophages. Instead, the majority of PML-RARα-expressing cells were clearly arrested at the promyelocytic stage of myeloid differentiation (Figure 5.2.3C), thus confirming the strong transforming potential of PML-RARα in these experimental settings as previously reported by a number of other groups (Minucci et al., 2002; Sternsdorf et al., 2006; Zhu et al., 2005). In line with the previous results, Δ2-expressing cells displayed intermediate levels of differentiation. In fact, a fraction of cells differentiated into granulocytes/macrophages, whereas, a small fraction remained in an immature state, reminiscent of PML-RARα blasts (Figure 5.2.3C).

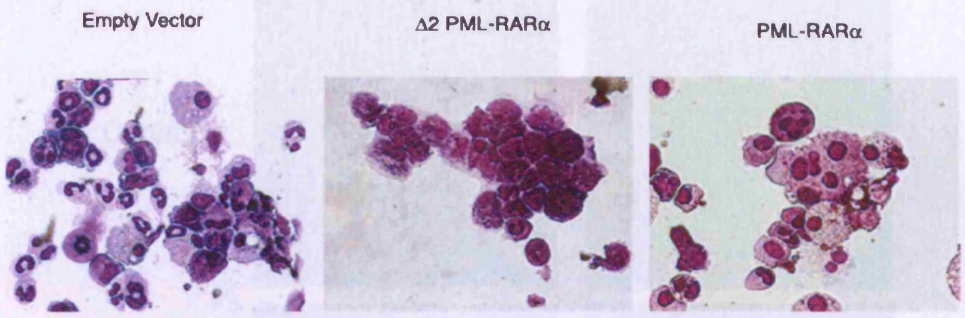


Figure 5.2.3C Cytoplasmic PML-RARα expressing cells display an intermediate state of differentiation. Empty vector, Δ2 and PML-RARα cells were recovered from the methylcellulose after being cultured for 10 days in the presence of high concentrations of G-CSF. Cells were subjected to cytological analysis by means of May Grunwald/Giemnsa staining. Light microscope images of vector (left), Δ2 (middle) and PML-RARα infected cells.

Alongside, confocal/immunofluorescence analysis of the cells was performed using an anti-human PML antibody in order to detect the expression of the fusion proteins (Figure 5.2.3D). As expected vector cells displayed a faint background staining, whereas, the majority of cells expressing PML-RARα showed the accumulation of PML-positive nuclear microspeckles (Figure 5.2.3D). Conversely, Δ2-expressing cells displayed a PML punctuated cytoplasmic staining reminiscent of that observed in transduced U937 cells (Figure 5.2.3D).

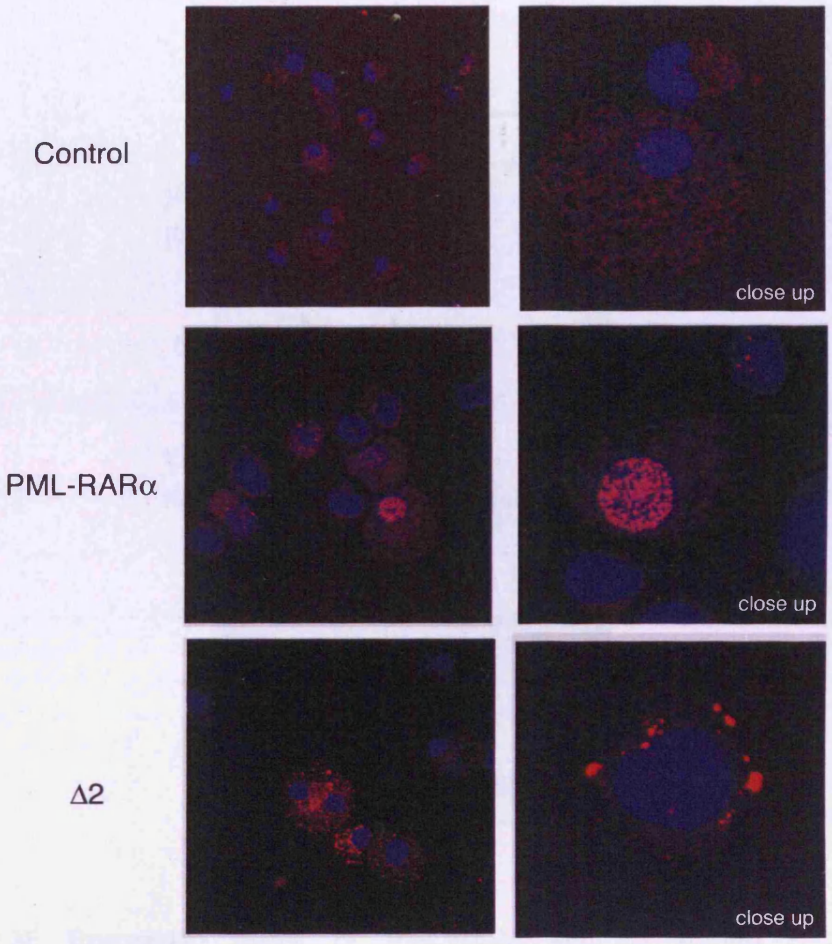


Figure 5.2.3D Δ2 accumulates in the cytoplasm of progenitors cells. Confocal images of vector, PML-RARα and Δ2 cells cultured for 10 days in the presence of G-CSF. Cells were stained with an anti-PML antibody (red). Nuclei were counterstained with DAPI (blue).

Immunostaining of endogenous PML using an anti-mouse PML antibody indicated that the protein normally accumulated in nuclear bodies in these cells (not shown). At the same time, levels of the transduced proteins were analyzed in the extracts prepared from vector, PML-RARα and Δ2 cells after different passages (Figure 5.2.3E). PML reactive bands corresponding to PML-RARα and Δ2 were readily detected in the extracts using an anti-human PML antibody (Figure 5.2.3E). Remarkably, the expression of the proteins was diminished but not completely depleted during differentiation. Moreover, the basal levels of PML-RARα were significantly lower than Δ2 (Figure 5.2.3E).

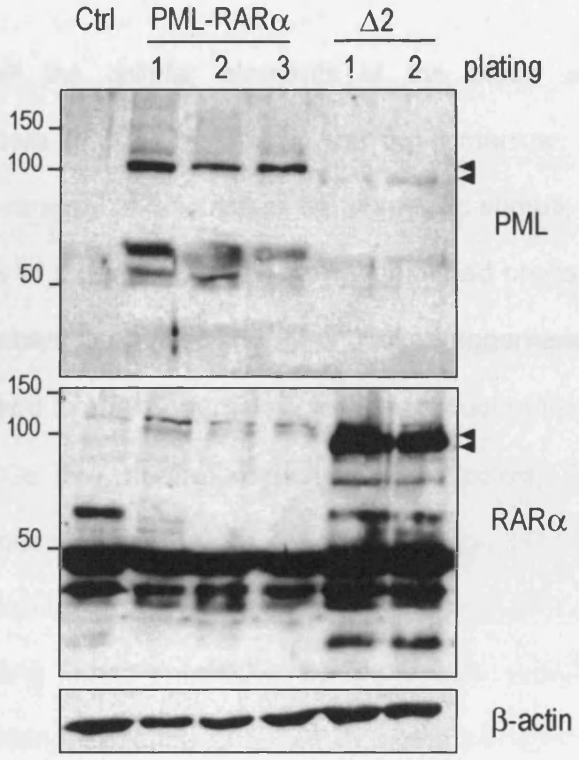


Figure 5.2.3E Expression levels of PML-RARα and Δ2 during G-CSF-induced differentiation of primary haematopoietic progenitors. The levels of PML-RARα and Δ2 were analyzed in primary progenitors cultured in methylcellulose in the presence of high concentrations of G-CSF at different platings. Proteins extracts were equally divided and subjected to independent SDS-PAGE. Membranes were probed with anti-PML (top) and anti-RARα antibodies (middle). Black arrows, on the right of each panel, indicate the bands corresponding to the fusion proteins. Notably, the anti-PML antibody used poorly recognized Δ2, which instead was expressed, as shown in the anti-RARα blot. In fact, the anti-PML antibody has the targets epitope within exons 5 and 6 of PML, which are in part deleted in the Δ2 mutant. β-actin was used as loading control (bottom).

This is in agreement with previous studies suggesting that low levels of PML-RARα are necessary to fully transform progenitors cells. Nevertheless, it was not possible to establish whether PML-RARα and Δ2 were cleaved in these cells, as no clearly detectable products were present in the extracts (Figure 5.2.3E).

5.3 Discussion

In adult mammals, all the cellular elements of the blood arise from somatic haematopoietic stem cells (HSCs) residing in the bone marrow. HSCs possess the unique property of self-renewal and, in response to specific stimuli, to divide in order to give rise to populations of progenitor cells. Then, committed progenitors further divide to generate the diverse haematopoietic lineages. Leukaemogenesis mediated by PML-RARα has been proposed to affect haematopoiesis by reducing the levels of cell death within the pool of HSCs and, at the same time, by blocking the myeloid lineage maturation, causing accumulation of malignant cells (Grignani et al., 1993a; Grignani et al., 1993b). The mechanisms underlying the pathogenesis of APL have been ascribed to chromatin remodelling effect mediated by the fusion protein in the nucleus. Nevertheless, accumulating evidence obtained by using transgenic animals and *ex-vivo* cultures of HSC suggest that the PML-RARα might have additional functions (Lane and Ley, 2003; Sternsdorf et al., 2006; Yoshida et al., 2007; Zhou et al., 2006; Zhu et al., 2007; Zhu et al., 2005). Limited information is available on the mechanisms that could promote transformation without a direct effect on chromatin. In this respect, data presented in chapter 4 indicates that cytoplasmic localization of PML-RARα blocks transcription and hampers RA-dependent differentiation in haematopoietic cells. In this section, the cytoplasmic functions of PML-RARα were investigated using more physiologically relevant systems: a mouse non-tumorigenic myeloid cellular model (32D cells) and murine primary haematopoietic progenitors. The coding sequence of PML-RARα and Δ2 were cloned into a retroviral expression vector to produce highly efficient viral particles and enable the transduction of the fusion proteins along with the GFP in 32D cells. GFP-positive cells were sorted and cellular clones encoding empty vector, PML-RARα and Δ2 were isolated. Nonetheless, we were unable to generate stable clones expressing full-length PML-RARα as the fusion protein was extremely

unstable in these cells. Accordingly, it has been shown that high levels of PML-RARα are toxic for the cells that counter-react downmodulating its expression (Ferrucci et al., 1997; Minucci et al., 2002; Westervelt et al., 2003). Furthermore, PML-RARα has been shown to be cleaved by neutrophil elastase, an enzyme highly active in promyelocytes (Lane and Ley, 2003). Although it has been proposed that PML-RARα cleavage is important for the development of the disease, it is not known whether the resulting cleavage products are involved in this process. Thus, future experiments using clones expressing cytoplasmic portions of PML-RARα could clarify this issue. In this regard, a PML-RARα clone (B23) expressed only a short cytoplasmic PML portion and appeared strongly refractory to undergo G-CSF-induced differentiation (C.Bellodi and P.Salomoni unpublished observations). Conversely, Δ2 was not cleaved and accumulated in the cytoplasm of 32D cells, thus suggesting that cytoplasmic localization greatly stabilizes the fusion protein and reduces toxicity even at very high expression levels (not shown). Accordingly, no morphological differences were noticed between control and Δ2 cells cultured in normal growing conditions. Thus, we tested whether Δ2 affected G-CSF-induced differentiation by comparing control and Δ2 stable clones. Cells were identically viable thus confirming that Δ2 does not alter growth and the survival of the cells during differentiation. Nonetheless, May Grünwald/Giemnsa preparation outlined striking morphological differences between control and Δ2-expressing cells as soon as after 5 days of G-CSF treatment. Indeed, we found the percentage of Δ2 cells terminally differentiated was reduced by almost 50% as compare to control cells. In keeping with this, we found that the expression levels of LF, ID1 and ID2, reported to increase during myeloid maturation and to be repressed by *bcr1,* were substantially downmodulated in the presence of Δ2. Specifically, *LF* showed the highest reduction, almost 50 % less than control cells. Interestingly, *LF* expression is regulated CCAAT-enhancer binding protein (C-EBP) family of proteins, which transcription is regulated by

PU.1, that has been shown to be repress by PML-RARα (Yoshida et al., 2007). Thus, there is the possibility that D2 also deregulate PU.1 activity, this aspect warrants more future investigations. Furthermore, we found that Δ2 levels are reduced upon G-CSF treatment. Previous studies have demonstrated that NE cleaves PML-RARα during myeloid differentiation (Lane and Ley, 2003); thus it is possible that Δ2 undergoes to a similar processing. Nonetheless, it remains to be established whether NE is also involved in Δ2 catabolism. Experiment using the specific NE inhibitor can clarify this aspect. Altogether these results suggest that Δ2 impairs G-CSF-induced differentiation of myeloid progenitor cells, possibly altering the transcription of important genes required for the correct timing of the maturation.

Subsequently, we investigated the consequences of Δ2 expression in primary haematopoietic progenitors, an elegant and powerful tool to study the transforming potential of a given protein in *ex-vivo* settings. Progenitor cells were infected with PML-RARα or Δ2 and serially re-plated in methylcellulose supplemented with high doses of G-CSF to induce differentiation until no colonies were detected in control cells due to terminal differentiation. In line with data in the literature, PML-RARα immortalized Lin- cells so that the number of colonies increased over passages (Minucci et al., 2002; Zhou et al., 2006). Interestingly, Δ2 was able to induce partial immortalization, thus confirming that the nuclear functions of the fusion protein remain indispensable to induce full-transformation. Notably, Δ2 showed a consistently reduced number of colonies at the first passage. These findings suggest that Δ2 may interfere with the cell cycle in primary cells however this aspect has not been analyzed in depth. Furthermore, morphological analysis revealed that Δ2 cells were a combination of terminally differentiated cells and immature blasts while in PML-RARα preparations the

majority of cells were blocked at the promyelocytic stage of maturation, thus demonstrating that Δ2-dependent block of differentiation is not complete.

Immunofluorescence and protein analysis confirmed that the expression levels of the two fusion proteins were strikingly different. Yet, in these cells Δ2 was found to accumulate at higher levels as compare to *bcr1*. Nonetheless, these data suggest that low levels of PML-RARα are sufficient to block differentiation and promote transformation *ex-vivo*. Accordingly, in transgenic animals low levels of PML-RARα are required to favour the acquisition of critical mutations that lead to the overt leukaemia (Minucci et al., 2002; Westervelt et al., 2003).

Overall, data presented in this chapter demonstrated that cytoplasmic PML-RARα is able to impair differentiation induced by G-CSF and promotes partial immortalization in non-transformed and primary haematopoietic progenitor cells. Nevertheless, transformation analysis suggests that the nuclear functions of PML-RARα remain indispensable to acquire the oncogenic potential necessary for the development of APL *in vivo*. Finally, our data suggest the possibility that the combination of nuclear and cytoplasmic functions of PML-RARα, as for *bcr3*, could lead to a more severe and aggressive leukaemia, however, only by generating suitable transgenic animals for *bcr3* could provide a final answer to this important question.

Chapter 6

General discussion

Nearly two decades of intense research has revealed that PML is a very intriguing and multifaceted protein. The vast majority of the studies on PML have focused on its growth suppressive and pro-apoptotic functions that are orchestrated from the PML-NB through the modulation of a number of key regulators of the cellular homeostasis including p53 and pRB (Salomoni and Pandolfi, 2002). In APL, PML is inactivated as consequence of the reciprocal and balanced translocation t(15;17), which leads to the generation of the oncogenic fusion protein PML-RARα. As opposed to PML, PML-RARα promotes survival as well as blocks myeloid differentiation possibly by exerting a dominant negative effect on PML and RARα functions. PML-RARα has been proposed to act at the level of the chromatin by deregulating gene expression. According to this model, PML-RARα promotes the formation of aberrant macromolecular nuclear receptor complexes that display increased affinity for transcriptional corepressors, thereby hampering the expression of genes regulating myeloid differentiation. Nevertheless, increasing evidence suggest that additional mechanisms might also by involved in PML-RARα-mediated leukaemogenesis (Sternsdorf et al., 2006). In keeping with this hypothesis, it has been shown that alternative splicing and/or mutations generate PML and PML-RARα transcripts, which are predicted to encode for cytoplasmic proteins. Nonetheless, it is still unclear whether these aberrant messengers are successfully translated into cytoplasmic proteins. Interestingly, atypical cytoplasmic accumulation of PML has been described in certain type of cancers such as hepatocellular, skin carcinomas and also in primary blasts derived from APL patients, however, it is currently unclear whether this is a primary or secondary event in tumorigenesis (Condemine et al., 2007; Daniel et al., 1993; Terris et al., 1995). Accordingly, mutations in the remaining *PML* allele resulting in aberrant cytoplasmic proteins have been described in two RA-resistant APL patients that experienced a very aggressive form of leukaemia. Furthermore, the overexpression PML-RARα in cell lines results in both nuclear and cytoplasmic

distribution, however, evidence demonstrating the same type of phenomena *in vivo* is still missing. This is in part due to the lack of suitable transgenic animal models for cytoplasmic isoforms of PML and PML-RARα. Hence, the work carried out in this thesis has aimed to establish whether PML-RARα and PML possess cytoplasmic functions that could possibly contribute to tumorigenesis.

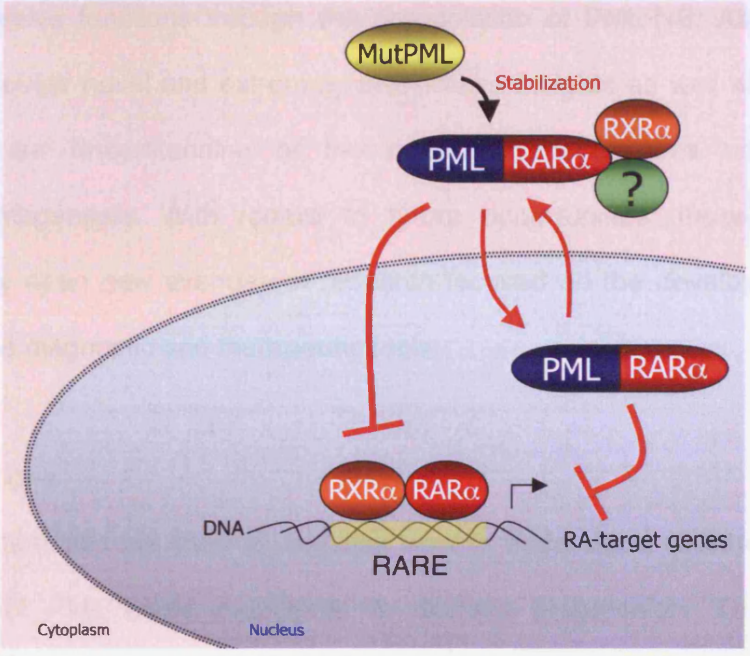


Figure 6A Summary of the key findings described in this thesis.
Data presented in this thesis suggest that PML-RARα drives tumorigenesis through nuclear as well as cytoplasmic repressive activities. On the one hand, PML-RARα functions in the nucleus through the formation of aberrant macromolecular complexes with RXRα and co-repressors, thus blocking the transcription of RA-target genes. On the other hand, PML-RARα represses the nuclear receptors function hijacking important factors such as RXRα in the cytoplasm. Furthermore, an APL-associated cytoplasmic mutant of PML inhibits RA-dependent down modulation of PML-RARα, thus augmenting its repressive potential.

Data presented in this thesis provide novel exciting findings and contribute to clarify some of the currently outstanding questions in the field:

i- *bcr3* PML-RARα isoform accumulates in cytoplasm of human primary leukaemic cells. I would like to emphasised that this is the first *in vivo* evidence demonstrating the cytoplasmic localization of the fusion protein;

ii- exogenous expression of a cytoplasmic mutant of PML-RARα inhibits RA-dependent transcription, differentiation and promote partial transformation of primary murine haematopoietic progenitors;

iii- expression of Mut PML in APL cells inhibits RA-mediated degradation of PML-RARα as well as augments its repressive functions;

iv- Cytoplasmic PML when transduced in non-APL cells, hampers p53 growth suppressive functions through the deregulation of PML-NB. Altogether these data provide novel and extremely provocative insights as well as contribute to broad our understanding of the molecular mechanisms underlying APL leukaemogenesis. With regard to future opportunities, these findings can possibly open new avenues of research focused on the development of more effective diagnostic and therapeutic tools.

Mut PML functions

The first question that we tried to address was to understand whether cytoplasmic accumulation of PML might participate in disease progression. To address this outstanding question we used as a tool cytoplasmic PML mutants recently identified in APL-resistant patients (Gurrieri et al., 2004b). Data obtained by expressing Mut PML in fibroblasts and haematopoietic cell lines was informative. Notably, Mut PML hampered the growth suppressive functions of p53 potentially by altering both its transcription-dependent and -independent functions. The importance of these findings is outlined by the fact that oncogene-induced senescence, a first key barrier against cellular transformation, is impaired in primary mouse fibroblasts expressing Mut PML. The main molecular mechanisms that we think is involved relies on the ability of Mut PML to promote the cytoplasmic relocation of nuclear PML and CBP, both of which function as important nuclear co-activators of p53 (Figure 6B).

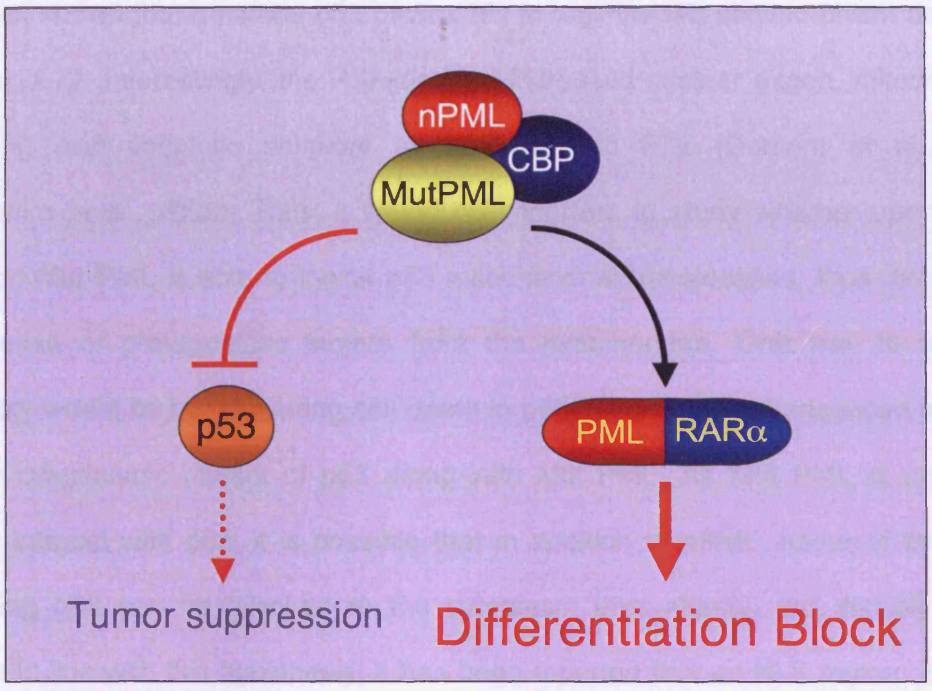


Figure 6B Working model summarizing the function of Mut PML. Our data suggest that Mut PML-dependent modulation of nPML, p53 and PML-RARα functions may contribute to the pathogenesis of APL. On one hand, Mut PML impairs p53 activation mainly through cytoplasmic relocation and inhibition of nPML. On the other hand, Mut PML potentiates PML-RARα inhibitory activity hampering its RA-dependent down-modulation. Overall, the net effect of Mut PML expression leads to a stronger differentiation block accompanied by an increased growth survival advantage of the leukaemic cells.

Nevertheless, many questions still remain unanswered. First of all, a more accurate investigation of p53 transcription in the presence of Mut PML is required. For instance, microarrays using RNA from control and Mut PML cells treated with ionizing radiation or subjected to oncogenic expression could reveal which p53 target genes are inhibited in the presence of Mut PML. Beside the effect on p53 transcription, Mut PML seems to inhibit also p53 transcriptional-independent functions. Interestingly, it has been shown that the cytoplasmic p53 possesses important roles in triggering apoptosis in response to different stress stimuli. In this regard, PUMA is essential in coupling and modulating nuclear and cytoplasmic pro-apoptotic functions of p53 (Chipuk et al., 2005). Thus, it would be important to analyze PUMA expression, mRNA and protein levels as well as its subcellular distribution in the presence of Mut PML. Importantly, it

has been shown that a natural p53 proline (P) to arginine (R) polymorphism occurs at aminoacid 72. Interestingly, the R72 displays increased nuclear export, mitochondrial trafficking and apoptotic potential as compared to P72 (Dumont et al., 2003; Marchenko et al., 2000). Thus, it would be important to study whether upon stress condition Mut PML is able to inhibit p53 mitochondrial translocation, thus diminishing the release of pro-apoptotic factors from the mitochondria. One way to test this possibility would be by measuring cell death in p53-deficient cells transduced with p53 R72 or cytoplasmic mutant of p53 along with Mut PML. As Mut PML is unable to directly interact with p53, it is possible that in addition to nPML, some of the factor regulating p53 can be hijacked to the cytoplasm thus altering the stability of the protein. In line with this hypothesis, it has been reported that an NLS mutant of nPML relocates Mdm2 to the cytoplasm (Bernardi et al., 2004). Thus, it can be theorized that as a consequence of Mdm2 redistribution the cytoplasmic portion of p53 could be subjected to increased proteasomal degradation. Beside Mdm2, it is possible that Mut PML could also alter the activity of some positive regulators of p53. An interesting candidate would be the 14-3-3δ protein, that has been shown to promote p53 stability (Yang et al., 2003). Interestingly, 14-3-3δ is together with PML, a member of the TRIM family of proteins. Thus, it is conceivable that Mut PML could heterodimerize with δ 14-3-3 altering its function. Moreover, future experiments are needed to test whether Mut PML can affect endogenous cytoplasmic PML, cPML, which has been implicated in the modulation of TGF-β signalling pathway (Lin et al., 2004; Seo et al., 2006). In fact, some of our preliminary data suggest that the two proteins homodimerize through their RING domains (not shown). For this reason it would be very important to analyze how cells expressing both Mut PML and cPML respond to TGF-β treatment. This could possibly provide important insights to explain some of the molecular mechanisms underlying deregulation of TGF-β signalling pathway in cancer.

Effects of Mut PML expression on PML-RARα functions

We have studied the role of Mut PML in APL. Strikingly we found that Mut PML potentiates PML-RARα inhibitory functions. Data show that the proteins interact in the cytoplasm and favour the stabilization of the fusion protein in response to treatment with pharmacological concentration of RA. These findings suggest that Mut PML interferes with the activity of proteins involved in PML-RARα catabolism (Figure 6B). Nonetheless, the molecular mechanisms underlying this process remain in part to be clarified. For example, it would be important to assess whether Mut PML can possibly interfere with the recruitment of the proteasomal subunit 11S, which is believed to trigger the degradation of the fusion protein (Lallemand-Breitenbach et al., 2001). Alternatively, Mut PML could affect SUMOylation of the PML portion, which has also been described to induce the proteasomal degradation of PML-RARα (Lallemand-Breitenbach et al., 2001). In this regard, it has been shown that the mammalian homologues of *Drosophila Seven in Absentia* (SIAH) targets several TRIM family members including PML for proteasomal degradation (Fanelli et al., 2004). Accordingly, SIAH overexpression diminishes the number of PML-NB, thus leading to loss of PML pro-apoptotic functions. Similarly, when expressed in PML-RARα positive cells, SIAH triggers the degradation of the fusion protein and, partially rescues the differentiation block (Fanelli et al., 2004). Interestingly, *SIAH* transcription depends on p53. Thus, it is possible that Mut PML by inhibiting p53 activity, could also suppress SIAH, thus promoting the stabilization of PML-RARα. It can also be hypothesized that Mut PML interferes with the activity of the ubiquitin-activating E1-like protein (UBEL1), which has been shown to promote the ubiquitylation and proteasomal-dependent degradation of PML-RARα (Pitha-Rowe et al., 2004). UBEL1 is induced in APL cells after treatment with ATRA. Remarkably, PML-RARα counteracts UBEL1 function inhibiting its RA-induced transcription (Pitha-Rowe et al., 2004).

Cytoplasmic PML-RARα

Two major isoforms of PML-RARα, *bcr1* or *bcr3*, are found in APL. However, it is still controversial whether the presence of *bcr1* or *bcr3* could have a different impact on disease progression. Some studies suggest that the presence of *bcr3* correlates with a worse prognosis. This is a very intriguing aspect considering that: very few data have been generated using this isoform and that *bcr3* lacks PML NLS motif and is predicted to gain a more pronounced cytoplasmic localization than *bcr1*. Importantly, we were able to demonstrate that *bcr3* PML-RARα accumulates in the cytoplasm of primary human leukaemic cells. This evidence supported by the data obtained using Mut PML lead us to study the role of cytoplasmic PML-RARα. Our findings suggest that a cytoplasmic mutant of PML-RARα, Δ2, still exerts a strong repression of RA-dependent functions, suggesting that it could act through indirect or even chromatin-remodelling independent mechanisms. The use of HDAC inhibitors combined to the analysis of the chromatin status, hystones acetylation/methylation, in cells expressing Δ2 and subjected to treatment with RA would certainly provide useful clues. Alternatively, PML-RARα could promote the cytoplasmic trafficking of important nuclear factors. Indeed, we found that Δ2 and *bcr3* can titrate RXRα, thus inducing its cytoplasmic relocation *in vitro* and *in vivo*, therefore, it would be important to study *bcr3* and Δ2 movements between the different cellular compartments. This can be studied, for example, to tracking GFP-tagged versions of *bcr3* or Δ2 using a fluorescence microscope. It would also be interesting to test whether an impaired nuclear export of PML-RARα could sensitize APL cells to ATRA. Accordingly, we found that Δ2 is less sensitive to ATRA-dependent degradation, thus suggesting that the degradation rely in part on the nuclear localization. Nonetheless, future efforts are needed to assess what is the sensitivity of cytoplasmic PML-RARα to other therapeutic agents. A recent study

identified the specific the E3 ubiquitin ligase RNF4 as the key factor for the degradation of PML-RARα (*bcr1*) in response to As_2O_3 treatment. In this regard, the SUMOylation of the fusion protein appears to have a central role in this process. Thus, a more complete analysis of the SUMOylation levels of *bcr3* is needed to understand whether this isoform is also subjected to RNF4-dependent degradation. Importantly, SUMOylation of PML-RARα has been shown to be required for transformation. In fact, the SUMOylation of K160 in the PML portion allows binding of DAXX which promotes the aberrant recruitment of nuclear co-repressor complexes (e.g. HDACs) (Zhu et al., 2005). Nevertheless, it needs to be established whether SUMOylation and DAXX sequestration/recruitment are also required for the cytoplasmic function of the fusion protein. This is a very important point that can be addressed using SUMOylation-deficient mutant of Δ2 that can be generated by single aminoacid mutagenesis of the crucial K160. Interestingly, we found that cytoplasmic localization increases the stability of the PML-RARα during myeloid differentiation in U937, 32D cells and also in mouse haematopoietic progenitors. Remarkably, the analysis of Δ2 levels in myeloid progenitor cells undergoing G-CSF-dependent differentiation demonstrated that the protein was downmodulated. However, the enzyme responsible for the cleavage has not been identified yet. It is possible that NE could be also responsible for the cleavage of Δ2. The use of specific inhibitors for NE (i.e. PMSF) or for other proteases expressed during myeloid differentiation such as cathepsin G and proteinase 3 could contribute to address this issue. Furthermore, it is still not clear the exact contribution (if any) of the cleavage products to the pathogenesis disease. Lane and colleagues by using NE-deficient animals suggested that the cleavage might be important for the progression of the disease (Lane and Ley, 2003). Furthermore, it can also be theorized that PML-RARα while in the cytoplasm, may interfere with the synthesis of key regulators of cellular homeostasis and myeloid differentiation. Increasing evidence suggest that deregulation of microRNAs (miRNAs) activity, which function as

translational repressor of specific RNA messengers, can contribute to tumorigenesis (Kumar et al., 2007a). Interestingly, it has been reported that a specific miRNA, referred to as miR-223, is implicated in the regulation of granulopoiesis and that its deregulation may play a role in the pathogenesis of myeloid disorders including APL (Fazi et al., 2005). Nonetheless, the analysis of the specific miRNA expression profile in APL cells has not been carried out yet, and more future investigation are needed to explore this intriguing hypothesis. Alternatively, it could be speculated that cytoplasmic PML-RARα interferes with the translation of messengers stored in cytoplasmic ribonucleoproteic complexes, for example the G-bodies (Liu et al., 2005a; Liu et al., 2005b). This process is very important in response to specific stress stimuli when translation of specific transcript needs to be rapid and efficient in order to allow cell homeostasis. In fact, it has been shown that amid these messengers some encode for important proteins involved in the regulation of the cell cycle, apoptosis (Liu et al., 2005a; Liu et al., 2005b). The determination of the number and morphology of G-bodies in control and Δ2-expressing cells will give an indication on whether PML-RARα can affect this pathway. Different laboratories have reported that *bcr1* PML-RARα blocks of differentiation and promotes immortalization of primary haematopoietic progenitors in clonogenic assays (Minucci et al., 2002; Zhou et al., 2006). Nevertheless, it has also been shown that immortalization is not always accompanied by transformation (Minucci and Pelicci, 2007; Sternsdorf et al., 2006; Zhu et al., 2007; Zhu et al., 2005). As PML-RARα has a mild leukaemogenic potential *in vivo,* it is conceivable that additional genetic events, for example inactivation of other tumour suppressor genes, could be required to develop overt leukaemia (Westervelt et al., 2003). Our findings suggest that nuclear functions of PML-RARα are essential to promote full transformation of haematopoietic progenitors. Thus, It would be important to investigate whether, for instance, cytoplasmic functions of PML-RARα are favouring the accumulation of secondary leukaemogenic events. Insinga and co-workers

demonstrated that *bcr1* inhibits p53 activity in a PML-dependent manner (Insinga et al., 2004). It is possible that PML-RARα impairs p53 but also other tumour suppressors hijacking nPML and/or other PML-NB components into the cytoplasm. In this regard, affinity purification of PML-RARα cytoplasmic bodies in combination with mass spectrometry could lead to the identification of potential new targets. Altogether, data presented in this thesis support the possibility that *bcr3* PML-RARα nuclear and cytoplasmic repressive functions may contribute to a more severe form of leukaemia, however, only the generation of transgenic animals for *bcr3* and Δ2 will provide the essential genetic tools to answers these fascinating questions.

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Cytoplasmic function of mutant PML and PML-RARα.
Bellodi C, Kindle K, Bernassola F, Dinsdale D, Melino G, Cossarizza A, Heery D, Salomoni P. **J Biol Chem. 2006 May 19;281(20):14465-73.**

A cytoplasmic PML mutant inhibits p53 function.
Bellodi C, Kindle K, Bernassola F, Dinsdale D, Melino G, Cossarizza A, Heery D, Salomoni P. **Cell Cycle. 2006 Nov;5(22):2688-92.**

New insights into the cytoplasmic function of PML.
Salomoni P, ***Bellodi C.*** **Histol Histopathol. 2007 Aug;22(8):937-46. Review.**

Effect of cytoplasmic PML-RARα on differentiation of mouse 32D myeloid precursor and primary haematopoietic progenitor cells.
Bellodi C, Calabretta B, Salomoni P (Manuscript in preparation)

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