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" FEDERICO II "

DOTTORATO IN

GENETICA E MEDICINA MOLECOLARE

XVII CICLO

"Molecular and functional characterization of the interaction between c-Myc and the MCM helicase"

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CHAPTER I
Introduction

1.1 c-Myc: the origins

In 1911 Peyton Rous observed that chicken sarcoma could be transmitted through cell-free extracts from the tumors, 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, which induces myeloid leukemia, sarcomas, liver, kidney, and other tumors in chickens, led to the identification of the v-*myc* oncogene. Its cellular homolog, the c-*myc* gene, was discovered more than 20 years ago (Crews et al., 1982; Dalla-Favera et al., 1982).

The c-*myc* gene is located on human chromosome 8q24. It was discovered soon after its identification that activated oncogenic c-MYC was instrumental in the progression of human Burkitt's lymphoma, as a result of a translocation between chromosome 8 and one of the three chromosomes that contain antibody-encoding genes (Taub et al., 1982; Dalla-Favera et al., 1982). Elevated or deregulated expression of c-*MYC* has been detected in a wide range of human cancers, and is often associated with aggressive, poorly differentiated tumours. Such cancers include breast, colon, cervical, small-cell lung carcinomas, osteosarcomas, glioblastomas, melanoma and myeloid leukaemias (Dang, 1999; Nesbit et al., 1999)

1.2 Who is c-Myc

Four transcriptional promoters have been identified, but RNA initiated at the P2 promoter usually contributes to 80-90% of total c-*myc* steady-state RNA in normal cells (Taub *et al.*, 1984). A shift in the transcription starting point has been documented in Burkitt's cell lines, where transcription of the translocated c-*myc* is preferentially initiated further upstream at promoter P1 instead of at P2 (Strobl and Eick, 1992; Strobl *et al.*, 1993; Taub *et al.*, 1984). The cause of this promoter shift is not known.

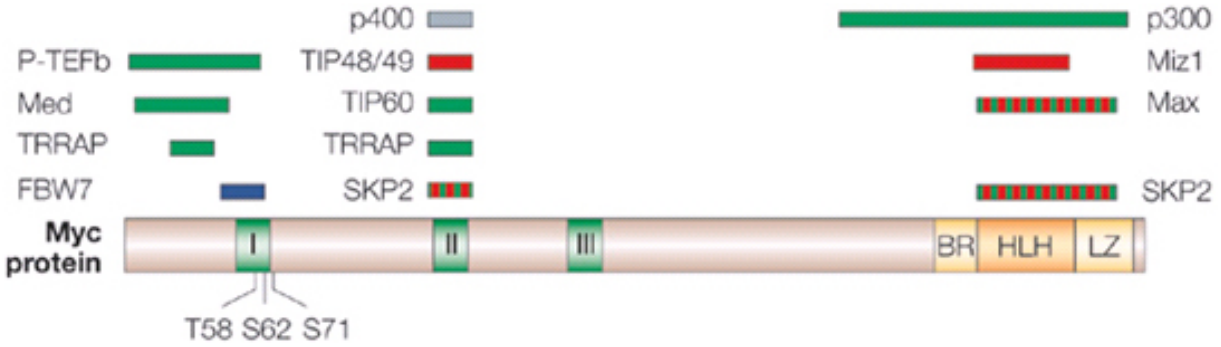
The c-*myc* gene comprises three exons. Exon 1 contains two promoters and is non coding. Exons 2 and 3 encode the Myc protein resulting in the the major 64-kDa

polypeptide with translation initiation at the canonical AUG start codon nucleotide 16 of exon 2. A longer polypeptide of 67 kDa results from translation initiated 15 codons upstream of the AUG at a CUG codon (exon 1) (Hann et al., 1992). An internal translationally initiated c-Myc 45-kDa polypeptide was recently recognized (Spotts et al., 1997).

In mammals, there are four related genes in the family, c-*Myc*, N-*Myc*, L-*Myc* and S-*Myc*. 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., 2002,; Eisenman, 2001). The N-terminal 143 amino acids and the C-terminal 140 amino acids of c-Myc are required for neoplastic transformation and inhibition of cellular differentiation (Dang, 1999). These two regions correspond to the N-terminal transactivation domain and the C-terminal DNA-binding and HLHzip dimerization domain The N terminus of Myc has three highly conserved elements, known as Mycboxes. Of these, MycboxI has been implicated in Myc turnover (Bahram et al., 2000). MycboxIII also regulates protein stability but is essential for Myc function *in vivo*, and is required for full transactivation and transrepression of many target genes (Herbst et al., 2004, 2005). Most attention has focused on understanding the function of MycboxII, the second Mycbox in the N terminus of Myc, as it is required for all the known biological functions of Myc. MycboxII is not involved in the binding of Myc to Max or to DNA, but is required for activation and repression of most, but not all, Myc target genes. Immediately N-terminal to the dimerization domain there is a domain rich in basic amino acids which directly contacts specific DNA sequences within the DNA major groove (Dang et al., 1992; Dong et al., 1994; Ferre-D'Amare et al., 1993) (**Figure 1**).

1.3 c-Myc and friends (molecular partners)

Several proteins can bind directly to MycboxII, raising the question of whether they bind simultaneously or whether Myc forms separate complexes with each of these proteins. One is TRRAP, which is a core subunit of the TIP60 and GCN5 Histone Acetyl Transferase (HAT) complexes (McMahon et al., 1998) and the recruitment depends on the integrity of MycboxII (Bouchard et al., 2001; Frank et al., 2001). Most probably,



(Adhikary S. and Eilers M.; *Nat Rev Cell Biol 2005*)

Figure 1: Domains of Myc and their binding proteins. The N terminus of Myc has three highly conserved elements, known as Mycboxesl–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.

therefore, transcriptional activation of some target genes depends on the recruitment of HAT activity by Myc. TRRAP is also part of a complex containing the p400 E1A-binding protein, which is devoid of HAT activity (Fuchs et al., 2001). This second complex is also found in association with Myc, which indicates that the Myc–TRRAP interaction has other roles in addition to the recruitment of HAT activity. Such roles might include the capacity to exchange histones, in particular histone H2A *(*Kusch T et al., 2004).

Two other proteins that are found in the TIP60 complex bind to MycboxII independently of TRRAP; these are TIP48 and TIP49, two highly conserved hexameric ATPases (Wood et al., 2000). Both proteins are found in several chromatin remodelling complexes. The *Xenopus* homologues of TIP48 and TIP49 have recently been implicated in transcriptional repression by the Myc–Miz1 complex (Etard et al.,2005).

MycboxII is required for interaction with SKP2 of the E3 ubiquitin ligase, SCF^{SKP2}, and Myc recruits SKP2 to its target genes *in vivo* (Kim et al., 2003; von der Lehr et al., 2003). Recruitment of SKP2 is required for the transactivation of several Myc target genes. Surprisingly, Myc is also a substrate of SKP2, which indicates that ubiquitylation of Myc might be required for transcriptional activation. Ubiquitylation by SCF^{SKP2} probably allows Myc to recruit proteasomal subunits that have a role in transcriptional activation that is proteolysis independent.

As mentioned above, not all Myc target genes require the integrity of MycboxII for activation, which shows that there are other mechanisms of Myc-dependent activation and, most likely, repression. For example, CREB-binding protein (CBP) and p300 have been identified as other histone acetyl transferases that interact with Myc and that are recruited to target genes *in vivo*. Both interact with the C terminus of Myc, which shows that the classic separation of Myc domains into DNA-binding and transcriptional-effector domains might be obsolete.

Also Brca1 interacts with the C terminus of Myc and inhibits its transcriptional and transforming activity in cells (Wang et al., 1998).

In vivo, Myc recruits Mediator complexes to its target promoters in a MycboxII-independent manner (Bouchard et al., 2004). The same is true for cyclin-dependent kinase-9 (CDK9), a subunit of the positive transcription elongation factor b (P-TEFb)

complex (Kanazawa et al., 2003). *In vitro*, both Mediator and P-TEFb complexes bind to the extreme N terminus of Myc.

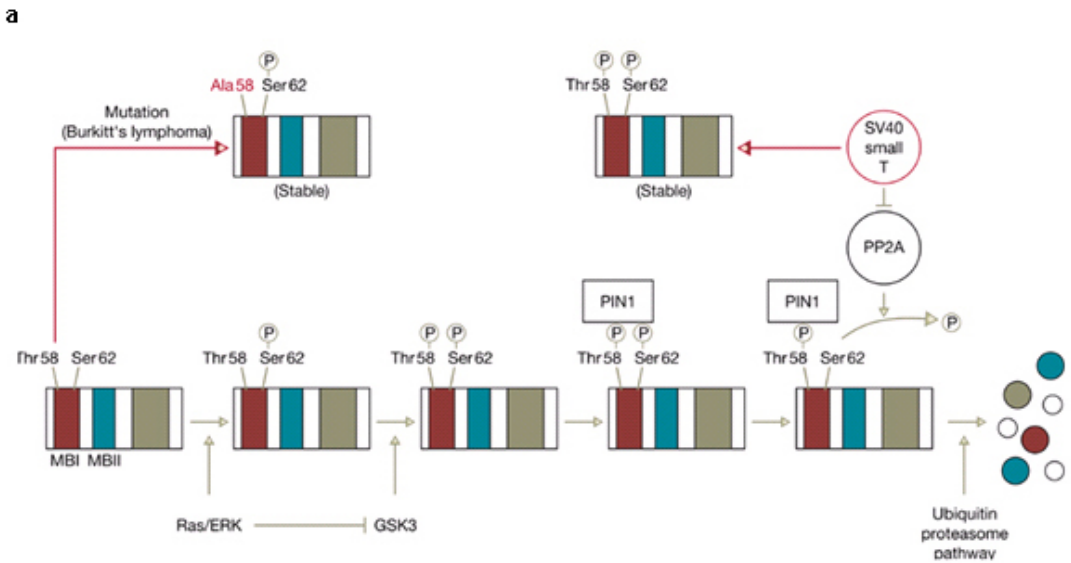
Finally, Myc recruits a DNA methyltransferase, DNMT3a, to the Myc–Miz1 complex, indicating that Myc-dependent gene repression could at least partly be mediated by methylation of its target promoters (Brenner et al., 2005).

1.4 c-Myc regulation

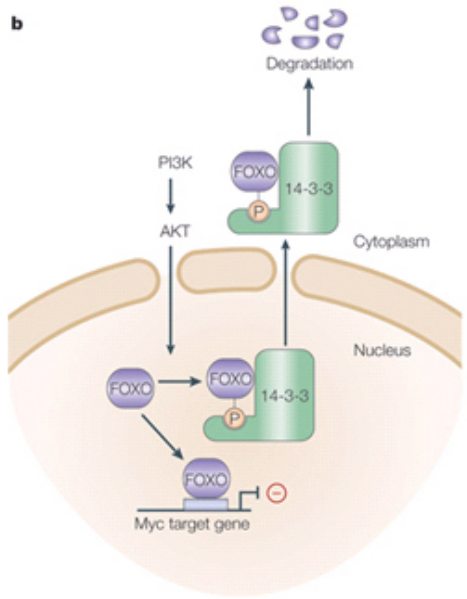
c-Myc activity is normally tightly controlled, at transcription level, by external signals including growth factors, mitogens and β-catenin, which promote and factors such as TGF-β, which inhibit. Its expression also undergoes a negative autoregulatory circuit (Grignani et al., 1990; Lombardi et al., 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; Hayday *et al.*, 1984; Nishikura *et al.*, 1983), 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 2**).



Dominguez-Sola D. and Dalla-Favera R.; *Nat Cell Biol 2004*



Adhikary S. and Eilers M.; *Nat Rev Cell Biol 2005*

Figure 2: Ras-mediated regulation of Myc. a) Myc is stabilized by Ras-dependent ERK-mediated phosphorilation at Ser 62. This modification is necessary for the subsequent phosphorilation at Thr 58 that leads to degradation: PIN1 indice conformational modifications that allow PP2A to defosphorilate Ser 62 and consequent recognition by the proteasome pathway. c-Myc mutation found in Burkitt's lymphoma or in retoviral v-myc oncogene (top let) and SV40 smal T antigen (top right), directly affect this pathway, resulting in stabilization of the c-Myc protein.
MBI: Mycbox I; MBII: Mycbox II; grey box: bHLHZip DNA binding domain.
b) | Several target genes that are transactivated by Myc are repressed by FOXO transcription factors. Activation of the PI3K pathway leads to the AKT-dependent phosphorylation of FOXO proteins and their nuclear export. Nuclear export is mediated by binding of phosphorylated FOXO to the 14-3-3 protein in the nucleus. In the cytosol, FOXO proteins are ubiquitylated by the E3 ubiquitin ligase SCFSKP2 and degraded by the proteasome. ERK, extracellular signal-regulated kinase; MAPK, mitogenactivated protein kinase; Ub, ubiquitin.

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-3βand 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 very 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 degradation of c-Myc.

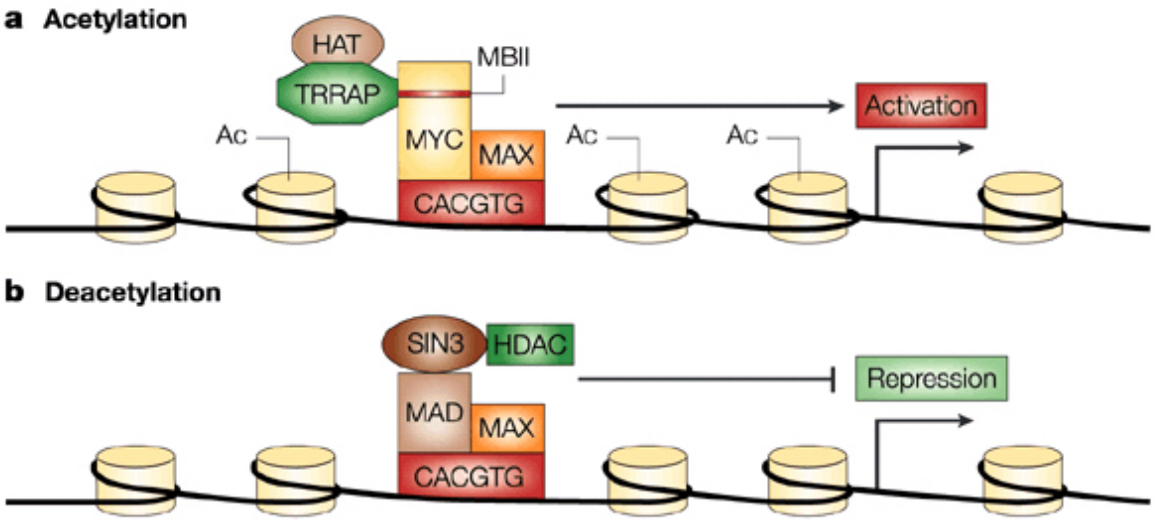
Considerable evidence suggests that Thr 58 phosphorylation is critical for ensuring the transient and timely degradation of c-Myc. All v-*myc* genes recovered in transforming retroviruses harbour mutation at Thr 58. Similarly, a large number of c-*myc* genes amplified in Burkitt's lymphoma carry a mutation at Thr 58, as well as in other residues between amino acids 57 and 63 (Pulverer et al., 1994; Henriksson et al., 1993; Salghetti et al., 1999). Assay of these mutants generally demonstrates their increased oncogenic potential in both cell transformation assays and animals (Chang et al., 2000). In example the c-MycT58A mutant is no longer a substrate for ubiquitination *in vivo* (Sears et al., 2000) and replaces SV40 small T antigen, which inhibits the protein phosphatase-2A (PP2A) and is a strong activator of PI3K, in transforming human fibroblasts in the presence of SV40 large T antigen (SV40T) and telomerase (Yeh et al., 2004).

Another mechanism by witch Ras regulates Myc activity is through the FOXO family 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.

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

1.5 The c-Myc network

No monomeric Myc proteins have been found *in vivo*. Instead, Myc is bound to a partner protein, Max, through a basic-region/helix–loop–helix/leucine-zipper (BR/HLH/LZ) domain (Blackwood et al., 1991, 1992). Max is present in stoichiometric excess to Myc, due to e constitutive gene expression and high stability at mRNA and protein level. Max is now recognized as the central and shared dimerization partner of a rather large network of related b-HLH-Zip transcription factors that function, as transcriptional repressors (Grandori *et al.*, 2000) Indeed it can also form homodimers or heterodimers with several related proteins, known as Mad1, Mxi1 (also known as Mad2), Mad3, Mad4 and Mnt (also known as Rox), as shown by *in vitro* binding experiments (Ayer and Eisenman., 1993; Hurlin et al., 1996,1997). The dimers all bind directly to the same DNA sequence (CACA/GTG), which is a subset of the general E-box sequence (CANNTG) that is bound by all bHLH proteins (Blackwell et al., 1990). *In vivo*, Myc–Max complexes activate transcription through interactions with transcriptional coactivators (such as TRRAP and BAF53) and their associated histone acetyltransferases (HATs, e.g., GCN5) and/or ATPase helicases (TIPs, e.g., TIP49) (McMahon *et al.*, 1998, 2000; Dugan *et al.*, 2002) and are often predominant in proliferating cells (**Figure 3)**. Instead Mad–Max or Mnt–



Pelengaris S., Khan M. and Evan G.; *Nat Rev Cancer 2002*

Figure 3: MYC–MAX and MAD–MAX complexes regulate gene activation through chromatin remodelling. a | MYC–MAX heterodimers binds to an E-box sequence (CACGTG) near the promoter of a c-MYC target gene. Co-activator TRRAP (transformation/transcription domain-associated protein), a component of a complex that contains histone acetyltransferase (HAT) activity, is then recruited to the MYC box II (MBII) domain of c-MYC and acetylates (Ac) nucleosomal histone H4 at the E-box and adjacent regions. Nucleosomal acetylation alters chromatin structure, allowing accessibility of MYC–MAX transcriptional-activator complexes to target DNA, resulting in expression of the target gene. **b** | Induction of MAD during terminal differentiation results in the MAD–MAX heterodimer binding to an E-box of a c-MYC target gene. Corepressor SIN3 and histone deacetylases (HDACs) are then recruited to MAD, resulting in local nucleosomal histone deacetylation and repression of target-gene expression.

Max complexes are predominant in resting or differentiated cells (Ayer and Eisenman., 1993) where actively repress transcription through direct protein-protein interactions with the general transcriptional corepressors Sin3a-3b (Ayer et al., 1995) and, with Sin3's corepressors (e.g., N-Cor and the SkiSno proteins) and histone deacetylases (HDACs) (Alland et al., 1997; Heinzel et al., 1997). Histone deacetylation is currently thought to be the major mode of transcriptional silencing by the Mad proteins. The Sin3-intacting domain motif, when tethered to an HLH/LZ transcriptional factor, TFEB, that binds Myc DNA sites, is able to inhibit c-Myc-mediated cellular transformation (Harper et al., 1996).

Detailed knowledge about the amino-acid residues that dictate leucine-zipper interactions has made it possible to design reciprocal mutants of Myc and Max that heterodimerize with each other, but not with the endogenous proteins (Amati et al., 1993). The analysis of these mutants shows that binding to Max is required for the transforming properties of Myc and the ability of Myc to induce cell-cycle progression and apoptosis. Not surprisingly, heterodimerization with Max is required for the binding of Myc to DNA. Surprisingly, however, this is true not only for the E-box sequence, to which the Myc–Max heterodimer binds directly, but also for the 'non-consensus' binding sites to which Myc is recruited through protein–protein interactions with other DNA binding factors (Mao, 2003). One potential explanation for this finding is that binding to Max might be required for the correct folding of the Myc protein.

A second surprising finding is that Myc and Max exist as an antiparallel tetramer in the crystal structure; tetramerization is mediated by interactions between specific amino acids on the 'outside' of the Myc and Max leucine zippers (Nair and Burley, 2003). The dissociation constant is so low that even the few Myc–Max complexes present in normal cells are expected to exist as tetramers *in vivo*.

When bound to E-box sequences, Myc–Max heterodimers activate transcription, whereas Mad–Max and Mnt–Max heterodimers repress transcription (Ayer et al., 1993, Hurlin et al., 1997). Several experiments have addressed the biological significance of this model. For example, if transcriptional activation is the key biological function of Myc, the model predicts that knockout of the Mad or Mnt repressor proteins should have a similar biological effect as the overexpression of Myc. Indeed, deletion of Mnt in primary mouse

embryonic fibroblasts induces proliferation in the absence of Myc, and mammary carcinomas develop in *Mnt*–/– animals (Nilsson et al., 2004; Hurlin et al., 2003). Similarly, deletion of Mad1 delays the terminal differentiation of granulocytes, and deletion of Mxi1 enhances proliferation in several cell types (Schreiber-Agus et al., 1998; Foley et al., 1998). However, there is little evidence that Mad or Mnt proteins function as tumour suppressor proteins in human tumours. Therefore, interactions of the HLH domain with proteins other than Max contribute to the biological function of Myc. One such protein is Miz1, a zinc-finger protein that binds to the 'outside' of the helix–loop–helix domain of Myc, but does not interact with Max, Mad or any other Myc family members (Herold et al., 2002; Peukert et al., 1997).

1.6 c-Myc target genes

The advent of technologies to study *in vivo* DNA-binding sites of Myc has yielded a number of important advances as well as surprises in the field. The known gene targets of MYC, however, have been far more difficult to assign to pathways that have obvious links to cellcycle progression or malignant transformation. Furthermore, none of the known targets of Myc, including the gene encoding cyclin D2, are able to completely substitute for any specific Myc function (Berns et al., 2000). In addition to its role in regulating cell proliferation, c-*myc* products regulate cell mass (Iritani and Eisenman, 1999; Johnston et al., 1999; Shuhmacher et al., 1999). Although a link between an increase in cell mass and cell-cycle progression has been known for many years, the direct mechanism by which these are related is not understood (Conlon and Raff, 1999). Myc has been shown to control many genes encoding products that regulate ribosome biogenesis and protein translation, which can ultimately affect cell mass and proliferation.

Several recent studies have shaken paradigm this, the model that Myc behaves like other transcription factors to regulate a handful of specific genes. In these studies Myc has been shown to bind not a few, but to thousands of potential target genes (Li et al., 2003; Mao et al., 2003; Fernandez et al., 2003; orian et al., 2003). 10–25% of the Myc targets that are identified are repressed rather than activated (Zeller et al., 2003).

In early studies, putative gene targets of Myc were identified by empirical methods (Dang, 1999). Typically, these studies identified a role for Myc either based on the function of the putative target or because the promoter region of the target gene contained a potential binding site for Myc.

Recently, different microarray-based screens have added over 600 genes to the list of potential Myc targets (Coller et al., 2000). This number might be artificially high, as most of the microarray screens do not distinguish between direct and indirect targets of Myc. In a few cases, a third approach to identifying Myc targets has been used. This approach has relied on screens for genes that can compensate for loss of Myc function. With a few notable exceptions, these kinds of studies have not identified genes that have obvious links to the biological processes typically associated with Myc function (for example, cell-cycle progression, malignant transformation and apoptosis).

Several groups have recently published important studies aimed at more precise identification of the genes that are regulated by Myc. These efforts were based on the wellfounded assumption that Myc, like most other transcription factors, simply binds to specific genes and alters their levels of transcription. The somewhat surprising result from some of these studies is that Myc only alters transcription levels of a minority of the genes it binds to. Fernandez and co-workers have performed a large-scale analysis of Myc binding to promoters that contain the E-box consensus element CACGTG in live human cells (Fernandez et al., 2003). As in the accompanying study of *Drosophila* Myc (Orian et al. 2003), the data reveal that the protein associates with a strikingly large number of genomic loci, suggesting significant diversity in the ensuing transcriptional response. The conserved core of high-affinity Myc-target genes represents roughly 11% of all cellular promoters. This number of target genes is most likely an underestimate, because the screen performed by Fernandez and co-workers was based solely on the "canonical" E-box element CACGTG, whereas another E-box (CACATG or CATGTG) and variant sites (e.g., CACGCG) can also be bound by Myc/Max (Grandori et al. 2000; Oster et al. 2002). A second reason is that Myc and Max are recruited to non-consensus binding sites through the interaction with other transcription factors. One example of such a 'tethering factor' is Miz1, which can recruit Myc and Max to core promoter sequences that lack a CACGTG sequence (Herold et al., 2002; Mao et al., 2003). So,

there is a class of core promoters at which both Myc and Miz1 are bound *in vivo* (Wanzel et al., 2005). As some core promoters have been found at which Myc, but not Miz1, is bound, there must be other proteins that recruit Myc to their cognate DNA-binding sites (Barsyte-Lovejoy et al., 2004).

The direct binding of Myc to such a large number of sites both in humans and *Drosophila* (Orian et al. 2003) was not anticipated and is not a general feature of eukaryotic transcription factors. The Myc-binding patterns that we have observed in our cell lines suggest that target sites in chromatin compete for limiting Myc protein levels. The peak expression of Myc in serum-stimulated human fibroblasts has been estimated at 3–6000 molecules per cell (mpc), whereas cycling cells expressed 1–3000 mpc. Thus, there may rarely, if ever, be enough Myc in normal cells to bind all potential targets, assuming that 11% of cellular genes means >4000 target loci (Hogenesch et al. 2002), many of which possess multiple E-boxes.

The analysis also included a small number of promoters that did not contain E-boxes. In cells with high levels of endogenous Myc, only 7% of non-E-box promoters were occupied. When Myc was ectopically expressed in cells, however, it bound to almost 100% of E-box-containing genes. Surprisingly, ectopic expression of Myc caused it to bind to 88% of these non-E-box promoters.

Among the best predictors of whether a given E-box would be occupied by Myc was its proximity to a CpG island (Fernandez et al., 2003). The Fernandez and co-workers also observed that the chromatin around the Myc-bound loci was highly acetylated, even before Myc binding. This was unexpected, as Myc has been shown to recruit histone acetyltransferase enzymes. These results indicate that Myc might preferentially bind to genes that have a chromatin structure that is already poised for transcription. Equally puzzling, an examination of the Myc-target-gene database reveals that only a minority (10.4%) of Myc bound genes show a transcriptional response to Myc activation (Zeller et al., 2003).

Myc, Max and Mnt were each shown to bind to a large number of genes that were not bound by the other family members (Orian et al., 2003). In fact, only about 34% (96/287) of the genes that bound to Myc were also found to be bound by its partner Max.

In another study Li *et al.* examined Myc binding to proximal promoter regions of 4,839 genes in a Burkitt's lymphoma cell line (Li et al., 2003). They demonstrated that Myc bound to 876 of these genes (~15%), 776 of which were also bound by Max. However, comparing this data to the Myc-target-gene database, only 68 of the 876 Myc-bound loci (7.8%) have been reported to be Myc-responsive genes.

These studies indicate that Myc binding to a given gene might not always correlate well with its level of transcription. So not all Myc-bound genes are necessarily effectors of Myc function. Moreover, many loci to which Myc is bound *in vivo* are not located close to a protein-coding gene. Myc also regulates transcription of both RNA polymerase III (RNA pol III)-dependent genes and RNA-pol-I-dependent, ribosomal RNA genes, and binds to the promoters of these genes *in vivo* (Grewal et al., 2005; Grandori et al., 2005; Arabi et al., 2005). Finally, DNA-binding sites of Myc are found 5′to the transcription start site of a novel class of non-coding RNA molecules, for which no catalytic function has been identified so far. Myc might therefore also regulate these non-coding RNAs, and the biological significance of this finding as well as the function of these RNAs remain to be determined.

1.7 c-Myc functions in Cell Growth and Proliferation

Targeted gene disruption of both c-*Myc* alleles in embryonic stem cells leads to embryonic lethality at day 9.5–10.5 with a lack of primitive hematopoiesis, which highlights the crucial role of c-*Myc* in normal growth control during mammalian development. And in *Drosophila* reduced expression of the orthologue *dmyc* results in smaller but developmentally normal flies and his level of expression directly correlates with the cell size (Johnston et al., 1999), while in Eμ-*Myc* transgenic mice its overexpression results in cell growth in the absence of cell-cycle progression (Iritani et al., 1999; Shuhmacher et al., 1999).

c-*Myc* transcripts are detected in a wide variety of tissues during development. In the mid-gestation mouse, enhanced c-*Myc* expression correlates well with active proliferation, and its downregulation accompanies mitotic arrest and onset of differentiation (Schmid et al., 1989).

More recently, the cell-cycle effects of ablating c-*Myc* have been investigated. A rat fibroblast cell line in which both alleles were ablated shows greatly reduced rates of cell proliferation, accompanied by cell-cycle defects in G1 that include significantly delayed phosphorylation of the retinoblastoma protein (Rb) (Mateyak et al.,1997). Intriguingly Rb phosphorilation occured when c-*Myc*-deficient cell reached the same size as control cells. The first notion that c-Myc influenced cell growth came from the correlation between c-Myc and the expression of the rate-limiting translation initiation factors eIF4E and eIF2α (Rosenwald et al., 1993), which are now known to be direct c-Myc targets (Coller et al.,2000). Important information is accumulating as to how c-MYC may be mediating effects on cell growth. RNA polymerase III (pol III) is involved in the generation of transfer RNA and 5S ribosomal RNA required for protein synthesis in growing cells and is activated by c-MYC via binding to TFIIIB, a pol III-specific general transcription factor (Gomez-Roman et al., 2003). It is indeed plausible that c-MYC's role in regulating cell proliferation could at least in part be mediated through its effects on cell growth.

G1–S progression of eukaryotic cells is controlled by the activities of the cyclin-dependent kinase (CDK) complexes cyclin-D–CDK4 and cyclin-E–CDK2. c-Myc induces cyclin-E–CDK2 activity early in the G1 phase of the cell cycle, which is regarded as an essential event in MYC-induced G1–S progression (Berns et al., 1997). But how does c-Myc activate cyclin-E–CDK2? It was recently shown that *CCND2* (which encodes cyclin D2) and *CDK4* are direct target genes of c-Myc (Bouchard et al., 1999; Hermeking et al., 2000). Expression of *CCND2* and *CDK4* leads to sequestration of the CDK inhibitor KIP1 (also known as p27) in cyclin-D2–CDK4 complexes (Perez–Roger et al., 1999). The subsequent degradation of KIP1 has been shown to involve two other c-Myc target genes, *CUL1* and *CKS* (O'Hagan et al. 2000). By preventing the binding of KIP1 to cyclin-E–CDK2 complexes, c-Myc allows inhibitor-free cyclin-E–CDK2 complexes to become accessible to phosphorylation by cyclin-activating kinase (CAK) (Perez–Roger et al., 1999). Increased CDK2 and CDK4 activities would result in Rb hyperphosphorylation and subsequent release of E2F from Rb.

Recent studies support the idea that c-Myc may also exert important influences on the cell cycle by repressing genes, such as the CDK inhibitors P15 and P21—that are involved in cell cycle arrest, through the Myc– Max heterodimer interacting with

positively acting transcription factors such as Miz-1 and Sp1. The suppression of *p21CIP1* expression by Myc is responsible for the failure of Myc-transformed cells to arrest in G1 phase after DNA damage. For instance, in the case of colorectal cancer, increased ß-catenin/TCF4 activity correlated with increased levels of c-Myc, leading to repression of p21 and ultimately to a proliferative phenotype of the cells. The interaction of Myc–Max with Miz-1 blocks the association of Miz-1 with its own co-activator (P300 protein), with the subsequent down-regulation of P15 and P21 (Staller et al., 2001).

Recent studies carried out in *Drosophila* (Moreno and Basler, 2004; de la Cova et al., 2004) show that Myc has a role in the regulation of cell proliferation beyond its ability to induce transcription of growth-promoting genes. Cells with high levels of Myc expression can act as supercompetitors that are capable of both out-growing and inducing death in nearby cells with lower Myc levels. Although the mechanism of apoptotic induction is unclear, it involves the activation of genes that are known to trigger apoptosis in *Drosophila* cells, such as *hid*. Super-competition appears not to be a general property of a relative increase in growth rate, because the activation of other growth-promoting pathways did not cause apoptosis in neighboring cells (de la Cova et al., 2004). These observations, together with the wellestablished evidence that Myc plays a prominent role in the induction of growth-promoting gene expression, specifically the ribosomal protein genes, suggest a mechanism by which deregulated Myc expression can initiate tumor formation. Oncogenic mutations that lead to increased Myc expression (Nesbit et al., 1999; Salghetti et al., 1999) enable cells to increase their biosynthetic capacity and clonally expand faster than their wild-type neighbors, while simultaneously inducing cell death in those wild-type cells. This could allow cells with an inappropriately high level of Myc to become tumorigenic by rapidly overpopulating a tissue while readily acquiring secondary oncogenic lesions.

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1.8 c-Myc and tumors

In contrast to the tightly regulated c-myc gene in normal cells, which only express the gene when cells actively divide, cancer cells may express the gene in an uncontrolled fashion as the result of genetic aberrations.

Alteration of the *c-myc* gene, via retroviral transduction, retroviral insertion, gene amplification (Dalla-Favera et al., 1982), chromosomal translocation (Dalla-Favera et al., 1982; Taub et al., 1982) and point mutation (Pasqualucci et al., 2001), represent one of the most common genetic lesions associated with cancer in multiple animal species and multiple tissues (Grandori et al., 2000). The common consequence of these alterations is the deregulated expression of the c-Myc protein in term of amount, time or cell context. Despite extensive studies and findings, fundamental questions regarding the role of Myc in tumorigenesis remain open.

One of the most striking findings of the past years has been the discovery that the enhanced expression of Myc proteins contributes to almost every aspect of tumour cell biology (Pelengaris et al., 2002). Whereas the ability of Myc to drive unrestricted cell proliferation and to inhibit cell differentiation had long been recognized, more recent work shows that deregulated expression of Myc can drive cell growth (Iritani and Eisenman,

1999; Johnston et al., 1999) and vasculogenesis (Baudino et al., 2002), reduce cell adhesion (Frye et al., 2003), and promote metastasis (Pelengaris et al., 2002) and genomic instability (Felsher and Bishop,1999). Conversely, the loss of Myc proteins not only inhibits cell proliferation and cell growth (Mateyak et al., 1999; Trumpp et al., 2001), but can also accelerate differentiation19, increase cell adhesion (Knoepfler et al., 2002) and lead to an excessive response to DNA damage (Herold et al 2002).

Recent results from several laboratories suggest that MYC inactivation in some cancers will not revoke tumorigenesis; rather, it will induce a transient loss of neoplastic character, marked by normal phenotype, that can be fully restored by Myc reactivation (Jain et al., 2002; Shachaf et al., 2004). The consequences of Myc inactivation and reactivation seem to depend upon the particular type of cancer. Myc inactivation in lymphoma commonly results in a permanent loss of a neoplastic phenotype. Thus, Myc-induced skin and pancreatic islet cell tumours undergo regression upon Myc inactivation and that tumours reform quickly after Myc reactivation (ref). Similarly, Myc-induced breast cancer undergoes regression upon Myc inactivation that is fully revoked by Myc reactivation (Pelengaris et al., 2004). The precise target genes through which Myc functions in each of these pathways have remained elusive in some, but not all, cases.

1.9 c-Myc and apoptosis

Meanwhile several studies were discovering how Myc functioned as an oncogenic transcription factor Myc was discovered to actually trigger rapid apoptosis (Evan et al., 1992), an endogenous and conserved program of cell suicide. Further, what rapidly became clear was that Myc was the rule, rather than the exception, as other oncogenes such as E1A (Lowe and Ruley, 1993) and E2F-1 (Kowalik et al., 1995) were then shown to behave in a similar fashion. This raised the hypothesis that apoptotic pathways must be disabled for oncogenes to promote transformation.

Myc-induced apoptosis requires its DNA binding functions and dimerization with Max (Evan et al., 1992; Amati et al., 1993). Since so fare there are no identified mutants lacking the transactivation activity alone, it's non easy to define which Myc function (transactivation or repression) is necessary for Myc role in apoptosis: likely both are important.

Apoptosis is an important safeguard that protects the organism from tumour cells: an intrinsic response, an immune surveillance mechanism, that somehow senses things are amiss in the mutated cell and instructs its suicide, protecting the organism from being overcome by a few bad actors. At its most basic level, apoptosis is controlled by intrinsic survival pathways (**Figure 4**) that are necessary to block the cell death program, and those invoked by extrinsic signals that actively trigger the demise of the cell. Extrinsic apoptotic pathways are direct and efficient signals that provoke cell suicide, and are induced following ligation of the Fas/TNF-a family of death receptors with their ligands. Although is not clear yet at which level it occurs, Myc does sensitize some cell lines to TNF-a- and Fas-induced death (Hueber et al., 1997; Klefstrom et al., 1997), and in some T-cell hybridomas c-Myc is required for activation induced death, which in peripheral T cells is Fas dependent. There are evidences that Myc somehow blocks activation of NF-kB by TNF-a (Klefstrom et al., 1997; You et al., 2002), which provides an essential survival function by regulating genes such as the antiapoptotic Bcl-2 family member bcl-X. And in the Fas pathway Myc can target or components of the DISC, such as the RIP serine/threonine kinase (Klefstrom et al., 2002). MEFs and myeloid cells derived from lpr or gld mice, which have inactivating mutations in the Fas and Fas ligand genes,

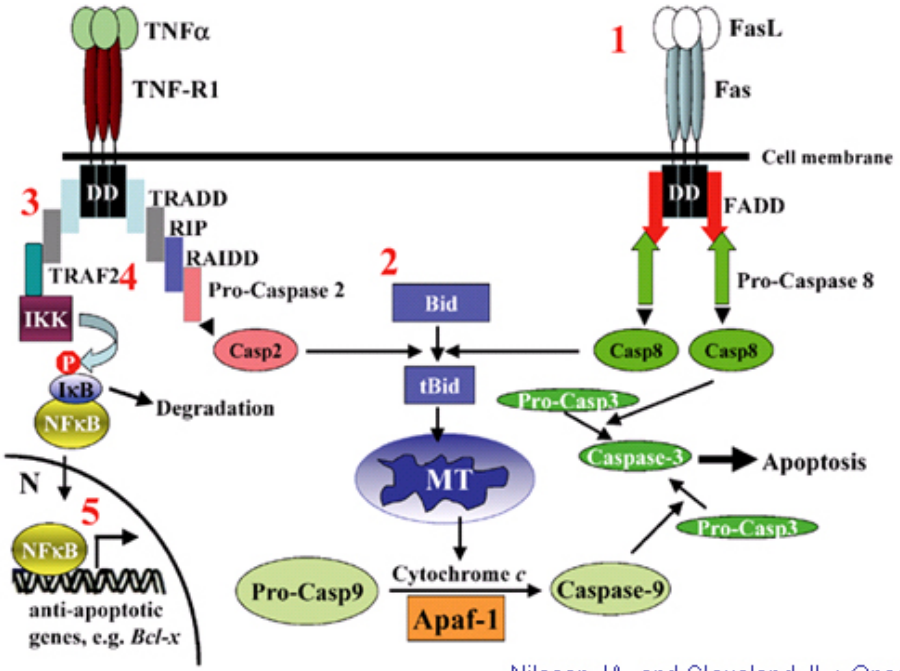

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Figure 4 Death receptor pathways and points of regulation by Myc. The trimeric death receptors Fas and TNF-R1 interact with their respective ligands, which induces death domain (DD)-mediated associations with the adaptor molecules FADD and TRADD and other components of the death-inducing signaling complex (DISC) (e.g., RIP, RAIDD, TRAF4, and the procaspases-8 and -2). This association triggers cleavage of pro-caspases-8 and –2, leading to cleavage of downstream effector caspases (caspase-3) or to cleavage of the proapoptotic BH3-only Bcl-2 family member Bid, which generates truncated Bid (tBid). tBid activates other proapoptoticBc l-2 family members Bax and Bak (not shown) and results in the release of proapoptotic factors from mitochondria (MT), most notably cytochrome c, which binds to the scaffold protein Apaf-1. The resulting apoptosome (not shown) that is formed then binds and induces cleavage of procaspase-9, which then cleaves and activates effector caspases, thus amplifying the apoptoticresponse. TNF-a- ediated pathways also induce signaling cascades that result in the activation of NF-kB, which provides an essential survival function by regulating genes such as the antiapoptoticBc l-2 family member bcl-X. Mycinterfac es with death receptor pathways at several levels: (1) Myc can activate expression of FasL, which could participate in the direct suicide of the cell or, alternatively, could trigger the death of Fas-expressing immune cells that target the cancer cell, a scenario that would explain the immune privilege state of Myc- xpressing cancer cells; (2) Myc provokes association of Bid with mitochondria; (3) Myc affects components of the DISC (e.g., RIP) to sensitize cells to TNF-a- ediated apoptosis; (4) Myc compromises the function of TRAF2, effectively disrupting activation of NF-kB; and (5) Myc inhibits the induction of bcl-X. N denotes nucleus

respectively, are resistant to Myc-induced death (Hueber et al., 1997; Amanullah et al., 2002).

Another way Myc can induce apoptosis is through Bax. *Bax* is a proapoptotic bcl-2 family member whose apoptotic function is antagonized by *bcl-2* expression (Oltvai et al., 1993). Bax along with Bak inserts into mitochondrial membranes and forms channels for the release of cytochrome *c*. And Bax-deficient (but curiously not Bak-deficient) MEFs are remarkably resistant to Myc-induced apoptosis (Soucie *et al.*, 2001; Juin *et al.*, 2002).

Is still controversial whether Myc directly activate Bax transcription, may be due to tissue or species specificity, but Myc is necessary (Soucie *et al.*, 2001) and sufficient (Juin *et al.*, 2002) for Bax activation. Myc can directly (human) or indirectly (mouse) induce the expression of Puma, leading to his association with the antiapoptotic proteins Bcl-2 and Bcl-X_L, functionally sequestering these proteins and releasing Bax and Bak to continue the chain of destruction. Myc can also activate Bid (another BH3 only factor Bcl2-family member) with consequent binding and activation of Bak bound to mitocondria (Wei *et al.*, 2000), or repress the transcription of *bcl-2* or *bcl-X*, but the way it occurs is still unresolved.

Myc is able to induce apoptosis also activating the Arf-p53 pathway. Arf and p53 are both tumor suppressor protein. Mutation, deletion or silencing of the *INK4a*/*ARF* locus and *p53* gene are the two most alterations in human cancer (Zambetti and Levine, 1993) and impairs Myc-induced apoptosis (Zindy et al., 1998). Myc activation in primary cells is associated with a profound induction of both Arf and p53 protein levels. This response occurs at several levels. First, in some cell types, *p53* is a transcription target of Myc and indeed the gene harbors an E-box in its promoter-regulatory region (Reisman et al., 1993). However, in most cells, p53 protein levels are more profoundly induced following Myc activation (Zindy et al., 1998), usually through the agency of Arf in blocking p53-inhibitor Mdm2 functions, but perhaps also via effects of Myc on the Atm pathway (Lindstrom and Wiman, 2003).

How Myc induces *Arf* remains unclear. Since *de novo* protein synthesis is required (Zindy et al., 1998), Myc likely cooperates with other transcription factors or signaling proteins that regulate *Arf* transcription. One of these can be E2f1, as Myc induces *E2f1*

expression in primary cells (Sears et al., 1997; Baudino et al., 2003; Fernandez et al., 2003) and E2f1 has been suggested to induce *Arf* directly (Bates et al., 1998). Even if there are evidences that Myc can clearly activate Arf and p53 expression and kill cells in the absence of *E2f1* (Baudino et al., 2003). Another candidate as a mediator of the Arf response to Myc is the calcium-regulated serine-threonine kinase DAP. DAP expression is augmented by Myc, the kinase is sufficient to activate *Arf*, and loss of *DAP* impairs Myc's ability to induce Arf and p53 and to trigger cell death (Raveh et al., 2001).

Bmi-1, Twist, Tbx2 and Tbx3 are transcription factors that repress *Arf* transcription and block Myc-induced apoptosis (Jacobs et al., 1999a; Maestro et al., 1999; Brummelkamp et al., 2002; Lingbeek et al., 2002), but no effect of Myc on the expression and/or activity of these Arf repressors have never been properly evaluated.

1.10 c-Myc and genomic instability

There are evidences that link Myc to genomic instability. The term *genomic instability* refers to genetic changes that affect the normal organization and function of genes and chromosomes. These alterations may be structural (point mutations, deletions, translocations, inversions…) or numerical (that leads to changes in the number of chromosomes).Genomic instability has frequently been associated with malignant transformation of cells. However, it is also found in normal and (pre)malignant cells. For example, normal mammalian lymphocytes use a form of genomic instability during normal recombination of their immunoglobulin (*Ig*) and T-cell receptor genes. Moreover, gene amplification occurs normally as part of development-specific programs in other species as Xenopus, Drosophila and Tetrahymena (Stark, 1993).

c-Myc/Max heterodimers are involved in the genomic instability of gene whose products are essential for DNA syntesis. Many, but not all, of these genes are also transcriptional targets of c-Myc/Max. For instance intrachromosomal as well extrachromosomal locus-specific amplification of the *dihydrofolaate reductase* (DHFR) gene (Mai, 1994) occurs 72 hours after *c-myc* upregualtion and following enhanced bindig of c-Myc/Max heterodimers to the *DHFR* 5' flanking E-boxes. This was proven both in tissue culture and in mouse models. Furthermore c-Myc is associated to locus-specific genomic

instability of other genes as *cyclin D2* (Mai et al., 1999), *Ribonucleotide Reductase R2* (Kuschak et al 1999) and *CAD* (Fukasawa et al., 1997).
Inducible system, as the fusion protein Myc-estrogen receptor (Myc-ER) responsive to hydroxytamoxifen, revealed that continued Myc activation *in vivo* and *in vitro* leads to structural and numerical chromosome alterations (karyotypic instability) that directly contributes to the tumorigenic potential of the cells (Feisher and Bishop, 1999). c-Myc-induced genomic instability is transient if c-Myc activation is induced only for a single time, as demonstrated in transgenic mice where malignant c-Myc induced T cell lymphomas, acute myeloid leukemias, hematopoietic tumors regressed when *c-myc* deregulation was removed.
Open question is how c-Myc induces gene amplification. A model proposed is the *replication-driven* amplification (or *onion skin* model) that postulates that re-replication of a particular gene occurs within the cell cycle, whereas usually each gene replicates only once in each cell division cycle (Hamlin JL and Ma C., 1990). The finding of several rounds of amplification of *R2* within a single cell cycle as consequence of *c-myc* overespression (Kuschak et al 1999) supports the idea of a *replication-driven* model for c-Myc induced genome instability.

A role for c-Myc in DNA replication was first reported by Classon et al. in 1987. The authors showed that lymphocytes with high level of c-Myc protein supported the replication of *Simian Virus 40* (*SV40*) better than lymphocytes with lower level of the protein. Later it was demonstrated that c-Myc was the limiting factor for ionizing-induced SV40 gene amplification in semi-permissive Chinese hamster embryo cells. Furthermore, it became evident that c-Myc was instrumental in the binding of protein complexes at the minimal origin of SV40 (Classon et al., 1993).
Is c-Myc then a replication-licensing factor?

1.11 MCM proteins and DNA replication

DNA replication is a highly controlled and coordinated process required for maintenance of the genome, that must be duplicated precisely once per cell cycle.

Since the human genome is not one long continuous thread of DNA but is divided into 46 chromosomes per diploid cell, each chromosome contains thousands of starting points of replication (origins of replication). The activity of these origins is coordinated as they fire in a defined temporal fashion during S phase. Different replication origins fire at different times during S phase, creating a 'replication timing program' whereby different segments of chromosomal DNA are replicated at distinct times. To avoid re-firing of the same origins eukaryotes have found a way to distinguish between replicated and unreplicated DNA (Rao and Johnson, 1970). Experiments in *Xenopus laevis* eggs suggest a model whereby replication origins were 'licensed' for replication during late mitosis and G1, but the license is removed as the DNA was replicated (Blow and Laskey, 1988). In the licensing of the origins, the Mcm complex plays a central role. Mcm2–7 proteins form an essential component of the pre-replicative complex (pre-RC) of proteins that are found at replication origins during G1 phase. Replication origins are licensed by stably binding complexes of the Mcm 2–7 proteins, and then they can initiate a pair of replication forks. Ones the DNA replication is started Mcm 2-7 proteins are displaced from the origins, probably traveling ahead of the replication fork (Blow and Hodgson, 2002; Nishitani and Lygerou, 2004). In this way the license is never associated with replicated DNA.

The MCM protein family was originally isolated from a genetic screen in budding yeasts which were defective in minichromosome maintenance, showing a high rate of loss of plasmids that contained a cloned centromere and replication origin (Sinha et al., 1986). It includes only the six proteins Mcm2 through Mcm7 that show sequence homology with hexameric DNA helicases and that share a region of sequence similarity called the MCM box that includes two ATPase consensus motifs (Koonin, 1993). The MCM family is conserved in all eukaryotes, but not in prokaryotes, even if MCM-related proteins exist in *Archaea* (Kearsey and Labib, 1998). Physical interactions were identified in vivo by using two-hybrid, co-immunoprecipitation, and biochemical purification (Adachi et al., 1997) suggesting that the bulk of MCMs in vivo associate in a heterohexamer with 1:1:1:1:1:1 stoichiometry, although there are likely to be small amounts of single MCMs and MCM subcomplexes in the cell. Since no mutants that disrupt complex assembly are viable (Lei et al., 2005), their assembly is necessary, however non sufficient, for their function (e.g. they require their NLS or ATPase domain). Chromatin

immunoprecipitation experiments have revealed that the MCM proteins are associated with replication forks as they elongate along chromosomal DNA (Aparicio et al., 1997), whereas inhibition of Mcm2–7 function during S phase causes a rapid cessation of DNA synthesis (Labib et al., 2000; Shechter et al., 2004), indicating that Mcm2–7 function is required for fork progression as well as initiation. These observations all indicate that Mcm2–7 functions as a helicase that unwinds DNA ahead of the replication fork. *In vitro* experiment already showed that the Mcm4,6,7 core will itself dimerize to form a dimertrimer (Mcm4,6,7)2 that is associated with DNA helicase activity (Ishimi et al., 1996). Moreover the structure of the N-terminal region of the *Methanobacterium thermoautotrophicum* MCM protein (MtMCM) has been solved (Fletcher et al., 2003). This archaeal species has only one MCM protein, which assembles into two head-to-head homohexameric rings, that have helicase activity (Shechter et al., 2000), with a large positively charged channel running through the centre that is wide enough to surround double-stranded DNA (**Figure 5**). The C-terminal helicase domain that is missing from the crystal structure could form an extra ring facing away from the dimer interface. This conclusion is supported by electron microscopy of the full-length archaeal MCM protein, which shows a bilobed hexameric structure with a large central channel (Pape et al., 2003).

1.12 Prereplication Complex and licensing reaction

Replication initiation depends on identification and activation of origins of replication distributed throughout the genome. The replication origin is marked by the binding of a complex of proteins called ORC (for "origin recognition complex") (Bell, 2002). ORC recruits two further proteins, Cdc6 and Cdt1, which are required for the loading of Mcm2–7 in order to form the prereplication complex (pre-RC) and the functional licensing of the origin although the mechanism is poorly understood (Blow and Hodgson, 2002; Nishitani and Lygerou, 2004) (**Figure 6**). As the ring-shaped structure of MCM2-7 encircles the DNA, it suggests that ORC and Cdc6 might act as an ATP-dependent clamp-loader. Among the pre-RC components in metazoans, Orc1, Orc4, and Cdc6 have both Walker A and Walker B motifs, whereas Orc5 has only a Walker A motif. The Walker A motif is critical for ATP binding and the Walker B motif is required

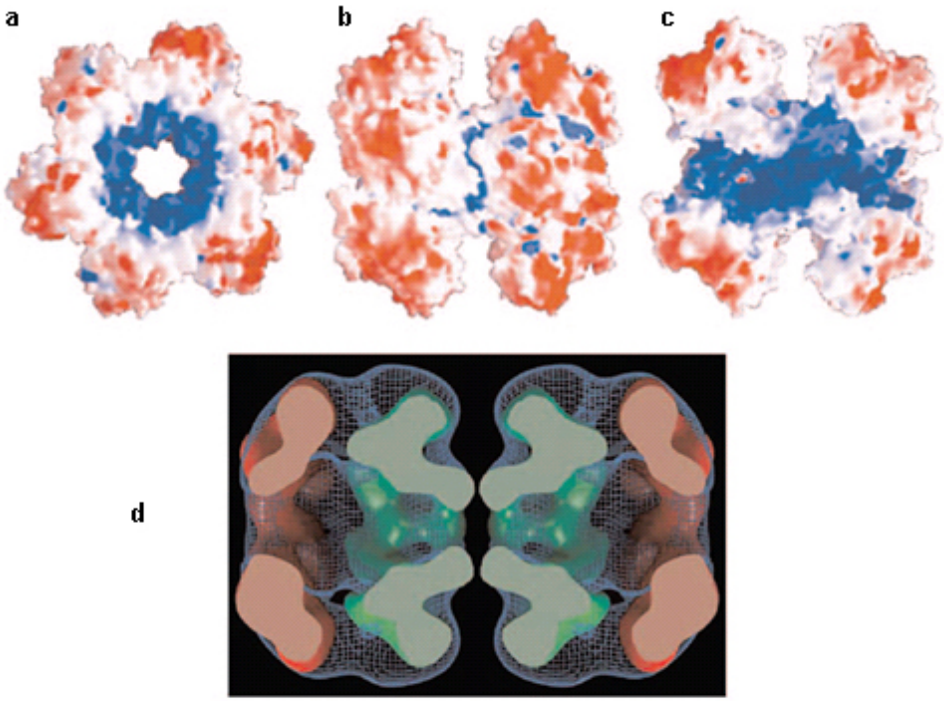

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Figure 5: Crystal structure of the N terminus of the *Methanobacter thermoautotrophicum* MCM. a–c Different views of the N terminus of the minichromosome maintenance (MCM) dodecamer, highlighting the central channel that runs through it. Positive charges are shaded in blue, negative charges in red. **a**, end view; **b**, side view; **c**, the same view as **b**, but with the two front monomers removed to reveal the central channel. Arrows indicate the side channels that pass between the exterior and the interior. Figure reproduced with permission from *Nature Structural Biology* REF. 15 © (2003) Macmillan Magazines Ltd. **d** A side view of the electron density of full-length *M. thermoautotrophicum* MCM, aligned to approximately correspond to the side view of the crystal structure shown in **b**. The crystal structure of the N terminus has been fitted into the electron density (green). A hexameric model of the core AAA+ domain of RuvB, which has homology to the C terminus of the Mcm2–7 proteins, has been fitted into the electron density (red).Figure reproduced with permission from *EMBO Reports* REF. 16 © (2003) the European Molecular Biology Organization.

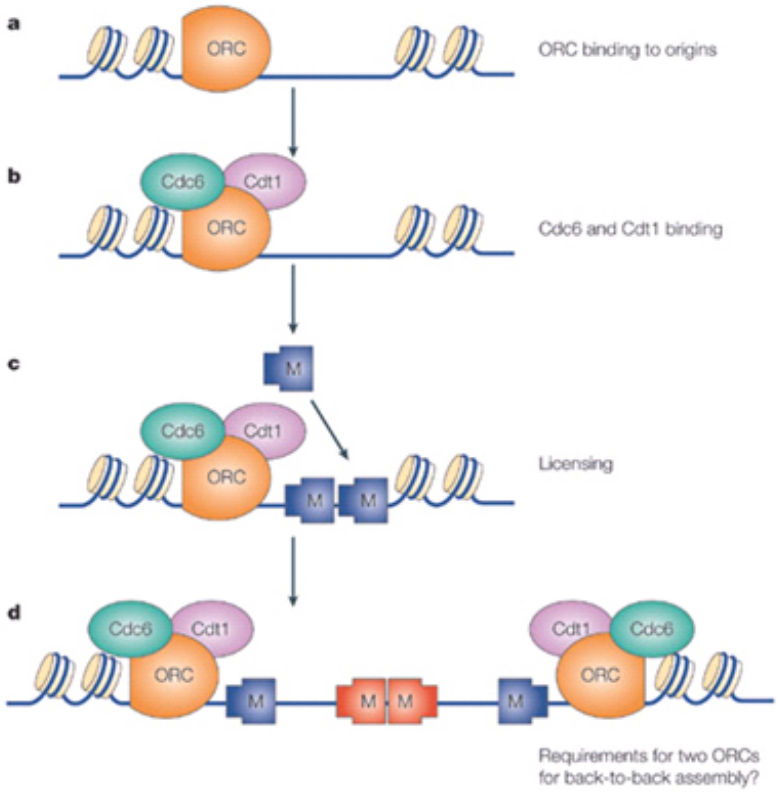

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Figure 6: Stepwise assembly of pre-replicative complex proteins during origin licensing. a | The origin recognition complex (ORC) is first recruited to the replication origin. **b** | ORC recruits Cdc6 and Cdt1. **c** | ORC, Cdc6 and Cdt1 act together to load multiple minichromosome maintenance (Mcm)2–7 protein hexamers onto the origin, which licenses the DNA for replication. **d** | Initiation-competent complexes are probably formed by the back-to-back assembly of two Mcm2–7 complexes. As the ORC is asymmetrical, this might require deposition of a second ORC molecule to load Mcm2–7 in the opposite orientation.

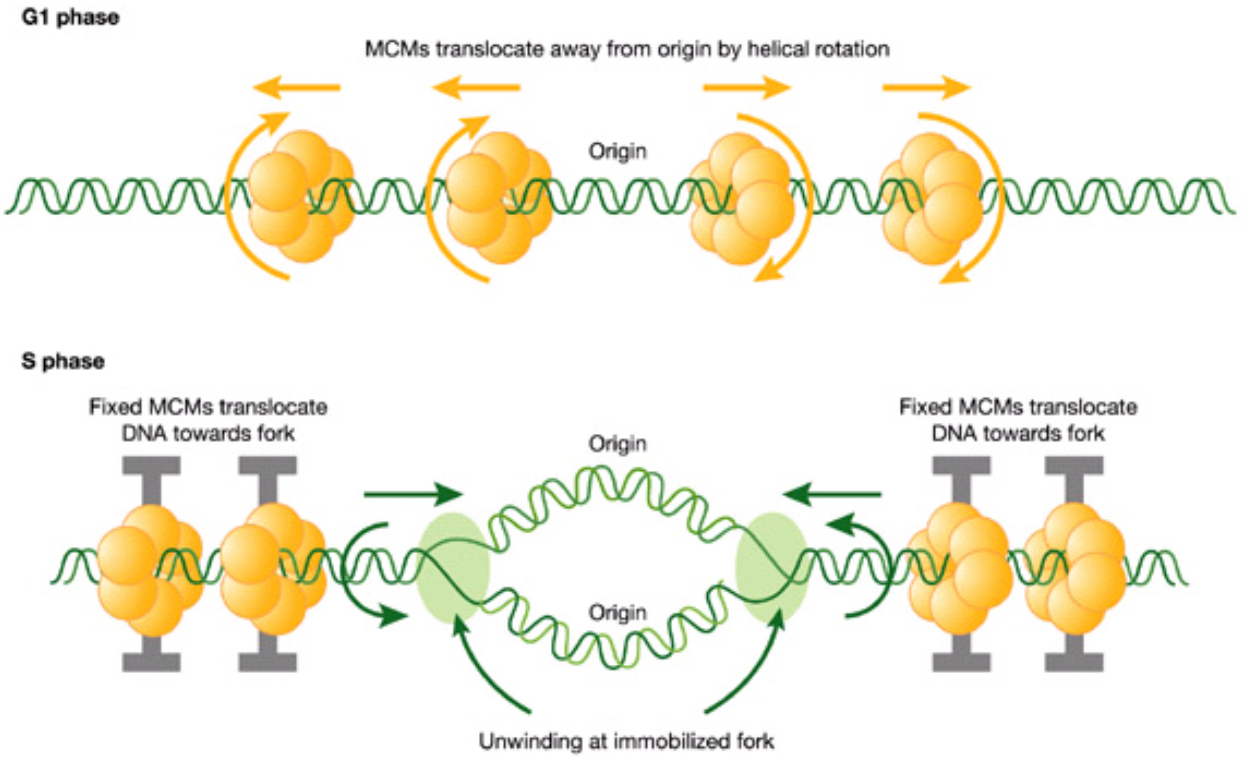
for ATP hydrolysis. Given that a nonhydrolyzable ATP analog inhibits the loading of MCM2-7, but not loading of ORC and Cdc6 (Harvey and Newport, 2003b), it seems likely that ATP hydrolysis is used to load MCM2-7 on chromatin. This model is consistent with the observation that ORC, Cdc6 and Cdt1 are only required for the loading of Mcm2–7 onto DNA (licensing), but are not required for the continued association of Mcm2–7 on DNA once this has occurred (Maiorano et al., 2000).Cdt1 directly binds members of the Mcm2-7 complex, and this interaction is enhanced by Cdc6 that must already be bound to the chromatin before Cdt1 can join the complex in an active form (Cook et al., 2004; Tsuyama et al., 2005).

Mcm2-7 complex show low helicase activity *in vitro.* Consistent with this observation, binding of Mcm2-7 to the DNA is not enough to initiate replication, but it requires an activation step in witch critical seems to be the loading of Cdc45 (Pacek and Walter, 2004). Cdc45 is required for both activation of MCM2-7 on origins and chromosome unwinding at the replication forks.

The Mcm2-7 not only can be activated, but also derepressed at transition from G1 to S phase. Mcm7 interacts with a tumor suppressor protein, pRB with consequent inhibition of DNA replication in a Xenopus *in vitro* DNA replication assay system. Active cyclin D1/CDK4 catalyzes the dissociation of a pRB-Mcm7 complex (Gladden and Diehl, 2003). Thus, pRB may suppress MCM2-7 helicase activity until activation of Cdk in a manner similar to how it represses E2F-mediated transcription until S phase.

1.13 MCM paradox

MCM proteins are in vast excess compared to the number of fired origins (Edwards et al., 2002). Although in *Escherichia coli*, two helicases per origin seem sufficient to allow bidirectional DNA replication, with each helicase acting at a single replication fork, in Eukaryotes 10-40 Mcm2-7 hexamers are present at each replication origin (Edwards *et al*., 2002).Furthermore most of Mcm2-7 are distributed all over the chromatin and don't co-localize with replication forks or sites of DNA synthesis (Edwards et al., 2002; Lei et al., 1996). One possible explanation for this paradoxal distribution is that the Mcm2-7 complex doesn't function as typical DNA helicase but as a fixed pump that unwind the DNA in fixed replication forks during S phase (Laskey and Madine, 2003) (**Figure 7**).



Laskey RA, Madine MA; *EMBO Rep. 2003*

Figure 7: A hypothetical rotary pump model showing two stages in the distribution and function of MCM hexameric ATPase complexes. First, in G1 phase, MCM hexamers move spirally along the helical grooves of unreplicated DNA, away from ORC, which is required for their loading and orientation. Second, in S phase, MCMs become immobilized, so that exactly the same rotary mechanism moves the DNA instead of the MCM proteins. This would result in translocation of DNA towards the replication forks. As DNA is twisted by fixed MCMs in S phase, it would become unwound at the distant replication fork, which is itself immobilized in fixed clusters

Another possibility is that all those Mcm2-7 don't work in DNA synthesis, but are loaded for contingency or other function. For instance one proposal is that Mcm7, interacting with ATRIP (a protein that activate the ATR kinase), is a critical regulator of S-phase check point (Cortez et al., 2004). It is also possible that the 10–40 Mcm2–7 hexamers that are loaded onto each origin form a number of back-to-back dimers, each of them could provide a different site at which initiation could occur. This might provide redundant origins for use if various components of the replication machinery were to fail.

1.14 Once and only once

How does the cell control DNA replication to occur only once per cell cycle, avoiding extra-copies of chromosomes leading to genome instability and, as last consequence, to cancer? To prevent re-firing of replicated origins during the S phase, it is important that the ability to license new replication origins is downregulated before entry into S phase. Mcm2-7 remains inactive in G1 phase, due to the low activity of CDK. Once DNA replication is initiated following Mcm2-7 activation, no additional Mcm2-7 complexes are loaded onto origins as combinatory effect of CDK activity and functional inactivation of their loading factors.

Orc1 (the ATPase subunit of ORC) in S phase is targeted of proteasomal degradation by SCF(Skp2) ubiquitination, probably due to Cdk2 activity. Excess of Cdc6 is exported out of the nucleus after Cdk2-mediated phosphorilation. Furthermore, phosphorylation of Mcm4 dramatically reduces its affinity for the chromatin in *Xenopus* extract and is associated to low level of DNA synthesis (Ishimi et al., 2003).

But downregulation of Cdt1 activity is the main strategy adopted by metazoans in preventing licensing during S and G2 phase. Cdt1 overexpression in human, *Drosophila* and *Xenopus* leads to re-replication, enhanced by co-expressing Cdc6 (Maiorano et al., 2005). The levels of Cdt1 are regulated during the cell cycle, being stable in G_1 when pre-RCs are formed and degraded in S phase when pre-RCs are fired and disassembled (Nishitani et al., 2001). Regulation is primarily at the protein level, as mRNA levels of Cdt1 remain constant throughout the cell cycle. Cdt1 is phosphorylated *in vivo* by Cdk2 or Cdk4 and its phosphorylation is required for interaction with the F-box protein Skp2 and proteasomal degradation (Liu et al., 2004). There is also another mechanism for Cdt1

degradation that is Skp2-indipendent, but triggered by replication and that is interestingly required for normal progression of S phase (Takeda et al., 2005).

Another key regulator of Cdt1 activity is geminin. Geminin is destabilized during G1 phase and accumulates during S, G2, and M phases of the cell cycle. At the metaphase-to-anaphase transition, geminin is ubiquitinated by APC and degraded by the proteasome to allow pre-RC formation in G1 phase. Geminin binds to Cdt1 and inhibits Cdt1 binding to both DNA and to Cdc6 and MCM2-7 subunits (Cook et al., 2004). But geminin also stabilizes Cdt1 during G2 and M phase, as RNAi experiment demonstrated in mammalian cells (Ballabeni et al., 2004). This stabilization seems to be important because the decrease in Cdt1 protein levels at mitotic exit cannot be recovered later by *de novo* protein synthesis. For this reason, the geminin-dependent accumulation of Cdt1 during mitosis is essential for pre-RC formation and DNA synthesis in the following cell cycle.

Like geminin, also Cdks seem to have a positive role in pre-RC formation, as experiments show Cdk2 to phosphorylate Cdc6 preventing it from poliubiquitination by APC. This stabilization is critical when cells enter the cell cycle from a quiescent state (Mailand and Diffley, 2005).

What happens if re-replication occurs? Structural problems that take place after origins re-firing, as stalled forks or DNA damage, rather than re-licensing itself, trigger checkpoint kinases ATM/ATR activation. These proteins in turn activate many downstream kinases as Chk1 and Chk2. ATM/ATR pathways inhibit Cdk2 and Cdc7 activity respectively. Cdc7 is required for initiation of DNA replication. In combination with Cdk2 protein kinase, Cdc7 promotes binding of Cdc45 to the pre-RC (Jares and Blow, 2000). In this way ATM/ATR prevent origin firing by inhibiting loading of Cdc45 on the chromatin in presence of DNA damage (Costanzo et al., 2000, 2003). Also subunits of Mcm2-7 are directly phsphorilated by ATM/ATR after DNA damage (Cortez et al., 2004; Yoo et al., 2004), but the meaning of this modification is still not well known.

Overexpression of Cdt1 or Cdc6, and consequent re-replication, leads to p53 activation and apoptosis in addition to cell cycle arrest (Vaziri et al., 2003). Instead downstream effector of geminin depletion is checkpoint kinase Chk1 that leads to G2 arrest, and

ultimately, in case this block is over-ridden, to p53-indipendent apoptosis (Zhu et al., 2004).

CHAPTER II

Aim and Results

Aim

Although a number of proteins binding c-Myc have been identified, their physiologic role remains unclear. The aim of this work is to isolate the protein complex containing c-Myc, to identify its components and to determine the contribution of selected ones on the c-Myc influence on the cell biology.

Results

2.1 Complex purification

To isolate the c-Myc containing protein complex from cell, we used an epitope-tagging strategy that was already successfully adopted in the lab. We used a human lung carcinoma H1299-derived cell line (H/HF2), which stably express low-level of a hemagglutinin (HA)/ Flag double tagged form of c-Myc. This cell line was already available in the lab. c-Myc is a very unstable protein, with an half-life of 30 minutes, that is ubiquitinated and degraded by he proteasome pathway (Salghetti et al., 1999). The MG132 proteasome inhibitor was added directly to the cell cultures before harvest in order to increase the overall c-Myc level by inhibiting the degradation pathway. To isolate complex containing the epitope-tagged protein, nuclear extracts from the cell line and from native cells as control were processed by affinity chromatography in batch. The co-purified proteins were separated by SDS-PAGE and stained by Blue Comassie (**Figure 8**). Comparing the protein pattern between the c-Myc stable cell line and the parental H1299 cell line, specific bands were cut from the gel and analyzed by microsequencing by mass spectrometry (MALDI-TOF and MS-MS). The recorded mass signals were used for protein database search. Among the protein identified in the c-Myc associated complex, Mcm7 and Mcm5 were found as novel c-Myc protein partners. Minichromosome Maintenance (MCM) proteins were originally identified in yeast (Maine et al., 1984) and are conserved in all eukaryotes. They are proposed to be the

DNA helicase and play a key role in DNA replication (see introduction). Those two proteins catalyzed our interest since recently the MCM proteins are becoming a diagnostic and prognostic marker for many tumor (breast, lung, brain, prostate, bladder etc.) (for review: Tachibana et al., 2004) substituting the more conventional proliferation marker as Ki67 and PCNA.

2.2 c-Myc interacts with the Mcm7

In order to validate the interaction between c-Myc and the new protein, Mcm7 cDNA was cloned in a eukaryotic vector expressing the protein as HA tagged. The Mcm7 cDNA was amplified by PCR from a Ramos cDNA library. 293T cell line were co-transfected with plasmid expressing c-Myc, Max and Mcm7-HA. The total cell lysates were immunoprecipitated with specific antibodies recognizing c-Myc or Max and tested for Mcm7 co-immunoprecipitation by western blot using an HA antibody. Mcm7 was detected in both c-Myc and Max immunorpecipitations, but not when irrelevant IgG were used as control for co-immunoprecipitation specificity (**Figure 9**). The different amount of Mcm7 that co-immunoprecipitates with Max directly correlates with the level of c-Myc (lanes 2, 4 of IP), suggesting that Max interaction with Mcm7 is c-Myc mediated.

To verify that this interaction was physiologic and not due to c-Myc overexpression or tagging, the interaction between the endogenous proteins was tested in native cell.

Protein extracts from different cell lines were separated by SDS-PAGE tested by Western Blot with specific antibodies for the expression of c-Myc and Mcm7 (**Figure 10 A,B**). Three different cell lines where chosen: MCF7 and Raji, expressing low and high level of c-Myc respectively, and the parental cell line H1299. Endogenous c-Myc and Max were immunoprecipitated from nuclear extract with specific antibodies. Mcm7 co-immunoprecipitated with c-Myc and Max in all the cell lines tested, but non when irrelevant IgG as control antibody (from the same species of Myc-Max antibodies) were used. This confirmed that Mcm7/c-Myc interaction was real.

2.3 c-Myc interacts with all the components of the MCM helicase

Since Mcm7 belongs to a complex of six proteins (Mcm2-7), and among them Mcm5 already co-purifies with c-Myc complex, we sought to determine whether c-Myc interacts

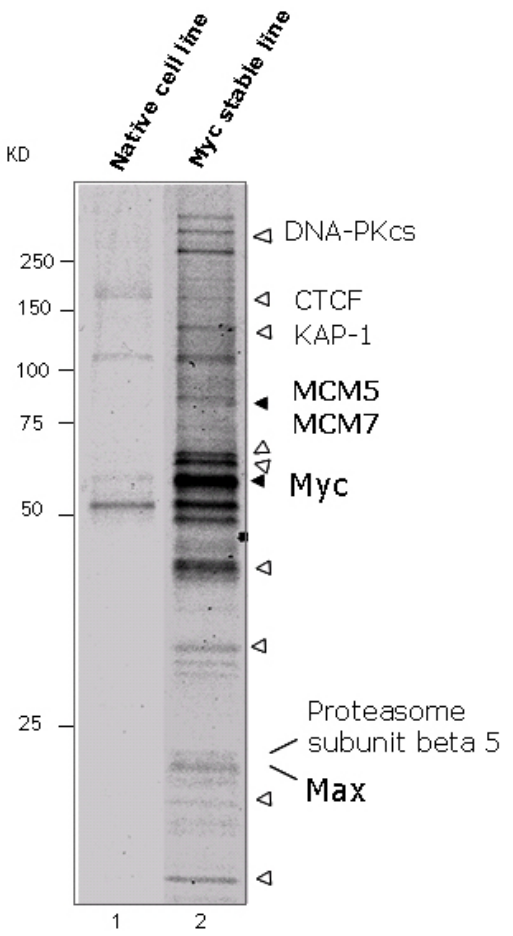


Figure 8: **Complex purification**. Nuclear extracts from H/HF2 and H1299 cell line were control were processed by affinity chromatography in batch. The co-purified proteins were separated by SDS-PAGE and stained by Blue Comassie. Black empty arrowheads indicates the band that were sequenced. Among the proteins identified were found also Mcm7 and Mcm5 (black arrowheads)

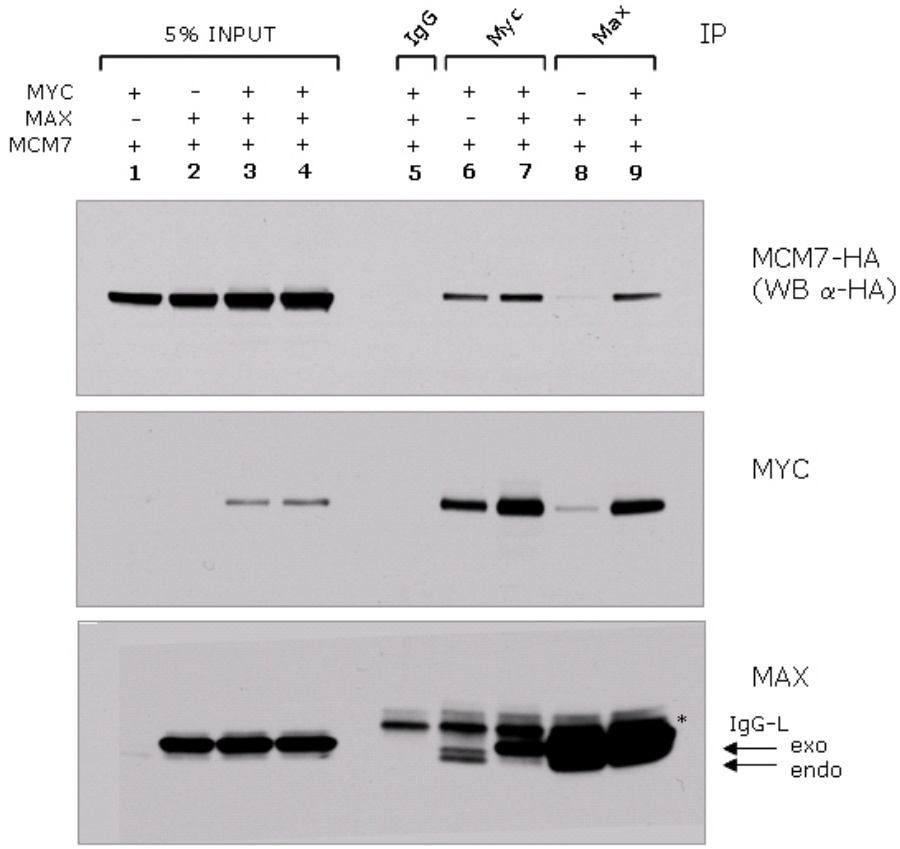


Figure 9: Exogenous Mcm7 co-immunoprecipitate with c-Myc. 293T cell line were transiently co-transfected (+) with plasmids encoding c-Myc, Max and/or MCM7-HA (lanes 1,2,3,4). The total cell lysates and were immunoprecipitated with specific antibodies recognizing either c-Myc (N262) or Max (C17). As negative control were usec irrelevant IgG. Western blot with anti-HA antibody shows Mcm7 co-immunoprecipitation with both c-Myc and (6 and 7) and Max (8 and 9).

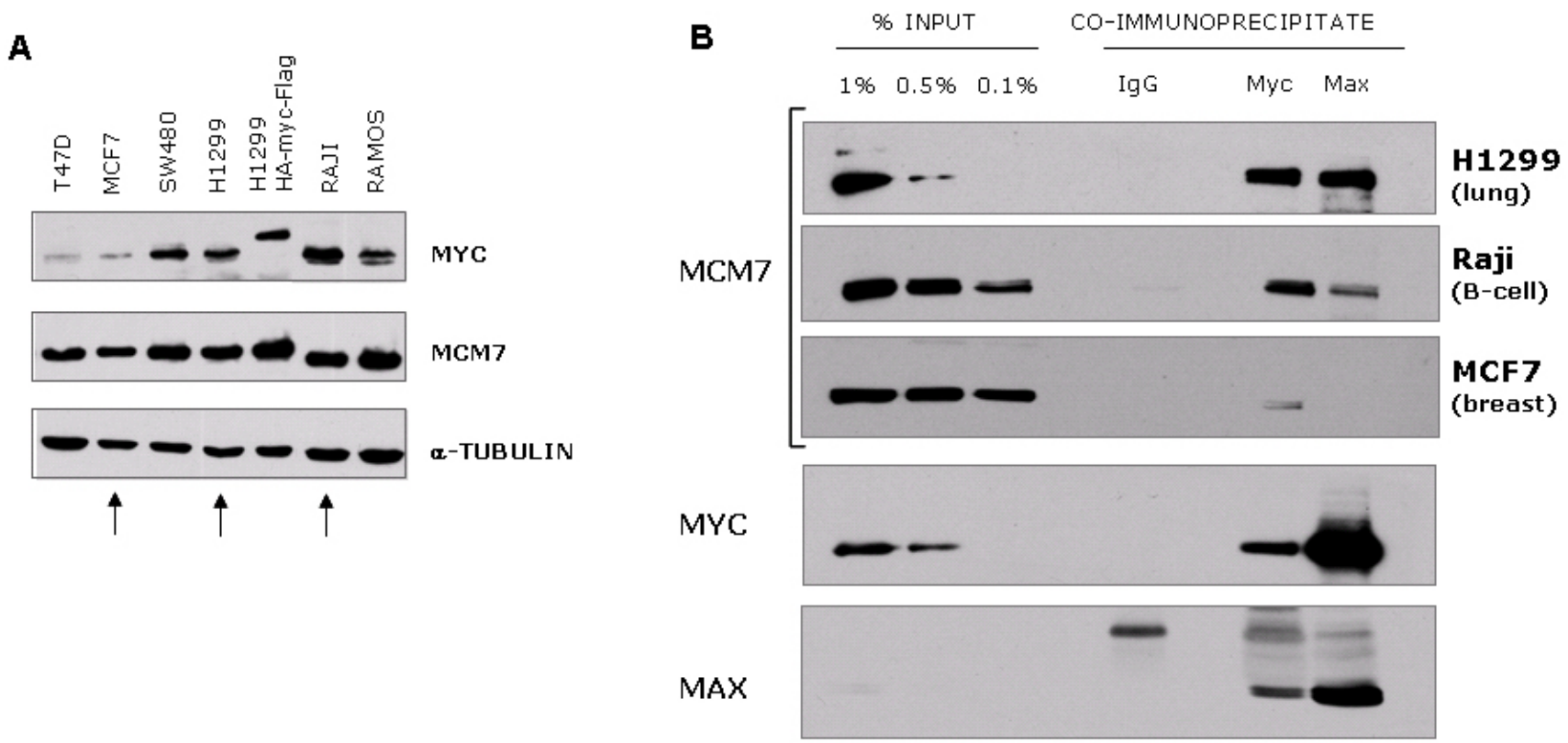


Figure 10. **Endogenous Myc and Mcm7 interact in vivo. A** different cell lines were tested for the levels of Mcm7 and c-Myc expression. Arrows shows the cell lines used to confirm the interactions between these two proteins. **B** Western blot analyses of Myc immunoprecipitations from H1299, Raji and MCF7 whole cell extracts, confirmed the interactions Myc-Mcm7. 1%, 0.5% and 0.1% indicates the amounts of total cell extracts used for the IP. Irrelevant IgG were used as negative control for the IP, while as positive control was performed a western blot with anti-Max antibody.

with the other components of the MCM complex. The cDNA of the other MCM proteins (2, 3, 4, 5 and 6) was cloned in vector expressing them as HA-tagged proteins. As for Mcm7, the interaction was tested in 293T cells by co-transfection and co-immunoprecipitation. A possible cooperative role of Mcm7 (the protein originally identified in the cMyc complex) was also tested. **Figure 11-13** show that all the MCM proteins co-immunoprecipitate with c-Myc independently from the presence of Mcm7. Again, the recruitment of the MCM proteins to the heterodimer c-Myc/Max is Myc dependent, as suggested by the direct correlation between the level of MCMs and of c-Myc, coming down along with Max. The interaction is specific since no detectable binding of the HA-tagged proteins is present in the control lanes.

Next we sought to confirm this interaction between c-Myc and the endogenous MCMs. The c-Myc complex from the stable cell line expressing a tagged form of c-Myc previously purified was separated by SDS-PAGE. The presence of all the endogenous proteins was verified by Western Blot using specific antibody (**Figure 14 A**). All the MCM proteins were found co-immunoprocipitated along with c-Myc.

Furthermore, we wanted to validate these interactions in absence of proteasome inhibitor. Nuclear extract from H1299 cell line was immunoprecipitated with c-Myc specific antibody or aspecific IgG as control. Western blot following SDS-PAGE separation of the samples revealed the effective co-immunoprecipitation at list of some of the MCM proteins (**Figure 14 B**). Not all the MCMs were detectable, likely due to the sensibility of the antibodies.

These data confirm that c-Myc interacts with all the MCM proteins and suggest that these interactions are physiologic.

2.4 Direct interaction between Myc and the MCM proteins

In order to test whether c-Myc interacts directly or not with some or all the component of this complex, the cDNAs of the MCMs were subcloned in a vector expressing them in bacteria as proteins tagged with six histidins (His6) at the N-terminus and HA at the C-terminus. In the lab was already available a prokaryotic expression vector for a double tagged c-Myc protein (HA-Myc-Flag). The recombinant protein were expressed in bacteria (*E coli*) and purified from cell lysates using a double-tag sequential purification

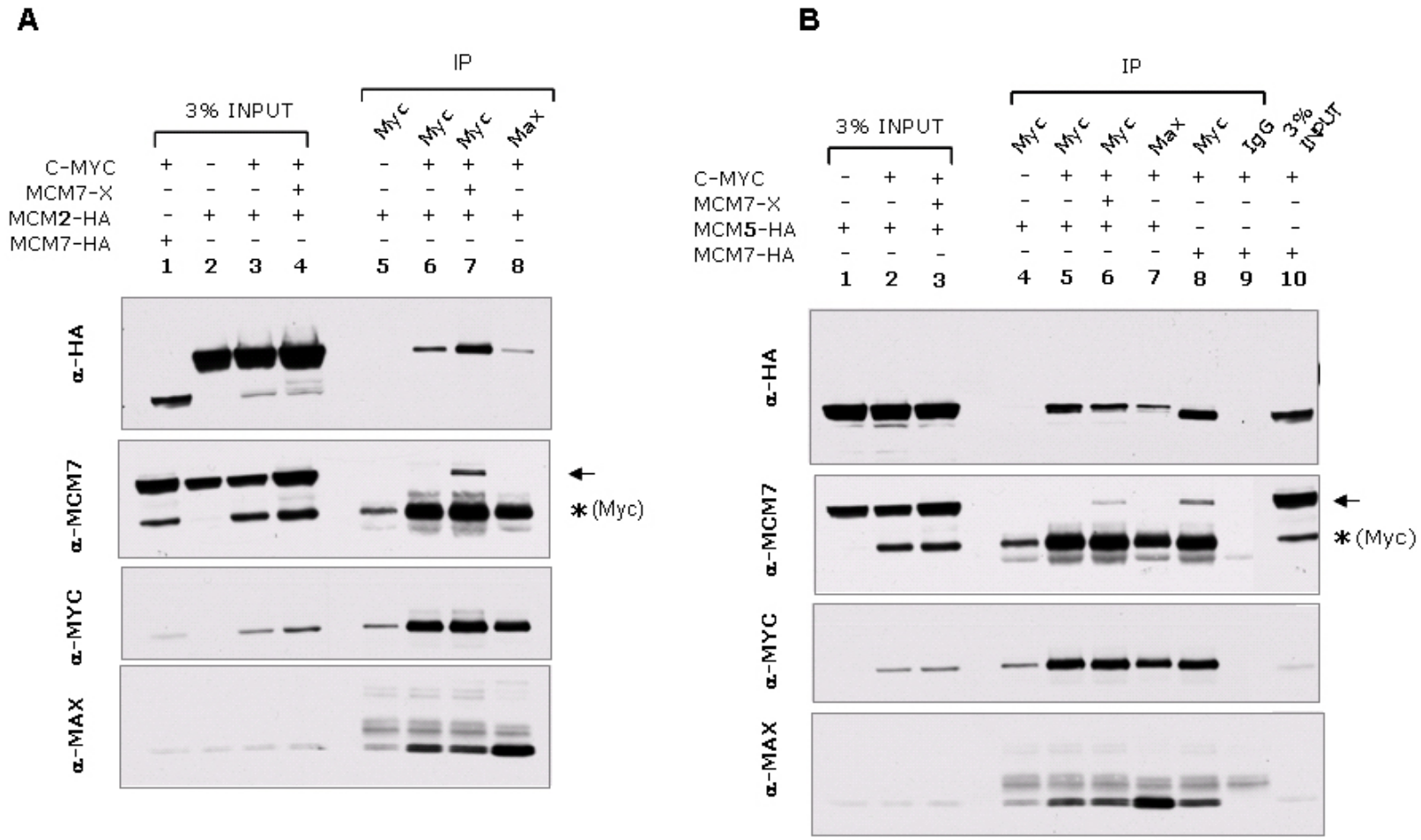


Figure 11: *In vivo* interaction between c-Myc and Mcm2/5. (A-B) 293T cell were co-transfected (+) with vectors expressing c-Myc, Mcm2-HA, Mcm5-HA, Mcm7-HA and Mcm7 no tagged (Mcm7-X). Whole cell extracts were immunoprecipitated with antibody against c-Myc (N262), Max (C17) or irrelevant IgG as control. Western blot performed with indicated antibody shows co-immunoprecipitations of Myc-Mcm2 (A) and Myc-Mcm5 (B). Black arrows indicate Mcm7. The star indicates c-Myc staining from the previous blot. Mcm7-HA is used as control for IP (B.lanes 8-10). Co-transfection of Mcm7-X indicates that the interactions between c-Myc and Mcm2 (A. 4,6,7) or Mcm5 (B. 3,5,6) are Mcm7-indipendent.

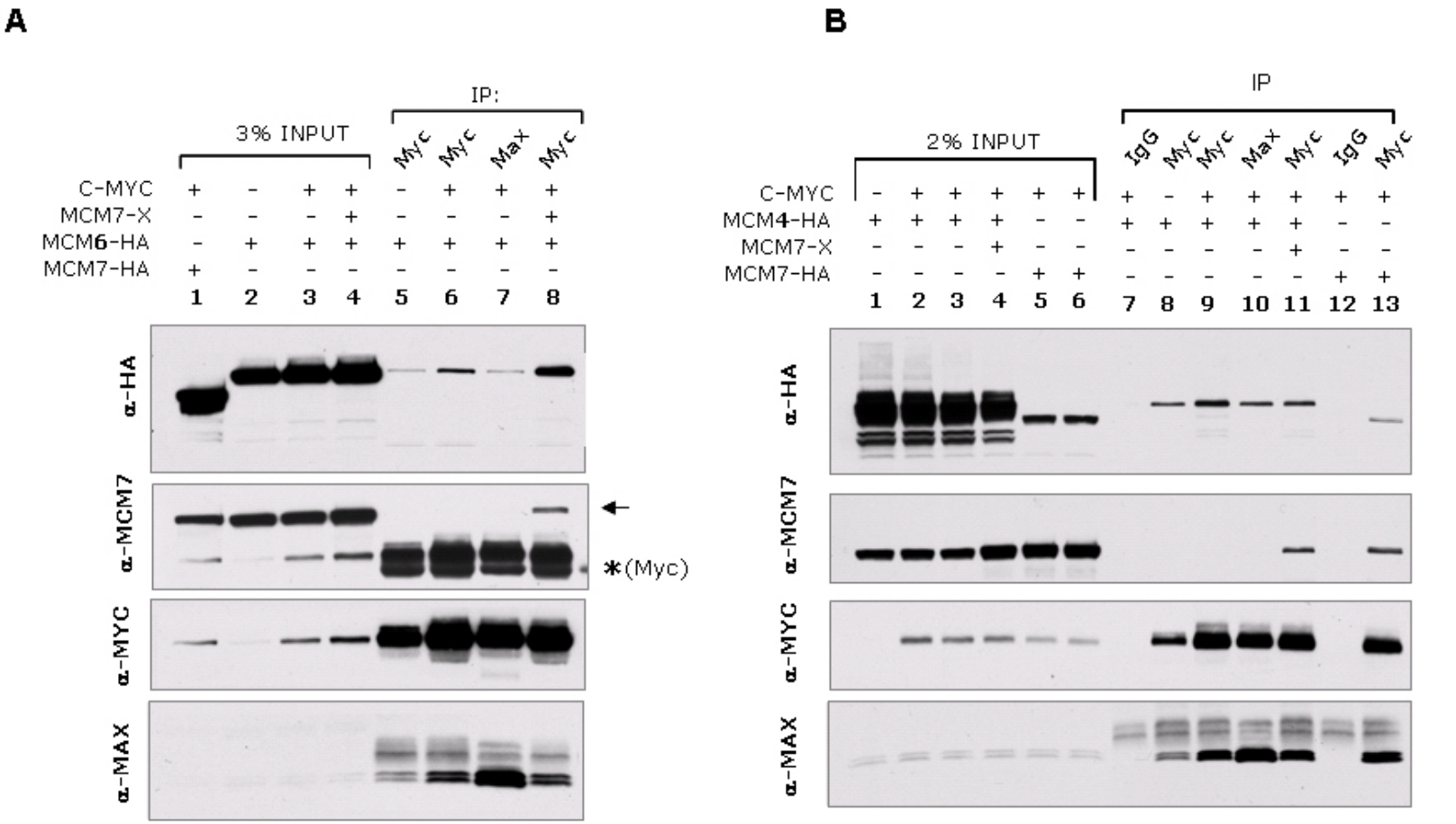


Figure 12: *In vivo* interaction between c-Myc and Mcm6/4. (A-B) 293T cell were co-transfected (+) with vectors expressing c-Myc, Mcm6-HA, Mcm4-HA, Mcm7-HA and Mcm7 no tagged (Mcm7-X). Whole cell extracts were immunoprecipitated with antibody against c-Myc (N262), Max (C17) or irrelevant IgG as control. Western blot performed with indicated antibody shows co-immunoprecipitations of Myc-Mcm6 (A) and Myc-Mcm4(B). Black arrow indicates Mcm7. The star indicates c-Myc staining from the previous blot. Mcm7-HA is used as control for IP (B.lanes 8-10). Co-transfection of Mcm7-X indicates that the interactions between c-Myc and Mcm2 (A. 4,6,7) or Mcm5 (B. 3,5,6) are Mcm7-indipendent.

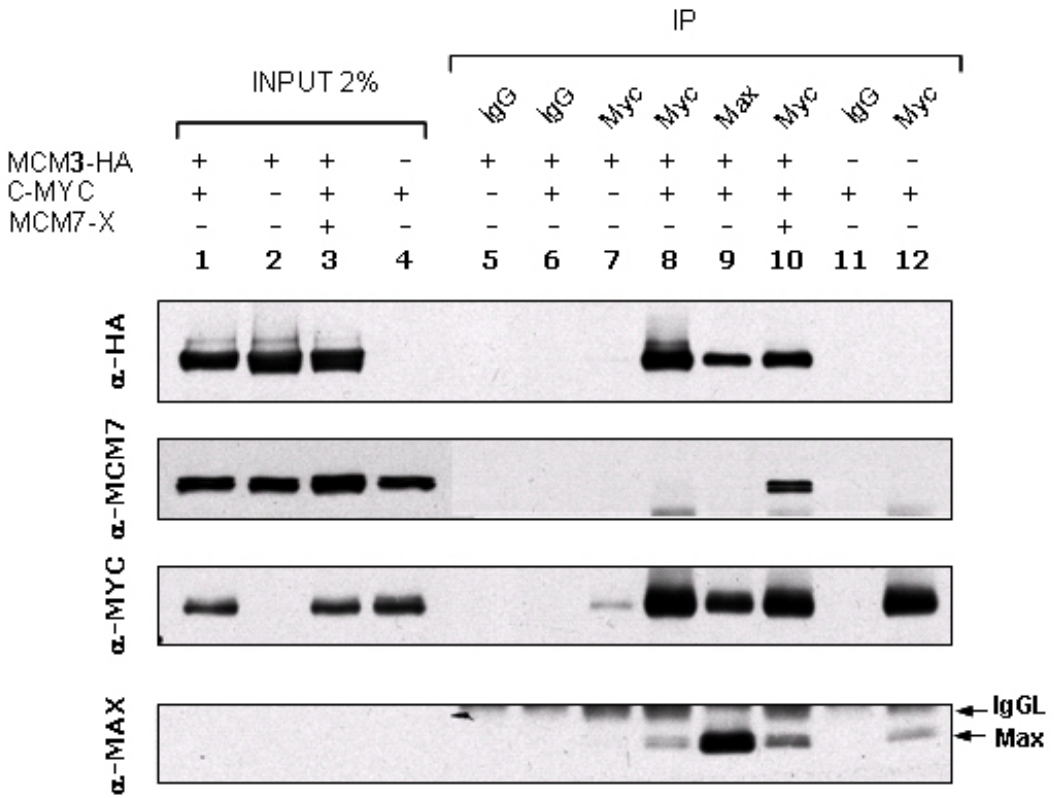


Figure 13: *In vivo* interaction between c-Myc and Mcm3.) 293T cell were co-transfected (+) with vectors expressing c-Myc, Mcm3-HA and Mcm7 no tagged (Mcm7-X). Whole cell extracts were immunoprecipitated with antibody against c-Myc (N262), Max (C17) or irrelevant IgG as control. Western blot performed with indicated antibody shows co-immunoprecipitations of Myc-Mcm3. Co-transfection of Mcm7-X indicates that the interaction between c-Myc and Mcm3 is Mcm7-indipendent (lanes 3, 8, 10).

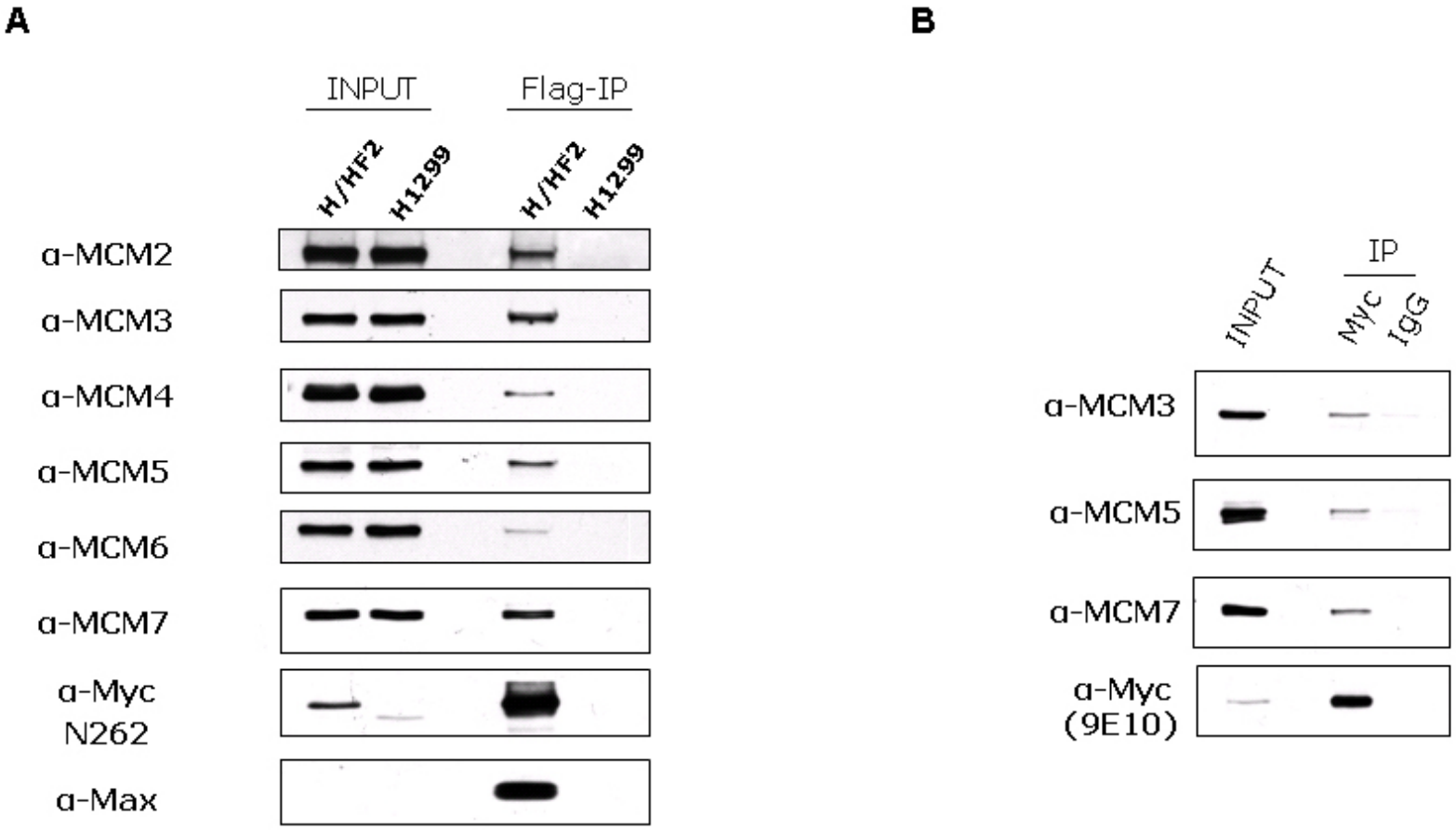


Figure 14: c-Myc interacts with the MCM helicase. A Nuclear extracts from H1299 or H/HF2 cell lines were immunoprecipitated with anti-Flag antibody and c-Myc associated complexes were resolved by SDS-PAGE. Western blot with the indicated antibody show c-Myc to interact with the entire MCM complex. **B** Nuclear extract from native H1299 was immunoprecipitated with anti-Myc antibody (Upstate) and assayed with the indicated antibodies.

in order to obtain full length products and no cleaved or degraded forms. The recombinant proteins were analyzed on SDS-PAGE for purity and quantitated by Comassie staining using a bovine serum albumin (BSA) curve as standard. *In vitro* pull down experiments were first performed incubating the recombinant c-Myc with Mcm7. After Myc immunoprecipitation with M2 Flag antibody the protein were separated by SDS-PAGE and the co-immunoprecipitation of Mcm7 was revealed by western blot. Max was used as control for the pull down (**Figure 15 A**). Next all the other MCMs were separately incubated in presence of or absence of Myc and a direct interaction between the two proteins tested was observed (**Figure 15 B,C**).

The *in vitro* pull down experiments suggest a direct interaction of c-Myc with all the MCM proteins.

2.5 Mapping the interaction between c-Myc and MCM complex

We were then interested in to identify the protein domains that were involved in these interactions. In order to map the region within Mcm7 that binds directly to c-Myc, we employed different truncated mutants containing various domains of Mcm7 molecule generated by PCR amplification and cloned in bacterial GST expressing vectors. Each protein after bacterial purification was subjected to SDS-PAGE followed by Comassie staining. The proteins, so quantitated, were incubated separately with equal amount of HA-Myc-Flag. After incubation with glutation-sepharose beads, the samples were analyzed with antibody directed against the HA tag or the GST. The experiment revealed that the C-terminal region of Mcm7 binds c-Myc. Surprisingly, interaction between the GST-full length Mcm7 and HA-Myc-Flag was not detectable, probably due to problem in the folding of the GST-fusion protein in a bacterial environment (**Figure 16**). Then we adopted the same strategy to map the c-Myc binding region on Mcm7 and Mcm2. A panel of truncated mutants of c-Myc fused to GST was generated by PCR amplification and the recombinant proteins purified from bacteria were incubated in presence of His6-Mcm7-HA first, and His6-Mcm2-HA later. As reported in **Figure 17** we identified two regions of c-Myc directly interacting with Mcm7: one N-terminal encompassing the MycboxII, and another C-terminal including the HLH/LZ domain. Only the C-terminal domain of c-Myc is involved in the binding of Mcm2 (**Figure 18**). We also tested

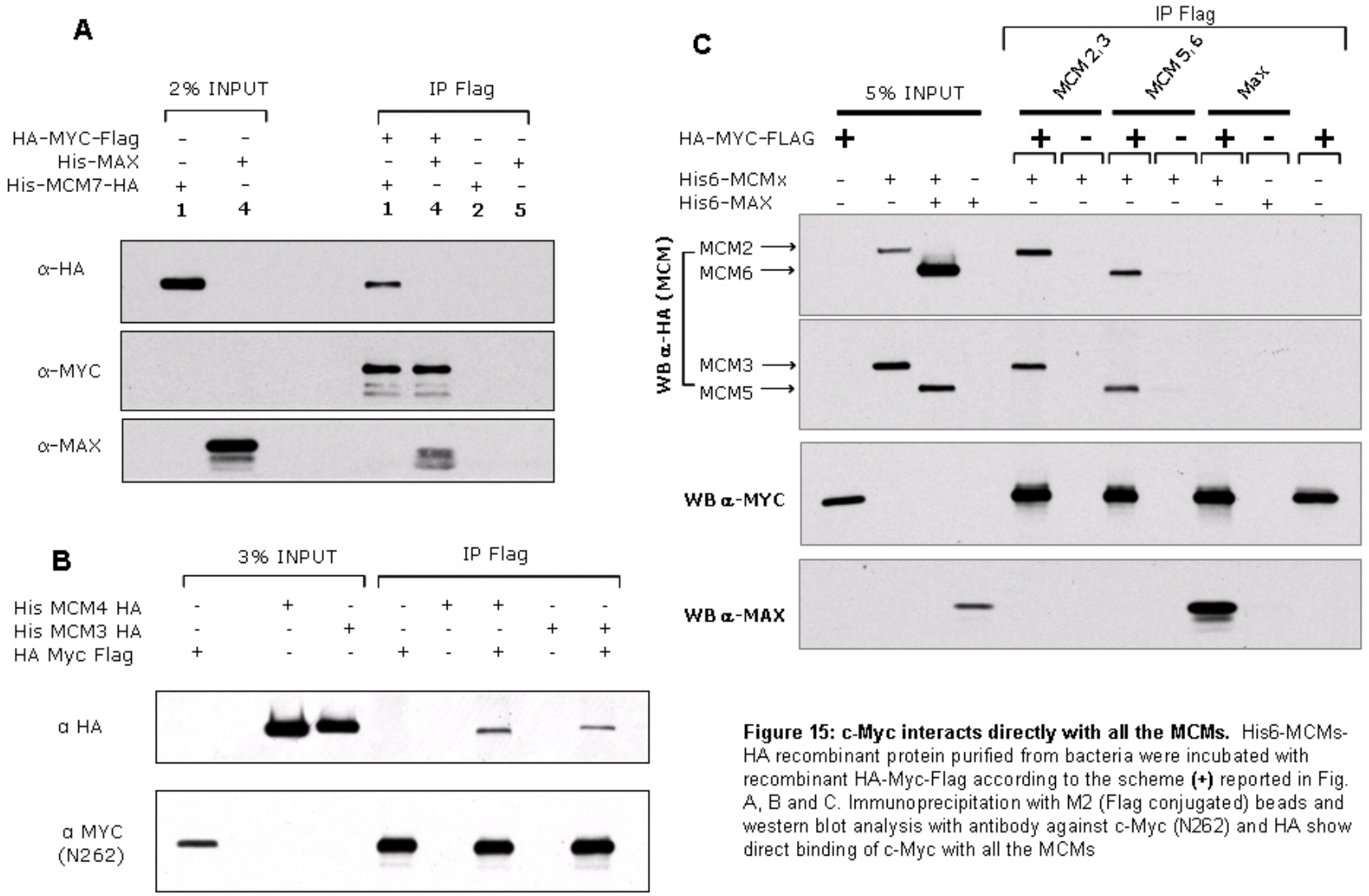


Figure 15: c-Myc interacts directly with all the MCMs. His6-MCMs-HA recombinant protein purified from bacteria were incubated with recombinant HA-Myc-Flag according to the scheme **(+)** reported in Fig. A, B and C. Immunoprecipitation with M2 (Flag conjugated) beads and western blot analysis with antibody against c-Myc (N262) and HA show direct binding of c-Myc with all the MCMs

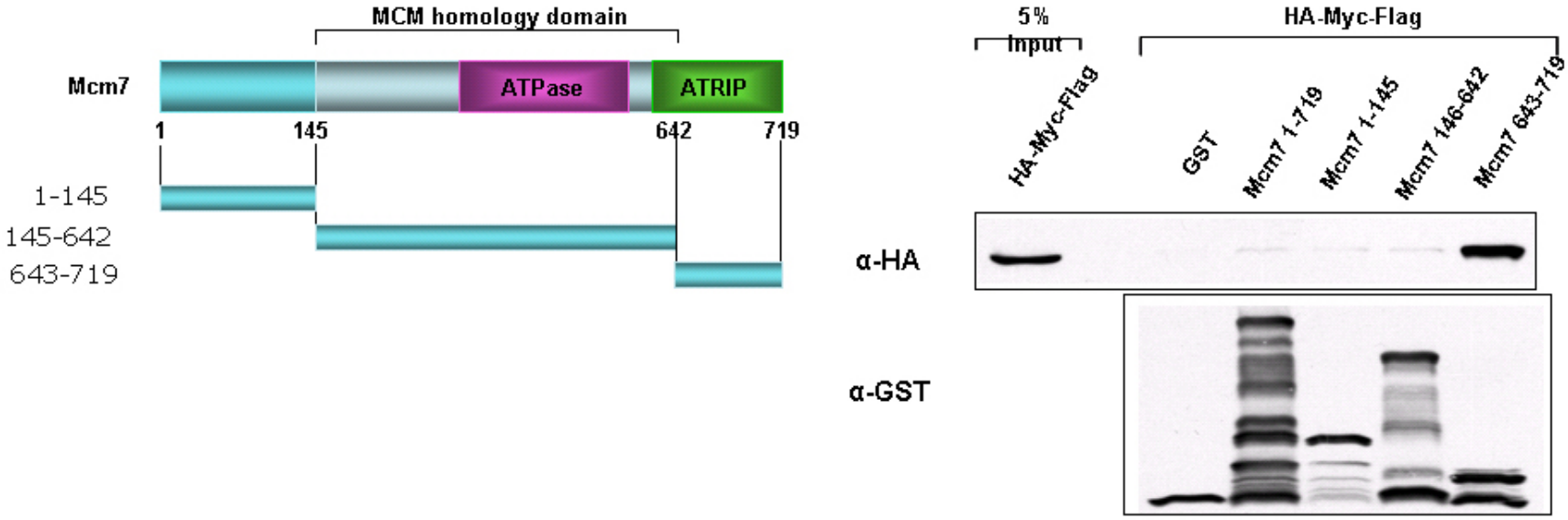


Figure 16: c-Myc interacts directly with the carboxi-terminal domain of Mcm. Mcm7 mutants were expressed in bacteria as GST-fused proteins and incubated with recombinant HA-Myc-Flag. GST-pull down assay and western blot analysis with the indicated antibodies shows that Mcm7 C-terminal domain directly interacts with c-Myc.

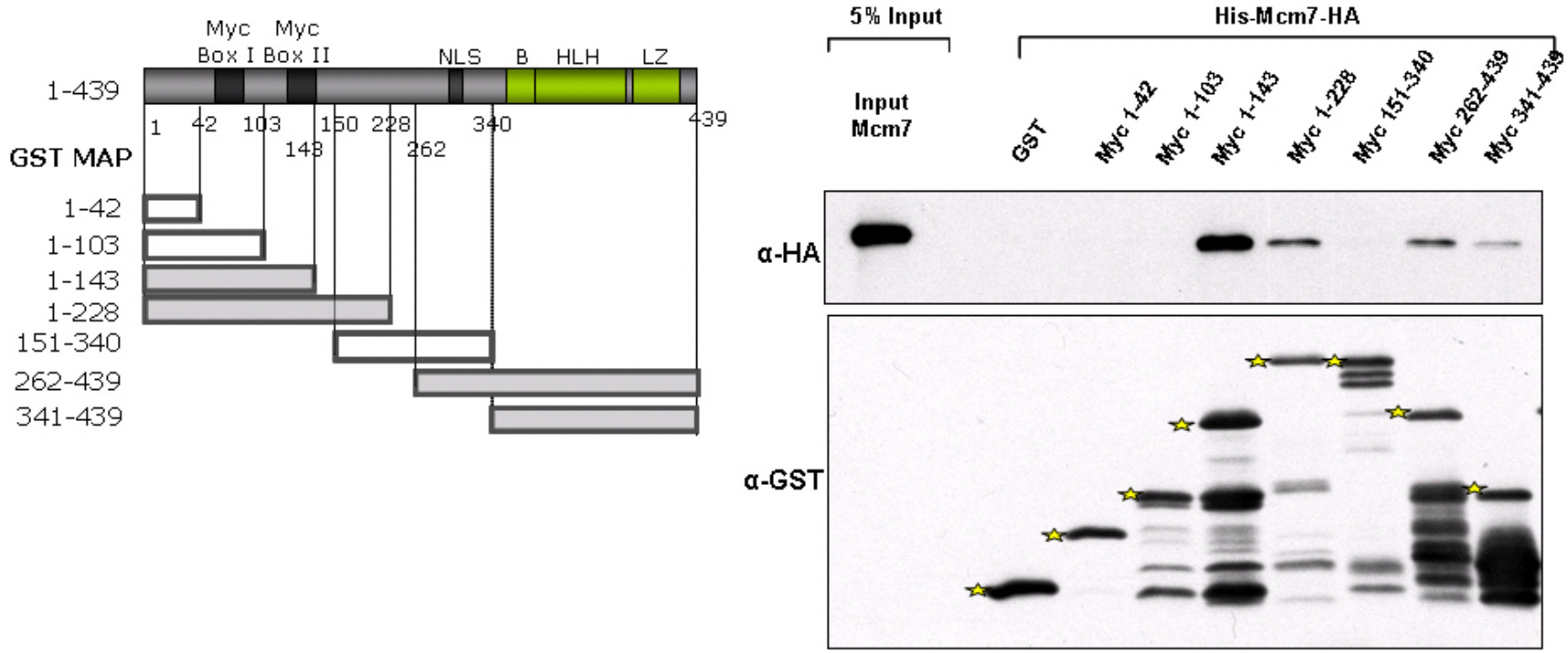


Figure 17: Two domains of c-Myc are involved in the interaction with Mcm7. Different mutants GST-Myc, as reported in the scheme, were used in a GST pull down assay with full length His6-Mcm7-HA. Grey bars in the scheme indicate the Mcm7 interacting Myc mutants. The yellow stars indicate the GST-Myc mutants over degradation products.

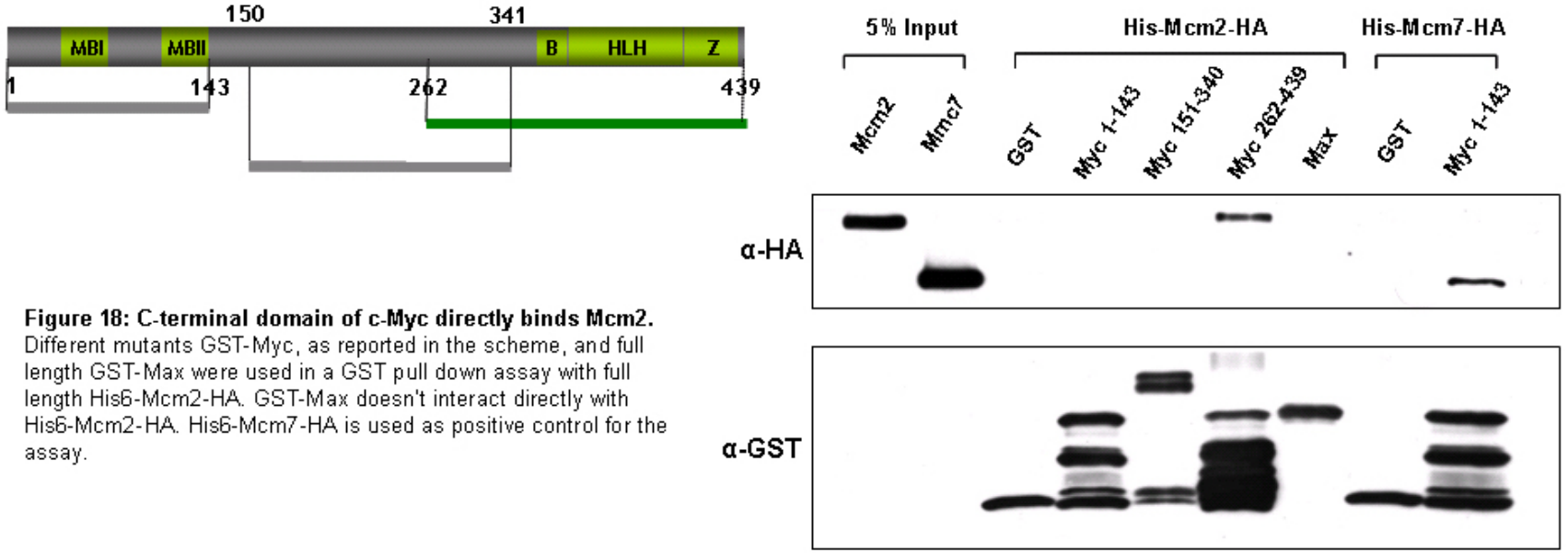


Figure 18: C-terminal domain of c-Myc directly binds Mcm2. Different mutants GST-Myc, as reported in the scheme, and full length GST-Max were used in a GST pull down assay with full length His6-Mcm2-HA. GST-Max doesn't interact directly with His6-Mcm2-HA. His6-Mcm7-HA is used as positive control for the assay.

whether Max is able to directly interact with Mcm7, but consistently with the data from the *in vivo* interaction, no binding was found (**Figure 19**).

To confirm the mapping *in vivo* we generated a set of vectors expressing truncated portions of full length c-Myc protein tagged with HA. These constructs were co-transfected in 293T cells with a vector expressing the Mcm7 full length no tagged. The cell lysates were analyzed by immunoprecipitation using an antibody against HA (for c-Myc fragments) followed by western blot with an antibody specific for Mcm7. The result showed in **Figure 20** suggests that c-Myc binding site is restricted to an N-terminal region including the mycboxII. Further mapping to validate this data with a different set of c-Myc mutants showed that deleting just that region is not sufficient to impair the binding to Mcm7, but a bigger N-terminal portion was needed to be deleted (**Figure 21**).

Not conclusive was also our attempt to better characterize the interaction between c-Myc and Mcm2. The panel of truncated mutants of c-Myc was co-transfected in 293T cells with a vector expressing the Mcm2 full length no tagged. Mcm2 co-immunoprecipitated with an internally inconsistent pattern of c-Myc mutants (**Figure 22**).

As reverse strategy we generated truncated mutants of Mcm7 tagged with HA according to the scheme adopted for the *in vitro* mapping, and examined their interaction with full length c-Myc. Despite the clear result obtained *in vitro*, we could not identify a specific region of Mcm7 that binds c-Myc since all the mutants co-immunoprecipitated with c-Myc, as reported in two complementary experiments in **Figures 23.**

2.6 c-Myc poorly co-localizes with Mcm7

To better characterize the interaction between c-Myc and Mcm7, we investigated whether the two proteins were associated in the same sub cellular compartment. Since both the proteins are nuclear, we checked if they co-localize on the chromatin. Considering that c-Myc is degraded in S phase (Lehr et al., 2002) and Mcm7 is released from chromatin after S phase, H1299 cells were synchronized in G1/S transition with a double thymidine/mimosine block. Due to the considerable nucleoplasmic pool of Mcm7, in order to avoid a diffuse staining for this protein, after fixing the cells the nuclei were permeabilyzed with triton. The treatment releases the entire nucleoplasmic protein fraction. The chromatin-bound fraction was hybridized with specific antibody against

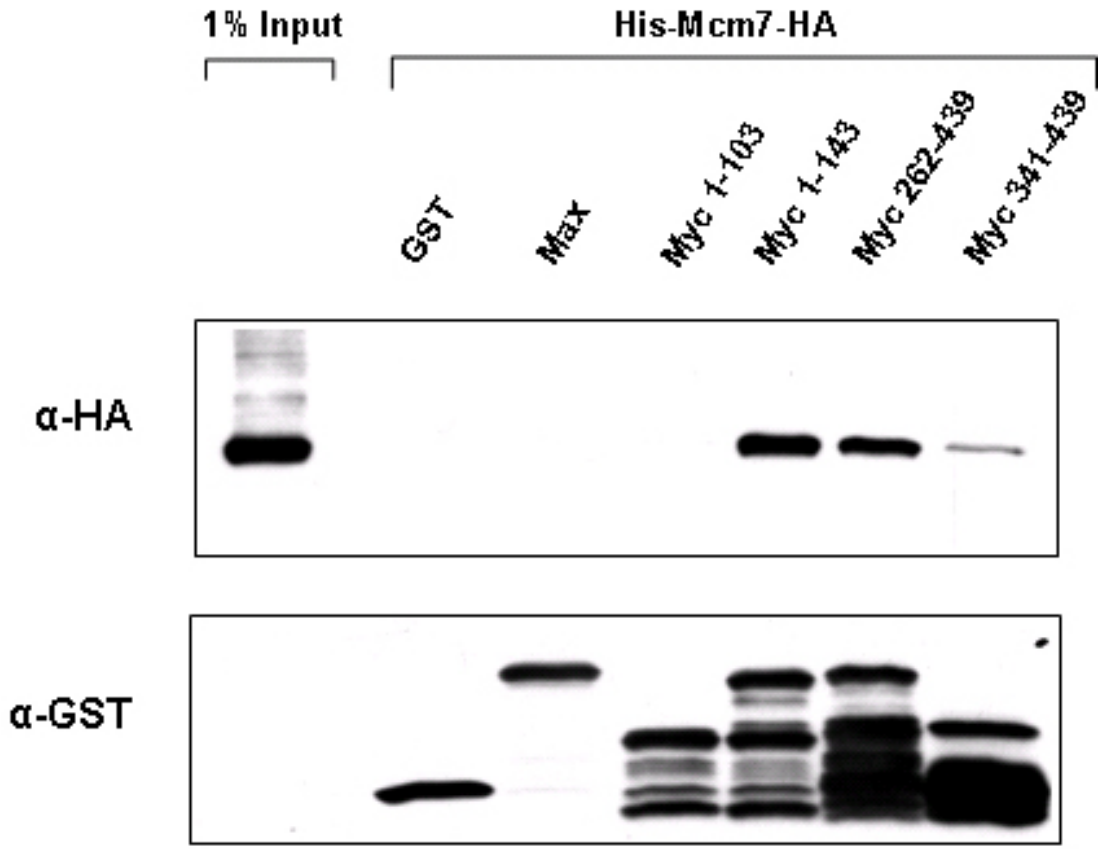


Figure 19: Max doesn't interact directly with Mcm7.
Full length GST-Max was used in GST pull down assay with His6-Mcm7-HA. Different GST-Myc mutants were used as control for the assay.

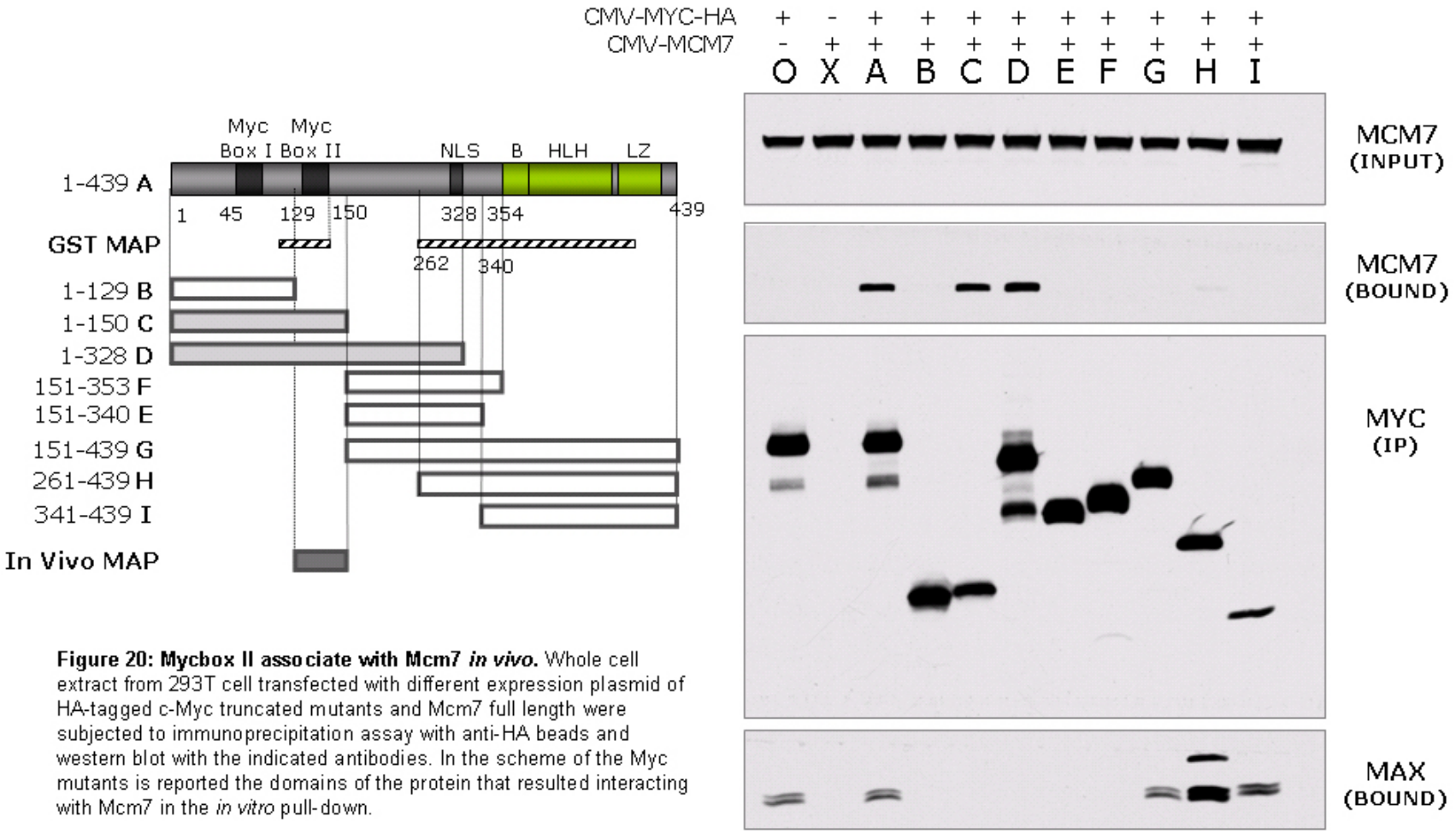


Figure 20: Mycbox II associate with Mcm7 *in vivo*. Whole cell extract from 293T cell transfected with different expression plasmid of HA-tagged c-Myc truncated mutants and Mcm7 full length were subjected to immunoprecipitation assay with anti-HA beads and western blot with the indicated antibodies. In the scheme of the Myc mutants is reported the domains of the protein that resulted interacting with Mcm7 in the *in vitro* pull-down.

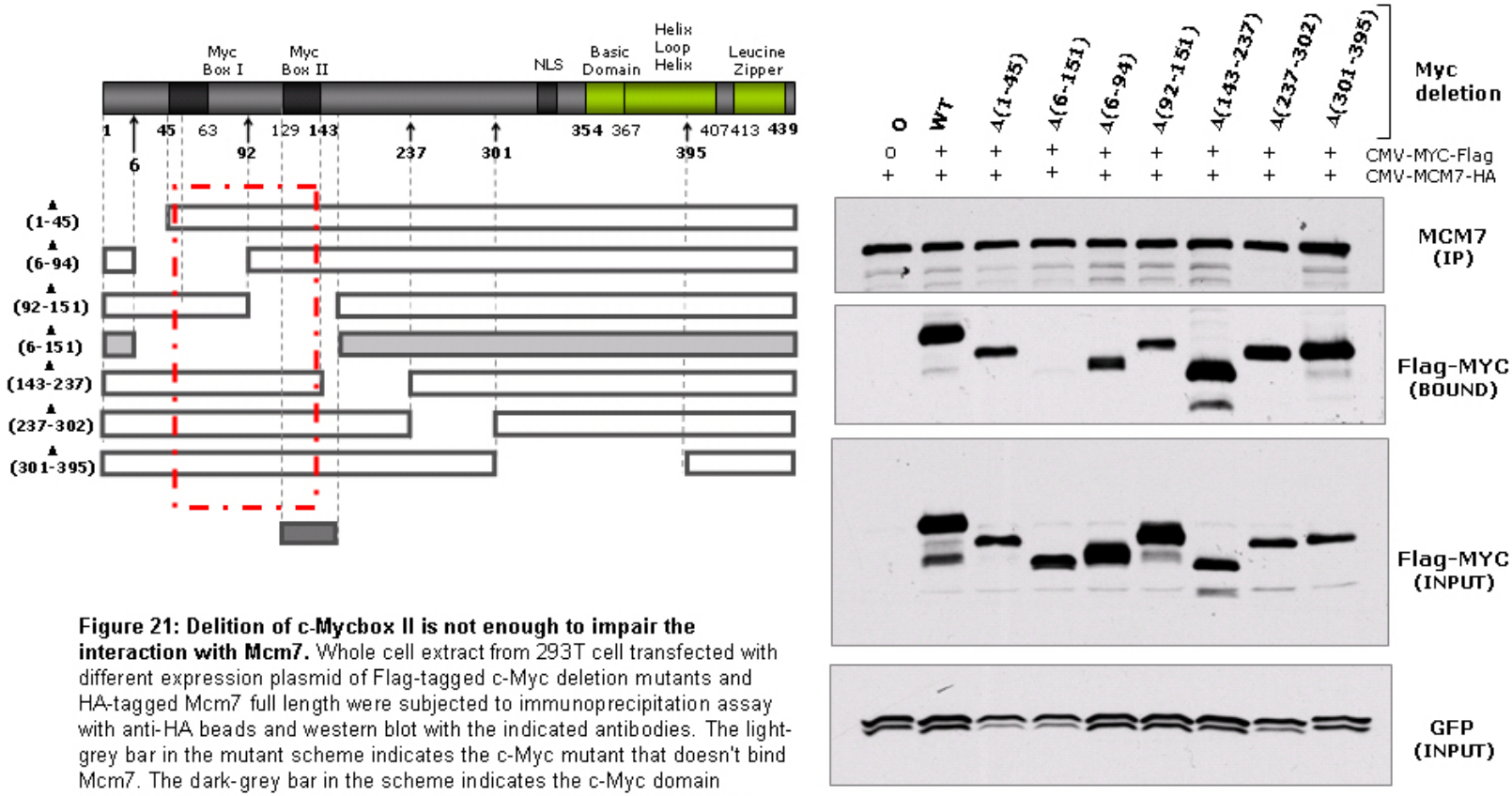


Figure 21: Delition of c-Mycbox II is not enough to impair the interaction with Mcm7. Whole cell extract from 293T cell transfected with different expression plasmid of Flag-tagged c-Myc deletion mutants and HA-tagged Mcm7 full length were subjected to immunoprecipitation assay with anti-HA beads and western blot with the indicated antibodies. The light-grey bar in the mutant scheme indicates the c-Myc mutant that doesn't bind Mcm7. The dark-grey bar in the scheme indicates the c-Myc domain responsible for the bind with Mcm7 defined in the mapping assay with c-Myc truncated mutants. The red-dashed square indicates the minimum c-Myc domain responsible for the interaction with Mcm7 defined in this assay.

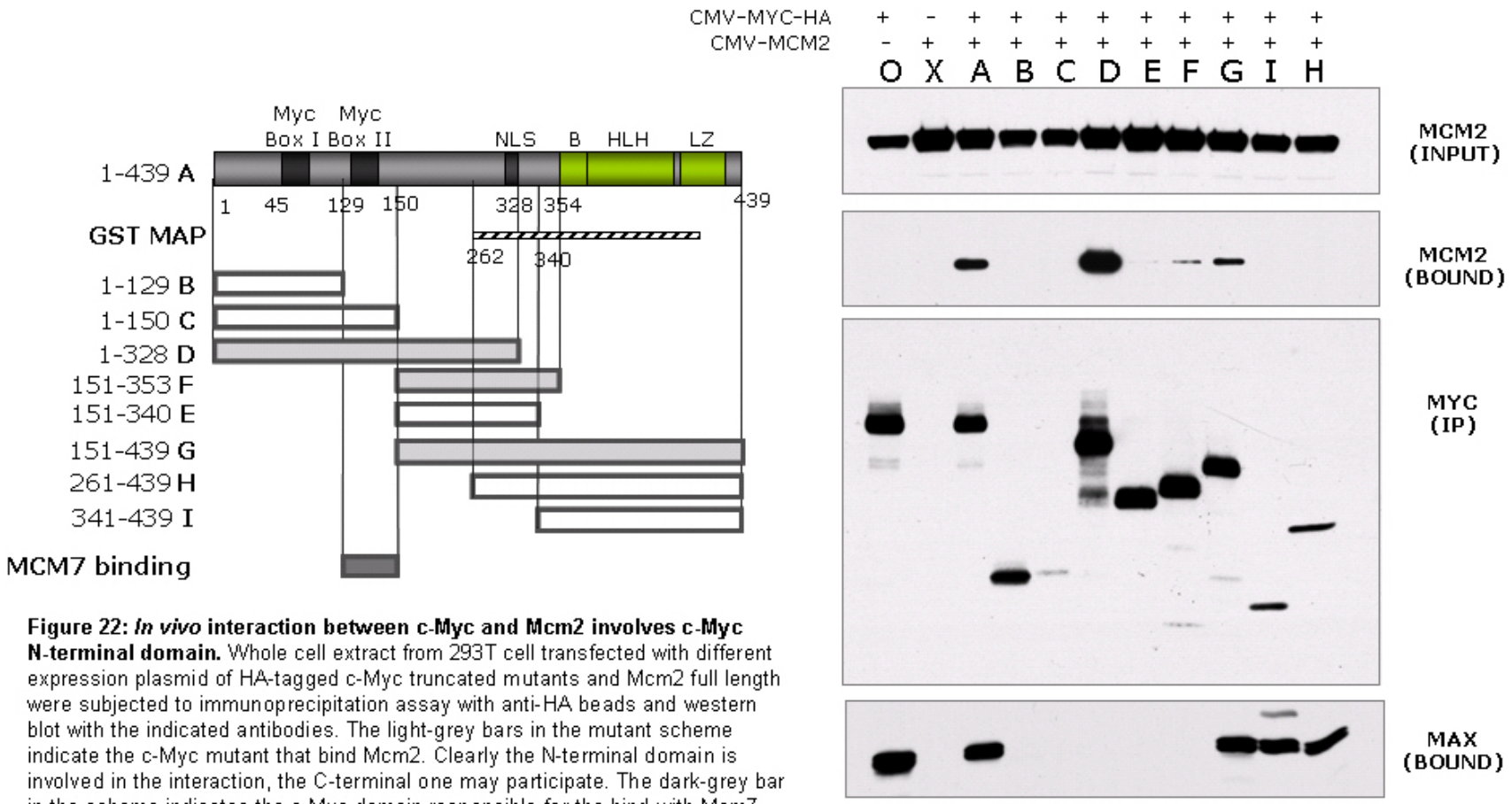


Figure 22: *In vivo* interaction between c-Myc and Mcm2 involves c-Myc N-terminal domain. Whole cell extract from 293T cell transfected with different expression plasmid of HA-tagged c-Myc truncated mutants and Mcm2 full length were subjected to immunoprecipitation assay with anti-HA beads and western blot with the indicated antibodies. The light-grey bars in the mutant scheme indicate the c-Myc mutant that bind Mcm2. Clearly the N-terminal domain is involved in the interaction, the C-terminal one may participate. The dark-grey bar in the scheme indicates the c-Myc domain responsible for the bind with Mcm7 defined in the same assay. The c-Myc domain that resulted interacting with Mcm2 in the *in vitro* pull-down, is reported.

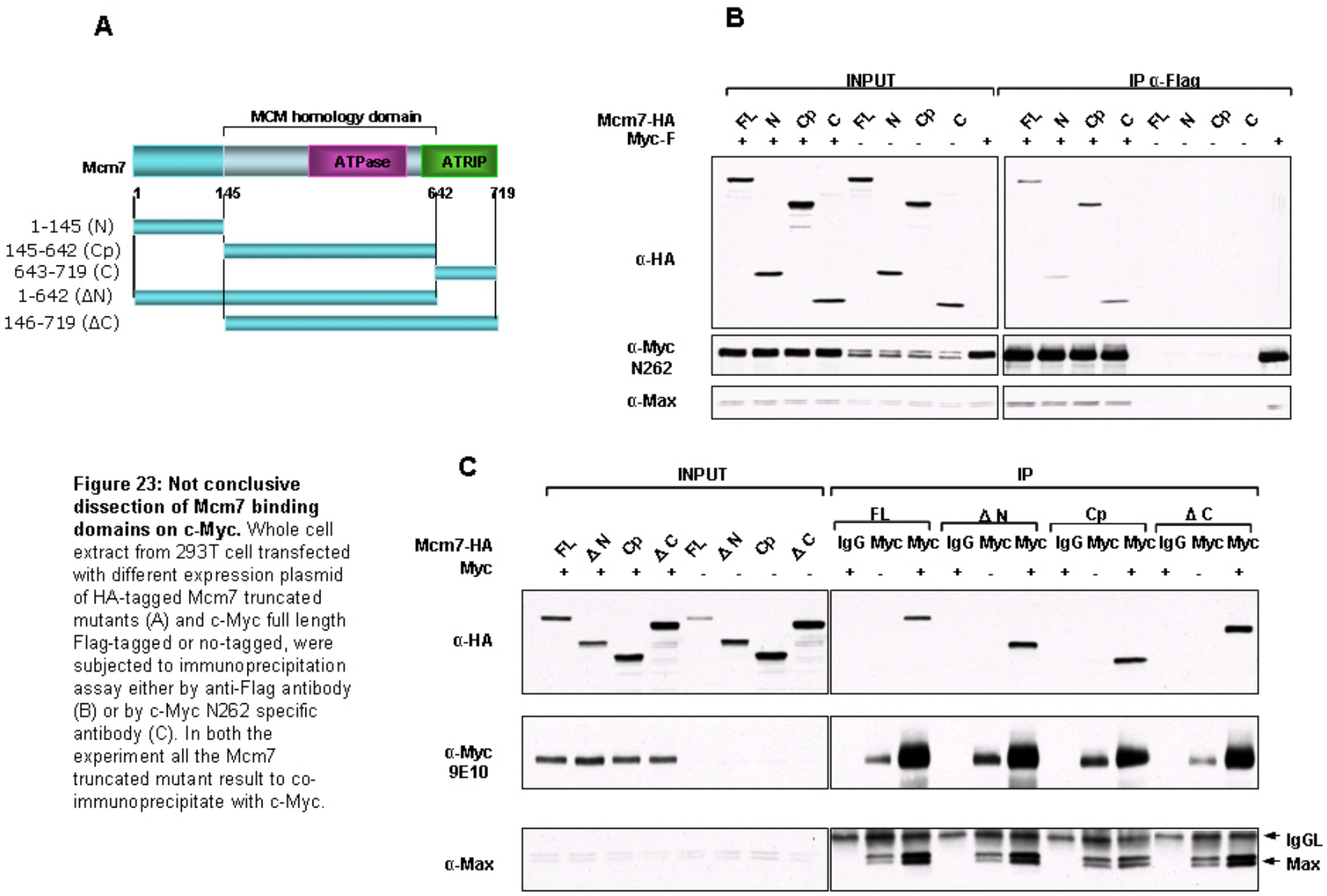


Figure 23: Not conclusive dissection of Mcm7 binding domains on c-Myc. Whole cell extract from 293T cell transfected with different expression plasmid of HA-tagged Mcm7 truncated mutants (A) and c-Myc full length Flag-tagged or no-tagged, were subjected to immunoprecipitation assay either by anti-Flag antibody (B) or by c-Myc N262 specific antibody (C). In both the experiment all the Mcm7 truncated mutant result to co-immunoprecipitate with c-Myc.

c-Myc and Mcm7. Analysis with a confocal microscope revealed that the level of co-localization of the two proteins is very low, roughly 15% of the spots co-localized (**Figure 24**).

2.7 c-Myc associates with the MCM complex along the cell cycle

To further characterize the association between c-Myc and the MCMs we wanted to study this interaction along the cell cycle. H1299 cells were synchronized in G1/S by a double thymidin/mimosine block. After the block was released in fresh medium, the cells were harvested at different time points. The cell cycle profile of each sample was determined by FACS analysis. c-Myc was immunoprecipitated from cell lysates using a specific antibody. The complex of proteins co-immunoprecipitated with c-Myc was analized by western blot. Using a specific antibody against Mcm7 we found that the association between the two proteins is stable all along the cell cycle (**Fugure 25**).

2.8 Synchronous recruitment on the chromatin

The MCM complex is thought to be the DNA helicase that unwinds the double helix allowing the DNA polymerase to bind one strand of the DNA and to move along it during the replication. The MCMs binding on chromatin is tightly regulated: they are recruited during G1 and are released after S phase. c-Myc is also a protein that binds the DNA, but up to date there are non clear study about its cycle regulation on chromatin. We wanted to verify whether there is any temporal correlation during the cell cycle between the loading on the chromatin of c-Myc and of the MCMs. H1299 cells were synchronized in mitosis with a thymidine-nocodazole block. After "shake off" the floating mitotic cells were collected and re-plated. Cells were harvested at different time and chromatin fractionation was obtained lysing the samples in CSK buffer (10mM HEPES-Na pH 7.9; 100mM NaCl; 1.5 mM $MgCl_2$; 300 mM Sucrose; 50 mM NaF; 0.1 mM Na_3VO_4; Proteinase inhibitor) (Mendez & Stillman, Mol Cell Biol 2000). Western blot analysis with antibody against Myc, Mcm2 and Mcm7 confirmed that the loading of the MCMs occurs during late mitosis/G1, and showed that c-Myc, although detectable already during the mitosis, has a boost of loading on the chromatin starting from the end of mitosis and during the G1. An antibody specific against Max revealed that the protein behaves like c-Myc

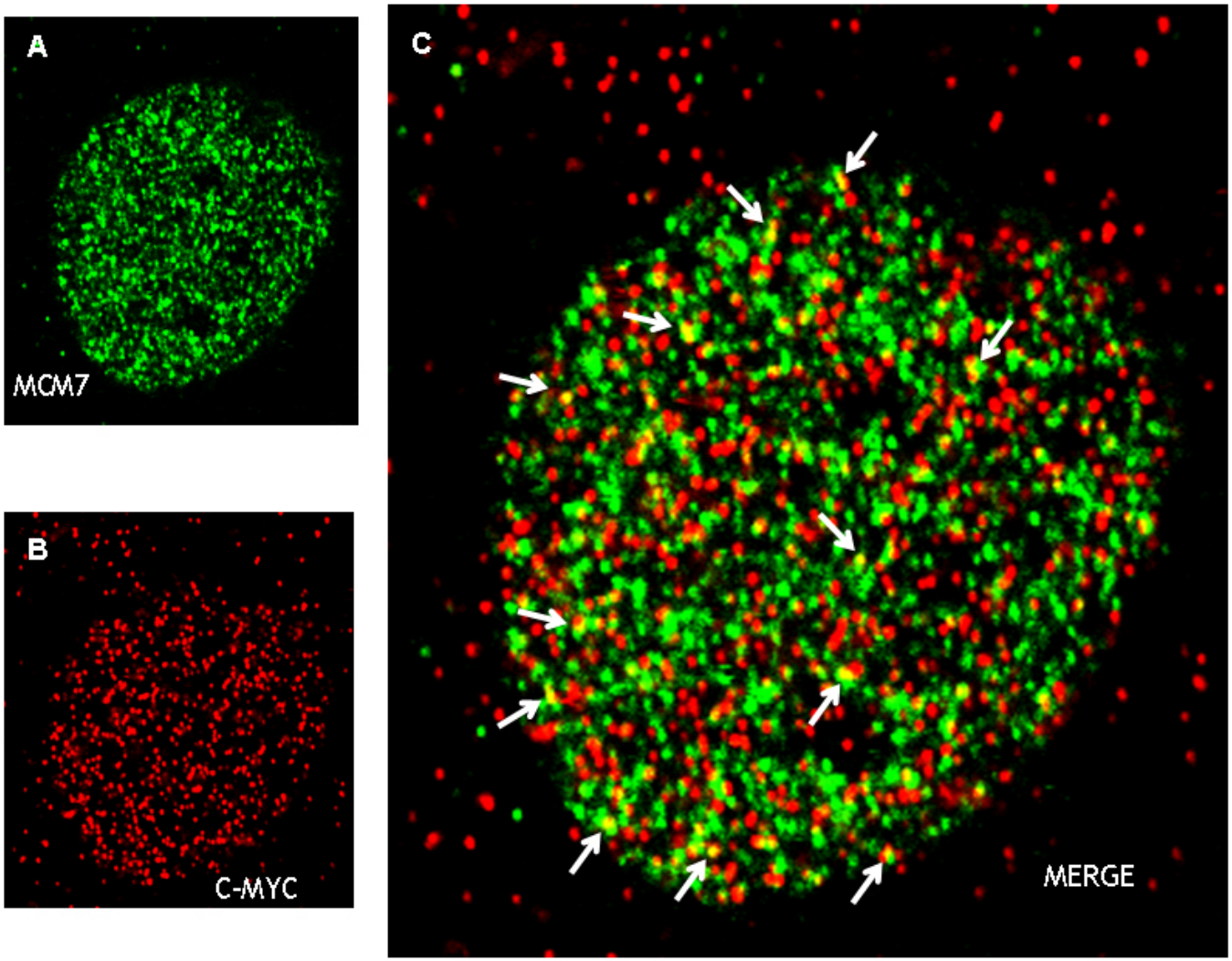


Figure 24: c-Myc poorly co-localize with Mcm7 in the G1/S transition. H1299 cells grown on coverslips were fixed after a double thimidine/mimosine block and labeled with antibodies against c-Myc (red, B) and Mcm7 (green, A). Immunofluorescence staining was analyzed by confocal laser scanning microscopy. 15% of total c-Myc spots in a planar confocal section co-localize with Mcm7 resulting in the yellow dots indicated by the arrows (C)

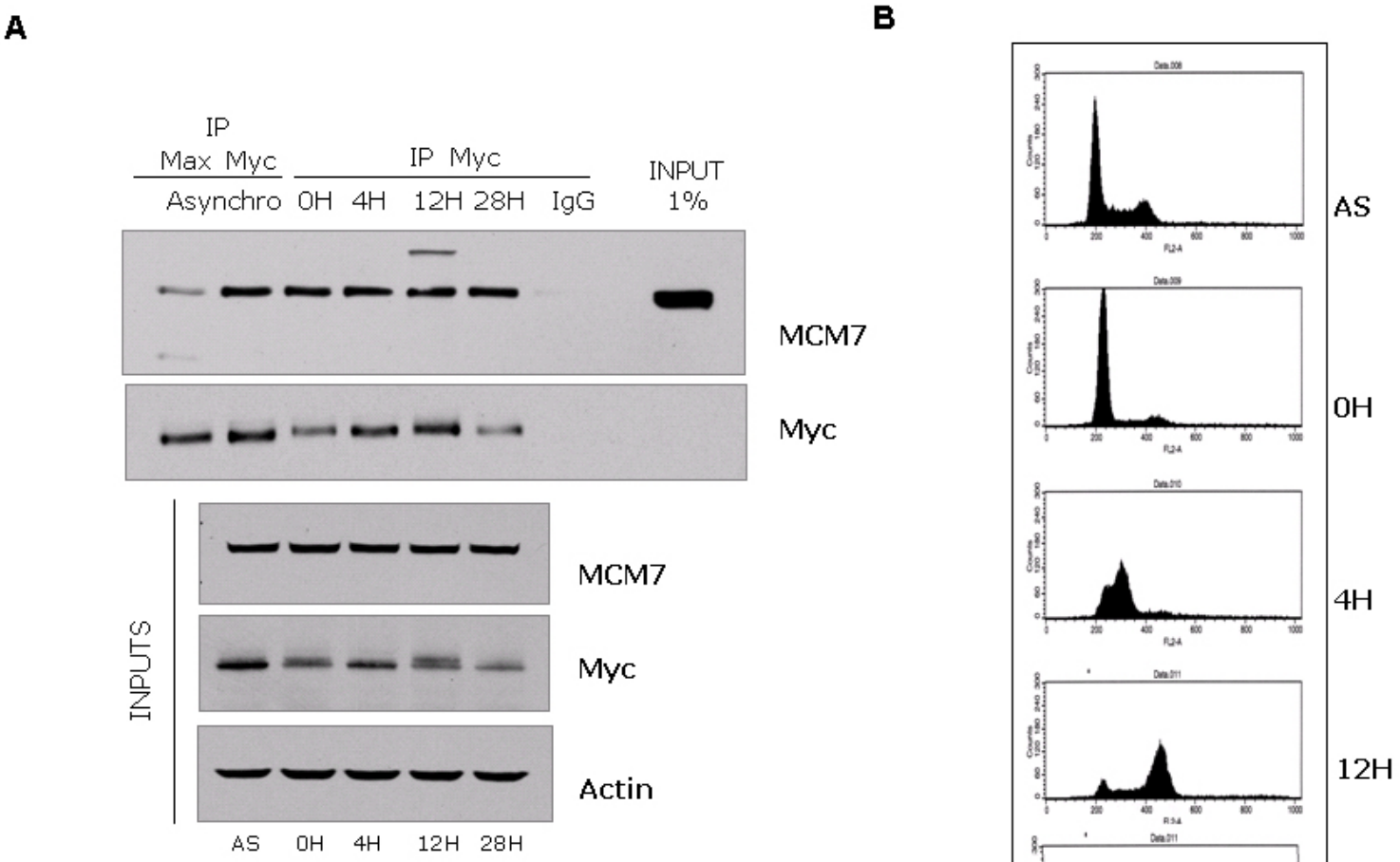


Figure 25: Stable interaction between c-Myc and Mcm7 along the cell cycle. H1299 cell were Synchronized in G1/S by double Thymidine/miosine block. Nuclear extracts from cells harvested at different time point were subjected to immunoprecipitation assay by c-Myc N262 specific antibody. Western Blot with anti-Mcm7 antibody reveals a stable interaction between c-Myc and Mcm7 at different phases of the cell cycle (A). Average cell cycle profile of the sampled population was verified by Propidium Iodide staning and FACS analysis (B).
AS = asynchronous population

although its level on chromatin after the end of mitosis is more stable compared to the c-Myc one. The same western blot performed on the Triton-soluble fraction reveal that the cytoplasmic-nucleoplasmic level of the three proteins is stable during the mitosis-G1. Blotting for phospho-histone H3, a marker for the mitosis, revealed the effective block in M phase, and the synchronous re-entry the cell cycle of the cell populations (**Figure 26**).

2.9 Sizing the complex

After we demonstrated c-Myc interacting with all the components of the MCM complex, we sought to further characterize the interaction between these proteins in order to determine whether these interactions are individual and mutually exclusive, or alternatively, if c-Myc interacts with the entire MCM complex. The c-Myc interacting complex was freshly purified from the H/HF2 stable cell line and separated according to the size by a gel filtration chromatography in physiological conditions. The collected fractions were analyzed by SDS-PAGE and western blot using antibody against c-Myc, Max and Mcm7. The corresponding size of the fractions was determined by comparisons with molecular marker run in the same conditions. c-Myc was found widely distributed along the fractions, with a pick in fraction corresponding to molecular weight around 400kD, and another one at higher molecular weight. Max distribution coincided with the first pick of c-Myc, but was not found in the higher molecular weight fractions. Unfortunately we failed in detecting any Mcm proteins (**Figure 27**). Since the presence of the proteins in the c-Myc complex was confirmed by western blot (data not shown), we thought that the no detectable level of the Mcm proteins was due to technical problem that occurred in the fractionation, as proteins adsorption by the resin of the column. To avoid any possible effect due to the column, the complex of proteins associated with c-Myc was separated by ultracentrifugation in glycerol gradient. A protein marker was run along with the sample and the distribution of the known proteins was checked separating the collected fractions by SDS-PAGE and following blue comassie staining. Western blot analysis of the fractions confirmed the wide distribution of c-Myc along the gradient, mostly in two picks. More importantly we were able to detect Mcm7 and Mcm3 distribution in high molecular weight fractions. Comparing with the molecular marker, the corresponding size of the complexes in which c-Myc and Mcm3/7 were found

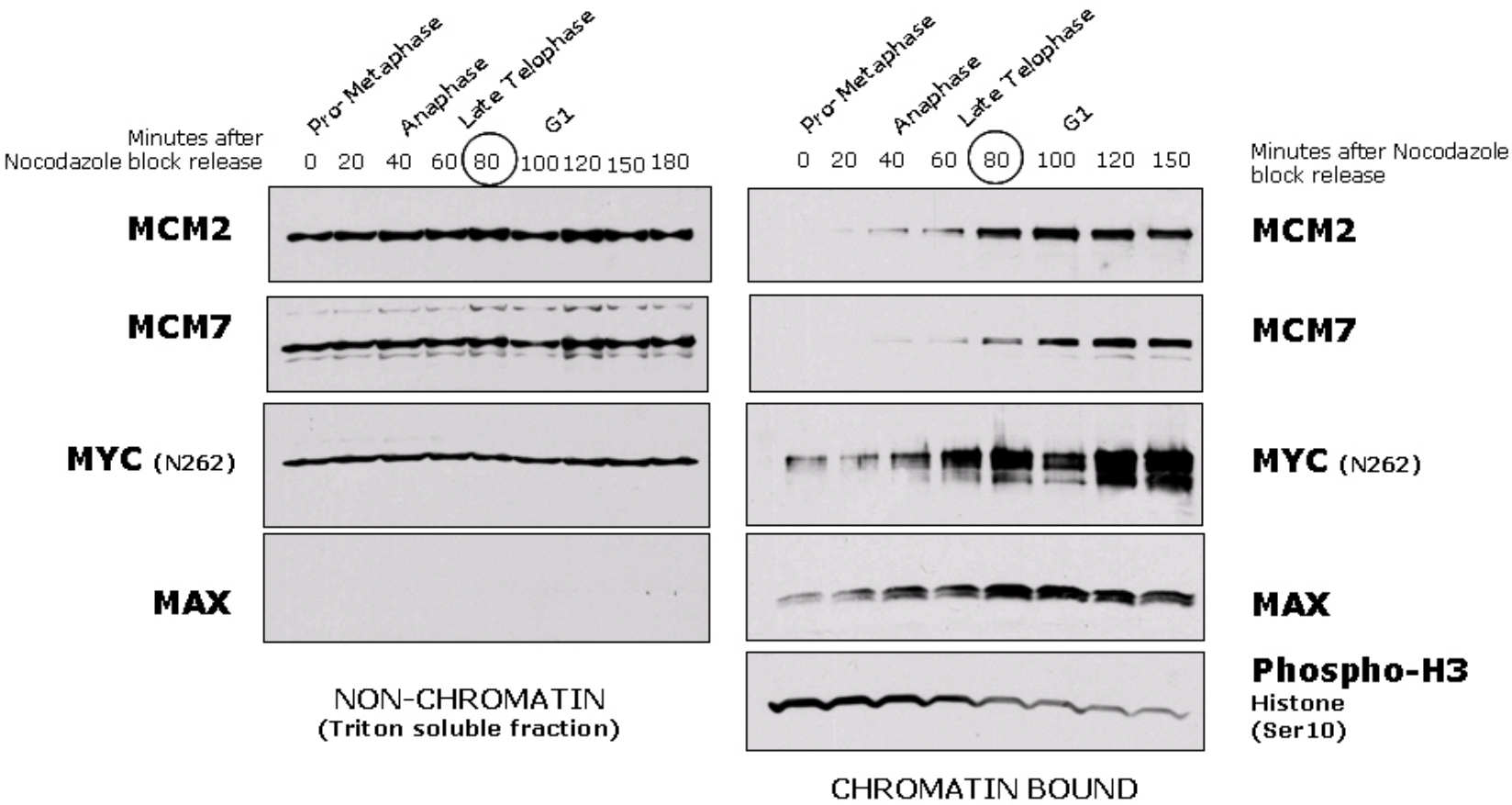


Figure 26: Parallel recruitment of c-Myc and MCM helicase to chromatin. H1299 mitotic cells were obtained by "shake-off" after first block in G1/S with Thymidine and a second one with Nocodazole to accumulate cells in pro-metaphase. Fractionated extracts collected at indicated time points were analyzed by western blot with indicated antibody. The grey circle at 80 minuts after Nocodazole block release represents the end of the mitosis. Cell cycle progression is monitored by anty-Phospho-H3 Histone (Ser10) antibody.

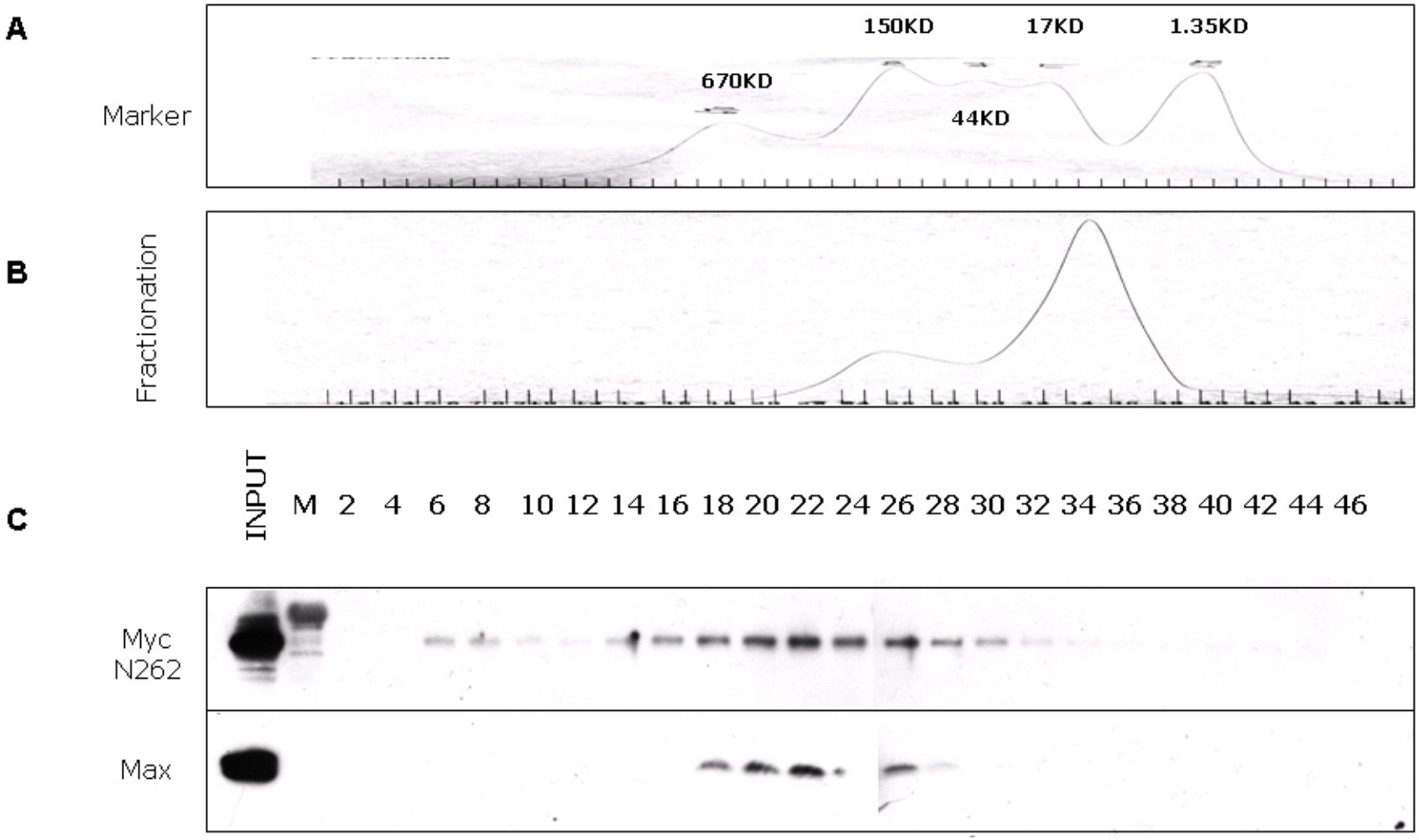


Figure 27: c-Myc forms high molecular weight complex independently from Max. c-Myc complexes from H/HF2 were separated on a Superose 6 SMART system gel filtration column. Fractions were collected and c-Myc and Max expression in each fraction was monitored by immunoblotting (C). Molecular size marker proteins were run on the same column under identical buffer conditions (A). The big pick that fractionation profile (B) is due to the Flag-peptide used for the elution of the complex from the beads.

associated exceed the molecular weight of a possible complex consisting in c-Myc, Max and the MCMs. The MCMs, at least in the initial steps of DNA replication, are recruited on the DNA by association with other proteins in a bigger complex, the pre-RC. Therefore we checked whether other members of the pre-RC complex were separated, by glycerol gradient, in the same fractions with c-Myc, Mcm3 and Mcm7. Western blot with specific antibody against Cdt1 and Cdc6, detected the presence of these two proteins in high molecular weight fractions, suggesting that c-Myc is associated in high molecular weight complex with different elements of the pre-RC (**Figure28**).

2.10 No transcriptional implication in c-Myc-MCMs interaction

c-Myc has been extensively studied for its role in regulating the transcription of its target genes. Although further validations are needed, weakly activated, or even repressed, the list of c-Myc target gene includes around 15% of the genome. Consistently with the emerging idea of an interaction between transcription and replication apparatus (for review Murakami and Ito, 1999) the MCMs have been implicated in several aspects of transcriptional control (Holland et al., 2002; Zhang et al., 1998Fitch et al., 2003). We were interested in verify whether the interaction between c-Myc and the MCMs was functional in c-Myc transcriptional activity. More specifically, we tested the influence of Mcm7 on the c-Myc mediated activation of a lucifarase gene under the control of *tert* promoter (as previously described in Wu KJ et al., 1999). 293T were transiently co-transfected with the reporter gene, fixed amount of a vector expressing c-Myc and increasing amount of a vector expressing Mcm7 full length. Measurement of the luciferase activity showed that Mcm7 doesn't positively affect the c-Myc activation of *tert* promoter. Instead a slight repression is relievable when high dose of Mcm7 are transfected. Western blot analysis confirmed the expression level of the proteins (**Figure 29**)

2.11 Mcm7 displaces c-Myc-Max from the E-box

In order to assess whether the effect of Mcm7 on c-Myc-mediated transcriptional activation was a consequence of an impaired capability of the heterodimer c-Myc/Max to bind the DNA, we performed an oligonunleotide pull down assay. Vectors expressing

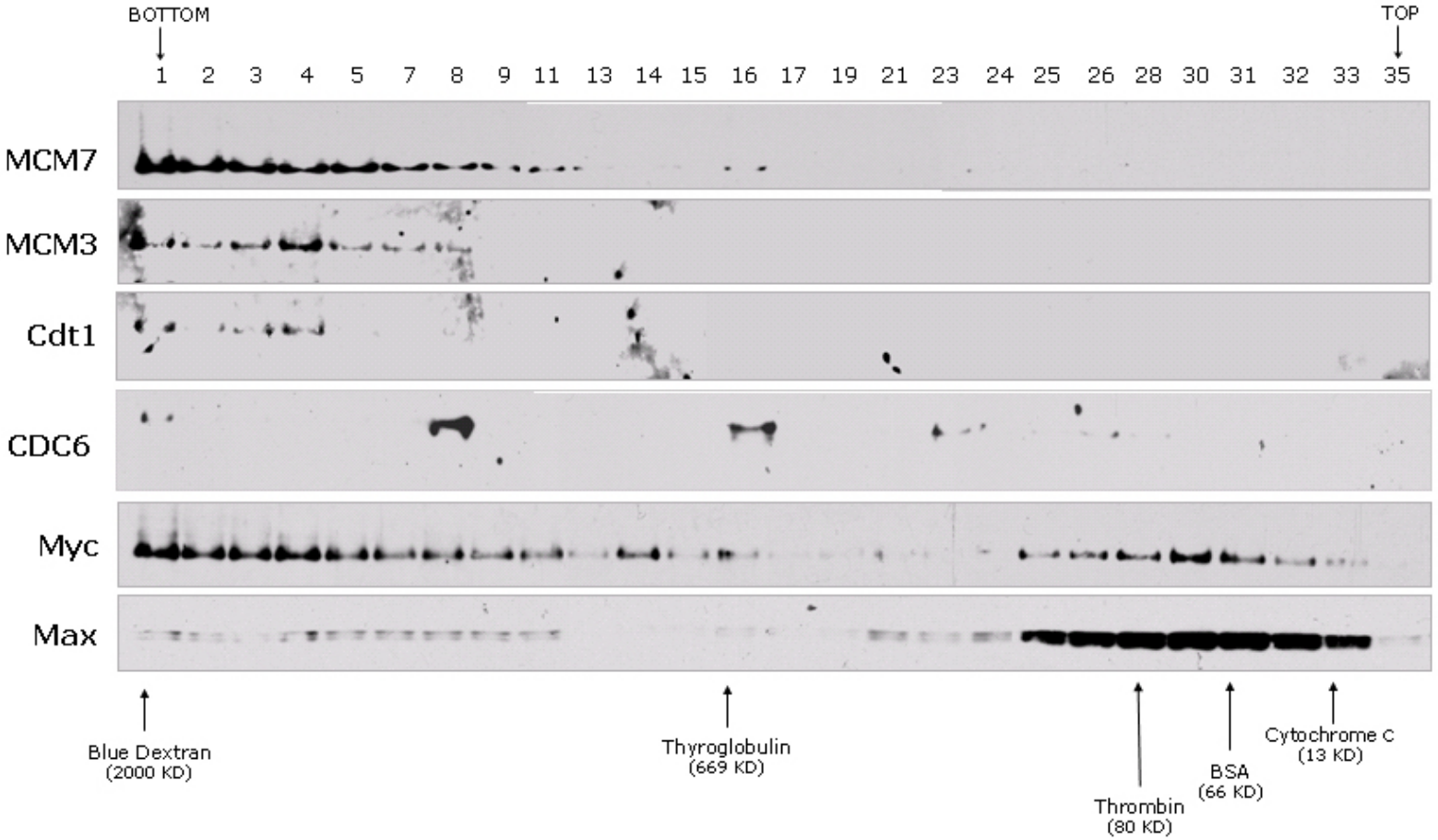


Figure 28: c-Myc co-sediments in high molecular weight fraction with members of the pre-Replication Complex. c-Myc associated proteins were sedomented by 30-60% glycerol gradient centrifugation at 48,000 rpm for 20 h woth the molecular weight markers. The fraction were collected and anylised by immunoblotting with the reported antibodies. The position of the molecular weight markers are indicated, as well the number of the fractions.

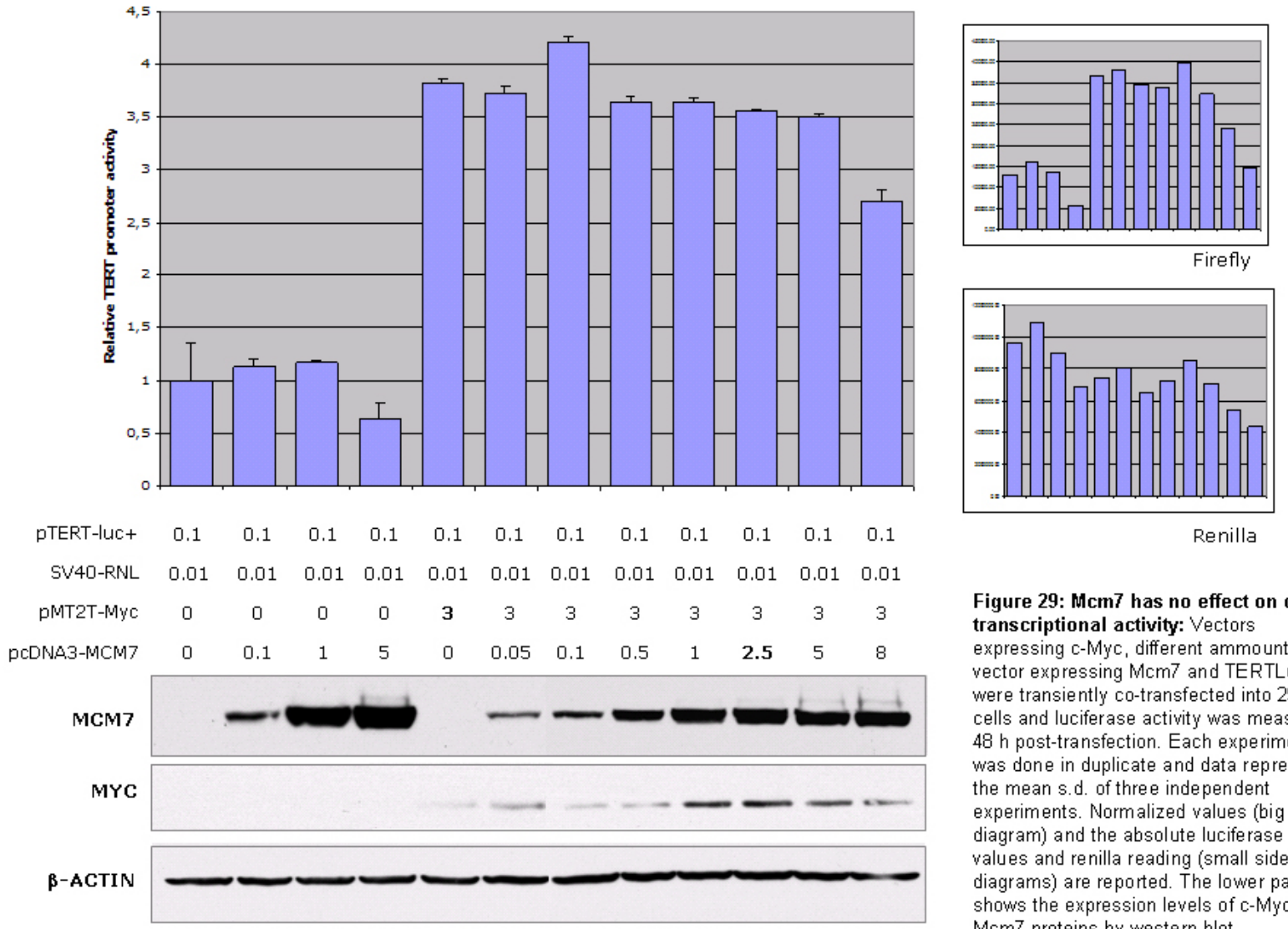


Figure 29: Mcm7 has no effect on c-Myc transcriptional activity: Vectors expressing c-Myc, different ammountof a vector expressing Mcm7 and TERTLuc800 were transiently co-transfected into 293T cells and luciferase activity was measured 48 h post-transfection. Each experiment was done in duplicate and data represent the mean s.d. of three independent experiments. Normalized values (big diagram) and the absolute luciferase values and renilla reading (small side diagrams) are reported. The lower panel shows the expression levels of c-Myc and Mcm7 proteins by western blot.

c-Myc, Max and Mcm7 were co-transfected in 293T cell in different combinations. Protein extracts were incubated with 38bp double strand biotinylated oligonucleotide encompassing the BN51-intron1 E-box previously described by Amati e co-workers (Greasley et al., 2000). The oligos were pulled down with streptavidin-conjugated agarose beads and the proteins interacting with the DNA were released from the binding boiling directly the samples in the SDS loading buffer. Western blot analysis showed that while Max can bind E-box in absence of c-Myc, c-Myc requires heterodimerization with Max to bind the consensus sequence, as it was already demonstrated. C-Myc binding to the E-box is impaired in presence of Mcm7. This happened even when a mutant of Mcm7 lacking the C-term domain, that *in vitro* data show to be the region involved in the direct binding with c-Myc, was used (**Figure 30**). As control for aspecific interaction with the probe we designed another couple of oligonucleotides carry a mutation in the E-box (CACGTG to TACGTG). As expected the mutated E-box is not efficiently bound by c-Myc/Max heterodimer.

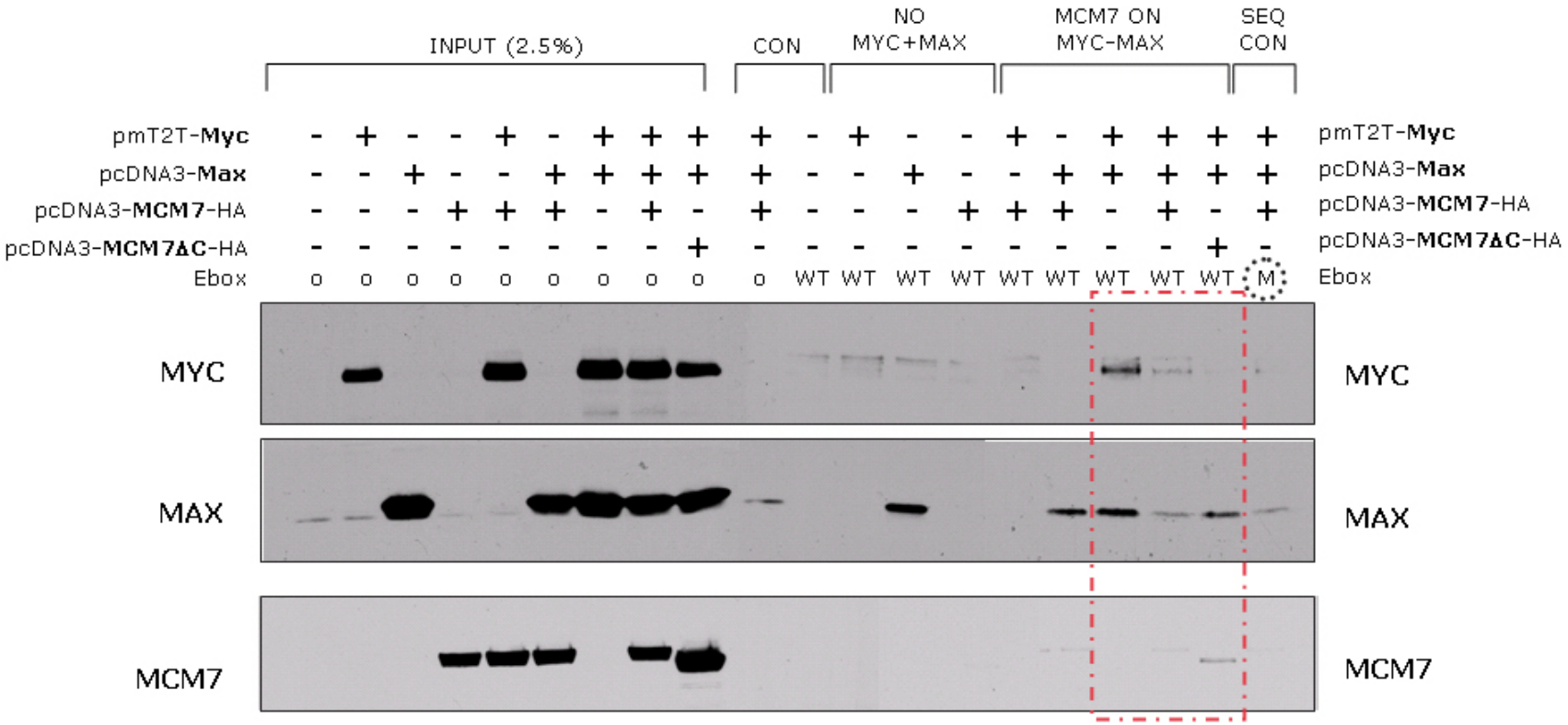


Figure 30: The Mcm7 interaction with c-Myc impairs E-box recognition by the c-Myc/Max heterodimer. 293T were transiently co-transfected with vector expressing c-Myc, Max, Mcm7 and Mcm7 mutant lacking the C-terminal domain (Mcm7ΔC) in different combinations. Nuclear extracts were prepared as indicated in "Materials and Methods," and incubated with the biotinylated BN51-E-box oligonucleotide or with a single point mutated one (CACGTG to TACGTG).Bound protein was precipitated with streptavidin-coupled agarose and analyzed after extensive washing by SDS-PAGE and immunoblotting for the transfected proteins. On the left side of the panel are reported the inputs for the pull down, shown on the right side. The red-dashed rectangle highlights the lanes that shows the impairing role of Mcm7 on c-Myc/Max heterodimer binding to DNA CON = control . The circle indicates the mutated E-Box.

CHAPTER III

Discussion

3.1 Complex purification

The link between c-Myc and cancer was immediately clear since in 1982 its gene was discovered as the human homolog of retroviral gene responsible for the avian myelocytomatosis (Crews et al., 1982; Dalla-Favera et al., 1982). Deregulation of gene expression due to translocations, amplifications, or enhanced translation or protein stability has been found involved in nearly all types of tumor (Sears et al., 1999; Stoneley et al., 2000; Noguchi et al., 2001; Alarcon-Vargas et al., 2002; Channavajhala and Seldin, 2002; Popescu and Zimonjic, 2002; Ruggero and Pandolfi, 2003; Tonini and Romani, 2003). Enforced c-Myc expression is sufficient to provoke the entry and continuous, mitogen-independent, proliferation of cells (Cavalieri and Goldfarb, 1987, 1988; Eilers, 1991), and it effectively blocks terminal cell differentiation (Coppola and Cole, 1986; Maruyama et al., 1987; Freytag, 1988). On the other side, c-Myc triggers rapid apoptosis in the cells (Askew et al., 1991; Evan et al., 1992). At the beginning c-Myc was thought to be a transcriptional activator. Interacting with Max, it recruits multiple co-activator and protein complexes to the promoter like Mediator complex, P-TEFb, TIP48/49, P300, GCN5 and others. But after the first empirically identified target genes, use of microarrays and a genome-wide survey has found that the number of gene that are bound by c-Myc and that are its potential target for regulation, is incredibly high (~15% of the genome)for any transcriptional factor so far studied. Moreover c-Myc not only activates, but also represses transcription of some target genes. After almost a quarter of century since its discovery, extensively studied, parsley-c-Myc exerts its influence in almost every aspect of cell biology, but the precise mechanisms how it does it, are still not well understood. In order to shed new light on its role in the cell fate, or to open new unexpected possibility to prevent Myc-mediated cancer insurgence, we wanted to identify proteins that interact with c-Myc under physiologic conditions. Previous studied have described several c-Myc interacting proteins that were isolated using ether strategies based on an *in vitro* formation of c-Myc containing complexes (MacMahon et al., 1998;

Wood et al., 2000), or two hybrid strategies that involved the formation of a non-physiologic complexes. In addition these studies typically used deletion mutants of the c-Myc protein, further allowing for the formation of non-physiological complexes. Although in each case the interaction with c-Myc was confirmed *in vivo* by co-immunoprecipitation analysis, it is conceivable that these approaches have missed a number of proteins that are present in physiologic-formed c-Myc complexes. Based on these observations we decided to isolate the *in vivo*-formed complexes directly from cells using as a bait physiological level of a tagged full-length c-Myc protein. c-Myc is a low abundant protein, whose level is strictly regulated. In order to increase the intracellular level of the protein we used MG132, an inhibitor of the proteasome. Using this strategy, and high salt condition to increase the stringency of protein-protein interactions, we isolated several proteins that were not before described as associated to c-Myc. Intriguingly we have no evidence that among the protein co-immunoprecipitated in our experiment, there are also other known partners of c-Myc as TRAAP or TIP48/49. The presence, at least for TRAAP, was discarded by western blot analysis (data not shown). It is conceivable that the presence of MG132 is somehow selecting for some complexes in which c-Myc is associated rather than others, but the purified c-Myc protein was mainly non-ubiquitinated, as assessed by its migration on SDS-PAGE, by Western Blot with specific anti-ubiquitin antibodies, and mass spectrometry microsequencing (data not shown). So our complexes of proteins was not co-purified with a modified form of c-Myc. Moreover the interactions between c-Myc and the proteins identified were confirmed in native conditions and without MG132 treatment.

Among the proteins that were identified by mass-spectrometry analysis we focused our attention to Mcm7, Mcm5. These two proteins belong to a complex of six proteins (Mcm2-7) that were originally isolated from a screening in yeast for mutants defective in minichromosome maintenance (Maine et al., 1984). Member of the MCM family have been found in all the eukaryotic by genetic and biochemical methods. It has been demonstrated that the MCM play a key role in DNA replication. Loading of the MCM on the origin is a critical step in order to start replication. According to sequence homology, *in vitro* data and crystal structure, they are thought to be the helicase that unwind the double strand of DNA (Ishimi, 1997; Fletcher et al., 2003). MCMs are expressed in

abundance in all phases of the cell cycle but they are degraded in cell that have exited the cell cycle, such as quiescent, senescent or differentiated cells (Madine et al 2000). This has recently led to their potential clinical application as marker for cancer screening as good alternative to the conventional KI-67 and PCNA (less effective and specific for proliferation). Given the involvement of c-Myc in tumorigenesis, we wondered whether the suggested interaction between c-Myc and Mcm7 could be functional in this process. Mcm7 has already been demonstrated to interact with Rb, and this interaction inhibits DNA replication in a *Xenopus* system (Sterner et al., 1998). Our finding would allow a scenario in which a tumour repressor and oncogene compete for binding the same protein with important consequences on DNA replication and then on the cell fate (normal cell vs. tumour cell). Moreover the MCMs are a distinct subgroup of AAA ATPase proteins. c-Myc has already been demonstrated to interact with two proteins with ATPase activity: TIP48 and TIP49, that are essential cofactors for c-Myc mediated transformation (Wood et al., 2000). These two proteins associate with each other as a double hexamer and are necassary for the catalytic activity of Ino80 complex involved in chromatin remodeling (Jonsson et al., 2004) with specific helicase and transcriptional activity (Shen et al., 2000). Taken together these observations suggesting relevant analogies between these proteins and the MCMs, we thought it could have been worthy to study the interaction between c-Myc and the Mcm7 protein identified in our complex.

3.2 Difficult mapping of the interaction between c-Myc and MCMs

We validated the interaction between c-Myc and Mcm7, and demonstrated that c-Myc actually interacts with the entire MCM complex. We found that the interaction is direct with each component of the MCMs.

It is not intuitive to imagine a model in which a single molecule of c-Myc interacts at the same time with the six subunits of the complex. A help comes from the analysis of the sequence of the MCMs. It has been suggested that the C-terminal region of MCM proteins may have a conserved helical structure. And it is known that domains containing α-helices are protein-protein interaction surfaces for contacting other proteins. Consistent with this structural data, the in vitro data show that the recombinant Mcm7 protein tested interacts with c-Myc through its C-terminal domain. The ability of c-Myc to interact with

all the MCM proteins could be essential to dynamically preserve the interaction between c-Myc and the MCM complex even in the presence of steric impairments due to other proteins binding the complex.

Considering the high abundance of the MCMs, the small fraction of them interacting with c-Myc (<1%) and assuming the capability of c-Myc of interacting with all the MCMs, we can also explain the impossibility to define in experiment of *in vivo* mapping the binding region of Mcm7 on c-Myc.

We do not exclude the possibility that c-Myc may have preferential partners among the MCMs in physiologic conditions, may be due to the ring shape conformation of the complex. This hypothesis is supported by the finding that in the complex of proteins isolated with c-Myc, all the Mcm proteins are not equally represented. As shown in **Figure 14** Mcm3 and Mcm7, among the other subunits, have the relatively higher affinity for c-Myc. It is reported that the MCMs have a different affinity for one another forming different sub-complex, among them Mcm4/6/7 with helicase activity *in vitro* and Mcm2/3/5 with regulatory activity (Ishimi, 1997; Ishimi et al., 1998; Lei, 1996). Data from the crystal structure show that c-Myc/Max form antiparallel tetramers (Nair and Burley, 2003). This would allowed a model in which in the cell a tetramer c-Myc/Max can interact with a MCM complex through preferential direct binding with Mcm3 and Mcm7, moving to others member of the complex in order to face conformational changes of the ring following interactions with other putative partners.

Unfortunately we failed in finding a point mutant of c-Myc that doesn't interact with the MCM complex. The *in vivo* mapping of the interaction between c-Myc and Mcm2 and Mcm7 was never conclusive, possibly due to the same reason discussed above. Given the data from the *in vitro-in vivo* interactions showing binding between c-Myc and all the subunits of the complex, and the sequence homology among them, we expected the same result from the mapping with the other MCMs. Even more troublesome was the dependence on the MycboxII domain of c-Myc for interaction with the MCMs that put a severe limitation in finding a mutant that exclusively impairs a specific Myc function without affecting all the others associated to its transcriptional activation domain (TAD).

3.3 Implication in transcription

The MCMs have been implicated in several aspect of transcriptional control. Several MCM proteins have been shown to associate with the carboxy-terminal domain (CTD) of RNA polymerase II (Yankulov et al., 1999). The CTD is essential for transcription initiation and elongation in the context of chromatin (Sims et al., 2004). Evidences suggest that the MCM proteins associate with specific transcription factors. Mcm3-Mcm5 heterodimer associates with a transcription factor stimulated by gamma interferon, STAT1α, for transcriptional activation (DaFonseca et al., 2001). In yeast Mcm7 regulates the transcription activity of Mcm1 by promoting Mcm1 binding to and activating its targets *in vitro* (Fitch et al., 2003).Well documented is the transcriptional activity of the heterodimer c-Myc/Max that is carried out recruiting to the E-box multiple co-activator and protein complexes. Among them there is Gcn5, a HAT. These observations suggested as functional meaning of the interaction between c-Myc and the MCMs, possible role in c-Myc-mediated transcriptional activation. Our results seem to discard this hypothesis. Not only Mcm7 failed in enhancing the transcriptional activation of *tert* promoter mediated by c-Myc, buy also seem to determine a slight reduction. More than possible involvements in c-Myc mediated transcriptional repression, a promoter specificity effect in determine the outcome of this experiment should be considered. In the specific context of the *tert* promoter is possible that Mcm7 can compete with the physiologic co-activators for the binding with c-Myc. Other possible explanation of this result comes from another experiment that shows the impairment of the c-Myc/Max heterodimer in binding the E-box determined by Mcm7. This data can be considered consistent with the one deriving from the ChIP studies of c-Myc genome binding. Fernandez et al. reported that in physiologic conditions endogenous c-Myc normally binds 7% of non E-box promoters. This number would rise up to 88% in case of ectopic expression of c-Myc (Fernandez et al., 2003). Together with the finding that there is a lean correlation between binding and transcription among the c-Myc bound genes (Zeller et al., 2003). These observations lead us to consider alternative functional meaning for the interaction c-Myc/MCM complex other than transcription.

3.4 Implication in replication

It is well characterized that MCMs play an important role in replication. There are also compelling evidences that link c-Myc to replication. One of the key biological functions of c-Myc is to promote G0 to G1 and G1 to S transition (Eilers, 1999). And overexpression of the d-Myc in *Drosophila* has been shown to shorten the G1/S transition. It also allows the replication of SV40 in human lymphoma cells (Classon et al., 1987) and *in vitro* replication system, using HL-60 cells nuclear extract, is blocked by addition of anti-c-Myc antibody (Iguchi-Ariga et al., 1987). Furthermore overexpressed c-*myc* was shown to be able to uncouple DNA replication from mitosis in rodent and human cells (Li and Dang, 1999), and to promote locus-specific amplification of a number of target genes (DHFR, Cyclin D2, Ribonucleotide Reductase R2) (Mai and Mushinski, 2003). But a clear proof that links c-Myc to replication control, explaining the mechanism, is still missing.

It is therefore intuitive to imagine the MCMs as possible link between c-Myc and control of DNA replication. This could represent a new function for a protein, c-Myc, that already exerts its influence at several levels in the cell biology, including interaction with new partners, new regulatory pathways. On the other hand we cannot exclude the possibility that this is a new outcome of the well characterized c-Myc transcriptional activity. Some evidences support this second hypothesis. Mcm2 has been demonstrated to interact biochemically and genetically with MYST family protein Hbo1 whose associated histone acetyltransferase (HAT) activity has been suggested to play a direct role in the process of DNA replication (Burke et al., 2001). c-Myc also recruits for its function as a transcriptional activation some histone acetyltransferases as GCN5 and TIP60. It is conceivable imagine that c-Myc and MCMs synergistic interact to bring their respective partner (HAT) on the chromatin inducing that structural remodeling in the double helix that allows the replication and/or transcription apparatus to seat on the DNA. This model would be consistent with the emerging opinion about transcription and replication as tightly interconnected (Murakami and Ito 1999).

More than an indirect role, a direct involvement of c-Myc in controlling replication is supported by our work. Here we have demonstrated that c-Myc interacts with a complex that is directly involved in DNA replication. Loading of MCMs on the origin is necessary

step for origin to fire. During S phase, when the forks start, different mechanisms by which cells avoid re-replication are basically to avoid MCM complex re-loading on the chromatin. In developing eukaryotes, MCM proteins are more abundant in tissues undergoing rapid cell divisions. Although there are no evidences of a direct role of MCMs in cancer occurrence, these observations may suggest that increasing the effective concentration of the MCM complex, or deregulating their normal cycle on the DNA, may result in the activation of a larger set of replication origins. Is c-Myc the "deregulating" element? In yeast it has been demonstrated that deregulation in the level of a single Mcm leads to genomic instability (Liang et al., 1999), since Mcm4,6 and 7 are reported as transcriptional targets in c-Myc database, by analogy, it is thus conceivable that deregulated c-Myc may also cause genomic instability. Our results show a direct interaction between c-Myc and MCMs that is stable along the cell cycle. We also show that these proteins are loaded on the chromatin at the same time, and data from the glycerol gradient purification of the complex suggest the possibility that c-Myc interacts with other component of the pre-replication complex (pre-RC). Among them Cdt1 and Cdc6 that are necessary to recruit the MCM complex in order to license the origins. It is tempting to hypothesize that c-Myc is somehow involved in the loading of the ring helicase on the chromatin, or at least that in pathologic conditions c-Myc overexpression can lead somehow to a deregulated amount of MCM complex on the origin or override their normal cycling. Further investigations are needed to verify these hypotheses that link c-Myc to origin firing and finally to genomic instability. Understanding the role of c-Myc in these processes may help to explain the impressive (compare to a classical transcription factor) number of genes that have been found bound by c-Myc, and also its involvement in almost every form of tumors.

The work presented in this thesis, the c-Myc complex purification and the characterization of the interaction between c-Myc and the MCM complex is the starting point of a bigger project that aims to assign a role of c-Myc in replication that is the current interest of our lab.

CHAPTER IV

Materials and Methods

4.1 Cell lines and Culture condition

293T are human embryonic kidney fibroblasts expressing SV 40 large T antigen that allows expression of plasmids with SV 40 origin. H1299 are p53-null human lung carcinoma cell line. MCF7 and T47D cell lines originate from human breast carcinoma pleural effusions. These cells were maintained in Dulbecco's modified Eagles medium (GIBCO) supplemented with 10% fetal bovine serum, penicillin (100 I.U./ml), and streptomycin (100 µg/ml).

Colon carcinoma cell line SW48 and Burkitt's lymphoma cell lines Raji, Ramos were maintained in Iscove modified Dulbecco medium (GIBCO) supplemented with 10% fetal bovine serum, penicillin (100 I.U./ml), and streptomycin (100 µg/ml).

The H1299 cell line stably expressing the full length c-Myc HA/Flag double tagged (H/HF2), was already available in the lab.

4.2 Plasmid preparation

Plasmids expressing the wild-type proteins used in this work, their tagged form or mutans, were generated by PCR sequence amplification form a library of Ramos cDNA and insertion in appropriated vecrtor as listed:

pcDNA3 (Invitrogen):

Mcm2, Mcm7

Mcm2-HA, Mcm3-HA, Mcm4-HA, Mcm5-HA, Mcm6-HA, Mcm7-HA

Mcm7 (1-146)-HA, Mcm7 (146-642)-HA, Mcm7 (146-719)-HA,

Mcm7 (1-145)-HA, Mcm7 (146-642)-HA, Mcm7 (643-719)-HA

Myc, Myc-Flag, Myc-HA

MycΔ(1-45)-Flag, MycΔ(6-94)-Flag, MycΔ(6-151)-Flag, MycΔ(92-151)-Flag, MycΔ(143-237)-Flag, MycΔ(237-302)-Flag, MycΔ(301-395)-Flag

Myc (1-129)-HA, Myc (1-150)-HA, Myc (1-328)-HA, Myc (151-353)-HA,

Myc (151-340)-HA, Myc (151-439)-HA, Myc (262-439)-HA, Myc(341-439)-HA

pProEX HT (Life Technologies):

Max

Mcm2-HA, Mcm3-HA, Mcm4-HA, Mcm5-HA, Mcm6-HA, Mcm7-HA

pGEX-4T (Amersham Bioscience):

Max

Myc(1-42), Myc(1-103), Myc(1-143), Myc(1-228), Myc(151-340), Myc(262-439), Myc(341-439)

Mcm7(1-719), Mcm7(1-145), Mcm7(146-642), Mcm7(643-719)

PCR reactions were performed with Platinum P*fx* DNA Polymerase (Invitrogen). Oligo sequences are available upon request.

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

4.3 Transfection

The cells 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, or 2×10^6 H1299 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.

4.4 Reporter assay

293T cells in 6 cm dishes were co-transfected, according to the calcium-phosphate method previously described, with different combination of plasmid as reported in Figure 25. As reporter gene we used a construct carrying the Luciferase gene under the control of 800br of the *tert* promoter (TERTLuc800) described in Wu KJ et al., 1999. 48 hours after the transfection, cells were harvested and lysed. Lyses of the cells and reporter assay were performed using the Promega Dual-Light system, according to the manufacturer instructions.

4.5 Protein extracts preparations

Nuclear extract preparation for complex purification

The cells were treated with MG132 (Sigma) 100μM for 4 hours, harvested in cold PBS (Dulbecco's Phosphate Buffered Salt Solution, Cellgro), and spun down by centrifugation at 2.500 rpm for 5 min at 4°C (Eppendorf centrifuge 5810 R). The cellular pellet was resuspended in brf A (10mM KCl, 10mM HEPS pH 7.9, 0.1 EDTA, 10mM NaF, 10mM β-glycerolphosphate, 1,5 Na_3VO_4, 1mM PMSF, Protease Inhibitors cocktail from Sigma) and the cell were allowed to swell on ice for 15 min. Then were vortexed vigorously and spun down at 3000 rpm 5 min at 4°C. The supernatant including the cytosolic fraction was discarded and the nuclear pellet resuspended in approximately 10 fold brf C (20mM HEPES pH 7.9, 400mM NaCl, 1mM EDTA, 10mM NaF, 10mM β-glycerolphosphate, 1,5 Na_3VO_4, 1mM PMSF, Protease Inhibitors). The final concentration 400mM NaCl was adjusted, according to the size of the pellet, by adding 5M NaCl. After adding NP-40 to a final concentration of 0.2%, the samples were vortexed on ice for 1 hr. Bfr D (20mM HEPES ph 7.9, 1mM EDTA 10mM NaF, 10mM β-glycerolphosphate, 1.5 Na_3VO_4, 1mM PMSF, Protease Inhibitors) was added to adjust the salt concentration to 300mM. The samples were ultracentrifuged at 25,000 rpm fot 1.5 hr at 4°C (Sorvall T-647.5) and the supernatant filtered trough 0.45 μm (NALGENE)

Whole cell extract

293T or H1299 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 .

Chromatin fraction extract

This protocol is a modification of the one reported in Mendez & Stillman, Mol Cell Biol 2000.

The cells are harvested in cold PBS. After centrifugation, the cellular pellet was resuspended in CSK bfr (10mM HEPES pH 7.9, 100mM NaCl, 1.5mM $MgCl_2$, 300mM Sucrose, 50mM NaF, 0.1mM Na_3VO_4, PMSF, Protease Inhibitors) and spun down to wash. The cell were resuspended in CSK-Triton bfr (CSK + 0.5% Triton X100) and were

incubated 10 min on ice. The samples were spun down at 5000 rpm for 3 min at 4°C. The supernatant contains cytoskeleton, membranes, cytosol and nucleoplasmic proteins. The pellets were resuspended in EDTA-EGTA bfr (20mM HEPES-Na pH 8.0, 3mM EDTA, 0.2mM EGTA, 1mM DTT, PMSF, Protease Inhibitors) and were incubated 4 min on ice. The samples were spun down at 5000 rpm for 3 min at 4°C. The supernatants were discarded. The pellet is the Chromatin enriched fraction.

4.6 Antibodies

For Western Blot analysis and immunoprecipitations, anti-Max (C-17) and anti-Myc (N262) polyclonal antibodies were purchased from Sancta Cruz. Anti-Mcm2 and anti-Mcm4 polyclonal antibodies were purchased from BD Pharmigene. Polyclonal anti-Mcm3 and monoclonal anti-Mcm6 antibodies were purchased from BD Transduction Laboratories. Anti-Mcm5 and anti-Mcm7 monoclonal antibody were purchased from Lab Vision. Anti-cdc6 monoclonal antibody was purchased from Upstate. Anti-Cdt1 antibody was kindly provided by Hideo Nishitani. Anti-Flag monoclonal antibody was purchased from Sigma. Anti-HA High Affinity (3F10) Rat monoclonal antibody was purchased from Roche. Anti-phospho-H3 histone on Ser10 antibody was purchased from Upstate.

To immunoprecipitate endogenous c-Myc from cell lysates was used anti-Myc rabbit polyclonal antibody purchased from Upstate.

In the Immuno-colocalization assay, anti-mouse FITC-conjugated antibody and streptavidin-Cy3 antibody were purchased from Jackson ImmunoResearch, while the anti-rabbit biotin-conjugated was purchased from Vector.

4.7 Immunoprecipitation

The protein extracts were precleared with protein G Sepharose 4 fast flow (Amersham) for 2 h at 4°C. After removing the beads, 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 samples were centrifuged for 10 min at 14,000 rpm, 4°C. 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 immunoprecipitation of Flag/HA-tagged proteins:

Flag conjugated M2-agarose beads (Sigma) were added in appropriate amount, according to manufacturer instructures, to cell lysates and left over night (O.N.) rotating at 4°C. The day after the beads were collected by centrifugation and the supernatant was discarded. The beads were washed 5 times in the same buffer used for the binding, and the proteins were eluted from the beads by affinity competition using Flag peptide (Sigma) 0.5µg/µl O.N. at 4°C.

For HA-tagged proteins were used Monoclonal Anti-HA Agarose Conjugate (Sigma) for immunoprecipitation and HA peptide (Sigma) 1µg/µl, 4 h - O.N. at 4°C

4.8 Cell synchronization

Double block Thymidine/Mimosine (G1/S)

After overnight incubation with 2 mM thymidine (Sigma) H1299 cells were washed with fresh medium without thymidine and released for 10 h. then the cells were subjected to the second block with 2 mM thymidine + 400 µM Mimosine (Sigma) for 14 h. The cells where washed twice in PSB and fixed with 4% paraformaldehyde in PBS for 20 min at T room.

Double block Thymidine/Nocodale (mitosis)

The cells were first trated O.N. with Thymidine for a first block. Then they were washed with PBS and incubated in fresh medium with 50ng/ml Nocodazole (Sigma) for 12 h. Loosely attached, rounded mitotic cells were shaken off by gently knocking the plate. The cells were counted, replated in same amount abd sampled at different time points. Fractionated protein extracts were obtained according the CSK buffer extraction described above.

Propidium iodide staining for FACS analysis

The cells were harvested and resuspended in PBS. In order to fix them, absolute ethanol was added drop by drop to the cells until a final concentration 70%. The samples were left O.N. at 4°C. Before FACS analysis wash the cells twice in PBS and stained O.N. at

4°C in PBS + 15mM Propidium Iodade + 1mM sodium citrate + 280µg/ml RNase. The day after the cell were washed, resuspended in PBS and analysed by flow cytometry.

4.9 Immunofluorescence

H1299 cells were seeded on cover slips and were synchronized with a double block thymidine/mimosine. After the second block, the cells were washed twice with PBS, and treated with CSK-Triton bfr for 5 min on ice. The nucleoplasmic, cytosolic and cytoskeleton proteins were washed away with PBS and then the cells were fixed. The cellular membranes were permeabilized with PBS + 0.2% Triton for 10 min at T room. After washing with PBS the cells were blocked with PBS + 0.1% Tween + 3% BSA for 1 h at T room. The cells were incubated 2 h in the same blocking brf with antibody against c-Myc and Mcm7, 1:100 N262 (Sigma) and 1:150 CDC47 / MCM7 Ab-2 (Lab Vision) respectively. Then the cell were washed three times with PBS for 5 min and incubated with the secondary antibody for 45 min at T room in blocking bfr. For Mcm7was used a mouse secondary antibody (Jackson ImmunoResearch) conjugated with FITC diluted 1:200, and for c-Myc a rabbit secondary antibody (Vector) biotin-conjugated diluted 1:400. After incubation the cells were washed three times in PBS to detect c-Myc was used streptavidin Cy3 (Jackson ImmunoResearch) 1:500 in blocking bfr for 15 min. The cells were washed three times in PBS and the cover slips mounted on glass for immunofluorescence analysis.

4.10 Glycerol gradient

30-60% glycerol gradient in BC300 buffer (300mM NaCl, 20% glycerol, 20mM Tris-HCl ph 7.9, 0.1% NP-40, 1mM PMSF) was generated with a gradient former in 5ml Backman ultracentrifuge tube. 250µl of sample (corresponding to the c-Myc protein complex purified from 12 15cm-dishes of H/HF2 cell line) were layered on the top of the tube. The tube was ultracentrifuged in a Backman swinging bucket rotor SW 50.1 at 48,000 rpm for 20 h at 4°C. From the bottom of the tube, 200µl fractions were collected and 1/3 of them run on SDS-PAGE for western blot analysis.

4.11 SMART gel filtration chromatography system

The complex of proteins associates with c-Myc was separated directly by a Precision Column PC 3.2/3.0 pre-pached with Superose 6 (Pharmacia Biotech). This is a highly cross-linked, 6% agarose-bases medium, optimized for high performance gel filtration.

4.12 Recombinant proteins purification and GST-pull down

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 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).

GST-fusion proteins were purified incubating the extracts O.N. with beads Glutathion Sepharose 4B (Amersham Biosciences). The day after the beads were washed 3 times in the same buffer and twice in BC100 buffer (100mM NaCl, 20% glycerol, 20mM Tris-HCl ph 7.9, 0.1% NP-40, 1mM PMSF). The protein bound to the beads were eluted incubating the beads in BC100 + 20mM glutation O.N. at 4°C.

His-fusion proteins were purified incubating the extracts with Ni-NTA resin (Life Technologies) according to the manufacturer instructions.

HA-Myc-*Flag* recombinant protein was purified according a double step strategy: Flag-conjugated M2 Agarose beads were added to the bacteria lysate, and incubated O.N. at 4°C. The beads were washed 3 times in the same buffer and twice in BC100 buffer. The Flag-bound proteins were eluted in bfr BC100 + Flag peptide 0.5µg/µl 4 h at 4°C. HA-agarose beads were added to the eluted proteins and incubated O.N. at 4°C. The day after,

the beads were washed 5 times with BC100 buffer and the HA-bound proteins were eluted with bfr BC100 + HA peptide 1µg/µl, 4 h - O.N. at 4°C.

After purification the recombinant proteins were tested on SDS-PAGE and Blue Comassie staining in order to estimate the amount and presence of possible degradation product.

For *in vitro* pull down experiment, the protein were incubated 2 h at 4°C in binding bfr (50mM Tris-HCl pH 7.4, 200-300mM KCl, 1mM $MgCl_2$, 1mM DTT, 0.2% NP-40). According to the bait we were using, M2 beads, Glutation Sepharose 4B, or Ni-NTA beads were added to the reaction mix and incubated 1h at 4°C. The beads were washed and resuspended in SDS loading dye. The proteins were separated by SDS-PAGE and detected by Western Blot.

4.13 Oligonucleotides pull down

293T were transfected 48 h before in 10-cm dish according to the technique already described.

The cell were harvested and lysed in bfr F. The protein extracts (1-2 mg) were incubated 1-2H with 20-40 µL W/v of Streptavidin-Agarose beads (Sigma) as preclearing step. Subsequently were inqubated with the 10µg of the biotinylated probe in presence of 100µg of poly dI-dC (Sigma), as competitor, O.N. at 4°C. The samples were spun down at 1400 rpm for 15 min at 4°C. The supernatants were transferred in new tubes with 10-20µl w/v Streptavidin-Agarose beads, prewashed and equilibrated in bfr F, and were incubated for 30 min at 4°C. The supernatants were collected as "unbound fractions" and the beads were washed 5 times with bfr F and then resuspended in SDS loading buffer.

Probe preparation

Oligos of 38bp encompassing the BN51-intron1 E-box (GenBank accession no. AF142779), described by Amati and coworkers (Greasley et al, Nucleic Acids Res. 2000), were synthesized. The mutated probe carries a single point mutation in the E-box: CACGTG to TACGTG. Only the forward one was 5' biotinylated. For the annealing equimolar amounts of the two oligos were diluted in annealing buffer (20mM Tris pH 7.9, 50mM NaCl, 1mM EDTA), boliled at 95°C for 5 min and then gradually cooled down.

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CHAPTER V
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