The Promyelocytic Leukaemia gene product PML interacts with Myc and influences the expression of Myc target genes

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PhD thesis in Life sciences

The Promyelocytic Leukaemia gene product PML interacts with Myc and influences the expression of Myc target genes

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September 2004
To my family.
Abstract

c-myc is a well-known proto-oncogene encoding a transcription factor that needs to be tightly regulated in order to preserve cell homeostasis. The Promyelocytic Leukaemia gene product PML plays an important role in cell growth and survival, and resides in discrete sub-nuclear structures called Nuclear Bodies (NB). PML is largely involved in gene regulation via recruitment of several transcription factors and co-factors to the NB. In this report, I show that Myc partially localizes to the NB and physically interacts with PML, and I demonstrate that PML over-expression affects Myc-mediated transcription of a reporter gene. Comparative analysis of the expression of 40 Myc target genes and of Myc binding to their regulatory regions in wild type and PML knockout mouse embryo fibroblasts was performed. The data show that if PML is absent, although Myc binding to the DNA regulatory sequences is unchanged, the expression profile of several Myc target genes is altered. As deregulation of both activated and repressed Myc target genes occurs, I propose that PML influences Myc transcriptional activity through a mechanism that involves the control of Myc post-translational modifications.
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Abbreviations

aa: aminoacid
APL: Acute Promyelocytic Leukaemia
ATP: adenosine triphosphate
bp: base pair
CBP: CREB (cAMP response element Binding) Binding Protein.
cDNA: complementary DNA
ChIP: Chromatin ImmunoPrecipitation
CMV: Cytomegalovirus
KDa: Kilo-Dalton
DAPI: 4’,6-diamidine-2-phenylindole, hydrochloride
dNTP: deoxyribonucleotide triphosphate
DNA: deoxyribonucleic acid
DTT: dithiothreitol
E.Coli: Escherichia Coli
GST: Glutathione S-Transferase
HDAC: Histone Deacetylase
HAT: Histone Acetyltransferase
IFN: interferon
Inr: Initiator Element
MEFs: Mouse Embryo Fibroblasts
NB: Nuclear Bodies
PCR: Polymerase Chain Reaction
PIC: Pre-Initiation Complex
PMSF: phenylmethylsulfonyl fluoride
SDS: sodium dodecyl sulphate
SDS/PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis
Tris: Tris (hydroxymethyl)-aminomethane

VIII
CHAPTER 1: Introduction

1.1-The myc family of proto-oncogenes

The c-myc gene was originally identified as the cellular homologue of v-myc, the oncogene captured by the avian MC29 myelocytomatosis transforming virus (Alitalo et al., 1983a; Alitalo et al., 1983b; Sheiness et al., 1978). c-myc belongs to the family of myc genes, which includes B-myc, L-myc, N-myc and s-myc (Ingvarsson et al., 1988; Legouy et al., 1987; Schwab et al., 1984; Sugiyama et al., 1989). The structural and biochemical features of Myc proteins mark them as direct regulators of gene expression.

1.1.1-Myc is a member of the basic-Helix-loop-Helix-leucine Zipper (bHLHZ) superfamily of transcription factors

The c-myc gene structure consists of three exons that encode for a polypeptide of 439 aa with two conserved regulatory motifs at the N terminus (Myc Box I and Myc Box II), and a C-terminal basic-helix-loop-helix leucine zipper (bHLHZ) domain, in which the basic region (b) is required for specific DNA sequence recognition and binding, while the helix-loop-helix and the leucine zipper motifs (HLHZ) determine the specific dimer formation between Myc and its obligate partner Max, another bHLHZ protein, in order to bind a six-nucleotide E-Box sequence CACGTG, and activate transcription of several genes (Fig.1.1 and 1.4) (Baudino and Cleveland, 2001; Luscher and Larsson, 1999).
The presence of a conserved Myc-like bHLH domain is a hallmark of the transcription factors of this protein family. Apart from the canonical E-Box consensus sequence, Myc-Max can also bind many types of non-consensus E-Box elements, such as CATGTG, CACCTG, CATGCG, CACGCG, CAACGTG, and CACGAG (Blackwell et al., 1993). Statistically, the consensus and non-consensus E-boxes are distributed throughout the genome at a frequency of 1/800bp, however, Myc seems to bind preferentially the E-Box sequences located in the vicinity of gene regulatory sequences, suggesting that Myc binding might be sensitive to unique structural features of gene promoters, like the presence of CpG islands (Fernandez et al., 2003). Myc-binding to the E-box targets is antagonized by Mad-1, Mxi-1, Mad-3, Mad-4 and Mnt bHLH proteins, which compete with Myc in binding with Max to the E-Box sequences, although providing transcriptional repression via an N-terminal mSin3 corepressor-binding domain that recruits co-repressors and histone deacetylases (HDACs) (Grandori et al., 2000).
The Max bHLHZip Superfamily

Fig1.1. Schematic representation of the Max Superfamily of transcription factors. All of the proteins indicated in yellow are obligate partners of Max in order to bind DNA and to either activate or repress transcription.
1.1.2-Myc-mediated transcriptional activation

Once bound to the target E-box sequences (Fig. 1.2), Myc can activate gene expression through multiple regulatory events. Particularly, Myc can activate transcription either via the recruitment of transcription co-factors such as histone acetyltransferases and of ATP-dependent chromatin remodelling complexes, or at the level of transcription initiation and elongation, via interaction with the RNA Polymerase II complex (Bouchard et al., 2001; Cheng et al., 1999; Eberhardt and Farnham, 2001; Frank et al., 2001; Xu et al., 2001). A head-to-tail pair of Myc-Max dimers may form a heterotetramer that is capable of bridging distant E-boxes, which contribute to gene regulation (Nair and Burley, 2003).

1.1.3-Myc-mediated transcriptional repression

On the other hand, Myc may also act as a repressor, as it lowers the expression levels of its target genes by direct interference either with the transcriptional machinery, or with the enhancers required to induce gene expression (Fig. 1.3). As a matter of fact, Myc binds the TATA-box binding protein TBP along an auxiliary pathway to control gene expression; moreover, several targets seem to be repressed by Myc through the initiator sequence (Inr), and others may undergo modification of expression on an ad hoc basis through the direct interaction of Myc with other transcriptional elements, like Sp1, NF-Y, Miz-1, Smad, YY-1, AP2, and other proteins (Fig. 1.4) (Facchini and Penn, 1998; Feng et al., 2002; Gartel et al., 2001; Izumi et al., 2001; Staller et al., 2001).
Mechanisms of Myc Transcriptional activation

a) Myc-Max dimer binds to an E-box sequence within the first intron.

b) Myc-Max dimer binds to an E-box sequence within the first exon.

c) Myc-Max dimer binds to an E-box sequence within the promoter region.

Fig. 1.2. Mechanisms of Myc-mediated transcriptional activation:

- \[ \text{target gene} \]
- \[ \text{E-Box} \]
Mechanisms of Myc Transcriptional repression

Fig. 1.3. Mechanisms of Myc-mediated transcriptional repression:

a) Myc-Max dimer binds to the PIC and blocks its transcriptional activity.

b) Myc-Max dimer binds to a transcription factor and impairs its activity.
Fig. 1.4. Myc structure, functional domains and sites of interaction. Myc box I (MbI) is mainly involved with transcriptional activation, whereas Myc box II (MbII) is indispensable for Myc-mediated repression.

Many of the interactions of Myc with its partners occur in the N-terminal or C-terminal domains.
1.1.4-Myc and chromatin remodelling

Besides its activity as a basic transcription factor, Myc is currently being investigated as modulator of the chromatin status. Indeed, several evidences have connected Myc with the major chromatin remodelling complexes, as elucidated by the physical interaction of Myc with both the SWI/SNF ATP-dependent remodelling complex, which uses energy to modify chromatin structure in a non-covalent manner, and the histone acetyl-transferase (HAT) core, a multiprotein complex that mobilizes nucleosomes by acetylation of the H3 and H4 histone tails (Frank et al., 2003; Liu et al., 2003). Finally, a clear involvement of Myc in DNA repair has been proposed (Chiang et al., 2003; Partlin et al., 2003).

1.1.5-Myc function in normal cells

Myc regulates diverse cellular processes integral to cell growth, survival and development. Due to its involvement in such important matters, Myc activity is tightly controlled by a precise and combined regulation of mRNA and protein expression levels. c-myc is an early serum-response gene, as its expression is induced by mitogenic signals provided by extra-cellular stimuli. Myc expression peaks in the early G1 phase, and returns to a basal level as cells enter the S phase (Henriksson and Luscher, 1996). Moreover, Myc protein half-life is extremely short, 15-20 minutes, and its rapid degradation occurs in a proteasome-dependent manner (Salghetti et al., 1999). Myc expression is a prerequisite for cellular proliferation, since rodent cells where both c-myc alleles are depleted (c-myc-/-) show an extremely increased
doubling time (Mateyak et al., 1997), and c-myc/- mouse embryos die at embryonic
day 9.5-10.5 (Davis et al., 1993).

1.1.6-Myc and Cancer

Myc misregulation leads to cancer, and its transforming potential can be
elicted by both genetic and epigenetic events, such as chromosomal translocation or
deregulated expression caused by constitutive activation of growth factor receptors,
respectively (Nesbit et al., 1999). Myc over-expression determines uncontrolled cell
proliferation but also increased apoptotic cell death, which provides a mechanism that
counterbalances myc oncogenic potential. The transformation of a normal cell into a
cancer cell requires that sustained Myc-induced proliferation and defective Myc-
induced apoptosis simultaneously occur.

In addition, Myc deregulated expression strongly induces genomic instability,
mainly by positive regulation of genes that promote cell growth and proliferation, and
by negative regulation of genes that induce cytostatic effects and DNA surveillance
(Chiang et al., 2003; Grandori et al., 2003; Seoane et al., 2002).

1.1.7-Myc regulatory pathways

Due to the multiple regulatory mechanisms it takes part in, it has been
suggested that Myc might be induced to discriminate the choice of the partner by post-
translational modifications. Myc protein is subjected to several modifications, namely
phosphorylation, glycosylation, ubiquitination, and, most recently described,
acetylation (Kamemura et al., 2002; Salghetti et al., 1999; Sears et al., 2000; Vervoorts et al., 2003). These modifications seem to play antagonistic roles on the Myc protein fate and function. As an example, glycosylation occurs at Threonin 58, which is also a well-known target of phosphorylation strictly connected with Myc activation and degradation. Likewise, ubiquitination of Myc, which preferentially occurs at the N terminus, seems to be antagonized by acetylation (Vervoorts et al., 2003). It has been recently shown that ubiquitination of transcription factors is commonly required for their functional activation (Salghetti et al., 2001), therefore it is possible to hypothesize that the regulation of Myc ubiquitination levels is important not only to induce its degradation process but also to modulate its transcriptional properties.

1.2-The PML gene

The Promyelocytic Leukaemia (PML) gene was originally identified in patients affected by Acute Promyelocytic Leukaemia (APL), where it is fused to the retinoic acid receptor α (RARα) gene, due to t(15;17) chromosomal translocation that generates the chimeric proteins PML/RARα and RARα/PML (Fig.1.5) (de The et al., 1991; Goddard et al., 1991). This fusion event accounts for 99% of Acute Promyelocytic Leukaemia (APL) cases (Ruggero et al., 2000).

The chimeric protein PML/RARα is insensitive to physiological retinoic acid concentration, resulting in the block of myeloid haematopoietic cell terminal differentiation and in their uncontrolled proliferation (Lin et al., 1999).
1.2.1-PML belongs to the TRIM protein family

PML is a nuclear phosphoprotein belonging to the TRIM protein family (Reymond et al., 2001), with a N-terminal RING finger domain (R) followed by two additional zinc fingers named respectively B-Box1 and B-Box2 (BB), a coiled-coil region (CC) and a C-terminus that shows different aminoacidic sequences due to multiple alternative splicing variants (Fig.1.6) (Jensen et al., 2001). Genes of this family are implicated in a variety of processes, such as development and cell growth, and are involved in several human diseases. PYRIN/MARENOSTRIN, MID1 and MUL are mutated in Familial Mediterranean Fever, X-linked Opitz/GBBB syndrome, and Mulibrey nanism, respectively (The International FMF, 1997a; The French FMF, 1997b; Avela et al., 2000; Quaderi et al., 1997), whereas PML, RFP and Tif1 acquire oncogenic activity when fused to RARα, RET or B-raf, respectively (Grignani et al., 1994; Le Douarin et al., 1995; Ruggero et al., 2000; Takahashi et al., 1988).
Fig.1.5. 99% of Acute Promyelocytic Leukaemia are due to a balanced translocation between chromosomes 15 and 17. This event occurs at the level of PML and RARα gene loci, and determines the formation of the two chimeric proteins PML/RAR α and RAR α/PML.
Fig.1.6. PML belongs to the TRIM protein family. The signature of this family is the Tripartite Motif, which consists of a RING, a B-box1, a B-box 2 and a coiled-coil domain at the N-terminus of the protein. In each protein, the single domains, when present, always respect this definite order within the aminoacidic sequence.
1.2.2-PML and the Nuclear Bodies

PML can homo-multimerize, and localizes within discrete nuclear structures called nuclear bodies (NB), PML oncogenic domains (PODs) or nuclear domain 10 (ND10) (Fig. 1.7). Cells typically contain 5-30 NB of 0.2-1 μm, even if the number and size may vary according to the cell cycle phase, the external signals and the cell type (Doucas and Evans, 1996; Everett et al., 1999; Koken et al., 1995). PML protein levels and NB number and size are increased in inflammatory tissues and as a cellular response to interferon, and NB disruption is an early event caused by several viral infections (Gongora et al., 1997; Guldner et al., 1992; Terris et al., 1995). PML localization to the NB is a prerequisite for the formation and maintenance of their macromolecular structure (Ishov et al., 1999; Zhong et al., 2000). In APL blasts, PML/RARα protein targets and delocalises PML, causing the destruction of the NB into micro-speckled structures; treatment of APL patients with all-trans retinoic acid induces dramatic differentiation of the leukemic blasts with rapid degradation of PML/RARα and recovery of normal NB (Melnick and Licht, 1999).

1.2.3-The functional role of the NB

The NB act like nuclear compartments where several proteins committed to different functions stably or transiently localize (Fig. 1.7). Moreover, they are sites of intense protein post-translational modification events, like acetylation, sumoylation, ubiquitination and phosphorylation (D'Orazi et al., 2002; Doucas et al., 1999; Everett et al., 1997; Hofmann et al., 2002; Muller et al., 1998).
The plethora of biochemical events of cellular and viral origin that associate with the NB topology and composition support the view of the NB as integrators of multiple extra-cellular stimuli. Indeed, PML is involved in multiple different biological functions, like cell growth, proliferation, differentiation, and apoptosis, and the functional significance of PML employment has been investigated in depth during the last few years. Interestingly, several lines of evidence have shown that PML and the NB intervene in the regulation of gene expression and in DNA stability. PML is involved in both the activation and repression of several target genes, as it physically interacts with several transcription factors, like Spl, p53, pRB and c-Jun (Alcalay et al., 1998; Best et al., 2002; Fogal et al., 2000; Vallian et al., 1998a; Vallian et al., 1998b), and it influences gene expression by preferential recruitment of HATs, like CBP and p300 (Doucas et al., 1999; LaMorte et al., 1998), or HDACs and corepressors (Khan et al., 2001; Langley et al., 2002; Wu et al., 2001) at the NB. Moreover, PML interacts with several proteins that control DNA genomic stability and repair (Naka et al., 2002; Zhong et al., 1999) and with the proteasome (Lallemand-Breitenbach et al., 2001), even if the functional significance of the interaction with the degradation machinery is still obscure.

1.2.4-PML acts like a tumour suppressor

There is growing evidence that PML acts like a tumour suppressor. In addition to the ability to preserve genomic stability, PML over-expression inhibits cell growth and proliferation, suppresses lymphomagenesis in mice (Wang et al., 1998a), and induces apoptosis (Guo et al., 2000; Koken et al., 1995; Liu et al., 1995; Quignon et
al., 1998; Wang et al., 1998b). Moreover, PML belongs to the pathway that leads cells to oncogenic Ras-dependent and independent senescence. Upon cell-entry into senescence, both PML levels and NB number and size increase (Ferbeyre et al., 2000; Jiang and Ringertz, 1997; Pearson et al., 2000), and PML may elicit induction of senescence by enhancing p53 and pRB activity (De Stanchina et al., 2004; Fang et al., 2002; Gottifredi and Prives, 2001) and by promoting both transcriptional activation and repression via modulation of several transcription factors activity.
The functional role of the NB

Fig. 1.4. PML localizes to the Nuclear Bodies (NB), and interacts with several proteins. Due to the diversity of the roles played by the proteins that are recruited to the NB, PML is involved in several cellular processes, such as cell growth, proliferation and apoptosis, acting either as a co-activator or as a co-repressor.
1.3-Myc and PML play antagonistic roles in several cell pathways

Several lines of evidence account for the involvement of Myc and PML in common cellular regulatory events, where they seem to play antagonistic roles.

1.3.1-Transformation assay

One of the most classical approaches to classify a novel gene as a potential oncogene is to investigate its ability to transform a primary culture of Rat Embryo Fibroblasts, when co-transfected with the constitutively activated oncogenic Ha-Ras. Following co-transfection with Ras, Myc induces cell transformation, which defines it as a proto-oncogene (Lee et al., 1985). Conversely, using the same functional approach, PML suppresses growth of Rat Embryo Fibroblasts transformed by co-expression of the Ha-Ras oncogene and c-Myc, therefore showing tumour suppression properties (Mu et al., 1994).

1.3.2-Cellular proliferation

Other indications arise from the recent insights on the Ras pathway. As mentioned above, PML is up-regulated by Ras, and leads to premature cell senescence through the induction of the p21^{WAF1} gene, a cell cycle inhibitor, and the functional activation of p53 tumour suppressor (Fogal et al., 2000; Pearson et al., 2000). As a proof, PML-/- Mouse Embryo Fibroblasts (MEFs) show complete loss of Ras induced senescence and increased resistance to apoptosis (Guo et al., 2000; Wang et al., 1998a). Moreover, in human cells, PML contributes to Ras mediated cell cycle arrest.
by engaging also the pRB suppression pathways via up-regulation of the intracellular levels of the cell cycle inhibitor p16\textsuperscript{INK4}. Conversely, Myc antagonizes p16 and p21 cytostatic effect and pRB- and p53-mediated tumour suppression pathways (Ferbeyre et al., 2000; Obaya et al., 1999).

1.3.3-Cell-cycle progression

Myc and PML oppositely influence the cell cycle progression. Both genes expression profile show a peak in the G1 phase (Chang et al., 1995; Rabbitts et al., 1985); however, while Myc over-expression induces S-phase entry, an increased amount of PML results in a delayed cell exit from G1. Consistently, the extremely delayed G1 phase observed in c-myc \textsuperscript{-/-} MEFs is also found in cell lines over-expressing PML (Mu et al., 1997), whereas PML\textsuperscript{-/-} MEFs and cells over-expressing Myc undergo opposite effect (Grandori et al., 2000; Wang et al., 1998a).

1.3.4-Chromatin remodelling

The role that PML plays in chromatin remodelling mainly relies on PML-dependent recruitment of both histone acetylation and deacetylation complexes to the NB (Ruggero et al., 2000; Wu et al., 2001). Given the emerging involvement of Myc in chromatin remodelling (Amati et al., 2001), it is possible to hypothesize that the reciprocal influence of Myc and PML in this mechanism may regulate gene transcription within defined chromatin regions.
1.3.5-Gene expression

Evidence of the interaction of PML with the Myc network comes from the involvement of PML in Mad1 mediated repression (Khan et al., 2001). PML physically binds HDAC1, and directly assists Mad1 to target genes repression (Wu et al., 2001). From a general point of view, it is known that Myc and Mad protein levels determine cell fate (Henriksson and Luscher, 1996; Queva et al., 1998). For example, the onset of the differentiation pathway of haematopoietic precursors is determined by the switching from Myc:Max to Mad1/Max binding to target genes (Grandori et al., 2000). It was recently proven that this switching correlates with the histone acetylation status of the telomerase reverse transcriptase promoter in the promyelocytic leukaemia human cell line HL60 (Xu et al., 2001). The mad genes share with Myc the majority of their target genes (Luscher, 2001; Nikiforov et al., 2003); this implies that, from a more general perspective, PML may antagonize Myc activity at least in an indirect way, for example by inducing histone deacetylation, which could promote nucleosome assembly and render gene promoters inaccessible to Myc.

Accordingly, it was recently described the discovery of a novel trans-repression pathway of Myc via the recruitment of a Trichostatin-A (TSA) sensitive transcriptional co-repressor complex to Myc through interaction with the adaptor protein MM-1 (Satou et al., 2001). TSA is a drug that inhibits the activity of HDAC protein family, and the discovery that Myc-mediated transcriptional activation dramatically increases after treatment of cells with TSA opens new questions on the mechanism by which Myc mediates transcriptional regulation. In view of the fact that the HDAC-mediated repression activity is associated to Myc via the TRIM protein
TIF1β, which is indirectly recruited to Myc via physical interaction of MM-1 with both proteins, it would be interesting to investigate if PML could also directly or indirectly recruit HDAC complexes to Myc as well.

1.3.6-PML interacts with several Myc transcription partners

In parallel to this, other insights suggest that PML might antagonize Myc function by influencing Myc transcriptional activity. In fact, as the NB have been proposed to serve as depots for the deployment and decommission of various proteins, PML could regulate the availability of specific transcription co-factors that cooperate with c-Myc.

One example comes from the interferon pathway. As mentioned above, PML expression is up-regulated by interferons (IFNs) (Heuser et al., 1998) and correlates with IFN-induced antiviral and antiproliferative effects (Ruggero et al., 2000; Turelli et al., 2001). Of note, c-myc shares many target genes with IFN-γ, and c-myc overexpression results in a global deregulation of multiple IFN-responsive genes (Nesbit et al., 2000). The regulatory region of many Myc target genes contain Sp1-binding sites that contribute to basal expression, so that genes might undergo cooperative regulation by Myc and the transcription factor Sp1 (Boyd and Farnham, 1997). Sp1 is known to interact with PML (Vallian et al., 1998a), and therefore could be actively sequestered in the PML bodies.

In addition, PML interacts with cyclin T1, a subunit of the positive transcription elongation factor b (P-TEFb), both at the NB and associated to a target promoter, suggesting that PML could regulate transcription by modulating the
availability of cyclin T1 and through a direct interaction with the transcription machinery (Marcello et al., 2003). Noteworthy, it was recently shown that Myc activates transcription of the CAD gene by recruitment of the P-TEFb to its promoter (Eberhardy and Farnham, 2002; Kanazawa et al., 2003), and this enrollment is mediated by Myc physical interaction with cyclin T1. Since Myc and PML bind two distinct cyclin T1 regions, cyclin T1 could simultaneously interact with Myc and PML, not only at the NB, but also at Myc target genes regulatory regions.

1.3.7-Myc is a target of multiple post-translational modification pathways that occur within the NB.

Finally, the influence of PML on Myc activity could occur at the level of Myc post-translational modifications. As mentioned above, it was recently proposed that ubiquitination of Myc might compete with CBP-mediated acetylation (Vervoorts et al., 2003). In particular, Myc ubiquitination enhances Myc transcriptional activity via recruitment of proteasome subunits (Kim et al., 2003; von der Lehr et al., 2003). Proteasome subunits directly participate in the activation of Myc target genes, having been found together with Myc on the promoter of the gene encoding for cyclin D2. Interestingly, PML interacts with the proteasome and recruits CBP at the NB as well, suggesting that it could be involved in the regulation of Myc post-translational status (Lallemand-Breitenbach et al., 2001).

1.4-Aim of the project

The aim of this project was to assess the involvement of PML in the Myc pathway. To this end, I focused in parallel on two main goals: the evaluation of PML
physical interaction with Myc, and the analysis of the influence of PML on Myc target genes regulation.

The physical interaction between the two proteins was investigated by co-localization and co-immunoprecipitation experiments, whereas the functional assay consisted of the evaluation of PML influence on Myc transcriptional activity. To this purpose, I selected several known or putative Myc direct target genes, and I performed a comparative analysis of Myc binding to their regulatory regions and of their expression levels in wt and PML knock out (-/-) Mouse Embryo Fibroblast (MEFs). In order to perform this comprehensive analysis, I split my efforts into two main parts:

1) Validation of Myc binding to target genes by chromatin immunoprecipitation (ChIP) in the two cell lines. This technique has been proven very powerful to study the activity of mammalian transcription factors (Boyd and Farnham, 1999). The specific immunoprecipitation of DNA/Myc cross-linked regulatory regions with α-Myc antibodies, from wt and PML^-^- synchronized MEFs at defined time points, spanning the whole G1 phase, was performed in order to evaluate if a selected set of known Myc targets is differently occupied by the Myc:Max dimer in presence or absence of PML protein.

2) Analysis of the expression profile of the validated Myc target genes by Real Time quantitative PCR in the two synchronized cell types at the same time points, in order to reveal if a subset of Myc target genes is differentially expressed in absence of PML, and if this can be related to Myc binding to their regulatory regions.
CHAPTER 2: Materials and Methods

2.1-Cloning of Myc and PML into constructs suitable for expression in eukaryotic cells

2.1.1-Production of insert and vector DNA fragments

The full-length of the murine c-Myc and human PML IV cDNA were cloned into pCMV-2-Flag (SIGMA) and pCDNA3-HA vectors, which are designed for protein expression in mammalian cells (Invitrogen).

To clone the two genes, Polymerase Chain Reaction (PCR) was performed using primers with restriction site-containing tails; particularly, the forward primer carries an EcoRI site upstream of the ATG start codon, which keeps the genes translationally in frame with the tag sequence, and an XhoI site downstream of the stop codon.

In order to generate the insert DNA, PCR was performed in final 50μl samples, using 100ng of DNA template, 500nM forward and reverse primers, 5μl of 10x Vent DNA Polymerase buffer, 0.2mM dNTPs, and 0.5μl (1U) of the high fidelity Vent DNA Polymerase (New England Biolabs). The amplification reaction was carried out with as follows:

1. 1 min at 95°C
2. 1 min at 95°C
3. 30 sec at 56°C
4. 4 min at 75°C for PML amplification, 3 min at 75°C for Myc amplification (about 1 min/500bp of cDNA length).
5. Steps 2 to 4 are repeated 30 times

6. 1 cycle: 10 min at 75°C

In order to check the efficiency of the PCR reaction, $5\mu l$ of the samples were loaded on a 1% agarose gel, consisting of 1 % weight/volume (w/v) agarose and final 0.2$\mu g/ml$ Ethidium Bromide diluted in TAE (40mM Tris-acetate, pH 7.5, 2mM EDTA), and checked under a UV lamp after electrophoretic run. Positive samples were subjected to protein extraction by adding 1 volume (Vol) of phenol/chlorophorm, and centrifuging 1 min at 14,000rpm in a bench centrifuge at room temperature (RT). The upper phase was collected, and DNA was precipitated in 2.5Vol 100% ethanol and 1/10Vol of 3M Sodium Acetate pH 4.8. After centrifuging 5min at 14,000rpm at RT, the pellet was washed with 100$\mu l$ of 70% ethanol, and let dry at RT.

### 2.1.2-DNA restriction

In order to restrict the fragment tails at the EcoRI and Xhol sites, DNA pellet was resuspended in 43$\mu l$ of distilled water, $5\mu l$ of 10x EcoRI buffer, which is compatible also with the Xhol restriction enzyme, were added, and the samples were incubated at 37°C for 1 hr with $1\mu l$ of EcoRI and Xhol 20,000U/ml restriction enzymes, in 50$\mu l$ final volume. All the restriction enzymes were from New England Biolabs. Then, the digestions were loaded on a 1% agarose gel, and the DNA bands that showed the expected size were cut from the gel with a razor blade, extracted from the gel using Qiaex II extraction kit (Qiagen) according to the manufacturer’s protocol, and resuspended in 25$\mu l$ of 1x TE buffer (10mM Tris-Cl, pH 7.5, 1mM EDTA).
Extraction efficiency was determined by loading 5μl on an agarose gel. Parallel to this, pCDNA3-HA and pCMV-2-FLAG were opened by digestion with EcoRI-XhoI and EcoRI-Sall restriction enzymes, respectively, purified by gel extraction as reported above (DNA digestion with Sall and XhoI enzymes provide compatible, not re-cleavable, ends), and quantified on gel.

2.1.3-DNA ligation

After diluting insert and vector DNA at the final concentration of about 100ng/μl, the ligation reactions were carried out in final 10μl sample volume, adding 1μl 10x ligation buffer, 1μl vector DNA, 2.5-5μl insert DNA, and 0.5μl T4 DNA Ligase (New England Biolabs), and incubating 1hr at RT. 5μl of each reaction was used for transformation of bacterial cells.

2.1.4-DNA transformation

In order to insert the constructs into the bacterial host cells, 50μl of chemical competent DH5α E. Coli cells, with an efficiency of at least 10⁶ colonies/μg DNA/μl cells, were incubated for 20 min on ice with 5μl of ligation samples, followed by 2 min thermal shock at 42°C, then 1ml Luria Broth (LB: 1 % bactotryptone, 1 % NaCl and 0.5 % Bacto-yeast extract) was added, and cells were incubated 45 min at 37°C in a water bath, centrifuged 2 min at 5,000rpm, resuspended in 20μl LB and plated on 100mm diameter B-agar Petri dishes containing 100μg/mg ampicillin, which allows the
selection of the transformed colonies, as the used vectors confer resistance to this antibiotic.

2.1.5-Clonal selection

Plates were incubated over night (o.n.) at 37°C, and then the grown colonies were picked and grown o.n. at 37°C in liquid LB medium. After plasmid DNA purification, the constructs were analysed by restriction digestion with EcoRI-XhoI enzymes, and the positive clones were subjected to direct sequence analysis by the sequencing core at TIGEM.

2.2-Purification of plasmid DNA and DNA quantification

Plasmid DNA preparations were performed using the Qiagen Midi or Mini preparation kits, according to the manufacturer’s instructions (Qiagen), which provided large and small amounts of purified DNA, respectively. DNA concentration was determined by spectrophotometer analysis at the fixed 260nm UV wavelength. DNA concentration is determined by the formula $[C] = \varepsilon A$, where $[C]$ =DNA concentration, $A$ =absorbance, and $\varepsilon$ = double strand DNA molar extinction coefficient, which is 50$\mu$g/ml (RNA = 40, single-strand DNA = 33).

2.3-Cell culture and synchronization

Mouse Embryo Fibroblasts (MEFs)(Wang et al., 1998a) were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 20% Fetal Bovine Serum (FBS, Invitrogen), 50$\mu$M $\beta$-mercaptoethanol, 100U/ml penicillin/streptomycin
and 2mM Glutamine, and split 1:3 every three-four days. For ChIP and Real-time experiments, cells at passage 9 were used, and 80% confluent MEFs were synchronized by 72-hour-starvation in DMEM supplemented with 0.1% FBS, 50μM β-mercaptoethanol, 100U/ml penicillin/streptomycin, and 2mM Glutamine, followed by re-addition of DMEM with 20% FBS and harvesting at the selected time points. Cos-7, HeLa, U2OS, HEK 293, HepG2 and NIH-3T3 cell lines were grown in DMEM supplemented with 10% FBS (SIGMA), 100U/ml penicillin/streptomycin, and 2mM Glutamine.

2.4-Transient transfection and transactivation assay

For these studies, I used murine c-Myc cloned into a pCMV-2-Flag vector and human PML isoform IV cloned into a pCDNA3-HA.

Plasmid DNA was purified with the Qiagen Midi Kit and used to transfect HeLa, Cos-7, HepG2, U2OS, HEK 293 and NIH-3T3 cell lines with Polyfect (Qiagen) according to the manufacturer's instructions. MEFs were transfected with Effectene (Qiagen). In transactivation experiments, 1.2x10^5 HeLa, HepG2, or HEK 293 cells/well were seeded in 12-well-plates and transfected with M4-tk-luc (carrying 4 E-box sequences in the promoter region) or tk-luc (kindly provided by B. Lüscher). Luciferase activity was assayed 48hrs post-infection according to the manufacturer’s instructions (Promega). Transactivation assays were performed in triplicate and repeated at least three times.
2.5-Antibodies

Rabbit polyclonal anti-Myc N-262 (Santa Cruz), rabbit polyclonal anti-PML H-238 (Santa Cruz), monoclonal anti-Flag M2 (SIGMA) and monoclonal anti-HA (Boehringer) were used for immunoprecipitation; anti-Myc N-262 and anti-PML H-238 were utilized for Western blot analysis. For immunofluorescence staining, in addition to the antibodies reported above, I used anti-Myc C-33 (Santa Cruz), anti-PML PGM3 (kind gift of P.G. Pelicci), anti-20S proteasome PW-8265 (Affiniti Research Products) and anti-CBP/p300 NM11 (BD Biosciences) monoclonal antibodies to perform co-localization experiments.

2.6-Chromatin Immunoprecipitation (ChIP)

MEFs at passage 9 were plated 7-9x10^5 in 100mm Petri dish, in 8ml DMEM 20% FBS. When 80% confluent, cells were synchronized by starvation in DMEM 0.1% FBS, for at least 72 hrs; then 20% FBS DMEM was re-added and ChIP was performed at 0, 6, 12, 16 and 20 hrs post re-induction. When using NIH-3T3, cells were grown in 10% FBS, synchronized in low serum for 48 hrs, and harvested before or 3 hrs post-reinduction. The cell extract from 1 dish was used at each time point.

2.6.1-Protein/DNA cross-linking

Cells were fixed by adding 800μl (1/10Vol) of cross-linking solution (11% formaldehyde, 0.1M NaCl, 1mM Na-EDTA pH 8, 0.5mM Na-EGTA pH 8, 500nM Hepes pH 8), and by leaving cells in the incubator at 37°C for an additional 20 min. The cross-linking reaction was stopped by addition of 10ml of quenching solution
(0.125M Glycine in Phosphate-Buffered Saline, (PBS)) and 2-3 minutes incubation at room temperature (RT). Plates were put on ice and washed once with ice-cold 0.5mM PMSF in PBS.

All of the steps reported below were performed on ice. 1ml buffer A (0.25% Triton X-100, 10mM Na-EDTA pH 8, 0.5mM Na-EGTA pH 8, 10mM Tris-Cl pH 8, 0.5mM PMSF, 10µg/ml aprotinin, 10µg/ml leupeptin, 5mg/ml pepstatin, 0.1mM sodium orthovanadate, 1mM tetra-Sodium pyrophosphate, 1mM NaF) was added, cells were scraped and collected in final 10ml buffer A/dish in a 15ml tube. After 10 min incubation at 4°C on a rotating wheel, cells were centrifuged for 5 min at 1,500 rpm, and the pellet was resuspended in 10ml of buffer B (0.2M NaCl, 10mM Na-EDTA pH 8, 0.5mM Na-EGTA pH 8, 10mM Tris-HCl pH 8, 0.5mM PMSF, 10µg/ml aprotinin, 10µg/ml leupeptin, 5mg/ml pepstatin, 0.1mM sodium orthovanadate, 1mM tetra-Sodium pyrophosphate, 1mM NaF). Incubation and centrifugation steps were then repeated, and the pellet was resuspended in 440µl of sonication buffer (0.2M NaCl, 10mM Na-EDTA pH 8, 0.5mM Na-EGTA pH 8, 10mM Tris-Cl pH 8, 0.5mM PMSF, 10µg/ml aprotinin, 10µg/ml leupeptin, 5mg/ml pepstatin, 1mM sodium orthovanadate, 10mM tetra-Sodium pyrophosphate, 10mM NaF). Samples were sonicated for 3x30 sec, yielding genomic DNA fragments with a bulk size of 500-2000 base pairs, and the sonication buffer was adjusted to a RIPA buffer by adding 1% Triton X-100, 0.1% Sodium Dodecyl Sulphate (SDS), and 0.1% Sodium Deoxycholate (DOC) final concentration. Samples were incubated for 10 min on a rotating wheel at 4°C and then centrifuged for 10 min at 10,000 rpm. 50µl/sample were kept to recover total DNA input, and the lysates were subjected to
immunoprecipitation (Ip).

2.6.2-Pre-clearing

A 50% protein A-sepharose (Pharmacia)/PBS slurry was incubated in Ip buffer without anti-proteases and anti-phosphatases, with 1mg/ml BSA and 0.25mg/ml sheared Salmon sperm final concentration, for 2hrs to o.n. at 4°C on a wheel. 80µl of blocked protein A beads were then added to the lysates and incubated for 30 min-1hr at 4°C on a wheel and centrifuged for 2 min at 5,000 rpm at 4°C.

2.6.3-Immunoprecipitation

Supernatants were transferred to new tubes and incubated o.n., either with 2µg of anti-Myc N-262 polyclonal antibody (Santa Cruz), or without antibody, at 4°C on a wheel. Immune complexes were recovered by adding 80µl of blocked protein A-beads and incubated 1hr at 4°C on a wheel. Beads were washed for 5 times in RIPA buffer, once with LiCl buffer (0.25M LiCl, 0.5% NP-40, 0.5% DOC, 10mM Tris-Cl pH 8, 1mM Na-EDTA pH 8, 0.1mM PMSF, 10µg/ml aprotinin, 10µg/ml leupeptin, 5µg/ml pepstatin or cocktail inhibitor SIGMA, cat.n.P8340, 0.1mM sodium orthovanadate, 1mM tetra-Sodium pyrophosphate, 1mM NaF) and twice with TE buffer. DNA-protein complexes were eluted by adding 250µl of elution buffer (1% SDS, 0.1% NaHCO<sub>3</sub>) and incubated for 15 min on a wheel at RT. After 1 min centrifugation at 5,000 rpm at RT, supernatant was transferred to a new tube and elution was repeated.

2.6.4-Reversal of cross-linking

In order to revert cross-linking, NaCl 0.2M final concentration was added to
ChIP samples and DNA input, and incubated o.n. at 65°C. Proteins were then digested by incubating for 1hr at 45°C after adding 20μM Tris-Cl pH 6.5 and 0.8mg/ml proteinase K final concentration, and extracted once with phenol chloroform and once with chloroform. DNA was precipitated in 2.5Vol ethanol 100%, 1/10Vol 3M Na-Acetate pH 4.8, and 20μg glycogen, washed with ethanol 70%, centrifuged for 2 min at 12,000 rpm and resuspended in TE, 50μl for input DNA samples and 100μl for ChIP DNA.

2.6.5-PCR reaction on ChIP samples

1-2μl of ChIP DNA was used for PCR analysis. PCR was performed in final 25μl sample containing 2.5μl of 10x PCR buffer, 0.25μl of HotStarTaq (Qiagen), 0.2mM dNTPs, 800nM forward and reverse primers, and, when required, 5μl of 5x Q solution (Qiagen). The following primer pairs were used: (gene name: forward primer and reverse primer) Adm: cagggcaggctggttttag and tccaagattgactgcagatga; B23: gttgggaggatacatctgc and gttggctagagtcggagagc; Bax: gcggctggtctagttccttg and gaggctctagggttcttg; BN51T: ggtgacggagtccaaagcta and tcctccgacatgtctcta; Cad: tgcgtggaacctatctag and cactggaaccaagcagcagcc; Cav-1: cctctgtgcaacacttgtg and tccegtggctgatgttag; Cdc25a: tgcattcagaaacacagag and ggggtgaacaagagagatg; Cdk4: gcccttcgaagacccatag and ggggaagacagctgtgttota; Cul1: gcctctcttggtggtct and atcagagcatggatat; Ccnb1: gaaacgcattctacgggaac and aatcagaggtcttccgatg; Ccn2: accaccctcctggttaag and ccgactctcctctctcaac; Ccnd2: taagccttcgtcctcattcagcccgtc and egtttcctcaacactctcctc; Eca39: atcactggaggggagacag and cgggtgcgaatgtgtgct; Eca40: aaccatgacgacacaggaagcag and aaccatgagcttcctgct; Eif2a: gggcagtgactcttcagct and ggggtgaacaagagagatg; or...
aaaagactccacttcccagaa and gttccacagtcgccattttag; Fth: cacactcacacaggctcctc and caagcactgttgaagcagga; Gadd45: caactctgccttgctttggt and ctagcctcgctggggaact; Hsp70.1: gagacatggacaagcaagca and ggtggtagaggtggaacct; 1sgf3g: cccaaggtgtactgtgatc and ettaaccttgacgctgaaaa; LDH-A: cttctgaggctgaggagcat and ggggccttaaatggaagc; Ddx18: attgcttccaaggggaaac and catccgcttattctcttc; Neu: ggcacaaaccccatctgg and tgcctccagctccttctt, or agtgggtgagaactgcaagc and ctcagctccacactcaeg; Nucleolin: ggaagagaggccaacctta and etgctttccactttctctcg; Odc: caccgtgactgtgaggtgga and gcaagtggagctgact; Cdkn2b: caccgaagctactgtgcctc and gttcaggcgtttggatct; Cdkn2a: ttctctgcgctgtgacttc and cttctgctccggaacctt; Cdkn1a: cggctggtgacaagaagata and ctgcagctcctctctctgc; Cdkn1b: agcttactgctgctgcttggt and agttctgacgctcagcaacag; Trp53: cttcattttctgtcctcagac and gttgggaccttagttagc; Pdgfr: gggggaaaagaagaagagagga and acaccattctgactccttcttc; Pdfr: atccctgagccaaactct and eggaggagacagacaac, or etggtatgaaagcggacctc and cctgcctaggaccccact; RCL: acggggagagtacagataaa and cccgagagttacacagacagac, or gttacttctggtgggagcat and ctagttagccgtcctccttc; Tert: tgtgtctagagcctgctcatt and ctcacttgacgcaacctag; or ttaagacacacccttctgc and gcaaccaagttcggttagat; TMP: cttgcttggagccaaactt and aagcaacacacagcctggt; Thbs1: taaggtttctttaggtgcttccccc and acgtcttgggtggaagtga; PML.1: gcagctcagattctgttgc and gttttctgtcctgtgag; PML.2: ccagctttctctctctcttcg and gcagagaaacctcgcagag.

2.7-RNA extraction and Real-Time PCR analysis

Total RNA for each time point was extracted from 80% confluent synchronized MEFs cells in a 100mm Petri dish with RNeasy mini Kit (Qiagen). Total RNA was quantified by spectrophotometer analysis, diluted to 100ng/µl final
concentration, and checked again by UV detection after running 4µl of each sample with 1µl of 5x RNA loading buffer (10ml solution: 80µl of 500mM EDTA pH 8, 720µl of 37% formaldehyde, 2ml glycerol, 3084µl formamide, 20mM 3-[N-morpholino] propanesulfonic acid (MOPS), 5mM sodium acetate, 1nM EDTA, and some bromophenol blue powder, in 10ml final volume of RNAse-free water) on a formaldehyde gel (1.2g agarose, 20mM 3-{N-morpholino}propanesulfonic acid (MOPS), 5mM sodium acetate, and 1nM EDTA in 100ml final volume of RNAse-free water, and brought at pH 7.0 with NaOH) run in a formaldehyde gel running buffer (20ml of 37% formaldehyde, 20mM 3-{N-morpholino}propanesulfonic acid (MOPS), 5mM sodium acetate, 1nM EDTA, and 980ml of RNAse-free water). cDNA was prepared using 1µg total RNA, 200pmol random hexamers (Invitrogen) and Omniscript reverse transcriptase (Qiagen) according to manufacturer’s instructions, in 30µl final volume. Gene expression profile was achieved by performing Real-Time quantitative PCR on an Applied Biosystems 7000 machine, in 20µl final sample, containing 1:3 dilution of each cDNA sample, 2x Sybr Green Master Mix (Applied Biosystems), and 300nM forward and reverse primers, which were designed on different exons in order to avoid that potential genomic contamination could alter gene quantification. Every sample was loaded in triplicate.

The following primer pairs were used: (gene name: forward primer and reverse primers;) Adm: gacctggtgatgagacgaca and tagatctgcttgcccaattt; B23: cctcatggaagactcgatgg and tcattgcttctgcctctacg; Bax: agaggcagcggcagtgat and ccctggatgaaaccctgtagc; BN51T: cgaagagaccaagcagatcc and gcacgtccacctccatatct; Cad: taaagagaagccaccggcga and gcttgagcacagaggtgcg; Cav-1: atgtctgggggcaatac and
acgtcgtggtggatgtctt; Cdc25a: gactgtccccgtcacaac and egtcagggaggattcaggt; Cdk4: 
ggcctttggaacatatccaaat and cagccagcctaggaactgac; Cul1: tctctctggggaagcaga and 
tgacttgggaaggaggac; Cenb1: cgctcaggggtctagaggaaca and agegttttgcgcttctttc; Cend1: 
ggcgtcctgtggaaccaaatct and cctctgccactttcctcct; Cend2: tctatgtggaacctggag and 
atgctatgtggaaccaaat; Eca39: actctacattgcacccagaggag and acgggaccaggagag; Eca40: 
actgtcctggaaccaaatct and tgcctgagccggaagatctct; Eif2a: egcagaacgacgatccttctatga 
and caacatggcgaagaatgcta; Eif4e: gcacaagcagcctggttctga and egctcctgtgctgcaattc; Fh: 
ggcgaggcagggagac and tgcctgagccggaagatctct; Gadd45: gaagacgagggagatggac and 
tgaggtggaaggtgagtc; Hsp70: gtcagagagac and garagaggagagagagag; Id2: cccccagaacaaggtgac and 
aggttggaggaggagag; Isgf3g: gaccaggatgctgccatatt and gtcctgctggcagtattcg; LDH-A: agctgggaccatttttggaga and egctcctgtgctgcaattc; 
Odc: tggctctggggcgtgggttctg; Cdkn1a: cttgtggctgctgctttgctgtctga and egcctcggggcgggagag; Cdkn2a: 
gtcctgctggcagtattcg; Nucleolin: tgcagagcagcctggttctga and ggtgctgctggcagtattcg; 
Ptma: ggtgctgctggcagtattcg; Rcl: agctgggaccatttttggaga and egctcctgtgctgcaattc; 
Tert: agtgacacagcctggttctgctgtctga and egcctcggggcgggagag; Ybx1: ggtgctgctggcagtattcg and 
gtctgctggcagtattcg; Thbs1: ctcctggtgtctgctgtctgctgtctga and egcctcggggcgggagag; JPO-1: 
acgtcctggtgtctgctgtctgctgtctga and cagttgcaaaagctggaacccaggtcaggt; Tk1: aatgtcaagcagtattcg and 

35
actgtactgggcatctgg; PML: cagaggaacctcgaagac and ccaggagctttcagag; Myc: ctcctcgagtttggaagg and agcgctgaatttcca; Gapd: ggtgaggttcgggtgaacgg and gtggtcagaggtgcttg, or Gapd: ggtgctagtatgtctgga and ctaacagttgggtgctcag.

Gene expression profiles were obtained by Real-Time relative quantification, and Gapd was used as a reference gene to quantify the expression levels of the gene analysed. The expression profile of each gene is the mean of at least three independent experiments. I could not analyse the expression profile of Hsp70.1, as this gene is present in the mouse genome in multiple copies, most of which are probably pseudogenes that are not distinguishable by RT PCR analysis, and thus failed to undergo quantitative assay. Instead, I decided to include in my analysis Tk1, which is a downstream target of Myc, even if Myc binding to its promoter region has been demonstrated so far only by bandshift (Pusch et al., 1997; and www.c-mycancergene.org).

2.8-Co-immunoprecipitation assay

Cells were transfected as reported above, at the concentration of 1x10^6 cells/100mm Petri dish. After 36-48 hrs of transfection, cells were washed twice with ice-cold PBS, harvested in 0.5mM PMSF in PBS and centrifuged for 1 min at 1,000 rpm at 4°C. Pellets were resuspended in 500μl PTG buffer (10mM Tris-Cl pH 8, 2mM DTT, 10% glycerol, 1mM MgCl2, 1mM PMSF, cocktail inhibitor SIGMA cat.n.P8340, 1mM sodium orthovanadate, 10mM tetra-Sodium pyrophosphate, 10mM NaF) and incubated for 10 min on ice; then 0.5% NP-40 final concentration was
added, samples were incubated for 3-4 min on a wheel at 4°C and centrifuged for 5 min at 800 rpm at 4°C. Nuclei were resuspended in Ip buffer (0.25% NP-40, 2mM DTT, cocktail inhibitor SIGMA, 1mM PMSF, 1mM sodium orthovanadate, 10mM tetra-Sodium pyrophosphate, 10mM NaF in PBS), sonicated for 3x5 sec on ice, incubated for 30 min on a wheel at 4°C and centrifuged for 5 min at 14,000 rpm at 4°C. Total lysates were immunoprecipitated for 3 hrs at 4°C on a wheel with the required antibodies, followed by 1 hr incubation with 40μl of 50% protein A-sepharose/PBS.

Beads were washed three times with Ip buffer, resuspended in final 1x Laemli buffer, and loaded onto 7.5% SDS-polyacrylamide gel. Biorad mini-gel equipment was used in accordance with the manufactures instructions. Proteins were transferred on PVDF membranes (Pharmacia), subjected to Western blot analysis using 1:500 final concentration of anti-Myc N-262 antibody or anti-PML H-238 antibody, and revealed by chemiluminescence using Super Signal Pico (Pierce).

2.9-GST pull-down

2.9.1-Constructs for GST pull-down

Three different constructs were used for the GST pull-down assay. These constructs corresponded to three different portions of Myc, corresponding to aa 1-178, 173-349 and 104-349, fused with its 5’ to the Glutathione S-Transferase moiety. These Myc fragments were obtained by PCR amplification with Vent polymerase using specific primers, and subcloned into a pGEX-4T1 vector (EcoRI-Xhol), so that the protein products are fused at N-terminus to the GST protein. The three constructs and
the empty vector were used to transform *Escherichia Coli* strain DH5α on LB plates with 100μM ampicillin.

**2.9.2-Expression and purification of GST-Myc chimeric proteins**

Colonies were inoculated into 5ml LB medium with ampicillin and incubated overnight at 37°C with vigorous shaking. The morning after the cultures were diluted 1:20 in 100ml LB medium with ampicillin and grown at 37°C until their OD₆₀₀ was 0.5-0.8. After removing 2ml as a control, the bacterial cultures were induced to express the fusion proteins by adding 0.1mM IPTG. Cells were grown for 3 hours at 37°C. Bacterial samples (2ml of uninduced and 1 ml of induced bacteria) were centrifuged to pellet cells, which were then resuspended in 100μl of 2x Final Sample Buffer (FBS) (250mM Tris-HCl pH 6.8, 35% glycerol, 100mM DTT, 2% SDS, 0.2% bromophenol blue). Samples were boiled for 5 min, and fractionated by SDS-PAGE and the protein gel was stained with Blue-Comassie (0.1% Comassie Blue, 40% Methanol, 10% acetic acid in H₂O) for 15 min and destained with a destaining solution (40% Methanol, 10% acetic acid in H₂O).

Once checked that the fusion proteins were induced, all the cultures were centrifuged 10 min at 3,500g to pellet the cells. Cells were resuspended in 1.8ml lysis buffer (50mM Tris-HCl pH 8, 200mM NaCl, 1mM EDTA, 1mM DTT, 1mM PMSF, 20μl cocktail inhibitor SIGMA), and sonicated 30x5 sec. Then 1% Triton x-100 final concentration was added, the lysates were incubated 30 min at 4°C on a rotating wheel, and centrifuged 30 min at 14,000 rpm at 4°C. Supernatants were collected, transferred to new tubes and supplemented with 100μl of Glutathione-Agarose slurry.
and incubated overnight at 4°C on a rotating wheel. Beads were washed 4 times with 1ml washing buffer (1xPBS, 1mM PMSF, cocktail inhibitor SIGMA), and 20μl were checked through SDS/PAGE and Comassie staining.

2.9.3-GST pull-down assay

Three Petri dishes of HeLa cells were transfected with human PML isoform IV cloned into a pCDNA3-HA as reported above, at the concentration of 1x10⁶ cells/plate. Cells were collected and resuspended in 600μl lysis buffer (50mM Tris-HCl pH 7.5, 1mM EDTA, 200mM NaCl, 0.5mM DTT, 1% Triton x-100, 1mM PMSF, 30μl cocktail inhibitor SIGMA), sonicated 3x5 sec and kept on a rotating wheel for 30 min. The sample was centrifuged at 14,000 rpm for 30 min, and the supernatant was divided into 4 new tubes and supplemented with the agarose beads bound to GST alone or to the specific GST-fusion proteins, and incubated overnight at 4°C. Samples were then centrifuged at 1,500g for 4 min and beads were washed 4 times in the lysis buffer. After the last wash, beads were resuspended in 2xFBS, boiled for 5 min, and subjected to by SDS/PAGE. Proteins were transferred on PVDF membranes (Pharmacia), and Western blot analysis was performed using 1:500 final concentration of anti-PML H-238 antibody, and revealed by chemiluminescence using Super Signal Pico (Pierce).

2.10-Immunoprecipitation of endogenous Myc in MEFs

Endogenous Myc protein levels at each time point were determined through immunoprecipitation. Two properly synchronized MEFs Petri dishes were used at
each time point. Cells were harvested, subjected to nuclear extraction (see above), and the nuclei were resuspended in RIPA buffer, sonicated three times for 5 sec on ice, incubated 30 min on a wheel at 4°C and centrifuged 5 min at 14,000 rpm at 4°C. Following protein quantification (see below), equal amounts of lysates were immunoprecipitated 3 hrs at 4°C on a wheel with 5μl anti-Myc N-262 antibody, followed by 1 hr incubation with 40μl of 50% protein A-sepharose slurry. Beads were recovered after centrifuging 1 min at 1,000 rpm and resuspended in Laemli buffer 1x final concentration, loaded onto a 7.5% SDS polyacrylamide gel and transferred on a membrane as described above.

Western blot analysis was performed using 1:500 final concentration of anti-Myc N-262 antibody.

2.11-Proteasome inhibition

Proteasome inhibitor MG-132 (Sigma) was stocked at the concentration of 10mM in DMSO. When added, it was diluted to the final concentration of 50μM in the culture medium 5 hours before immunoprecipitation or immunofluorescence assays were performed.

2.12-Protein quantification

Protein concentrations were determined using Bradford’s method (Bradford, 1976). Protein samples were mixed with Bradford’s reagent (Biorad) and the absorbance at 595 nm was measured on a spectrophotometer. Protein absorbance was converted to mg/ml
concentration using a standard curve constructed by measuring the absorbance of a range of bovine serum albumin (BSA) concentrations.

2.13-Myc stability assay

Proliferating cells were induced to undergo block of translation by adding cycloheximide at the final concentration of 10μg/ml, in the culture medium. At the established time points, cells were harvested, and endogenous Myc was immunoprecipitated and revealed as described above.

2.14-Western Blot analysis

Polypeptides separated by SDS/PAGE were transferred to a Hybond-P polyvinylidene difluoride (PVDF) transfer membrane optimised for protein transfer (Amersham Pharmacia Biotech), using a wet blotter (Biorad). The membranes were blocked with PBS containing 5% non-fat dry milk powder and 0.1% Tween-20, then incubated with monoclonal or polyclonal antibodies diluted in blocking buffer in PBS containing 0.1% Tween-20. Horseradish peroxidase conjugated anti-mouse IgG and anti-rabbit IgG (Amersham) were used as secondary antibodies. Protein detection was obtained by chemiluminescence using Super Signal Pico (Pierce).

2.15-Immunofluorescence staining

In order to perform immunofluorescence assays, 20,000 cells/well were plated in 8-well-chamber slides. When transfected, 200ng of Myc, PML and CBP expression vectors were used. After transfection, cells were grown on cover slips for 36-48 hrs,
washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 5 min at RT. Cells were rinsed twice with PBS, permeabilised in 0.2% Triton X-100 in PBS for 30 min at RT, and blocked for 1 hr in 0.1% Triton X-100 in PBS containing 10% heat-inactivated goat serum. Cells were incubated for 3 hrs at RT (or o.n. at 4°C) with primary antibodies diluted in blocking buffer, followed by 1 hr incubation at RT (or 30 min at 37°C) alternatively with FITC-conjugated anti-mouse and/or TRITC-conjugated anti-rabbit antibodies (Dako Glostrup) or with Cy2-conjugated anti-rabbit antibodies and/or Cy3-conjugated anti-mouse antibodies (Jackson ImmunoResearch), followed by two washes with PBS. In some cases, to detect nuclei, cells were stained using DNA specific stain DAPI (Roche).

2.15.1-Biotin-streptavidin amplification system

To detect endogenous Myc protein, a biotin-streptavidin amplification method was used. In this system, after incubation for 2 hrs at RT with polyclonal anti-Myc N-262 antibody, cells were incubated first with Biotin-conjugated goat anti-rabbit antibodies (SIGMA) for 1 hr, followed by 1 hr with Cy3-conjugated streptavidin (SIGMA). Cells were then stained for 5 min at RT with Hoechst 33258, washed once with PBS, mounted with Vecta Shield (Dako Glostrup), and observed under a Zeiss-Axioplan 2 microscope or Leica TCS SP2 AOBS laser scanning confocal microscope.
CHAPTER 3: Results

3.1-Myc partially co-localizes with PML

Myc and PML are two proteins that exhibit different nuclear distributions. As mentioned above, PML localizes to the NB, whereas Myc shows a diffuse speckled sub-nuclear pattern (Yin et al., 2001). In order to investigate whether Myc localization might overlap with that of PML, murine c-Myc and human PML IV were co-expressed in U2OS, Cos-7 and HeLa cell lines, and immunofluorescence assays were performed and analysed by fluorescence microscopy (Fig.3.1). In order to rule out the possibility of artefacts, I co-expressed the two proteins with different viral promoters and at different concentrations, so that I could finally reproduce the subcellular localization of the endogenous proteins.

I observed that the pattern of distribution of each protein is very heterogeneous in the cell nuclei, which probably depends on the cell cycle stage and the expression levels. Myc localization is mostly diffuse throughout the nucleoplasm, although in some cells it seems to concentrate in discrete sub-nuclear structures (Fig.3.1a, d, g). In contrast, PML was localized in the NB, which differ in number and size from cell to cell, often accompanied by a weak diffuse nuclear staining (Fig.3.1b, e, h). Combined immunofluorescence revealed a subset of cells where Myc and PML signals appear to overlap partially in several NB (Fig.3.1 c, f, i).
Fig. 3.1. Co-localization of transfected Myc and PML in different cell types by fluorescence microscopy. U2OS (a-c), Cos-7 (d-f), and HeLa cells (g-i) were co-transfected with pCMV-2-Flag-Myc and pCDNA3-HA-PML IV. Myc protein shows a diffuse nucleoplasmic pattern (red), whereas PML is preferentially organized into the NB (green). Co-localization of the two proteins is observed at some NB (yellow).
The localization assay was also performed on untransfected cells (Fig.3.2). Due to the low and diffuse Myc signal, it is very difficult to evaluate a co-localization between the two proteins. However, several cell nuclei show a co-distribution of Myc with both punctuate and diffuse PML in definite subnuclear areas, suggesting that an interaction between the two endogenous proteins may occur. To verify this hypothesis, I decided to investigate further the co-localization between Myc and PML by confocal microscopy (Fig.3.3). Particularly, I looked at the frequency of co-localization of the two endogenous proteins. HeLa cells were immunostained with PGM3 anti-PML monoclonal antibody and N262 anti-Myc polyclonal antibody, using the biotin-streptavidin amplification system to increase Myc signal. Confocal analysis on the endogenous proteins revealed co-localization in about 10-15% of the cells, in which only a small but consistent fraction of Myc protein localizes to the NB (Fig.3.3 c, f, and enlarged squares).
Fig. 3.2. Localization of the endogenous Myc and PML proteins. Immunofluorescence performed on HeLa cells. In some cells, Myc localization (red) concentrates in discrete areas of the nucleus, possibly the nucleoli, where an enhanced co-localization with the PML bodies and with the diffuse fraction.
Fig.3.3. Analysis of the rate of co-localization between Myc and PML by confocal microscopy. (a-c and d-f) Co-localization of the endogenous proteins in HeLa cells. The white arrows into the enlarged squares (c’, f’ and f”) indicate the site where PML (green) and Myc (red) signals overlap; being this co-localization in most cases unable to cover the entire NB, we argue that Myc localizes only to definite areas of the NB.
3.2-Myc physically interacts with PML

3.2.1-Myc and PML co-immunoprecipitate

In light of the partial co-localization observed by immunofluorescence microscopy, I investigated the association between Myc and PML by immunoprecipitation. To this end, Cos-7 (Fig. 3.4) and HeLa cells (not shown) were co-transfected with two constructs expressing Flag-tagged Myc and HA-tagged human PML IV. Cell lysates were immunoprecipitated with specific antibodies, and the final samples were loaded on a polyacrylamide gel and subjected to SDS/PAGE. Under these conditions, the two proteins migrate as 63 and 85 KDa polypeptide chains, respectively.

As shown in Fig. 3.4, when lysates were immunoprecipitated with anti-Flag or anti-Myc antibody, a small portion of PML was recovered (Fig. 3.4a, lanes 2 and 3). Likewise, immunoprecipitation of PML by addition of anti-PML or anti-HA antibody to the samples determined co-immunoprecipitation (co-IP) of Myc (Fig. 3.4b, lanes 1 and 4). Immunoprecipitation with the control antibody failed to recover either Myc or PML proteins (Fig. 3.4a and b, lanes 5 and 6).

To investigate the interaction further, I performed co-IP experiments in cells that over-expressed only one of the two proteins. When transfected in NIH-3T3 murine fibroblasts, PML was recovered after immunoprecipitation of endogenous Myc (Fig. 3.5, lane 5). However, I could not reproduce the complementary result by immunoprecipitating endogenous PML in Myc transfected cells. This may be due to the stoichiometry of the complex. In fact, it may be that immunoprecipitation with
anti-Myc antibody co-precipitates not only PML protein directly involved in the interaction, but also PML contained in the NB where Myc docks, thus enriching PML recovery. This could also explain why I failed to perceive a detectable amount of Myc protein after immunoprecipitation with anti-PML. Also, the very low extent of endogenous Myc-PML co-localization, and presumably the dynamic nature of their interaction, impeded the co-IP of the endogenous proteins. Due to the ability of both Myc and PML to bind multiple partner proteins, and considering that all of the co-immunoprecipitation experiments were conducted in low stringency buffers, I cannot rule out the possibility that Myc and PML association might not be direct, but rather mediated by the presence of cofactors.

3.2.2-Myc and PML interact by GST pull-down assay

The interaction between the two proteins was also assessed via GST pull-down assay. In this experiment, three different fragments of Myc, spanning the entire protein length, were fused to a GST moiety, expressed in E.Coli, and affinity-purified with a glutathione-sepharose slurry, as confirmed by Comassie staining after loading an aliquot of each sample on gel (Fig 3.6a). The resins were then incubated with cellular extracts from HeLa cells transfected with PML, loaded on a gel, and immunoblot analysis was performed with anti-PML antibody. As shown in Fig.3.6b, PML specifically interacts with the N-terminal portion of Myc, but neither with the other protein fragments, nor with the GST moiety alone (not shown). The protein fragment interacting with PML includes both Myc N-terminal domains, Myc Box I and Box II,
respectively. As Myc Box II is also part of a protein segment that does not show interaction with PML, it is probable that the interaction between the two proteins is mediated by the Box I, or that both domains are required for the interaction with PML.
Fig. 3.4. Myc and PML interact *in vivo*. (a, b) pCDNA3HA-PML IV and pCMV-2-Flag-Myc were co-transfected in Cos-7 cells and immunoprecipitated with antibodies that specifically recognize the two proteins or the two tags, or with unrelated antibodies. Samples were subjected to 7.5%-SDS-PAGE and electroblotted on a Hybond nylon membrane. Western blot was performed with anti-PML H-238 (a) and anti-Myc N-262 (b) specific antibodies.
Fig. 3.5. Transiently expressed PML is immunoprecipitated by endogenous Myc. NIH-3T3 mouse fibroblasts were transfected with HA-PML IV and Flag-Myc together (lane 1 to 4) or separately (lane 5 and 6) and immunoprecipitated with anti-Myc, anti-PML or unrelated antibodies. Western blot was performed with anti-PML antibody. Transfected PML is immunoprecipitated by both transfected (lane 1) and endogenous Myc (lane 5).
Fig. 3.6. PML interacts with Myc by GST pull-down assay. Three recombinant GST-Myc fusion proteins, bearing different domains of Myc and covering the entire protein length, were produced in E.Coli, purified on a Glutathione-sepharose slurry (a), and incubated with a protein lysate obtained from PML-transfected HeLa cells.

Samples were loaded on a polyacrylamide gel, electro-blotted on a membrane, and analysed by Western Blot with an anti-PML antibody (b).
3.3-Myc-PML association increases upon proteasome inhibition.

Besides its diffuse nucleoplasmic staining, Myc can also distribute around the nucleoli. When over-expressed, the rate of Myc localization to the nucleoli increases, and the same effect is observed if cells are treated with proteasome inhibitors (Arabi et al., 2003). Recent evidences have demonstrated the recruitment of PML to the perinucleolar zone following proteasome inhibition (Mattsson et al., 2001). I observed that the nucleolar distribution of over-expressed Myc induced PML sequestration at the nucleoli in several cells (Fig.3.7A). Therefore, I hypothesized that Myc-PML interaction might be sensitive to the turnover determined by the proteasome-mediated degradation. To test this, HeLa, Cos-7, and U2OS cells where transfected with Myc, and incubated for 5 hours with 50μM MG-132 in DMSO or with DMSO alone. Over-expressed Myc showed the classical nucleoplasmic diffuse and nucleolar pattern, both in untreated or DMSO-treated cells. However, cells treated with the proteasome inhibitor showed an increased distribution of Myc around the nucleoli and in well-defined nuclear speckles (Fig.3.7B, a-c). As the proteasome co-localizes with Myc at the nucleoli after proteasome inhibition, I checked whether this localization is also extended to Myc-containing nuclear speckles. We observed a strong re-localization of the proteasome in Myc nuclear inclusions, which was undetectable in untransfected cells (Fig.3.7B, d-f). To further confirm the specificity of the effect observed, cells were co-transfected with Myc and CBP, or with PML and CBP. After proteasome inhibition, Myc re-localized in the nuclear speckles, whereas CBP does not (Fig.3.7B, g-i); furthermore, this effect was never observed in PML and CBP co-transfected cells.
Upon Myc transfection, endogenous PML showed a remarkable redistribution into Myc-enriched structures. PML strongly co-localized with Myc in the perinucleolar region and almost completely in Myc-containing speckles, whereas PML localization was not affected by proteasome inhibition in untransfected cells (Fig.3.7C, a-c). Then, I checked if Myc re-localization with PML after cell treatment with the proteasome inhibitor corresponded to increased interaction. HeLa cells co-transfected with Myc and PML, and treated with MG-132, showed a clear enrichment of Myc-PML co-immunoprecipitation, using either anti-Myc or anti-PML antibodies (Fig.3.8), thus confirming that proteasome inhibition stabilizes the Myc-PML complex.
Fig. 3.7A. **Myc-PML association increases at the nucleoli.** Perinucleolar accumulation of Myc (green) in a small percentage of Myc-transfected HeLa cells induces mislocalization of endogenous PML (red) at the nucleoli.
Fig.3.7B. Myc nuclear localization is influenced by inhibition of protein degradation. (a-c) Myc nuclear localization in Cos-7 cells untreated (a) or treated as indicated (b-c). (d-l) U2OS cells were transfected with Myc alone (d-f), or co-transfected with Myc and CBP (g-l), or with PML and CBP (j-l), treated with MG132, and subjected to immunofluorescence staining as indicated. pr.inh.: proteasome inhibitor; 20S: 20S proteasome subunit.
Fig. 3.7C. Myc-PML association increases upon proteasome inhibition. HeLa cells transfected with Myc and subjected to immunofluorescence assay as indicated. PML shows increased relocalization with transfected Myc at the nucleoli and at well-defined nuclear inclusions.
Fig. 3.8. Myc-PML physical interaction is increased by proteasome inhibition. HA-PML and Flag-Myc were co-transfected in HeLa cells, immunoprecipitated with the indicated antibodies in presence or absence of the proteasome inhibitor, and analysed by Western Blot.
3.4-PML enhances Myc induced expression of an E-Box driven reporter gene

As mentioned above, PML is deeply involved in the regulation of gene expression by modifying transcription factor activity. Therefore, I investigated the role of PML in the main tasks of Myc: DNA binding and regulation of target gene expression.

As a preliminary assay, I tested the ability of PML to influence Myc transactivation properties. HeLa and HepG2 cells were transfected with Myc and PML constructs, and with a reporter vector that bears 4 E-boxes upstream of the minimal Tk promoter that drives expression of the firefly luciferase reporter gene (Vervoorts et al., 2003). As shown in Fig.3.9, PML enhances the transcriptional response of Myc, resulting in a 2- to 3-fold increase of luciferase activity compared to the expression of the reporter gene by Myc alone. The same results were observed in HEK 293 cells (data not shown). PML showed negligible influence on the expression of the E-box driven luciferase in the absence of Myc. These results show that PML influences the transcriptional activity of Myc.
Fig. 3.9. Transactivation assay. HeLa and HepG2 cells were transfected with a luciferase reporter gene driven by 4 E-box elements, and luciferase activity was measured after co-transfection with pCMV-2-Flag-Myc and pCDNA3-HA-PML IV ( ), pCMV-2-Flag-Myc and pCDNA3-HA ( ), pCMV-2-Flag and pCDNA3-HA-PML IV ( ) or pCMV-2-Flag and pCDNA3-HA ( ).
3.5-Selection of the cell-system and of the Myc target genes to be analysed by ChIP and Real-Time PCR

In order to investigate more extensively the role that PML plays in Myc transcriptional activity, I decided to perform a widespread in vivo comparative analysis of expression levels and promoter occupancy of Myc direct target genes. Particularly, I took advantage of wt and PML-/- MEFs, where the PML gene has been knocked out (Wang et al., 1998a). In this way, I could compare the efficiency of Myc transcriptional properties in two cell types that differ only for the presence of the PML loci.

3.5.1-Myc and PML co-localize in MEFs

The suitability of MEF cell-system for our studies was evaluated by immunolocalization and gene-expression analysis (Fig.3.10 and 3.11, respectively). Immunofluorescence assay confirmed that, following co-transfection of Myc and PML in wt MEFs, Myc protein localizes to the NB (Fig.3.10).

3.5.2-wt and PML-/- MEFs show the same timing of cell-cycle re-entry from quiescence

C-myc is an early serum-response gene, being immediately expressed by cells that exit their quiescent state. C-myc expression peaks in the early G1 phase and rapidly decreases as cells enter the S phase (Henriksson and Luscher, 1996). Therefore, I was interested in monitoring the events that occur during that time span.
To do this, wt and PML-/- MEFs were synchronized by serum starvation and induced to re-enter the cell cycle. Cells were harvested while still in the G0 phase (t0 time point) and 6, 12, 16 and 20 hours after serum addition. This protocol allowed me to follow the whole of the G1 and part of the S phase.

The efficacy of synchronization and the comparable timing of cell phases between the two cell types were assessed by using Real-Time quantitative PCR to monitor the expression profile of c-myc, and of Ccnb1, the gene that encodes for cyclin B1. c-myc expression peaks in the early G1 and returns to basal levels in the late G1/S phase, while Ccnb1 is strongly expressed by the late S/G2 phase. In my cell culture conditions, the expression profile of these genes accounts for a comparable length of the G1 phase that follows the exit from G0 in the two cell lines (Fig.3.11). Finally, I monitored the proper expression of PML mRNA in wt MEFs, which has been reported to be expressed during G1 and to decrease by the S phase (Chang et al., 1995).

To ensure that Myc was equally represented in both wt and PML-/- MEFs, I checked c-myc transcription and translation in the two cell types. As Fig.3.11 shows, c-myc mRNA expression levels are comparable in the two cell types. In order to analyse Myc protein levels, Myc was immunoprecipitated from wt and PML-/- synchronized MEFs at the selected time points. In parallel, I also surveyed Myc protein stability in the two cell types. Proliferating wt and PML-/- MEFs were incubated with 10μg/ml cycloheximide to block protein synthesis, and subjected to immunoprecipitation before adding cycloheximide (nt), and 30 or 60 minutes after drug addition. As shown in Fig.3.12A and B, both experiments didn’t show significant
differences, even if, at a more careful analysis, Myc seems slightly more stable in PML-/- cells.

3.5.3-Choice of Myc target genes

Once I had ensured that MEFs provided a good cell system for my studies, I decided to look for candidate Myc direct target genes to be chosen for chromatin immunoprecipitation (ChIP) and Real-Time PCR experiments. In order to obtain an unbiased analysis of Myc transcriptional properties, I decided to include in my study genes with different cellular functions, and for which Myc-mediated transcriptional activation or repression had already been reported. Primers were designed taking advantage of the Primer3 software, developed by the Whitehead Institute for Biomedical Research (Cambridge, MA), in order to amplify 200-300bp fragments. On Myc up-regulated genes, the selected primer pairs would amplify regulatory regions that are very close, or overlapping with the Myc-responsive E-box elements. On the other hand, potential down-regulated target genes were analysed by amplification of the promoter regions covering the TATA-box, the Inr, or other promoter sequences bound by transcription factors negatively influenced by Myc. A schematic view of the promoter and of the 5' structure of Myc target genes is reported in Fig.3.13.
Fig. 3.10. Co-localization of Myc and PML in MEFs. Co-staining (yellow) of transfected pCMV-2-Flag-Myc (red) and pCDNA3-HA-PML IV (green) is detectable in nearly all of the NB.
Fig. 3.11. Expression profile in wt and PML-/- MEFs. Cells were synchronized by serum starvation and harvested before (t0) or 6, 12, 16, and 20 hours after cell cycle re-entry was induced by serum addition. After cDNA synthesis, single gene expression analysis was performed by Real-Time PCR using Gapd as reference gene, and repeated at least four times. The time point with the lowest expression level was used to normalize each assay.

x-axis: time points; y-axis: fold expression; wt MEFs: ■; PML-/- MEFs: □
Fig. 3.12. Myc protein quantification and stability in wt and PML-/- MEFs at the time points selected for ChIP and Real-time analysis.

(A) Myc protein levels at the selected time points. The different levels of Myc protein at t6 and t16 in the two cell types were not reproducible, suggesting that this may be introduced by the experimental procedure. The low level of Myc protein in the t20 -/- sample is due to a problem of sample loading, and not to a reproducible difference compared to wt.

(B) Myc protein stability before (nt = not treated) and after 30 or 60 min incubation with 10µg/ml cycloheximide.
Fig. 3.13. Schematic representation of the 5’ region of the selected Myc target genes.
3.6-PML does not affect Myc binding to DNA, yet it influences the expression profile of several Myc target genes

3.6.1-Chromatin immunoprecipitation of Myc targets

Myc binding to regulatory regions was assessed for 36 genes that are proven or candidate Myc direct targets (www.c-mycancergene.org and Table 1) by performing chromatin immunoprecipitation (ChIP). Myc-DNA complexes were immunoprecipitated at the time points reported above after induction of cell cycle re-entry. As negative controls, immunoprecipitation was performed without antibody as well as at t0, where Myc is functionally inactive and Myc protein levels are very low. PCR analysis on the immunoprecipitated DNA confirmed that Myc binds to all of the genes analysed, some of which had not been previously confirmed by ChIP (Table 1). Moreover, when PCR was performed using couples of primers designed outside the Myc-responsive region of genes like Ddx18 and Cdknb2, no enrichment was detected, further confirming the specificity of the ChIP assay (data not shown).

Fig.3.14 reports the ChIP profile of 6 out of 22 Myc up-regulated genes and of 5 out of 14 Myc down-regulated genes. I could not find any substantial difference in Myc binding levels between the two cell types, with the exception of a few targets where PCR performed on Myc-immunoprecipitated samples at t0 revealed a faint band in PML-/- MEFs that was absent in the wt counterpart, suggesting that some residual Myc activity may escape G0 block.
Fig. 3.14. Analysis of Myc binding to target promoters by chromatin immunoprecipitation (ChIP). Wt and PML-/— MEFs were synchronized by serum starvation. DNA-protein cross-linking and cell harvesting was performed before (t0) and 6, 12, 16, and 20 hours after induction of cell cycle re-entry by serum addition. An aliquot of extract for each time point was set apart to quantify total DNA. (a): immunoprecipitation with anti-Myc N-262 antibody; (-): no antibody. Immunoprecipitated samples were subjected to reversal of cross-linking and DNA purification, and the enrichment in the promoter region of Myc target genes was assayed by performing PCR analysis with specific primers (see Materials and Methods). Both genes target for up-regulation (up) and down-regulation (down) were analysed (see Table1).
3.6.2- Analysis of Myc target genes by Real-Time PCR

In parallel with the ChIP analysis, I monitored the expression profile of 40 genes (Table I), including all of the genes analysed by ChIP, and others for which the direct influence of Myc has already been proven, by performing quantitative PCR analysis at the same time points. Significantly, in the G1 phase (t6 and t12), which coincides with the peak of c-myc expression and Myc protein activity, many of the target genes, both activated and repressed, showed an increased expression in PML-/- MEFs compared to wt.

Table I summarizes the differences observed in the gene expression profile between the two cell types at the selected time points. For all of the genes, the expression differences between the two cell types at each time point were analysed by performing the paired Student’s t-test. This test focuses on the difference between paired data, and reports the probability that the mean difference is consistent with zero. This statistical function proved suitable for my study, as I needed to pair the expression data obtained from wt and PML-/- MEFs of every single experiment, when evaluating the consistency of the mean differences.

As shown, 21 out of 40 genes analysed display a statistically significant increase of expression in PML-/- G1 cells (Student’s t<0.05), and many of the remaining 19 genes show this same trend (see Fig.3.15 and 3.16). In the table, genes are divided into subgroups corresponding either to significantly increased expression in wt or PML-/- cells, or to no increase in the G1 phase (t6 and t12). Fig.3.15 shows the altered expression profiles of TMP and Pdgfr, two genes that are reported targets for up- and down-regulation by Myc, respectively. Of the genes analysed, p21 is the
only one that is more expressed in wt cells. p21 is also a direct target of p53, which shows defective activity in PML-/- MEFs (Fogal et al., 2000; Guo et al., 2000). It is possible that p21 gene misexpression is determined by an epistatic effect of p53. The expression profile of the other genes analysed is reported in Fig.3.16.
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<th>Real Time*</th>
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Table 1. Myc target gene analysis by ChIP and Real Time PCR.

a: + = positive result; ?= unclear; n.p.= not performed

b: wt= genes more expressed in wt cells; -/-= genes more expressed in PML/-/cells; G1 = G1 phase; n.d.= no statistical difference; *= the asterisk indicates expression profiles opposite to what reported in literature.
3.6.3-Analysis of the expression data

Fig.3.17 summarizes the expression data, and shows the number of genes that are differentially expressed in the two cell types at each cell phase. The diagrams illustrate the distribution of genes up- or down-regulated by Myc, separately or together. At t0, while cells are kept in the G0 phase, most genes do not show differences in the expression levels between the two cell types. At t6+t12, corresponding to the G1 phase, there is a strong increase in the number of genes, both activated and repressed by Myc, which are more expressed in PML-/- cells. This number decreases as cells enter the S phase (t16), notwithstanding the difference between the outlines of the two gene groups. In fact, the expression levels of the up-regulated genes return to become comparable between the two cell types as soon as cells exit the G1 phase. In contrast, the augmented expression in PML-/- cells shown by down-regulated genes during G1 is mostly retained toward the S phase, indicating a difference of sensitivity between the mechanisms that regulate transcription in the two gene subpopulations.

The results obtained by ChIP and Real-Time PCR, together with the co-localization and interaction data, strongly indicate a direct involvement of PML in Myc-mediated transcriptional activity.

The observation that the two oppositely regulated gene categories share the same kind of alteration in their expression profile after PML withdrawal suggests an epistatic role of PML on Myc activity.
Fig. 3.15. Expression profile of Myc target genes by Real-Time PCR (see fig. 3.9). Each value is the mean of four independent experiments, and the differences were validated by Student's t test (t<0.05). x-axis: time points; y-axis: fold expression; wt MEFs: ■; PML-/- MEFs: □
Fig. 3.16

- **B23 expression**
- **Bax expression**
- **BN51 expression**
- **Ced expression**
- **Cdc25a expression**
- **Cdki4 expression**
- **Cul1 expression**
- **Cyclin D2 expression**
Legend:
- wt
- +/-
x-axis: time points
y-axis: fold induction
Red numbers: Student's t<0.05
Panels 1 to 3: up-regulated gene-expression profiles
Panels 4 and 5: down-regulated gene-expression profiles
Fig. 3.17. Comparative analysis of gene expression levels in wt and PML -/- cells throughout cell cycle. Genes are divided in groups that show increased expression in MEFs wt (grey), PML-/- (black), or that show no difference (white), and compared in the G0 (t0), in the early and middle G1 (t6+t12), and in the late G1/early S phase (t16). The three diagrams display the result of the comparative analysis of the three gene groups in up-regulated, down-regulated or total genes.
3.7-PML is a Myc target gene

As shown in Fig.3.10, c-myc and PML gene expression profiles show opposite trends throughout the cell cycle. PML is highly expressed in G0, where c-myc displays a basal level of expression; during the early G1 phase, c-myc expression is strongly induced, whereas PML expression curve drops dramatically, and is re-induced by late G1. I hypothesized that PML might be a Myc target gene, and that Myc might participate to PML gene repression in G1. Using the rVISTA analysis, (Loots et al., 2002), I compared the mouse and human PML promoter regions, and we identified a highly conserved promoter region, spanning 100bp 5' of the Inr sequence. Particularly, this sequence contains several Sp1 putative binding sites (Fig.3.18A). As Myc can inhibit gene expression by physical interaction with both the Inr-complex and Sp1 (Gartel and Shchors, 2003), I checked whether Myc binds to the PML promoter. ChIP was performed using NIH-3T3 cells starved by 48-hour serum withdrawal, and harvested before (t0) or 3 hours after serum was re-added to the culture medium (t3), in order to obtain two cell populations in G0 and in the early G1 phase, respectively. ChIP successfullness was confirmed by performing PCR using nucleolin-specific primer pairs (Fig.3.18B). PCR analysis was performed using two different primer pairs, one of which including the conserved sequence and the Inr, and the other spanning -500 to -300bp 5' of the Inr. As shown in Fig.3.18, a strong enrichment of the Inr-containing sequence was observed. As the chromatin sonication step preceding immunoprecipitation produced 300-2000bp fragments, a lower but consistent enrichment of the adjacent region was also detected. Myc binding to the PML
promoter strongly indicates that Myc is directly involved in PML transcriptional control.
Fig. 3.18. **PML is a Myc target.** (A) Schematic representation of the comparison between the murine and the human PML promoter. The pink-coloured area indicates the most conserved region between the two species. PML.1 and PML.2 indicates the regions amplified by PCR following ChIP assay. (B) ChIP performed on synchronized NIH-3T3 murine fibroblasts. a = anti-Myc antibody; - = no antibody.
CHAPTER 4: Discussion

In metazoans, cell growth, survival and commitment are major regulatory functions that need to undergo severe combinatorial regulation to keep every cell system in the designed equilibrium. Cells are sensitive to a variety of extra-cellular stimuli that trigger specific intra-cellular signalling pathways and that play crucial roles in cell fate maintenance. The integration of these diverse signals by proteins that regulate cell proliferation, differentiation, and apoptosis, ultimately determines the response of individual cells. Mutations that perturb this system often predispose or determine genetic diseases and cancer. Myc and PML are two essential integrators of extracellular stimuli that seem to play antagonistic and overlapping roles in cellular response. The data I have presented show for the first time that Myc and PML physically interact and that Myc partially localizes within the NB. Moreover, these results indicate that this interaction seems to have functional consequences on Myc activity, as the absence of PML alters the expression of numerous Myc target genes.

4.1-PML influences the expression of Myc target genes

In PML-/- MEFs, Myc target genes display a general up-regulated expression during the G1 phase, when Myc reaches its peak of transcriptional activity, suggesting that Myc function is somehow altered. The genes selected for this study belong to diverse cell functions, such as growth, proliferation, and survival. I could not associate the observed differences of expression to any of these subgroups, further suggesting that a general deregulation of Myc-mediated transcriptional activity occurs.
The proto-oncogene c-myc executes multiple activities mostly through the transcriptional regulation of target genes. Myc can activate genes through multiple regulatory events both at the level of transcriptional initiation and elongation (Bouchard et al., 2001; Cheng et al., 1999; Eberhardy and Farnham, 2001; Frank et al., 2001; Xu et al., 2001). By contrast, Myc seems to repress transcription by interfering with the transcriptional machinery or with the enhancers that are required for the activation of the target genes (Feng et al., 2002; Gartel et al., 2001; Izumi et al., 2001; Staller et al., 2001; Watanabe et al., 2002). As both activated and repressed Myc target genes share the same tendency in PML-/- cells, PML probably does not influence the two distinct pathways; rather, it might affect some upstream regulation of Myc activity. More than 50% of the genes show a statistically significant increase of expression in PML-/- G1 cells compared to wt. Likewise, many of the remaining genes reveal the same trend. In some specific cases, such as for p21 expression, the genes selected might also be controlled by other transcription pathways, the function of which would be possibly affected by the lack of PML as well. It is also possible that the result obtained in murine fibroblast may vary in different tissues, depending on the two proteins abundance or on the involvement of additional specific partners.

4.2-PML does not affect Myc binding to chromatin targets

The effect of PML on Myc transcriptional activity does not seem due to interference with Myc DNA-binding properties. The PCR reactions that I performed on ChIP samples are semi-quantitative, as I could clearly observe the enrichment of
Myc binding to the regulatory regions of the target genes throughout the G1 phase. My results indicate that Myc binding to DNA is independent of the presence of PML.

4.3- Myc involvement in PML transcription

The discovery that PML is a Myc target gene suggests that the two genes might undergo reciprocal transcriptional regulation. PML has been recently involved in the β-catenin and plakoglobin transcription pathways, which regulate the transcription of proliferation-related genes, including c-myc (Shtutman et al., 2002). Moreover, PML-RARα fusion protein up-regulates the expression of the c-myc gene (Muller-Tidow et al., 2004). It would be interesting to verify this hypothesis within pathways that require a proper regulation of both genes, as for example the interferon (IFN) pathway. In fact, as already mentioned, PML is upregulated by interferons (Heuser et al., 1998); this upregulation correlates with IFN antiviral and antiproliferative effects. Consistent with this hypothesis, NB disruption is a prerequisite for viral infection (Ruggero et al., 2000; Turelli et al., 2001). Conversely, c-myc shares many target genes with IFN-γ and c-myc overexpression correlates with a global deregulation of multiple IFN-response genes (Nesbit et al., 2000), possibly by positive or negative interaction with several INF-related transcription factors, such as Sp1.

4.4-Trisomy of the c-myc locus is frequently associated to APL

As known, Myc over-expression is a marker of poor prognosis for several types of cancers (Pelengaris et al., 2002). NB are strongly involved in tumour suppression
(Salomoni and Pandolfi, 2002), and PML localization to the NB is a prerequisite for the formation and the maintenance of their macromolecular structure (Ishov et al., 1999; Zhong et al., 2000). In APL blasts, the PML/RARα protein targets and delocalises PML, causing the destruction of the NB into micro-speckled structures. Abrogation of the NB structure might lead to the de-regulation of Myc activity and predispose to cellular transformation. Intriguingly, 15-40% of patients with APL and t(15;17) have complete or partial trisomy of chromosome 8 as secondary defect, and the smallest region of gain corresponds to bands 8q23-24, where c-myc gene is mapped (Le Beau et al., 2002; Le Beau et al., 2003; Zimonjic et al., 2000). As well, PML/RARα transgenic mice develop recurring chromosomal alteration in leukemic cells, the majority of which associates with trisomy of chromosome 15. Murine c-myc maps on chromosome 15D2-3, and these bands are frequently associated to mice leukaemias and lymphomas. These data suggest that Myc tumorigenic pathway might be preferentially selected following NB disruption, which would further account for the requirement of intact NB for the surveillance of Myc activity.

4.5-PML directly influences Myc transcriptional activity

Transactivation assays further suggest that PML directly influences Myc-mediated transcription. However, the increased transcriptional activity of Myc when co-expressed with PML is in contrast with the results obtained in vivo, as Myc target genes are upregulated when PML is absent. This divergency is probably due to the over-expression of the two proteins, as Myc and PML expression levels are crucially related to their transcriptional role (Eisenman, 2001; Salomoni and Pandolfi, 2002).
As in this assay the balance between the two proteins is lost, the transcriptional assay only indicates that a cross-talk between the two proteins exists, whereas the absolute transcriptional result is not informative.

4.6-Does PML play a role in Myc post-translational modification?

The finding that Myc is recruited in the NB further enforces the idea that the PML bodies are key co-factors in modulating cell transcriptional activity, and opens questions concerning the mechanism by which PML influences Myc transcriptional activity.

4.6.1-Myc function is regulated post-translationally

Myc protein is subjected to several post-translational modifications, particularly phosphorylation, glycosylation, ubiquitination and most recently observed, acetylation (Kamemura et al., 2002; Salghetti et al., 1999; Sears et al., 2000; Vervoorts et al., 2003). These modifications play diverse roles on protein fate and function. In fact, glycosylation antagonizes phosphorylation of Threonin 58, which is strictly related to Myc degradation and is a well-known mutation hotspot, which renders cells tumour prone (Gregory and Hann, 2000). Also, ubiquitination has recently emerged as a wide mechanism that regulates the functional activation of transcription factors (Salghetti et al., 2001). Besides targeting Myc degradation (Salghetti et al., 1999), ubiquitination enhances Myc transcriptional activity via recruitment of proteasome subunits, which have been found together with Myc on the
promoter of the gene encoding for cyclin D2 (Kim et al., 2003; von der Lehr et al., 2003). It was recently proposed that ubiquitination of Myc protein might compete with acetylation by CBP (Vervoorts et al., 2003). Interestingly, other than recruiting CBP at the NB, PML interacts with the proteasome as well (Lallemand-Breitenbach et al., 2001), even if the functional significance of this interaction is still obscure.

4.6.2-Post-translational modifications at the NB

In the localization experiments, I observed that only a small amount of Myc localizes to the NB. This is consistent with the data reported for other transcription factors that functionally interact with PML (Fang et al., 2002; Fogal et al., 2000; Wu et al., 2002). It is possible to hypothesize that the NB regulate just a small sub-pool of Myc protein, or that the turnover of the interaction is extremely rapid. As known, NB are sites of regulation of transcription factor post-translational modifications, such as phosphorylation, acetylation and sumoylation (Best et al., 2002; Moller et al., 2003; Pearson et al., 2000), and are engaged in gene expression via preferential recruitment of transcription co-factors such as histone acetyltransferases (HATs) like CBP and p300 (Doucas et al., 1999; LaMorte et al., 1998), or histone deacetylases (HDAC) and co-repressors ((Khan et al., 2001; Langley et al., 2002; Wu et al., 2001), represented in Fig.4.1).
4.6.3-Inhibition of Myc degradation pathway induces increased interaction with PML

As Myc and PML are both engaged by the proteasome, we investigated if Myc recruitment to the NB could be affected by blocking the proteasome degradation pathway. Immunolocalization and immunoprecipitation performed in cells treated with proteasome inhibitor show that the pool of Myc protein co-localizing and interacting with PML dramatically increases. This enrichment suggests that the amount of Myc that is recovered at the NB strongly depends on its post-translational turnover. I observed that a small amount of Myc localizes at the NB, suggesting transient and dynamic interaction. This is consistent with the data reported for other transcription factors that interact with PML, as well as for other PML interactors, such as CBP (Borden, 2002). Myc and PML belong to a subset of proteins that translocate to the nucleoli when overexpressed or by inhibition of the proteasome-mediated degradation (Arabi et al., 2003) (Mattsson et al., 2001). In HeLa cells, PML takes several hours to localize in the nucleolus following proteasome inhibition; however, when I overexpress Myc, strong co-localization of the two proteins with the proteasome at the nucleolus and in Myc nuclear inclusions is detected after 3 hours. As Myc localization to the nucleoli is a dynamic and reversible process that engages the proteasome, I hypothesize that Myc accumulation induced by overexpression and proteasome inhibition also stabilizes the dynamic interaction with PML. These data further suggest that the interaction between Myc and PML may depend on Myc post-translational status.
4.6.4-Proposed model

Myc recruitment in the NB and physical interaction with PML opens questions concerning the mechanism by which PML influences Myc transcriptional activity. Therefore, we hypothesize that PML might recruit Myc to the NB and influence its activity, modulating the status of Myc protein (compare figure Fig4.1a and b). Due to the multiple regulatory mechanisms it takes part in, we propose that Myc selectively binds distinct partners depending on its post-translational status. If PML is lacking, this balance is perturbed, and Myc protein modifications might render Myc more efficient as a transcriptional activator of the up-regulated genes than as a mediator of repression of the down-regulated ones.
Fig. 4.1. Schematic representation of the PML and Myc pathway, before (a), and after (b) this study was performed. The transient recruitment of Myc to the NB could modify Myc post-translational status and contribute to address Myc toward its biological functions.
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CHAPTER 5: Bibliography


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