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"Physical and Functional interaction between Myc and ARF"

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Background

Myc overview.

The c-myc proto-oncogene is a senior administrator of the cell helping to allocate resources and direct proliferation, growth, differentiation and apoptosis. As an oncogene, Myc is a high-ranking member of the gang of molecular thugs that hijacks cells and perpetrates cellular terror.

In 1911 Peyton Rous observed that chicken sarcoma could be transmitted through cell-free extracts from tumor, suggesting that a virus could be the etiologic agent of these sarcomas. On the basis of the work by Bishop and coworkers, studies of a specific subgroup of avian retrovirus, witch induces myeloid leukemia, sarcomas, liver, kidney and other tumor in chicken, led to the identification of the v-myc oncogene. The c-myc gene is the its cellular homolog.

For 25 years, from its discovered, 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 tumor cells.

Since v-Myc was a nuclear protein (Eisenman 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 (Ihle at al., 1987; Cole et al., 1989) 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 (Baltimore et al., 1989; McKnight et al., 1988).

Myc became firmly established as a transcription factor with the discovery of Max. Max can homodimerize and bind to DNA directly, whereas Myc cannot homodimerize and must form a heterodimer with Max in order to bind the specific DNA sequence CACGTG (the E-box) (Eisenman et al., 1991). Max is a small, ubiquitously expressed protein that can bind to a whole collection of B-HLH-LZ proteins (Cleveland 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; Land at al., 1993). Max also forms etherodimers with the bHLH-Zip proteins Mad1, Mxi-1 (or Mad2), Mad3, Mad4 and Mnt. These alternative dimers bind the E-box and actively repress transcription and therefore antagonize both the transcriptional and transforming activities of Myc (**Figure 1**) (Eisenman at al., 1993). In addition myc and mad genes are generally regulated in opposite modes in growth control and development (Luscher at al., 1996).

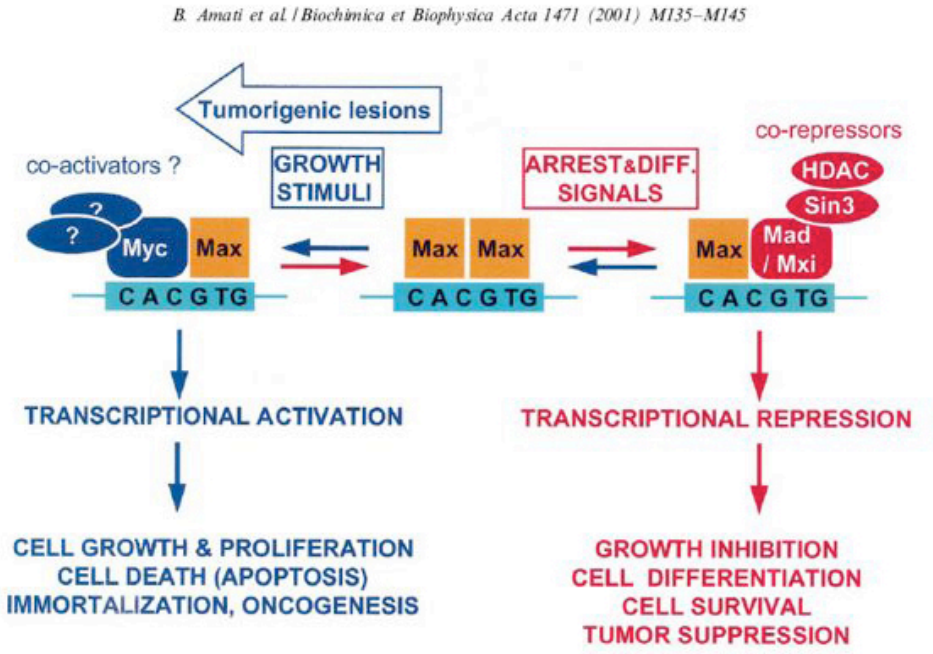


Figure 1. Transcription-competent Myc/Max dimers are the active form of Myc in inducing cell-cycle progression, apoptosis and malignant transformation**.** Max also forms etherodimers with the bHLH-Zip proteins Mad1, Mxi-1 (or Mad2), Mad3, Mad4 and Mnt. These alternative dimers bind the E-box and actively repress transcription and therefore antagonize both the transcriptional and transforming activities of Myc.

Myc family members.

The Myc family members, c-Myc, N-Myc, L-Myc and S-Myc, all function as oncogenes in different tumors and have a high degree of sequence conservation (Cole, 1986). 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 (Cole at al., 2002). Furthermore, their coding regions can substitute for each other in mouse development (Alt at 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.

Myc regulation.

c-Myc activity is normally tightly controlled, at transcription level, by external signals including growth factors, mitogens and β-catenin, which promote and factors such as TGF-β, which inhibit. Its expression also undergoes a negative autoregulatory circuit (Grignani et al., 1990; Lombardi et al., 1990; Penn et al., 1990): expression of one c-Myc allele leads to the downregulation of the other. The normal c-*myc* allele is usually transcriptionally silent in Burkitt's lymphomas (ar-Rushdi *et al*., 1983; Cory, 1986), and thus the only Myc protein in most Burkitt's cells is derived from the translocated c-*myc* allele; as well mice constitutively expressing a transgenic allele, downregulate the endogenous gene. This autoregulation is active in normal and immortalized cells derived from multiple tissues, but is inactivated in fully transformed tumor cells (Grignani et al., 1990).

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 2A**). 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.

Considerable evidence suggests that Thr 58 phosphorylation is critical for ensuring the transient and timely degradation of c-Myc. All v-*myc* genes recovered in transforming retroviruses harbour mutation at Thr 58.

Another mechanism by witch Ras regulates Myc activity is through the FOXO family of transcription factors (Bouchard et al. 2004). In their non-phosphorylated state, FOXO factors directly bind to and repress many Myc target genes that are involved in cell proliferation by inhibiting the formation of the preinitiation complex on these genes. Following Ras activation, the PI3K pathway leads to the AKT-dependent phosphorylation of FOXO proteins and their nuclear export mediated by binding to the 14-3-3 protein. Moreover also the DNA-binding domain of Miz1 is a target for phosphorylation by the AKT kinase (Wanzel et al., 2005). After phosphorylation, 14-3-3 PROTEINS bind to Miz1 and inhibit DNA binding and transcriptional activation of $p21^{CIP1}$. As a result, the activation of AKT cooperates with Myc in compromising the ability of Miz1 to inhibit cell proliferation (**Figure 2B**).

These post-translational controls of Myc function partially explain the requirement for Ras in cellular transformation induced by Myc.

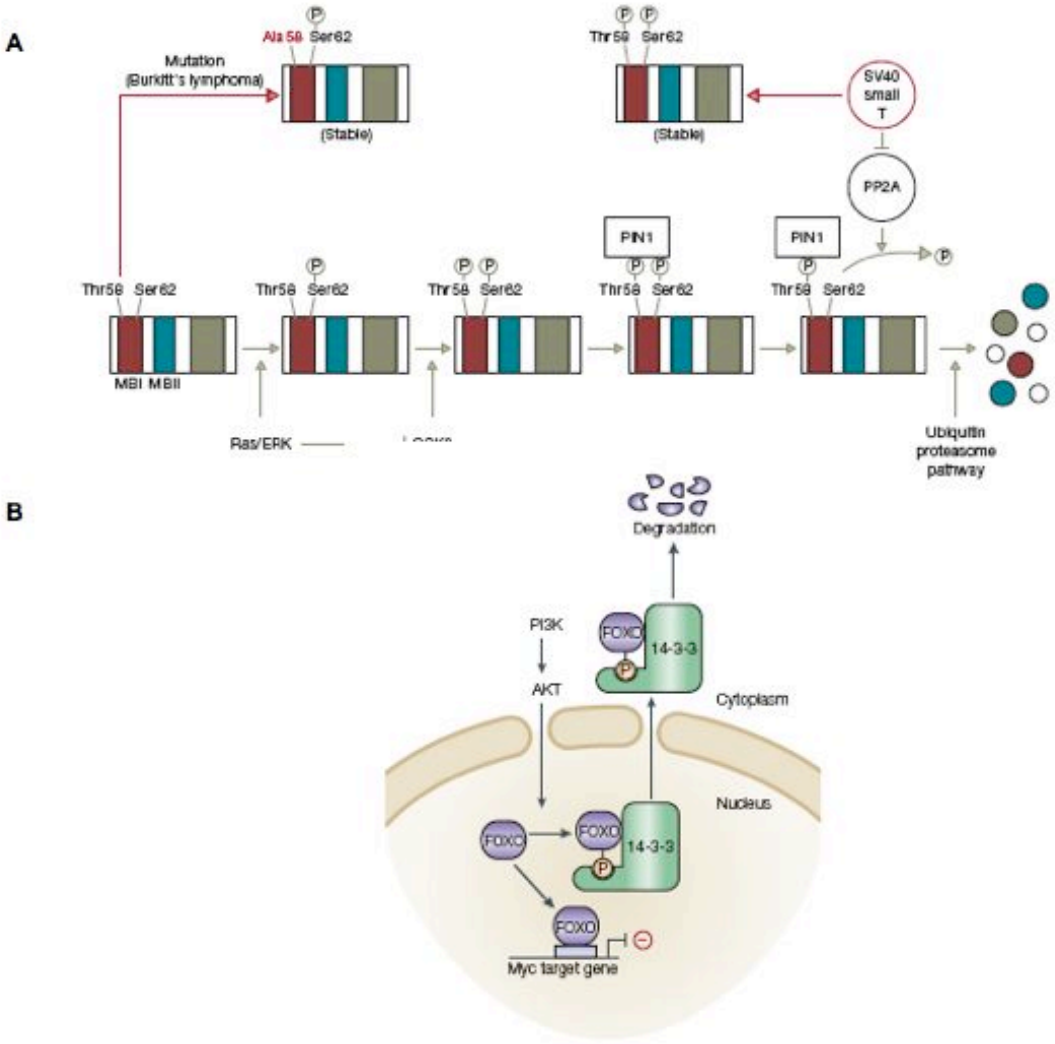


Figure 2. Ras-mediated regulation of Myc. A) Myc is stabilized by Ras-dependent ERK-mediated phosphorylation at Ser62. This modification is necessary for the subsequent phsphorilation at Thr58 that lead to degradation. PIN1 induce conformational modification that allow PP2A to dephosphorilate Ser62 and consequent recognition by the proteasome pathway. c-Myc mutation found in Burkitt's lymphoma of in retroviral v-myc oncogene (top left) and SV40 small T antigen (top right). Directly affect this pathway, resulting in stabilization of the c-Myc protein. MBI: Myc BoxI; MBII: Myc BoxII; in grey: bHLHZip BNA binding domain. **B**) Several target genes are transactivated by Myc are repressed by FOXO transcription factor. Activation of Pi3K pathway leads to the AKT-dependent phosphorylation of FOXO proteins and their nuclear export. Nuclear export is mediated by binding of phpsphorylated FOXO to the 14-3-3 protein in nucleus. In the cytosol, FOXO protein are ubiquitinated by the E3 ubiquitin ligase SCFSKP2 and degraded by the proteasome. ERK: extracellular signal-regulated kinase; MAPK: mitogenactivated protein kinase; Ub: ubiquitin.

Biologically Functional Myc Domains.

Sequence-specific transcription factors are usually modular, with a well-defined,

evolutionarily conserved DNA binding domain and a more loosely defined effector domain that either activates or represses transcription when tethered near a basal promoter. Before Myc was definitively shown to be a DNA binding protein, it was found that fusion of the Myc N-terminus to the Gal4 DNA binding domain created a potent transactivator (>100-fold; Kato et al. 1990). However, this work raised two puzzles that remain unresolved. First, there was not a good correlation between domains within the N-terminus that were biologically important with those that promoted transcriptional activation (Kato et al. 1990). Second, once the Max protein and consensus DNA binding sites were available, it was found that Myc/Max heterodimers were much less potent at transcriptional activation (typically 3- to 4-fold) than Gal4-Myc fusion proteins, even using concatamerized binding sites (Kretzner et al. 1992). The latter, relatively weak activity has now been confirmed in vivo through the analysis of chromosomally localized target genes which have Myc/Max binding sites (Bush et al. 1998). Several broad studies of Myc target genes using microarrays find an average transcriptional activation of chromosomal targets of approximately twofold (Coller et al. 2000; O'Connell et al. 2003). Moreover, only a small number of the Myc responsive genes identified in these studies are verified as in vivo targets, but the average response of verified targets is quite comparable (2- to 3-fold). Hence, while virtually all studies agree that Myc is a direct activator of transcription, its activity is inevitably quite modest and pales in comparison to potent transactivators like NF-κB (100- to 1,000-fold in comparable assays).

It remains a distinct possibility that Myc has transcription independent activity. Mapping of the transactivation domain revealed discordance between biologically significant domains and those required for transactivation (Dang at al., 1990). In addition, it is a mystery why some tumors express Myc at 100-fold over the endogenous level, when a much lower quantity of Myc protein achieve the same transactivation.

Characterization of the Myc protein has revealed several domains that are critical for different activities. The C-terminal basic region/helix-loop-helix/leucine zipper (bHLHZip) domain is central to Myc function since it provides the DNA binding activity. This domain is composed of three different elements, the basic, the helix - loop - helix, and the leucine zipper regions. The bHLHZip domain is characteristic of a class of transcription factors binding to the called E box DNA recognition sequences

with the core motif 5'-CANNTG. The function of this domain is to specify homo- or heterodimerization through the HLHZip region and interaction with DNA through the basic region (**Figure 3**) (Larsson at al., 1999).

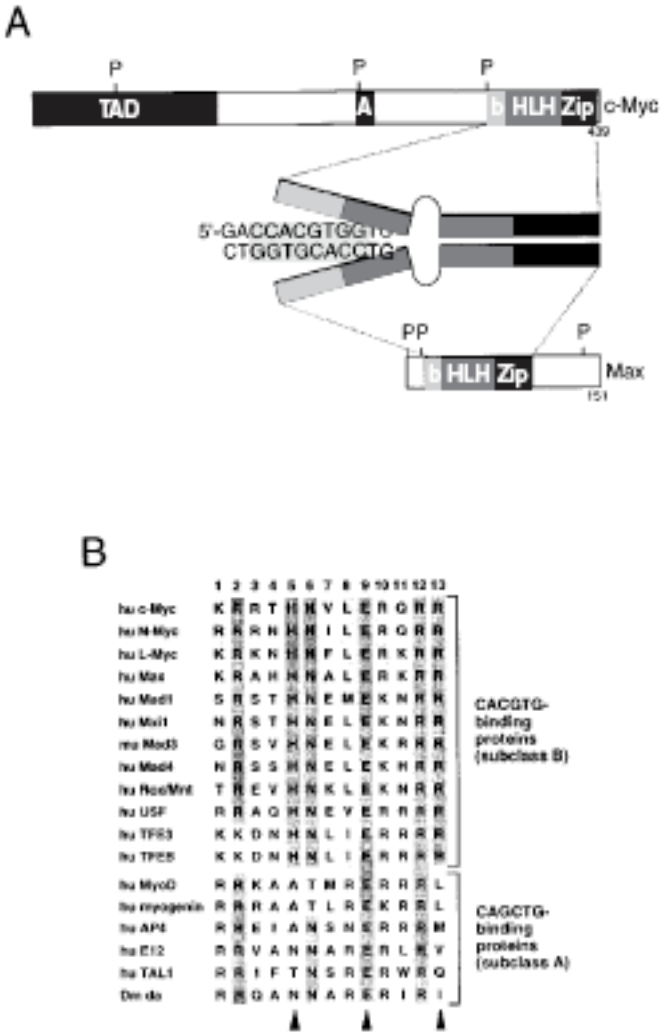
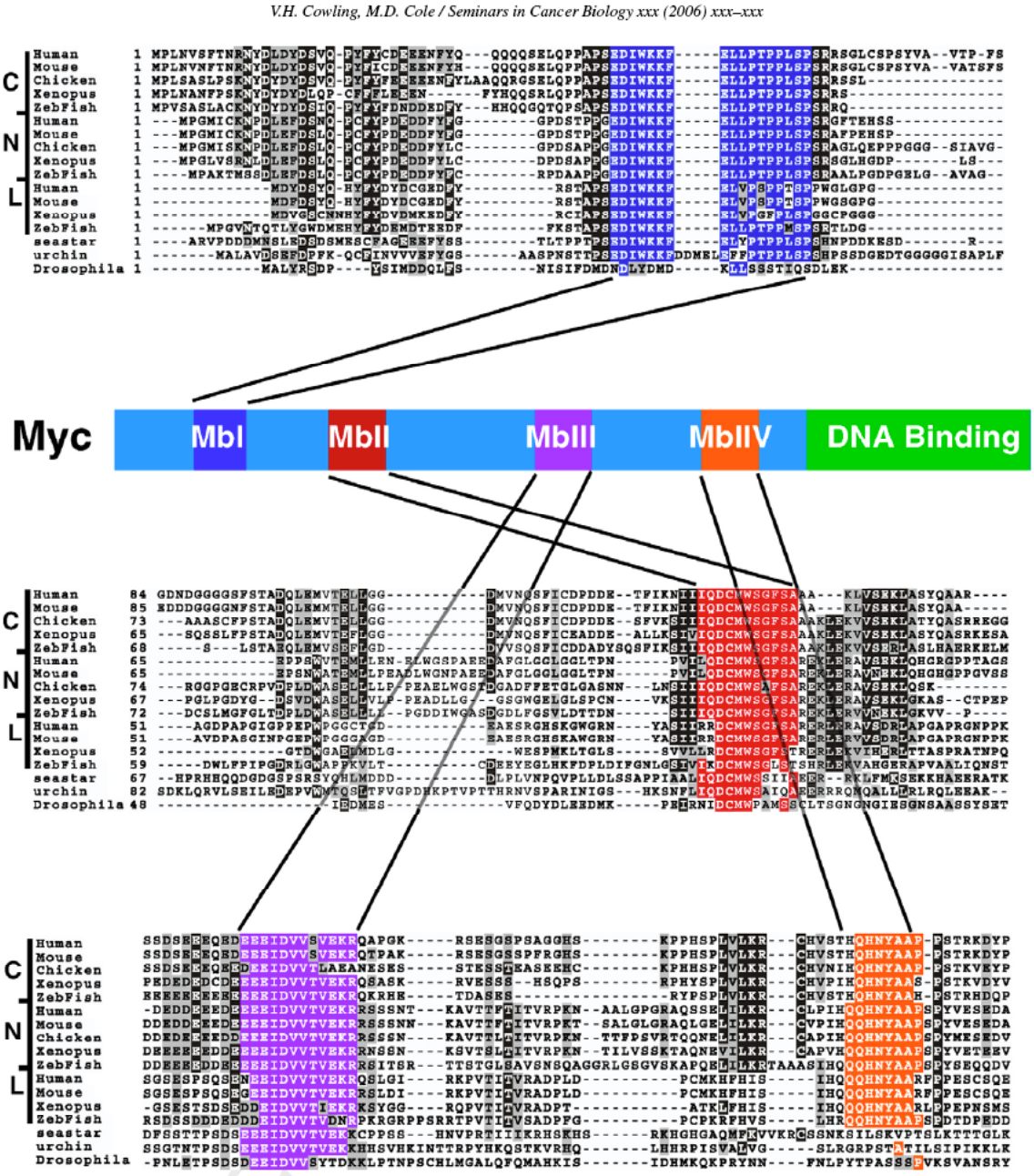


Figure 3. DNA binding by Myc/Max/Mad complexes and comparison of basic regions of E box binding proteins. Schematic representation of a Myc/Max heterodimer. The structural elements in c-Myc and in Max are indicated: transactivation domain (TAD), acidic region (A), basic region (b), helix ± loop ± helix region (HLH), and leucine zipper (Zip). P indicates regions of in vivo phosphorylation. The bHLHZip domain is enlarged to indicate that the basic region and helix 1 as well as helix 2 and the leucine zipper form two a-helices connected by the loop region. The interaction of these four a-helices allows the precise positioning of the basic region to interact with the E box sequence 5'-GACCACGTGGTC. The nbases that are specified by protein-base contacts in the Max/Max-DNA co-crystal are shaded.

(B) Basic regions of E box-binding proteins. Sequences of the basic regions (numbered 1 ± 13) of the indicated E box-binding proteins are displayed. Subclass A and B refers to proteins that preferentially bind to 5'-CAGCTG and 5'-CACGTG E boxes, respectively. Identical and highly conserved amino acids are indicated in dark.

In the N terminus, the prominent evolutionarily conserved domains are called Myc homology boxes (MBs). Of particular note is MBII, which is necessary for full transcriptional activation and repression. Mutation of this domain inhibits the binding of most Myc coactivators and also inhibits most Myc phenotypes (Hay at al., 2000; hancock at al., 1992; Lee at al., 1990; Cole at al., 2002; Penn at al., 2003; Lee at al., 1987). In fact, the MBII deletion phenotype is so severe that MBII may be interpreted to be necessary for the integrity of the N terminus as a whole rather than as a distinct domain. The MBI domain has a more complex phenotype which is dependent on the extent of the mutation. MBI is a site of phosphorylation and is the only consistent site of Myc protein mutation found in some tumors (Magrath at al., 1993; Hann at al., 1994). MBI mutations can affect Myc protein turnover (Hann at al., 2000; Nevins at al., 2000), and larger deletions indicate that this domain is necessary for full activity in transformation assays (Tansey at al., 2003; Lee at al., 1987). Early studies showed that deletion of MBI was not necessary for transactivation (Dang at al., 1990), and this has been confirmed for endogenous target genes in subsequent studies (Penn at al., 2003). Why MBI is necessary for cell transformation but not transcription remains an unsolved mystery of Myc biology, and contributes to the notion that Myc may have transcription-independent functions. Recent work has defined a third region of the Myc N terminus, MBIII, as a mediator of apoptosis, transformation, and tumorigenesis (Tansey at al., 2005). More recent work has characterized a domain conserved in c-, N-, and L-Myc from fish to humans (designed MBIV) necessary for full Myc transforming activity and apoptosis. Deletion of MBIV potentiates Myc-induced G2 arrest (**Figure 4**) (Cole at al., 2006). Moreover, deletion of MBIII or MBIV reduces Myc-dependent transactivation, although by not nearly as much as deletion of MBII (Cole at al., 2006).

Figure 4. Alignment showing conserved sequences of the Myc transactivation domain. Neither MbI or MbIV are conserved in insects. No specific function has been described for MbIII. The latter segment is not conserved in l-Myc and hence does not fit the criterion for a 'Myc homology box' as conserved among all Myc proteins.



Myc and Chromatin Modification.

The MBII domain has recently been shown to facilitate Myc binding to a novel large nuclear cofactor called TRRAP (*tr*ansactivation/*tr*ansformation-domain *a*ssociated *p*rotein), which 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). TRRAP binding to the N-terminus is directly correlated with

Myc oncogenic activity, since deletions or mutations in Myc that disrupt TRRAP binding are transformation-defective, and the weakly transforming L-Myc protein exhibits poor TRRAP binding (McMahon et al. 1998; Nikiforov et al. 2002). Furthermore, the disruption of endogenous TRRAP pools, by using antisense and ectopic expression of TRRAP fragments with dominant inhibitory activity, severely impairs Myc-mediated oncogenic transformation (McMahon et al. 1998). These data imply that the recruitment of TRRAP to cellular promoters is essential for Myc-mediated oncogenic transformation. 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 (*S*PT/*A*DA/*G*CN5/*a*cetyltransferase) 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). Myc binds directly to a small internal domain of TRRAP that is similar in location to the binding site on Tra1p for transcription factors in yeast (Brown et al. 2001; Park et al. 2001). Several recent studies have demonstrated that, in addition to TRRAP, many other components of the SAGA complex are also highly conserved from yeast to humans (Martinez et al. 2001;Ogryzko et al. 1998; Smith et al. 1998). 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. This model is appealing since the alternate Max heterodimeric partners, Mad and Mxi, can antagonize Myc function through recruitment of Sin3A/Sin3B and the histone deacetylases HDAC1/2 (Ayer 1999; Knoepfler and Eisenman 1999). A series of studies has found several other complexes that contain TRRAP in addition to the human ortholog of the yeast SAGA complex. The SAGA complex is related to the STAGA and TFTC coactivator complex defined in transcription assays (Brand et al. 1999; Martinez et al. 2001).

TRRAP is also found in a complex with the TIP60 H2A/H4 HAT (Ikura et al. 2000), and this complex shares many subunits with a complex containing the Swi2/Snf2-related p400 protein (Fuchs et al. 2001). However, the latter complex lacks HAT

activity. The p400 complex binds to c-Myc in U2OS extracts, presumably through TRRAP, but the functional consequences of this recruitment remain unclear. Alternately, it is possible that TRRAP-containing complexes may directly repress target gene expression, even though histone acetylation is usually linked to gene activation. In support of this concept, the ARG1 gene in yeast was shown be repressed through recruitment of GCN5 HAT activity and the SAGA complex (Ricci et al. 2002). Mutants of GCN5 that lack HAT activity fail to repress ARG1, and the SAGA complex is recruited directly to ARG1 through the ArgR/Mcm1 repressor complex. It is not clear if it is the acetylation of histones or some other substrate that mediates repression, but the direct involvement of TRRAP/TRA1-linked HAT activity to repression could explain the MBII-dependence of Myc repression (Claassen and Hann 1999). A general model of Myc-mediated histone acetylation as the basis of oncogenic transformation raises a number of important questions that must be resolved in the future. On one hand, Myc recruits HAT activity (McMahon et al. 2000), and TRRAP is recruited to the promoters of several Myc-responsive genes following serum stimulation in association with induction of H4 but not always H3 acetylation (Bouchard et al. 2001; Frank et al. 2001). It was recently shown that the silent TERT gene acquires both H3 and H4 acetylation in the course of being activated by c-Myc in primary human fibroblasts (Nikiforov et al. 2002). On the other hand, another study reported that activation of Myc target genes *cad* and *tert* in cell lines occurs without concomitant increases in histone H3 or H4 acetylation (Eberhardy et al. 2000). Furthermore, MBII mutants that fail to recruit HAT activity can still induce several Myc target genes in their native chromosomal context (Nikiforov et al. 2002). The MycS protein does not bind to TRRAP, but it can rescue the growth defect in *myc*-null fibroblasts (McMahon et al. 1998; Xiao et al. 1998). This suggests that MycS may interact with other cofactors to mediate cell cycle progression, anchorage-independent growth, and apoptosis. Finally, although the TIP60 H4 HAT is recruited to c-Myc target genes and promotes localized histone acetylation, abolishing TIP60 HAT activity had no impact on Myc target gene expression (Frank et al. 2003). Thus, it remains possible that localized HAT recruitment may well correlate with Myc target gene activation because this activity is recruited as part of TRRAP complexes. However, HAT activity itself may be dispensable and the critical function recruited by Myc is provided by TRRAP itself or

other associated proteins.

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). An ATPase-defective mutant of TIP49 was a potent inhibitor of Myc oncogenic transformation but had little effect in proliferation (Wood et al. 2000). This same mutation enhanced Myc-mediated apoptosis (Dugan et al. 2002), suggesting that apoptosis and transformation may result from different pathways. The biochemical function of TIP49/48 proteins remains unclear. 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 at al., 1999), suggesting a role for SWI/SNF in transcriptional activation by Myc. It remains unknown whether Myc associates with the whole SWI/SNF complex in vivo, recruits this complex to DNA and induces nucleosome remodeling at target promoters.

Other acetyltransferase activity factors that interacts with Myc (and many other transcription factors) are p300 and CBP (**Figure 5**). 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 at al., 2003; Faiola at al., 2005). Interestingly, Myc is also a substrate for the other TRRAP-associated cofactors with acetyltransferase activity, hGCN5 and TIP60 (Patel at al., 2004). Myc acetylation sites depend from the different acetyltransferases but there is a general agreement that acetylation stabilizes the Myc protein about three-fold in transient cotransfections. However, there is still no evidence that an acetylated, stable pool of Myc protein exists *in vivo* in cells expressing native levels of Myc and these cofactors.

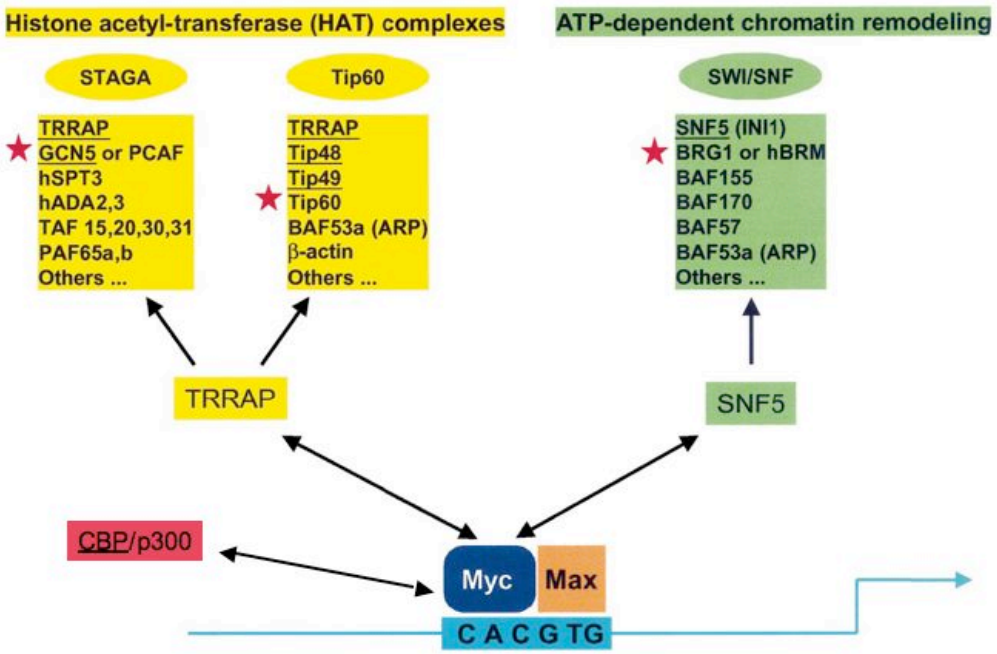


Figure 5. Myc associates with subunits of the chromatin-modifying complexes STAGA, Tip60, SWI/SNF and CBP/p300. The Myc-interacting proteins TRRAP, SNF5 and CBP are part of the indicated complexes. The names of subunits in each complex that were individually shown to interact with Myc are underlined. The catalytic activity of each complex is designed at the top, with each catalytic subunit indicated by a red star.

Transcriptional Elongation and Myc.

Activation of Myc target genes in some cell systems occurs independently of an increase in histone acetylation (Eberhardy at al., 2000). Deletion of MBII inhibits Myc binding to TRRAP and dramatically reduces transcription of most Myc target genes, but some genes can still be activated in response to Myc MBII (Cowling at al., 2006).

Thus, some Myc target genes are TRRAP and/or histone acetylation independent.

Investigation into the HAT independent activation of Myc target genes revealed that RNAPII is engaged but stalled at the promoters of some Myc target genes in the absence of Myc (Eberhardy at al., 2002). Following serum stimulation of fibroblasts or differentiation of U937 cells, while RNAPII binding to the *cad* promoter was not obviously altered despite large changes in Myc binding and *cad* transcription, RNAPII was found at the 3' end of the *cad* gene only when Myc was bound and transcription was active. In the case of the *cad* gene, Myc binding could regulate RNAPII promoter clearance. To corroborate these findings it was found that Myc was

able to bind to both subunits of P-TEFb, Cyclin T1 and CDK9 (Eberhardy at al., 2002). These experimental results support the idea that Myc regulates RNAPII promoter clearance via recruitment of an RNAPII CTD kinase (**Figure 6**). However, evidence for direct Myc recruitment of any RNAPII kinase *in vivo* is still lacking.

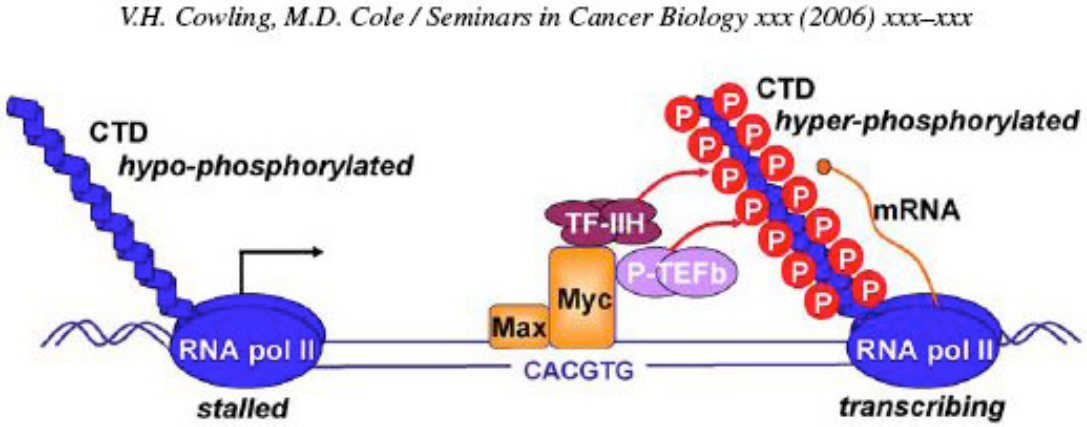


Figure 6. Myc-dependent recruitment of P-TEFb. Model for Myc recruitment of kinase complexes that phosphorylate the carboxy-terminal domain of RNA PII to promote early steps in transcription initiation, elongation and mRNA processing. Myc recruits TFIIH and/or P-TEFb complexes to target genes with stalled RNA polII to promote phosphorylation of the polII carboxy-terminal domain.

Inhibitors of Myc Activity.

The cofactors mentioned in the previously paragraph mediate the activation of Myc target genes. A number of other proteins that interact with Myc have the opposite effect: they inhibit Myc functions and/or block Myc-mediated transactivation.

One example is the cdr2 protein that is normally expressed in cerebellar Purkinje neurons. Cdr2 binds to c-Myc, but do not interact Max, and the binding sequesters c-Myc into the cytoplasm. Cdr2 is an antigen associated with perineoplastic cerebellar degeneration (PCD), a disorder in which the onconeural antigen cdr2 is expressed in breast and ovarian cancers and the anti-cdr2 antibodies promote neural degeneration. PCD antisera block the cdr2-Myc interaction in vitro and could theoretically free Myc to promote unscheduled cell-cycle entry in Purkinje neurons, subsequently leading to cell death. However, only 20% of Purkinje neurons express both c-Myc and cdr2, whereas all Purkinje neurons express cdr2 itself (Okano et al. 1999), making it unclear if targeting of only a subset of neurons could account for the disease.

Two other inhibitors of c-Myc function have also been described. The differentiation

and interferon inducible p202a protein can inhibit c-Myc transcriptional activity and dimerization with Max. p202 is induced in differentiated cells and its overexpression inhibits cell proliferation. p202a is able to interact with (and inhibit) a number of other transcription factors, such as c-Fos, c-Jun, AP2, E2F, myoD, and NF-κB (Wang et al. 2000). Overexpression of p202a can both reduce dimerization of c-Myc with Max and inhibit Myc-dependent transactivation. c-Myc does not interact with the oncogenic fusion protein BCR-ABL since the binding domain with in BCR is C-terminal to the junction. BCR appears to suppress c-Myc activity by competing with Max for c-Myc binding to the C-terminal B/HLH/LZ (basic region/helix-loophelix/leucine zipper) domain. BCR is able to suppress the ability of c-Myc to activate the expression of an artificial reporter construct as well as the endogenous cyclin D2 gene. It can also suppress the ability of c-Myc to cooperate with H-Ras (G12V) in the transformation of NIH3T3 cells.

Even if it is well documented that all the proteins mentioned can form distinct complexes with Myc interesting question remain unsolved. What fraction of the endogenous c-Myc protein is inhibited by any of these proteins at native levels of expression? Since the binding is mutually exclusive with Myc/Max dimerization, one might expect a variable pool of c-Myc protein complexed with these inhibitors rather than with Max.

Myc regulates the RNA polymerase I-dependent transcription.

The most distinctive signatures of target genes downstream of Myc are the genes involved in ribosome and protein biogenesis (Schlosser at al., 2003; Boon at al., 2001). This compartment is one of the only consistent features of the Myc target gene response in both mammals and flies. 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 at al., 2005 – Arabi at 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 polI transcription factors (Grandori at al., 2005). If Myc stimulates rDNA transcription, the ribosome content per cell should also increase, which was demonstrated directly in two studies (Grewal at al., 2005; Grandori at al., 2005). These observations have a profound implication for interpreting the Myc target gene response. The traditional method for normalizing RNA samples has always been through rRNA quantitation, and rRNA makes up >90% of total cellular RNA. If the rRNA content per cell differs substantially in response to Myc levels, then every mRNA whose level parallels the rRNA must also be Myc responsive. For example, if rRNA content is increased two-fold, then the fact that 90% of all genes parallel rRNA means that 90% of all genes in the cell are actually Myc regulated two-fold as well. If these observations hold up, then Myc is a far more profound transcription factor than previously appreciated.

Myc regulates the RNA polymerase III-dependent transcription, too.

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 at al., 2003). 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 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 Pol III cofactor TFIIIB occurs. Determining how Myc binding to TFIIIB and RNA Pol III can stimulate activity will require further investigation.

N-Myc.

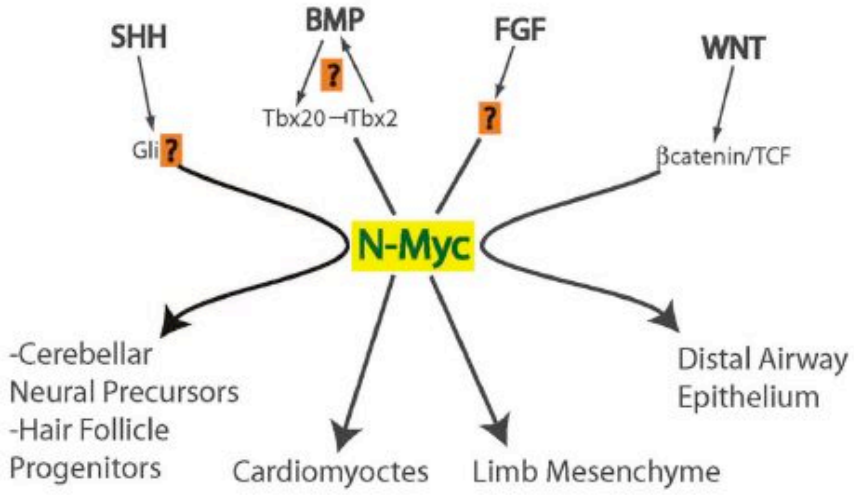
N-Myc is another member of the Myc family prominently expressed in undifferentiated subsets of cells in the lung, heart, central and peripheral nervous system, kidney, visceral arches, limb buds, and eye (Zimmerman et al., 1986; Mugrauer et al., 1988; Downs et al., 1989; Hirvonen et al., 1990; Hirning et al., 1991). There is considerable sequence divergence among c-Myc, N-Myc, and L-Myc (Nau et al., 1985), complementation experiments performed in Rat1a 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 c-Myc and N-Myc are expressed in highly complementary patterns during embryonic development (Hurlin et al., 1997). Nearly everywhere N-Myc is expressed, c-Myc is not expressed, and vice versa, but collectively they encompass most, if not all, proliferating cell types.

In most tissues and organs, N-Myc is normally expressed in cell compartments comprised of progenitor populations. Then, as the cells advance to more differentiated states concomitant with the progressive maturation of tissues and organs, N-Myc expression is turned off. Collectively, these results imply that N-Myc and the broad transcriptional program it directs, function in a general manner to maintain cells in a proliferative and undifferentiated state. In this capacity, N-Myc serves as an essential downstream target of various key signaling pathways (e.g., SHH, Wnt, TGF, and FGF pathways) to help coordinate morphogenesis (**Figure 7**).

These activities are consistent with a great deal of evidences indicating that deregulated N-Myc expression promotes tumor formation by locking cells into a progenitor-like phenotype.

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 neuroblastomas.

Figure 7. N-Myc is a target of a variety of signaling pathways that are involved in promoting proliferation of progenitor populations in different organs and tissues.



ARF *(Alternative Reading Frame) tumor 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 at al 2003). Because the inactivation of these genes allows cells that sustain oncogenic insults to survive and proliferate, their loss of function through deletion, mutation or epigenetic silencing is detected in most forms of cancer. 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 8**). 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) that encodes p16INK4a (Quelle at al., 1995).

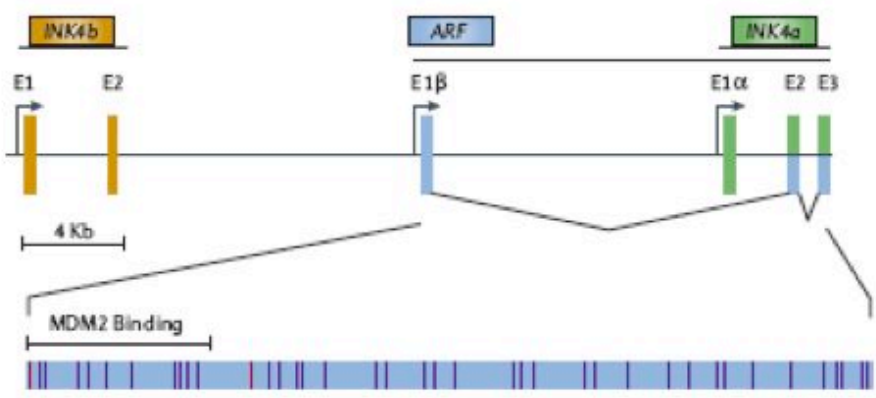


Figure 8. The *INK4b–ARF–INK4a* locus include s three tumour-suppressor genes in close proximity to one another. Numbered exons (E) are indicated by coloured rectangles and the promoters of the genes are designated by arrows. Both INK4a (green) and INK4b (orange) encode inhibitors (p16INK4a and p15INK4b) 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).

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), an abundant nucleolar phosphoprotein of 37 kDa. NPM is responsible not only for protecting ARF from degradation, but also for its nucleolar compartmentalization.

A recent view is that ARF is stored in an inactive state within the nucleolus in complexes with NPM, but can be displaced to the nucleoplasm where it can enter into more dynamic complexes with MDM2 or other targets (Brady at al., 2004).

It has been found that ARF has an associated sumoylating activity that can lead to the modification of proteins to which it binds, including MDM2 and NPM (Xirodimas at al., 2002).

Even more surprisingly, the ARF protein is also a potent tumor suppressor that blocks cell-cycle progression by directly binding to, and interfering with, the p53 negative-

regulator MDM2, thereby stabilizing and activating p53 (Kamijo at al., 1997; Scott at al., 1998). In turn, by antagonizing the E3 ubiquitin ligase activity of MDM2, ARF stabilizes p53 and increases its transcriptional activity (**Figure 9**). The most accepted view was that the tumor-suppressor functions of ARF was mediated through p53.

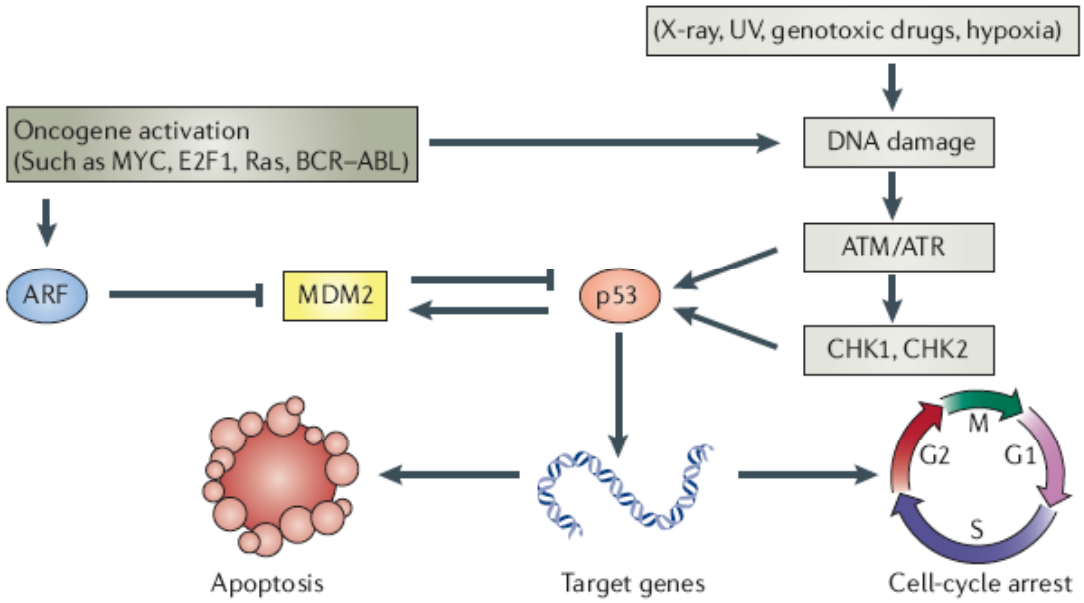


Figure 9. Sustained and increased signalling thresholds induced by mutated or overexpressed oncogenes activate the ARF promoter. Once expressed, the ARF protein interferes with the activity of MDM2, leading to p53 stabilization and triggering a complex p53-dependent transcriptional programme mediated 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. Interestingly, oncogene-induced stress can also activate protective DNA damage responses early in precancerous lesions. Depending on the nature of the inducing signal, these 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. Although ARF is not generally thought to be induced in response to DNA damage, its MDM2-inhibitory activity can still modify the DNA-damageinduced p53 response. 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, recent evidences are now suggesting that ARF might have additional p53-independent tumor-suppressor activities (Weber at al., 2000).
ARF has now been reported to physically interact with more than 25 other proteins, at least some of which have been postulated to be responsible for its p53-independent functions. These include proteins involved in unrelated cellular processes, including ribosome biogenesis, transcriptional regulation, the DNA-damage response, apoptosis and autophagy (Grisendi at al., 2006).

ARF and regulation of gene expression.

In order to induce cell-cycle arrest ARF must functionally antagonize gene expression governed by transcription factors such as E2F and Myc, the activities of which are required for cell-cycle progression. Members of the E2F family are best known for their role in driving the G1–S transition and DNA replication, but they also govern the expression of many other target genes, *ARF* among them, that are not periodically expressed during the cell cycle. Certain E2F isoforms assemble into complexes that repress *ARF* transcription, a process that is relieved by *RB* inactivation or E2F overexpression, both of which occur in many cancer cells. Therefore, *ARF* is positively and negatively regulated by different E2F complexes. An abnormal surfeit of Myc activity can also induce *ARF*, but the mechanism is likely to be indirect. By inducing p53, ARF blocks the growth-promoting activities of both Myc and the E2Fs, whereas their oncogenic potential is unleashed by the disruption of the ARF–MDM2–p53 pathway (Lowe at al., 2003). However, several groups of investigators have argued that ARF functions independently of p53 in directly binding to E2F1 and Myc and attenuating their transcriptional activity (Eeymin at al., 2001; Datta at al., 2005; Qi at al., 2005). It has been shown that ARF can bind to E2F1 and to its dimerization partner DP1, but not to assembled E2F1–DP1 complexes. Because ARF binding requires the DP1 segment required for E2F heterodimerization, ARF inhibits the formation of the active transcriptional complex. Although the unassembled E2F1 and DP1 subunits could be imported together with ARF into the nucleolus their relocalization might not be necessary for the interference of E2F-mediated transcription (Eymin at al., 2001).
It has been shown that the co-transfection of ARF and E2F1 into *p53*-null human cells inhibited E2F1 transactivating activity, and ARF sequences encoded by exon 1β were

necessary and sufficient for this effect. In an *ARF*-inducible U20S cell line engineered to co-express a dominant-negative, interfering form of p53, *ARF* activation reduced the expression of cyclin A, an E2F-responsive gene, before any inhibition of S-phase progression; conversely, siRNA-mediated knockdown of *Arf* in MEFs that lacked both *p53* and *Mdm2* led to increased cyclin A transcription (Datta at al., 2005). When ARF levels were reduced, chromatin immunoprecipitation (ChIP) analysis showed increased binding of DP1 to the *DHFR* promoter (another E2F-responsive gene). Although the ChIP findings show that ARF can attenuate the E2F transcriptional programme in a p53-independent manner.

Aim

The aim of this thesis has been focused on the c-Myc and N-Myc transcriptional regulation. In particular, I focus myself on the research of the molecular mechanism by which the onco-suppressor p14ARF factor inhibits the transcriptional activity of c-Myc and if this inhibition can be extended to the N-Myc family member.

Results

Biochemical characterization of the c-Myc/p14ARF interaction.

p14ARF directly interacts with c-Myc.

Recent studies have indicated that mouse p19ARF interacts and negatively regulates Myc's transcriptional activity. The structural differences between the murine p19ARF and the human p14ARF proteins prompted me to determine if also the human p14ARF tumour suppressor protein associated with the human 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 10A**, 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 most importantly to determine a putative mutually exclusive interaction of p14ARF and Max with Myc. 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 10B** lane 2, shows that the GST-p14ARF associated with both Myc and Max, demonstrating that p14ARF interact with the Myc-Max complex and that the binding of p14ARF does not interfere with Myc-Max interaction *in vitro*.

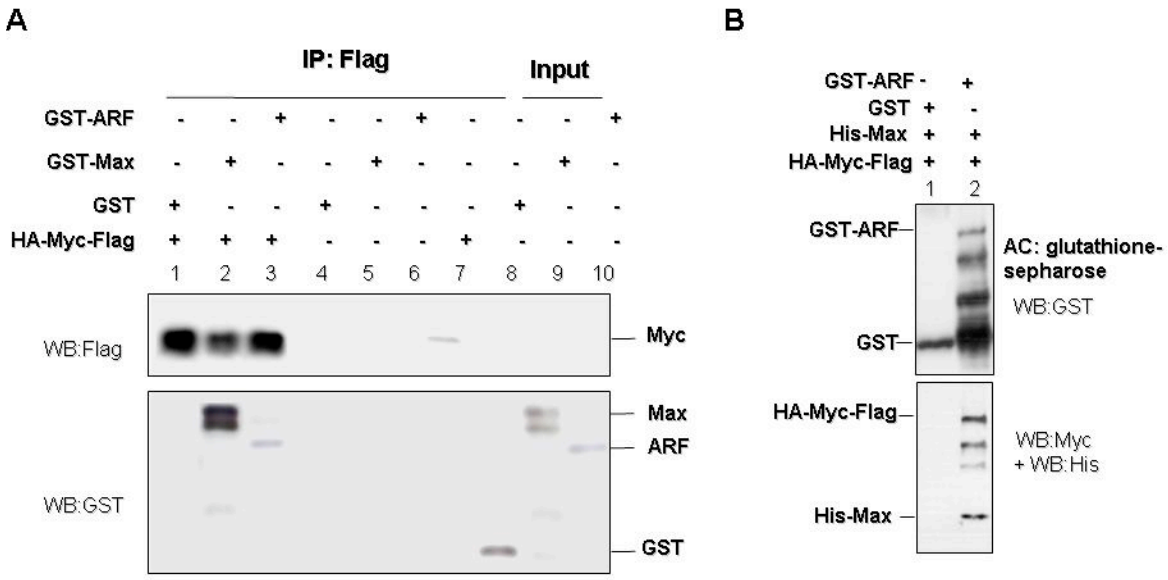


Figure 10: p14ARF directly interacts with Myc *in vitro*. (**A**) The GST, GST-Max or GST-p14ARF proteins (600ng) were incubated in presence (lane 1, 2, 3) or absence (lane 4, 5, 6) of HA-Myc-FLAG bait protein (600ng). Protein complexes were recovered by immunoaffinity with ANTI-FLAG M2-Agarose and analyzed 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).

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

To identify 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 11**, 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 in Figure 13 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*.

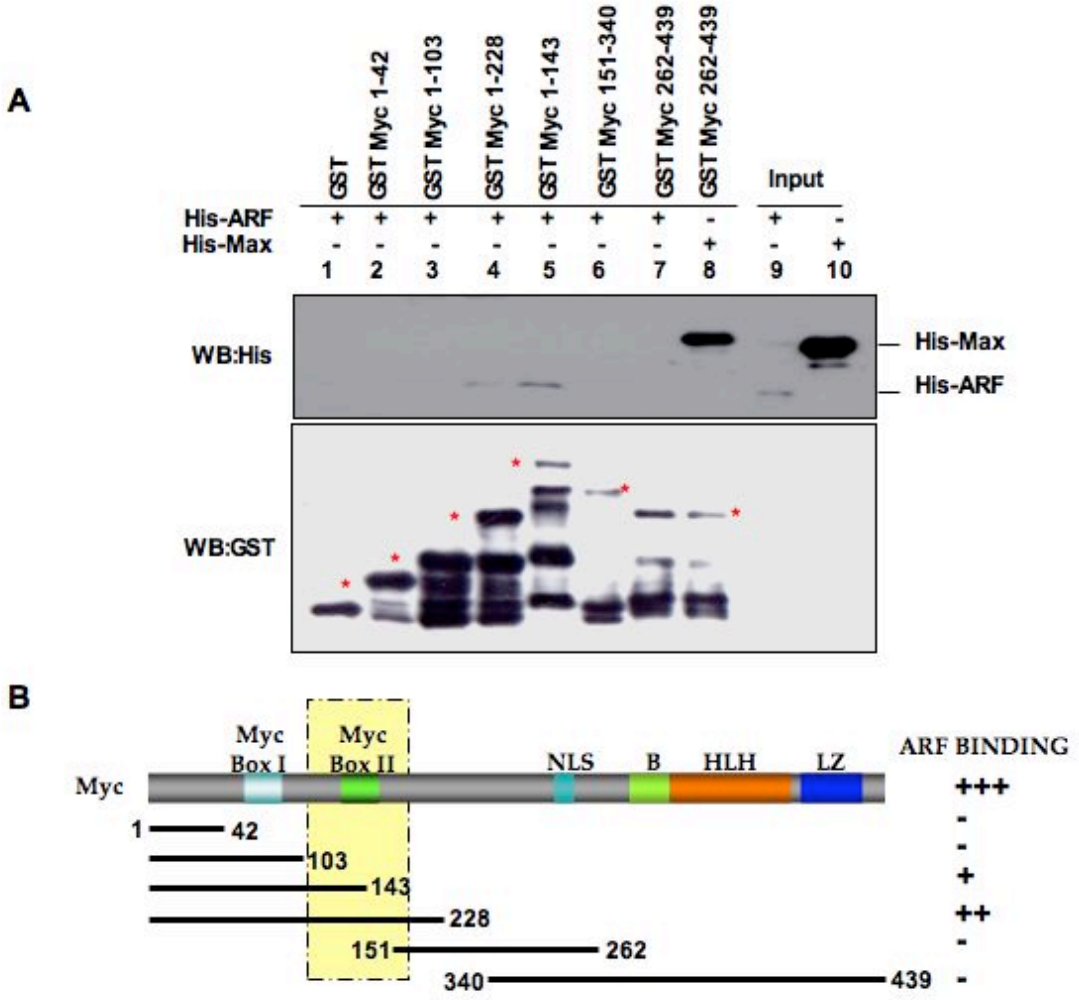


Figure 11: Mapping of the Myc domains involved in ARF interaction. (**A**) His-p14ARF was incubated with GST and GST-Myc deletion mutants (lane 1, 2, 3, 4, 5, 6, 7) proteins. In lane 8, as positive control of Myc interaction, GST-Myc 262-439 was incubated with the His-Max. The protein complexes were 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 12A** 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 12B**, 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, were 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 12C lane 6**).

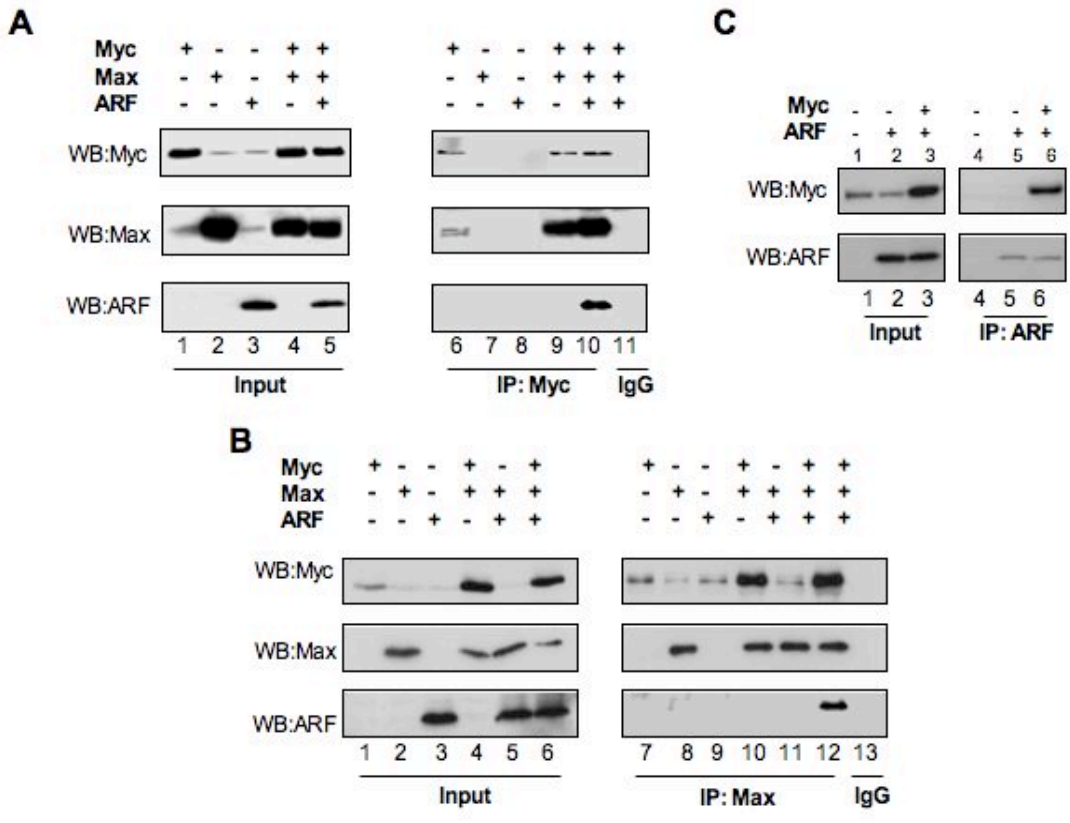
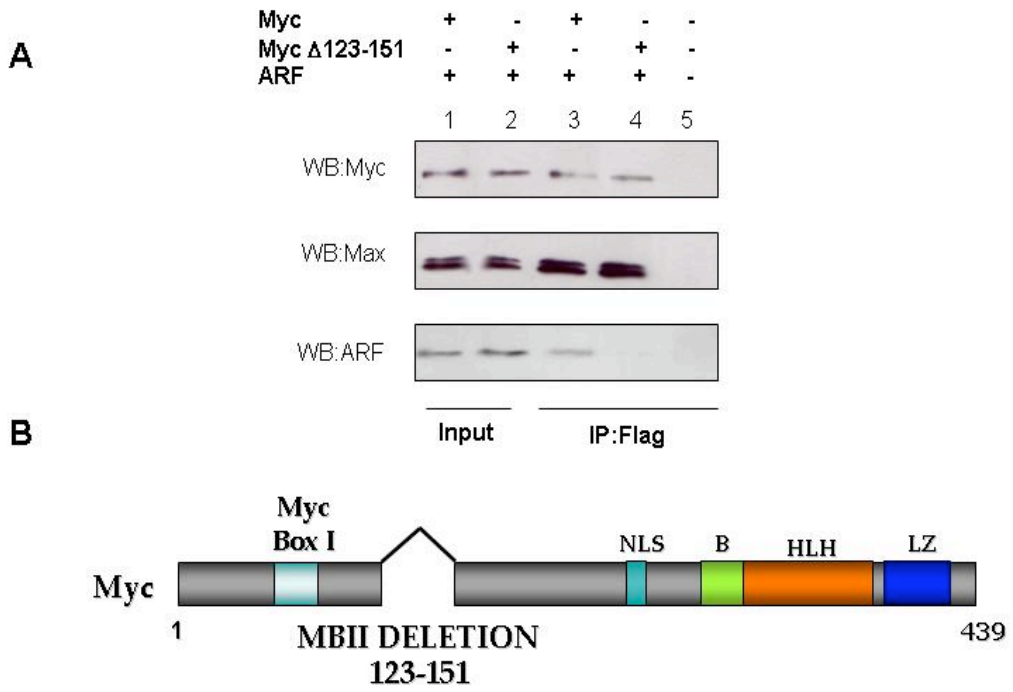


Figure 12: p14ARF interacts with Myc *in vivo.* (**A**) 293T cells were co-transfected by the calcium–phosphate method with pcDNA3-FLAG-Myc, pcDNA3-Max and pcDNA-ARF-HA as indicated. Protein extracts were immunoprecipitated with the anti-Myc N262 antibody (lane 6-10), and IgG antibody (lane 11). The 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 result shown in **Figure 13**, 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*.

Figure13: 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.



Myc-p14ARF nucleolar colocalization 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 nucleoplamatic localization. When ARF and Myc were cotransfected in a 1-to1 ratio, 82% of the cotransfected cells exhibited co-localization of Myc and ARF protein into the nucleoli (**Figure 14A**). 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**

14B). These findings underlie the relevance of the Myc BoxII domain in the physiological interaction between the ARF and Myc protein.

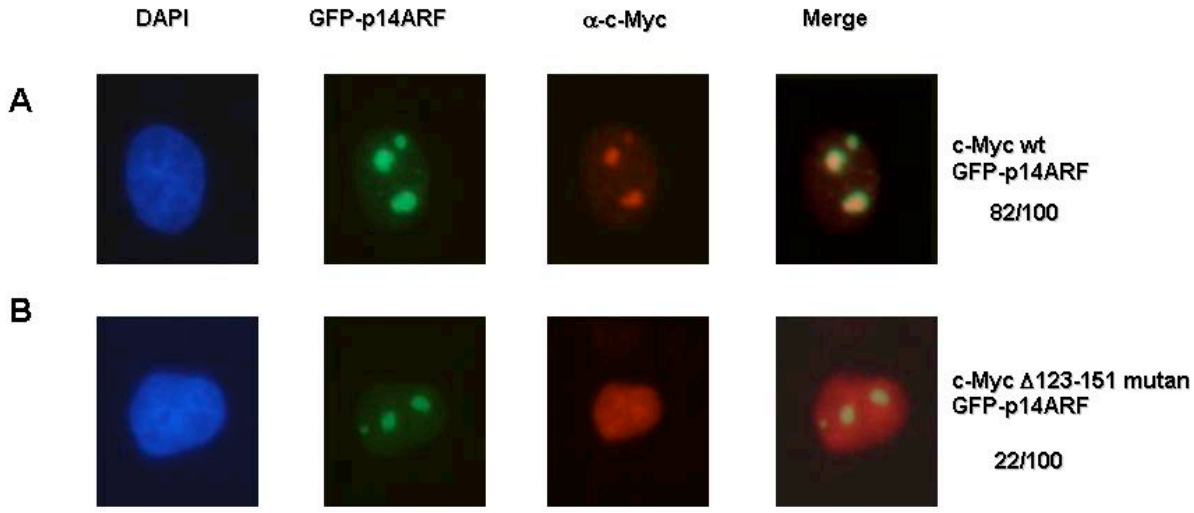


Figure 14: Myc-ARF nucleolar colocalization is impaired by Myc BoxII deletion. U2OS cells were co-transfected with GFP-p14ARF and pcDNA3-FLAG-Myc (**A**) or pcDNA3-FLAG-MycΔ123-151 (**B**) by Lipofectamine 2000 as indicated. An example of immunofluorescence microscopy of the cells immunostained with 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.

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 15** 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.

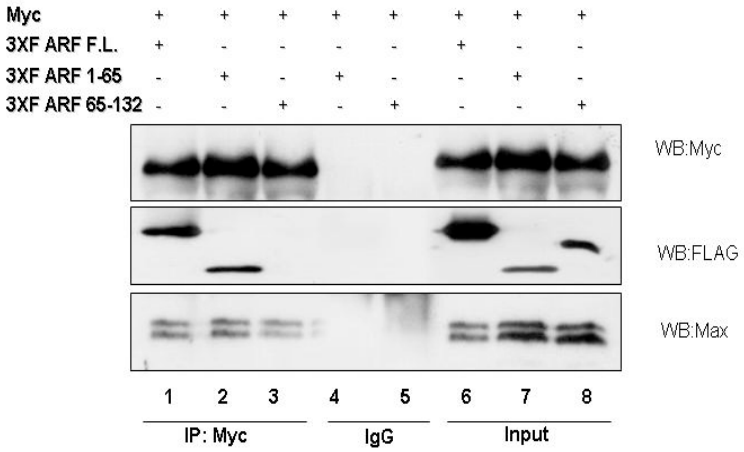


Figure 15: Myc interacts with the N-terminal region of p14ARF. 293T cells were co-transfected with pcDNA3-FLAG-Myc, p3xFLAG-ARF full length or p3xFLAG-ARF1-65 or p3xFLAG-ARF65-132 as indicated. Protein extracts were immunoprecipitated with the anti-Myc N262 antibody (lane 1-3), and IgG antibody (lane 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.

Biochemical characterization of the N-Myc/p14ARF interaction.

N-Myc associates with p14ARF *in vivo*.

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 an 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 16** and immunoprecipitations were performed using the N-Myc antibodies. The results show that p14ARF co-immunoprecipitates with N-Myc (**Figure 16A** 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 contained the Myc protein (**Figure 16B** lane 4).

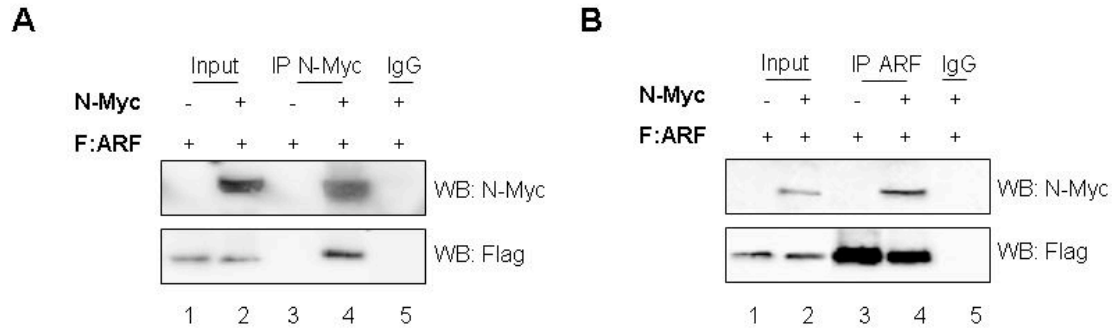


Figure 16: p14ARF interacts with N-Myc *in vivo*. (**A**) 293T cells were transfected with 3xFLAG-ARF or co-transfected with N-Myc and p3XFLAG-ARF F.L. as indicated. Protein extracts were immunoprecipitated with the N-Myc antibody (lane 3, 4) and anti IgG (lane 5) as control and the co-purified complexes 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.

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 17B**. 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 17A**, the N-Myc deletion mutant d(1-300) loose 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 reside in the region from aa 140 to aa 300 containing the MBIII conserved domain.

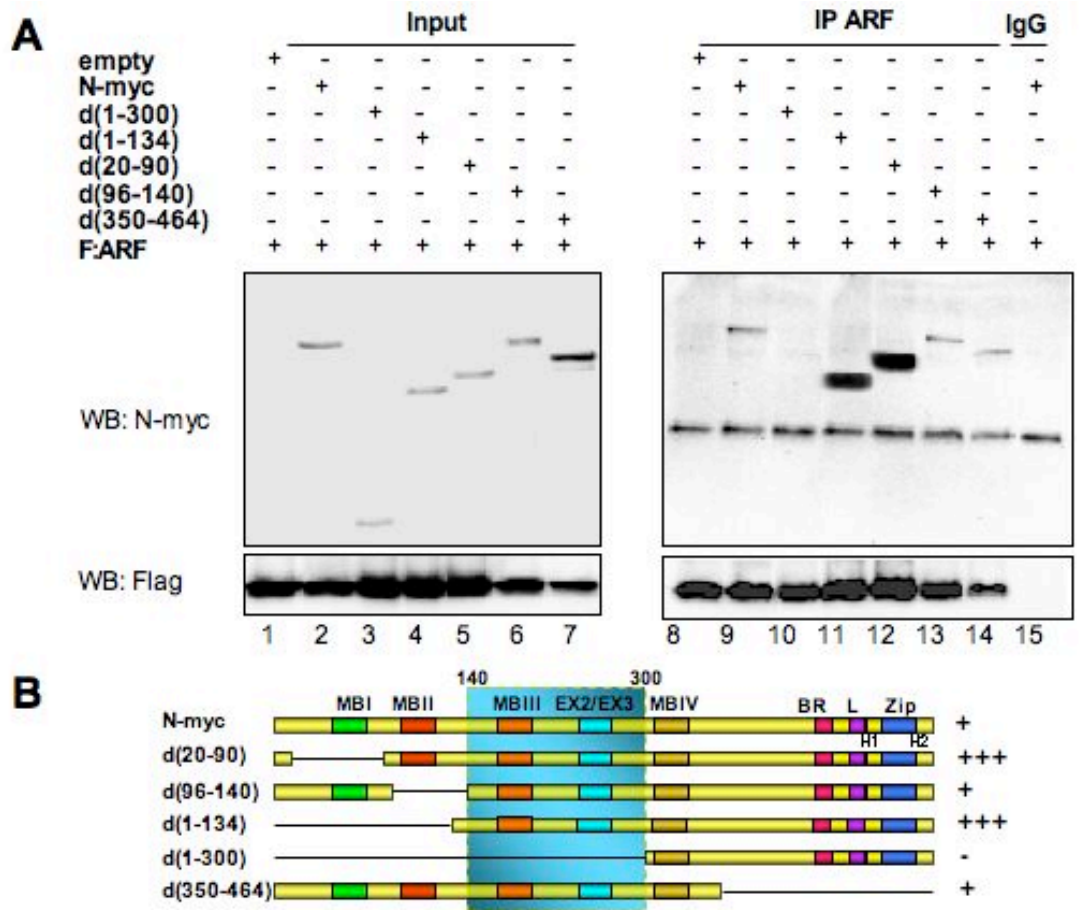


Figure 17: p14ARF interacts with the MBIII of N-Myc *in vivo*. (A) 293T cells were co-transfected with 3xFLAG-ARF and different N-Myc delection mutants as indicated. Protein extracts were immunoprecipitated with the ARF antibody (lane 8-14) and anti IgG (lane 15) as control and the co-purified complexes 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.

N-Myc co-localize 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 relocalize ARF from the nucleolus to the nucleoplasm or c-Myc co-localize 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 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 detectable 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 show a predominantly nucleolar localization of the exogenous ARF protein.

In cells co-transfected with the two expression vectors, the GFP-p14ARF protein retain 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 18A**. 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 18B**, in the cells co-transfected with both GFP-p14ARF and N-Myc d (1-300) mutant, the GFP-ARF protein is found in the nucleoli and the mutated N-Myc protein is found in the nuclear compartment.

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

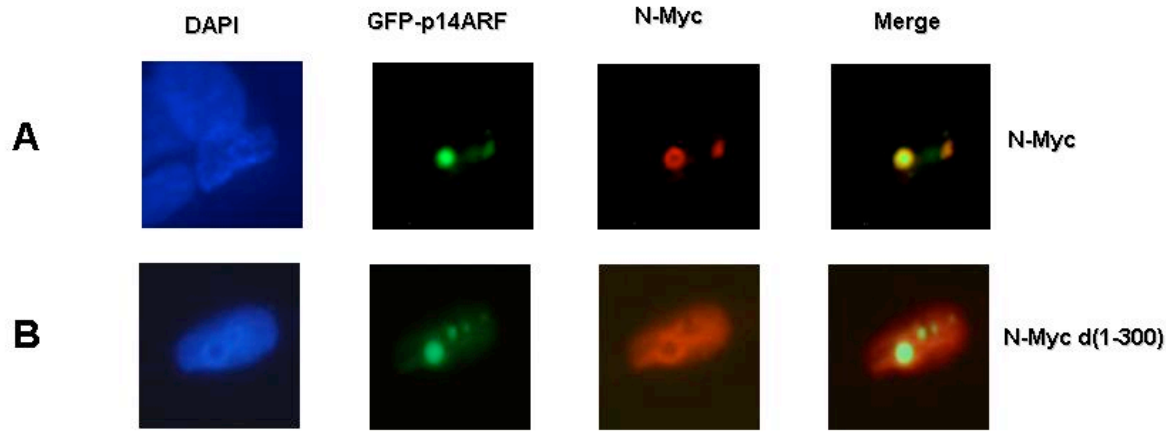


Figure 18: N-Myc-ARF nucleolar colocalization is impaired by Myc BoxIII deletion. SKNBE cells were co-transfected with GFP-p14ARF and N-Myc (**A**) or N-Myc d(1-300) mutant (**B**) as indicated. An example of immunofluorescence microscopy of the cells immunostained with anti-N-Myc and analyzed by fluorescence microscopy as previously described, is shown. At least 150 cells were analyzed in each experiment. Values are means from three independent experiments.

The p14ARF N-terminal domain is involved in interaction with N-Myc.

In the attempt to define the ARF region involved in N-Myc interaction, I performed similar experiments already utilized to map the ARF domain involved in c-Myc binding. The 3xFLAG-ARF1-65 and 3xFLAG-ARF65-132 constructs, described in previously paragraph, were transfected alone or in combination with the N-Myc expression vector into 293T cells. The protein extracts from the transfected cells were immunprecipitated with the N-Myc antibody and the coimmunoprecipitated proteins analyzed by WB with N-Myc, Max and FLAG antibodies. The results shown in **Figure 19** demonstrate that either the WT ARF protein then the protein encoding for the first 65 aa co-immunoprecipitate with N-Myc. In contrast the 65-132 ARF C-terminal domain is impaired in N-Myc binding. As control of N-Myc immunoprecipitation, WB with the Max antibody confirms the presence of the endogenous Max protein in all the Myc immunopreciptated extracts. From these experiments I conclude that the same p14ARF domain is involved in c-Myc and N-Myc binding.

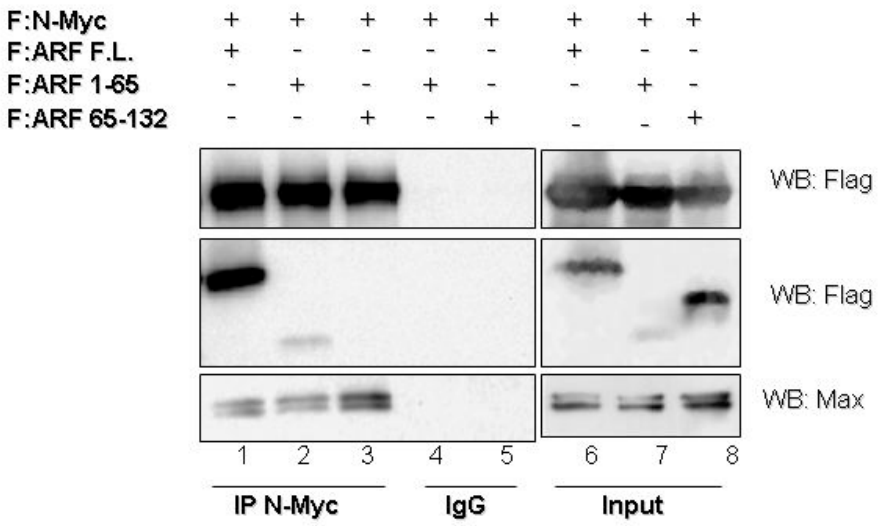


Figure 19: N-Myc interacts with N-terminal of p14ARF. 293T cells were co-transfected with 3xFLAG-N-Myc, p3xFLAG-ARF or p3xFLAG-ARF1-65 or p3xFLAG-ARF65-132 as indicated. Protein extracts were immunoprecipitated with the anti-N-Myc antibody (lane 1-3), and IgG antibody (lane 4 and 5) and the copurified complexes analyzed by WB with anti-FLAG and anti-Max antibodies, as indicated. 5% of the proteins inputs were loaded in lanes 6-8.

Functional characterization of the Myc-p14ARF interaction.

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, human homolog, was able to inhibit both c-Myc and N-Myc transcriptional activity. First of all, I investigated if p14ARF expression 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 p14ARF vector. As reported in **Figure 20A**, Myc exogenous expression in U2OS cells activates three fold the hTERT-Luc promoter expression (lane 2) and cotransfection 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 22A (lane 6), p14ARF does not have any influence of hTERT promoter transcription in the absence of exogenous c-Myc.

In order to extend these finding to N-Myc mediated transcriptional activation, I took advantage of the Tet21N cell line (kindly provided by Prof G. Della Valle) that stably express 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 20B**, 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 is able to repress both c-Myc and N-Myc transcriptional activities.

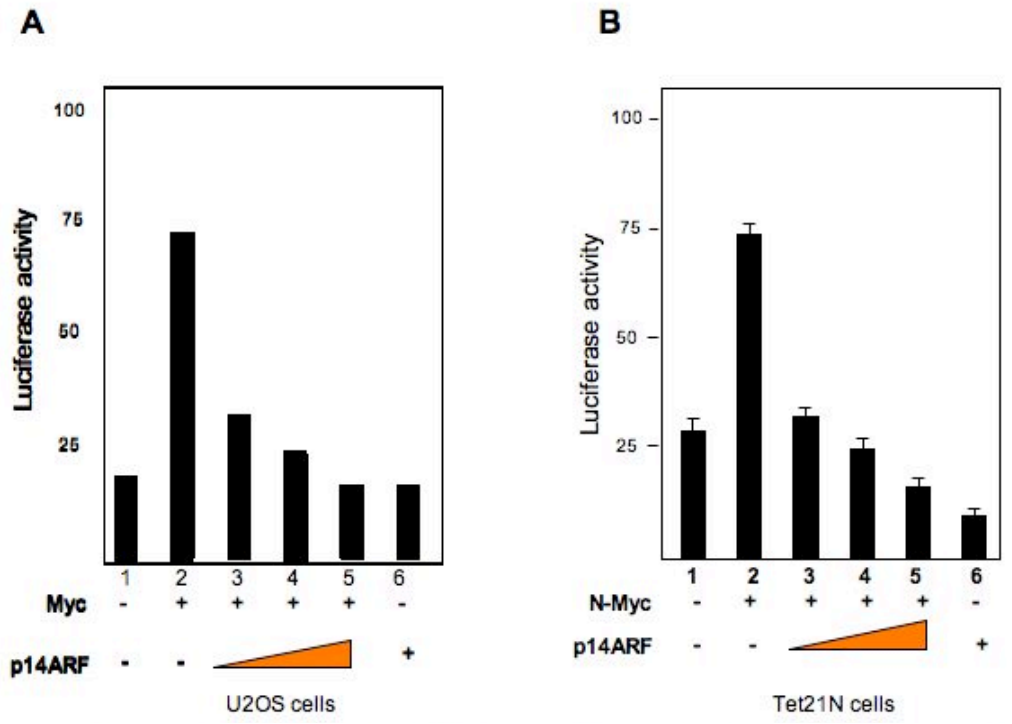


Figure 20. ARF expression inhibits c-Myc and N-Myc activated transcription. A) U2OS cells were cotransfected with 100ng of hTERT-Luc, 200ng pMT2T-Myc and different amounts (0,1; 0,5 and 1µg, respectively) of pcDNA-p14ARF-HA as indicated. Each histogram bar represents the mean of three independent transfections 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%.

p14ARF does not possess an intrinsic repressor domain.

In the background chapter I reported several models by which ARF might repress Myc mediated transcription. For example, ARF could possess an intrinsic repressor domain through which it can inhibit c-Myc and N-Myc transcriptional activities. In order to evaluate if the p14ARF protein is able to repress transcription when artificially recruited on a targeted promoter, the human ARF cDNA was fused to the yeast GAL4 DNA binding domain and the activity of the resulting plasmid, GAL4-p14ARF, assayed on a targeted promoter bearing 5xGAL4 DNA binding sequences. As control, the GAL4-KRAB expression vector, bearing the well-described KRAB repressor domain was used. As shown in **Figure 21A**, artificial recruitment of GAL4-p14ARF has no significant effects on G5-83HIV-Luc expression (lane 1 and 2), while GAL4-KRAB effectively repressed the G5-83HIV-Luc promoter's activity (lane 4

and 5). Immunofluorescence of transfected cells with a GAL4 antibody indicated that both GAL4 fusion proteins are expressed at comparable levels (percentage of GAL4 positive cells) and both proteins display a prominent nuclear localization (**Figure 21B**). Thus, the ability of p14ARF to repress Myc mediated activation cannot be addressed to a putative intrinsic repression capability.

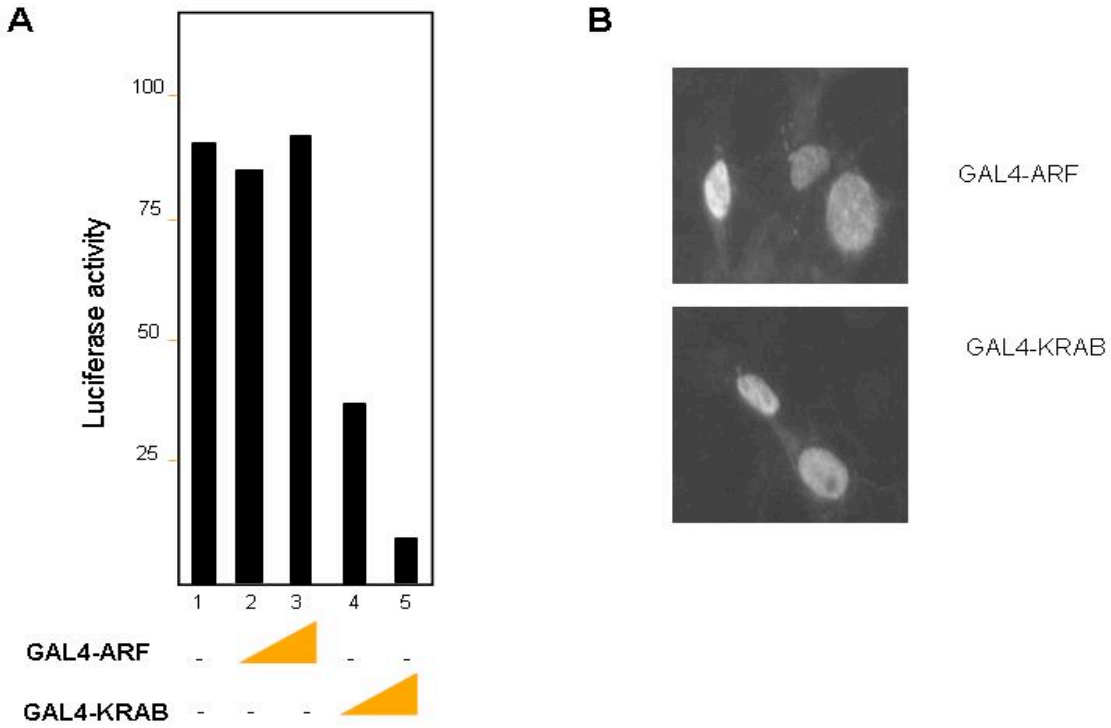


Figure 21. GAL4-p14ARF fusion protein artificial recruitment. A). Cos cells were cotransfected with 100ng of the G5-83HIV-Luc vector, GAL4-p14ARF (0,5 and 1μg) and GAL4-KRAB (0,5 and 1μg) as indicated. **B**) immunostaining with anti-GAL4 of transfected cells is shown. Each histogram bar represents the mean of three independent transfections made in duplicate with a standard deviation less than 10%.

p14ARF does not interfere with Myc-Max binding to E-boxes.

Another hypothesis by which ARF could inhibit Myc-mediated transcription is that ARF may interfere with the Myc-Max binding to E-box. To address this issue, I decided to perform an EMSA assay by using the radiolabelled Myc-Max consensus oligonucleotide, containing the canonical Ebox, (Santa Cruz Biotechnologies). In particular, I assayed whether endogenous or exogenous ARF was able to interfere with the heterodimer Myc-Max binding to Ebox. To address if the endogenous ARF

interferes with the Myc-Max binding to Ebox, I used nuclear extracts from 293T cells, expressing low amount of ARF, and from the U2OS cell line, not expressing p14ARF, in gel retardation experiment. Furthermore I also used nuclear extracts from both cell line (293T and U2OS) transfected with CMV driven p14ARF vector. The results shown in **Figure 22A**, demonstrated that the Myc-Max binds the E-boxes (see the arrow, lane 1, 4 and 7, 9) both in untransfected and in transfected cells. The binding specificity of Myc was verified by competition of Myc-Max binding with an unlabelled consensus oligonucleotide (C) (lane 2, 5 and 8, 10) and unlabelled non-consensus oligonucleotide (NSC) (lane 3, 6). Comparing the Myc-Max binding in lane 1 and 7 with the Myc-Max complex in lane 4 and 9 Based on this result, I reasoned that p14ARF does not interfere with Myc-Max binding to E-boxes. Immunoblotts confirmed the presence of the transfected ARF protein in the nuclear extracts used in EMSA assay (**Figure 22B** lane 2, 4).

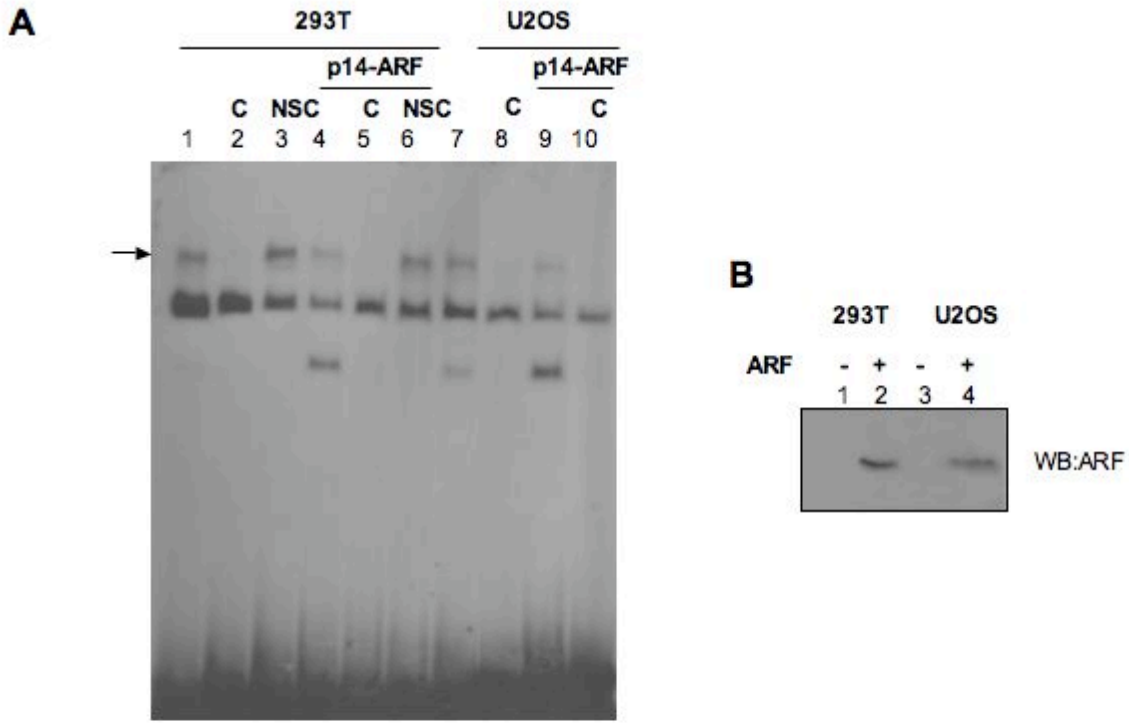


Figure 22. p14ARF does not interfere with Myc-Max binding to E-boxes. A) Gel mobility shift analysis was performed by using a labeled Myc-Max consensus oligonucleotide (Santa Cruz) and nuclear extracts from 293T and U2OS cells. The arrow indicates specific complexes for Myc and Max. Nuclear extracts from 293T and U2OS cells, wild type (lane 1-3, 7-8) or transfected with CMV based p14ARF vector (lane 4-6, 9-10), were incubated with labeled consensus oligonucleotide in the absence (lane 1,4,7,9) or in the presence of 150-fold molar excess of unlabeled consensus oligonucleotide (lanes 2, 5, 8, 10) and unlabelled mutant oligonucleotide (Santa Cruz) (lane 3, 6). B) Nuclear

extracts, from 293T and U2OS not trasfected (-) and transfected (+) with CMV based p14ARF vector and used in EMSA, were tested by WB anti ARF to confirm the overexpression of ARF.

p14ARF competes with P-TEFb for Myc binding.

As described in background chapter, ARF is able to inhibit the Myc-mediated transcription interfering with the binding of the Myc co-activators. As shown in **Figure 23**, many co-activators bind Myc through the Myc BoxII and the Myc BoxI and their each one binding to Myc could be disturbed by ARF binding. Among these other partners, I focused on P-TEFb that has been in the last years the major topic in Lania's laboratory. Moreover, it is described that CycT1 component of the P-TEFb complex interacts with Myc, through the Myc BoxI, and in this way promotes RNAPII promoter clearance in the *cad* gene expression activation.

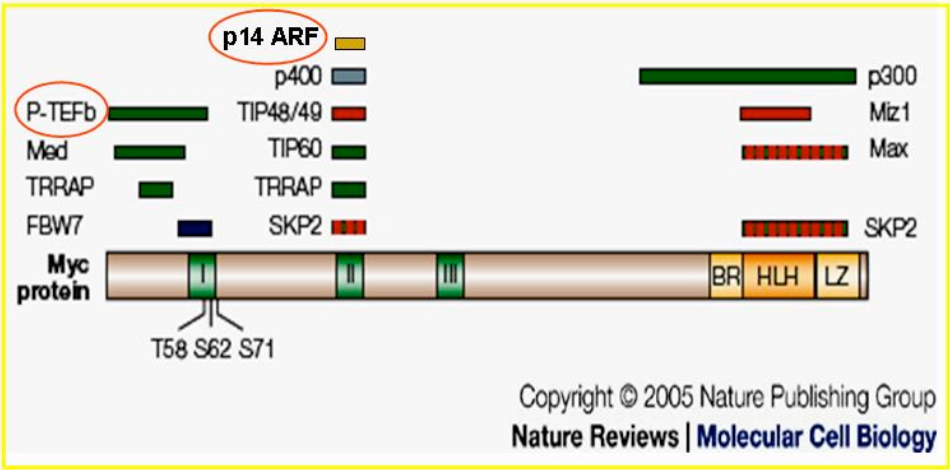


Figure 23. Domains of Myc and their binding proteins. The N terminus of Myc has three highly conserved elements, known as MycboxesI–III. The C terminus contains the basicregion/helix–loop–helix/leucine-zipper (BR/HLH/LZ) domain. T58, S62 and T71 are known phosphorylation sites of Myc, and are targeted by glycogen synthase kinase-3 (T58), MAP kinase (S62) and Rho-dependent kinase (T71), respectively. The domains of Myc that interact with specific binding proteins are shown above the full-length protein structure. If the interaction results in Myc-dependent transactivation, the domain is represented in green. If the interaction results in Myc-dependent repression, the domain is shown as a red bar, and if protein interaction results in the repression of Myc function, the domain is represented in blue. Interactions that mediate both transcriptional activation and repression by Myc are indicated by a dashed bar. Domains of Myc that bind with partner proteins for which a role has not yet been determined are shown in grey. FWB7 is not a transcriptional cofactor, but is part of an E3

ubiquitin ligase that regulates Myc protein stability. SKP2 functions as part of an E3 ubiquitin ligase and as a cofactor for Myc. p300 is a histone acetyltransferase and p400 is a histone exchange factor. TIP48 and TIP49 are hexameric ATPases that are part of chromatin remodelling complexes, whereas TIP60 is a histone acetyltransferase complex. TRAPP, an adaptor protein, is the core subunit of the TIP60 and GCN5 complexes. Med, Mediator.

I have chosen to investigate if ARF repress the Myc-mediated transcription interfering with the P-TEFb binding to Myc. It is possible to hypotize that *in vivo* there could be two complexes binding in a mutually exclusive way. The overexpression of ARF could shift the equilibrium from the Myc-P-TEFb complex to Myc-p14ARF complex. In order, I decided to address this hypothesis with a biochemical approach. To this end, I performed Co-IP assays with protein extracts from transiently transfected 293T cells. The cells were transfected with constant amount (7μg) of CMV-based Myc and CycT1 expression vectors in the absence and presence of increasing amount (1, 5 and 10 μg) of p14ARF. The extracts were immunoprecipitated with anti-Myc antibody. Immunoblot analysis was then performed using Myc, Max, CycT1 and FLAG (for ARF) antibodies. The results reported in **Figure 24A** that Myc is able to form a separates complex with CycT1 (lane 1) and with p14ARF (lane2). Moreover, in Myc's immunocomplex was possible to observe that increasing amount of p14ARF determine decreasing amount of CycT1 (lane 3, 4, 5). Hence, the overexpression of ARF had shifted the equilibrium by Myc-P-TEFb complex to Myc-ARF complex. Max helps me to exclude artefacts being constantly present in Myc complex (lane 3, 4, and 5). These results demonstrated that ARF binding may alter the association of Myc to P-TEFb co-activator. In order to corroborate these finding, I performed a similar experiment by using the ARF1-65 mutant that contains the Myc binding region and ARF 65-132 that does not contains the Myc binding. The 293T cells were transiently transfected with CMV-based Myc and CycT1 expression vectors in presence of the mutants ARF1-65 and the ARF65-132, respectively. The results reported in **Figure 24B**, lane 3, show that only overexpression of ARF1-65, containing the Myc binding, is able to antagonize the CycT1 binding to Myc. Finally, these finding strongly support the hypothesis by which p14ARF may inhibit the Myc transcriptional activity competing with the binding of the P-TEFb to Myc.

Figure 24. p14ARF competes with the CycT1 Myc-binding. (**A**) 293T cells were co-transfected with constant amount (7μg) of pcDNA3-FLAG-Myc and p3xFLAG-cycT1 and increasing amount of

p3xFLAG-ARF and as indicated. Protein extracts were immuno-precipitated with the anti-Myc N262 antibody (lane 1-5) and IgG antibody (lane 6). The copurified complexes were analyzed by WB with anti-Myc 9E10, anti-Max, anti CycT1 and anti-FLAG (ARF) antibodies, as indicated. 5% of the proteins inputs were loaded in lanes 7-9. **B**) 293T cells were co-transfected with (7μg) of each pcDNA3-FLAG-Myc, p3xFLAG-ARF1-65 or p3xFLAG-ARF65-132 and p3xFLAG-CycT1 as indicated. Protein extracts were immunoprecipitated with the anti-Myc 9E10 antibody (lane 1-4) and IgG antibody (lane 5-6). The copurified complexes analyzed by WB with anti-Myc 9E10, anti-Max, anti-CycT1 and anti-FLAG (ARF deletes) antibodies, as indicated. 5% of the proteins inputs were loaded in lanes 7-9.

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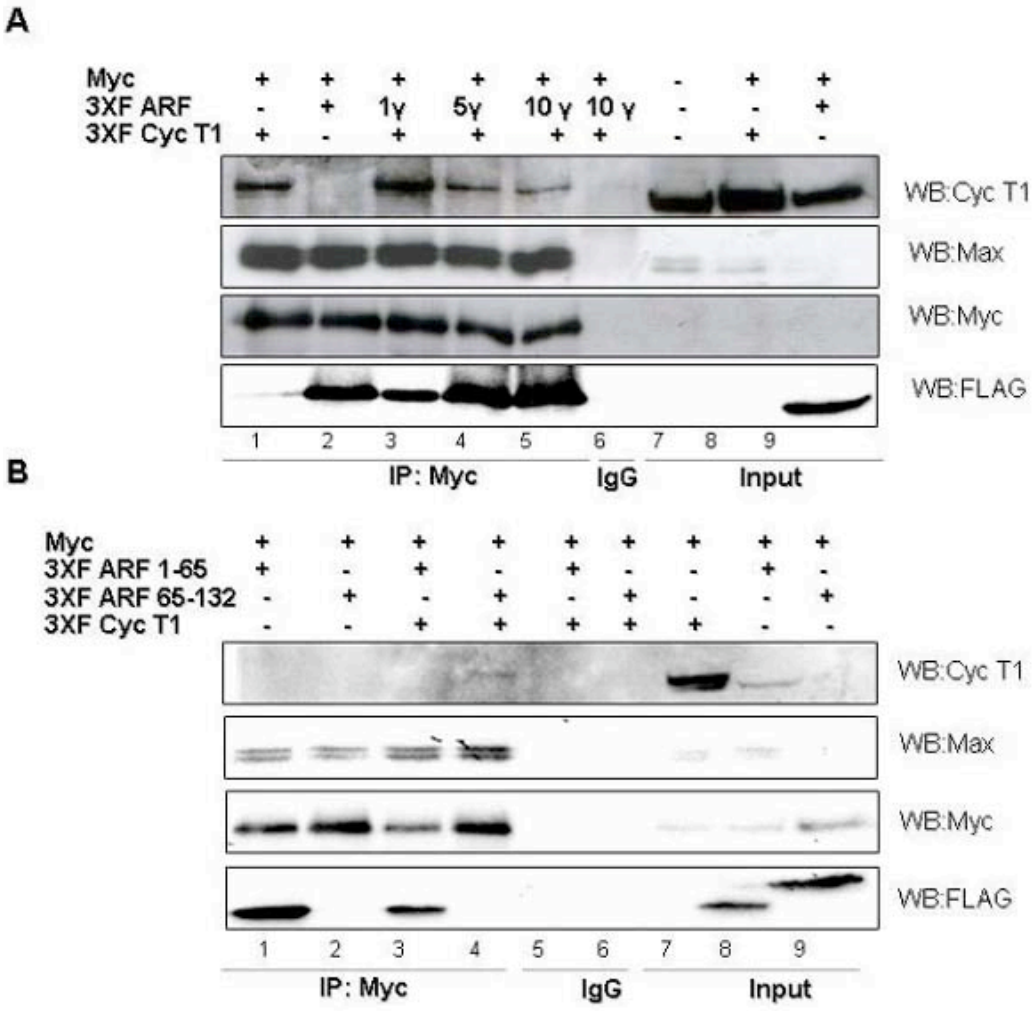


CycT1 recruitment on *cad* promoter is Myc-dependent.

I speculated that ARF binding might alter the association of c-Myc to dedicated

cofactors either through sterical hindrance or to a mutually exclusive interaction due to binding to the same domain. It is pertinent to note that ARF inhibits c-Myc-mediated *cad* activation. Previous findings demonstrated that *cad* gene is activated by c-Myc in a TRRAP independent manner. It has been suggested that in the case of *cad* genes c-Myc regulates RNA pol II clearance and elongation, thus c-Myc regulates some target genes by recruitment of RNA pol II CTD kinase. Accordingly, it has been shown that P-TEFb interacts *in vitro* with c-Myc (Eberhardy at al., 2001 and Eberhardy at al., 2002). I have obtained strong biochemical evidences that c-Myc interacts with P-TEFb through interaction with CyclinT1 and, through CoIPs experiments, I have demonstrated that c-Myc forms *in vivo* a complex with P-TEFb and that ARF interferes with binding of P-TEFb to Myc-Max complexes.

It is quite unlikely that ARF could affect recruitment of c-Myc-Max heterodimer on DNA target promoters. As I have demonstrated through gels retardation experiments, the expression of the p14ARF protein in the U2OS cells does not reduce the association of the Myc-Max complex to the target DNA sequence. Accordingly, ChIP analysis on Myc targeted promoters indicate that Myc binds to the elF4E and nucleolin genes promoters whether or not p19ARF is present, indicating that p19ARF does not affect recruitment of Myc on these target genes. However, it remains to be shown if c-Myc, P-TEFb and ARF are actually present on c-Myc-target genes.

To this end, I plan to use the well-described Rat1-Myc-ER cells expressing the inducible c-Myc-ER chimera (kindly provided by Bruno Amati IEO, Milano). This cell line, expressing the inducible c-Myc-ER chimera, can be synchronized in the G0-G1 (quiescence) cellular phase in which it is well documented that c-*myc* expression is virtually undetectable. After mitogenic or serum stimulation, c-*myc* mRNA and endogenous c-Myc 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. Moreover, after treatment with hydroxytamoxifen (OHT) the exogenous c-Myc-ER 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.

To investigate if ARF overexpression could be able to compete for CycT1 or other Myc coactivators binding a Myc target promoters, first of all, I characterized the Rat1-Myc-ER cell cycle through FACS analysis. To this end, the cells were synchronized in G0-G1 or quiescent phase in medium serum-free for 48h. After, the block was released in fresh medium with 10% serum or 600nM OHT, the cells were harvested at different time points. The cell cycle profile of each sample was determined by FACS analysis. As shown in **Figure 25**, after starvation almost 92% for the cells are in G1 phase. After serum stimulation, in almost 40 hours the cells conclude one cell cycle. Moreover, after OHT stimulation the cells do not conclude the cell cycle, but it is possible to observe a percentage of the cells that re-entry in G1-S phases, confirming that Myc activation is sufficient to drive quiescent cells into cell cycle.

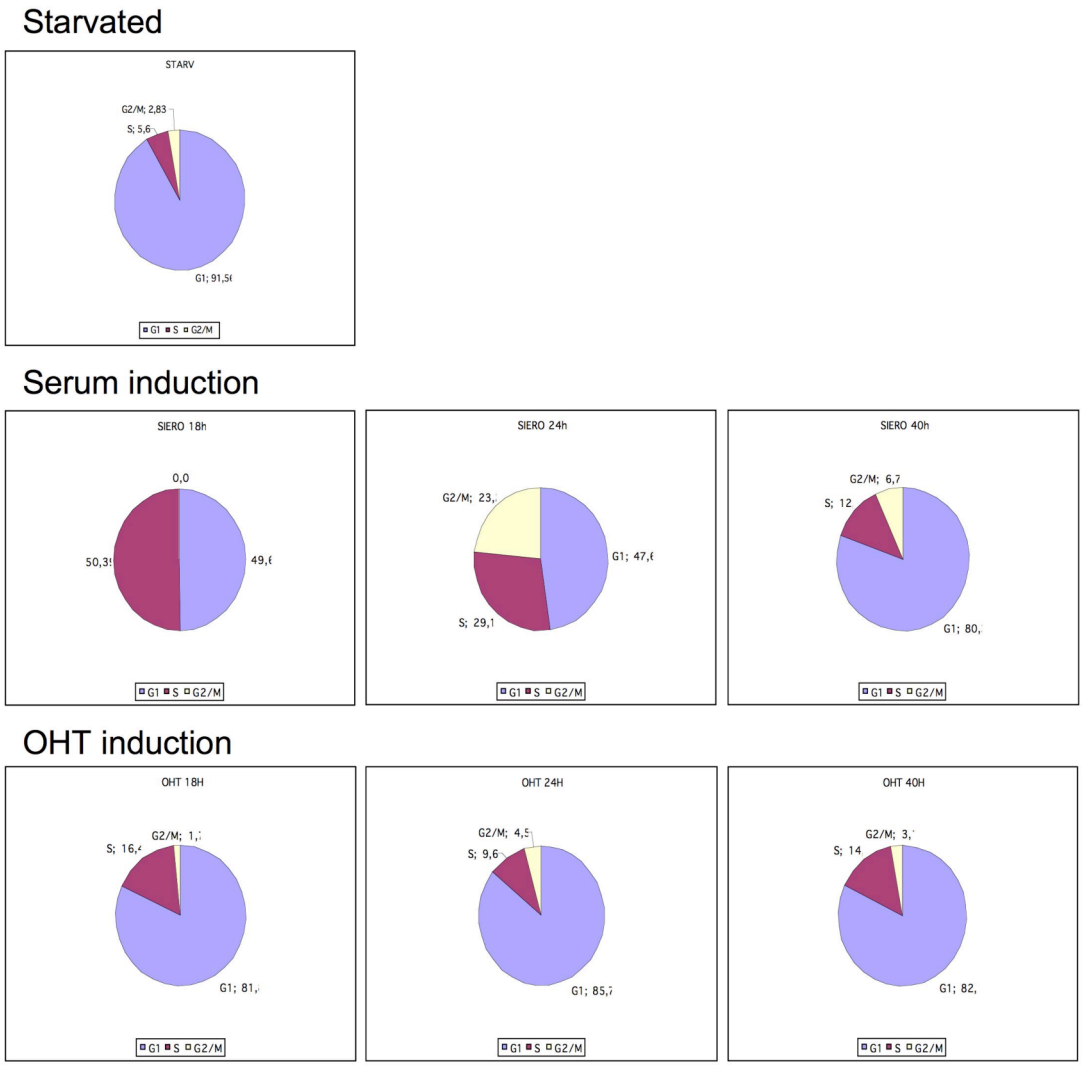


Figure 25. FACS analysis of RAT1-Myc-ER cells. A) It is reported the FACS profile of RAT1-Myc-ER cells starved for 48h in medium serum-free. B) After starvation, the cell cycle were induced adding serum in medium, the cells were harvested at different time as indicated. C) After starvation, the cell cycle were induced adding 600nM OHT in medium, the cells were harvested at different time as indicated. The experiment was performed twice and resulted in similar data.

To confirm that serum or OHT stimulation leads to the recruitment of Myc on its target genes and consequently their activation, the cells, synchronized by serum deprivation and then stimulated by serum or OHT, were collected and lysed for mRNA extraction with the TRIZOL (Sigma). Retrotrascription of RNA has been performed with Reverse Transcriptase reaction (Invitrogen).). cDNA was diluted 1:3 prior to use in quantitative PCR (qPCR). Quantitative analysis of the expression of *cad* and *nuc* genes, Myc's target genes, was performed by using the AbiPrism 7500 sequence detector system (Perkin-Elmer Applied Biosystems). As shown in **Figure 26,** I have found that the *cad* gene response to Myc induction is more efficient than *nuc.* Since, I decide to use *cad* in subsequent experiments.

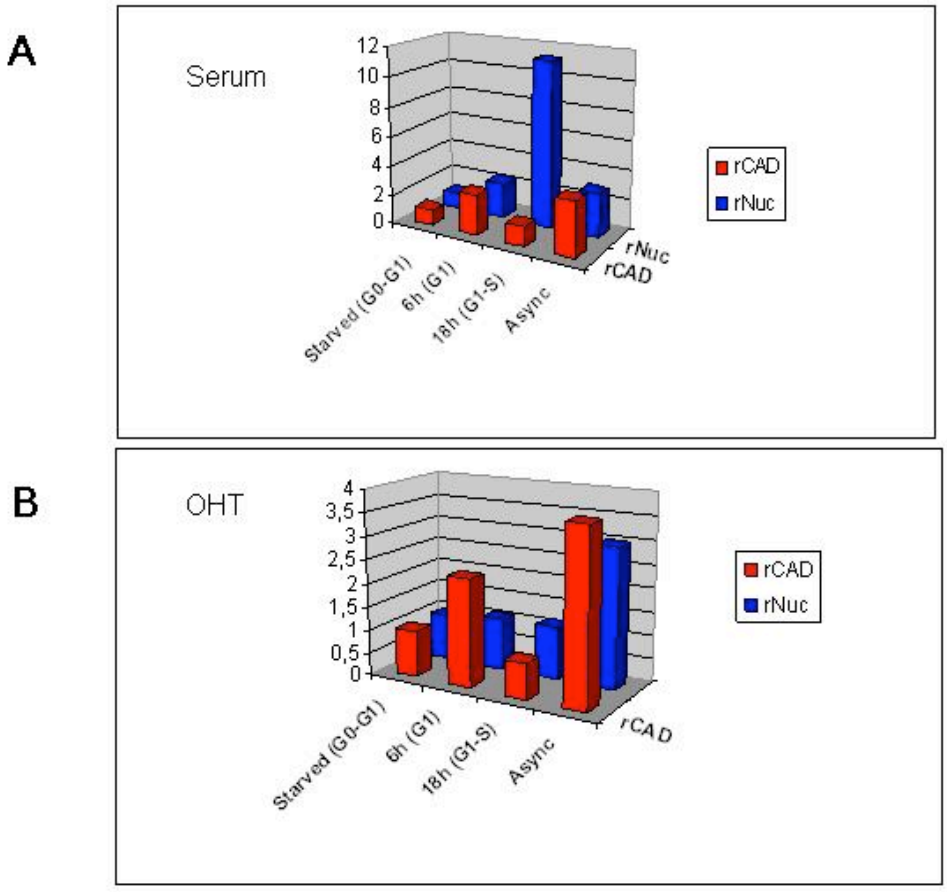


FIGURE 26. *cad* and *nuc* mRNA quantification. **A)** Total RNA was prepared at the indicated times from RAT1-Myc-ER cell cycle induced with serum. *cad* and *nuc* mRNA levels were quantified using qPCR. The mRNAs levels are presented relative to RNA levels of RAT1-Myc-ER cells at 0 time (Starved). **B)** Total RNA was prepared at the indicated times from RAT1-Myc-ER cell cycle induced with 600nM OHT. *cad* and *nuc* mRNA levels were quantified using qPCR. The mRNAs levels are presented relative to RNA levels of RAT1-Myc-ER cells at 0 time (Starved). This experiment was performed two times with similar results.

To determine whether c-Myc and P-TEFb were recruited on *cad* gene, quiescent Rat1 cells that expressed a conditionally active Myc-estrogen-receptor (ER) chimera were treated or not with OHT and analyzed by chromatin immunoprecipitation (ChIP). ChIP is a powerful tool for identifying proteins associated with specific regions of the genome by using antibodies that recognize a specific protein or a specific modification of a protein. The initial step of ChIP is the cross-linking, with formaldehyde, of protein-protein and protein-DNA in live cells. After cross-linking, the cells are lysed and crude extracts are sonicated to shear the DNA. Proteins together with cross-linked DNA are subsequently immunoprecipitated with the antibody of interest. Proteins-DNA cross-linked in the immunoprecipitated material are then reversed and the DNA fragments are purified and PCR amplified with selected oligos.

By using the RAT1-Myc-ER cells I performed ChIP assays and chromatin was immunoprecipitated with Myc or CycT1 antibodies. DNA fragments, obtained as described above, were purified and PCR amplified by using oligos specifics for the Myc target *cad* and the unrelated *achr* (acetylcholine receptor gene) promoters. As shown in **Figure 27,** both Myc (lane 4) and CycT1 (lane 6) binds to the *cad* promoter but not to the unrelated *achr* promoter. As negative control of immunoprecipitation, chromatin was incubated without the specific antibody (lane 1 and 2). B represents the negative control of PCR in which the oligo have been tested in absence of DNA. In conclusion, in this experiment I can address that Myc and CycT1 are recruited to *cad* promoter after mitogenic stimulus and Myc induction suggesting that Cyclin T1 is recruited on *cad* DNA only after Myc induction.

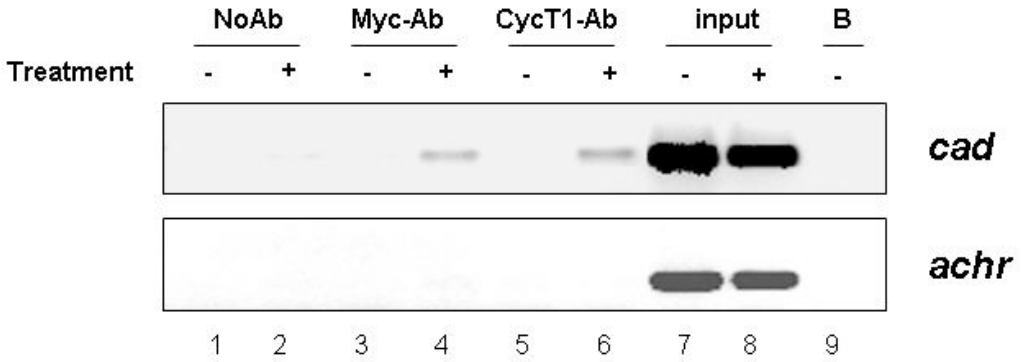


Figure 27. ChIP analysis of Myc and CycT1 on *cad* promoter. Quiescent Rat1 cells expressing a Myc-estrogen-receptor (ER) chimaera were analyzed by chromatin precipitation with Myc and CycT1 antibodies before (-) or after (+) treatment with serum plus 4-hydroxytamoxifem (OHT). PCR analysis was performed with primers that recognize Myc-bound E-boxes in *cad* or with primers that amplify the *ACHR* promoter, which was used as a control.

What is interesting is that also CyclinT1 binding to *cad* promoter appears to be dependent from OHT induction. To further investigate if P-TEFb could be recruited on *cad* promoter by Myc, I performed a ChIP-re-ChIP assay on *cad* promoter. ChIP-re-ChIP assay is used to ascertain whether two proteins can simultaneously associate with the same genomic region *in vivo*. Protein–DNA complexes recovered from the first immunoprecipitation are subjected to an additional immunoprecipitation with a second antibody of a different specificity. The crosslinks of these doubly immunoprecipitated protein–DNA complexes are then reversed, and the DNAs are analyzed by quantitative PCR in an analogous manner to conventional ChIP samples.

The ChIP-re-ChIP was performed using chromatin from both the Rat1-Myc-ER cells starved (-) and the Rat1-Myc-ER cells starved and stimulated by serum and OHT (+). Chromatin was immunoprecipitated (IP) with the Myc antibody or with the CycT1 antibody. In particular, the DNA-proteins obtained from the CycT1 IP, after elution from the beads, were re-immunoprecipitated with Myc antibody. The DNA recovered from both Myc and CycT1 immunoprecipitation and from Myc re-immunoprecipitation was used as template in PCR assay by using oligos specific for the *cad* and unrelated the *achr* promoters. As shown in **Figure 28**, results by the first ChIP confirm that Myc and CycT1 were recruited on *cad* gene by serum and OHT

stimulation (lane 6 and 8). Furthermore the re-ChIP results demostrate that CycT1/Myc complex was present on *cad* promoters (lane 10). As negative control of immunoprecipitation or re-immunoprecitation, chromatin was incubated without the specific antibody (lane 1, 2, 3 and 4). B represents the negative control of PCR in which the oligo have been tested in absence of DNA. This finding strongly supports the idea that CyclinT1 is recruited on the *cad* promoter only after serum and OHT induction and that CyclinT1 is recruited on promoters by Myc direct binding.

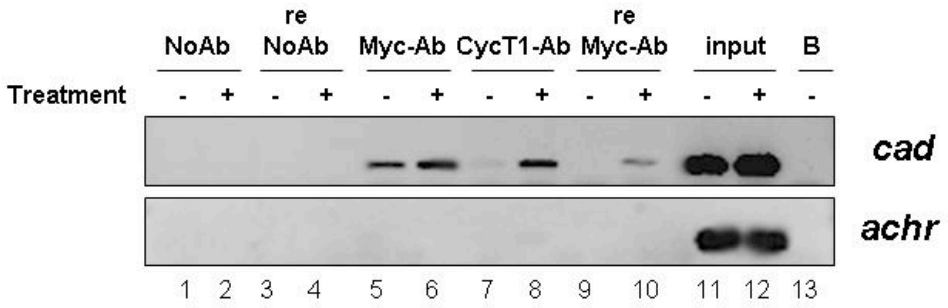


Figure 28. ChIP and ChIP-re-ChIP analysis of Myc and CycT1 on *cad* promoter. Quiescent Rat1 cells expressing a Myc-estrogen-receptor (ER) chimaera were treated (+) or not (-) with serum plus 4-hydroxytamoxifem (OHT). The cells were analyzed by chromatin precipitation with Myc (lane 5, 6) and CycT1 (lane 7, 8) antibodies. The chromatin immunoprecipitated with CycT1 antobody were re-immunoprecipitated with Myc antibody (lane 9, 10). PCR analysis was performed with primers that recognize Myc-bound E-boxes in *cad* or with primers that amplify the *ACHR* promoter, which was used as a control.

Finally, I plan to investigate if ARF overexpression can compete with the occupancy of the Myc-CycT1 complex on *cad* promoter. To this end, I needed to overexpress p14ARF in quiescente Rat1-Myc-ER cells and then stimulate Myc recruitment on *cad* promoter by serum and OHT induction and the co-localization of c-Myc, P-TEFb and over-expressed p14ARF protein would be then determined by ChIP and Chip-re-Chip experiment. However, after several attempts to transfect the Rat1-Myc-ER I have found that this cell line is poorly transfectable; hence, I am constructing a Lentivirus vector expressing the p14ARF protein, that is suitable to infect quiesciente Rat1 c-Myc-ER cells with high efficiency.

Discussion

Biochemical characterization of the c-Myc and N-Myc/p14ARF interaction.

Myc is the first oncogene identified to regulate ARF function. Overexpression of Myc in B-lymphocytes augments cell proliferation which is counteracted with 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 observations suggest that this pathway is not so simple. ARF induction requires very high and sustained Myc activity and a physiological level of Myc do not activate the ARF promoter. Cells with p53-null or p53-mutated status have marked upregulation of p19ARF which is suppressed by overexpression of p53 and p53 also inhibits Myc expression. Thus, there is a feedback regulation among these proteins the balance of which determines the ultimate fate of the cells (**Figure 29**). Adding to this complexity is the recent findings that p19ARF directly interacts with Myc and interferes with Myc's transcriptional activity independent of Mdm2 and p53. Interaction of Myc with p19ARF relocates p19ARF from the nucleolus to necleoplasm in both wild type and p53-null MEFs or conversely p19ARF relocalize Myc into the nucleolus in U2OS cells.

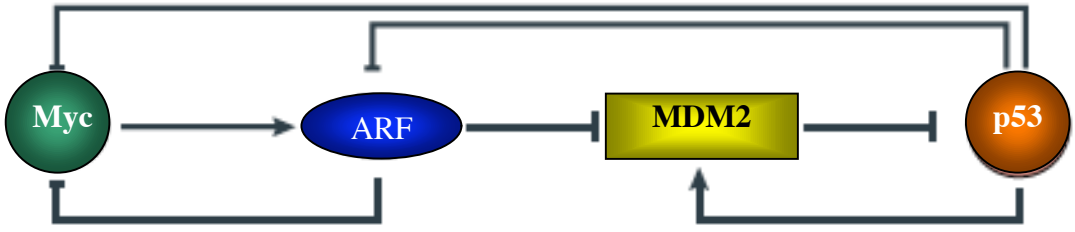


Figure 29. Functional interactions of Myc, ARF and p53. Myc is one of several ongogenes that can induce ARF expression when overexpressed, which in turn antagonizes the function of MDM2 to stabilize p53. The p53 protein not only feeds back to induce the transcription of MDM2 but also negatively regulates 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.

ARF inhibits Myc-induced transcriptional activation of target genes, such as, eIF4E, nucleolin, TERT, Cdk4 and Cul1, but does not affect Myc-induced transcriptional repression of GADD45 and INK4B genes. ARF thus blocks Myc-induced hyperproliferation and transformation by blocking transactivation of key Myc-target genes. Although p19ARF and p14ARF are the ARF gene products in mouse and human, respectively, they share limited sequence homology at the levels of cDNA and protein. p19ARF is a 169 a.a. protein while p14ARF is a 132 a.a. protein. While p19ARF is induced during Ras-mediated senescence, p14ARF is not.

These potential differences indicate that findings observed with p19ARF need to be experimentally validated for p14ARF before they can be generally attributable to ARF gene product as a whole.

In this study I wished also to demonstrate if the two members of the Myc family, c-Myc and N-Myc, were both involved in ARF binding. Despite the considerable sequence divergence among c-Myc and N-Myc (Nau et al., 1985), complementation experiments performed in Rat1a 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 c-Myc and N-Myc are expressed in highly complementary patterns during embryonic development (Hurlin et al., 1997). Nearly everywhere N-Myc is expressed, c-Myc is not expressed, and vice versa, but collectively they encompass most, if not all, proliferating cell types. Among the members of the family, the N-Myc oncogene is implicated in the pathogenesis of neural crest derived tumours including neuroblastoma, the most frequent solid malignancy in infants. Amplification of N-Myc gene is the major negative prognostic marker in human neuroblastomas. Hence, given the sequence divergence among c-Myc and N-Myc and the great interest for N-Myc implicated in the pathogenesis of the neuroblastoma tumour, it was important to investigate if also N-Myc is able to contact p14ARF.

The data that I have presented in this study, I demonstrate that p14ARF interacts with

c-Myc *in vivo*. I have shown by in vitro pull down assays that c-Myc binds directly to p14ARF and that c-Myc, Max and p14ARF are able to form a trimeric complex *in vitro* suggesting that there isn't a mutually exclusive interaction between these three proteins. In addition, through *in vitro* pull down assays and with *in vivo* CoIP, I demonstrate that the Myc Box II domain is critical for the interaction with p14ARF.
Moreover, I demonstrate with in vivo CoIP that also N-Myc is able to bind p14ARF. I also individuate, with in vivo CoIP, the Myc Box III as the domain through which N-Myc contacts p14ARF.
It is pertinent to note that these studies demonstrate that c-Myc and N-Myc interact p14ARF through different conserved domains. Actually the Myc Box II and the Myc Box III have been demonstrated indispensable for many aspects of Myc function among which also his transcriptional activity (Frank at al., 2003 and Herbst at al., 2005).
Previous studies have shown that, depending from the cell line in which the Myc-ARF interaction has been investigated, upon ectopic expression of p19ARF, Myc colocalizes with p19ARF in nucleoli. In U2OS cells and in SKNBE (neuroblastoma) cells I demonstrate that, while both c-Myc and N-Myc proteins are nucleoplasmatic proteins, the p14ARF protein has a predominantly nucleolar localization. When c-Myc and p14ARF or N-Myc and p14ARF were co-expressed I found that the majority of cotransfected cells exhibited colocalization of the c-Myc/p14ARF complex and of the N-Myc/p14ARF complex into the nucleoli. Moreover, when p14ARF and the c-Myc ΔMBII mutant or p14ARF and the N-Myc ΔMBIII mutant were co-expressed only a little percentage of co-transfected cells exhibited colocalization of the c-Myc ΔMBII mutant and p14ARF proteins and N-Myc ΔMBIII mutant and p14ARF proteins into the nucleoli. These findings underlie the relevance of the MBII and MBIII domains in the physiological interaction between the ARF and Myc protein.
Finally, by in vivo CoIP analysis, using two p14ARF deletion mutants, I demonstrate that the same N-terminal domain of p14ARF is responsible for the binding to both c-Myc and N-Myc.

Functional characterization of Myc/ARF interaction.

It has been demonstrated that p19ARF could associate with Myc on chromatin, antagonizing the transactivation of selected Myc target genes without impairing its

transrepression of others (Gregory at al., 2005).

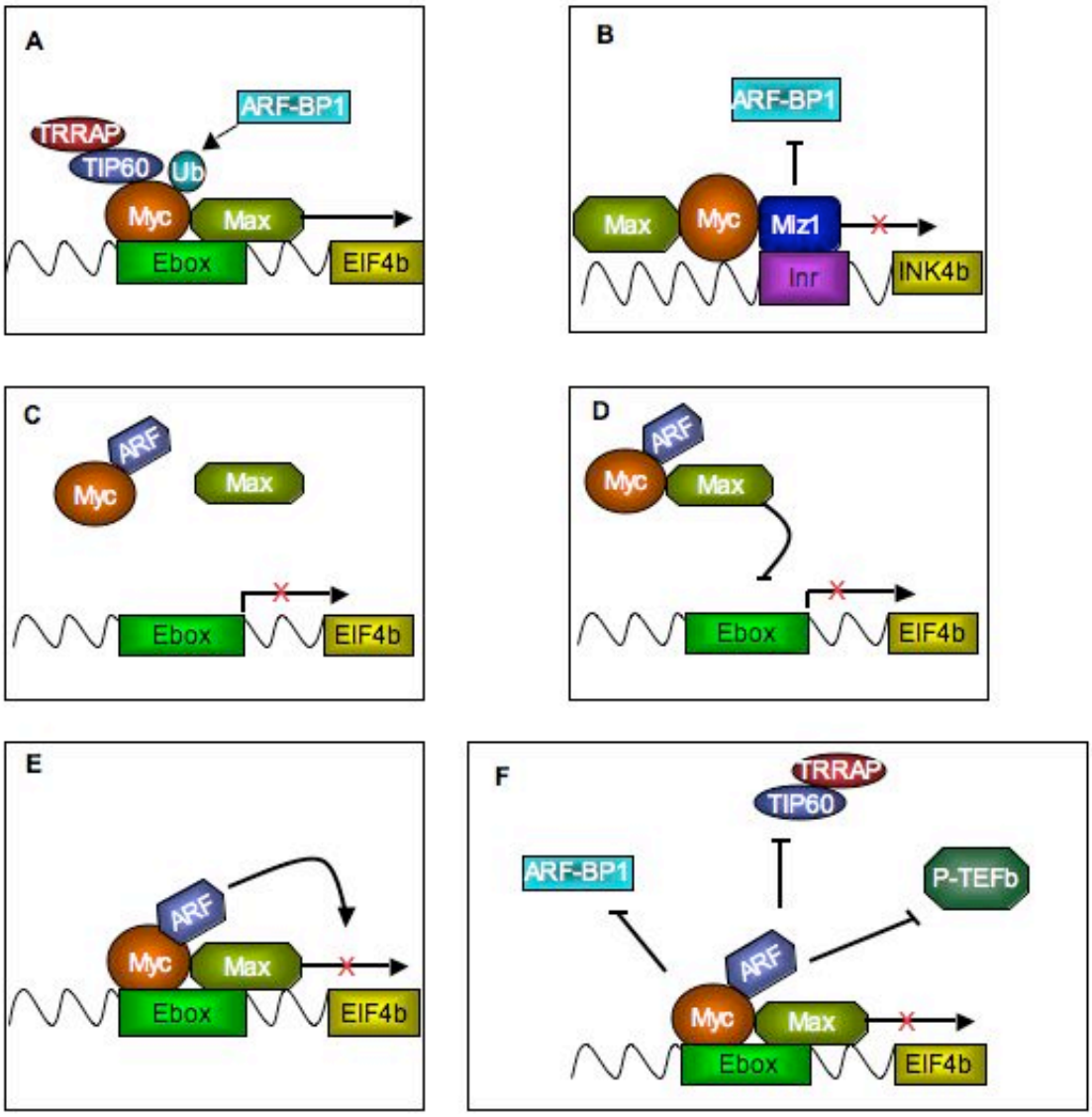
There are several ways that ARF binding to Myc might inhibit its transactivating functions. One mechanism might involve ARF-induced sumoylation of Myc containing complexes or of neighboring histones (Shiio at al., 2003).

As show in **Figure 30** 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 at 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 at al., 2005) (**A**). By contrast, the Myc transrepressing cofactor Miz1 antagonizes this modification (**B**). ARF strongly inhibits the ubiquitin ligase activity of ARF-BP1, which might contribute to the selective dampening of Myc transactivating activity by ARF.

The dampening effects of p19ARF on Myc-regulated transcription may result from interference with Myc binding to its heterodimerization partner Max (**C**), from interference with Myc/Max heterodimer binding to Ebox (**D**) or from an intrinsic repression domain (**E**).

Moreover ARF can also inhibit Myc interfering with the binding of cofactors as the histone acetyl transferase TIP60 or P-TEFb (**F**).

Figure 30. Putative molecular mechanisms by which ARF repress Myc activity. The Myc–Max heterodimer binds to Ebox (CACGTG) consensus sequences to activate transcription. Activation depends on the recruitment of cofactors such as TRRAP, TIP60 and P-TEFb and on Myc ubiquitylation (Ub) by ARF-BP1 (**A**). 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 (**B**). ARF is show to inhibit Myc preventing Max binding (**C**) or Myc-Max binding to Ebox (**D**) or from a intrinsic repressor domain (**E**). ARF is shown to interact directly with DNA-bound Myc to inhibit activation by these different cofactors. Transcriptional activation and antagonism (**F**) 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. The target spectrum for Myc–Max–Miz1 repression is different because of the requirement for Inr elements at the promoter, and includes genes such as INK4b (shown) and GADD45, neither of which can be regulated by ARF.



First of all, I performed different assays in which I demonstrate that p14ARF is able to repress the c-Myc's transactivation as well as the N-Myc's transcriptional activity mediated on the *htert* promoter.

Different hypothesis, as reported above, can be advanced to explain the molecular mechanism by which ARF repress the Myc-mediated transcription. The findings reported in this work discard same of this hypothesis and suggest a hypothetical mechanism for ARF mediated inhibition of Myc activity.

To understand the molecular mechanism by which ARF represses Myc's transactivation I have performed several approaches.

First of all, I have found that p14ARF protein is not able to repress transcription when artificially recruited on a targeted promoter demonstrating that ARF does not possess

an intrinsic repressor domain. In order to investigate if ARF could affect recruitment of Myc-Max heterodimer on DNA target promoters, I performed gels retardation experiments. These experiments have indicated that expression of the p14ARF protein in the 293T and U2OS cells does not reduce the association of the Myc-Max complex to the target DNA sequences. Accordingly, ChIP analysis on Myc targeted promoters indicate that Myc binds to the elF4E and nucleolin genes promoters whether or not p19ARF is present indicating that p19ARF does not affect recruitment of Myc on these target genes (Qi at al., 2004). Hence, ARF does not interfere with the Myc-Max binding to Ebox.

From the data that I have presented I can assess that the Myc Box II and the Myc BoxIII are critical for c-Myc and N-Myc binding, respectively, with ARF. In particular for c-Myc, I have identified 30 amino acids encompassing the Myc Box II domain required for p14ARF interaction and colocalization in vivo. A large number of evidences demonstrated that Myc Box II is required for activation and repression of most target genes (Adnikary at 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 at al., 1998) and the ATPases TIP48 and TIP49 found in chromatin remodelling complexes (Frank at al., 2001). Moreover, it has been found that the Myc BoxI is required for P-TEFb binding and activation of the *cad* gene expression. However, it is not known if these proteins bind Myc simultaneously or Myc forms different complexes with some of them.

It is pertinent to speculate whether ARF binding may alter the association of Myc to these cofactors either through sterical hindrance or to a mutually exclusive interaction due to binding to the same domain.

Hence, I investigated if ARF could interfere with the binding the Cyclin T1, component of the P-TEFb complex, to Myc demonstrating that overexpression of ARF or a deletion mutant expressing only the ARF domain involved in Myc binding (ARF1-65) is sufficient to antagonize the CycT1 binding to Myc. These findings strongly support the hypothesis by which p14ARF may inhibit the Myc transcriptional activity by competition with the P-TEFb binding to Myc (**Figure 31**).

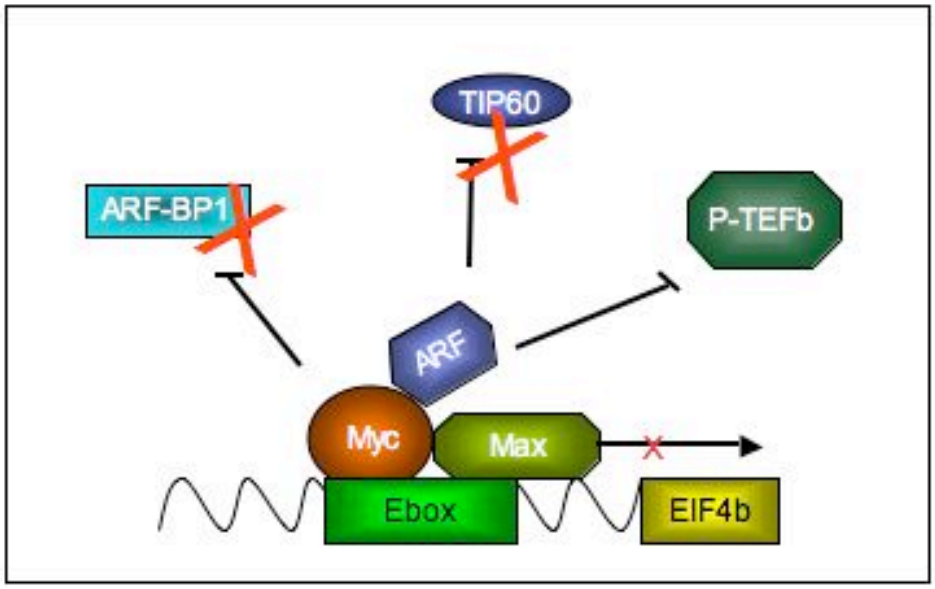


Figure 31. Putative molecular mechanism by which ARF could repress Myc transactivation activity. The molecular mechanism by which ARF could repress Myc-mediatedtranscription impairing P-TEFb binding to Myc-Max complex. is shown

Finally, I demonstrate that c-Myc/CycT1 complex is seated on *cad* promoter, it remains to be shown if c-Myc, P-TEFb and ARF binds simultaneously to target genes or Myc form different complex with them on target genes.

Even if the region involved in ARF binding seems to be different among c-Myc and N-Myc it has to be noted that the Myc BoxIII has reported to be important in the regulation of the N-Myc transcriptional activity (Herbst at al., 2005). Moreover, it still remains to understand the molecular mechanism by which ARF repress the N-Myc transcriptional activity.

After 25 years by which Myc was discovered it still remains a great enigma.

Methods

Plasmids.

GAl4-KRAB and G5-83HIV-Luc plasmids were already available in lab. Gal4-p14ARF was constructed by inserting an EcoRI/SalI fragment containing the ARF cDNA obtained from the GFP-p14ARF into the pSG424 vector. The same insert was subcloned in pPROEX HTa vector (GIBCO Life Technologies) to obtain 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). p3xFLAG-CycT1 plasmid was already available in lab. PCR reactions the cDNA were performed with PFU TURBO DNA Polymerase (Stratagene). Oligo sequences are available upon request.

The integrity of all the plasmid used in this study was confirmed by sequence analysis

In vitro proteins binding assays.

BL21 bacteria were transformed with prokaryotic expression vector carrying the cDNA of the protein of our interest. Singles colonies were inoculated in 20 ml LB medium with 100 μg /ml Ampicillin which was placed in a 37°C shaker overnight. The next day these starter cultures were used to inoculate 200 ml LB medium which contained 100 μg/ml Ampicillin. Protein induction was carried out with the addition of 0.5mM IPTG after the cells reached an OD_{590} of 0.4-07 and incubated O.N. at 30°C. Bacteria were spun down at 5,000 rpm for 10 min at 4°C and resuspended in PBS + 1mM PMSF + 5mM β-mercaptoethanol. After adding Sarkosil to a final concentration

of 1.5%, the samples were sonicated on ice for 15 min with 2 sec pulses every 2 sec. Triton to final concentration of 1% was added to the lysates, and the NaCl concentration increased up to 250mM. The samples were vortexed vigorously and put rotating 30 min at 4°C. The lysates were centrifuged at 25,000 rpm for 30 min at 4°C and then passed through 0.45μm filter (NALGENE).

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: in the first step the recombinant protein was affinity purified with ANTI-FLAG M2-Agarose Affinity Gel (Sigma) and subsequently eluted by incubation with the FLAG peptide; in the second step the resulting protein was incubated with Monoclonal Anti-HA Agarose Conjugate (Sigma) and eluted from these beads by using HA Peptide (Sigma). 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 Laemmli buffer, separated on SDS PAGE followed by Western Blotting.

Cell culture, luciferase assays and immunofluorescence.

Human 293T, SKNBE, U2OS, RAT1-Myc-ER cell lines were grown in DMEM supplemented with 10% fetal calf serum.

For the luciferase assay, U2OS cells were transfected with lipofectamine 2000 (Invitrogen Technologies) and pRLCMV (Promega) was co-transfected for normalization. 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.

TET21N cells were maintained in presence of tetracycline (1μg/ml) for at least one week before transfection. $1x10^5$ cells/well were plated and grown an additional 24 h in 6 well plates in the presence of tetracycline. Cells were washed with 1X PBS and then transfected with the appropriate hTERT-Luc construct (100 ng) and different amounts (0,1; 0,5 and 1µg, respectively) of pcDNA-p14ARF-HA constructs by the PEI method. After transfection, cells were incubated for 48h in presence or absence of tetracycline as required. Activity of firefly or Renilla luciferase was measured with the dual luciferase assay kit (Promega) according to the manufacturer's instructions by using a T20/20 luminometer (Turner Design).

Cos cells were cotransfected by Lipofectamine with 100ng the G5-83HIV-Luc vector, GAL4-p14ARF (0,5 and 1µg) and GAL4-KRAB (0,5 and 1µg).

For immunofluorescence analysis U2OS and SKNBE cells were transfected with lipofectamine 2000 with 200ng of the pcDNA3-FLAG-Myc, pcDNA3-FLAG-MycΔ123-151, GAL4-KRAB, Gal4-ARF, GFP-ARF, pcDNA-N-Myc and pcDNA-N-Myc d1-300 plasmid and cells processed as described16 using the anti-GAL4 (Upstate Bio,Inc) and anti-Myc (9E10, Santa Cruz Biotechnology, Inc.) antibodies.

Calcium phosphate transfection.

The cells used in CoIP experiments were transfected according a modification of the calcium phosphate precipitation technique described by Wigler et al. (1977). Briefly, one day before transfection, 2.3×10^6 293T cells, were seeded in 10-cm dish. One day later, a calcium-phosphate precipitation of DNA (20-25 µg of plasmid) was prepared and added to the medium covering the cell. The cells were incubated for 24 hours, and the medium was replaced. After 40 hours from the transfection the cells were harvested.

Whole cell extract.

Cells were harvested and washed in cold PBS. After centrifugation the cellular pellet was resuspended in bfr F (10mM Tris pH 7.05, 150mM NaCl, 30mM Na Pyrophosphate, 50mM NaF, 5µM $ZnCl_2$, 1% Triton, 0.1mM Na_3VO_4, 1mM PMSF, Protease Inhibitors) and vortexed 30 min on ice. The lysates were then centrifugated at 14.000 rpm for 30 min at 4°C.

Antibodies and coimmunoprecipitations.

The following antibodies were used for the immunological techniques: anti-Myc (N262 for IP and 9E10 for WB, Santa Cruz Biotechnology, Inc.), anti-Max (C17, Santa Cruz Biotechnology, Inc.), anti-ARF (C-18, Santa Cruz Biotechnology, Inc.), anti-FLAG M2 Monoclonal Antibody-Peroxidase Conjugate (Sigma), anti-GST (B-14, Santa Cruz Biotechnology, Inc.), 6xHis Monoclonal Antibody (BD Biosciences), anti-GST (B-14, Santa Cruz Biotechnology, Inc.), anti Gal4 (Upstate Bio,Inc.), anti N-Myc (2,Santa Cruz Biotechnology, Inc.), anti CycT1 (H245 for immunoprecipitation, C-20, T18 and N19 for WB, Santa Cruz Biotechnology, Inc.).

Co-immunoprecipitations from transiently transfected cells was 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 and resuspended in SDS loading bfr (10% glycerol, 60m Tris-HCl pH 6.8, 2% SDS, 0.025% BrPh Blue, 50mM DTT).

For c-Myc IP, 5 μg of antibody or IgG plus 25 μl of pre-equilibrated protein G-Sepharose 4 Fast Flow (Amersharm Biosciences) was used; for Max IP, 3 μg of antibody or IgG was used plus 25 μl of pre-equilibrated protein G-Sepharose 4 Fast Flow (Amersharm Biosciences) was used. For FLAG IP, 25 μl of pre-equilibrated anti-FLAG M2-Agarose Affinity Gel (Sigma) was used. The interactions were carried out overnight at 4°C. Beads were washed at least five times 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 SDSPAGE.

Electrophoretic mobility shift assays.

Gel shift assays were performed with radiolabelled GC binding sites of the E-box. Santa Cruz Biotechnology supplied the Myc-Max consensus oligonucletides and the Myc-Max mutated olignucleotides. Binding reactions for band shift assay used a 20 ml volume of reaction mix consisting of 10 mM HEPES pH 7.9, 25 mM NaCl, 0.05 mM EDTA, 2.5% glycerol, 2 mM $MgCl_2$ and 1 μg poly (dI-dC). Proteins (20 μg) were added to the reaction mix and pre-incubated for 10 min on ice. Labelled

oligonucleotide (2,1 pmol) was then added and the reaction incubated for 20 min at 20'C. 150-fold of molar excess of the unlabelled oligonucleotide was added together with the labelled DNA following the pre-incubation period. DNA-protein complexes were loaded on 5% polyacrylamide gels containing 0.5 x TBE and electrophoresed at 200 V for 2-3 h. The gels were then dried and subjected to autoradiography.

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. All oligoprimers were described in Frank at al., 2002. 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 genes (large subunit of RNA pol II and HPRT). After normalization the data are presented as fold change relative to the 0 point.

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.

ChIP-re-ChIP analysis.

Nuclear proteins were cross-linked to genomic DNA by adding formaldehyde for 10 min directly to the medium to a final concentration of 1%. Cross-linking was stopped by adding glycine to a final concentration of 0.125mM 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.

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Appendix I

Regulation of transcription elongation has been a major topic of the Lania's lab since several years. In the first two years of my PhD, I have been involved in a proteomic study aimed to identify the CTD-phosphatase FCP1-interacting partners (Licciardo et al, 2003, Amente et al, 2005).

The carboxyl-terminal domain (CTD) of RNA polymerase II (RNAPII) is essential for transcription and has a fundamental role as a critical platform for recruiting and coordinating the actions of proteins involved in mRNA maturation and export of the mature transcript to the cytoplasm (Conaway at al., 2000; Kobor at al., 2002; Palancade at al., 2003). The phosphorylation pattern of the CTD undergoes defined changes during transcription; specifically, the phosphorylation of serine 5 occurs between transcription initiation and promoter clearance and modification of serine 2 is found only when RNAPII is associated with the coding region of the gene (Komarnitsky at al., 2000). Protein serine kinases and protein serine phosphatases are implicated in CTD modification by reversible phosphorylation during the transition from initiation to elongation (Palancade at al., 2003; Archambault at al., 1998; Majello at al., 2001). Stimulation of RNAPII promoter clearance and efficient transcription elongation is associated with the RNA pol II kinases, TFIIH (serine 5) and Positive Transcription Elongation Factor-b (P-TEFb, serine 2), respectively (Silver at al., 2001).

Among the CTD phosphatases, FCP1 has been demonstrated to facilitate recycling of the hyperphosphorylated form of RNAPII allowing RNAPII to reenter another round of transcription. FCP1 has been found to be associated with promoter and coding regions, suggesting a constant requirement for FCP1 during transcription elongation. FCP1 is a phosphoprotein in vivo and phosphorylation of FCP1 is important for its optimal CTD phosphatase and transcription elongation activities.

In addition to its CTD phosphatase activity, FCP1 is involved in transcription regulation as a stochiometric component of the elongation complex (Archambault at al., 1997; Kimura at al., 2002).

In order to isolate FCP1 partners I have generated a stably transfected cell line expressing physiological levels of a FLAG-epitope tagged FCP1 protein. I

demonstrated that in addition to RAP74, a previously known FCP1 interacting factor, FCP1 interacts with the RNAPII itself independently from the status of phosphorylation of the CTD, suggesting the presence of the FCP1/TFIIF/RNAPII complex in vivo (Licciardo et al, 2003).

By mass spectrometry (MALDI-TOF) of affinity-purified extracts, I have identified as novel FCP1 partners, i.e. MEP50, PRMT5 and the enhancer of rudimentary homologue (ERH) proteins. Intriguingly, these proteins are interacting partners of a different transcription elongation factor: the SPT5 protein, suggesting a putative role of these proteins in controlling transcription elongation (Licciardo et al, 2003, Amente et al, 2005). PRMT5 is type II arginine methyltransferase also involved in transcriptional repression of the Myc/Max/Mad target gene *cad* (Pal at al., 2003), while the MEP50 protein is one of three components (together with PRMT5 and pICln) of the methylosome required for the methylation and assembly of components of the snRNP core particles. Interestingly, I found that the MEP50 and PRMT5 proteins associated with FCP1 specifically in the nucleus as a complex distinct in size and subcellular localization from the methylosome complex. Moreover, I found that FCP1 is a genuine substrate of PRMT5-methylation both in vivo and in vitro, and FCP1-associated PRMT5 can methylate histones H4 in vitro. ERH, instead, is the product of the erh gene, a putative transcriptional repressor (Pogge von Strandmann at al., 2003). In drosophila, mutations in the rudimentary (r) gene (orthologue of the human *cad* gene) determine in the adult drosophila the phenotype of rudimentary flies. This aberrant phenotype is enhanced by the contemporary mutation in the enhancer of rudimentary (er) gene (orthologue of the human erh gene) (Wojcik at al., 1994). Given *cad* a gene typically Myc-regulated and on the basis of the genetic interaction between r/*cad* and er/erh, it is also possible to indicate ERH as a putative new transcriptional repressor of Myc.

Reassuming, PRMT5 and ERH can regulate the Myc-mediate transcription. However, while for PRMT5 there are evidences that show so, for ERH these evidences are still lacking.

Appendix II