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"Positive and negative regulation of Myc-mediated transcription"

Tutore

Ch.mo Prof. Luigi Lania

Candidato

Dott.ssa Barbara Gargano

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CHAPTER I

Introduction

5.8 The Myc oncogene

The *c-myc* proto-oncogene encodes the c-Myc transcription factor, and was originally identified as the cellular homologue to the viral oncogene (*v-myc*) of the avian myelocytomatosis retrovirus (Vennstrom et al., 1982).

The *c-myc* gene is located on human chromosome 8q24. It was discovered soon after its identification that activated oncogenic *c-myc* was instrumental in the progression of the human Burkitt's lymphoma, as a results of a translocation between chromosome 8 and one of the three chromosomes that contain antibody-encoding genes (Dalla Favera et al., 1982).

For 25 years, from its discovery, *c-myc* has been a pioneer in the oncogene field. Among the first cellular homolog of genes cloned from acute oncogenic viruses, it is also the first site of proviral integration at an oncogene, the first oncogene mapped to a chromosomal translocation breakpoint and the first oncogene found amplified in tumour cells.

The *c-myc* gene is induced by a wealth of growth factors and is essential for most normal cells to proliferate. Following the deregulation of *c-myc* expression by translocation, gene amplification or aberrant signalling, c-Myc becomes a potent oncoprotein that promotes unrestrained cell proliferation (Evan et al., 2001). Approximately 70% of human tumours have elevated *c-myc* expression, and suppression of *c-myc* expression can lead to regression of tumours (Felsher et al., 1999).

1.2 Myc family members

In mammals there are four related genes in the Myc family*, c-myc, N-myc, L-myc* and *S-myc*, all function as oncogenes in different tumors and have a

high degree of sequence conservation. A fifth gene, *B-myc*, encodes a protein that shows significant homology to the N-terminus, but lacks essential domains in the C-terminus, of the other Myc proteins, and its biology is poorly understood (Levens et al., 2003,; Eisenman, 2001).

c-myc and *N-myc* are particularly well conserved and have equivalent oncogenic activities. Furthermore, their coding regions can substitute for each other in mouse development (Malynn et al., 2000). Myc proteins are also well conserved across species, which is reflected in the observation that the Drosophila myc gene, *dmyc*, can functionally substitute for mammalian *c-myc.*

Since the viral oncogene (*v-myc*) of the avian myelocytomatosis retrovirus was a nuclear protein (Hann et al., 1983) several groups began to investigate whether Myc was a transcription factor by measuring the transcriptional response of individual genes to Myc expression (Dean et al., 1987). Around the same time, the Myc C-terminus was found to contain a leucine zipper (LZ) and a helix-loop-helix (HLH) motif, both of which were previously found in sequence specific DNA-binding proteins (Murre et al., 1989; Landschulz et al., 1988).

Myc dimerizes with the basic helix-loop-elix (bHLH)/Leu-zipper protein Myc-associated factor-X (Max) through a C-terminal HLH/Leu-zipper domain to facilitate DNA binding.

The N-terminus of MYC protein contains a transactivation domain and a number of evolutionarily-conserved motifs known as MYC boxes (**figure 1**). MYC boxes are well conserved across species (Cole and Cowling 2008). In particular, MYC box II (MBII) is highly conserved and is the most important region of the transactivation domain. MBII is necessary for MYC binding to most cofactors, for the transactivation and repression of most MYC target genes and for the efficient execution of the biological effects of MYC.

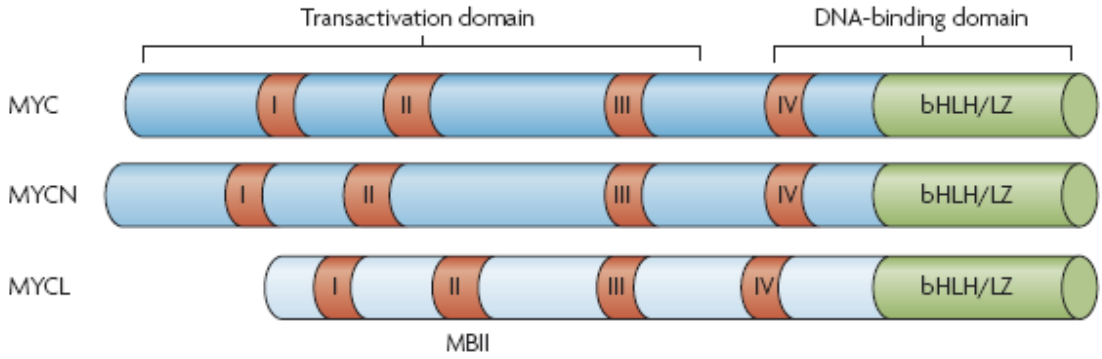

Figure 1. The conserved regions of MYC. The three MYC proteins (MYC, MYCN and MYCL) are encoded by separate genes with distinct developmental regulation, but all three have been directly implicated in cancer. The N terminus of MYC contains the transactivation domain and the C-terminus contains the DNA-binding domain. The MYC boxes I, II, III and IV are indicated in red. The basic helixloop-helix/Leu zipper (bHLH/LZ) domain is indicated in green. MYC box II (MBII) has been shown to have a crucial role in most of the biological activities of MYC. The MBIV is not a component of the minimal DNA-binding domain but does influence DNA binding *in vivo*.

The MBI and MBII were also found to be necessary for Myc to induce apoptosis and block differentiation. Two further Myc homology domains have been characterized. MBIII is necessary for cell transformation and deleting MBIII potentiates Myc-induced apoptosis (Herbst et al., 2005). MBIV is also necessary for full Myc transforming activity and apoptosis, and deleting MBIV potentiates Myc-induced G2 arrest (Cowling et al., 2006).

N-myc is another member of the Myc family prominently expressed in undifferentiated subsets of cells in the lung, heart, central and peripheral nervous system, kidney, visceral arches, limb buds, and eye (Zimmerman et al., 1986; Mugrauer et al., 1988; Downs et al., 1989; Hirvonen et al., 1990; Hirning et al., 1991).

There is not considerable sequence divergence among *c-myc* and *N-myc*, infact complementation experiments performed in Rat1 fibroblasts lacking Myc suggest that they are largely functionally redundant (Berns et al., 2000; Nikiforov et al., 2000). Furthermore, mice in which the *c-myc* gene was replaced with the *N-myc* gene exhibited few developmental defects and

were viable (Malynn et al., 2000). Although they share a high degree of functional redundancy, *N-myc* and *c-myc* have strikingly distinct patterns of gene expression. Whereas *c-myc*, is expressed during embryonic development and in adult tissues, *N-myc* is expressed almost exclusively in embryonic tissues. It is also intriguing that *N-myc* and *c-myc* are expressed in highly complementary patterns during embryonic development (Hurlin et al., 1997).

In most tissues and organs, *N-myc* is normally expressed in cell compartments comprised of progenitor populations. Collectively, *N-myc* maintains the cells in a proliferative and undifferentiated state. In this capacity, *N-myc* serves as an essential downstream target of various key signaling pathways (SHH, Wnt, TGF, and FGF pathways) to help coordinate morphogenesis.

Among the members of the family, the *N-myc* oncogene is implicated in the pathogenesis of neural crest derived tumors including neuroblastoma, the most frequent solid malignancy in infants. Amplification of *N-myc* gene is the major negative prognostic marker in human neuroblastoma.

1.3 Myc as transcription factor binds chromatin modifying complexes

Myc is able to bind a partner protein, Max, through a basic-region/helix-loop-helix/leucine-zipper (BR/HLH/LZ) domain (Blackwood et al., 1991).

While Max can homodimerize and bind to DNA directly, Myc cannot homodimerize and must form an heterodimer with Max in order to bind the specific DNA sequence CACGTG (the E-box) (Blackwood et al., 1991). Max is a small, ubiquitously expressed protein that can bind to a whole collection of B-HLH-LZ proteins (Baudino et al., 2001). Transcription-competent Myc/Max dimers are the active form of Myc in inducing cell-

cycle progression, apoptosis and malignant transformation (Henriksson and Luscher, 1996; Amati and Land, 1994; Amati et al., 1993).

Max factor can also form homodimers or heterodimers with several related proteins, known as Mad1, Mxi1 (also known as Mad2), Mad3, Mad4 and Mnt (also known as Rox), as shown by *in vitro* binding experiments (Ayer and Eisenman., 1993; Hurlin et al., 1997). The dimers all bind directly to the same DNA sequence (CACA/GTG), which is a subset of the general E-box sequence (CANNTG) that is bound by all bHLH proteins (Blackwell et al., 1990). *In vivo*, Myc–Max complexes activate transcription through interactions with transcriptional coactivators (such as TRRAP and BAF53) and their associated histone acetyltransferases (HATs, e.g., GCN5) and/or ATPase/helicases (TIPs, e.g., TIP49) (McMahon *et al.*, 1998, 2000 ; Dugan *et al.*, 2002). This interactions are often predominant in proliferating cells (**Figure 2A**). Instead Mad–Max or Mnt–Max complexes are predominant in resting or differentiated cells (Ayer and Eisenman., 1993) where actively repress transcription through direct protein-protein interactions with the general transcriptional corepressors Sin3a/-3b (Ayer et al., 1995) with Sin3's corepressors (e.g., N-Cor and the Ski/Sno proteins) and histone deacetylases (HDACs) (Alland et al., 1997; Heinzel et al., 1997). Histone deacetylation is currently thought to be the major mechanism of transcriptional silencing by the Mad proteins (**Figure 2B**). The Sin3-intacting domain motif, when tethered to an HLH/LZ transcriptional factor, TFEB, that binds Myc DNA sites, is able to inhibit c-Myc-mediated cellular transformation (Harper et al., 1996).

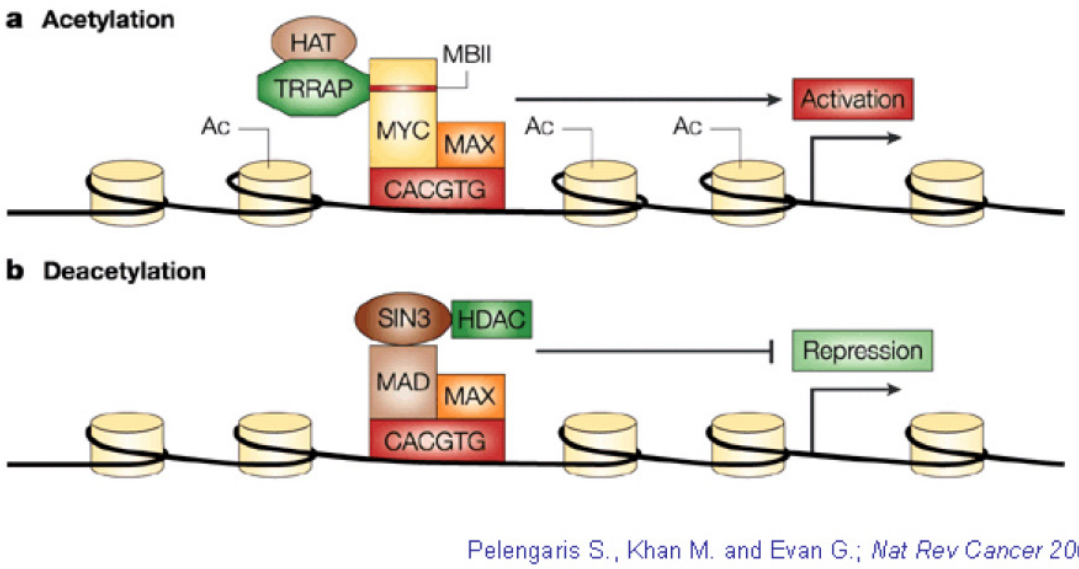

Figure 2. MYC-MAX and MAD-MAX complexes regulate gene activation through chromatin remodelling. **A)** MYC-MAX heterodimers binds to an E-box sequence (CACGTG) near the promoter of a c-MYC target gene. Co-activator TRRAP (transformation/transcription domain-associated protein), a component of a complex that contains histone acetyltransferase (HAT) activity, is then recruited to the MBII domain of c-MYC and acetylates (Ac) nucleosomal histone H4 at the E-box and adjacent regions. Nucleosomal acetylation alters chromatin structure, allowing accessibility of MYC-MAX transcriptional-activator complexes to target DNA, resulting in expression of the target gene. **B)** Induction of MAD during terminal differentiation results in the MAD-MAX heterodimer binding to an E-box of a c-MYC target gene. Corepressor SIN3 and histone deacetylases (HDACs) are then recruited to MAD, resulting in local nucleosomal histone deacetylation and repression of target-gene expression.

The nuclear cofactor TRRAP (transactivation/transformation-domain associated protein), was purified by affinity chromatography using the c-Myc N-terminal transactivation domain (McMahon et al. 1998). TRRAP is a 3,830-amino-acid protein with limited homology to the phosphoinositide (PI)-3 kinase/ATM family, although TRRAP lacks the kinase catalytic residues present in other members of the family (McMahon et al. 1998). Myc was found to bind directly to an internal domain of TRRAP. Deletions in the Myc MBI and MBII regions which inhibit transformation also inhibit TRRAP binding (McMahon et al. 1998; Nikiforov et al. 2002). The identification of TRRAP as an essential cofactor provided an important mechanistic insight into the function of the Myc N-terminal domain when

TRRAP was found to be part of the SAGA complex (Grant et al. 1998a; Saleh et al. 1998; Vassilev et al. 1998). SAGA (SPT/ADA/GCN5/acetyltransferase) is a 1.8-Mda complex containing approximately 20 proteins, which has been implicated in transcriptional regulation, primarily through genetic screens in yeast (Grant et al. 1997).

Among the many proteins contained in SAGA, the only one with a clearly defined biochemical function is the histone acetyltransferase GCN5 (Georgakopoulos and Thireos 1992; Marcus et al. 1994; Wang et al. 1997). Histone acetylation by transcription cofactors has frequently been associated with gene activation (Grant et al. 1998b), making this an attractive mechanism for Myc-mediated transactivation **(Figure 3)**.

TRRAP is also found in a complex with the H2A/H4 histone acetylase TIP60 (Ikura et al. 2000).

Overexpression of a catalytically inactive TIP60 HAT delays the induction of H4 acetylation of target genes by Myc and also reduces Myc binding to chromatin, although no reduction in target gene induction was found. The subunits in these TRRAP complexes largely overlap those in a complex containing the Swi/Snf-related p400 protein in mammalian cells. Myc binds to this complex through TRRAP, but the consequences of Myc binding remain unclear since the p400/TRRAP complex is reported to lack histone acetyltransferase activity (Fuchs et al., 2001).

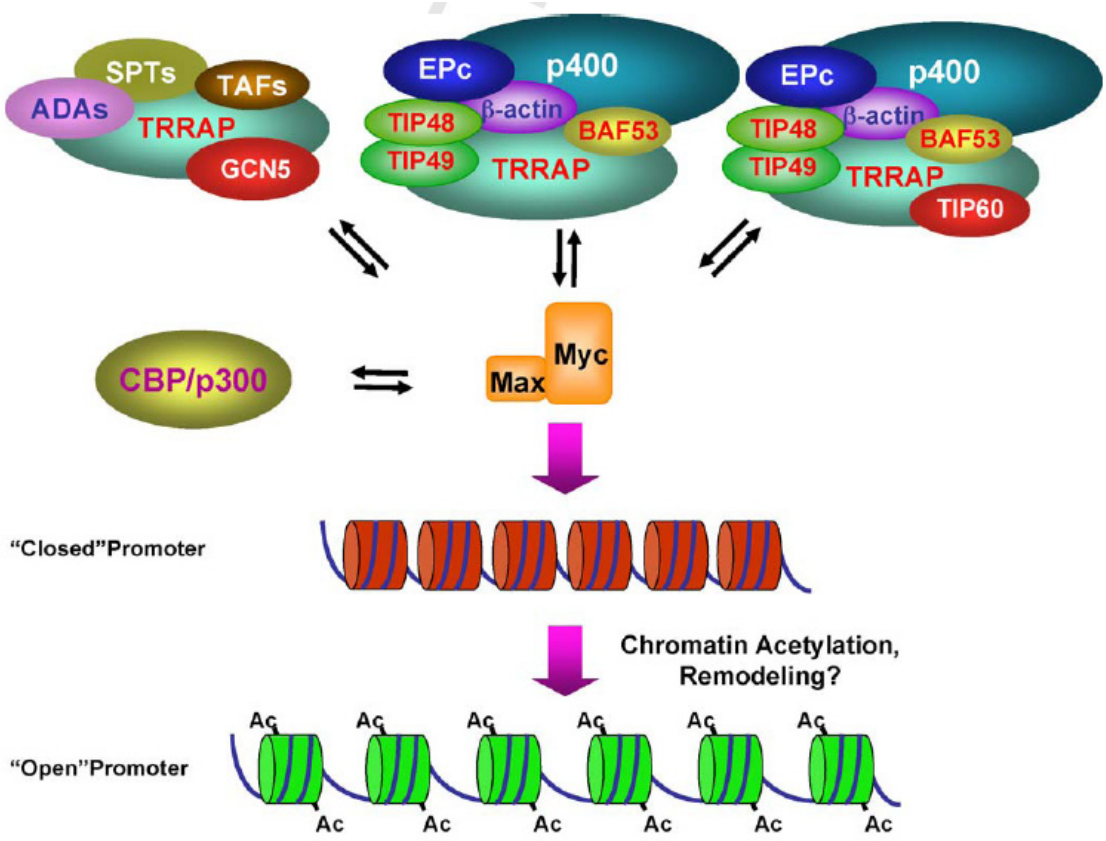

Figure 3. Model for Myc recruitment of histone acetyltransferases that open chromatin through acetylation of nucleosome. The Myc protein recruits several complexes that can promote localized modification and remodeling of chromatin. These complexes may alter the acetylation around Myc target genes or perturb chromatin in some other undefined way.

Another set of cofactors recruited by Myc are evolutionarily conserved proteins called TIP49 and TIP48, which contain ATPase motifs (Wood et al. 2000). These proteins are found as part of the TRRAP : TIP60 HAT complex in mammalian cells (Ikura et al. 2000), but some mutations in Myc retain TIP49/48 binding while losing TRRAP binding, suggesting that these proteins may interact with Myc independently (Wood et al. 2000). They are not components of the analogous H4 histone acetyltransferase complex in yeast (Allard et al. 1999), although they are found in other yeast chromatin remodeling complexes (Shen et al. 2000).

Another evidence that link Myc to chromatin remodeling is its interaction with SNF5 (also known as INI1/BAF47), a subunit of the human Swi/Snf

complex. Co-expression of a dominant-negative mutant of Brg1, the catalytic subunit of Swi/Snf, suppressed reporter gene activation by Myc in a transient transfection assay (Cheng et al., 1999), suggesting a role for Swi/Snf in transcriptional activation by Myc.

Other acetyltransferase activity factors that interacts with Myc (and many other transcription factors) are p300 and CBP (**Figure 3**). Cotransfection of CBP with Myc stimulates transactivation and CBP is weakly recruited to Myc target genes *in vivo*. One of the substrates for CBP/p300 is Myc itself, with several distinct sites acetylated in transient cotransfection assays (Vervoorts et al., 2003; Faiola et al., 2005).

1.4 Myc-induced transactivation is also regulated at the level of transcriptional elongation

A recent global genome analysis reports the presence of paused RNA pol II at specific promoters, including those of heat shock and MYC genes (Saunders 2006). This finding suggests that regulation of transcription also occurs at the level of transcriptional elongation and not just at transcriptional initiation. RNA pol II undergoes a cycle of phosphorylation and dephosphorylation during transcription and, with its C-terminal domain (CTD) in a hypophosphorylated form, RNA pol II is recruited to promoters. Phosphorylation of the CTD occurs during transcription initiation and elongation, whereas the CTD must be dephosphorylated to allow RNA pol II to be recycled for another round of transcription (**Figure 4**). RNA pol II has been found to pause on most promoters after transcribing approximately 20–40 bases. Specific signals and cofactors then stimulate transcriptional elongation and further RNA pol II phosphorylation (Price 2008).

This model fits well with the earlier finding that MYC does not induce transcription of the target gene CAD (carbamoylphosphate synthetase-2,

aspartate transcarbamylase, dihydroorotase) by driving RNA pol II recruitment, but rather stimulates the release of paused RNA pol II from the promoter and stimulates subsequent transcriptional elongation (**Figure 5**) (Eberhardy and Farnham 2001). Thus, some Myc target genes are TRRAP and/or histone acetylation independent.

Investigation into the HAT independent activation of Myc target genes revealed that RNA pol II is engaged but stalled at the promoters of some Myc target genes in the absence of Myc (Eberhardy and Farnham 2002). In the case of the CAD gene, Myc binding must regulate RNA pol II promoter clearance. Stimulation of RNApol II promoter clearance and efficient transcription elongation is associated with the RNA pol II kinases, TFIIH and positive transcription elongation factor b (P-TEFb). GST-Myc was found to bind to both subunits of P-TEFb, cyclin T1 and CDK9. In a separate study, MycER stimulated the recruitment of mediator, TFII-H, and P-TEFb to the cyclin D2 promoter (Bouchard 2004).

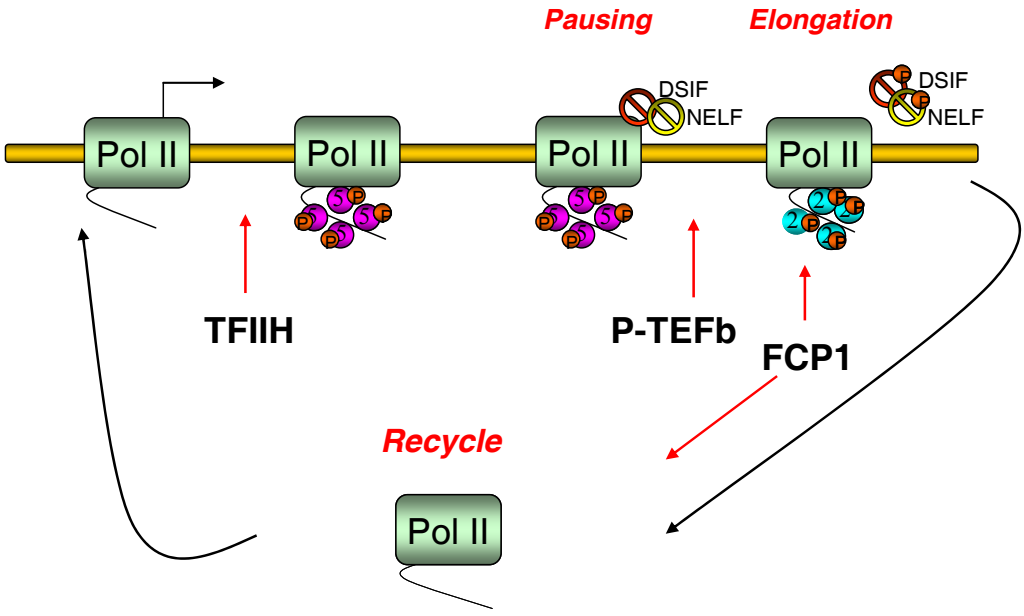

Figure 4: Transcription elongation – The Pol II CTD phosphorylation cycle . After RNAPII has been recruited into a pre-initiation complex, the CTD repeat is phosphorylated on Ser 5 by the CDK-7 subunit of the GTF TFIIH. This phosphorylation is required for Pol II to transcribe beyond the immediate promoter region (clearance), and for recruitment of the mRNA capping enzyme. Subsequently, phosphorylation of CTD Ser 2 by CDK-9 facilitates elongation and is required for mRNA termination,

cleavage, and processing . CDK-9 is a subunit of the GTF P-TEFb (positive transcription elongation factor b)

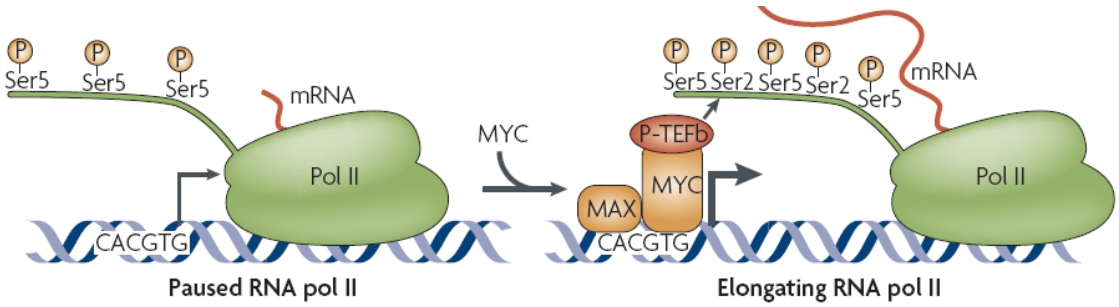

Figure 5. MYC recruits basal transcription factors and promotes the clearance of promoters through RNA polymerase (pol) II. RNA pol II is frequently paused on promoters after phosphorylation of Ser5 on the RNA pol II C-terminal domain (CTD) and synthesis of a short (20–40 base) segment of mRNA28. The MYC protein can promote a paused RNA pol to continue transcription of the mRNA by recruiting the P-TEFb (positive transcription-elongation factor-b) complex, which phosphorylates the CTD on Ser2 and promotes transcriptional elongation.

1.5 The P-TEFb complex

The positive transcription elongation factor b (P-TEFb) is a cyclin-dependent kinase that controls the elongation phase of transcription by RNA pol II (Peterlin and Price 2006). The RNA pol II C-terminal domain (CTD) is hypophosphorylated when initially recruited to genes, and undergoes sequential phosphorylation at Ser5 during promoter clearance and at Ser2 by P-TEFb at start of elongation (Price 2000).

Recently it has been shown that P-TEFb influences multiple steps in gene expression, from transcription elongation and co-transcriptional control of mRNA processing and export through the CTD, to mRNA translation in the cytoplasm. Therefore P-TEFb has been defined a multi-tasking complex (Bres et al., 2008).

P-TEFb complex exists in the cells in two forms in dynamic equilibrium between them. The catalitically active form (small comlex) is a heterodimeric complex and comprises two subunit, cyclin-dependent kinase-9 (CDK9) and Cyclin T1, T2 or K. The other half of P-TEFb exists

in a calitically inactive form (Nguyen et al., 2001; Yang et al., 2001, Michels et al., 2003), the large complex, that comprises 7SK small nuclear RNA (7SKsnRNA) and HEXIM1 (Michels et al., 2003; Yik et al., 2003; Chen et al., 2004; Nguyen et al., 2001; Yang et al., 2001) or HEXIM2 protein (Blazek et al., 2005). It has shown that active and inactive P-TEFb complexes are in rapid equilibrium, either a transcriptional arrest, genotoxic insults and UV or RNase treatments, triggers dissociation of 7SK and HEXIM1 from CDK9/Cyclin T1 resulting in a subsequent accumulation of kinase active P-TEFb complex (**Figure 6**).

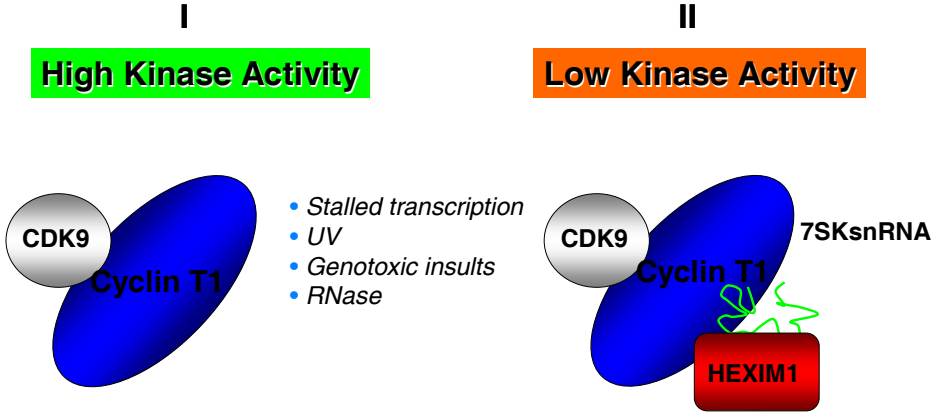

Figure 6: Active and inactive complexes of P-TEFb. P-TEFb is regulated by its reversible association with HEXIM1 and 7SK RNA. When P-TEFb is in this RNA-protein complex , its kinase activity is inhibited.

Notably, the core active P-TEFb complex, is likely also associated with the positive regulator bromodomain Brd4 (Jang et al., 2005; Yang et al., 2005). In fact, besides 7SK and HEXIM1, Brd4 has recently been identified as a major factor associated with CycT1/CDK9 heterodimer (Jang et al., 2005*)*. Brd4 is a bromodomain protein that binds highly acetylated chromatin (Wu et al., 2007) and interacts with mediator complex. Brd4 may therefore link P-TEFb recruitment with histone acetylation at induced genes.

Many studies has shown that P-TEFb is not only essential for the expression of most cellular protein-encoding genes, but also it is indispensable for the replication of human immunodeficiency virus type 1

(HIV-1) (Jones et al., 1997; Cullen et al., 1998). Transcription of HIV-1 proviral DNA by RNAPII is controlled primarily at the level of elongation by the viral Tat protein (Barboric and Peterlin 2005). Tat is a protein encoded by HIV-1, transcribed from multiply spliced viral RNA molecules expressed at early stages of viral gene expression. It is composed of the two exons of the viral Tat gene and encodes a protein of approximately 101 amino acids and in the late stage of the infection cycle, a carboxy-terminally truncated, encoded for Tat protein of 72 aminoacids also sufficient to transactivate the HIV-1 promoter.

Cyclin T1 was originally identified as a direct binding partner of the HIV-1 Tat protein in HeLa nuclear extracts, and Tat and Cyc T1 cooperate to recruit P-TEFb to the viral 5' TAR RNA (Price 2000; Saunders et al., 2006). An equally conserved arginine-rich motif is essential for direct contact of Tat with TAR RNA. Tat and cyclin T1 bind TAR RNA cooperatively and induce phosphorylation of the C-terminal domain of RNA pol II by CDK9 (**Figure 7**).

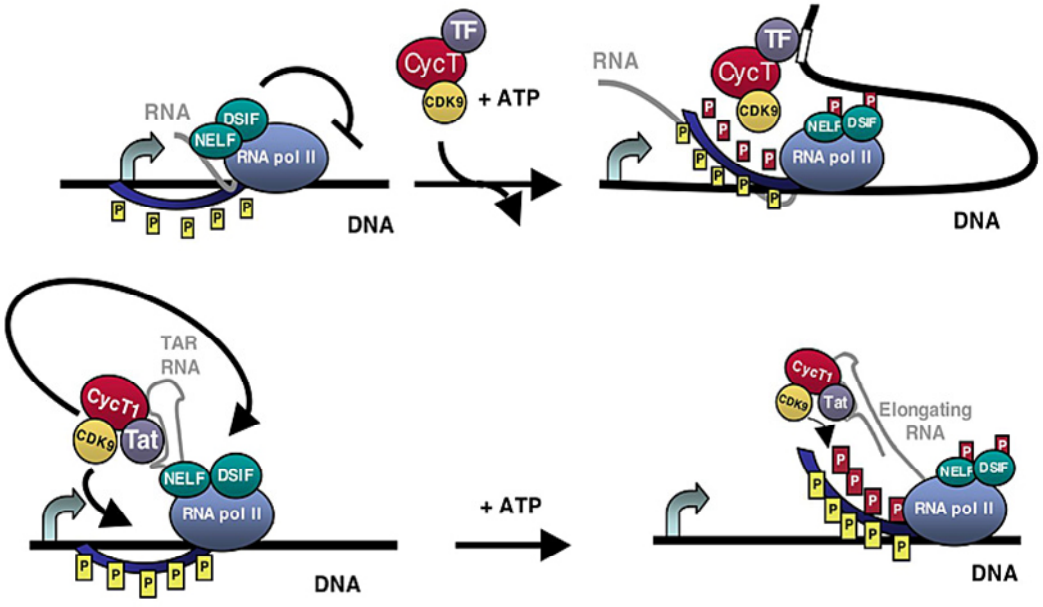

Figure 7. HIV-1 Tat transactivation involves the human P-TEFb complex. Tat interacts with cellular proteins as well as either a highly structured RNA element, transactivation-responsive TAR RNA, which is located at the 5' end of nascent viral transcripts. Tat binds CyclinT1 by a conserved domain cysteine-

rich region, which is part of trans-activating domain. An equally cis-region is essential for direct contact of Tat with TAR RNA cooperatively and induces phosphorylation of the C-terminal domain of RNAPII by CDK9. Neither CycT1 nor the P-TEFb complex binds TAR RNA in the absence of Tat, signifying that binding to RNA is highly cooperative for both Tat and P-TEFb. Since most of the P-TEFb are sequestered in the catalytically inactive and active complexes in cells, Tat could in principle modulate their configurations to increase the pool of P-TEFb for efficient HIV-1 transcription.

1.6 c-Myc regulation and turnover

c-myc activity is normally tightly controlled, at transcription level, by external signals including growth factors, mitogens and β-catenin.

In its physiological role, *c-myc* is broadly expressed during embryogenesis and in tissue compartments of the adult that possess high proliferative capacity (such as skin epidermis and gut). Its expression strongly correlates with cell proliferation. In quiescent cells *in vitro*, *c-myc* expression is virtually undetectable. However, after mitogenic or serum stimulation, c-Myc mRNA and protein are rapidly induced and cells enter the G1 phase of the cell cycle. Thereafter, the mRNA and protein decline to low, but detectable, steady-state levels in proliferating cells. If serum or growth factors are removed, c-Myc levels decline to undetectable levels and cells arrest. Temporal regulation of c-Myc protein accumulation is essential for normal cell proliferation.

c-Myc protein is stabilized after activation of Ras, allowing it to accumulate to high levels (Sears et al., 1999). Ras promotes stability of c-Myc through at least two effector pathways: the Raf–MEK–ERK kinase cascade, and the phosphatidylinositol-3-OH kinase (PI(3)K)–Akt pathway that inhibits glycogen synthase kinase-3β (GSK-3β) (**Figure 8**). The ERK and GSK-3β kinases phosphorylate two sites near the amino terminus of c-Myc that are highly conserved in all mammalian c-Myc isoforms. These phosphorylation sites, Thr 58 and Ser 62, exert opposing control on c-Myc degradation through the ubiquitin- proteasome pathway (Sears et al., 2000).

Thus, after a growth stimulatory signal, c-Myc gene transcription is increased and newly synthesized c-Myc protein is phosphorylated on Ser 62, via the Raf–MEK–ERK pathway, resulting in its stabilization. Phosphorylation at Ser 62 is also required for the subsequent phosphorylation of c-Myc at Thr 58 by GSK-3β, which is associated with c-Myc degradation (Sears et al., 2000; Pulverer et al 1994). During early G1 phase, however, GSK-3β activity is regulated by Ras-mediated activation of the PI(3)K/Akt pathway (which phosphorylates and inhibits GSK-3β), facilitating stabilization of c-Myc. Later in G1 phase, Ras activity declines after cessation of the growth stimulus, PI(3)K and Akt activities also decline, resulting in reactivation of GSK-3β and phosphorylation of c-Myc on Thr 58 which is important for c-Myc turnover. Phosphorylation of Thr 58 is important for recognition of c-Myc by the Pin1 prolyl isomerase. Pin1 facilitates c-Myc dephosphorylation at Ser 62 by PP2A, which then promotes c-Myc turnover by the ubiquitin-proteasome pathway through E3 ligase SCF^{FBW7} that recognizes Phospho-Thr 58. Thus, the mechanism that stabilizes and amplifies c-Myc accumulation, c-Myc phosphorylation at Ser 62, also triggers the subsequent phosphorylation at Thr 58 and the series of events that culminate in c-Myc degradation.

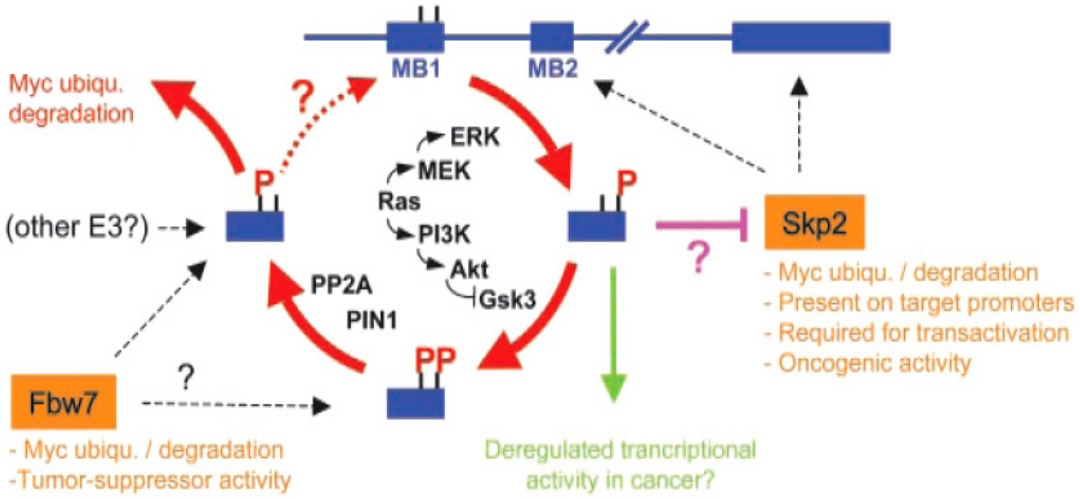

Figure 8. ‘‘Myc modification cycle’’ regulating protein turnover and activity. A series of posttranslational modifications in MBI regulates Myc’s interactions with ubiquitin ligases and may impact its transcriptional activities.

Recently it has been demonstrated (Bonetti et al., 2008) that Nucleophosmin, NPM/B23 protein regulate c-Myc turnover through Fbw7γ, a nucleolar ubiquitin ligase previously implicated in the ubiquitination/degradation of c-Myc (Yada et al., 2004).

NPM is a nucleolar protein that shuttles continuously between the nucleus and the cytoplasm (Grisendi et al., 2006). NPM has been proposed to regulate ribosomal RNA transcription/processing and the transport of preribosomal particles to the cytoplasm and *in vivo* interacts with many growth regulators, including the tumor suppressors p53 and ARF, and the HDM2 (Mdm2 in mouse) oncogene.

This protein is well known to regulate ARF tumor suppressor. In NPM-null cells, ARF loses its physiological localization in the nucleolus and becomes unstable, which suggests that NPM is critical for the proper localization and stability of ARF (Colombo et al., 2005). Notably, this function of NPM is lost for AML associated NPM alleles (NPM-mut), which compete with wild type NPM for ARF binding but target ARF to the cytoplasm, where it becomes more susceptible to degradation.

In the absence of NPM or in the presence of NPM-mut, cells express increased levels of the c-Myc proto-oncogene. NPM interacts with FBW7γ and in the absence of NPM, FBW7γ loses its nucleolar localization and is rapidly degraded by the proteasome. As a consequence, ubiquitination of c-Myc is defective and the protein is stabilized. NPM-mut maintains the property of interacting with FBW7γ but delocalizes it to the cytoplasm, where it is degraded, thus leading to accumulation of c-Myc and increased c-Myc signaling (**Figure 9**). Thus, mutations of NPM seem to

simultaneously dampen a tumor-suppressor pathway (p53 – ARF) and enhance an oncogenic c-Myc pathway.

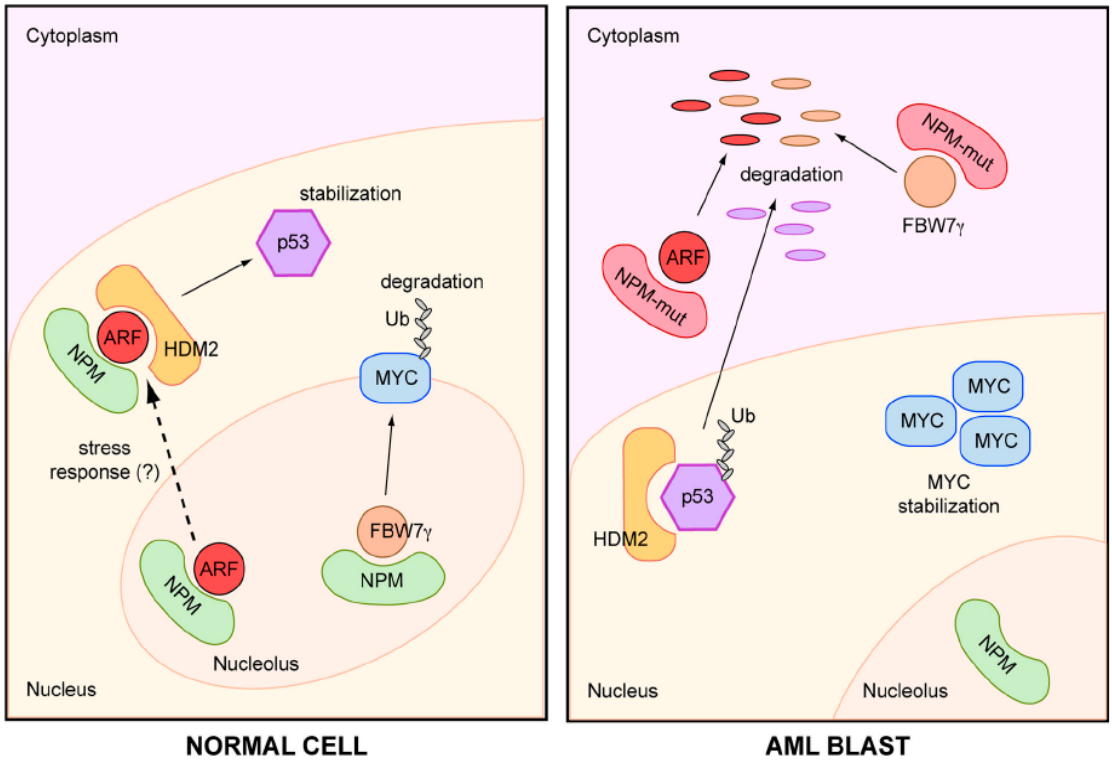

Figure 9. Mutated NMP attenuates an oncosuppressor pathway and enhances an oncogenic one. Normal cell: NPM is mainly localized in the nucleolus and is required for nucleolar accumulation and stability of FBW7γ and ARF. This is relevant for the control of MYC turnover and provides an active pool of ARF ready to inactivate the HDM2-mediated p53 degradation in response to cellular stress. AML blast: NPM-mut is mainly localized to the cytoplasm and causes cytoplasmic delocalization and degradation of ARF and FBW7 γ. As a consequence, HDM2 can induce ubiquitination/degradation of p53, and MYC accumulates and activates its target genes.

1.7 Myc regulates the RNA polymerase I and RNA polymerase III dependent transcription.

The most distinctive signatures of target genes downstream of Myc are the genes involved in ribosome and protein biogenesis (Schlosser et al., 2003; Boon et al., 2001). Regulation of the protein synthesis machinery is a critical component of growth regulation since a cell must double its protein mass before division. An important recent finding is that Myc stimulates rDNA transcription in both mammals and Drosophila (Grewal et al., 2005 – Arabi et al., 2005). Elevated Myc expression increases rDNA transcription

and nucleolar size in both mammalian cells and Drosophila embryos. Myc is able to bind to the rDNA repeats in mammalian cells, but not in Drosophila, although the sites of interactions are still controversial. Induction of rDNA transcription has been found to be dependent on MBII, and binding was accompanied by recruitment of TRRAP and histone acetyltransferases as well as RNA polI transcription factors (Grandori et al., 2005). If Myc stimulates rDNA transcription, the ribosome content per cell should also increase, which was demonstrated directly in two studies (Grewal et al., 2005; Grandori et al., 2005). These observations have a profound implication for interpreting the Myc target gene response.

For protein synthesis, the translation apparatus needs tRNA and 5S RNA in addition to ribosomes and translation cofactors. Therefore, it is consistent with Myc's prominent influence on translation, that it also enhances RNA polymerase III activity (Gomez-Roman et al., 2003). RNA Pol III transcribes tRNA and 5S RNA genes using a distinct set of cofactors than those used by RNA Pol I and RNA Pol II. Overexpression of Myc or activation of Myc-ER by tamoxifen leads to a rapid induction of Pol III activity and binding of Myc to Pol III-transcribed genes, even though these genes do not have consensus Myc/Max binding sites. The mechanism of RNA Pol III activation remains unclear, but the Myc transactivation domain (amino acids 1–110 or 106–143) is required. By using pull down assays, it has been found that interaction between the Myc transactivation domain and the basal RNA Pol III cofactor TFIIIB occurs. Determining how Myc binding to TFIIIB and RNA Pol III can stimulate activity will require further investigation.

1.8 Emerging novel function of Myc: regulation of translation

A number of experimental findings have suggested that Myc might have biologically significant, transcription-independent functions.

First, Myc biological activity can be uncoupled from the regulation of transcription by mutant analysis. Mutations near the DNA-binding domain can reduce the DNA-binding activity of Myc with no effect on Myc dependent cell proliferation and rat embryo fibroblast cell transformation (Cowling et al., 2006). Second, Myc mutants that cannot dimerize with MAX or lack DNA-binding activity can promote cell proliferation (Cowling and Cole 2007). These findings imply that inherent DNA binding and transcriptional activation are not required for every biological activity of Myc.

Recently it has been found that Myc can increase protein abundance by directly regulating the translation of individual mRNAs. This novel Myc mechanism came from the observation that the protein levels of several cyclin and cyclin-dependent kinases (CDKs), which are required for cell-cycle progression and transcription, abundantly increased in response to Myc expression without any change in their mRNA levels or in their requirement for the DNA-binding domain of Myc (Cowling and Cole 2007). Conversely, reducing the level of Myc in normal fibroblasts by small interfering RNA led to a suppression of cyclin and CDK protein levels without causing a suppression of mRNA levels, demonstrating that endogenous Myc protein has an activity that is comparable to the Myc mutants that lack direct DNA-binding activity.

Myc was found to increase the translation of specific mRNAs by promoting the methylation of the 5′ mRNA guanine or ‘cap’ (Cowling and Cole 2007), which is an essential step for protein-coding gene expression. Genes that are subject to Myc-dependent cap methylation, for example, cyclin T1 and CDK9, represent a novel set of Myc responsive genes.

During the early stages of transcription mRNA is capped and methylated. Cap methylation is necessary for the binding of translation factors to the mRNA and thus is required for translation (Bentley, 2005; Shuman, 2002).

Both capping enzyme and RNA methyl transferase are recruited to mRNA after transcription initiation by binding specifically to the RNA pol II CTD, which has been phosphorylated on Ser5 by the kinase TFIIH and on Ser2 by P-TEFb complex.

Myc can stimulate the methylation of specific mRNA modulating the levels of RNA pol II phosphorylation by elongation factors recruitment (**Figure 10**).

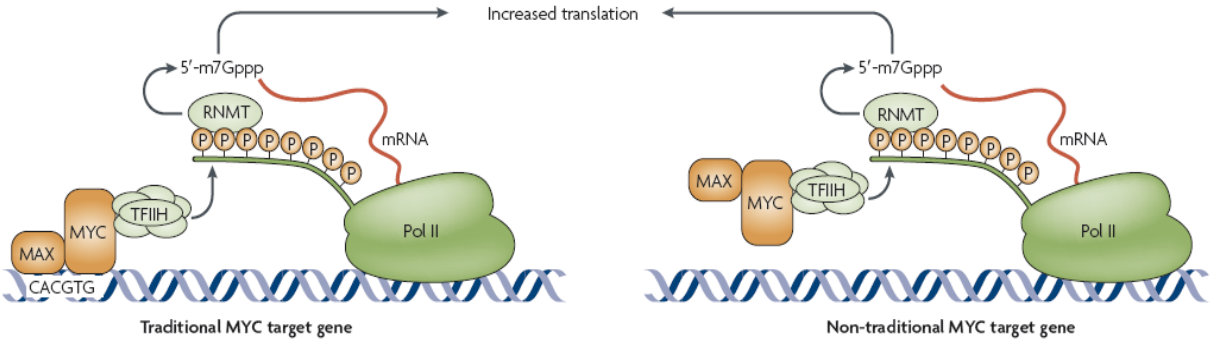

Figure 10. Mechanism of MYC-induced mRNA cap methylation. MYC promotes recruitment of the transcription factor TFIIH kinase to promoters and RNA polymerase (pol) II phosphorylation. Increased RNA pol II phosphorylation increases cap RNA methyltransferase (RNMT) recruitment and/or activity, which correlates with MYC-dependent mRNA cap methylation. At direct MYC target genes (left), TFIIH enhances the recruitment or activity of the cap RNMT to increase the fraction of cap methylated mRNA. At other promoters (right), MYC stimulates mRNA cap methylation through TFIIH stimulation by the MYC transactivation domain and through the subsequent recruitment or activation of RNMT by C-terminal domain phosphorylation. In doing so, MYC functions as a transcription-independent factor. Activation of direct targets is MYC-associated factor-X (MAX)-dependent (left), whereas activation of transcription-independent targets is MAX-independent (right).

1.9 Emerging novel function of Myc: control of DNA replication

In DNA replication the genome must be faithfully replicated at each cell cycle and the chromosomes must be segregated to the daughter cells. Disruption of any step in this process, such as a stalled replication fork or DNA damage incurred during S phase, activates a checkpoint that halts the cell cycle until the lesion can be repaired (Machida et al., 2005).Failure to correct this damage leads to a mutation and/or genomic instability.

A recent study describes a direct, non-transcriptional role for Myc in the initiation of DNA replication (Dominguez-Sola et al., 2007) Myc was found to bind to numerous components of the pre-replicative complex, including MCM proteins, ORC2, CDC6 and CDT1, and localize to early sites of DNA replication. These observations suggested that Myc might directly control the initiation of S phase and that Myc effects on genomic instability might not depend on the transcriptional induction of S-phase-promoting genes.

Levels of Myc protein seem to govern the number of active replication origins in both Xenopus and mammalian cells, suggesting that Myc functions to control origin selection.

Because this activity is dependent on the integrity of both the N-terminal and C-terminal domains of Myc, it suggests that Myc directly binds to DNA to recruit factors that govern the firing of replication origins.

1.10 Antagonism of Myc functions by p14ARF

Myc was the first oncogene recognized to activate ARF (Alternative reading frame) gene expression (Zindy et al., 1998). The ARF tumour suppressor is transcriptionally induced in response to the overexpression or mutational activation of growth-promoting genes, including MYC and RAS, and responds in turn by inhibiting the p53-specific ubiquitin ligases MDM2 (Korgaonkar et al., 2002) and ARF-BP1 (Chen et al., 2005), leading to the initiation of a p53-dependent cell growth arrest and apoptosis program. In order to induce cell-cycle arrest ARF functionally antagonizes gene expression governed by transcription factors such as E2F and MYC, the activities of which are required for cell-cycle progression.

However, several groups of investigators have argued that ARF functions independently of p53 in physically binding to E2F1 and MYC and attenuating their transcriptional activity (Eymin et al., 2001; Qi et al., 2004;

Datta et al., 2004). As for E2F1, the interactions between ARF and MYC highlight a p53-independent negative feedback mechanism (**Figure 11**). In some cell lines in which MYC expression was enforced, MYC binding relocalized ARF from the nucleolus to the nucleoplasm, whereas in other cell types ARF was found to import MYC into nucleoli (Qi et al., 2004; Datta et al., 2004). However, more striking were observations that p19ARF could associate with MYC on chromatin, antagonizing the transactivation of selected MYC target genes without impairing its transrepression of others (Gregory et al., 2005). The dampening effects of ARF on MYC-regulated transcription did not result from interference with MYC binding to its heterodimerization partner MAX, did not depend on MDM2 and p53 and, in Trp53-null cells, preceded the inhibition of S-phase progression.

ARF does not interfere with apoptosis induction by Myc, suggesting a role of ARF as a checkpoint for Myc-induced oncogenesis. Similarly, ARF antagonizes the activities of other transcription factors, including the forkhead box (Fox) family member FOXM1B, B-cell lymphoma 6 (BCL6), p63 and HIF-1α (Suzuki et al., 2005; Calabro et al., 2004; Fatyol et al., 2001).

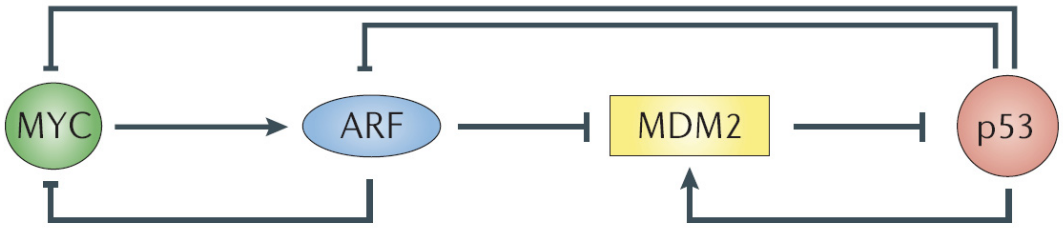

Figure 11. Functional interactions of MYC, ARF and p53. MYC is one of several ongogenes that can induce ARF expression when overexpressed, which in turn antagonizes the function of MDM2 to stabilize p53. The p53 protein not only feeds back to induce the transcription of MDM2 but also negatively regulates *ARF* and endogenous MYC expression through as yet ill-defined mechanisms. The ARF protein reportedly binds directly to MYC to inhibit its transactivation functions selectively.

1.11 ARF (Alternative Reading Frame) tumour suppressor

The *INK4a–ARF* locus (*CDKN2A* in humans) encodes two intimately linked but distinct tumor-suppressor proteins, p16INK4a and p14ARF (p19ARF in the mouse), that indirectly govern the activities of the retinoblastoma protein (RB) and the p53 transcription factor, respectively. These four proteins comprise part of a complex signaling network that regulates checkpoint responses to oncogenic stress by halting cell division and/or eliminating cells that have sustained irreparable damage (Lowe et al 2003). The intercalation of an additional exon (designated exon 1β) between the *INK4a* and *INK4b* genes enables the production of an alternatively spliced mRNA that also incorporates sequences encoded by exons 2 and 3 of *INK4a* (**Figure 12**). Unexpectedly, this transcript was found to specify an entirely unrelated protein, the exon-2-derived segment of which is translated in an alternative reading frame (ARF) (Quelle et al., 1995).

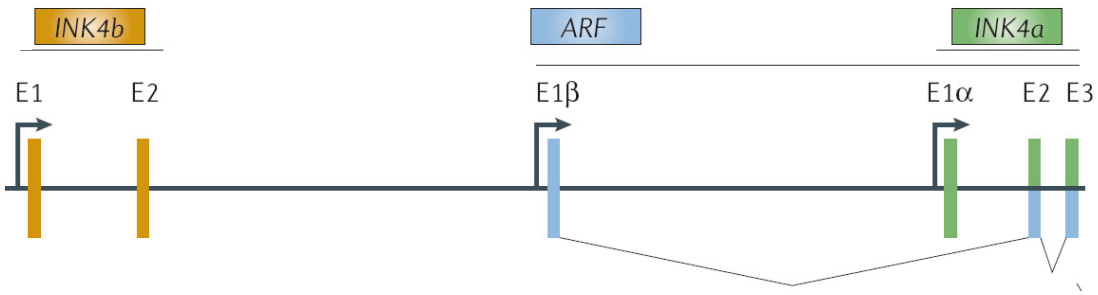

Figure 12. The *INK4b–ARF–INK4a* locus includes three tumour-suppressor genes in close proximity to one another. Numbered exons (E) are indicated by coloured rectangles and the promoters of the genes are designated by arrows. Both INK4a (green) and INK4b (orange) encode inhibitors (p16INK4a and p15INK4b) of the cyclin D-dependent kinases CDK4 and CDK6. The two INK4 genes flank ARF exon 1β, the encoded RNA of which is spliced (indicated by connecting lines below the linear schematic) to the exon-2- and exon-3-encoded segments of the INK4a gene (ARF-encoding exons in blue). The initiator codons in the ARF and INK4a mRNAs open alternative reading frames in INK4a exon 2 (from which the ARF gene got its name).

The ARF protein has an unusual amino-acid composition, being highly basic (pI>12, despite a paucity of lysine residues); from all the evidences

present in the literature it can be hypothesized that p14ARF is probably unstructured unless bound to other targets and highly promiscuous in its binding (Sherr 2006).

During the last years many efforts have been attempted in search ARF partners. The ARF interactors ‘‘harem’’ consists of something like 30 different proteins involved in various cellular activities (**Figure 13**): proteins involved in transcriptional control, such as E2Fs, DP1, c-Myc, p63, Hif1a, Foxm1b, nucleolar proteins such as nucleolin/C23 and nucleophosmin (NPM/B23), viral proteins such as HIV-1Tat, proteins involved in copper metabolism like COMMD1, proteins involved in chromosomal stability and/or chromatin structure such as Topoisomerase I, Tip60, and WRN helicase, ubiquitin ligases like Ubc9 (the E2 ligase required for sumoylation), MDM2 and ARF-BP1/Mule, (E3-ubiquitin ligases). Although the mechanisms by which ARF affects the activity of its partners are still unclear, the functional consequence is, quite invariably, inactivation.

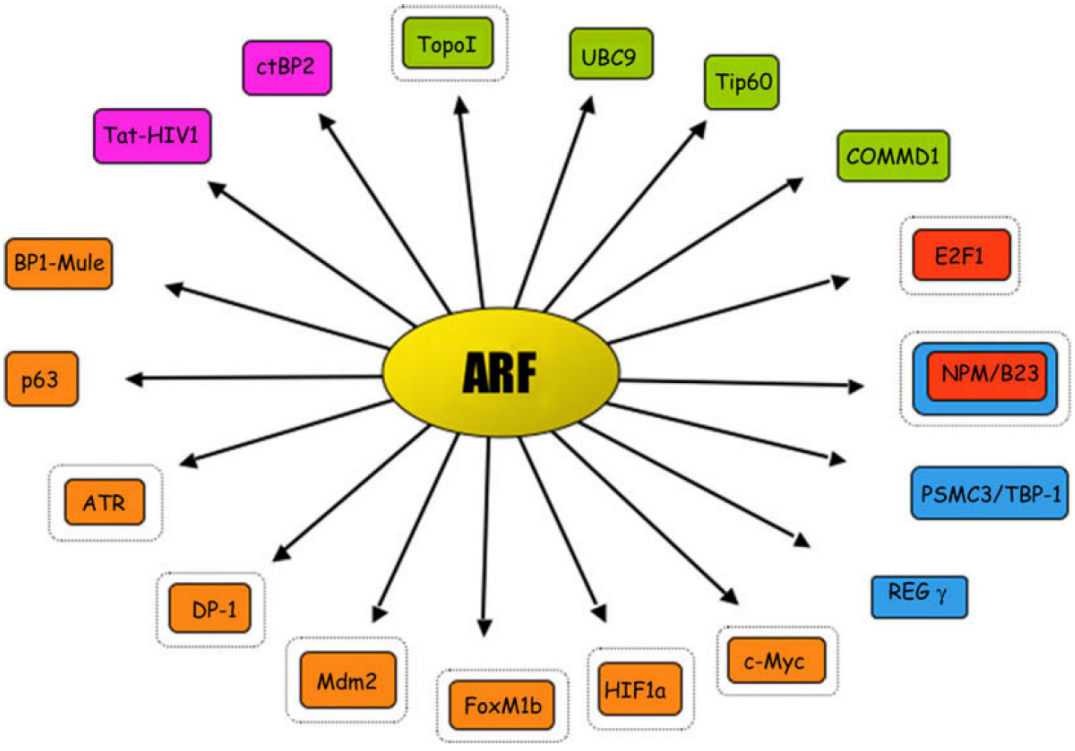

Figure 13. A schematic view of the ''ARF harem''. Orange is for partners whose activity is blocked by ARF. Red is for partners that are induced to proteasome and ubiquitin-dependent degradation by ARF. Pink is for partners that are induced to proteasome and ubiquitin-independent degradation by ARF. Green is for partners whose activity or stability are positively regulated by ARF. Blue is for partners that regulate ARF protein turnover. A second black circle indicate nucleolar sequestration.

The discovery of multiple ARF interactors and the observation that, aside oncogenic stimuli, also viral, genotoxic, hypoxic and oxidative stresses activate an ARF-dependent response, suggest that ARF could exert a wider role to protect the cell (Eymin et al., 2006; Fatyol et al., 2001; Garcia et al., 2006; Menendez et al., 2003).

It has recently been shown that the p19ARF mRNA can produce a short isoform of the ARF protein by internal initiation of translation at methionine 45 (Reef et al., 2006 and Sherr 2006). This isoform, dubbed short mitochondrial ARF or smARF, lacks the ARF NH2-terminal region that contains the MDM2 and ARF-BP1 binding domains required for ubiquitin ligase inhibition and, consequently, for p53-dependent ARF function. smARF also lacks the p19ARF nucleolar localization signal and is therefore excluded from the nucleolar compartment, localizing to mitochondria instead. This isoform induces cell death by autophagy, a cellular process associated with type II programmed cell death and characterized by the formation of cytosolic double-membrane vesicles, called autophagosomes, that engulf cellular content and fuse with lysosomes to digest it (Levine et al., 2004). Autophagy has been implicated in tumor suppression (Jin 2005) via full-length ARF in both p53-dependent and p53-independent manners, depending on cellular context (Abida and Gu 2008).

1.12 ARF exerts p53-dependent or p53-independent tumour suppressor functions

ARF protein is a potent tumour suppressor that blocks cell-cycle progression by directly binding to, and interfering with, the p53 negative-regulator MDM2 (HDM2 in human), thereby stabilizing and activating p53 (Kamijo et al., 1997; Stott et al., 1998). In turn, by antagonizing the E3 ubiquitin ligase activity of MDM2, ARF stabilizes p53 and increases its transcriptional activity (**Figure 14**). The proto-oncoprotein HDM2 also interacts with HIV-1 Tat protein and mediates its ubiquitination *in vivo* and *in vitro* (Bres et al., 2003). HDM2 is a positive regulator of Tat-mediated transactivation, indicating that the transcriptional properties of Tat are stimulated by ubiquitination.

The most accepted view was that the tumor-suppressor functions of ARF was mediated through p53.

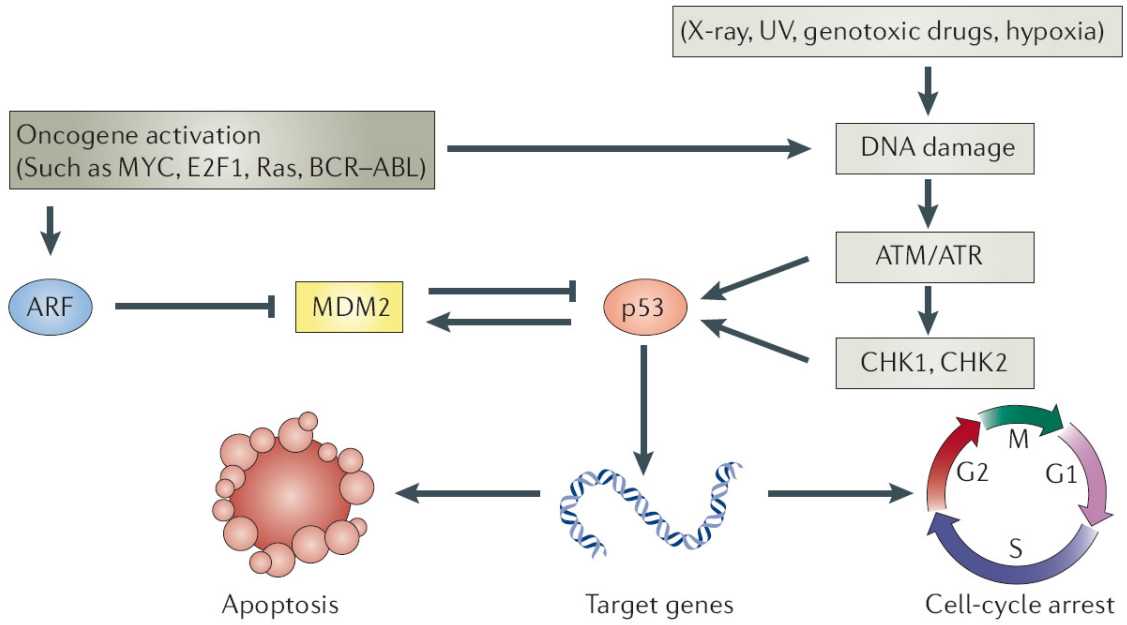

Figure 14. The ARF-MDM2-p53 pathway. Once expressed, the ARF protein interferes with the activity of MDM2, leading to p53 stabilization and triggering a complex p53-dependent transcriptional programme mediated by hundreds of target genes MDM2 is not only a negative regulator of p53-dependent transcription and turnover, but is also a canonical p53-activated gene that has a key role in negative-feedback regulation of the p53 response. The activation of p53 classically occurs in response to many other cellular stresses that produce DNA damage. DNA-damage responses activate the kinase

mutated in the ataxia telangectasia syndrome (ATM) and/or the ATM and RAD3-related kinase (ATR). These kinases phosphorylate p53 directly and also indirectly through the agency of the CHK kinases. These phosphorylations have an important role in increasing the transcriptional activity of p53. Target genes induced by p53 can generate different biological outcomes depending on the tissue type and convergence of different activating signals. ARF induction primarily tends to trigger cell-cycle arrest, but oncogene-induced signals conveyed through collateral pathways (not shown) can shift the response from growth arrest to apoptosis.

Despite this neat paradigm, there is evidence to suggest that ARF might have additional tumour-suppressor activities.

The reintroduction of p19ARF into primary *Trp53*-null or TKO murine embryonic fibroblasts (MEFs) can arrest their proliferation, albeit much less efficiently than in cells that retain the expression of MDM2 and p53 (Weber et al., 2000 and Carnero et al., 2000).

Roles for p14ARF in triggering the growth arrest or apoptosis of p53-deficient human tumour cell lines in culture and in inhibiting their growth as xenografts in nude mice have also been described (Eymin et al., 2001 and Eymin et al., 2003).

These observations raised the idea that ARF has an MDM2- and p53-independent role as a tumour suppressor.

1.13 ARF NPM/B23 interaction

On induction, the ARF protein accumulates within the nucleolus, an intranuclear organelle primarily concerned with ribosome biosynthesis. Here, the ARF protein associates in high-molecular-mass complexes with nucleophosmin (NPM, also known as B23, numatrin or NO38) (Itahana et al., 2003 and Korgaonkar 2005), an abundant nucleolar phosphoprotein of 37 kDa. Although most NPM resides within the nucleolus, the protein contains a nuclear export signal (NES) and shuttles between the nucleus and cytoplasm. NPM has been implicated in diverse cellular processes, including ribosome biogenesis, centrosome duplication, DNA-damage

responses, transcription and nucleosome remodelling (Grisendi et al., 2006).

The complexes including ARF and NPM are much more abundant than ARF–MDM2 complexes. ARF is stable when expressed within the nucleolus, but turns over more rapidly in the nucleoplasm. ARF proteins are polyubiquitylated at their free (non-acetylated) N-termini and are degraded by the proteasome (Kuo et al., 2004). In response to increased levels of NPM, the turnover of p19ARF is retarded, therefore, the ARF protein assumes a stable structure when bound to NPM.

NPM is also responsible for ARF nucleolar compartmentalization. Recently it has been found the identification of leukaemia-associated cytoplasmic NPM mutants (AML NPMc+ proteins) that delocalize ARF to the cytoplasm and attenuating the ability of ARF to stabilize p53 and to sumoylate both NPM and MDM2 (den Besten et al., 2005 and Colombo et al., 2006).

The ability of NPM to shuttle between the nucleus and cytoplasm, its association with maturing pre-ribosomal particles and its effects in promoting the processing of ribosomal RNA precursors implicate NPM in ribosome biogenesis. ARF retards rRNA transcription and processing, interferes with NPM nucleocytoplasmic shuttling and impedes ribosome export from the nucleus to the cytoplasm (Brady et al., 2004 and Yu et al., 2006). (**Figure 15**)

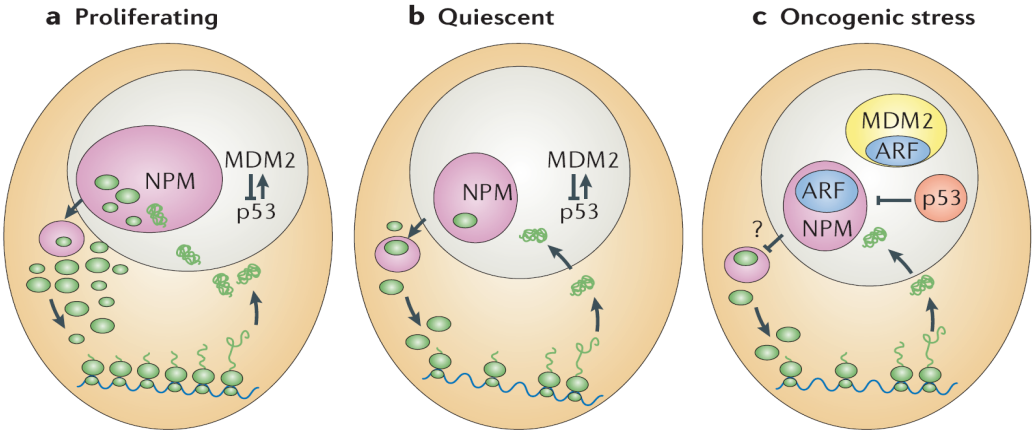

Figure 15. ARF–NPM interactions and ribosomal biogenesis. A | High levels of nucleophosmin (NPM) (pink) are expressed in rapidly proliferating cells. Although most NPM is compartmentalized within the nucleolus, NPM dynamically shuttles between the nucleus and cytoplasm. NPM is assumed to have a key role in ribosomal biogenesis, by facilitating the transport of large (60S) and small (40S) ribosomal subunits (green circles) into the cytoplasm, where, together with mRNAs, they form polyribosomes required for protein synthesis. **B** | In cells made quiescent by mitogen deprivation, ribosome biogenesis is attenuated and the level of NPM falls. **C** | In response to oncogenic stress, induced ARF protein (blue) binds to MDM2 (yellow), which leads to p53 activation and cell-cycle arrest. ARF also enters the nucleolus to form distinct, stable complexes with NPM. One effect of p53 is to inhibit the transcription of RNA polymerase I and slowing ribosome biogenesis.The ARF protein antagonizes the shuttling of NPM and attenuates ribosome trafficking to the cytoplasm .

1.14 ARF: proteasome and protein turnover

The discovery that p14ARF can directly interact with regulative components of the proteasome multi-protein complex, such as TBP-1/PMSC3 of the 19S subunit (Pollice et al., 2004; Pollice et al., 2007) and REG-γ of the 11S lid (Takaoka et al., 2003) offers a new key to interpret the mechanisms through which ARF is regulated and regulates cell growth and proliferation. The first evidence of a link between ARF and the proteasome is the observation that both human and mouse ARF accumulate following treatment with proteasome inhibitors suggesting that ARF degradation depends, at least in part, by the proteasome (Kuo et al., 2004).

A very recent report describes a direct involvement of the REG-γ proteasome in a ubiquitin-independent regulation of the ARF turnover (Chen et al., 2007) (**Figure 16**).

The feature of proteins targeted to the REG-γ pathway is the lack of ubiquitination, usually due to the absence of lysine residues. Both p16 and human p14ARF are naturally lysine-less proteins. Interestingly, viral proteins constitute a substantial subset of naturally lysine-less proteins. This raises the hypothesis that the REG-γ pathway might play a role in the control of viral pathogenesis. This is particularly interesting, given that ARF activation has been linked to viral response (Garcia et al., 2006).

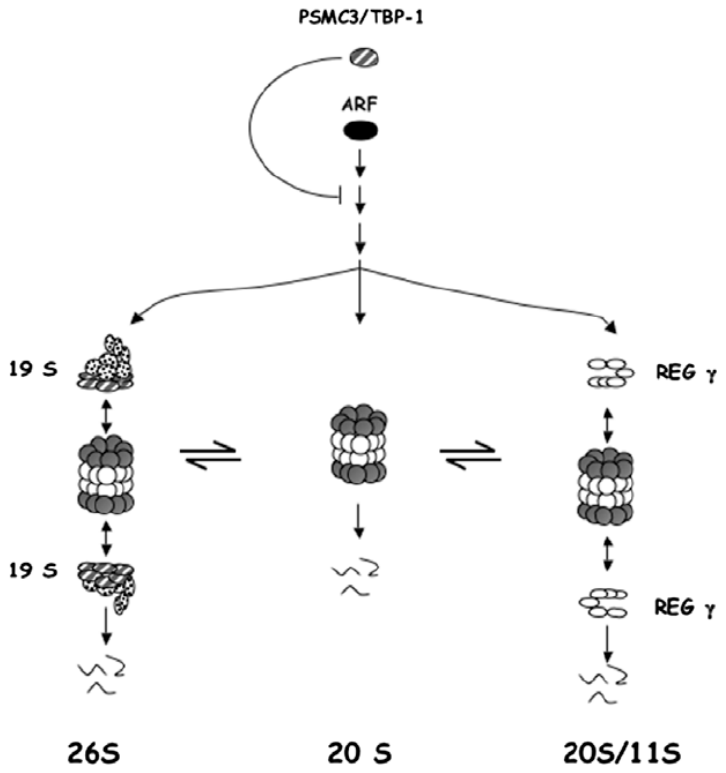

Figure 16. A model for the regulation of ARF turnover. ARF can be degraded by the proteasome through ubiquitin-independent (by the 20S or 20S/REG-c complex) or dependent (by 26S complex) mechanisms. Binding to the 19S subunit PSMC3/TBP-1 protects ARF both in vitro and in cells.

ARF also causes alteration of stability for some binding partners.

For example, B23/NPM and E2F become degraded by the proteasome in an ubiquitin-dependent manner, while the CtBP2 antiapoptotic transcriptional co-repressor become degraded by the proteasome in an ubiquitin-independent manner (Paliwal et al., 2006). Most of other partners become sumoylated although a precise function to this modification has not yet been assigned (Rizos et al., 2005; Tago et al., 2005; Liu et al., 2007).

In some cases, ARF is able to stabilize its partners from proteasomal degradation. It has been described the ARF's ability to induce a non-classical poly-ubiquitination of interacting partners, like Tip60, Topo I and COMMD1 (a multifunctional protein involved in copper metabolism and apoptosis) that leads to a stabilization of this factors (Huang et al., 2008).

Therefore, ARF interaction with the proteasome could serve dual roles: on one side it is necessary to regulate ARF protein turnover, while, on the other side, it could play a role in bringing ARF interacting partners in contact with the ubiquitin/proteasome machinery.

CHAPTER II

Aim

Myc is a well known proto-oncogene: deregulation of Myc gene expression due to amplification or translocation is present in a wide variety of human tumours. Since 25 years, from its discovery, Myc has been a pioneer in the oncogene field. Myc over-expression induces cell proliferation, cell growth and inhibition of cell differentiation. The Myc protein is a transcription factor; it exerts all these different effects in the cellular context by modulating the gene transcription. It has been extimated that about 1500 genes in the human genome are Myc-responsive tagets and they are compiled in the Myc target gene database.

The aim of this thesis has been focused to analyze the positive regulation of Myc-mediated transcription by P-TEFb elongation complex, and the negative control exerted by p14ARF onco-suppressor on Myc's transactivation functions.

Moreover in a parallel line of research I investigated the p14ARF involvement on Tat-mediated transcriptional transactivation of HIV-1(Human Immunodeficiency Virus 1) gene.

CHAPTER III

Results

3.1 P-TEFb regulates positively c-Myc transactivation

3.1.1 Myc interacts exclusively with catalytically active P-TEFb complex.

Previous works (Eberhardy and Farnham 2002; Kanazawa et al., 2003) have shown that Myc, as transcription factor, in addition to recruit histone acetylation activity, binds the positive transcription elongation factor b (P-TEFb) which consists of the cyclin-dependent kinase CKD9 and its regulatory subunit cyclin T1. The highly conserved Myc Box I (MBI) interacts directly with Cyclin T1. P-TEFb phosphorylates the carboxyl-terminal-domain (CTD) of the larger subunit of RNA polymerase II as well as negative elongation factors allowing efficient transcription elongation.

It has been demonstrated that the Myc's ability to activate transcription of *cad* gene promoter correlates with binding of cyclin T1.

Moreover it has been shown (Kanazawa et al., 2003) that the inhibition of P-TEFb complex blocks the transcriptional activation of Myc target gene as well as cellular proliferation and apoptosis induced by Myc.

The P-TEFb complex is object of studies in the laboratory in which I have worked since several years. The P-TEFb complex exists *in vivo*, in the cells, essentially in two forms in dynamic equilibrium between them. A light complex with high kinase activity, in which the active P-TEFb is composed by CDK9 and Cyclin T1, and the larger complex with low kinase activity, in which the two proteins are associates with an inhibitor protein called Hexim and with the snRNA 7SK.

To determine if Myc interacts with the core active or the large inactive P-TEFb complex, I have performed experiments of co-immunoprecipitation.

Transient transfections were performed in human 293T cells using CMV-Flag-Myc and CMV-CycT1 expression vectors. Cellular extracts were prepared and subjected to CoIP experiment with anti-CycT1 or anti-Myc antibodies, respectively. As expected Myc, Max, CDK9 and Hexim proteins were found in the immunoprecipitated Cyc T1 materials, while anti-Myc antibody co-precipitated the associates partner Max as well as Cyc T1/CDK9 proteins, but no Hexim protein was detectable in the anti-Myc cointaining complex (**Figure 17 A** lane 2-4). These data demonstrated that Myc interacts exclusively with 'core' active CycT1/CDK9 complex.

Next, to corroborate these results I have investigated if also the endogenous Myc interacts with the P-TEFb complex. I carried out a CoIP analysis with cell extracts from 293T cells; as shown in **Figure 17 B**, the anti-Myc antibody co-precipitated Max as well as P-TEFb (CycT1 and CDK9), but no HEXIM1 was detectable in the Myc-IP materials. Reciprocally, anti-CycT1 coprecipitated endogenous Myc/Max, as well as the expected partners CDK9 and HEXIM1, whereas the IgG, used as control, did not.

Moreover, the absence of HEXIM1 protein in the Myc-associated materials suggests that Myc interacts, in association with Max, only with the catalytic active P-TEFb complex.

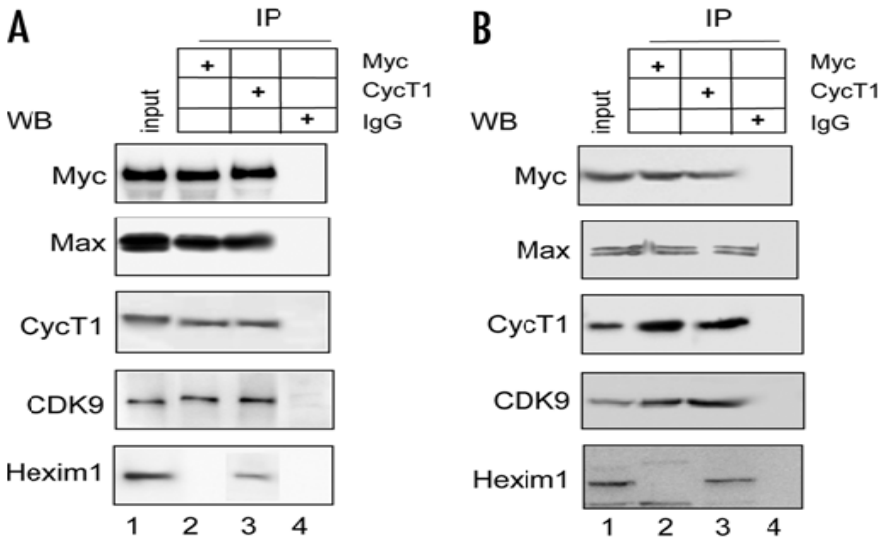

Figure 17. Myc/Max interacts with P-TEFb. (A) 293T cells were transiently transfected with pcDNA3-Myc and pcDNA3-CycT1 constructs. Protein extracts were immunoprecipitated with anti-Myc or anti-CycT1 as indicated, and preimmune antiserum IgG as negative control. Co-IP complexes were analyzed by WB with the indicated antibodies. Five percent of the protein input was loaded in lane 1. (B) 293T cellular extracts were precipitated with anti-Myc or anti-CycT1, as indicated; and inputs (10%) and precipitates (Ips) were analyzed by immunoblotting with the indicated antibodies.

3.1.2 Myc directly recruits P-TEFb to chromatin templates

The physical interaction between P-TEFb and Myc/Max suggests that Myc might recruit P-TEFb at the chromatin of responsive genes upon binding of the Myc/Max complex to the E-box (the promoter region). To value this hypothesis, I planned to use the well-described Rat1-MycER cell line expressing the inducible c-Myc-ER chimera (kindly provided by prof. Bruno Amati IEO, Milano). This cell line expresses the inducible c-MycER chimera and can be synchronized by starvation in the G0-G1 (quiescence) cellular phase, in which it is well documented that c-*myc* expression is virtually undetectable. After mitogenic and/or serum stimulation, c-*myc* mRNA and endogenous c-Myc protein are rapidly induced and cells enter the G1 phase of the cell cycle. Moreover, after treatment with 4-hydroxytamoxifen (OHT) the exogenous c-MycER chimera can be activated. Expression of exogenous Myc in cultured fibroblasts promotes S-phase entry and shortens G1 phase of the cell cycle, while activation of a conditional Myc is sufficient to drive quiescent cells into cell cycle.

In order to demonstrate if Myc directly recruits P-TEFb to chromatin templates of both NUC and CAD genes target, I performed chromatin immunoprecipitation (ChIP) assays using Rat1-MycER cells, made quiescent by contact inhibition followed by serum removal for two days. After two days the cells were treated with 4-hydroxytamoxifen (OHT) to determine, during MycER-mediated activation, the *in vivo* binding of Myc, P-TEFb and RNAPII to the E-box of the CAD gene. I assessed their presence by using antibodies against Myc and CycT1. As shown in **Figure**

18, upon Myc induction (only 90' of 4-OHT treatment), I observed a concomitant presence of both Myc and P-TEFb at E boxes of CAD promoter.

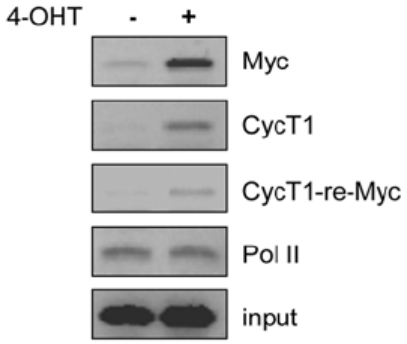

Figure 18. Co-occupancy of Myc and P-TEFb at CAD E-Box. Quiescent Rat-MycER were treated with either vehicle or 4-OHT for 90' and subjected to chromatin immunoprecipitation (ChIP) assays. The assays were carried out with the indicated antibodies using amplicon spanning the CAD E-box.17 To determine co-occupancy, the CAD E-box region was immunoprecipitated with CycT1 antibody and re-immunoprecipitated with Myc antibody. The data shown are from a single experiment, and similar results were seen in three separate experiments.

Accordingly with previous findings (Frank et al., 2001; Frank et al., 2003), I determined that also RNAPII was loaded onto the CAD promoter before Myc induction.

The semi-quantitative nature of these assays was taking in account by performing PCR amplification using serial dilutions of DNA template as well as by repeating the experiments (2–3 times) using different chromatin preparations. In addition, the ACHR promoter was constantly used in all experiments as negative control.

Moreover to determine whether a unique complex containing both Myc and P-TEFb is associated to the CAD promoter, I sought to examine possible co-occupancy of both factors on CAD E-box. I performed re-ChIP experiment in which the CAD E-box region first was immunoprecipitated with CycT1 antibody and after re-immunoprecipitated with Myc antibody. As shown in **Figure 18**, co-occupancy of Myc and CycT1 was seen at the

CAD promoter in response to 4-OHT treatment. I concluded that Myc recruits *in vivo* P-TEFb at the CAD promoter.

3.1.3 The DRB affects the Myc transactivation functions and blocks Myc-induced proliferation and apoptosis

The binding between Myc/Max and active core P-TEFb together with the clear evidence of their recruitment on NUC and CAD chromatin templates, strongly suggests that Myc/P-TEFb interaction is functional relevant.

To analyze the involvement of the CDK9 kinase in transcriptional regulation driving by Myc, I used 5.6-di-chloro-1-b-D-ribofuranosyl-bensimidazole (DRB), the pharmacological specific inhibitor of CDK9 kinase activity .

To test such premise, I performed the quantitative Real-Time qPCR using Rat1 cells expressing a 4-hydroxytamoxifen (OHT)-inducible MycER chimera were made quiescent by contact inhibition followed by serum removal for two days. After two days the cells were treated with 4-hydroxytamoxifen (OHT) in the presence or absence of DRB (20 and 50 μM), and I evaluated the relative levels of expression of two Myc-responsive target genes, nucleolin (NUC) and CAD as well as two housekeeping control genes beta-2M and RPS9 (**Figure19**).

All samples were normalized using as control genes (CGs) the betaglucuronidase (GUS) and 18 sRNA (Beillard et al., 2003). As represented in **Figure 19**, both NUC and CAD gene expression were up-regulated by Myc and DRB treatments (at 50 μM) effectively reduced NUC and CAD activities.

Importantly, DRB did not block the expression of the MycER chimera following 4-OHT treatment.

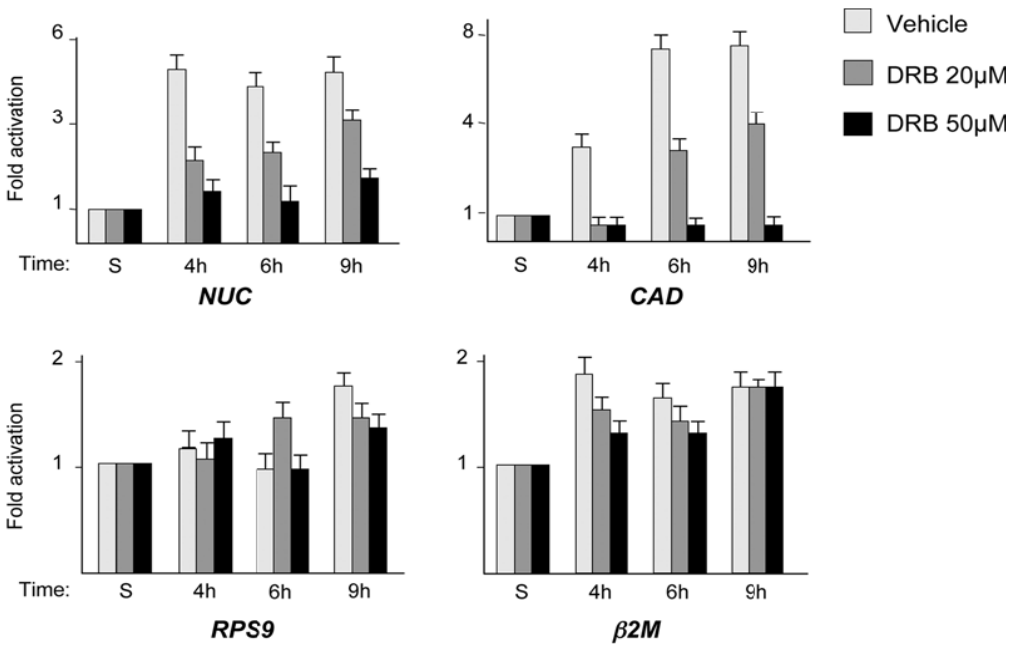

Figure 19. DRB blocks expression of Myc-target genes. Quiescent Rat-MycER cells were treated with 4-OHT in the absence or presence of DRB at 20 μM and 50 μM, and total RNA was prepared at the indicated times and NUC, CAD RPS9 and beta2M mRNA levels were quantified using qPCR. mRNA levels were normalized to GUS mRNA levels. The values are presented relative to RNA levels in quiescent Rat-MycER cells.

It has been shown (Kanazawa et al., 2003) that the specific CDK9 inhibitor DRB blocks cellular proliferation and apoptosis induced by Myc.

To determine the functional effects of DRB treatments on the cellular changes that occur upon Myc-activation, I performed cell cycle distribution by Facs analysis of Rat1 MycER cells.

It is well known that Myc induces S-phase and apoptosis of quiescent cells grown in low serum. As shown in **Figure 20**, after 18 hrs of 4-OHT treatment it is evident the activation of Myc, and Myc induces proliferation (percent of S-phase) and apoptosis (percent of sub-G1 cells), in black, while DRB treatments inhibits both Myc-induced proliferation and apoptosis. In particularly, treatment with DRB at 50μM affects cell viability. These results strongly suggest that CDK9 is crucial for the induction of Myc-responsive gene as well as for Myc-induced cellular outcomes.

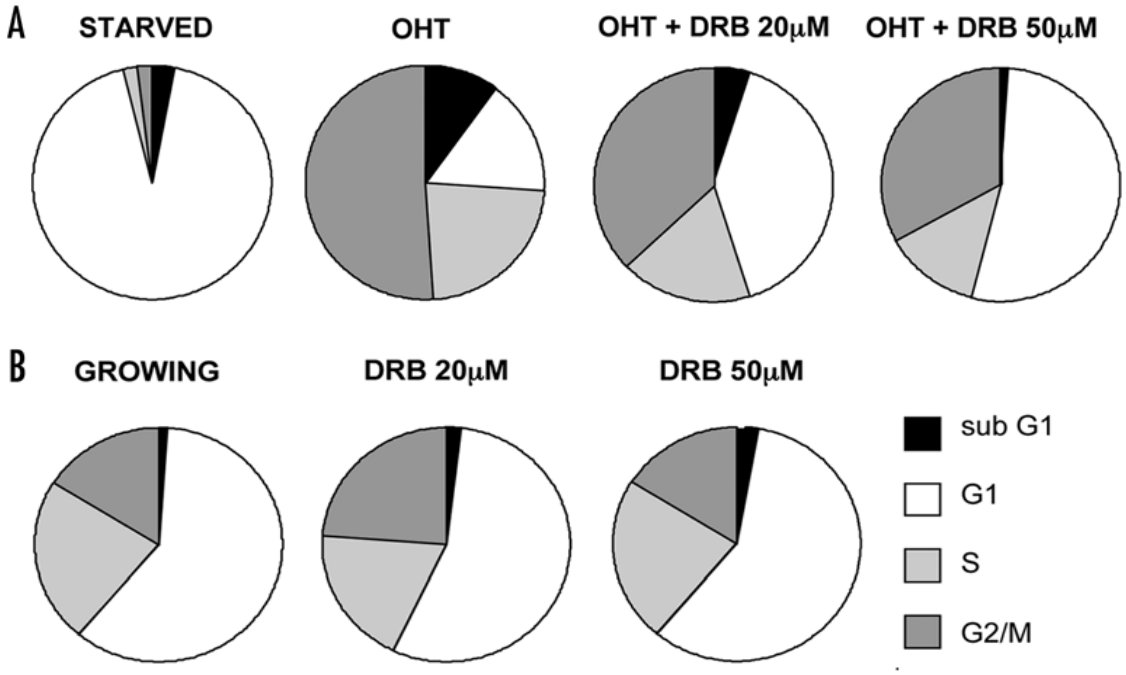

Figure 20. DRB affects Myc-induced proliferation and apoptosis in Rat cells. (A) Quiescent Rat-MycER cells were treated with 4-OHT in the absence or presence of DRB at 20 μM and 50 μM as indicated. After 18 hrs of treatment cells were collected and cell cycle distribution was analyzed by FACS. (B) Actively growing Rat-MycER cells were treated with DRB and cells were collected after 18 hrs and analyzed by FACS.

3.1.4 The distribution of RNA Pol II, Myc and P-TEFb in vivo are differentially impaired by DRB treatment

DRB treatment provided circumstantial evidence that Myc/P-TEFb interaction is functional relevant. Both Myc-induced cellular physiological changes and expression of Myc-target genes were effectively and specifically inhibited by DRB. It is pertinent to note that previous studies showed that DRB effectively blocks CDK9 activity, and, to lesser extent, other CDKs (Dai et al., 2003). Then, the contribution of other kinases to the inhibition of Myc-responsive genes cannot be strictly excluded.

To assess the role of CDK9 activity, I investigated the distribution and the relative presence of Myc, P-TEFb and Pol II at the E-box promoter sequences as well as in the coding regions of CAD and NUC genes after DRB treatment.

To this end, I performed chromatin immunoprecipitation (ChIP) experiments that were analyzed by semiquantitative PCR, and quantitative Real-Time PCR, and both methods gave similar results. For RNA Pol II immunoprecipitation, I used three different anti Pol II antibodies (8WG16, H14 and H5) which recognize different epitopes of the CTD. 8WG16 was used to follow total Pol II, while the phosphorylated Pol II was detected by H14, recognizing phosphor-Ser-5 CTD. The distribution of the Ser-2 residues of the CTD was assessed using the H5 antibody.

As represented in **Figure 21 A** and **B**, either 4-OHT and DRB treatment led to the same results at the E-box region of both CAD and NUC genes, in the similar distribution of Myc, Pol II and P-TEFb. This results suggest that recruitment of these factors to chromatin do not require CDK9 catalytic activity. In contrast, clearly DRB treatment reduces the density of Ser-2 CTD, but does not affect the density of Ser5 CTD. This result corroborates the results obtained by expression analysis of these genes after DRB treatment. This demonstrate that P-TEFb-mediated phosphorylation of Ser-2 CTD is a key control step for transcription of Myc-target genes.

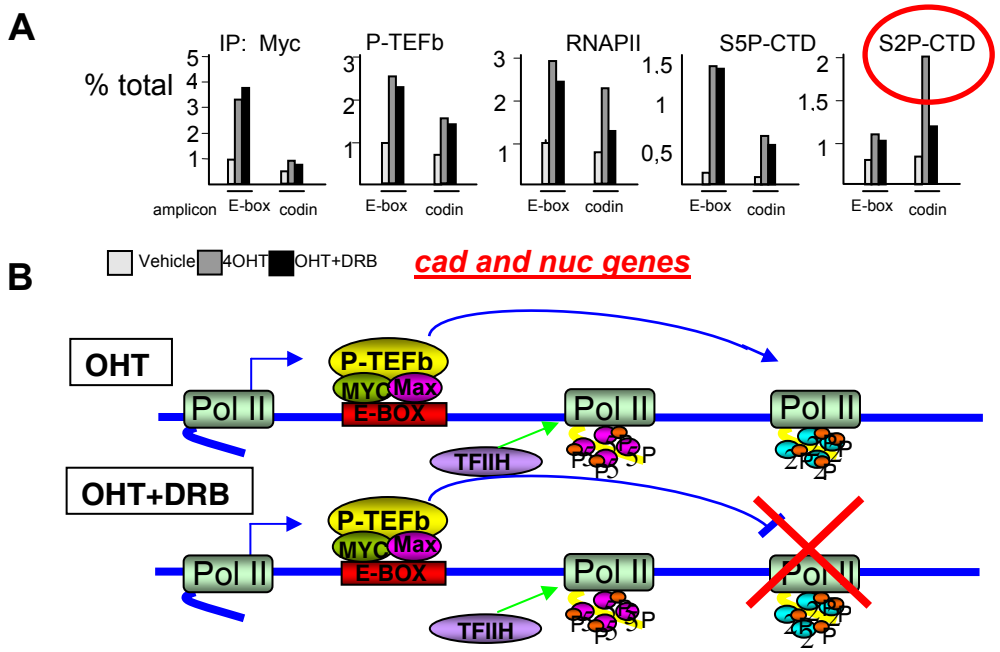

Figure 21. DRB affects CTD phosphorylation. **A.** Quiescent Rat-MycER were treated with either vehicle or 4-OHT in the presence or absence of DRB (50 μM) for 90' and subjected to chromatin immunoprecipitation (ChIP) assays using the indicated antibodies. Immunoprecipitated material was analyzed by Real Time PCR using sets of primers against regions of NUC encompassing E-Box (+574) and coding region (+1500) and CAD gene E-Box and coding region (+3258).**B.** Schematic representation.

3.2 Myc/p14ARF interaction impairs Myc functions

3.2.1 Biochemical characterization of the c-Myc/p14ARF interaction

The first oncogene identified to regulate ARF tumour suppressor function is Myc (Zindy et al., 1998). Overexpression of Myc in B-lymphocytes augments cell proliferation which is counteract by the ARF-p53-Mdm2 axis. Inhibition of this axis suppresses Myc-induced apoptosis and facilitates B cell lymphoma formation (Eischen et al., 1999). This findings indicate that Myc-induced cell growth and proliferation is balanced by simultaneous activation of p53 via ARF. However, several observations suggest that this pathway is not so simple. ARF induction requires very high and sustained Myc activity and physiological level of Myc does not activate the ARF promoter (Cleveland and Sherr 2004). Cells with p53-null or p53-mutated status have marked upregulation of murine p19ARF, which is suppressed by overexpression of p53 and p53 also inhibits Myc expression (Sherr et al., 2000). Thus, there is a feedback regulation among these proteins the balance of which determines the ultimate fate of the cells. Adding to this complexity, it has been demonstrated (Qi et al., 2004; Datta et al., 2004) that mouse p19ARF interacts with c-Myc independently of MDM2 or p53 and negatively regulates its transcriptional activity. Interaction of Myc with p19ARF relocates p19ARF from the nucleolus to nucleoplasm in both wild-type and p53-null MEFs. The structural differences between the murine p19ARF and the human p14ARF proteins prompted me to determine if also the human p14ARF tumour suppressor protein was associated with the human c-Myc protein. To this end, I

performed *in vitro* interaction assays using highly purified bacterial expressed HA-Myc-FLAG and GST-p14ARF proteins. Equal amounts of the two purified proteins were incubated for 3 hours and subsequently the HA-Myc-FLAG protein complex was recovered with M2-FLAG beads and analyzed by western blotting with the GST antibody for the presence of the GST-p14ARF co-purified protein. The HA-Myc-FLAG was incubated with GST and GST-Max proteins as negative and positive interaction controls, respectively (**Figure 22 A**, lane 1 and 2). In addition, equal amounts of the GST, GST-Max and GST-ARF were incubated, in absence of the bait Ha-Myc-FLAG, with M2-FLAG beads as control of aspecific purification (lane 4, 5 and 6). The result demonstrated that Myc directly interacts with p14ARF (lane 3).

Interaction between Myc and p19ARF has also been shown to alter the transcription activity of Myc, as described in the background chapter. Since Myc binds to target promoters as heterodimer with Max, I sought to determine if p14ARF was able to bind to the Myc-Max heterodimer and if this interaction was putative mutually exclusive. Purified GST-p14ARF, HA-Myc-FLAG and His-Max proteins were mixed together and then the proteins subjected to GST-pull down. The GST-affinity-purified complex (AC) was then analyzed for the presence of Myc and Max proteins by immunoblotting with anti-Myc and anti-His antibodies, respectively. The result in **Figure 22 B** lane 2, shows that the GST-p14ARF associated with both Myc and Max, demonstrating that p14ARF interacts with the Myc-Max complex and that the binding of p14ARF does not interfere with Myc-Max interaction *in vitro*.

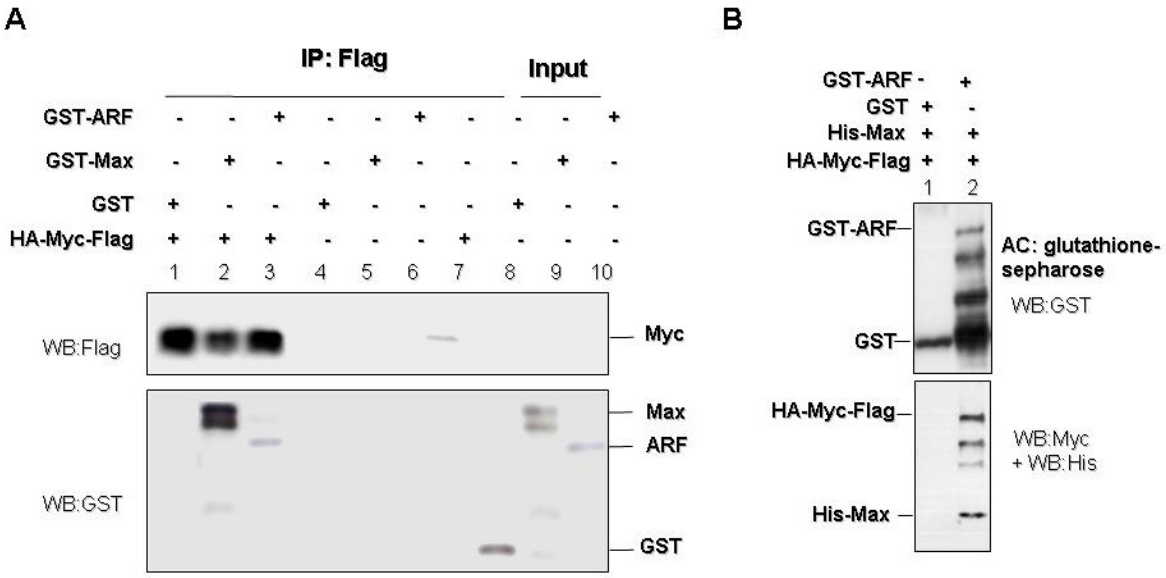

Figure 22: p14ARF directly interacts with Myc *in vitro*. (**A**) The GST, GST-Max or GST-p14ARF proteins (600ng) were incubated in presence (lane 1, 2, 3) or absence (lane 4, 5, 6) of HA-Myc-FLAG bait protein (600ng). Protein complexes were recovered by immunoaffinity with ANTI-FLAG M2-Agarose and analyzed by WB with anti-FLAG (top panel) and anti-GST (bottom panel) (Santa Cruz Biotechnology, Inc.). In lanes 8, 9, 10, 5% of the inputs were loaded. (**B**) GST-p14ARF and GST-Max proteins (600ng) were incubated with equal amounts of GST (lane 1) or HA-Myc-FLAG (lane 2). Affinity complexes (AC) were analyzed by WB using anti-GST (top panel) and a mixture of anti-Myc plus 6xHis Monoclonal Antibody (BD Biosciences, bottom panel).

3.2.2 Myc Box II is required for Myc-ARF interaction in vivo and in vitro

To identify c-Myc protein sequences that are essential for association with p14ARF, I performed *in vitro* GST pull down assays using the His-p14ARF protein and various GST-Myc deletion mutants. The different GST-Myc deletion mutants purified, described in **Figure 22**, were mixed with the His-p14ARF protein and the complexes were affinity-purified by GST beads; the presence of p14ARF was monitored by immunoblotting with His antibody. Aliquots of each sample were assayed with the GST antibody for the presence of the different GST-Myc mutants used as baits. The results represented in **Figure 23** show that the Myc deletion mutants 1-143 and 1-228 (lane 4-5) retain the ability to bind the His-p14ARF protein.

In particular, the GST-Myc 1-228 shows stronger interaction (lane 5). In contrast, the N-terminal deletion mutants, GST-Myc 151-340 and GST-Myc 262-439 fail to bind His-p14ARF (lane 6, 7). As positive control of interaction the GST-Myc 262-439 interacts with His-Max (lane 8). Thus, the Myc residues 103 to 151, including the Myc BoxII domain, are required for association with p14ARF *in vitro*.

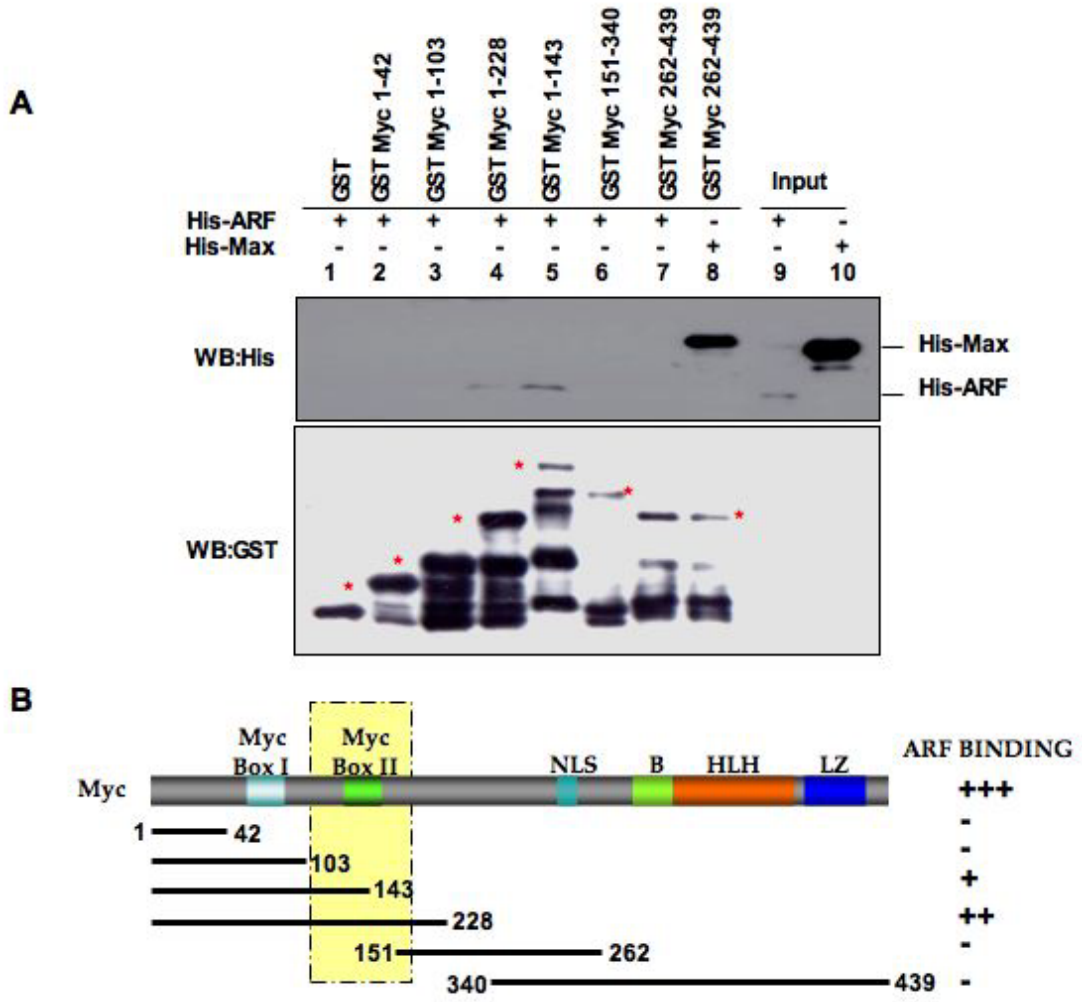

Figure 23 : Mapping of the Myc domains involved in ARF interaction. (**A**) His-p14ARF was incubated with GST and GST-Myc deletion mutants (lane 1, 2, 3, 4, 5, 6, 7) proteins. In lane 8, as positive control of Myc interaction, GST-Myc 262-439 was incubated with the His-Max. The protein complexes were recovered using glutathione-sepharose and the copurified proteins revealed with anti-His (top panel) and anti-GST (bottom panel). In lane 9 and 10, 5%of the proteins inputs were loaded. (**B**) Schematic representation of the Myc full-length protein and deletion mutants. The relative strengths of interactions with p14ARF are indicated.

To determine whether Myc/Max heterodimer interacts with p14ARF *in vivo*, Co-IP assays were performed with protein extracts from transiently transfected 293T cells that express low levels of endogenous Myc and ARF proteins. The cells were transfected with CMV-based Myc and Max expression vectors in the presence and absence of p14ARF, and immunoprecipitations performed using anti-Myc or anti-Max antibodies. Immunoblot analysis was then performed using ARF antibody. The results reported in **Figure 24 A,** show that either the p14ARF (lane 10) and Max (lane 9, 10) proteins co-immunoprecipitated with Myc. In parallel the extracts were immunoprecipitated with the Max antibody followed by immunoblotting with Myc, Max and ARF antibodies, respectively. As shown in **Figure 24 B**, Max immunoprecipitated extracts contained both the Myc and the ARF proteins (lane 12). Collectively these findings demonstrate that p14ARF associates with Myc/Max heterodimer *in vitro and in vivo* and that Myc binding to Max and p14ARF is not mutually exclusive. To further validate the Myc-ARF interaction the U2OS cell line, which does not express the ARF protein, was transfected with CMV-based Myc and ARF expression vectors and cellular extracts were immunoprecipitated with the ARF antibody. Immunoblotts confirmed the presence of the Myc protein in the immunoprecipitated extracts (**Figure 24**

C lane 6).

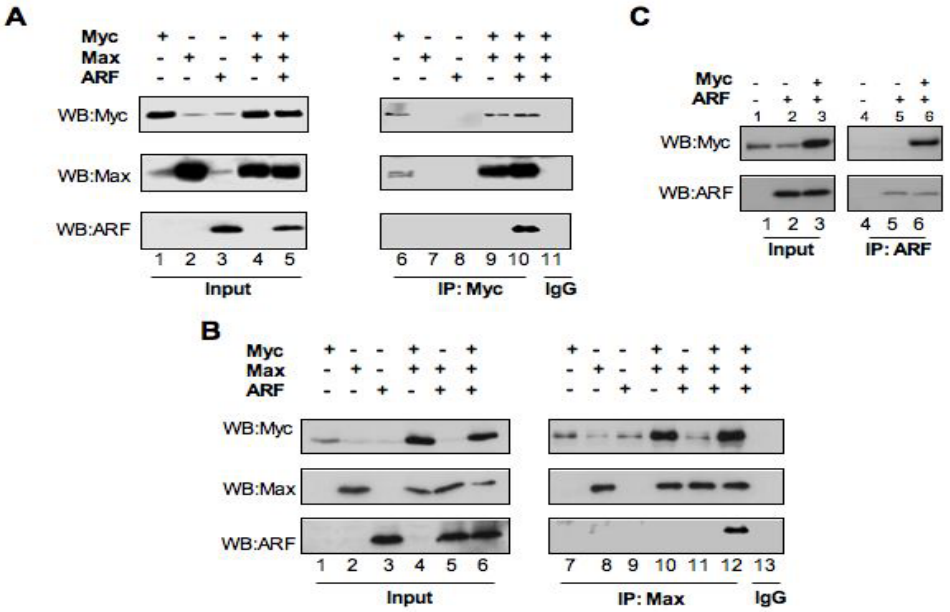

Figure 24: p14ARF interacts with Myc *in vivo*. (**A**) 293T cells were co-transfected by the calcium–phosphate method with pcDNA3-FLAG-Myc, pcDNA3-Max and pcDNA-ARF-HA as indicated. Protein extracts were immunoprecipitated with the anti-Myc N262 antibody (lane 6-10), and IgG antibody (lane 11). The copurified complexes analyzed by WB with anti-Myc 9E10, anti-Max and anti-ARF antibodies, as indicated. 5% of the proteins inputs were loaded in lanes 1-5 (**B**) 293T cells were co-transfected by the calcium–phosphate method with of pcDNA3-FLAG-Myc, pcDNA3-Max and pcDNA-ARF-HA as indicated. Protein extracts were immunoprecipitated with the anti-Max antibody (lane 7-12), and IgG antibody (lane 13). The copurified complexes analyzed by WB with anti-Myc 9E10, anti-Max and anti-ARF antibodies, as indicated. 5% of the proteins inputs were loaded in lanes 1-6. (**C**) U2OS cells were co-tranfected with the indicated vectors and protein extracts were IP with anti-ARF antibody (lane 4, 5, 6) and the Co-IP complexes analyzed by WB with anti-Myc (top) and anti-ARF (bottom). 5% of the proteins inputs were loaded in lanes 1- 3.

To corroborate the requirement of the Myc Box II in the interaction with p14ARF, I performed Co-IP assays with protein extracts prepared from 293T cells that were transiently co-transfected with a CMV driven p14ARF expression vector along with the pcDNA3-FLAG-Myc vector expressing the full-length protein or an isogenic vector, pcDNA3- FLAG-MycΔ123-151, expressing a protein with an in-frame deletion of the Myc BoxII domain. Protein extracts were immunoprecipitated with the FLAG antibody

followed by immunoblot with the ARF and Max antibody, respectively. The results shown in **Figure 25**, illustrate that while the full-length Myc protein interacts with both p14ARF and Max (lane 3), the deletion of aa 123 to 151, including the Myc BoxII, severely invalidates the Myc-ARF binding, without significant effects on Max binding (lane 4). Collectively, these results substantiated the physical interaction between ARF and Myc and demonstrate that the region encompassing the Myc BoxII is involved in the interaction between Myc and p14ARF both *in vitro* and *in vivo*.

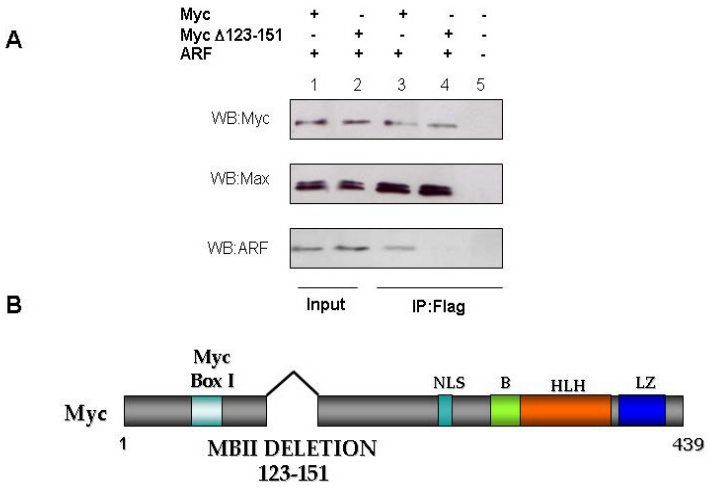

Figure 25. Myc Box II is required for the Myc-ARF interaction *in vivo.* (A) 293T cells were co-transfected with pcDNA-p14ARF-HA along with pcDNA3-FLAG-Myc or pcDNA3-FLAG-MycΔ123-151 as indicated. Protein extracts from the transfected cells were IP with ANTI-FLAG M2-Agarose followed by WBs with anti-Myc (top), anti-Max (middle) and anti-ARF (bottom). (B) Schematic representation of Myc deletion mutant is shown.

3.2.3 Myc-p14ARF nucleolar co-localization is abrogated by MBII deletion

The Myc protein localizes in the cellular nucleus while the p14ARF protein has a predominantly nucleolar localization. Overexpression of Myc-induced relocalization of p19ARF out of the nucleolus and into the nucleoplasm in MEF cells, or conversely p19ARF could delocalize Myc into the nucleolus in U2OS cells. Even though the functional meaning is

still obscure, this behaviour can be instrumental to determinate if the Myc BoxII domain has *in vivo* sub-cellular relevance in the Myc-ARF interaction. Consequently, I sought to analyze the contribution of the Myc BoxII region, required for in *vivo* and *in vitro* binding, in the sub-cellular co-localization of Myc and p14ARF. U2OS cells, that do not express the p14ARF, were co-transfected with a green fluorescent protein (GFP) fusion, GFP-p14ARF, along with the Myc expression vector or the deletion mutant MycΔ123-151. In agreement with previous observations, I found that the GFP-p14ARF accumulates predominantly into the nucleolus whereas Myc displayed typical nucleoplasmatic localization. When ARF and Myc were co-transfected in a 1 to 1 ratio, 82% of the co-transfected cells exhibited co-localization of Myc and ARF protein into the nucleoli (**Figure 26 A**). In contrast, a significant reduction of co-localization into the nucleoli (22%) was observed when GFP-p14ARF was co-transfected with the MycΔ123-151 deletion mutant (**Figure 26 B**). These findings underlie the relevance of the Myc BoxII domain in the physiological interaction between the ARF and Myc protein.

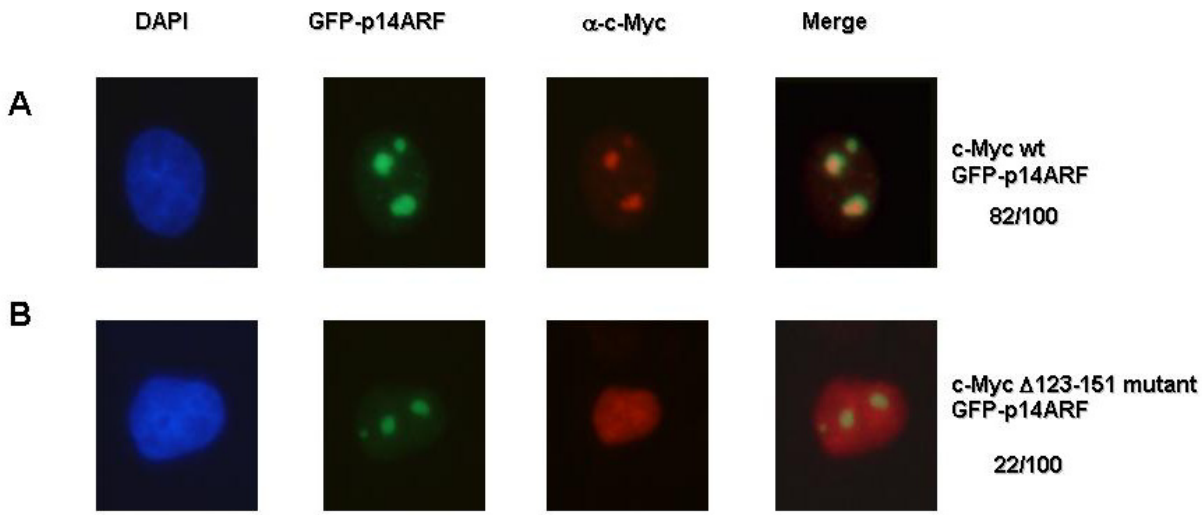

Figure 26. Myc-ARF nucleolar colocalization is impaired by Myc BoxII deletion. U2OS cells were co-transfected with GFP-p14ARF and pcDNA3-FLAG-Myc (**A**) or pcDNA3-FLAG-MycΔ123-151 (**B**) by Lipofectamine 2000 as indicated. An example of immunofluorescence microscopy of the cells immunostained with anti-Myc 9E10 and analyzed by fluorescence microscopy as previously described, is

shown. At least 150 cells were analyzed in each experiment. Values are means from three independent experiments.

3.2.4 p14ARF N-terminal domain interacts with c-Myc

In the attempt to define the ARF region involved in Myc interaction, I subcloned the ARF cDNA regions coding for aa 1 to 65 and for aa 65 to 132 in a FLAG epitope tagged CMV10 vector. These constructs and the wild type p14ARF vector were transfected alone or in combination with c-Myc expression vector into 293T cells. The protein extracts from the transfected cells were immunprecipitated with the c-Myc antibodies and the coimmunoprecipitated proteins analyzed by WB with c-Myc, Max and FLAG antibodies. The results shown in **Figure 27** demonstrate that either the WT ARF protein then the protein encoding for the first 65 aa co-immunoprecipitate with c-Myc (lane 1, 2 respectively). In contrast the 65-132 ARF C-terminal domain is impaired in binding to Myc protein (lane 3). As control of c-Myc immunoprecipitation, WB with Max antibody confirms the presence of the endogenous Max protein in all the Myc immunopreciptated extracts.

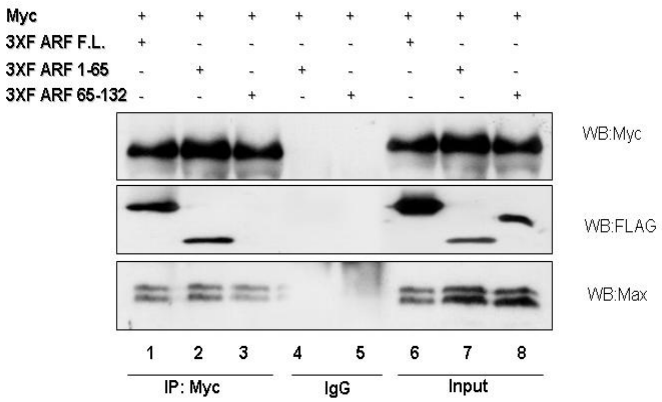

Figure 27. Myc interacts with the N-terminal region of p14ARF. 293T cells were co-transfected with pcDNA3-FLAG-Myc, p3xFLAG-ARF full length or p3xFLAG-ARF1-65 or p3xFLAG-ARF65-132 as indicated. Protein extracts were immunoprecipitated with the anti-Myc N262 antibody (lane 1-3), and IgG antibody (lane 4 and 5) and the copurified complexes analyzed by WB with anti-Myc 9E10, anti-Max and anti-FLAG antibodies, as indicated. 5% of the proteins inputs were loaded in lanes 6-8.

3.2.5 Biochemical characterization of the N-Myc/p14ARF interaction

The data reported in the previously paragraphs demonstrated that p14ARF directly associates with the c-Myc protein. Even if is general assumption that the interaction partners of c-Myc are also N-Myc partners, the failure of expression of either two Myc family members is not redundant and it cannot be excluded that they can form different complexes with their interactors.

In order to investigate if p14ARF is also a N-Myc partner, CoIP assays were performed with protein extracts from 293T transiently transfected cells. The cells were transfected with different combinations of CMV-based expression vectors for p14ARF and N-Myc as reported in **Figure 28** and immunoprecipitations were performed using the N-Myc antibodies. The results show that p14ARF co-immunoprecipitates with N-Myc (**Figure 28 A** lane 4). In parallel the extracts were immunoprecipitated with the ARF antibody followed by immunoblotting with Myc, and ARF antibodies, respectively. The results show that ARF immunoprecipitated extracts containing the Myc protein (**Figure 28 B** lane 4).

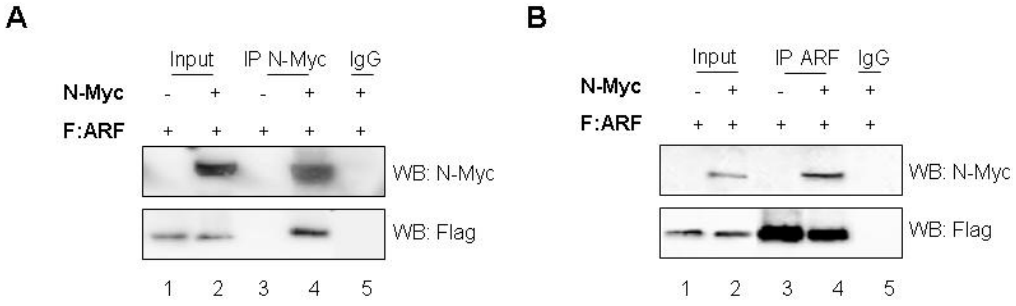

Figure 28. p14ARF interacts with N-Myc *in vivo*. A. 293T cells were transfected with 3xFLAG-ARF or co-transfected with N-Myc and p3XFLAG-ARF F.L. as indicated. Protein extracts were immunoprecipitated with the N-Myc antibody (lane 3, 4) and anti IgG (lane 5) as control and the co-purified complexes analyzed by WB with anti-N-Myc anti-FLAG antibodies, as indicated. 5% of the proteins inputs were loaded in lanes 1 and 2. **B**. The same protein extracts utilized in panel A were IP with the anti-ARF antibody (lane 3, 4) and anti IgG (lane 5) as control and the Co-IP complexes analyzed by WB with anti-N-Myc and anti-FLAG antibodies. 5% of the proteins inputs were loaded in lanes 1, 2.

3.2.6 Myc Box III is required for in vivo N-Myc-ARF interaction

To identify N-Myc sequences involved in N-Myc-ARF interaction, I performed CoIP analysis using protein extracts from 293T cells transiently co-transfected with a FLAG tagged p14ARF expression vector and several N-Myc deletion mutants expressing different domains of the N-Myc protein as indicated in **Figure 29B**. The protein extracts were immunoprecipitated with the ARF antibody and the co-immunoprecipitated proteins analyzed by WB with N-Myc and ARF antibodies. As shown in **Figure 29A**, the N-Myc deletion mutant d(1-300) lost the ability to associate with p14ARF (lane 10) while all the other mutants and in particular the N-Myc d (1-134), whose deletion covers part of the deletion of the N-Myc d(1-300), are able to bind p14ARF. From these data I can conclude that the N-Myc region involved in p14ARF interaction resides in the region from aa 140 to aa 300 containing the MBIII conserved domain.

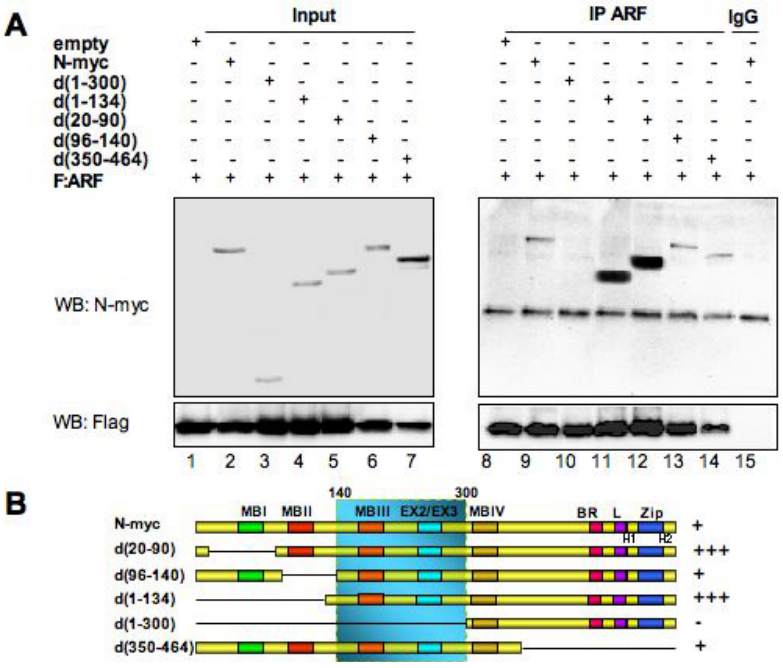

Figure 29. p14ARF interacts with the MBIII of N-Myc *in vivo*. A 293T cells were co-transfected with 3xFLAG-ARF and different N-Myc delection mutants as indicated. Protein extracts were immunoprecipitated with the ARF antibody (lane 8-14) and anti IgG (lane 15) as control and the co-purified complexes analyzed by WB with anti-N-Myc anti-FLAG antibodies, as indicated. 5% of the

proteins inputs were loaded in lanes 1-7. **B** Schematic representation of the N-Myc full-length protein and deletion mutants. The relative strengths of interactions with p14ARF are indicated.

3.2.7 N-Myc co-localizes with p14ARF in nucleoli upon ARF overexpression

As shown, c-Myc and ARF co-localize in the same cellular compartment upon ARF overexpression. Depending from the cell lines analyzed by different authors, Myc binding relocalizes ARF from the nucleolus to the nucleoplasm or c-Myc co-localizes with p14ARF into nucleoli. In order to investigate if also N-Myc could co-localize with ARF in the same cellular compartment, I transfected the neuroblastoma cell line SKNBE with the GFP-p14ARF expression vector and I analyze the localization of the GFP-ARF and N-Myc proteins. As expected the GFP-ARF protein in these cells shows a predominantly nucleolar localization while the endogenous N-Myc protein was barely detectabled by immunocytochemistry analysis. I therefore decided to restrict the analysis to co-transfected cells. SKNBE cells were transfected with the N-Myc expression vector alone or in combination with the GFP-p14ARF. In cells transfected with N-Myc the protein was found exclusively in the nucleolar compartment and cells transfected with the GFP-ARF alone showed a predominantly nucleolar localization of the exogenous ARF protein.

In cells co-transfected with the two expression vectors, the GFP-p14ARF protein retains the nucleolar localization while the N-Myc protein was found in the nucleolar compartment in 78% of the co-transfected cells as shown in **Figure 30A**. I then tested the ability of the N-Myc d (1-300) protein that I have found impaired in binding to the ARF protein, to be recruited by ARF in the nucleoli upon ARF overexpression.

As shown in **Figure 30B**, in the cells co-transfected with both GFP-p14ARF and N-Myc d (1-300) mutant, the GFP-ARF protein was found in

the nucleoli and the mutated N-Myc protein was found in the nuclear compartment.

These data corroborated the findings that the N-terminal region of the N-Myc protein was involved in binding with ARF and that the N-Myc protein, impaired in ARF binding, lost the capability to be recruited by ARF in the nucleoli.

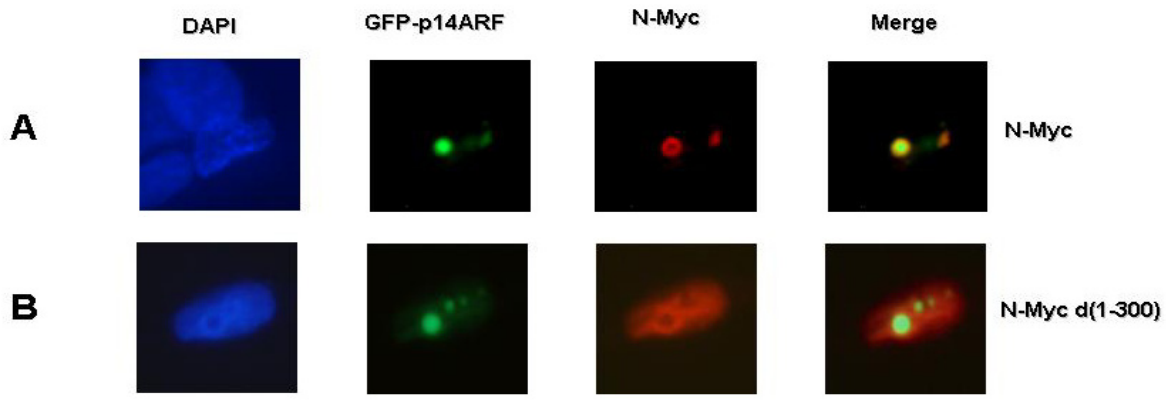

Figure 30. N-Myc-ARF nucleolar colocalization is impaired by Myc BoxIII deletion. SKNBE cells were co-transfected with GFP-p14ARF and N-Myc (**A**) or N-Myc d(1-300) mutant (**B**) as indicated. An example of immunofluorescence microscopy of the cells immunostained with anti-N-Myc and analyzed by fluorescence microscopy as previously described, is shown. At least 150 cells were analyzed in each experiment. Values are means from three independent experiments.

3.2.8 p14ARF inhibits c-Myc and N-Myc transcriptional activation

As mentioned in the background chapter, mouse p19ARF is able to block Myc's ability to activate transcription. Furthermore, I decided to investigate if p14ARF, the human homolog, was able to inhibit both c-Myc and N-Myc transcriptional activity. First of all, I investigated if p14ARF expression was able to inhibit Myc ability to transactivate the Telomerase Reverse Transcriptase (hTERT) promoter. To this end, I cotransfected the U2OS cell line with hTERT-luc construct, in which the luciferase gene is under hTERT promoter control and with c-Myc in presence of increasing amount of the p14ARF vector. As reported in **Figure 31A**, Myc exogenous expression in U2OS cells activates three fold the hTERT-Luc promoter

expression (lane 2) and co-transfection of p14ARF inhibits Myc-activation in a dose dependent manner (lane 3, 4 and 5). As control, p14ARF alone was co-transfected with hTERT-luc. As shown in **Figure 31 A** (lane 6), p14ARF does not have any influence of hTERT promoter transcription in the absence of exogenous c-Myc.

In order to extend these findings to N-Myc-mediated transcriptional activation, I took advantage of the Tet21N cell line (kindly provided by Prof G. Della Valle) that stably expresses the N-Myc protein in the absence of tetracycline. N-Myc expression can be down regulated, to the complete absence of expression, by addition of tetracycline in the medium for at least 48 hours. In this way N-Myc expression can be modulated by the different amount of tetracycline. Tet21N cells were grown in the presence of tetracycline for 2 weeks to abrogate N-Myc expression and co-transfected with the hTERT-Luc construct and an expression vector for p14ARF. Then tetracycline was removed from the medium for N-Myc expression. Cells were then left untreated or treated with tetracycline and extracts analyzed in luciferase assay for the human Telomerase promoter (hTert) driven luciferase expression. As reported in **Figure 31B**, N-Myc activates the hTERT promoter three fold (lane 2) and p14ARF was able to inhibit with a dose responsive effect the N-Myc mediated activation of the Telomerase promoter (lane 3, 4 and 5). Finally, altogether those findings demonstrated that p14ARF was able to repress both c-Myc and N-Myc transcriptional activities.

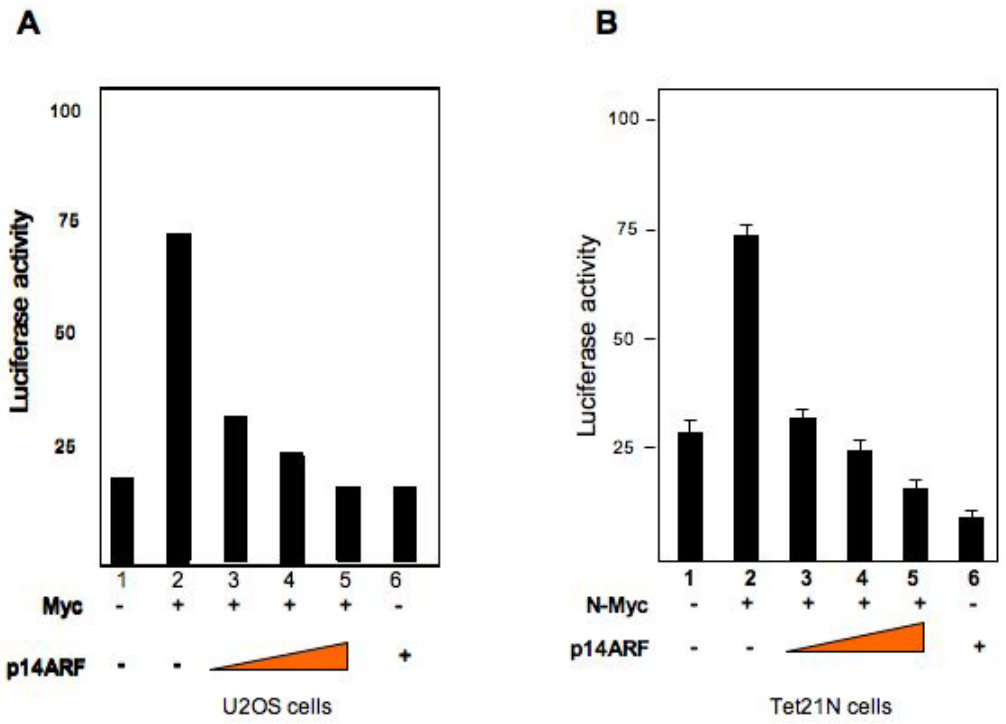

Figure 31. ARF expression inhibits c-Myc and N-Myc activated transcription. A U2OS cells were cotransfected with 100ng of hTERT-Luc, 200ng pMT2T-Myc and different amounts (0,1; 0,5 and 1μg, respectively) of pcDNA-p14ARF-HA as indicated. Each histogram bar represents the mean of three independent transfections made in duplicate with a standard deviation less than 10%. **B** Tet21N cells were grown in the absence (*High N-Myc, "+"*) or presence (*Low N-Myc, "-"*) of tetracycline for 2 weeks and co-transfected with the hTERT-Luc construct (100 ng) and different amounts (0,1; 0,5 and 1μg, respectively) of pcDNA-p14ARF-Haas indicated. Each histogram bar represents the mean of three independent transfections made in duplicate with a standard deviation less than 10%.

3.3 p14ARF antagonizes HIV-1 Tat protein functions

3.3.1 p14ARF affects Tat transactivation on the HIV-1 promoter

In a parallel line of research I have deepened a recently discovered and unexpected role of tumor suppressor ARF in viral infection surveillance.

ARF expression is induced by interferon and after viral infection. ARF protects against viral infection through a mechanism that involves ARF-induced release of PKR from nucleophosmin complexes (Garcia et al., 2006).

ARF is a potent tumour suppressor that blocks cell-cycle progression by directly binding to, and interfering with, the p53 negative-regulator Mdm2 (Hdm2 in human), thereby stabilizing and activating p53 (Kamijo et al., 1997; Stott et al., 1998). The proto-oncoprotein Hdm2 also interacts with HIV-1 Tat protein and mediates its ubiquitination *in vivo* and *in vitro* (Bres et al., 2003). The E3 ubiquitin ligase Hdm2 is a positive regulator of Tat-mediated transactivation, indicating that the transcriptional properties of Tat are stimulated by ubiquitination (post-translate modification). Because ARF interacts with Hdm2 and interferes with its activity leading to p53 stabilization, I sought to determine whether ARF could affect Tat transactivation of the HIV-1 promoter.

HIV-1 Tat-mediated transcription as well as P-TEFb complex are object of study in the Prof. Lania-Majello laboratories since several years. Transcription of HIV-1 proviral DNA by RNAPII is controlled primarily at the level of elongation by the viral Tat protein (Barboric and Peterlin 2005). Cyclin T1 was originally identified as a direct binding partner of the HIV-1 Tat protein in HeLa nuclear extracts, and Tat and Cyc T1 cooperate to recruit P-TEFb to the viral 5' TAR RNA (Price 2000; Saunders et al., 2006).

Tat is a protein encoded by the HIV-1, transcribed from multiply spliced viral RNA molecules expressed at early stages of viral gene expression. It is composed of the two exons of the viral Tat gene and encodes a protein of approximately 101 amino acids and in the late stage of the infection cycle, a carboxy-terminally truncated, encoded for Tat protein of 72 aminoacids also sufficient to transactivate the HIV-1 promoter.

The aim of this line of research it has been to demonstrate if the ARF tumour suppressor could affect Tat protein functions as the Tat transactivation of the HIV-1 LTR promoter.

To address this point, I carried out transient co-transfection experiments in H1299 cells, in HeLa HL6 cells (containing the Luc gene under the control of and integrated HIV-1 LTR), and in H358/Tet-On-ARF cells, in which doxycycline (Dox) treatment induces a strong expression of p14ARF protein (this cell line was kindely provided by Dr. S. Gazzeri). As shown in **Figure 32,** in all cell lines tested p14ARF over-expression inhibits Tat transactivation of the HIV-1 promoter.

Moreover to map the minimal region essential for Tat repression, I performed co-transfection experiments using the F:ARF(1-65), F:ARF(65-132) constructs which contain the p14ARF cDNA regions coding for aa 1 to 65 and for aa 65 to 132 in a FLAG epitope tagged CMV10 vector, respectively. I found that overexpression of F:ARF(1-65) protein negatively affected Tat transactivation, while the F:ARF(65-132) protein did not. These findings suggest that the N-terminal region of ARF is required for ARF-mediated Tat repression.

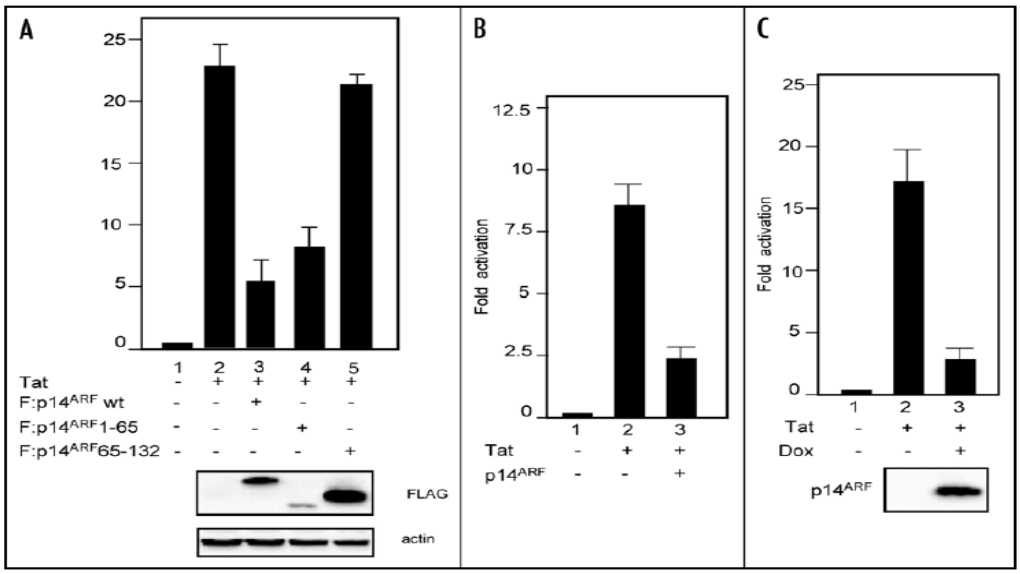

Figure 32. p14ARF expression inhibits Tat transactivation of the HIV-1 promoter. (**A**) H1299 cells were cotransfected with G5-83HIV-Luc (100 ng), Tat101-wt (10 ng) and F:p14ARF(1 μg), F:p14ARF (1–65) (1 μg) and F:p14ARF (65–132) (1 μg), as indicated. (**B**) HL6 cells were cotransfected with Tat-101 wt (1 μg) F:p14ARF(1 μg). (**C**) H358/Tet-On/p14ARF inducible cells were treated for 72 hr with or without 1 μg/ml doxycycline and then cotransfected with G5-83HIV-Luc (100 ng) and F:Tat-101 wt (10 ng). Each histogram bar represents the mean of three independent transfections made in duplicate.

3.3.2 p14ARF promotes Tat degradation

The discovery that p14ARF can directly interact with regulative components of the proteasome multi-protein complex, such as TBP-1/PMSC3 of the 19S subunit (Pollice et al., 2007) and REG-γ of the 11S lid (Takaoka et al., 2003) offers a new key to interpret the mechanisms through which ARF is regulated and regulates cell growth and proliferation. The first evidence of a link between ARF and the proteasome is the observation that both human and mouse ARF are accumulated following treatment with proteasome inhibitors, suggesting that ARF degradation depends, at least in part, by the proteasome (Kuo et al., 2004).

A very recent report describes a direct involvement of the REG-γ proteasome in an ubiquitin-independent regulation of the ARF turnover (Chen et al., 2007). REG-γ pathway plays a role in the control of viral pathogenesis and this is particularly interesting, given that ARF activation has been linked to viral response (Garcia et al., 2006).

It has been reported that Hdm2 interacts with Tat and mediates polyubiquitination of Tat *in vitro* and *in vivo* (Bres et al., 2003; Lassot et al., 2007) One highly conserved lysine, lysine 71, functions as the major, ubiquitination site in Tat. Moreover, Hdm2 overexpression enhances Tat activity, thus it functions as a positive Tat co-activator. Since Hdm2 is negatively regulated by p14ARF, a possible mechanism of p14ARF repression might involve an interference of Hdm2-mediated ubiquitination of Tat. Accordingly with previous data (Bres et al., 2003), I found that Hdm2 enhances Tat transactivation, while p14ARF represses Tat (**Figure 33A**). Overexpression of Hdm2 fails to relieve p14ARF repression of Tat activity, indicating that Hdm2 over-expression does not counteract the negative function of p14ARF.

The main function of Hdm2 in Tat-mediated transactivation is to attach covalently ubiquitin chain to Tat. The fusion construct in which a ubiquitin

chain is attached to Tat protein bypasses the requirement of Hdm2 in Tat transcriptional activation. Then I wished to demonstrate if p14ARF could interfere with Hdm2-mediated ubiquitination and the transactivation capabilities of Tat-Ub fusion protein should be refractory to p14ARF inhibition. To verify this hypothesis, I tested the relative transactivation abilities of Tat, Tat-Ub and TatK71R-Ub proteins in the presence or absence of p14ARF. The Tat-Ub vector presents a poliubiquitin chain fused to C-terminal domain of Tat protein, while TatK71R-Ub vector presents one mutation in lisine 71, replaced with arginine, that affects this main poliubiquitination site. I found that Tat wild-type as well as the Tat-Ub fusions were repressed by p14ARF (**Figure 33B**), suggesting that p14ARF-mediated repression of Tat transactivation is not dependent on ubiquitination process.

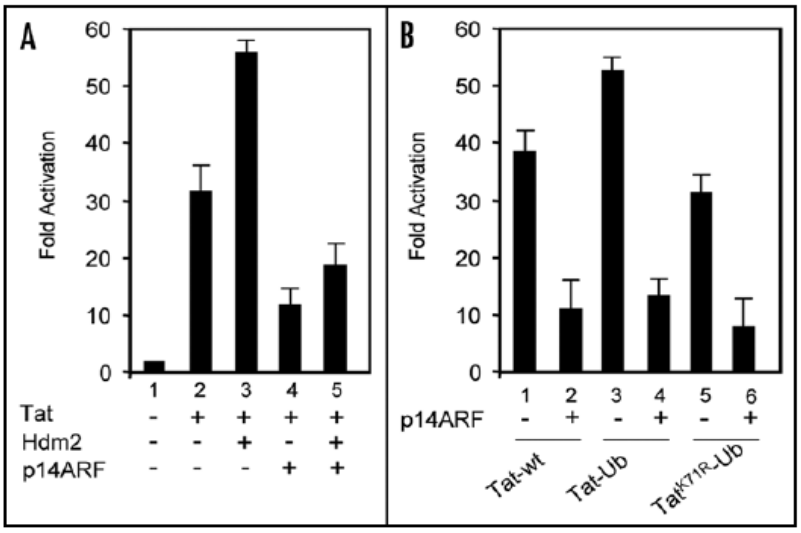

Figure 33. p14ARF expression inhibits Hdm2 enhanced Tat-mediated transactivation of the HIV-1 promoter. (**A**) 293T cells were cotransfected with G5-83HIV-Luc (100 ng), F:Tat-101 wt (10 ng), F:p14ARF (500 ng) and CMV-Hdm2 (500 ng), as indicated. (**B**) p14ARF expression down regulates F:Tat101-wt, pTatWt-Ub and pTatk71R-Ub fusion proteins. 293T cells were cotransfected with -83HIV-Luc (100 ng), F:Tat-101 wt, pTatWt-Ub fusion protein or pTatk71R-Ub fusion protein (10 ng in each case) and in the presence or absence of F:p14ARF (500 ng) as indicated. Each histogram bar represents the mean of three independent transfections made in duplicate.

ARF also causes alteration of stability for some binding partners.

For example, B23/NPM, E2F1, E2F3 and Mdm2 become degraded by induction of the proteasome (26S) in an ubiquitin-dependent manner, while the CtBP2 antiapoptotic transcriptional co-repressor becomes degraded by the proteasome in a ubiquitin-independent manner (Paliwal et al., 2006). Most of other partners become sumoylated although a precise function to this modification has not yet been assigned (Rizos et al., 2005; Tago et al., 2005; Liu et al., 2007).

On the contrary in some cases, ARF is able to stabilize its partners (Tip60, Topo I and COMMD1) through proteasomal degradation inducing a non-classical poly-ubiquitination (Huang et al., 2008).

Therefore, it is possible that p14ARF-mediated inhibition of Tat transactivation is due to reduced levels of Tat protein. In order to evaluate the influence of p14ARF on the accumulation of Tat protein, the relative amounts of the Tat protein were determined in the presence of increasing amounts of p14ARF. I co-transfected 293T cells with a F:Tat vector together with increasing amount of p14ARF expression vector and cell extracts were analyzed by immunoblotting. As reported in **Figure 34A,** Tat protein levels decrease in the presence of p14ARF and the reduction is inversely correlated to the amounts of co-transfected and expressed p14ARF protein. Quantitative RT-PCRs of Tat mRNA isolated from the transfected cells clearly showed that p14ARF does not affect Tat transcription (**Figure 34B),** suggesting that p14ARF inhibitory effect is exerted at posttranscription level. To determine whether p14ARF induced reduction of Tat levels was due to proteasome-mediated degradation and to avoid the inherent limitation of co-trasfection experiments, I used the H358/Tet-On-ARF cells in which endogenous p14ARF protein could be induced by Dox treatment.

p14ARF expression was induced in the presence of Dox for two days, then Tat vector was transfected into Dox-treated and untreated H358/Tet-On-

ARF cells and protein levels were determined in the absence o presence of the proteasome inhibitor MG132. As shown in **Figure 34C**, MG132, interfered significantly with the ability of p14ARF to destabilize Tat in the H358/Tet-On-ARF cells.

Next, I determined the stability of the Tat-Ub and TatK71R-Ub fusion proteins in the presence of p14ARF.

Dox-treated and untreated H358/Tet-On-ARF cells were transfected with Tat-Ub and TatK71R-Ub vectors and the relative amounts of Tat proteins monitored by immunoblotting. As shown in **Figure 34D** p14ARF-mediated reduction of Tat protein levels was observed with both Tat-Ub and TatK71R-Ub fusion proteins. Collectively, these results suggest that p14ARF-mediated reduction of Tat protein is unaffected by the ubiquitination status of the Tat protein.

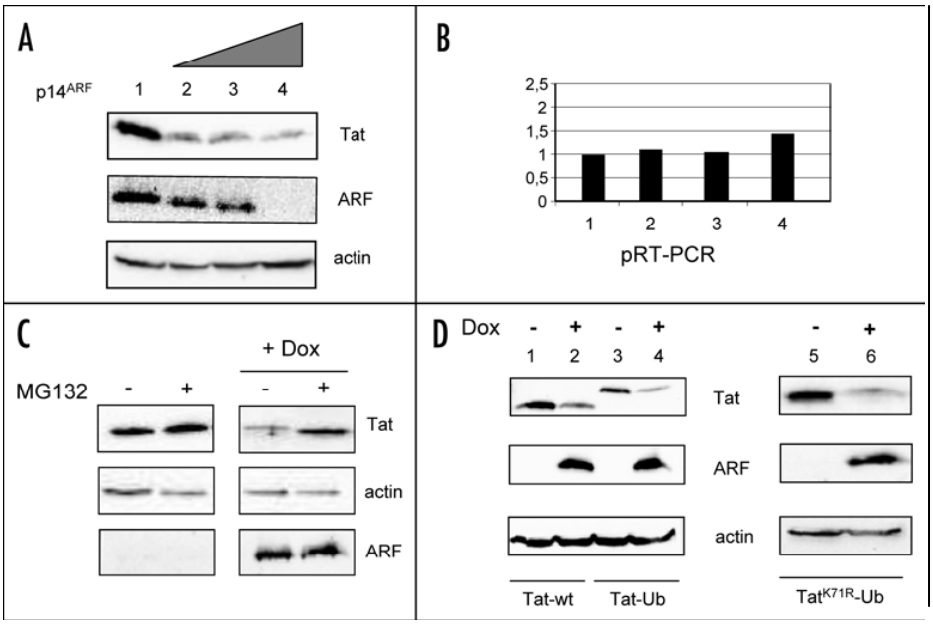

Figure 34. p14ARF targets Tat for degradation. (**A**) 293T cells were co-transfected whit Tat-101 wt (1 μg) and different amounts (1; 2 and 6 μg, respectively) of F:p14ARF. Extracts were analyzed by WB with anti-Tat, anti-ARF and anti-actin, serum as indicated. (**B**) Total RNA from the same transfected cells was prepared and Tat mRNA levels were quantified using qPCR (**C**) Expression level of Tatwt are affected by the proteasome inhibitor MG132. H358-p14ARF inducible cell line were treated for 72 h in presence (+) or in absence (-) of Dox, then the cells were transfected with Tat-101 wt (2 μg) and 24 h post-transfections, the cells were treated with 20 μM MG132 for 2 hr. Protein extracts were analyzed by WB with anti-Tat, anti-ARF and anti-actin antibodies, as indicated. (**D**) p14ARF reduces expression levels of

Tat-101 wt, Tat-Ub and Tatk71R-Ub fusion proteins. H358-p14ARF cells were treated for 72 h in presence (+) or in absence (-) of Dox, then the cells were transfected with the indicated Tat vectors and 24 hr post-transfection cell extracts were analyzed by WB with anti-Tat, anti-ARF and anti-actin antibodies, as indicated.

3.3.3 p14ARF affects the stability of Tat

Accordingly with previous studies, (Bres et al., 2003), in the absence of p14ARF, Tat protein is quite stable as the relative protein level is largely unaffected by MG132 treatment.

To examine the stability of Tat in the presence or absence of p14ARF, I transfected H358 cells in the presence or absence of doxycycline (Dox) (**Figure 35**). The Tat protein was quite stable, consistent with previous measurements of stability. However, p14ARF expression decreased Tat half-life, and the presence of covalently attached Ub-chain results in a modest increase of degradation of Tat-Ub. Collectively, these findings suggest that p14ARF induces de-stabilization of Tat via an ubiquitin-independent pathway.

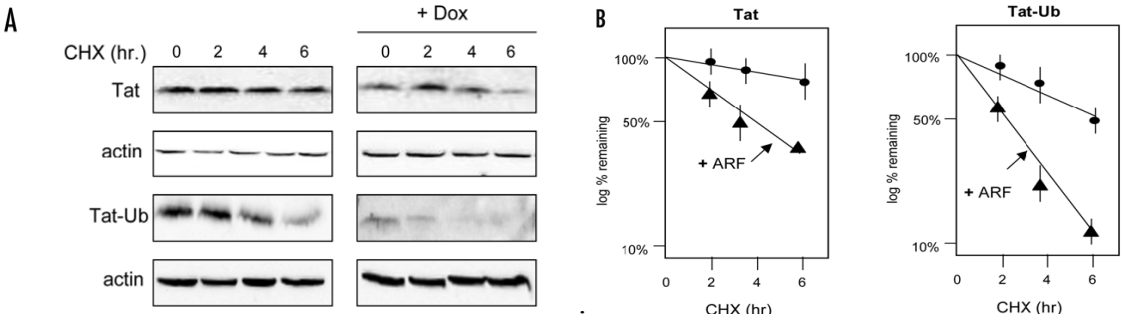

Figura 35. p14ARF affects the stability of Tat-wt and Tat-Ub fusion protein. (A) In H358-p14ARF cells the expression of p14ARF was carried out as described before, then the cells were transfected with Tat-101 wt or with pTatWt-Ub, as indicated. Twenty-four hours post-transfections, protein translation inhibition was achieved with addition of 80 µg/ml of CHX for 2–4–6 h, as indicated. Cellular extracts were analyzed by WB with anti-Tat and anti-actin antibodies. (B) The densitometric signals were normalized to actin as a loading control. A 100% value was arbitrarily assigned to the signal at zero time of treatment. The results shown in (B) are from three independent experiments.

3.3.4 ARF/Tat interaction

Experiments of gel filtration chromatography and glycerol density gradient sedimentation carried out in my laboratory, have demonstrate that the p14ARF protein association in high-molecular mass complexes (Berwistle et al., 2004) was counteracted by concomitant expression of Tat. Extracts from Tat-expressing cells clearly indicated the induction of a partial p14ARF redistribution in a low-molecular weight complex. The concomitant presence of both Tat and p14ARF in the same fractions prompted us to investigate a possible interaction between Tat and p14ARF proteins.

To analyze the putative association between Tat and p14ARF, 293T cells were co-transfected with p14ARF and a Tat expression vector. As control, we also co-transfected p14ARF and Hdm2. After transfections cell extracts were subjected to IP with p14ARF antibody followed by immunoblotting with Tat, p14ARF and Hdm2, respectively (**Figure 36**). As expected, Hdm2 was found to associate with p14ARF. Albeit at a lower efficiency, the Tat protein was found in p14ARF-IP material, while p14ARF-IP from untransfected cells did not. However, only a small fraction of Tat was detected in the ARF CoIP, suggesting that only a relative small amounts of Tat interacts with ARF. In conclusion, Tat induces a redistribution of ARF in a lower molecular weight complex and Co-IP results suggest that Tat can interact with ARF in the same complex.

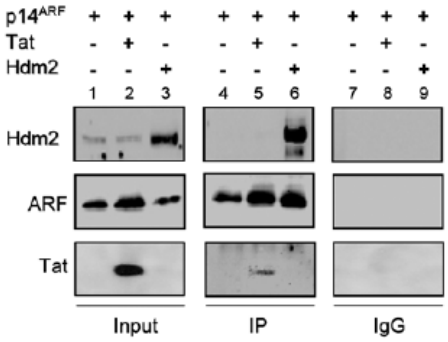

Figure 36: Tat and p14ARF interaction. 293T cells were co-transfected by calcium-phosphate method with Tat-101 wt (5 μg), F:p14ARF (5 μg) and CMV-Hdm2 (2 μg) in different combinations as indicated. Whole-cell extracts were immunoprecipitated with anti-ARF C-18 antibody (lanes 1–6), anti IgG antibody (lanes 7–9). Immuno-complexes were analyzed by WB with anti-Tat, anti-ARF and anti-HDM2 antibodies as indicated. Twenty percent of inputs (lanes 1–3) were loaded.

3.3.5 ARF does not inhibit Tat functions by sumoylation or by sub-cellular re-localization

It has been shown that ARF- induced sumoylation observed for some ARF-interacting proteins as WRN helicase, Hdm2, E2F-1, HIF-1α, TBP-1, p120E4F, might be a mechanism for ARF action through a common modification of different binding proteins.

There are no evidences that Tat is a bona-fide substrate for ARF sumoylation; in order to investigate if the overexpression of the CELO adenovirus protein, Gam1, which is known to block ARF-induced sumoylation, could have overt effect on the ability of ARF to repress Tat, I transfected 293T cells with G5-83HIV-Luc (100 ng), F:Tat-101 wt (10 ng), F:p14ARF (500 ng) and CELO Gam1(500 ng). Gam1 does not alter the p14ARF repression of Tat protein (**Figure 37**).

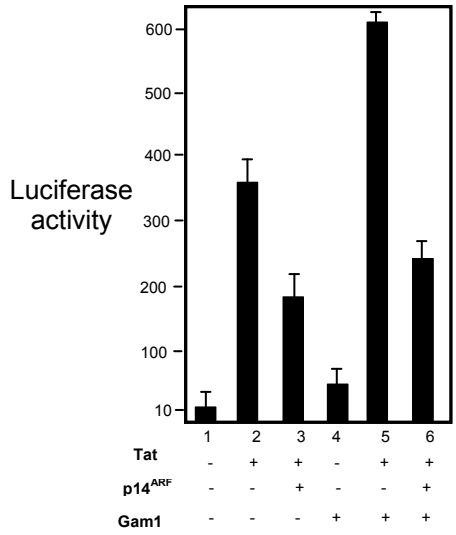

Figure 37. The p14ARF sumoylation does not involved the ARF repression of Tat functions. 293T cells were cotransfected with G5-83HIV-Luc (100 ng), F:Tat-101 wt (10 ng), F:p14ARF (500 ng) and CELO Gam1(500 ng). p14ARF expression down regulates F:Tat101-wt and concomitant expression of

CELO Gam1 does not alter the p14ARF repression of Tat protein. Each histogram bar represents the mean of three independent transfections made in duplicate.

Several studies have shown that ARF induces nucleolar re-localization of some of its binding partners. In order to test this hypothesis I transfected H358-ARF cells with GFP-Tat (2µg) and I treated with Dox (1 µg/ml) that induces the ARF expression

As shown in **Figure 38** by immunofluorescence assay I did not observe any significant difference in Tat sub-cellular localization upon ARF overexpression. Endogenus ARF protein presents as expected nucleolar sub-cellular localization and GFP-Tat has mostly nuclear localization.

ARF overexpression does not change Tat original localization.

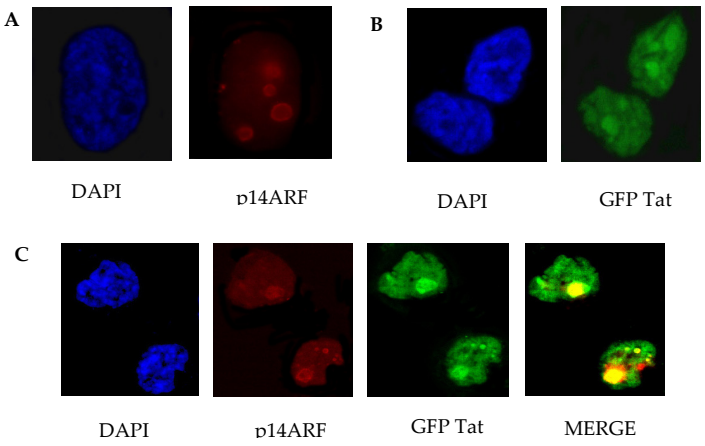

Figure 38. The ARF expression does not alter the Tat localization . Immunofluorescence assay carried out in H358-ARF cells. The cells were transfected with GFP-Tat (2µg) and treated with Dox (1 µg/ml).**(A)** The nucleolar p14ARF localization. **(B)** The localization of GFP-Tat. **(C)** ARF does not change Tat original localization when co-expressed.

CHAPTER IV
Discussion

4.1 P-TEFb regulates positively c-Myc mediated- transcription

The prevailing model of Myc-mediated transcription postulates that Myc increases local histone acetylation at promoters. Myc binds to histone acetyltransferase complexes including TRAAP (transformation/transcription –domain –associated protein) and either general control of amino-acid-synthesis protein-5 (GCN5) or TIP60, which preferentially acetylate histones H3 or H4, respectively (McMahon et al., 1998; McMahon et al., 2000; Frank et al., 2001). Activation of Myc target genes in some cell systems occurs independently of an increase in histone acetylation (Eberhardy et al., 2000). Some Myc target genes are activated completely independently of TRRAP. Deletion of MBII inhibits Myc binding to TRRAP and dramatically reduces transcription, but some genes can still be activated in response to MycΔMBII (Nikiforov et al., 2000).

Previous works have shown that Myc interacts with CycT1, the regulatory component of the P-TEFb complex (Eberhardy and Farnham 2001; Eberhardy and Farnham 2002; Kanazawa et al., 2003), a pivotal transcription factor that regulates elongation phase of transcription of RNA Pol II genes.

However, the contributory role of P-TEFb in Myc transactivation remained elusive. In my work I have demonstrated that the Myc/Max heterodimer binds to P-TEFb. Co-IP assays indicate that the Myc/Max heterodimer copurifies with CycT1/CDK9 proteins and the absence of HEXIM1 in the Myc IP-associated proteins, suggests that Myc forms a complex exclusively with the core catalytic active P-TEFb complex.

ChIPs assays clearly have demonstrated that Myc induction directly recruits P-TEFb to chromatin templates, because I have found co-occupancy of both factors (P-TEFb and the Myc/Max complex) at the E-boxes of CAD and NUC responsive genes.

To analyze the involvement of the CDK9 kinase in transcriptional regulation drived by Myc, I used 5.6-di-chloro-1-b-D-ribofuranosyl-bensimidazole (DRB), the pharmacological specific inhibitor of CDK9 kinase activity. Analysis of cell cycle distribution of Rat-MycER cells in the presence of DRB treatments has shown strongly reduction of both Myc-induced proliferation and apoptosis. These results suggested that CDK9 is crucial for the induction of Myc-responsive gene as well as for Myc-induced cellular outcomes. Moreover, I also evaluated the relative levels of expression of two Myc-responsive target genes, nucleolin (NUC) and CAD and I have found that DRB specifically inhibits the expression of Myc-target genes at the concentrations that marginally affect the expression of housekeeping control genes.

ChIPs analysis whit DRB treatment have demonstrated that kinases, likely CDK9, are required to phosphorylate Ser2-CTD of RNAPII when transiting at both NUC and CAD loci. DRB treatment during Myc activation did not alter the co-occupancy of both Myc and P-TEFb at the E-box promoter region, while a strong inhibition of Ser2-CTD was seen in the coding region of both Myc target genes. Conversely, DRB treatment did not change Ser-5 CTD phosphorylation by TFIIH at the E-box, thus phosphorylation of Ser-2 CTD by CDK9 kinase appeared to represent an important limiting step for transcription of Myc-target genes.

However, the contribution of other kinases to the inhibition of Myc-responsive genes cannot be strictly excluded. High levels of Myc have been shown to strongly stimulate genome-wide RNAPII Ser-2 and Ser-5 phosphorylation, and enhance mRNA cap methylation on target mRNAs,

even in the absence of the Myc DNA-binding domain. It has been shown that c-Myc can also bind to CDK7. These data highlight the strong interaction between Myc and CTD kinases and their effect on growth proliferation (Cowling and Cole 2007).

P-TEFb is a multi-tasking complex (Brès et al., 2008) because influences multiple steps in gene expression, from transcription elongation and co-transcriptional control of mRNA processing (splicing) and export through the CTD (Brès et al., 2005; Yoh et al., 2007), to mRNA translation in the cytoplasm (Rother et el., 2007).

Recently, it has been shown (Zippo et al., 2007) that c-Myc binds the Pim1 kinase through the MBII domain and recruits Pim1 to direct H3S10P at a site upstream of the c-FosL1 and ID2 target genes. Phosphorylation of H3S10 by JIL-1 kinase has been reported to be in Drosophila a prerequisite for recruitment of P-TEFb to heat shock genes (Ivaldi et al., 2007). Depletion of Pim1 blocks transcription as well as CTD Ser2P at c-FosL1 and ID2 genes (Zippo et al., 2007). Thus, H3S10P seems to be a necessary step for P-TEFb loading (**Figure 39**).

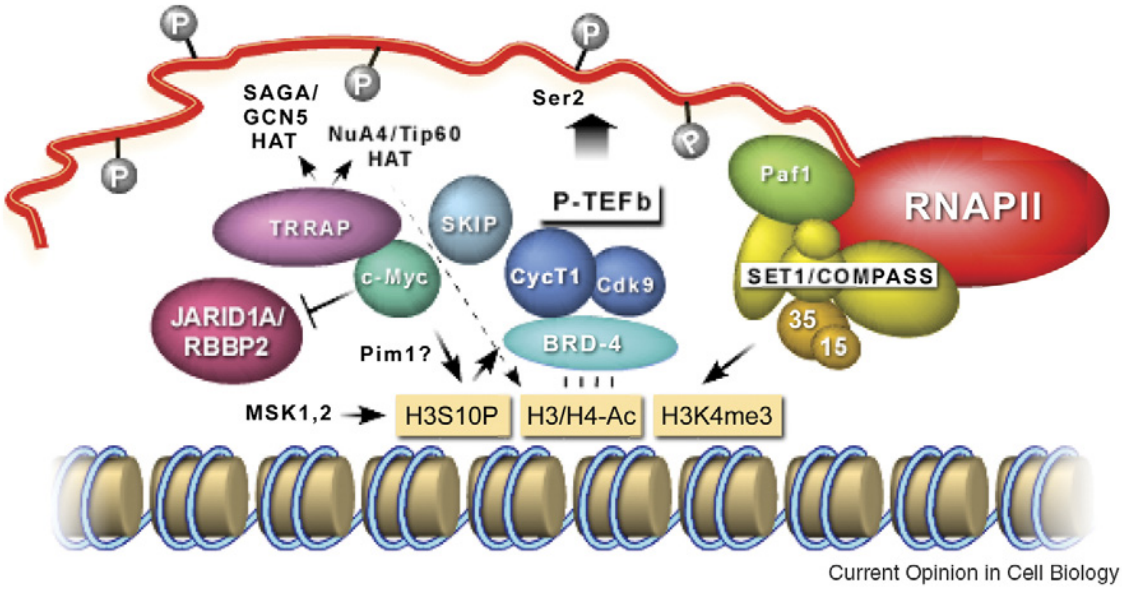

Figure 39. **Transcription factors implicated in P-TEFb recruitment and function**. Activators may recruit P-TEFb directly as c-Myc, or indirectly through binding to Brd4. P-TEFb recruitment is also linked to H3S10P, which can be mediated by Pim1, which can be recruited through c-Myc:TRRAP

complexes. TRRAP is a frequent target of DNA activators and associates with HAT complexes that acetylate chromatin and stabilize binding of Brd4. P-TEFb functionally cooperates with proteins like SKIP to activate transcription and RNAPII CTD phosphorylation links elongation with downstream events required for gene expression.

Collectively these studies together with the demonstration of P-TEFb / histone H2A:2B association in yeast (Wyce et al., 2007), propose the possibility that P-TEFb might influence nucleosome assembly or chromatin structure during elongation.

c-Myc also associates with highly modified chromatin and is linked to H3 acetylation and H3K4me3 and H3K79me3 (Guccione et al., 2006).

Then, these data strongly indicate that Myc transactivation involves additional mechanisms that influence the structure and dynamic of the elongating polymerase without to exclude mechanisms that involve modulation of the chromatin context surrounding the Myc-responsive genes.

4.2 p14ARF negatively regulates c- and N-Myc mediated transcriptional control

Myc is the first oncogene identified to regulate ARF tumor suppressor functions. Overexpression of Myc in B-lymphocytes augments cell proliferation which is counteracted by the ARF-p53-Mdm2 axis. Inhibition of this axis suppresses Myc-induced apoptosis and facilitates B cell lymphoma formation. These findings indicate that Myc-induced cell growth and proliferation is balanced by simultaneous activation of p53 via ARF. However, several groups have argued that ARF functions independently of p53 in physically binding to E2F1 and MYC and attenuating their transcriptional activity (Eymin et al., 2001; Qi et al., 2004; Datta et al., 2004). In both wild type and p53-null MEFs in which MYC expression was enforced, MYC binding re-localized ARF from the

nucleolus to the nucleoplasm, whereas in other cell types (U2OS cells) ARF was found to import MYC into nucleoli (Qi et al., 2004; Datta et al., 2004). However, more striking were observations that p19ARF could associate with MYC on chromatin, antagonizing the transactivation of selected MYC target genes as, eIF4E, nucleolin, TERT, Cdk4 and Cul1, without impairing Myc transrepression of of GADD45 and INK4B genes (Gregory et al., 2005).

p19ARF and p14ARF show limited sequence homology at the levels of both cDNA and protein. p19ARF is a protein of 169 a.a., while p14ARF of 132 a.a. p19ARF is induced during Ras-mediated senescence, while p14ARF is not.

These differences indicate that the data observed between p19ARF and Myc need to be experimentally validated for p14ARF.

The data that I have obtained, have demonstrated that the human p14ARF interacts with c-Myc*:* through *in vitro* pull down assays and with *in vivo* CoIP, I have also shown that the Myc Box II domain is critical for the interaction with p14ARF.

Moreover, I have demonstrated that another member of Myc family, N-Myc, is able to bind p14ARF and the Myc Box III is the domain through which N-Myc contacts p14ARF.

Although c-Myc and N-Myc share a high degree of functional redundancy, they have strikingly distinct patterns of gene expression. Whereas c-Myc is expressed during embryonic development and in adult tissues, N-Myc is expressed almost exclusively in embryonic tissues.

It is pertinent to note that my studies demonstrate that c-Myc and N-Myc interact with p14ARF through different conserved domains. The Myc Box II and the Myc Box III are indispensable for many aspects of Myc functions among which also their transcriptional activity (Frank et al., 2003 and Herbst et al., 2005). The immunofluorescence data obtained also

underlie and give relevance to the involvement of MBII and MBIII domains in the physiological interaction between ARF and Myc proteins. Moreover I have demonstrated that p14ARF inhibits c- and N-Myc transcriptional activation.

There are several ways that p14ARF binding to Myc might inhibit its transactivating functions. One mechanism might involve ARF-induced sumoylation of Myc containing complexes or of neighboring histones (Shiio et al., 2003). As show in **Figure 40A** another potential mode of regulation might be mediated by ARF-BP1 (also known as HECTH9), a HECT containing E3 ubiquitin ligase with which ARF directly interacts (Chen et al., 2005). ARF-BP1 catalyses the lysine-63-linked polyubiquitylation of Myc, a process that facilitates the recruitment of co-activators and the upregulation of Myc target genes (Adhikary et al., 2005). By contrast, the Myc transrepressing cofactor Miz1 antagonizes this modification (**Figure 40B**). ARF strongly inhibits the ubiquitin ligase activity of ARF-BP1, which might contribute to the selective dampening of Myc transactivating activity by ARF.

Gels retardation experiments have excluded the hypothesis that the dampening effects of p19ARF on Myc-regulated transcription may result from interference with Myc binding to its heterodimerization partner Max, or from interference with Myc/Max heterodimer binding to E-box. Moreover in the laboratory it has been demonstrated that p14ARF does not possess an intrinsic repression domain.

Then ARF might inhibit Myc's functions interfering with the binding to co-factors as the histone acetyl transferase TIP60 or P-TEFb (**Figure 40C**). A large number of evidences have demonstrated that Myc Box II is required for activation and repression of most target genes (Adnikary et al., 2005) and in addition to ARF, other proteins can bind directly to this region: the TRRAP, a core subunit of the TIP60 and GCN5 histone acetyltransferase

complex (HAT) (McMahon et al., 1998) and the ATPases TIP48 and TIP49 found in chromatin remodelling complexes (Frank et al., 2001).

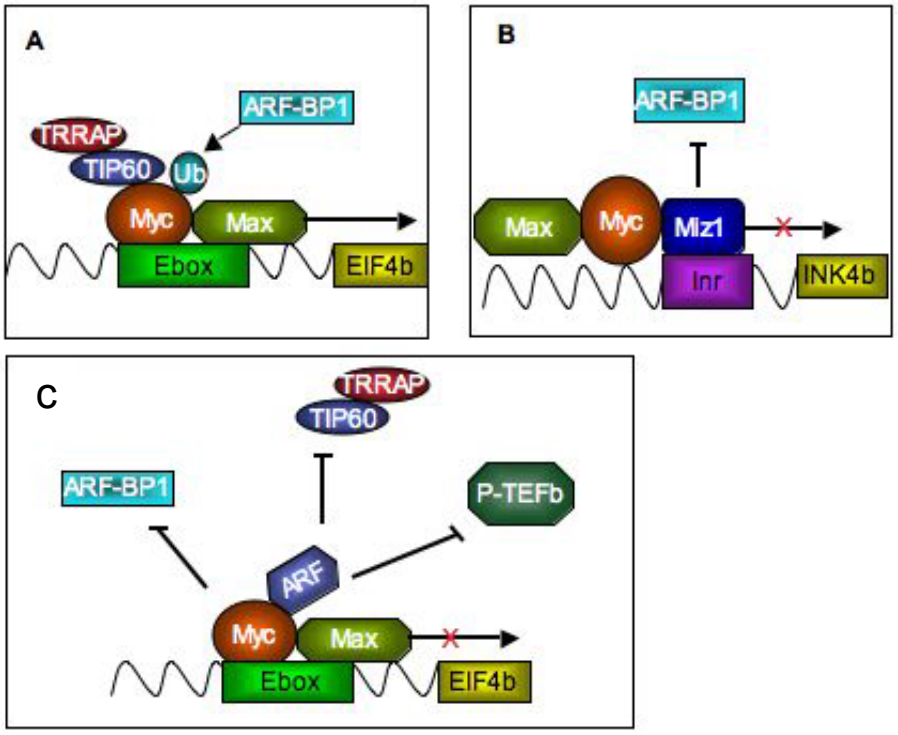

Figure 40. Putative molecular mechanisms by which ARF might repress Myc activity. (**A**) The Myc–Max heterodimer binds to E-box (CACGTG) consensus sequences to activate transcription. Activation depends on the recruitment of cofactors such as TRRAP, TIP60 and on Myc ubiquitylation (Ub) by ARF-BP1. (**B**) Myc–Max complexes can also repress transcription by interacting at initiating elements (Inr) with the zinc-finger protein Miz1. Among its activities, Miz1 opposes the activity of ARF-BP1. (**C**) Transcriptional activation and antagonism both depend upon Myc binding to CACGTG elements, thereby affecting a subset of Myc target genes, which include EIF4E (shown), nucleolin, telomerase reverse transcriptase, cyclin-dependent kinase 4 and cullin 1.

4.3 p14ARF antagonizes HIV-1 Tat protein functions

Another line of research in the laboratory investigates since several years mechanisms that involved the transcription activation of HIV-1 proviral DNA by RNAPII. This mechamisms are controlled primarily at the level of transcription elongation by the viral Tat protein (Barboric and Peterlin 2005). The P-TEFb elongation complex was originally identified as a direct binding partner of the HIV-1 Tat protein, and Tat and Cyc T1 cooperate to

recruit P-TEFb to the viral 5' TAR RNA (Price 2000; Saunders et al., 2006).

Moreover recently it has been demonstrated that ARF is a unexpected sensor of the viral infections, and in regard to this considerations I have supposed whether ARF could be able to negatively interfere with HIV-1 Tat- mediated transcription.

The tumor suppressor p14ARF, by antagonizing the E3 ubiquitin ligase Hdm2 activity, is known to inhibit cell-cycle progression and to stabilize p53 transcriptional activity. The proto-oncoprotein Hdm2 is also known to interact with HIV-1 Tat protein and mediates its ubiquitination *in vivo* and *in vitro* (Bres et al., 2003). Hdm2 is a positive regulator of Tat-mediated transactivation, indicating that the transcriptional properties of Tat are stimulated by ubiquitination (post-translate modification).

Since Hdm2 is negatively regulated by p14ARF, I wished to determine whether p14ARF could affect Tat transactivation of the HIV-1 promoter interfering with Hdm2-mediated ubiquitination of Tat.

The data I have obtained demonstrated that p14ARF enhanced expression inhibits Tat transactivation of the HIV-1 LTR promoter in transient transfections and that the N-terminus of p14ARF is required for ARF-mediated inhibition. I observed such effects in different cell lines that express or do not express the p53 factor, suggesting that the repression of Tat transactivation is p53-independent.

Moreover I have demonstrated that HIV-1 Tat protein levels are reduced in the presence of p14ARF in a proteasome-dependent manner and the induction of degradation is independent on the ubiquitin state of the Tat protein. Tat protein is quite stable and co-expression of p14ARF induces Tat protein half-life decrease.

Furthermore it has been shown in the laboratory that Tat induces a redistribution of ARF in a lower molecular weight complex, and that Tat can interact with ARF in the same complex.

ARF-mediated repression of Tat protein could occur by sumoylation mechanism. It has been shown that ARF-induced sumoylation for some ARF-interacting proteins as WRN helicase, Hdm2, E2F-1, HIF-1a TBP-1, p120E4F (Rizos et al., 2005; Tago et al., 2005). I have carried out other experiments that indicate to exclude this hypothesis because overexpression of Gam1 vector, which blocks ARF-induced sumoylation, had no overt effect on the ability of ARF to repress Tat.

Another mechanism by which p14ARF could counteract HIV-1 Tat protein functions could involve a change in Tat sub-cellular localization. Several studies in fact have shown that ARF induces nucleolar re-localization of some of its binding partners, but in my findings I did not observe any significant difference in Tat sub-cellular localization upon p14ARF overexpression.

Recent studies have shown that p14ARF induces proteasomal degradation in both p53-dependent and independent manner (Eymin et al., 2006; Rizos et al., 2007). My data clearly indicate that ARF is capable of inducing a proteasome-dependent degradation of Tat protein.

The first evidence of a link between ARF and the proteasome was the observation that both human and mouse ARF were accumulated following treatment with proteasome inhibitors, suggesting that ARF degradation depends, at least in part, by the proteasome (Kuo et al., 2004).

A very recent report describes a direct involvement of the REG-γ proteasome in an ubiquitin-independent regulation of the ARF turnover (Chen et al., 2007; Li et al., 2007).

REG-γ pathway plays a role in the control of viral pathogenesis and this is particularly interesting, given that ARF activation has been linked to viral

response (Garcia et al., 2006). Interestingly, both ARF and Tat physically interact with REG-γ complex, (Huang et al., 2002), also known as 11S or PA28. Then it is possible that functional interaction between ARF/Tat/REGγ might be responsible for p14ARF- induced degradation of Tat protein.

CHAPTER V

Material and Methods

5.1 Plasmids

pcDNA3-Myc and pcDNA3-CycT1 plasmids were already available in lab. G5-83HIV-Luc and Tat-101 wt plasmids were already available in lab. The insert obtained by GFP-p14ARF was subcloned in pPROEX Hta vector (GIBCO Life Technologies) to give the pHis-ARF vector.

GFP-p14ARF, GST-p14ARF, GST-Max, His-Max, pcDNA3-Max, pHA-Myc-FLAG, pcDNA3-FLAG-Myc, pcDNA3-FLAG-MycΔ123-151, pMT2T-Myc, pcDNA-p14ARF-HA, GST-Myc deletion mutants, pMT2T-Myc, hTERT-Luc were kindly provided by G. La Mantia and R. Dalla Favera.

p3xFLAG-N-Myc was kindly provided by G. Della Valle. pcDNA-N-Myc and his deletion mutant were kindly provided by T. Fotsis.

p3xFLAG-ARF F.L., p3xFLAG-ARF1-65 and p3xFLAG-ARF65-132 were constructed by inserting EcoRI/BglII fragment, obtained by PCR reaction and containing the ARF cDNA (full length, aa 1-65 and aa 65-132, respectively), in pCMV10 vector (Sigma). PCR reactions: the cDNA were performed with PFU TURBO DNA Polymerase (Stratagene).

pTat-Ub and pTatK71R-Ub plasmids were kindly provided by M. Benkirane.

5.2 Cell lines and treatments

Rat cells expressing a 4-hydroxytamoxifen (OHT)-inducible MycER chimera were cultured in DMEM medium supplemented with 10% fetal calf serum. Cells were made quiescent by contact inhibition followed by serum removal for two days. To induce entry into the cell cycle, the synchronized G1 arrested cells were treated with 4-OHT (600 nM) and harvested at the indicated times. Human 293T, SKNBE, U2OS, HL6,

H1299 cell lines were grown in DMEM supplemented with 10% fetal calf serum. Human H358/Tet-On/p14ARF inducible cell line (kindly provided by Dr. M. S. Gazzeri) was cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum. H358-p14ARF inducible cell line was treated for 72 h with o without 1 μg/ml doxycycline (Dox), then the cells were transfected with Lipofectamine (Invitrogen) and 24 h posttransfection, cells were treated with 80 μg/ml of cycloheximide (CX; Sigma) and harvested at the indicated times thereafter. Proteasome inhibition was achieved by treating the cells with 20 μM MG132 for 2 hr.

5.3 Luciferase assays and immunofluorescence

For the luciferase assay, the cells were transfected with lipofectamine or lipofectamine 2000 (Invitrogen Technologies) and pRLCMV (Promega) was co-transfected for normalization.

After 48 hrs from transfection the cells were lysed and assayed for activity of firefly or Renilla luciferase by measure with the dual luciferase assay kit (Promega) according to the manufacturer's instructions and using a T20/20 luminometer (Turner Design). Plasmids used in transient transfections: hTERT-Luc, pMT2T-Myc, pcDNA-p14ARF-HA, G5-83HIV-Luc vector containing the HIV-1 LTR sequences from -83 to +85, Tat-101 wt, F:p14ARF, F:p14ARF(1–65), F:p14ARF (65–132), pTatWt-Ub and pTatk71R-Ub, and pCMVHdm2.

For immunofluorescence analysis U2OS and SKNBE cells were transfected with lipofectamine 2000 with 200ng of the pcDNA3-FLAG-Myc, pcDNA3-FLAG-MycΔ123-151, GFP-ARF, pcDNA-N-Myc and pcDNA-N-MycΔ1-300 plasmids and the cells processed as described in Napolitano et al., 2003 using anti-Myc (9E10, Santa Cruz Biotechnology, Inc.) antibody.

5.4 In vitro proteins binding assays

BL21 bacterial cells were transformed with prokaryotic expression vector carrying the cDNA of the protein of our interest.

Bacterial cells were lysed in PBS 1X Buffer with 1mM PMSF and protease inhibitors and subsequently sonicated. The lysates were centrifuged and recombinant proteins were affinity purified: the GST-fusions (GST; GST-ARF; GST-Max; GST-Myc 1-42; GST-Myc 1-103; GST-Myc 1-143; GST-Myc 1-228; GST-Myc 151- 340; GST-Myc 262-349) were purified using glutathione-sepharose (Amersham Biosciences) and subsequently eluted from the beads by 20mM glutathione incubation. His-Max and His-ARF proteins were affinity purified by using Ni-NTA Agarose (Invitrogen life technologies) and subsequently eluted in Buffer C (20mM Tris-HCl; 100mM KCl; 5mM 2-mercaptoethanol, 10% glycerol, 100mM imidazole). The HA-Myc-FLAG protein was double purified in two steps. For the individual experiments 600ng of each recombinant protein were incubated in a final volume of 1 ml of Binding Buffer (50mM Tris-HCl pH7,4; 150-500mM NaCl; 1mM MgCl2; 1mM DTT; 0,2% NP40). After extensive washing in Binding Buffer, the bound proteins were eluted by 2X Laemli buffer, separated on SDS PAGE followed by Western Blotting.

5.5 Antibodies and co-immunoprecipitations

The following antibodies were used for the immunological techniques: anti-Myc (N262 for IP and 9E10 for WB, Santa Cruz Biotechnology), anti-Max (C17, Santa Cruz Biotechnology), anti-ARF (C-18, Santa Cruz Biotechnology), anti-FLAG M2 Monoclonal Antibody-Peroxidase Conjugate (Sigma), anti-GST (B-14, Santa Cruz Biotechnology), 6xHis Monoclonal Antibody (BD Biosciences), anti-GST (B-14, Santa Cruz Biotechnology), anti N-Myc (2,Santa Cruz Biotechnology), anti CycT1 (H245 for immunoprecipitation, C-20, T18 and N19 for WB, Santa Cruz

Biotechnology), anti-HEXIM1, and anti-CDK9 (H-169), anti-actin (I-19, Santa Cruz Biotechnology), anti-HDM2 (Calbiochem) and HIV-1 Tat Antiserum (NIH AIDS Research).

Co-immunoprecipitations from transiently transfected cells were so carried: each mg of protein extract was incubated O.N. at 4°C with 2-5 μg of specific antibody for the protein of interest. The day after, the antibodies were immunoprecipitated by incubating the supernatants with protein G Sepharose 4 fast flow for 2 h at 4°C. The beads were washed 5 times for 5 min each at 4°C using buffer F (10mM Tris-HCl pH7.5, 150mM NaCl, 30mM Na4O7P2, 50mM NaF, 5μM ZnCl2, 0.1mM Na3VO4, 1% Triton, 0.1mM PMSF) before loading on SDS-PAGE.

5.6 FACS analysis

Rat1-Myc-ER cells were trypsinised, collected by centrifugation and washed in phosphate-buffered saline (PBS). Cells were resuspended in hypotonic solution 0.1% Na-Citrate, 50 μg/ml propidium iodide, 6.25 μg/ml RNAse, and 0.00125% Nonidet P40 (Sigma Chemical Co), incubated in absence of light for 30-60 at room temperature. Cell cycle data acquisition and analyses were performed on a Becton Dickinson flow cytometer using CellQuest Pro and ModFit 3.0 software.

5.7 mRNA measurement by quantitative Real-Time PCR

Total RNA was isolated from cells using TRIZOL reagent according to the manufacturer's instructions (Invitrogen). The RNAs were treated with Dnase I (Invitrogen) and 2 μg of total RNA was reverse transcribed with 100U Super Script II Rnase H- Reverse Transcriptase (Invitrogen) in a volume of 40 μl, using 100 μM random hexamer primers (Roche) according to the manufacturer's instructions (Invitrogen). cDNA was diluted 1:3 prior use in quantitative PCR (qPCR). Quantitative analysis was

performed by using the AbiPrism 7500 sequence detector system (Perkin-Elmer Applied Biosystems). The PCR reactions were performed in a final volume of 15 µl using 1 µl of cDNA, 5 pmol of each primer and 7.5 µl of SYBR GREEN 2× PCR Master Mix (Applied Biosystems). Each sample was run in triplicate. PCR cycling profile consisted in 50°C for 2 min, 95°C for 10 min and 40 two-step cycles at 95°C for 15 s and at 60°C for 1 min. Quantitative real time PCR analysis was carried out using the 2(-Delta Delta C(T)) method (2^{-Ct}) (Livak and Schmittgen 2001). In all qPCR experiments the data were normalized to the expression of housekeeping beta-glucuronidase (GUS) and 18S RNA genes.

5.8 ChIP-re-ChIP analysis

Rat-MycER cells were serum starved for two days and treated with 4-OHT for the indicated hrs. After PBS wash, cells were cross-linked with a 1% formaldehyde/PBS solution for 10 min at room temperature. Cross-linking was stopped by adding glycine and incubating for 5 min at room temperature on a rocking platform. The medium was removed and the cells were washed twice with ice-cold phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4 and 8.1 mM Na2HPO4.2H2O). The cells were collected by scraping in ice-cold PBS supplemented with a protease inhibitor cocktail (Sigma). After centrifugation the cell pellets were resuspended in lyses buffer [1% SDS, 10 mM EDTA, protease inhibitors and 50 mM Tris–HCl (pH 8.1)] and the lysates were sonicated to result in DNA fragments of 300 to 600 bp in length. Cellular debris was removed by centrifugation and the lysates were diluted 1:10 in ChIP dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM NaCl, protease inhibitors and 16.7 mM Tris–HCl (pH 8.1)]. Non-specific background was removed by incubating the chromatin resuspension with a salmon sperm DNA/protein A-agarose slurry (Upstate Biotechnology, Lake

Placid, NY, USA) for 5 h at 4 C with agitation. The samples were centrifuged and the recovered chromatin solutions were incubated with 8 μg of indicated antibodies overnight at 4 C with rotation. The antibodies against c-Myc (N262), CycT1 (T18, T20 and H245) were obtained from Santa Cruz Biotechnologies. The immuno-complexes were collected with 60 μl of protein A-agarose slurry (Upstate Biotechnology) for 1 h at 4 C with rotation. The beads were pelleted by centrifugation at 4 C and washed sequentially for 5 min by rotation with 1 ml of the following buffers: low salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl and 20 mM Tris–HCl (pH 8.1)], high salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl and 20 mM Tris–HCl (pH 8.1)] and LiCl wash buffer [0.25 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA and 10 mM Tris–HCl (pH 8.1)]. Finally, the beads were washed twice with 1 ml TE buffer [1 mM EDTA and 10 mM Tris–HCl (pH 8.0)]. For re-ChIP the immunocomplexes were eluted by adding 100 μl re-ChIP elution buffer (10 mM DTT) at room temperature for 30 min with rotation, the supernatant was diluted 1:20 in ChIP dilution buffer and the antibody against the second protein of interest was added, the new immuno-complexes were allowed to form by incubating at 4°C overnight on a rocking platform, the immuno-complexes were collected by incubating with 60 μl protein A-agarose slurry at 4 C for 1 h on a rocking platform and finally washed as indicated above. In both cases the immuno-complexes were then eluted by adding 500 μl elution buffer (1% SDS and 100mM NaHCO3) and incubation for 15 min at room temperature with rotation. After centrifugation, the supernatant was collected and the cross-linking was reversed by adding NaCl to final concentration of 200 mM and incubating overnight at 65°C. The remaining proteins were digested by adding proteinase K (final concentration 40 μg/ml) and incubation for 2 h

at 55 C. The DNA was recovered by phenol/chloroform/isoamyl alcohol (25/24/1) extractions and precipitated with 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2 vol of ethanol using glycogen as a carrier.

Immunoprecipitated DNA was analyzed by PCR using sets of primers against regions of NUC encompassing E-Box (+574) and coding region (+1500) and CAD E-Box and coding region (+3258). The ACHR promoter ampicon was used as negative control in all experiments. PCR products were analyzed by semiquantitative and quantitative Real-Time PCR.

CHAPTER VI

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Appendix

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Publications

• p14ARF directly interacts with Myc through the Myc BoxII domain.
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† These authors contributed equally to this work.