ROLES FOR ORC1 AND CDC6 IN THE REGULATION OF HUMAN DNA REPLICATION

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"...Non vorrei che pensaste che mi sto montando la testa o, errore anche peggiore, che la mia è proprio una grossa scoperta. Né una cosa, n'è l'altra. Non mi monto affatto la testa e so perfettamente che quanto ho trovato non è che un piccolissimo spiraglio (…). Se gli sviluppi saranno limitati, come è probabile, poco importa. Ciò non toglie che io abbia passato delle ore di inverosimile gioia…".

I don't want you to think I'm full of myself or, even worse, that mine is really a great discovery. Not one nor the other. I'm not full of myself and I know perfectly well that what I found is just a small grain (...). And even if what will develop next will be small, it doesn't matter. Nonetheless, I spent some hours of true happiness..."

(Rita Levi Montalcini, Cantico di una vita).

to Massimo,

for his (un)patient waiting to share our lives

and

to Ramiro,

for his extraordinary genius.

CONTENTS

SECTION I

INTRODUCTION

1 DNA replication initiation

The exact duplication of a genome once per cell division is required for every proliferating cell. To achieve this goal, eukaryotes adopt a strategy that limits every replication origin to a single initiation event within a narrow window of the cell cycle by temporally separating the assembly of the pre-replication complex (pre-RC) from the initiation of DNA synthesis (Lei and Tye, 2001).

Eukaryotic genomes are very large and the process of DNA replication is restricted to the S phase of the cell cycle: as a consequence replication must start at thousands of different chromosomal locations that are specifically selected; these sites are referred to as origins of DNA replication. The initiation of DNA replication must be strictly controlled to ensure that DNA is replicated once and only once per cell cycle. Therefore, origins of DNA replication are the key points to understand the cell cycle controls that are imposed on the process of DNA replication.

The first essential event in the initiation of DNA synthesis is the local opening of the duplex to provide access to the template strands. Origins of replication serve to increase the efficiency of the replication process by providing loci for the assembly of multi-protein complexes that mediate DNA synthesis.

In the original replicon model proposed over 40 years ago, Jacob, Brenner and Cuzin postulated the existence of two important elements required for replication initiation: the replicator and the initiator (Jacob and Brenner, 1963). The replicator is where replication starts, namely the cis-acting sequence within the genome, whereas the initiator is what binds the replicator, the positive trans-acting factor able to recognize a specific sequence of the genome that overlaps with the replicator. In response to the appropriate cellular signals, the initiator directs the local unwinding of chromatin and recruits additional factors to initiate the process of DNA replication.

Compared to the simpler prokaryotic genomes, eukaryotic DNA replication is much more complex as genomes are larger and cell growth and differentiation have to be coordinated within a complex, developing, multi-cellular organism (Huberman, 1995). The main difference between prokaryotic and eukaryotic replication origins consists in the way in which chromosomal DNA is synthesized. In the latter case, in fact, genomes are larger and the duplication of chromosomal DNA relies on the activity of many different origins, the activation of which has to be strongly coordinated in space and time (DePamphilis, 1993; Kornberg, 1991). The advantage of this mechanism, besides reducing the overall time required to duplicate the entire genome, is that the generation of single-stranded DNA is much more localized and transient, helping preserving the genome integrity (DePamphilis, 1993). The initiation of DNA replication is mediated by a complex protein machinery that is assembled at each replication fork and that acts in concert to unwind the parental strands and carry out simultaneous synthesis of the two progeny strands (Bell and Dutta, 2002; Diffley, 1992; Diffley et al., 1995). The overall situation concerning the regulation of DNA replication in eukaryotic genomes appears to be far more complex than that in bacteria or DNA viruses, where replication occurs starting from a single origin. The initiation of DNA replication in lower eukaryotes is similar to that observed in bacteria in that it occurs at well-defined, site-specific origins of DNA replication that are recognized by specific initiator proteins (DePamphilis, 1993). However, although in the unicellular yeast *S. cerevisiae* the genome is duplicated from 250 to 400 replication origins, defined site-specifically, higher eukaryotic systems are expected to harbor a number of origins that is at least 100 times higher, and, at present, no sequence specific replicators have been found (DePamphilis, 1999; Gilbert, 2001; Todorovic et al., 1999). Nonetheless, in both simple and complex eukaryotes, replication origins are activated at each cell cycle, driving the replication of a limited region of the genome and leading to the formation of tandemly arranged replication units, each one considered an analog of the bacterial replicon (Stillman, 1996). In spite of the disparities, the proteins that regulate replication are highly conserved in function from yeast to humans, suggesting a common mechanism in the

replication function that does not depend on the origin sequence itself (Gerbi and Bielinsky, 2002; Gilbert, 2004; McNairn and Gilbert, 2003; Mechali, 2001; Pasero and Gasser, 2002).

1.1 The pre-Replication Complex (pre-RC)

Initiator proteins have been identified and extensively characterized during the past 15 years, since the Origin Recognition Complex (ORC) was first isolated in yeast cells (Bell and Stillman, 1992). Subsequently, the Cdc6 protein and the minichromosome maintenance (MCM) protein complexes were isolated (Koonin, 1993; Zhou and Jong, 1993) and altogether these factors have been shown to be evolutionary conserved in metazoans (Cocker et al., 1996; Fujita et al., 1999; Gavin et al., 1995; Kearsey and Labib, 1998; Saha et al., 1998; Tugal et al., 1998). More recently, a new member of the pre-RC has been isolated, CDT-1, and also found in all the different eukaryotes analyzed (Devault et al., 2002; Maiorano et al., 2000; Nishitani et al., 2000; Tada et al., 2001; Wohlschlegel et al., 2000). The conservation of all these factors in metazoans corroborates the notion that their function is required for origin activity in all the different organisms. A current model for the process of initiation of DNA replication is the following. Starting from late mitosis, the ORC, Cdc6, CDT-1 and other proteins cooperate to load the MCM proteins onto chromatin to form licensed pre-replication complexes (pre-RCs) at sites that have the potential to become origins of DNA replication (Bell and Dutta, 2002; Lei and Tye, 2001; Takisawa et al., 2000). At the beginning of the S phase, cyclin-dependent kinases (CDKs) and the Cdc7 kinase (also named Dbf4-dependent kinase, DDK) cooperate to signal initiation of DNA replication at a subset of the pre-RCs, mediating the unwinding of the double helix at the origin and the recruitment of additional essential factors responsible for the synthesis process (Blow and Hodgson, 2002; Diffley, 2001; Diffley, 2004). The ordered recruitment or the activation of these proteins is believed to be responsible for controlling the process of initiation of DNA replication in terms of both space and time, as well as their subsequent inactivation or removal is believed to be necessary to prevent re-replication

during a single S phase. For this reason, initiator proteins are crucial in regulating origin activity.

1.1.1 Origin Recognition Complex (ORC)

The Origin Recognition Complex (ORC) is a six-subunit complex (Orc 1-6) that acts as the initiator, likely selecting the sites for subsequent initiation of replication at eukaryotic origins of replication. First identified in *S. cerevisiae* as the ARS ACS binding factor (Bell and Stillman, 1992), it was subsequently found to be a very conserved element of chromosomal replication in all eukaryotes. Studies in *Xenopus* egg extracts demonstrated that the Xenopus analogue XlORC is required for initiation of replication in this organism (Carpenter et al., 1996). Similarly, recessive mutations in multiple *Drosophila* ORC subunits were shown to give rise to lethal phenotypes (Austin et al., 1999). In humans, although isolated a few years ago (Gavin et al., 1995; Tugal et al., 1998) and shown to be required for replication activity from the OriP of Epstein-Barr virus transfected in human cells (Chaudhuri et al., 2001; Dhar et al., 2001; Schepers and Diffley, 2001), hsORC has been only recently shown to be directly involved in the initiation of DNA replication (Abdurashidova et al., 2003; Ladenburger et al., 2002; Mendez et al., 2002; Todorovic et al., 2005). ORC drives the formation of the pre-RCs at replication origins, and one of its best-characterized features is its ability to bind DNA. In the yeast model, the ORC complex marks the origin throughout the cell cycle, binding to specific sites that map within the A and B1 elements of the yeast origin, spanning a region of ~30 bp (Bell et al., 1993; Micklem et al., 1993; Rao and Stillman, 1995). Conversely, in higher eukaryotes the ORC-DNA interaction is still unclear. Both *in vivo* and *in vitro* studies indicate that ORC is present at origins of replication (Kreitz et al., 2001; Mendez et al., 2002; Natale et al., 2000; Okuno et al., 2001). In *Drosophila*, ORC binds both the ori-β and ACE3 control elements, although its affinity seems to depend uniquely upon the presence of AT-rich DNA (Austin et al., 1999; Chesnokov et al., 2001; Schaarschmidt et al., 2004; Vashee et al., 2003). Similarly, the ORC complex has been shown to interact *in vivo* with the amplification origin II/9A of

the fly *Sciara coprophila* (Bielinsky et al., 2001). Chromatin immunoprecipitation (ChIP) studies demonstrated the association of hsORC with the OriP of the Epstein-Barr virus (Chaudhuri et al., 2001; Dhar et al., 2001; Schepers and Diffley, 2001). More recently, some authors have shown its interaction with human replication origins (Abdurashidova et al., 2003; Ladenburger et al., 2002; Todorovic et al., 2005). In contrast to the yeast model, some of the members of the ORC complex are believed to be displaced from the origin site after initiation of DNA replication (Kreitz et al., 2001) suggesting a more dynamic interaction between mammalian ORC and origin DNA. Both *Xenopus* and *Drosophila* ORC cannot be extracted as a stable complex (Natale et al., 2000; Thome et al., 2000). Biochemical studies have demonstrated that human ORC is formed by a core sub-complex of 4 subunits, Orc2-5 (Dhar et al., 2001; Vashee et al., 2001). Recent studies in mammalian cells suggest that not all ORC subunits remain tightly associated as part of the complex throughout the cell cycle (Bell and Dutta, 2002). Unlike ORC from budding yeast, *Drosophila*, and *Xenopus*, the subunits of the SpORC and mammalian ORC are difficult to extract as a stable complex (Moon et al., 1999; Natale et al., 2000; Thome et al., 2000). For example, SpOrc4p is retained on chromatin under conditions that elute the remainder of SpORC (Moon et al., 1999). Similarly, whereas mammalian Orc2p is found constitutively on the chromatin, mammalian Orc1p is removed from the chromatin at the end of S phase and rebinds only as cells re-enter G1 (Kreitz et al., 2001; Natale et al., 2000; Tatsumi et al., 2000). Studies in *Homo sapiens* suggest that Orc1 may be proteolyzed during S phase as a mechanism to prevent re-replication (Kreitz et al., 2001; Mendez et al., 2002); however, other studies have found HsORC1p to be stable throughout the cell cycle (Okuno et al., 2001; Saha et al., 1998) (T. Kelly, personal communication in (Bell and Dutta, 2002)). Yet another study has observed that Hamster Orc1p is stable through the cell cycle but is regulated in its association with chromatin by cell cycle regulated ubiquitination (Li and DePamphilis, 2002). These substantial differences are unlikely to be due to simple technical differences but instead might indicate variations in the regulation of this key factor in different cell lines. In *S. cerevisiae*, ORC binding to DNA requires the Orc1-5 subunits, four of which

(1,2,4 and 5) contact DNA directly. The Orc6 subunit does not seem to be required for DNA binding, but it is essential for replication (Lee and Bell, 1997). In *S. pombe*, ORC-origin binding is mediated uniquely by Orc4, which is able to recognize and bind specifically AT-rich sequences through its AT-hook DNA binding motif (Kong and DePamphilis, 2001; Kong and DePamphilis, 2002). The specificity of mammalian ORC binding to DNA is very low, due to its limited ability to distinguish specific sequences, as more recently reported (Remus et al., 2004; Schaarschmidt et al., 2004; Vashee et al., 2003). Moreover, the difficulties in identifying well-defined ORC binding sites in species other than yeast raise the possibility that other DNA binding factors may contribute and facilitate ORC localization and origin selection. In support to this hypothesis are some results obtained in *Drosophila*, where ORC has been shown to interact with the transcription factor E2F-1 and the disruption of such interaction reduced chorion amplification (Asano and Wharton, 1999; Royzman et al., 1999). ORC binding to DNA requires ATP. However, studies in both *S. cerevisiae* and *Drosophila* indicate that only ATP binding and not its hydrolysis is required for DNA binding by ORC. ATP binding is mediated by the Orc1 subunit in both yeast and fly, an observation that suggests conservation of function through evolution (Austin et al., 1999; Chesnokov et al., 2001; Klemm et al., 1997). Recent work has shown that ssDNA stimulates ATP hydrolysis, suggesting that, once bound to the origin, ORC is retained in an ATP-bound state and that DNA unwinding stimulates its hydrolysis (Lee et al., 2000). Additional data indicate that ATP binding might be needed for Cdc6 interaction (Klemm and Bell, 2001).

The so-named "ORC cycle" is therefore the premier step in preventing rereplication of DNA during a single cell division cycle: the ORC not only selects the sites where prereplication complexes are assembled and DNA replication begins, it is the first in a series of multiple coherent pathways that determines when prereplication complexes are assembled. Data from yeast, frogs, flies and mammals present a compelling case that one or more of the six ORC subunits undergoes cell cycle dependent modifications involving phosphorylation and ubiquitination that repress ORC activity during S, G2 and M-phases. ORC activity

is not restored until mitosis is complete and a nuclear membrane is present. In yeast, frogs and mammals, the same cyclin-dependent protein kinase [Cdk1(Cdc2)] that initiates mitosis also inhibits assembly of functional ORC/chromatin sites. In yeast, ORC remains bound to chromatin throughout cell division, but in the metazoa either ORC or the Orc1 subunit appears to cycle on and off the chromatin (DePamphilis, 2005).

ORC functions go beyond DNA replication: recent findings show that the complex is able to promote the formation of transcriptionally silent, late-replicating, chromosomal domains (see paragraph 1.5 for details).

1.1.2 Cell division cycle 6 (Cdc6)

Identified in a screen for proteins involved in controlling cell cycle progression, Cdc6 is a member of the AAA+ ATPases protein family, strictly related to Orc1 and, to a limited extent, to Orc4, Orc5 and to the MCM2-7 proteins (Lee et al., 2000). Cdc6 is essential in the formation of pre-RC at origins of DNA replication (Cocker et al., 1996; Liang et al., 1995) and requires ORC to associate with DNA (Blow and Tada, 2000; Romanowski et al., 2000) and is in turn required for the association of the MCMs (Cook et al., 2002; Kearsey et al., 2000; Mendez and Stillman, 2000; Yanow et al., 2001). Periodic transcription of the yeast Cdc6 gene in rapidly proliferating cells starts late in mitosis and this correlates with the appearance of the Cdc6 protein (Hateboer et al., 1998). It is believed that Cdc6 is synthesized at this stage of the cell cycle because it contributes to the inactivation of CDKs at the end of mitosis to inhibit cyclin B-CDK complexes (Calzada et al., 2001; Cook et al., 2002; Tanaka et al., 1997; Weinreich et al., 2001).

The ATPase domains of human Cdc6 and Orc1 are critical for DNA replication: recent work has established that an artificial recruitment Cdc6 or Orc1 to a DNA sequence can create a functional origin of replication (Takeda et al., 2005). Moreover, Randell and colleagues have recently found that Cdc6 is an ORC- and origin DNA-dependent ATPase that functions at a step preceding ATP hydrolysis by ORC: inhibiting Cdc6 ATP hydrolysis stabilizes CDT-1 on origin DNA and

prevents Mcm2-7 loading and, in contrast, the initial association of Mcm2-7 with the other pre-RC components does not require ATP hydrolysis by Cdc6 (Randell et al., 2006). Importantly, these coordinated yet distinct functions of ORC and Cdc6 ensure the correct temporal and spatial regulation of pre-RC formation.
The initiation protein Cdc6, like other initiator factors, is post-translationally modified in a cell-cycle dependent manner thereby preventing re-replication events to occur (see 1.4 paragraph for details).

1.1.3 Cdc10-dependent transcript 1 (CDT-1)

Originally identified in *S. pombe* (Hofmann and Beach, 1994), CDT-1 has been recently shown to be a key element in the formation of the pre-RC and, moreover, in the regulation of the "once per cell cycle" replication feature. It is periodically expressed under the control of the transcription factor Cdc10, which also controls the expression of Cdc6 in different species (Hofmann and Beach, 1994). In *S. pombe*, CDT-1 was shown to be an essential factor for origin licensing, similar to Cdc6, as its over-expression alone or together with Cdc6 induces high levels of re-replication (Gopalakrishnan et al., 2001; Nishitani et al., 2000; Tada et al., 2001; Yanow et al., 2001). At the same time, *Xenopus* CDT-1 was shown to be required for origin licensing in terms of MCM protein loading (Gillespie et al., 2001; Maiorano et al., 2000). In both cases, CDT-1 associated to DNA in an ORC-dependent manner. CDT-1 can be found in all organisms (Blow and Tada, 2000; Rialland et al., 2002; Wohlschlegel et al., 2000). Its identification in *S. cerevisiae* is relatively recent (Hodgson et al., 2002; Tanaka and Diffley, 2002); in this organism, it is required for proper MCMs loading, therefore to form a complete, functional pre-RC (Takahashi et al., 2003). Despite the apparent redundancy in their roles, CDT-1 expression differs from that of Cdc6. In fact, the factor peaks during the second half of G1, being its expression somewhat delayed with respect to Cdc6 expression (Ballabeni et al., 2004; Gopalakrishnan et al., 2001; Nishitani et al., 2001; Yanow et al., 2001). Moreover, its degradation does not occur until M phase, whereas Cdc6 is exported from the nucleus and degraded as soon as cells enter the S phase (Liu et al., 2004). The activity of CDT-1 is regulated by geminin, constituting a

second mechanism used by cells to control origin firing and prevent re-replication (Wohlschlegel et al., 2000). Geminin is a known inhibitor of DNA replication that acts by preventing MCM loading onto origins and displays its activity from S to M phase, thus preventing unwanted additional firing events (McGarry and Kirschner, 1998). Geminin was shown to interact with CDT-1 during the S phase, targeting it for degradation thereby preventing MCM loading until the following G1 and hence re-replication (Tada et al., 2001; Thomer et al., 2004; Wohlschlegel et al., 2000). CDT1 degradation, following ubiquitination, was shown to also occur in response to UV irradiation (Hu et al., 2004). In *Xenopus* egg extracts, CDT-1 is the key feature preventing re-replication of DNA (Li and Blow, 2005). XCDT-1 is downregulated late in the cell cycle by two different mechanisms: proteolysis, which occurs in part due to the activity of the anaphase-promoting complex (APC/C), and inhibition by geminin.

Recently, some authors have reported that replication-dependent proteolysis of CDT-1 requires its interaction with proliferating cell nuclear antigen (PCNA), a homotrimeric processivity factor for DNA polymerases. Moreover, mutation of the PCNA-interaction motif yields a stabilized Cdt1 protein that induces re-replication. DDB1, a component of the Cul4 E3 ubiquitin ligase that mediates human CDT-1 proteolysis in response to DNA damage, is also required for replication-dependent CDT-1 destruction. Thus, PCNA functions as a platform for CDT-1 destruction, ensuring efficient and temporally restricted inactivation of a key cell-cycle regulator (Arias and Walter, 2006).

1.1.4 Mini-Chromosome Maintenance proteins (MCMs)

MCM proteins have also been found in all eukaryotic cells and represent the functional analogs of bacterial dnaC helicases (Tye, 1999). Discovered as important factors for the maintenance of plasmids in cells (Sinha et al., 1986), they play a key role in the cell cycle control of chromosome replication as they distinguish replication competent (licensed) chromatin during the G1 phase from replication-incompetent chromatin during the G2 phase and mitosis (Labib et al., 2001; Labib et al., 2000). Moreover, MCMs have been shown to be part of the

active pre-RC, being loaded just before origin firing (Chong et al., 1995; Madine et al., 1995).

All eukaryotes appear to have exactly six MCM protein analogs, each one falling into one of the existing classes (MCM2-7). This observation argues, that each MCM protein has a unique and important function (Kelly and Brown, 2000). MCMs require the coordinate function of ORC, Cdc6 and CDT-1 to be loaded onto chromatin (Aparicio et al., 1997; Tanaka et al., 1997). Interestingly, once the MCMs have been loaded, the ORC and Cdc6 proteins can be displaced from chromatin without preventing replication initiation, suggesting that the primary role of these proteins is to load MCMs (Chong et al., 1995; Hua and Newport, 1998; Rowles et al., 1999). Moreover, in higher eukaryotes, in contrast to ORCs and Cdc6, MCMs do not appear to be displaced from origins as firing occurs and, by ChIP experiments, MCMs have been recently shown to localize with the replication machinery (Labib et al., 2001; Labib et al., 2000; Lee and Hurwitz, 2001; Zhou and Elledge, 2000). This implies that they play an active role as replicative helicases in both the initiation and the elongation processes. In S. cerevisiae, each MCM appears to be required for replication (Labib et al., 2000) whereas in higher eukaryotes only a subset of them, MCM4, MCM6 and MCM7, were shown to be required for DNA helicase activity (Ishimi and Komamura-Kohno, 2001; Ishimi et al., 2000; Schwacha and Bell, 2001; You et al., 2003). These same subunits were recently shown to display a preference for AT rich sequences, suggesting a possible role of these proteins in the recognition of the origin site (You et al., 2003). Recent work shows that the accumulation on chromatin of an additional member of the MCM protein family, human MCM8 (hMCM8), occurs during the early G(1) phase, before the hMCM2-hMCM7 complex binds. hMCM8 interacts *in vivo* with hCdc6 and hOrc2 resulting a crucial component for pre-RC assembly (Volkening and Hoffmann, 2005).

1.2 Assembly of the pre-RC

The process of building an active replication origin complex is believed to consist of two phases (Fig. 1). The first step (loading step), occurring at late mitosis and

early G1, involves the ordered assembly of the pre-replicative complex (pre-RC) at potential replication origins. The assembly of this multiprotein complex is initiated by the association of the Origin Recognition Complex (ORC1-6), which is required to recruit both Cdc6 and CDT-1 proteins that are then loaded on ORC-bound chromatin independently of one another (Blow and Tada, 2000). ORC, Cdc6 and CDT-1 are together required for the loading of the Minichromosome Maintenance (MCM2-7) proteins on the origins during G1. Extensive evidence supports a nucleotide-binding role of many of these factors. MCM2-7, Orc1/4 and 5 and Cdc6 have consensus motifs for nucleotide binding and mutations in these motifs result in nonfunctional proteins (Schepers and Diffley, 2001).

Moreover, *in vitro* studies indicate that there are at least two ATP-requiring steps in pre-RC formation: ORC association with origins and the subsequent recruitment of Cdc6 and MCMs (Harvey and Newport, 2003; Klemm and Bell, 2001; Schwacha and Bell, 2001; Seki and Diffley, 2000). The loading of all these factors results in the origins becoming "licensed" for DNA replication in the subsequent S-phase.

The second phase (firing step) involves the activity of numerous other proteins or protein complexes that associate with some of the pre-RC marked origins prior to successful initiation of DNA synthesis. These proteins include regulatory factors as well as components of the DNA replication fork, such as Cdc7-Dbf4, Cdc28, MCM10 and cyclin-dependent kinases (CDKs), that modulate the activity of the chosen origin by loading the Cdc45 protein and inducing the initiation of a pair of replication forks (Bell and Dutta, 2002). Being the factors involved in the assembly of the pre-RC and in the replication process very conserved among different organisms, all models of metazoan replication are largely based on the yeast paradigm just described. Recent advances in DNA microarray technology have enabled eukaryotic replication to be studied at whole-chromosome and genome-wide levels . These studies, in both *S. cerevisiae* and higher eukaryotes, have provided new insights into the mechanisms that influence origin selection and the temporally co-ordinated activation of replication initiation from these sites (MacAlpine et al., 2004). The comprehensive nature of the microarray-based studies has revealed clear connections between chromosome organization

and the pattern of replication. For example, in yeast, the centromeric proximal sequences are consistently early replicating and telomeric regions are consistently late replicating. The metazoan studies reveal a recurring theme of gene-dense transcriptionally active regions of the genome replicating before gene-sparse regions.

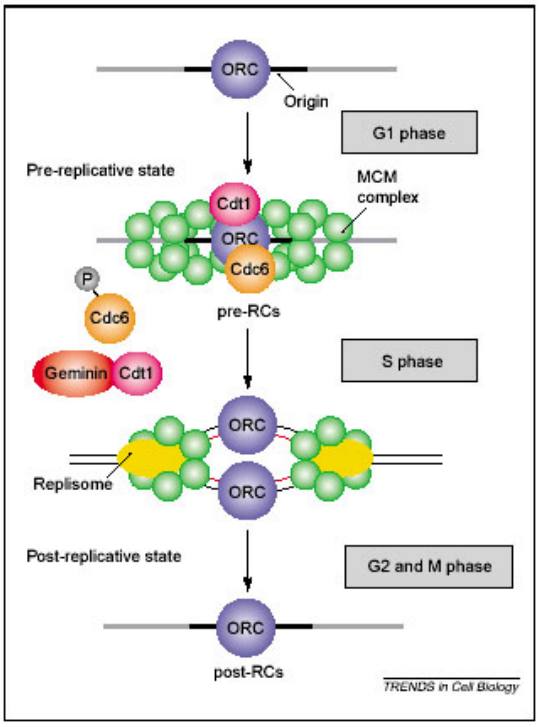

Figure 1. Assembly of replication-competent chromatin and the post-replicative state of origins. ORC, Cdc6, CDT-1 and MCM proteins bind to chromatin sequentially during G1 phase, licensing the DNA for replication. At the G1–S phase transition, pre-replicative complexes (pre-RCs) are activated and disassembled. ORC binds to DNA throughout the cell cycle in budding and fission yeasts, but in mammalian cells only some of the ORC subunits are tightly associated with chromatin at all stages. Cdc6 is phosphorylated and either degraded (yeasts) or exported from the nucleus (animal cells). Geminin binds to and inhibits CDT-1 (animal cells). The MCM complex has a role in both the initiation and elongation steps of DNA synthesis. The yellow oval schematically represents the replicative machinery (replisome) at the two forks moving in opposite directions (Pelizon, 2003).

However, the dynamic association of ORC and other replication proteins with origin DNA to form the pre-RC might be different in higher eukaryotes, especially given the lack of a target consensus sequence responsible for ORC-origin interaction (Gilbert, 2001; Gilbert, 2004; Mechali, 2001). As a matter of fact, the way in which these factors recognize or are recruited to the origin site is still to be uncovered (Blow and Tada, 2000; Cimbora and Groudine, 2001; Quintana and Dutta, 1999). Therefore, it can be concluded that the same function is performed by the same set of conserved factors in all eukaryotes while the mechanisms underlying these processes can vary depending on the organism.

1.3 Origin activity, cell-cycle progression and checkpoints

To ensure that each replication origins fires efficiently and only once per cell cycle, eukaryotic cells have evolved a remarkable molecular switch which, when turned on, promotes just a single initiation event from each origin per S-phase. The temporal separation of pre-RC assembly and origin activation steps is a key feature of the replication checkpoint that ensures that new pre-RC cannot assemble on origins which have already been fired (Diffley, 2001; Diffley, 2004). The heart of this mechanism is the tightly regulated assembly of the pre-RC complex; the activity of several cell-cycle regulated kinases is central to this regulation (Bell and Dutta, 2002). CDKs and DDKs (Dbf4-dependent kinases) are essential for triggering the initiation of DNA replication from origins that contain preassembled pre-RC. While DDKs seem to act on MCMs (Lei and Tye, 2001), CDKs appear to play a direct role in preventing the assembly of new pre-RCs. Because CDK activity remains high from S phase onset to the end of the following mitosis, re-licensing cannot occur until the beginning of the next cell cycle (Ballabeni et al., 2004; Diffley, 2004; Noton and Diffley, 2000; Tanaka and Diffley, 2002). At least three of the components of the pre-RC (ORC, Cdc6 and MCMs) are phosphorylated by CDKs to prevent re-replication and pre-RC assembly (Furstenthal et al., 2001; Lei and Tye, 2001). Moreover, CDKs have also been implicated in controlling the time of replication initiation at specific origins (Zou and Stillman, 1998). Cell cycle progression, as well as the control of

genomic integrity, is under continuously surveillance by cell cycle checkpoints (Nyberg et al., 2002; Zhou and Elledge, 2000). It is thus conceivable that one of the steps at which these checkpoint act by blocking cell cycle progression is DNA replication origin activation. Given the complexity and the importance of the S phase for the maintenance of genome integrity, many different checkpoint pathways are clearly active within this window of time, as demonstrated by studies in the yeast model (Bartek et al., 2004). In addition, and most importantly, the fact that the induction of genotoxic stresses during S phase causes a delay but not an arrest implies that replication origins are differentially regulated depending on the time of their firing (Merrick et al., 2004). Experiments carried out in the *Xenopus* cell-free system indicated that one of the main consequences of the induction of a replication checkpoint is the regulation of the recruitment of key members of the pre-RC to the origin site. Double-strand breaks allowed the assembly of complete pre-RCs, but prevented Cdc45 interaction with pre-RC in an ATM- and/or ATR- dependent, but Mre11-independent fashion (Costanzo and Gautier, 2003; Costanzo et al., 2001; Costanzo et al., 2000). In budding yeast, HU treatment blocked forks progression from early-origins and prevented the firing of late-origins, and this mechanism was shown to depend on Rad53 and Mec1, homologs of human ATM and Chk2 (Santocanale and Diffley, 1998). The same conclusion was obtained also following induction of double-strand breaks, and the protein involved in this regulation was shown to be yeast Orc2 (Shirahige et al., 1998). Recently, the *Drosophila* CDT-1 protein was reported to be phosphorylated and degraded in a Cyc E-Cdk2 dependent fashion, thereby preventing rereplication (Thomer et al., 2004). Moreover, also Cdc6 was recently reported to be directly involved in the control of rereplication (Mimura et al., 2004). Altogether, these data suggest the same factors involved in the formation of the pre-RC are also involved in the regulation of the replication process at different stages during the cell cycle, being the targets of many checkpoint proteins.

1.4 CDK-dependent regulation of human Cdc6 protein

As introduced in the previous paragraph, prior to cell division, the eukaryotic genome is duplicated during S phase of the cell-cycle. To ensure that only one single round of DNA replication occurs per cell cycle, a strict regulation is imposed. In G1/S phase, preRCs are assembled at origins of replication. During G1 the protein levels of both Cdc6 and CDT-1 increase, and they bind to the ORC complex. Since Cdc6 and CDT-1 are essential for the subsequent loading of the helicase MCM complex, these factors are thought to license the cell for replication. It has been established that not all origins are activated at the same time in S-phase. Some origins appear to be activated in early S phase, which has been shown to correlate with active transcription, whereas other origins are activated late in S-phase. Therefore, in the existing model preRCs are individually activated and fired (Duursma and Agami, 2005b). This firing is thought to be executed through phosphorylation of the preRC components by cyclin dependent kinases (CDKs) and the Cdc7-Dbf4 kinase (Lei and Tye, 2001). Apart from its role in activating DNA replication, CDK phosphorylation inhibits the formation of new replication complexes and inactivates components of the fired preRCs. Only at the end of mitosis CDK activity decreases due to degradation of mitotic cyclins, which allows new preRC formation in the G1 phase of the next cell cycle.

How exactly CDK activity both activates the initiation of replication at origins and inhibits the firing of origins that have already been activated is at present not clear. It has been proposed that the level of CDK activity is the primary determinant of this "replication switch" (Jallepalli and Kelly, 1997). Low kinases levels would be sufficient to trigger origin activation, whereas high CDK activity would result in disassembly of preRCs and inhibition of new preRC formation. However, this model would have the risk of aberrant regulation at intermediate levels of CDK activity.

Another possibility would be that the specific phosphorylation of replication proteins determines whether the origin is activated or inhibited. Whether these proteins are phosphorylated or not depends on their availability or their

accessibility. In this model, E3-ubiquitin ligase proteins like the anaphase promoting complex (APC) and the Skp1-cullin-F box SCF complex could play a central role in determining the availability of the initiation proteins by regulating their cell cycle dependent destruction.

At present several proteins of the mammalian preRC have been described to be phosphorylated by CDKs and as a result degraded or inactivated. First, human Orc1 was shown to be phosphorylated by cyclin A/CDK2 and degraded in a SCF^{Skp2}-dependent manner (Mendez et al., 2002). Second, it was demonstrated that cyclin A-dependent CDT1 phosphorylation in S-phase induces its SCF^{Skp2}-dependent degradation (Li et al., 2003; Liu et al., 2004; Sugimoto et al., 2004). Notably, CDT1 activity remained regulated by geminin in G2/M and G1-phase of the cell cycle. Third, CDK phosphorylation decreases the helicase activity of the MCM-complex. However, phosphorylation of an initiation protein by CDK2 that is essential for activation of DNA replication has not been revealed.

Intriguingly, in addition to Orc1, CDT1 and the MCM proteins also the licensing protein Cdc6 was previously recognized as a CDK target. Cdc6 phosphorylation by Cyclin-A/CDK2 was described to occur in S-phase and to result in its translocation from the nucleus to the cytosol and subsequent degradation (Delmolino et al., 2001; Herbig et al., 2000; Jiang et al., 1999; Petersen et al., 1999; Saha et al., 1998). However, these studies were all performed with ectopically expressed and tagged wild-type Cdc6 or Cdc6 that had been mutated in several phosphorylation sites. Later on, this model has been challenged by the finding that only ectopically expressed Cdc6 or the soluble endogenous form are translocated to the cytosol, whereas the chromatin-bound form persist through S and G2 phases (Coverley et al., 2000; Mendez and Stillman, 2000). Notably, Cdc6 phosphorylated on serine 54 was also shown to remain chromatin bound in S-phase (Alexandrow and Hamlin, 2004).

Interestingly, recent observations establish Cdc6 as the first example of a key replication initiation protein whose stability is increased by CDK phosphorylation. In a recent work, Agami and co-workers observed that Cdc6 is phosphorylated and thereby stabilized by CDK2/cyclin E activity (Duursma and Agami, 2005a). In particular, phosphorylation of one aminoacid (serine 54) protects Cdc6 from

APC^{Cdh1} mediated destruction. Moreover, in line with these results, Mailand and colleagues showed that phosphorylation of Cdc6 prevents its Cdh1-dependent ubiquitination (Mailand and Diffley, 2005). The fact that Cdc6 stability is controlled by CDK2 implies regulation through the p53 pathway in stress responses. DNA damage induces stabilization and activation of the p53 transcription factor, which results in increased synthesis of the CDK inhibitory protein $p21^{cip1}$ (Fei and El-Deiry, 2003). Morevoer, enhanced Cdc6 destruction was observed following DNA damage in a p53 and $p21^{cip1}$-dependent manner; Cdc6 is regulated in a p53-dependent manner in non stressed cells (Duursma and Agami, 2005a).

Both Cdc6 and CDT1 are the licensing factors of DNA replication. Therefore, this key step in initiation of DNA replication appears to be regulated by several independent pathways. Whereas the abundance of Cdc6 protein in G1/S phase is positively regulated by cyclin E/CDK2 phosphorylation through protecting it from APC^{Cdh1}-dependent degradation, CDT1 is negatively regulated during S-phase by Cyclin A phosphorylation in a SCF^{Skp2}-dependent manner (Li et al., 2003; Liu et al., 2004; Sugimoto et al., 2004).

However, this model is more complex since CDT1 activity is also inhibited by geminin (Sugimoto et al., 2004). As both Cdc6 and geminin (McGarry and Kirschner, 1998) are regulated by the APC a paradox emerged. How is the APC-dependent destruction of both an activator (Cdc6) and an inhibitor (geminin) of DNA replication coordinated to ensure that Cdc6 and CDT1 are present in the same time-frame to allow efficient preRC assembly? Based on the last results, a model for the assembly of the preRCs has been proposed (Fig. 2). Geminin is degraded in an APC-dependent manner at the end of G2/M and in early G1 phase of the cell cycle. Due to the degradation of geminin the levels of Cdt1 will rise during G1. Cdc6 transcription is regulated by E2F transcription factors (Hateboer et al., 1998; Yan et al., 1998), hence Cdc6 transcription increases in G1 phase. Yet, Cdc6 protein levels will only be stabilized in the course of G1 as the activity of CDK2/Cyclin E increases.

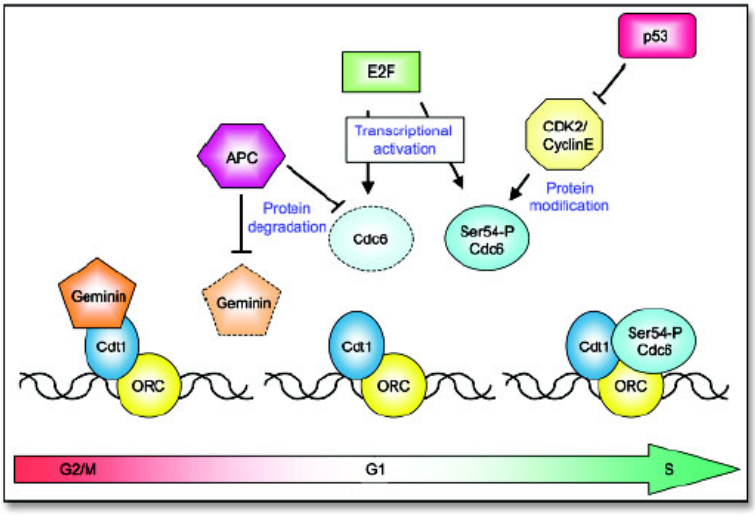

Figure 2. A schematic model of the regulation of DNA replication licensing in G1 phase of the cell cycle. Licensing of replication origins occurs in a time-frame where geminin is degraded by the APC, resulting in active CDT-1, and Cdc6 is protected from APC-dependent degradation by CDK2/cyclin E phosphorylation of serine 54 (Duursma and Agami, 2005b).

Phosphorylation of Cdc6 at serine 54 will protect Cdc6 from APC^{Cdh1}-dependent destruction and the protein is allowed to accumulate. This provides the cells with a period of time in which both licensing factors CDT-1 and Cdc6 are present. Binding of these proteins to the ORC complex at origins results in recruitment of the MCM-complex and formation of a preRC. Thus, phosphorylation of Cdc6 serine 54 could be the primary determinant of the timing of preRC formation at G1/S transition. Another question that arises is whether the CDK2-dependent phosphorylation of Cdc6 not only stabilizes the protein but also plays a role in recruitment of the MCM-complex. Interestingly, defective MCM loading was observed in mouse embryonic fibroblasts (MEFs) lacking both cyclin E1 and E2. Cells that reentered the cell cycle from quiescence showed chromatin bound Cdc6 but non MCM2 (Geng et al., 2003). In addition, it was shown that a phospho-mimicking mutant of Cdc6 that was coexpressed with CDT-1 under conditions where the endogenous licensing proteins were absent, could enforce chromatin loading of MCM6 (Mailand and Diffley, 2005). Together, a model can

de depicted whereby phosphorylation of Cdc6 plays a crucial role in MCM recruitment to the chromatin.

The regulation of DNA replication initiation in mammalian cells is based on the finding that Cdc6 is stabilized by CDK phosphorylation. As shown in the model (Fig. 3), Cdc6 phosphorylation was sufficient to load MCM6 onto the chromatin. Cdc6 is a key CDK2/cyclin E target in the replication complex for activation of origins in G1-phase. Stabilization of Cdc6 by phosphorylation together with the destruction of geminin results in binding of both Cdc6 and CDT-1 to the ORC complex at origins of replication leading to the loading of the MCM-complex. Next, independent of further CDK activity, the MCM complex recruits Cdc45, which in turn recruits DNA polymerase. Thus, opposing the hypothesis that CDK-dependent phosphorylation of MCM proteins results in origin firing, some authors have proposed that CDK activity is crucial for activating replication origins by phosphorylating Cdc6 (Duursma and Agami, 2005b).

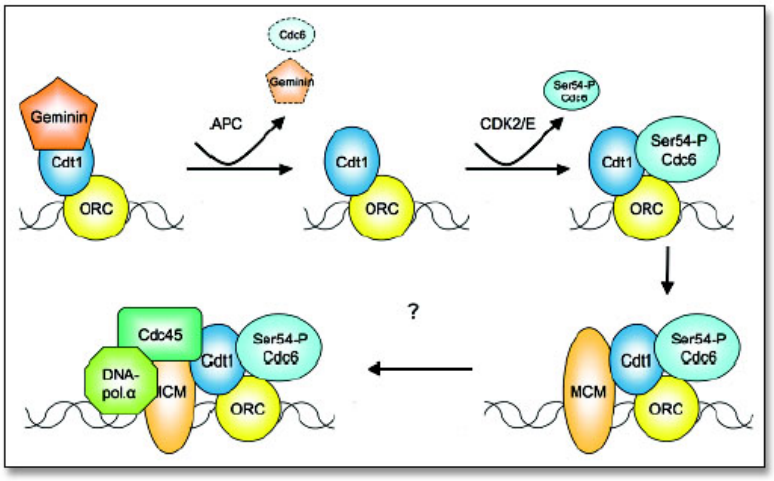

Figure 3. A proposed model for the regulation of initiation of DNA replication. Geminin and Cdc6 are both degraded by the APC, until Cdc6 is stabilized by CDK2/ Cyclin E activity in course of G 1 phase. This is the step in origin firing that requires CDK activity. Once Cdc6 and CDT-1 license the chromatin, the MCM-complex will be recruited. The subsequent loading of Cdc45 and DNA-polymerase a occur in a CDK independent manner, but what is required for this loading remains to be determined (Duursma and Agami, 2005b).

This model is consistent with the fact that all CDK-phosphorylated MCM proteins identified until now are negatively regulated by this modification (Hendrickson et al., 1996; Ishimi, 1997). However, this hypothesis disagrees with the suggested requirement of CDK activity between the assembly of MCM in the preRC and the loading of Cdc45, which was mainly based on studies in yeast and *in vitro* studies with *Xenopus* egg extracts.

In yeast it was shown that association of Cdc45 with chromatin correlated with activation of S-phase CDK activity at G1/S transition (Zou and Stillman, 1998). Nevertheless, chromatin binding of yeast Cdc45 in G1 was reported by others (Aparicio et al., 1997). Further, *in vitro* experiments with *Xenopus* egg extracts and sperm chromatin showed that addition of $p21^{cip1}$ or $p27^{kip1}$ could block Cdc45 in mammalian cells is lacking and therefore interesting to be determined.

CDKs were also proposed to fire individual origins, resulting in early and late replicating origins. Therefore, if CDKs do not play a role in activating individual preRCs, what then determines the activation of individual origins at different time-points during S-phase? One possibility is the activity of Cdc7-Dbf4 kinase which was shown to phosphorylate MCM2 and to be required for activation of DNA replication in mammalian cells (Jiang et al., 1999). Moreover, it has been proposed that this kinase acts locally at individual origins (Jares et al., 2000). Therefore, it would be interesting to find out what determines the activation of individual replication origins and whether the Cdc7-Dbf4 kinase is involved.

Recently, initiation of DNA replication has been found to be regulated by p53 through Cdc6 stability (Duursma and Agami, 2005a). Indeed, Cdc6 has been identified as a novel target of the p53 pathway. Activation of tumor suppressor p53 in response to genotoxic stress imposes cellular growth arrest or apoptosis. The authors show that p53 activation by DNA damage results in enhanced Cdc6 destruction by the anaphase-promoting complex. This destruction is triggered by inhibition of CDK2-mediated Cdc6 phosphorylation at serine 54. Conversely, suppression of p53 expression results in stabilization of Cdc6. The loss of p53 results in more replicating cells, an effect that can be reversed by reducing Cdc6 protein levels establishing a novel connection between DNA damage and the formation of pre-RCs in G1 phase (Fig. 4).

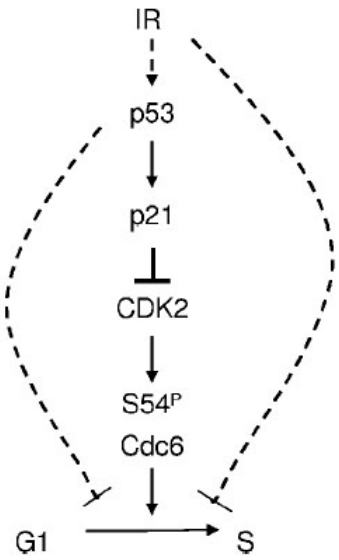

Figure 4. Schematic model depicting the p53 pathway that regulates Cdc6, and thereby S-phase entry, both under normal tissue culture conditions (solid lines) and following ionizing irradiation (IR, dashed lines) (Duursma and Agami, 2005a).

1.5 ORC and gene silencing

Histone modifying enzymes, chromatin-remodelling complexes and DNA methylation are thought to be components of intricate epigenetic mechanisms that help compact and organize genomes into discrete chromatin domains (Goll and Bestor, 2005; Jenuwein and Allis, 2001). This organization also underlies many aspects of chromosome behaviour, such as transcription, recombination and DNA repair (Kosak and Groudine, 2004).

A key feature of heterochromatin is its ability to propagate, and thereby influence gene expression in a region-specific, sequence-independent manner. When heterochromatin spreads across domains, it generally causes epigenetic repression of nearby sequences, in a process that is referred to as silencing (Grewal and Jia, 2007).

Although epigenetic gene silencing has become almost synonimous with heterochromatization, there are several reports in the literature in which heterochromatin formation is required for activation of gene expression (Lu et al., 2000; Weiler and Wakimoto, 1995; Yasuhara and Wakimoto, 2006). Histone H3 methylated at lysine 9 (H3K9me) and the heterochromatin protein HP1, which are necessary for the formation of heterochromatin, have been found in association with a subset of transcribed genes (Greil et al., 2003; Piacentini et al., 2003). Furthermore, it has been shown that heterochromatin proteins recruit factors that facilitate the access of RNA polymerase II (Pol II) to heterochromatin loci (Zofall and Grewal, 2006). An important emerging theme is that heterochromatin provides a mechanism for the recruitment and spreading of regulatory proteins (effectors) that are implicated in different aspects of chromosome biology. Although these effectors can be targeted to individual loci in a sequence-specific manner, the ability of heterochromatin to spread provides a sequence-independent platform to allow recruitment of these effectors at the level of chromatin domain. This might facilitate coordinated control of loci that are otherwise incapable of recruiting effectors themselves. From an evolutionary point of view, the current multipurpose character of heterochromatin might represent a series of co-optation events. Indeed, although all eukaryotes use epigenetic silencing mechanisms, different lineages have emphasized different aspects of heterochromatin in regulation, depending on the chromosomal contexts. It is therefore provocative, but non entirely unexpected, to find that many of the same histone modifications and proteins that are required to assemble silent heterochromatin structures are, in other circumstances, instead essential for gene activation. Multiple pathways of histone modifications and DNA methylation in higher eukaryotes contribute to how heterochromatin is assembled (Goll and Bestor, 2005; Jenuwein and Allis, 2001; Maison and Almouzni, 2004).

There are two types of eukaryotic gene silencing complexes: the Sir2-containing silencing complexes, so far only been studied in budding yeast (Aparicio et al., 1991; Gottschling et al., 1990; Hecht et al., 1995; Klar et al., 1981; Moazed,

2001; Nasmyth et al., 1981; Rine and Herskowitz, 1987; Strahl-Bolsinger et al., 1997) (Fig. 5), and the HP1 and Swi6 complexes that mediate silencing in metazoans and fission yeast, respectively (Aagaard et al., 1999; Eissenberg and Elgin, 2000; Eissenberg et al., 1990; Jenuwein, 2001; Kellum, 2003; Platero et al., 1995) (Fig. 6). Despite the divergence of molecular components, mechanisms of heterochromatic gene silencing in budding yeast, fission yeast, *Drosophila* and mammals are similar (Moazed, 2001) (Fig. 7).

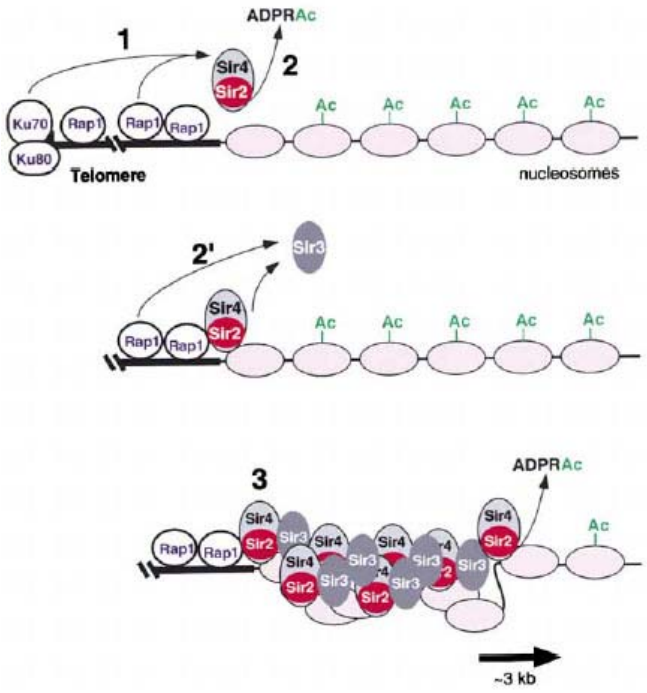

Figure 5. Model for Step-Wise Assembly of Silent Chromatin in budding yeast. Telomere binding proteins, the yKu70/yKu80 heterodimer and Rap1, ADP-ribose recruit the Sir2/Sir4 complex to DNA (step 1). Following deacetylation of histone tails by Sir2 (step 2), the Sir3 protein is recruited via interactions involving Rap1, Sir4, and histone tails and binds to nucleosomes (shown as purple ovals) by interacting with the deacetylated histone tails (step 2). Multimerization of Sir3 and Sir4 then results in additional rounds of modification and binding, and spreading of the complex along nucleosomes (step 3). Ac, acetyl group on amino-terminal lysines of histones; ADPRAc, O-acetyl-ADP-ribose (Moazed, 2001).

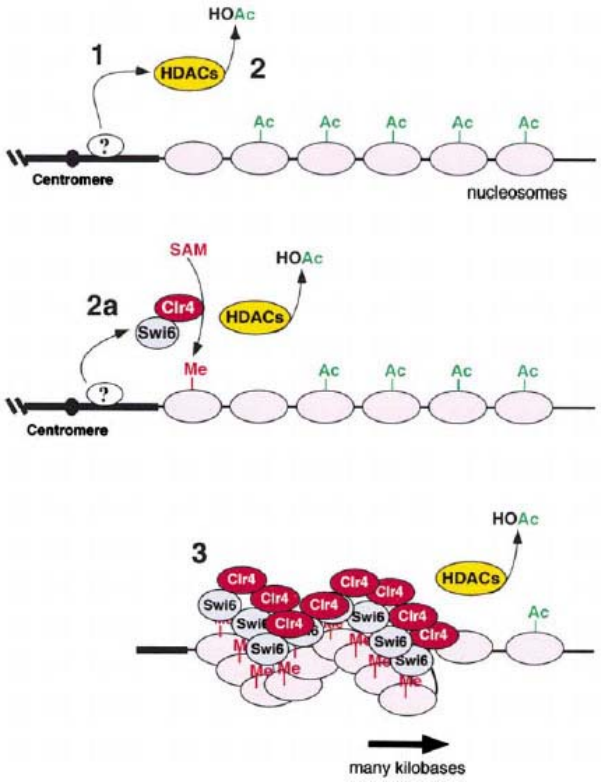

Figure 6. Model for step-wise assembly of silent chromatin domains in fission yeast. Following recruitment to DNA by protein(s) that have not yet been identified (step 1), histone deacetylases (HDACs, Clr3, and Clr6) deacetylate histone tails (step 2). The H3-specific methyltransferase, Clr4, then methylates lysine 9 of H3 and creates a binding site for the Swi6 protein (step 2a). Self-association of the Swi6 protein and subsequent rounds of modification and binding result in the spreading of the complex along nucleosomal DNA for several kilobases (step 3). Model adapted from Nakayama et al. (2001). Similar models have been proposed for mammalian HP1/SUV39H1 assembly (Bannister et al., 2001; Lachner et al., 2001). SAM, S-adenosyl-methionine; Me, methyl group on lysine 9 of histone H3; HOAc, acetate (Moazed, 2001).

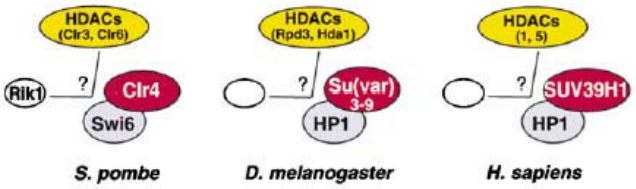

Figure 7. The Swi6/HP1 silencing complex is conserved in the fission yeast, *S. pombe*, and metazoans. The human HP1β protein has been shown to be associated with the methyltransferase protein SUV39H1. By analogy, Swi6 and the *Drosophila* HP1 are represented in association with SUV39H11 homologs Clr4 and Su(var)3-9; these interactions are strongly supported by genetic and colocalization experiments, but physical evidence for the association of Swi6 with Clr4 or HP1 with Su(var)3-9 is lacking. In *S. pombe*, HDACs and the zinc finger protein Rik1 are required for the association of Swi6 with silent chromatin, but it is unknown whether Rik1 and HDACs are physically associated with Swi6 or Clr4 (Moazed, 2001).

Originally identified in *Drosophila melanogaster*, HP1 belongs to a highly conserved family of chromatin proteins, with homologues that are found from fission yeast (Swi6, Chp2 and Chp1) to humans (HP1 α, HP1 β and HP1 γ) (Huisinga et al., 2006). These proteins contain an amino-terminal chromodomain, a short variable hinge region and, with the exception of Chp1, a chromoshadow domain. Each HP1 protein interacts with diverse factors that are involved in different aspects of heterochromatin structure and function. The diversification of HP1 isoforms is also indicated by their distinct localization patterns. Whereas HP1 α and HP1 β are distributed mainly at pericentric chromatin domains, HP1 γ is localized to discrete euchromatic sites (Huisinga et al., 2006). The binding of HP1 proteins to chromatin is believed to be highly dynamic (Cheutin et al., 2003; Festenstein et al., 2003). Histones and their modifications have crucial roles in the formation of heterochromatin (Jenuwein and Allis, 2001). Heterochromatin has a characteristic histone-modification profile, which is distinguished by hypoacetylation and H3K9 methylation; euchromatin is characterized by histone H4 acetylation and methylation of histone H3 at lysine 4 (H3K4me) (Cam et al., 2005; Grunstein, 1998; Litt et al., 2001; Nakayama et al., 2001; Noma et al., 2001). Histone methylation serves as a 'molecular anchor' recruiting proteins that either directly modify chromatin or

recruit others that do so (Martin and Zhang, 2005). Given the multimerization of Swi6/HP1 through the chromoshadow domain (Brasher et al., 2000; Cowieson et al., 2000), and the ability of swi6/HP1 to bind to numerous proteins that are implicated in heterochromatin formation, including histone deacetylases (HDACs) (Lechner et al., 2005; Smothers and Henikoff, 2000; Yamada et al., 2005; Zhang et al., 2002a), it has been suggested that Swi6/HP1, when bound to methylated H3K9, serves as an assembly platform for chromatin-modifying factors that are involved in stabilization (maintenance) and spreading of heterochromatin (Hall et al., 2002; Yamada et al., 2005).

Evidences accumulated in the last few years indicate that the function of ORC extends beyond DNA replication. The N-terminal region of the largest subunit of ORC, the Orc1p, is required for transcriptional silencing at the HM loci but is dispensable for DNA replication (Fig. 8).

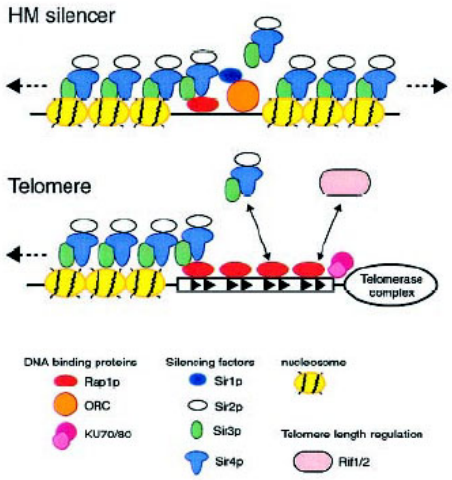

Figure 8. Molecular model for silencing at HM mating-type loci. Silencing is initiated by DNA-binding proteins [Rap1p and origin recognition complex (ORC), or Rap1p and Ku] that cooperate to recruit SIR protein complexes. These histone-interacting SIR complexes then assemble along adjacent nucleosomal DNA, masking or in some other way altering the nearby chromatin (Shore, 2000).

The N-terminal silencing domain of Orc1p (BAH domain) shares ~50% amino acid identity with the N-terminal region of Sir3p (Bell et al., 1995). Unlike Sir3p, however, the Orc1p domain interacts with Sir1p (Triolo and Sternglanz, 1996) and in *Drosophila*, the corresponding region of dORC1 interacts with HP1 (Pak et al., 1997).

The clearest example of an alternate function is the role of ORC in the transcriptional repression of the silent mating type loci, HMR and HML, in *S. cerevisiae* (Fig. 8). Paradoxically, in this case ORC participates in the assembly of the chromatin conformation that eventually suppresses the activity of the origin (Vujcic et al., 1999).

The ability of ORC to promote the formation of transcriptionally silent, late-replicating, chromosomal domains has been recently demonstrated also for the yeast rDNA locus by means of the dynamic molecular combing technique (Pasero et al., 2002). In both cases, transcriptional silencing and origin inactivation depend on the interaction of ORC with Sir proteins. The ability of ORC to interact with heterochromatin markers and to direct their recruitment to silent portions of the genome seems to be evolutionary conserved. However, there is no evidence so far that this holds true also in mammalian cells. In *Xenopus* and in *Drosophila*, Orc1p binds heterochromatin protein 1 (HP1), which is functionally analogous to Sir1p of budding yeast. *In vitro* studies have shown that both the yeast and the *Drosophila* Orc1p interact with Sir1/HP1 through their N-terminal portion (Pak et al., 1997; Zhang et al., 2002c).
This region overlaps the BAH domain that Orc1 proteins share with Sir3p and other chromatin associated proteins such as DNA methyl-transferases. Collectively these findings support the hypothesis that ORC could be involved also in the establishment and maintenance of chromatin domains.
Shareef and colleagues have identified a novel component of the HP1/ORC complex in *Drosophila*, named the HP1/ORC-associated protein (HOAP, Fig. 9) (Shareef et al., 2001). HOAP contains similarity to DNA sequence-specific HMG proteins and is shown to bind specific satellite sequences and the telomere-associated sequence *in vitro*. The protein is shown to have heterochromatin

localization in both diploid interphase and mitotic chromosomes and polytene chromosomes. Moreover, the gene encoding HP1/ORC-associated protein was found to display reciprocal dose-dependent variegation modifier phenotypes, similar to those for mutants in HP1 and the ORC2 subunit.

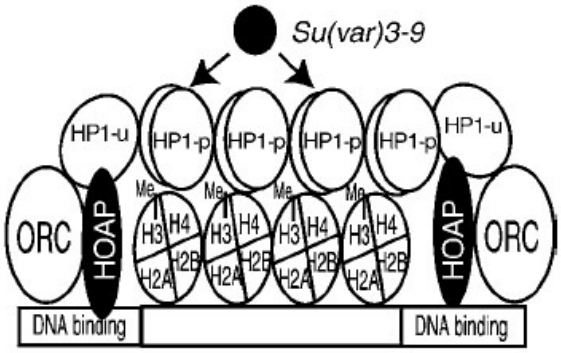

Figure 9. Model for *Drosophila* heterochromatin assembly. The DNA-binding activities of ORC and the HP1/ORC-associated protein (HOAP) are proposed to recruit underphosphorylated HP1, followed by (or concomitant with) recruitment of histone deacetylation activities (HDAC) and *Su(var)3-9* methyltransferase and highly phosphorylated HP1 (Shareef et al., 2003).

An interesting observation concerns the association of Orc1p with heterochromatin in mid and late G1. The association of ORC with transcriptionally silenced, late replicating portions of the genome has been observed in other organisms, where ORC seems to play an active role in the assembly of these chromatin conformations. In the yeast *S. cerevisiae,* ORC binding to an ARS element is required for the recruitment of Sir factors and hence for the transcriptional silencing of the HML locus (Vujcic et al., 1999). In *Drosophila* mutations of Orc2p were shown to perturb HP1 localization (Huang et al., 1998; Pak et al., 1997). Finally, it has been reported that human Orc2p binds *in vivo* to α-satellite sequences that compose pericentric heterochromatin (Keller et al., 2002).

Finally, a conserved protein kinase, Hsk1-Dfp1, which is implicated in DNA replication, interacts with Swi6 in *S.pombe* (Bailis et al., 2003). It is conceivable

that Swi6 recruits this kinase complex to activate replication origins within heterochromatic regions.

2 The E2F/Rb complex

Transition through the mammalian cell cycle requires an interplay of transcription factors that coordinately induce or repress gene expression in a temporally defined manner.

The E2F transcription factor is known to play a pivotal role in mediating gene expression during cell proliferation (Takahashi et al., 2000). E2F activity consists of a heterodimer containing one of six factors (E2F-1, E2F-2, E2F-3, E2F-4, E2F-5, and E2F-6) that pairs with a second subunit (DP-1 or DP-2) (Dyson, 1998). The transcriptional activation potential of E2F is counterbalanced by the retinoblastoma tumor suppressor protein (pRB) with which E2F tightly associates. E2F is under the control of the Rb (Flemington et al., 1993; Grana et al., 1998; Helin et al., 1993) and of CREB Binding Protein (CBP)(Ait-Si-Ali et al., 2000; Trouche and Kouzarides, 1996). E2F heterodimers not bound by the pRB family (free E2F) are thought to represent the active transcription factors.

In fact, it has been proposed that different E2F heterodimers might activate particular sets of growth-related gene targets, and a number of ectopic expression studies have suggested that this could be the case (DeGregori et al., 1997; DeGregori et al., 1995).

The pRB pocket family of inhibitors consists of pRB and the related proteins p107 and p130. pRB associates with each member of the E2F family, except E2F-5 and E2F-6, whereas p107 binds E2F-4 exclusively, and p130 binds both E2F-4 and E2F-5 (Dyson, 1998).

Rb physically interacts with E2F's transactivation domain (Flemington et al., 1993; Helin et al., 1993; Ross et al., 1999). It is thought that the masking of this domain participates in E2F inhibition. In addition, a second mechanism is based on active repression: Rb recruits `chromatin remodeling factors', including Histone Deacetylases (HDACs) (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998), members of the ATP-dependent chromatin remodeling

complex SWI/SNF (Dunaief et al., 1994; Trouche et al., 1997) and DNA methyltransferase 1 (DNMT1) (Robertson et al., 2000).

The mechanisms by which the pRB family represses transcription have been the subject of considerable interest. The role of HDAC recruitment in repression by pRB is thought to inhibit gene expression by altering chromatin structure, and the decreased acetylation of histones is associated with transcriptionally inactive chromatin (Kornberg and Lorch, 1999). Moreover, the recruitment may be promoter-specific, however, as HDAC is not strictly required for transcriptional inhibition of all promoters (Luo et al., 1998; Ross et al., 1999).

2.1 The E2F/Rb complex and the cell cycle

Complex formation between E2F and pRB families is cell cycle dependent: although these proteins form tight physical interactions in early-to-mid-G1 phase, cyclin-dependent kinases phosphorylate the pRB family in late G1, liberating free E2F. Subsequent phosphorylation of specific E2F family members by cyclin A-associated kinases could down-regulate E2F activity after entry into S phase (Dynlacht et al., 1994; Dynlacht et al., 1997; Krek et al., 1994).

As a matter of fact, E2F controls one of the critical moment in the cell cycle, the G1/S transition, by regulating the transcription of families of genes whose products are either required for DNA synthesis or involved in the regulation of S phase entry (Johnson et al., 1993).

Another aspect of E2F function—that of a transcriptional repressor—has emerged, reflecting the importance of E2F–pRB family complexes. A repressive role for E2F was suggested by studies in which mutation of an E2F site in several different promoters (*B-Myb*, *Cdc2*, *cyclin E*, and *E2F-1*) led to increased expression in quiescent and G1 cells (Dyson, 1998). Expression of these genes is therefore thought to result primarily from relief of repression (derepression) in G1 phase, although it is likely that other transcription factors also contribute to activation at the G1/S transition. Genomic footprinting experiments with the *B-Myb*, *cyclin A*, and *Cdc2* promoters further support this notion because potential E2F-binding sites in each promoter are occupied in quiescent and early G1 phase

cells, when the promoters are repressed, and largely unoccupied during the G1/S.

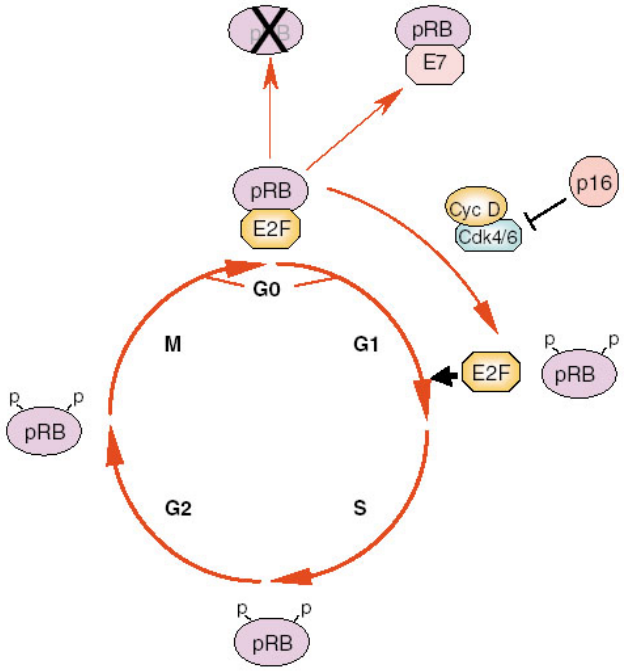

Figure 10. A simplified view of the pRB–E2F pathway. In this rendition, pRB binds and inhibits E2F in G0 and early G1. In proliferating cells, pRB phosphorylation by cyclin D-Cdk4/Cdk6 releases E2F, which then induces genes that mediate S phase entry. In tumor cells, the pRB–E2F interaction is disrupted by mutation of the RB gene (X), by pRB binding to DNA tumor virus oncoproteins such as human papilloma virus E7, or by inappropriate pRB phosphorylation due to overexpression of D cyclins, loss of the p16INK4A inhibitor of Cdk4/Cdk6, or mutation or overexpression of the Cdk4 or Cdk6 genes (Cobrinik, 2005).

transition when the genes are actively transcribed (Huet et al., 1996; Tommasi and Pfeifer, 1995; Zwicker et al., 1996). The observation that E2F-1 knockout mice develop tumors may further support this negative role for E2F and may be explained in part by the ability of E2F to act as a repressor of growth-related gene expression through the recruitment of pRB family members (Yamasaki et al., 1996).

Rb is regulated by phosphorylation: in non-cycling cells, or in early G1, Rb is hypophosphorylated and inhibits E2F activity; during G1, Rb is progressively phosphorylated by cyclin-CDK complexes (Harbour and Dean, 2000) and, as a consequence, loses its affinity for E2F. The release of Rb triggers the activation of E2F target genes, which allows the cells to proceed through the G1/S transition (Fig. 10).

2.2 The E2F/Rb complex and DNA replication

The initiation of DNA replication a the G1/S phase transition represents a key decision point in cell cycle control because the cell commits to duplication on traversing this boundary. The retinoblastoma protein (Rb) and the E2F transcription factors are crucial components of the cell machinery that control this G1/S phase transition. Fundamentally, Rb/E2F complex is known to regulate replication indirectly, by repressing genes that mediate S phase entry as well as genes that encode components of the replication machinery. Therefore, these transcriptional effects may be utilized to induce an intra-S phase block in response to DNA damage (Harrington et al., 1998; Knudsen et al., 2000; Kondo et al., 2001; Lan et al., 2002). Nevertheless, studies in *Drosophila* have shown that Rb/E2F complex could also directly affect DNA replication. These studies showed that the E2F1 and Rb homologs (dE2F1 and Rbf) bind the *Drosophila* origin recognition complex (DmORC) and the chorion gene cluster origin of replication, and thereby limit the physiological amplification of this cluster in ovarian follicle cells (Bosco et al., 2001; Royzman et al., 1999) (Fig. 11). Furthermore, recent studies suggest that Rb also have replicative functions in vertebrates. Rb was found to affect the spatial organization of replication in primary mammalian cells and the presence of Rb was crucial for the production of specific focal replication structures (Barbie et al., 2004).

Rb is also connected to replication through its role in preventing genomic rereplication. Rb inhibits poliploidy in cells experienced an S phase DNA damage, G2/M arrest and M phase block (Harrington et al., 1998; Niculescu et al., 1998). Moreover, in cells irradiated in early S phase, Rb was associated with an early

firing origin of DNA replication (Lamin B2) during the S phase block, and then bound to additional replication origins in the order in which they fired. The presence of Rb at origins, at about the time that they replicated, suggested that Rb might modify the origins in a manner that prevents rereplication (Avni et al., 2003). A second means by which Rb might suppress rereplication could be through effects on the replication licensing machinery, composed of MCM proteins, Cdc6, and others. This apparatus binds to replication origins in late M and G1, and prepares – or licenses – them to function after entry into S (Blow and Hodgson, 2002).

Licensing activity is normally suppressed in S, G2, and early M phase by cyclin A-Cdk activity (Yam et al., 2002). Rb might further suppress relicensing by interacting with MCM7 (Sterner et al., 1998), or by repressing genes that encode components of the licensing apparatus. In this regard, it is notable that Rb-deficient cells have increased expression of MCM2, 4, 5, 6, and 7 (Gladden and Diehl, 2003). Rb-deficient cells also have increased cyclin E, which could further promote relicensing and rereplication (Coverley et al., 2002; Spruck et al., 1999).

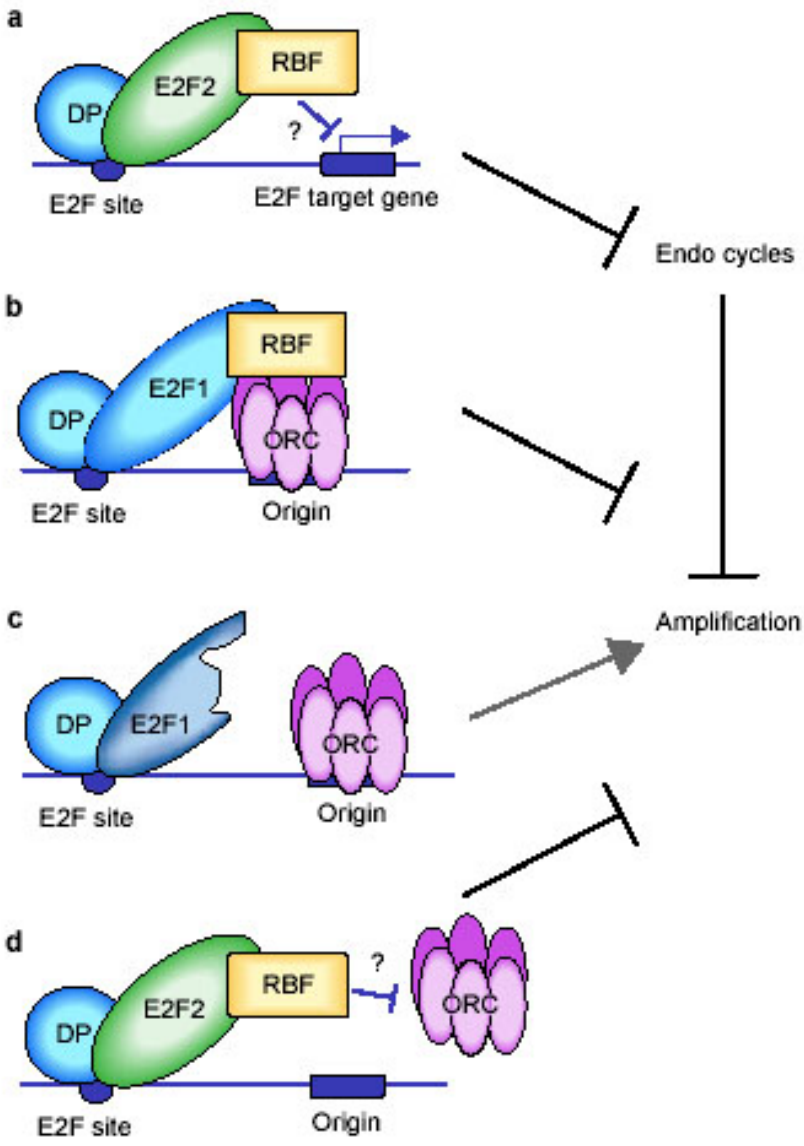

Figure 11. (For legend figure see next page).

Figure 11. Model for the dE2F–Rbf regulation of endo cycles and DmORC activity during gene amplification. a, *Drosophila* follicle cells undergo endo cycles that cease at stage 9 or 10A of egg-chamber development. Mutations in the dDP DNA-binding domain or Rbf should inactivate both dDP–dE2F1–Rbf and dDP–dE2F2–Rbf complexes, and in these mutants endo cycles continue to occur. No amplification occurs in the dDP mutant, possibly because ectopic endo cycles occurring in all follicle cells compete critical replication factors away from chorion origins, inhibit initiation and lead to low levels of amplification. In the Rbf female-sterile mutant low levels of wild-type Rbf protein allows some cells to transiently exit endo cycles, but these cells cannot maintain the prolonged gap in stages 10–13 and eventually resume endo cycles. Those Rbf (mutant follicle cells that have established a gap phase can initiate amplification and later may resume endo cycles, seeming to do both amplification and endo cycles. b, dDP–dE2F1–Rbf–DmORC form complexes, some of which may be bound to DNA. Complexes bound to replication origins are inhibited from origin initiation by the dE2F1–Rbf–DmORC interaction. Release of Rbf from the bound complex permits DmORC to initiate origin firing when follicle cells receive a developmental signal to commence amplification. c, The dE2F1i2 truncation mutant protein cannot interact with Rbf or DmORC, but DmORC is bound to the chorion amplicon. As dE2F1i2 and Rbf are not in a complex they cannot downregulate DmORC activity, leading to overamplification. Similarly, in the Rbf mutant low levels of Rbf fail to limit the number of DmORC initiation events. d, In dE2F1i1 mutants deficient for DNA binding, dE2F sites become available for dDP–dE2F2–Rbf binding. DmORC does not localize in these mutants and amplification does not occur, possibly because dDP–dE2F2–Rbf repels DmORC localization from the replication origin (Bosco et al., 2001).

Moreover, pRB may prevent polyploidy by enforcing the normal expression of mitotic checkpoint proteins such as Emi1 and Mad2. The Emi1 and Mad2 genes are regulated by E2F, and the proteins are overexpressed in Rb-deficient cells or tumors (Hernando et al., 2004; Hsu et al., 2002).

3 Protein acetylation

Histone acetylation promoted by histone acetyl-transferases (HATs) plays an important role in coordinating gene expression, cell-cycle progression and differentiation. Component of the cell cycle regulatory apparatus are both regulated and bind directly to HATs. Moreover transcription factors have been identified as substrates for HATs. Several are the enzymes, acetylases and deacetylases that can regulate transcription by modifying the acetylation state of histones or transcription factors and some of them are present in multisubunit complexes. Acetylation of histonic or nonhistonic proteins is a reversible process, and the balance between acetylation and deacetylation has been demonstrated to be important in regulating gene expression and it is thus linked to the control of cell fate. As a consequence, hyperacetylation of normally silenced regions or deacetylation of normally actively transcribed regions can lead to various disorders, including developmental and proliferative diseases.

Thus, protein acetylation, analogous to protein phosphorylation, may influence a wide range of biological processes, encompassing cellular proliferation, differentiation and tumorigenesis (Spencer and Davie, 1999).

3.1 Chromatin-modifying enzymes and histone acetylation

Chromatin structure is known to have profound effects on gene expression in eukaryotic cells. DNA in eukaryotes is tipically packaged in repeating arrays of nucleosomes, in which 146 bp of DNA are wrapped around a histone octamer. Each octamer includes four histone proteins (H2A, H2B, H3 and H4). Chromatin structure is dynamically regulated by proteins that remodel chromatin in an ATP dependent manner through post-translational modifications including phosphorylation, ADP ribosylation, methylation, ubiquitination and acetylation.

Histone acetylation involves the transfer of an acetyl group from acetyl-CoA to the ε–amino group of lysine side chain within the substrate. Co-translational N^{α}-terminal acetylation is one of the most frequent protein modifications, occurring on approximately 85% of eukaryotic proteins (Polevoda et al., 1999). A less common, but perhaps more important, form of protein acetylation takes place post-translationally on the ε–amino group, and as a result, has a significant impact on the electrostatic properties of the protein (Glozak et al., 2005).

The modification of the lysines groups of core histones by multiple post-translational modifications coincident with mitogenic signaling (Clayton et al., 2000) has led to a model in which the N-terminal substrates of histone acetyl transferase FAT (Factor Acetyl Transferase) might also function as signaling platforms in acetylation-phosphorylation cascades.

The acetylases so far identified have the ability to modify histones in free solution but only a subset of them are able to acetylate histones in a nucleosomal structure. Although H3 and H4 are in general preferred substrates over H2A and H2B, acetylases such as p300 and CBP are able to modify all four. In addition, the specific lysine residues modified by each acetylase may differ, sugesting this difference may be an indicator of differences in function between acetylases (Davie, 1998). It is also known that several HATs have a self-acetylating activity *in vitro*, including P/CAF, p300, Tip60, Gcn5 and MORF (Bannister and Kouzarides, 1996; Champagne et al., 1999; Col et al., 2001; Herrera et al., 1997; Ogryzko et al., 1996). However it is unknown whether these events have physiological relevance as self-regulation or not.

The acetylation reaction is both complex and specific since lysines are specifically acetylated by given HAT activities. How the post-translational modifications of histone activates gene expression remains unclear. The modification of lysine groups may disrupt electrostatic interactions between histones and DNA and increase accessibility of nuclear factors or coactivator complexes.

3.2 Histone acetyl-transferase families

HATs were historically classified as type A, located in the nucleus and known to acetylate nucleosomal histones within chromatin, and type B HATs, located in the cytoplasm. There are now six families of proteins known to exhibit a histone acetyltransferase activity (Table 1).

The GNAT (Gcn5-related N-AcetylTransferase) superfamily includes the best characterized yeast Gcn5, originally discovered in the ciliate *Tetrahymena thermophila* (Brownell et al., 1996). In mammals, the p300/CREB binding protein-associated factor (P/CAF) was identified on the basis of sequence homology to Gcn5 and was found to associate to the p300 co-activator protein (Yang et al., 1996). The role of P/CAF in transcription has been investigated by multiple studies, and its requirement as a HAT and co-activator has been described for myogenesis and nuclear receptor-mediated, and growth factor-signaled activation among other processes. Although P/CAF was originally identified as a HAT, a recent work has focused on its acetylation of various non-histone transcription related protein (Tables 2-8).

Another group of evolutionary related proteins that are known to possess HAT activity is the MYST family, named for its founding members: MOZ (Monocytic leukemia Zinc finger protein), Ybf2/Sas3, Sas2 and Tip60. Additional members have more recently been identified including human HBO1 (Histone acetyltransferase Bound to Orc1) and MORF (MOZ-related factor).

HAT	Histones
GNAT superfamily	
hGCN5	H3, H4 (Brownell et al., 1996)
P/CAF	H3, H4 (Yang and Larson, 1996)
Hat1	H4 (Verreault et al., 1998)
MYST family	
Tip 60	H2A, H3, H4 (Kimura and Horikoshi, 1998)
MOZ	ND (Borrow et al., 1996)
MORF	H2A, H3, H4 (Champagne et al., 1999)
HBO1	ND (Iizuka and Stillman, 1999)
p300/CBP	H2A,H2B,H3,H4 (Bannister and Kouzarides, 1996)
nuclear receptors co-activators	
SRC1	H3, H4 (Spencer et al., 1997)
ACTR	H3, H4 (Chen et al., 1997a)
TAFII250	H3, H4 (Mizzen et al., 1996)
TAFIIIC	H2A, H3, H4 (Kundu et al., 1999)

Table 1. Histone acetyl-transferase families.

Non histone chromatin proteins		
Substrates	**known function *in vivo***	**Known FAT enzyme *in vitro***
HMG1	chromatin component	P300/CBP
HMG2	chromatin component	ND
Yeast Sin1	transcriptional regulator	Gcn5
HMG14	nucleosome binding	P300/CBP
HMG17	nucleosome binding	P/CAF
HMGI(Y)	Enhanceosome component	P/CAF, p300/CBP

Table 2. Non histone chromatin proteins.

Transcriptional activators		
Substrates	**known function *in vivo***	**known FAT enzyme *in vitro***
P53	Tumour suppressor	P/CAF, p300/CBP
c-Myb	proliferation, differentiation	P300/CBP,Gcn5
GATA-1	blood cell differentiation	p300/CBP
Tal-1	blood cell differentiation	P/CAF
EKLF	globin gene expression	p300/CBP
MyoD	muscle differentiation	P/CAF
E2F (1,2,3)	cell cycle control	P/CAF
DTCF	developmental regulation	P/CAF
HIV Tat	HIV-1 transactivation	P/CAF
NF-κB (RelA subunit)	inflammatory response	p300/CBP

Table 3. Transcriptional activators.

Nuclear receptors co-activators		
Substrates	**known function *in vivo***	**Known FAT enzyme *in vitro***
ACTR	hormone signals	p300/CBP
SRC-1	transcriptional response	p300/CBP
TIF2		p300/CBP

Table 4. Nuclear receptors co-activators.

General transcription factors		
TFIIE	general transcription machinery component	P/CAF, p300/CBP, TAFII250
TFIIF	general transcription machinery component	P/CAF, p300/CBP
TAF(I) 68	rRNA transcription component	P/CAF

Table 5. Nuclear receptors co-activators.

Transcriptional repressors		
Rb	tumour suppressor	p300/CBP
MDM2	E3 ubiquitin ligase	p300/CBP
EVI1	Hematopoietic differentiation	P/CAF, p300/CBP
BCL6	transcriptional repressor	p300/CBP

Table 6. Transcriptional repressors.

DNA metabolism control proteins		
Substrates	**known function *in vivo***	**known FAT enzyme *in vitro***
Fen1	Chromatin remodeling	p300/CBP
TDG	DNA repair	p300/CBP

Table 7. DNA metabolism control proteins.

Other proteins		
importin-α 7, rCh1	Nuclear import	p300/CBP
α-tubulin	Microtubule component	ND
adenovirus E1A	Cellular transformation	P/CAF, p300/CBP

Table 8. Adapted from (Sterner and Berger, 2000)**.**

These proteins are grouped together on the basis of their close sequence similarities and their possession of a particular acetyltransferase homology region. Although containing regions similar in sequence, the members of the MYST family are involved in a wide range of regulatory functions in various organisms (Sterner and Berger, 2000).

After the discovery of histone acetyation by Gcn5 and P/CAF, the critical role of acetyl-transferases in trascriptional regulation was also demonstrated by the fact that the previously well-characterized co-activators of multicellular eukaryotes, p300 and its close homologue CBP (CREB binding protein), are themselves HATs (Bannister and Kouzarides, 1996; Ogryzko et al., 1996) and FATs. The interactions of p300/CBP (p300 and CBP are often referred as a single entity, since the two proteins are considered functional homologs) with nuclear receptor coactivators, are examples of transcriptional regulatory complexes with multiple acetyltransferase activities. Overall p300/CBP is one of the most potent and versatile through the acetyltrasnferases, consistent with its roles a global co-activator in higher eukaryotes. Like P/CAF, p300/CBP is known to acetylate and regulate various transcription-related proteins other than histones.

HAT proteins have also been directly implicated in transcriptional activation brought about by hormone signals. The HAT activities of human co-activators

ACTR, SRC-1 and TIF2, which interact with nuclear hormone receptors, demonstrate the involvement of acetylation in yet another system of transcriptional regulation. The three proteins are a part of an evolutionary and functionally related HAT family and all three interact with p300/CBP.

Another direct connection between acetylation and activated transcription was demonstrated with the discovery that one of the TAFII (TATA-binding protein TBP-associated factor human TAFII250) subunits of the general transcription factor TFIID is itself a HAT (Mizzen et al., 1996). The HAT activity of TAFII250 suggests a model for the initiation of assembly of a transcriptional complex at chromatin-packaged promoters. As part of TFIID, TAFII250 may facilitate TBP binding directly by acetylating histones at the TATA box allowing formation of pre-initiation complex.

Evidence that histone acetylation is a general employed mechanism in transcription is supported by the fact that also subunits of TFIIC, a general transcription factor in the RNA polymerase III basal machinery, were also recently identified as HATs (Kundu et al., 1999).

3.3 Acetylases in complexes

It si becoming increasingly apparent that acetylases are mostly present within large nuclear complexes: several human protein complexes have been purified and characterized. Subunit identification has shown that some of these complexes are remarkably analogous to known yeast HAT complexes, and in each case an involvement in transcription is also suggested by subunits besides the HAT protein. As described above, the TAFII250 is part of the well-characterized TATA-box binding (TBP)-containing, TFIID complex. Evidences from human and yeast cells indicated that P/CAF and GCN5 are also in large complexes (Struhl, 1997). Interestingly, some of the proteins in the P/CAF complex have turned out to be TBP-associated factors (TAFs) that are also present in the TFIID complex.

3.4 Acetylation and protein function

Some of the enzymes exhibiting histone acetyl-transferase activity are known to participate in transcriptional regulation by acetylating proteins other than the histones. FAT (Factor Acetyl Transferase) activities have been demonstrated for P/CAF, p300/CBP and TAFII250, with transcription-related substrates ranging from activators and co-activators to basal transcription machinery factors and non-histone chromatin proteins. Acetylation has been demonstrated to affect either positively or negatively the activity in a numbers of activators involved in various cellular and developmental processes other than transcription, as summarized in table 2. In the case of other DNA binding transcription factors (E2F1, p53 and GATA-1) the acetylation site also falls directly adjacent to the DNA binding domain and acetylation results in stimulation of DNA binding (Boyes et al., 1998; Gu and Roeder, 1997; Martinez-Balbas et al., 2000; Marzio et al., 2000; Zhang and Bieker, 1998). Differently the lysines acetylates within the HMGI(Y) transcription factor or Fen-1 (Hasan et al., 2001) fall within the DNA binding domain itself and result in disruption of DNA binding. Besides affecting DNA binding, acetylation might also regulates protein-protein interactions either positively or negatively, and protein stability. The growing list of proteins modified by acetylation, including transcription factors like EVI1 (Chakraborty et al., 2001) and NF-B RelA subunit (Chen et al., 2001), tumour suppressors as pRB (Chan et al., 2001) and MDM2 (Kawai et al., 2001), transcriptional repressors as BCL6 (Bereshchenko et al., 2002), transforming factors like E1A (Zhang et al., 2000), co-activators, general transcription machinery components as TAF(I)68 (Muth et al., 2001), and nuclear import proteins, suggests that acetylation may function as a mechanism which itself must be tightly regulated. A new class of HAT substrates, which is represented by Fen1 (Hasan et al., 2001) and TDG (Tini et al., 2002) that play a critical role in regulating DNA metabolic events, reveal a potential regulatory role for protein acetylation in maintaining genomic stability.

Lysine acetylation is known to occur in over 40 sequence-specific transcription factors (such as POP-1, FOXO proteins, MyoD) (Gay et al., 2003; Matsuzaki et

al., 2005; Polesskaya et al., 2000) and affect their DNA-binding affinity, coregulator association, nuclear localization, phosphorylation, ubiquitination and stability (Yang, 2004b). In most cases, this modification potentiates transcription. However, acetylation of some factors such as NF-*k*B, RelA, HMGI(Y) and ERα inhibits transcription, which may serve as a feedback mechanism to control the duration of transcription (Chen et al., 2002; Deng et al., 2003; Munshi et al., 1998). Lysine acetylation also occurs in transcriptional coregulators, general transcription factors and chromatin remodelers. Notably, acetylation of some factors such as TAFI68 and Brm turns off transcriptions and autoacetylation of the transcriptional coactivators PCAF and p300 regulates their acetyltransferase activities (Bourachot et al., 2003; Chen et al., 1999; Muth et al., 2001; Santos-Rosa et al., 2003; Thompson et al., 2004).

Acetylation also modifies non-histone eukaryotic proteins involved in DNA replication and repair, sister chromatid cohesion, cellular signaling and cell motility (PCNA, ku70, α-Tubulin) (reviewed in (Yang, 2004b)). Also interestingly, this modification regulates the functions of several viral and bacterial proteins such as E1A, HIV Tat and Alba (reviewed in (Yang, 2004b)). Therefore, lysine acetylation occurs not only in many nuclear proteins, but also in cytoplasmic, viral and bacterial proteins.

3.5 GCN5 acetyltransferase

The first cloning of a histone acetyltransferase gene, the yeast *HAT1* gene, was reported in 1995 (Kleff et al., 1994). Subsequently, it was suggested that HAT1 protein is cytoplasmic and involved in histone deposition (Parthun et al., 1996), although the lack of phenotypes of yeast *hat1* mutants as well as recent evidence that both the human and yeast enzymes are nuclear, makes the *in vivo* function of HAT1 unclear. A major breakthrough in this field was the purification and cloning of HAT A, a HAT from the macronucleus of the ciliate *Tetrahymena* (Brownell et al., 1996). The sequence of HAT A showed that it was similar to a

known yeast transcriptional coactivators, GCN5 (general control non-derepressible 5; Fig. 12) that belongs to the Gcn5/PCAF family.

Since then, numerous studies have demonstrated that GCN5 (and the related P/CAF) are conserved HATs whose activity on nucleosomes facilitates initiation of transcription (Mizzen and Allis, 1998). Interestingly, GCN5 by itself can acetylate free histones (particularly Lys-14 of H3) but not nucleosomes. Proteins of the GCN5 family have four highly conserved sequence motifs and a bromodomain that is absent in cytoplasmic HATs (Lin et al., 1999). Yeast Gcn5 possesses a HAT domain and a bromodomain and is highly homologous to the C-terminal halves of human PCAF and GCN5 L (mammalian GCN5 long form) (Georgakopoulos and Thireos, 1992; Smith et al., 1998; Wang et al., 1997; Xu et al., 1998; Yang et al., 1996). *in vivo*, yeast Gcn5 has been shown to exist in at least two high molecular weight protein complexes, a 1.8 Mda SAGA complex and a 0.8 Mda ADA complex (Grant et al., 1997; Saleh et al., 1997). In addition to acetylation activity, these *in vivo* complexes possess other activities, including interaction with activators for promoter targeting (Utley et al., 1998) and interaction with TBP for regulation of basal factor activity (Belotserkovskaya et al., 2000; Dudley et al., 1999). Taken together, an emerging model is that the SAGA complex is recruited to promoters by interaction with sequence-specific activator proteins, followed by GCN5-mediated acetylation of histones within the basal promoter of these genes and then general factor recruitment, culminating in heightened transcription from the associated promoters (Lo et al., 2000).

Like *Drosophila* GCN5, mammalian PCAF and GCN5L possess PCAF-specific N-terminal domains (Smith et al., 1998).

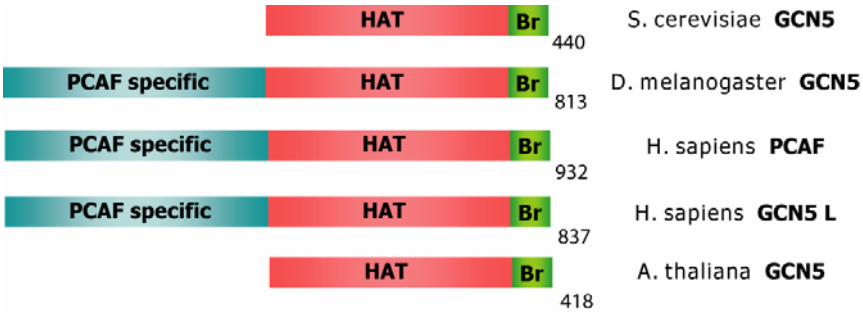

Figure 12. Schematic illustration of the Gcn5/PCAF HAT family. Br, bromodomain (Yang, 2004a).

The human P/CAF and GCN5 cDNAs were both identified based on their sequence similarity to yeast GCN5 (Candau et al., 1996; Yang et al., 1996). Two isoforms of the human GCN5 protein have been detected and are proposed to be the result of an alternative splicing event (Smith et al., 1998; Xu et al., 1998). The less abundant short form (GCN5 S, 476 amino acids) is similar in length to yeast Gcn5p (Candau et al., 1996; Yang et al., 1996), whereas the predominantly expressed long form (GCN5 L, 813 amino acids) contains an extended N-terminal domain similar to P/CAF, mouse GCN5 and *Drosophila* GCN5 (Smith et al., 1998; Xu et al., 1998). P/CAF, human GCN5 S and human GCN5 L copurify with multiprotein HAT complexes that are highly similar to SAGA (Ogryzko et al., 1998; Vassilev et al., 1998). For example, TFTC (TBP-free TAF_{II}-containing complex), a SAGA-related HAT, contains human GCN5 L, ADA3 and SPT3, the human counterparts of yeast Ada2p, Ada3p and Spt3p, and TRRAP (Ogryzko et al., 1998; Vassilev et al., 1998). The high degree of similarity between these human SAGA-related HAT complexes raises interesting questions regarding their specific roles in transcription. The observation that P/CAF and GCN5 mRNAs are differentially expressed in mouse tissues suggests that these HATs might have distinct cellular functions (Xu et al., 1998).

Acetylation of nucleosomes by GCN5 requires that it be in one of two large protein complexes called Ada and SAGA in yeast (Grant et al., 1997). The Gcn5-Adap complex was initially described as a transcriptional adaptor, based on the findings that it is needed by several acidic transactivators and basal transcription

proteins such as TBP (Barlev et al., 1995). Interactions between the acetyltransferase complex and DNA-binding transactivators suggest a mechanism by which these enzymes might be directed to specific regions of the genome for transcriptional activation. Moreover, mutations in GCN5 that eliminate Gcn5p acetyltransferase activity *in vitro* are defective in transcriptional activation *in vivo* (Candau et al., 1997; Wang et al., 1997), further indicating that histone acetylation is required for Gcn5p-mediated activation events. Recombinant Gcn5p (rGcn5p) displays a non-random specificity for acetylation of lysines in the N-termini of histones H3 and H4 (Kuo et al., 1996). The N-termini of these two histones are remarkably conserved across species, and each contains multiple conserved lysines that serve as sites of post-translational acetylation (Turner et al., 1992). In H4, acetylation occurs on lysines (K) 5, 8, 12 and 16. Acetylation of K5 and K12 is linked to histone deposition into newly synthesized chromatin during S phase in several species (Sobel et al., 1995), and acetylation of K16 is commonly enriched in transcriptionally active and potentially active chromatin (Roth and Allis, 1996; Turner et al., 1992). Acetylation of histone H3 occurs at lysines 9, 14, 18 and 23. Acetylation of K9 is correlated with histone deposition, whereas acetylation of the other sites in H3 and/or H4 in yeast confers specific transcriptional and cell growth defects (Grunstein, 1990; Roth and Allis, 1996; Smith and Stillman, 1991). Recombinant Gcn5p preferentially acetylates K14 of H3 *in vitro* and K8 and K16 of H4 (Kuo et al., 1996; Roth and Allis, 1996). In recent works is shown that Gcn5p acetylates primarily histones H3 and H2B (Suka et al., 2001; Zhang et al., 1998). The overwhelming evidence is that this HAT-containing complex functions as a co-activator and directly participates in the transcription process by being specifically recruited by activators to the promoter of many genes (Roth et al., 2001). For instance, activators such as Gal4 and Gcn4 interact directly with the SAGA complex *in vitro* (Brown et al., 2001; Drysdale et al., 1998; Klein et al., 2003) and *in vivo* (Bhaumik et al., 2004), and they recruit SAGA at target gene promoters *in vivo* (Kuo et al., 2000; Larschan and Winston, 2001), an event that can occur independently of transcription (Bhaumik and Green, 2001; Kuo et al., 2000; Topalidou and Thireos, 2003) and that precedes and is required for the

recruitment of the RNA polymerase II (Pol II) machinery at certain promoters (Bhaumik and Green, 2001; Bryant and Ptashne, 2003). Once recruited, the Gcn5 HAT subunit of SAGA locally acetylates histones, which is thought to facilitate transcription by loosening the chromatin structure or by generating specific binding sites for the recruitment of transcription factors (Roth et al., 2001). SAGA also serves an adaptor function to recruit the TATA-binding protein (TBP) through its Spt3 subunit (Bhaumik and Green, 2001; Larschan and Winston, 2001). Recent genome-wide location studies suggest that Gcn5-containing complexes may actually be recruited by regulatory proteins to the upstream activating sequence (UAS) of most active genes (Robert et al., 2004). In addition to its targeted coactivator function, Gcn5 also acetylates histones genome-wide, a phenomenon affecting most nucleosomes in yeast (Waterborg, 2000) and referred to as global acetylation (Kurdistani and Grunstein, 2003). This global activity results in a basal state of histone acetylation throughout the genome that varies among loci and over which targeted acetylation superimposes (Katan-Khaykovich and Struhl, 2002; Roh et al., 2004; Vogelauer et al., 2000). A recent work supports a role for global Gcn5 HAT activity in modulating transcription independently of its known coactivator function (Imoberdorf et al., 2006). Since the basal acetylation state is likely to differ between loci, such a global role for Gcn5 HAT activity might indirectly participate in the specificity of activator function by differentially modulating activity of the same or different activators depending on the gene's chromosomal location and/or promoter architecture. Global acetylation may therefore add an additional level of complexity to the mechanisms that contribute to the enormous divesity of gene expression.

As recently demonstrated, GCN5 interacts/acetylates a number of non histonic proteins that are involved in different cellular processes. For example, GCN5 acetylates the steroidogenic factor-1 (SF-1), an orphan nuclear receptor that plays an essential role in the development of the hypothalamic-pituitary-gonadal axis in both sexes, stimulating its transcriptional activity (Jacob et al., 2001). C-Myc, one of the most frequently overexpressed oncogens in human cancer, is a

substrate for GCN5/PCAF, and its acetylation increases c-Myc stability (Patel et al., 2004). GCN5 is also involved in viral infection, specifically interacting with and acetylating the human immunodeficiency virus type 1 (HIV-1) transactivator protein, Tat. Tat acetylation by GCN5 considerably enhances Tat-dependent transcription of the HIV-1 long terminal repeat (Col et al., 2001). The runt domain of AML1/MDS1/EVI1 (AME) protein interacts with P/CAF and GCN5; the co-expression of AME and either P/CAF or GCN5 abrogates the repression of an AML1-dependent reporter gene (Senyuk et al., 2003). Another report identified the first transcription factor target for human GCN5, the ubiquitous transcription factor NF-1. NF-Y plays a pivotal role in the cell cycle regulation of the mammalian cyclin A, cdc25C, and cdc2 genes and in the S phase activation of the ribonucleotide reductase R2 gene. Moreover, this factor has a critical role as a key proximal promoter factor in the transcriptional regulation of the albumin, collagen, lipoprotein lipase, major histocompatibility complex class II, and a variety of other eukaryotic and viral genes. In the report, the NF-Y complex has been shown to possess histone acetyltransferase activity through physical association with related histone acetyltransferase enzymes, human GCN5 and P/CAF *in vivo*. This association may serve to modulate NF-Y transactivation potential by aiding disruption of local chromatin structure thereby facilitating NF-Y access to its CCAAT box DNA binding sites (Currie, 1998). Recently, Puigserver and colleagues have reported that the nuclear hormone receptor coactivator PGC-1α, the key regulator of gluconeogenic genes during fasting, is acetylated by GCN5 and sequestered in nuclear foci thereby inhibiting gluconeogenesis (Lerin et al., 2006). GCN5 acetyltransferase activity plays therefore important roles in regulating different cellular processes. Many HATs share protein substrates (Glozak et al., 2005), this latter observation outlining a complex regulation network of cellular events similarly to other well-studied post-translational modifications of protein function such as phosphorylation.

3.5.1 GCN5 , P/CAF and cell cycle progression

As outlined in the previous paragraphs, in eukaryotes, the orderly cell cycle events are governed by number of cell cycle-related factors (proteins), such as

E2Fs, cyclins, Rb-related proteins, cdcs, cdks, etc. The molecular basis for functions of many of these factors has been clarified in kingdom starting from yeast to mammalian (Kikuchi et al., 2005). The orderly appearance and disappearance of the multiple factors to program the sequence of molecular events during normal cell cycle progression are manifested by positive and negative regulations of the cell cycle-related genes. On the other hand, knowledge concerning the involvement of acetylation of core histones in the regulation of cell functions has rapidly been accumulated (Carrozza et al., 2003; Pazin and Kadonaga, 1997).

The HAT and HDAC members play much diverse and broader roles in cell functions, such as transcription activation, gene silencing, cell cycle progression and/or arrest, cell differentiation and DNA repair in eukaryotes (Kikuchi et al., 2005).

GCN5 and PCAF show tissue (or cell type) specific expression characteristics, and therefore each is expected to play the distinct role, and may be at a particular time. For instance, the participation of these two HAT enzymes in the cell cycle progression has been identified in yeast (Burgess et al., 1999; Howe et al., 2001; Krebs et al., 1999; Zhang et al., 1998).

The activity of GCN5, appears essential for proper cell cycle progression during both the G1, S and G2/M phases in yeast (Burgess et al., 1999; Howe et al., 2001; Krebs et al., 1999; Zhang et al., 1998).

In late G1 phase of the cell cycle, GCN5 together with the SW1/SNF complex and its transcription coactivators Swi4p/Swi6p is required for expression of the HO gene (Krebs et al., 1999). The combined loss of GCN5 and SAS3 functions results in both an extensive global loss of H3 acetylation and cell cycle arrest in G2/M phase (Howe et al., 2001). GCN5 and Rpd3 play a distinct and opposing role in IME2 transcription during both meiosis and vegetative growth (Burgess et al., 1999). The deficiency of GCN5 in yeast cells leads to the accumulation in G2/M phase, indicating its impact on normal cell cycle progression (Zhang et al., 1998). In addition, Esa1p is found to be required for the cell cycle progression of

yeast, potentially through discrete transcriptional regulatory events (Clarke et al., 1999).

Genes associated with cell cycle progression are tightly controlled by E2Fs that are originally identified for their role in G1/S transition. In mammalian cells, GCN5 together with a cofactor TRRAP is required for the transcriptional activation of E2F4 that regulates the temporal activation of genes involved in the cell cycle progression (Lang et al., 2001). E2F family members responsible for the control of the cell cycle progression themselves are acetylated and regulated by p300/CBP (Marzio et al., 2000). Acetylation of E2F1 by PCAF, CBP and p300 has three functional potentialities: increasing its DNA binding ability, its transcriptional activation capacity and its protein half-life (Martinez-Balbas et al., 2000). Human GCN5 and PCAF are reported to be physiologically associated with the ubiquitously distributed transcriptional factor NF-Y, which itself plays a key role in the cell cycle progression through the transcriptional regulation of cyclin A, cdc25 and cdc2 genes (Currie, 1998). This notion was further supported by the fact that following the release of E2Fs/HDACs, a hierarchy of PCAF–NF-Y–p300 interactions and histones H3/H4 acetylations are required for the activation of cell cycle-related promoters as detected by chromatin immunoprecipitation assay (Caretti et al., 2003). Acetylation represents indeed a novel mechanism for transcriptional activation of several genes essentially required in coordinating cell cycle events (Kikuchi et al., 2005).

Although extensive studies with HATs and HDACs have provided valuable clues to their general functions as histone modifying enzymes, their participatory roles in the cell cycle progression have not been explored much. Furthermore, in spite of accumulation of extensive knowledge on functional nature and/or coordinated action of cell cycle related factors, the way by which amounts of all or most of these factors are maintained throughout the cell cycle progression is still unknown. This could be achieved by three possible ways as follows. Firstly, transcriptions of genes encoding all or most cell cycle-related genes are individually controlled. Secondly, transcriptions of genes for almost all of the factors is collectively and directly regulated by a putative master transcription

factor(s). Thirdly, all inclusive transcriptional regulation for almost all of the genes occurs through alterations in the chromatin structure surrounding them. A recent work describes the third the most likely to happen, since alterations in the chromatin structure have been known to be preferentially involved in DNA-utilizing processes, such as gene expression, DNA replication, recombination and repair. To clarify the role of GCN5 and P/CAF in the control of cell cycle progression, the authors generated two homozygous DT40 chicken cell mutants, lacking GCN5 and P/CAF enzymes, respectively. The GCN5 mutant gave a delay in the growth rate and was accompanied with alterations in the cell cycle distribution, such as the decreased or increased number of cells containing less than a diploid distribution as a result of the GCN5 deficiency may be the consequences of suppressed mitosis and/or the delayed progression from G1 to S phase. The authors examined then the expression levels of various cell growth and cell cycle-related genes, especially for G1/S phase transition-related genes. In the proposed model, GCN5 preferentially participates in G1/S phase transition of the cell cycle, though the transcriptional regulation of a number of genes involved. The molecular basis of the participation should be divided into two distinct ways: in DT-40 cells, normally, GCN5 up-regulates the expression of E2F-1, E2F-3, E2F-4, E2F-6, DP-2, cyclin A, cyclin D3, PCNA and cdc25B, but conversely down-regulates that of three putative counterparts, such as c-myc, cyclin D2 and cyclin G1. Similarly, it up-regulates the expression of p107 but down-regulates that of p27. Thus, GCN5 is directly involved positively or negatively in controlling the required amounts of a certain set or another set of G1/S phase transition-related factors, via no other HAT and HDAC functions, and thereby governs normal G1/S phase transition of the cell cycle (Fig. 13). GCN5 also participates in the positive or negative regulation for the expression of apoptosis-related gene bcl-xL or bcl-2 for the purpose of maintaining a desired considerable level so as to block apoptotic cell death. Although the former should directly be controlled by GCN5, the latter may be controlled indirectly through the P/CAF and HDAC 4 functions. To conclude, monitoring as a supervisor, GCN5 activates or suppresses collectively the transcription of a set of these cell cycle-related genes, and directs orderly progression of the normal cell

cycle. As expected, GCN5 increases or decreases global acetylation levels of various specific lysine residues of core histones H2A, H2B, H3 and H4, indicating that it takes part in transcriptional regulations, probably through alterations in the chromatin structure surrounding these widely distributing genes.

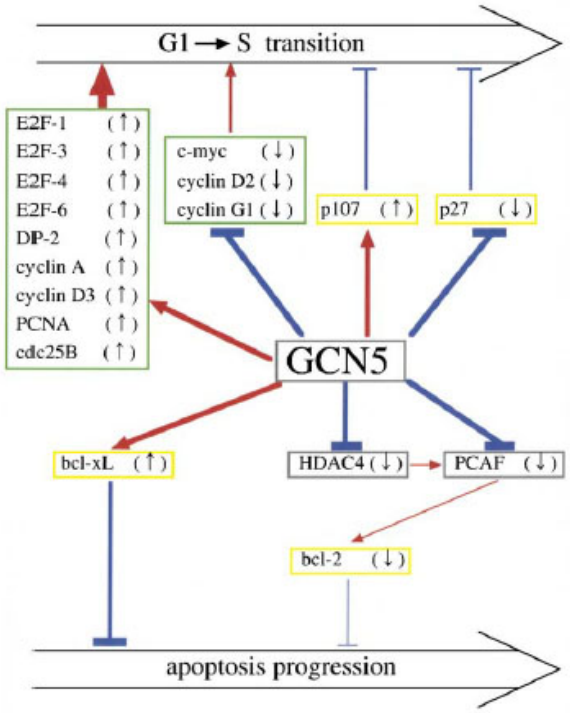

Figure 13. A model for GCN5 function as a supervisor in all inclusive control of cell cycle progression of vertebrate cells (Kikuchi et al., 2005).

3.6 Protein acetylation and DNA replication

In addition to factors directly involved in the formation of the preRCs, it has been suggested a potential role of chromatin structure in the control of initiation of DNA replication. Chromatin structure has been linked to the inititiation of DNA replication (Brown et al., 1991), and histone modification has been implicated in regulating replication timing in budding yeast (Aparicio et al., 2004; Vogelauer et al., 2002). Recent studies further suggest that histone acetylation is involved in origin activation at the chorion gene loci in *Drosophila* follicle cells (Aggarwal

and Calvi, 2004) and in *Xenopus* early development (Danis et al., 2004). In addition, the histone deacetylase Sir2 has been shown to negatively regulate pre-RC assembly in budding yeast (Pappas et al., 2004). These results suggest that histone acetylation is potentially involved in the control of pre-RC assembly.

The human Hbo1 (hHbo1), a MYST family histone acetyltransferase (Utley and Cote, 2003), was originally identified by Iizuka and Stillman (Iizuka and Stillman, 1999) through its binding to the human Orc1 protein; subsequently, Hbo1 was found to bind mouse Mcm2 as well (Burke et al., 2001). More recently, Hbo1 was found to associate with the latent replication origin of Kaposi's sarcoma-associated virus replication (Stedman et al., 2004). These physical and functional interactions suggest that the acetylase activity of Hbo1 might participate in pre-RC formation and replication licensing. In a recent work, it has been outlined that Hbo1 is a previously unrecognized positive regulatory factor for pre-RC assembly (Iizuka et al., 2006). Iizuka and colleagues have found that Geminin, CDT-1 and Orc2 (unpublished results) are co-immunoprecipitated with XHbo1. These results raise the possibility that one or more of the pre-RC components and their regulators may be physiological targets of Hbo1 acetylation. To test this, the authors assayed the ability of recombinant human Hbo1 to acetylate bacterially expressed human Orc2, Mcm2 and geminin and chinese hamster Cdc6. Hbo1 indeed acetylates *in vitro* Geminin and Orc2 and, with a lesser extent, Cdc6 and Mcm2. When Hbo1 expression was inhibited in human cells, Mcm2-7 failed to associate with chromatin even though ORC and Cdc6 loading was normal. In *Xenopus* egg extracts immunodepleted of *Xenopus* Hbo1 (XHbo1), chromatin binding of Mcm2-7 was lost, and DNA replication abolished. The binding of Mcm2-7 to chromatin in XHbo1-depleted extracts could then be restored by the addition of recombinant CDT-1.

The Py enhancer PEA1 and PEA3 sites are particularly important for stimulating Py DNA replication (Chen and Fluck, 2001; Chen et al., 1995; Guo et al., 1996; Ito et al., 1996; Martin et al., 1988; Mueller et al., 1988; Murakami et al., 1991; Piette and Yaniv, 1987; Rochford et al., 1990; Rochford et al., 1992; Tang et al., 1987). Jun, a member of the AP1 (PEA1) complex, recruits Py large T antigen

(PyLT) to the origin to stimulate DNA unwinding, particularly at early times after infection when PyLT is limiting (Guo et al., 1996; Ito et al., 1996; Martin et al., 1988; Mueller et al., 1988; Murakami et al., 1991; Rochford et al., 1992). The AP1 complex and its family proteins (that bind the PEA3 site) as well as Gal4VP16, NF-κB, E1a, Sp1, and p53, which also can stimulate Py DNA replication (Baru et al., 1991; Bennett-Cook and Hassell, 1991; Bennett et al., 1989; Guo and DePamphilis, 1992; Ishikawa et al., 1993; Kanda et al., 1994; Murakami and Ito, 1999; Nilsson et al., 1991; Wasylyk et al., 1990); reviewed in (DePamphilis, 1993; Murakami and Ito, 1999), interact with p300/CBP (Arany et al., 1994; Arias et al., 1994; Avantaggiati et al., 1997; Bannister and Kouzarides, 1995; Bannister et al., 1995; Gerritsen et al., 1997; Gu and Roeder, 1997; Jayaraman et al., 1999; Kundu et al., 2000; Lee et al., 1996; Lill et al., 1997; Lundblad et al., 1995; Martin et al., 1988; Perkins et al., 1997; Suzuki et al., 2000; Yang et al., 1998), P/CAF and GCN5 (Candau et al., 1996; Liu et al., 1999; Utley et al., 1998; Wang et al., 2000) and other coactivators that acetylate histones and nonhistone proteins involved in transcription, including HMG17, HMGI(Y), E2Fs, p53, c-Jun (Vries et al., 2001), MyoD, YY1, Tat, TFIIE, TFIIF and TFI68 (Chen et al., 2001; Roth et al., 2001; Sterner and Berger, 2000). Acetylation regulates these proteins' functions and interactions with other proteins (Chen et al., 2001; Kouzarides, 2000; Roth et al., 2001; Sterner and Berger, 2000; Strahl and Allis, 2000).

Other proteins directly involved in DNA replication also interact with acetyltransferases, including PyLT, which interacts with p300/CBP (Cho et al., 2001; Nemethova and Wintersberger, 1999) PCAF and GCN5 acetyltransferases, when tethered near the Py origin, bind PyLT and stimulate DNA replication *in vivo* and they concluded that the process is related to PCAF-dependent PyLT acetylation (Xie et al., 2002) (Fig. 14).

Another component of the pre-Rc complex, MCM3, is endogenously acetylated and the acetylated component of MCM3 is strictly chromatin-bound in late G1 phase. Moreover, MCM3 associated protein (MCM3AP), a protein isolated by two-hybrid screening using MCM3 as bait, is a specific MCM3 acetyltransferase of the

GNAT superfamily (Takei et al., 2001). The MCM3 acetylase activity of MCM3AP is required to inhibit initiation of DNA replication and the association of MCM3AP to chromatin alone is not sufficient for the inibition. The interaction between MCM3 and MCM3AP is essential for nuclear localization and chromatin binding of MCM3AP. Hence, MCM3AP is a potent natural inhibitor of the initiation of DNA replication whose action is mediated by interaction with MCM3.

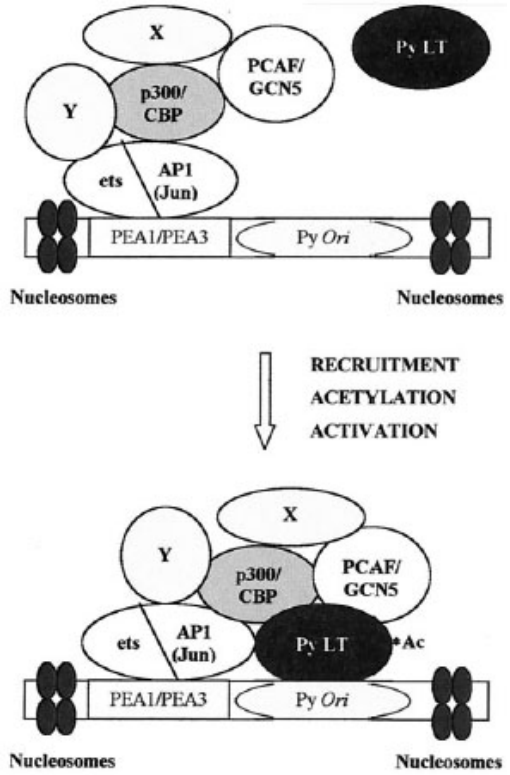

Figure 14. Model for Py enhancer-origin complexes containing accessory proteins PCAF, GCN5, and PyLT (Xie et al., 2002).

4 Multisite Protein Modification

Protein function is regulated in eukaryotes through the post-translational modification, a major mechanism. Most eukaryotic proteins are modified in one form or the other and many covalent modifications are transient and play important roles in regulating protein function.

Many proteins are modified at single and, most importantly, at multiple sites, a phenomenon referred to as multiple modification. The multiplicity of modification sites on a protein often correlates with its biological importance and the complexity of the corresponding organism. It is well known that multisite phosphorylation serves as a common mechanism for regulating protein function in eukaryotes (Cohen, 2000; Holmberg et al., 2002). One extreme example is RPB1, the largest subunit of RNA polymerase II. Dependent on the organism, the C-terminal domain (CTD) of RPB1 consists of 25-52 heptapeptide repeats with the consensus sequence YSPTSPS. Each repeat contains five phosphorylable sites (Buratowski, 2003; Hampsey and Reinberg, 2003; Kobor and Greenblatt, 2002). Ser 2 phosphorylation is seen in coding regions and coupled to 3'-RNA prodessing, whereas Ser 5 phosphorylation is detected primarily at promoter regions and linked to RNA capping (Fabrega et al., 2003; Komarnitsky et al., 2000; Meinhart and Cramer, 2004). Interestingly, both serines are phosphorylated during the M phase of the cell cycle to inhibit RNA splicing and promote gene silencing (Xu et al., 2003) (Fig. 15). Histones are subjected to regulation by different modifications, which include acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation and citrullination. For example, some authors have investigated the role of ser-10 phosphorylation and lysine 14 acetylation in histone H3 (Lo et al., 2000). They found that, *in vitro*, several HAT enzymes displayed increased activity on H3 peptides bearing phospho-Ser 10. this augmenting effect of Ser-10 phosphorylation on acetylation by yGcn5 was lost by substitution of alanine for arginine 164 (Gcn5 R164A), a residue close to Ser 10 in the structure of the

ternary tGcn5/CoA/histone H3 complex. Gcn5 (R164A) had reduced activity *in vivo* at a subset of Gcn5-dependent promoters, and, strikingly, transcription of this same subset of genes was also impaired by substitution of serine 10 to alanine in the histone H3 tail.

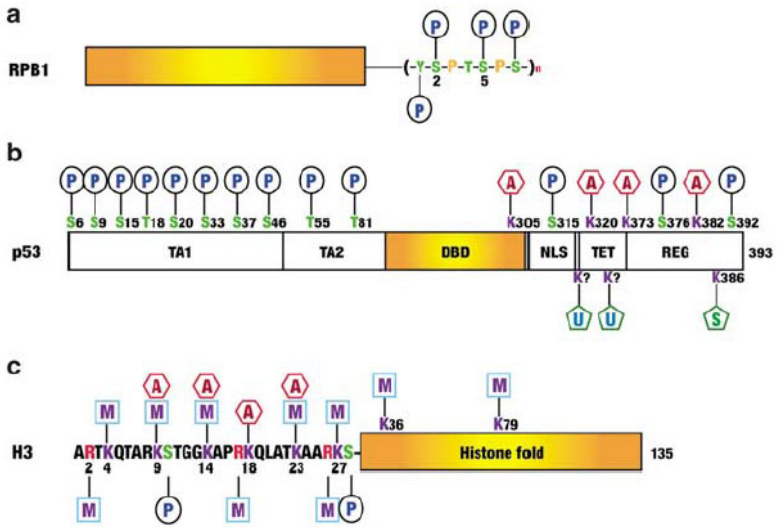

Figure 15. Multisite modification of representative proteins. (a) The CTD of RPB1 comprises heptapeptide repeats with the indicated consensus sequence. From yeast to humans, the number (n) of repeats increases from 26 to 52. In addition to phosphorylation, the CTD is subject to other modifications like proline isomerization and glycosylation. (b) p53 is composed of modular domains, including the N-terminal transcriptional activation domains (TA1 and TA2), DNA-binding domain (DBD), nuclear localization signal (NLS), tetramerization motif (TET) and C-terminal regulatory domain (REG). The number at the right refers to total residues of human p53. Modified residues are indicated by single letters along with their positions. p53 is ubiquitinated at its C-terminus (Brooks and Gu, 2003), but the sites are not so well de.ned. (c) Histone H3 comprises of a flexible N-terminal tail and a C-terminal histone-fold domain. Post-translational modifications are labeled with color letters: P in oval, phosphorylation; A in hexagon, acetylation; U in pentagon, ubiquitination; S in pentagon, sumoylation; M in square, methylation (Yang, 2005).

These observations suggest that transcriptional regulation occurs by multiple mechanistically linked covalent modifications of histones.

Multisite modification occurs in many other eukaryotic proteins, including DNA-binding transcritpion factors (e.g. Pho4, NF-AT, c-Jun and Elk-1), enzymatic transcriptional coactivators (e.g. p300 and CBP), DNA replication regulators (e.g. PCNA), cell cycle controllers (e.g. cyclins and Cdc25 phosphatases), apoptosis regulators (e.g. BAD), cytoskeletal proteins (e.g. tubulins and neurofilament proteins) and signalling molecules (e.g. Raf-1, MEK1 and tyrosine kinases). Therefore, multisite modification is a common but complex mechanism for regulating protein function in eukaryotic cells.

4.1 Histone code

Multisite modification constitutes a complex layer of molecular information beyond the amino-acid sequence of a given protein, so an intruiguing question is how such molecular information is conveyed to regulate its function. The "histone code" hypothesis (Fischle et al., 2003; Jenuwein and Allis, 2001; Strahl and Allis, 2000; Turner, 2000; Turner, 2002), "chromatin signalling" (Schreiber and Bernstein, 2002) and "histone interaction surface" models (Kurdistani et al., 2004) have been proposed to explain how different histone modifications regulate chromatin structure and function. In addition, hypotheses similar to that of "histone code" have been put forward for modifications of p53 (Appella, 2001; Appella and Anderson, 2000) (see paragraph 4.3.1 for detail), RPBI (Buratowski, 2003), p300/CBP (Legube and Trouche, 2003) and tubulins (Westermann and Weber, 2003). For cellular signaling, it is well known that multisite phosphorylation serves as an essential way for transducing molecular signals (Cohen, 2000; Holmberg et al., 2002). No matter which model or hypothesis is used, it is clear that modifications of a protein at multiple sites form a complex regulatory program for the qualitative and quantitative control of its function. Emerging evidence suggests that this program displays characteristics of a "dynamic molecular fingerprint or barcode".

4.2 Switch- and gauge-like effects of multisite modification

Multiple modifcation events on a protein frequently interplay with each other and their functional consequences are often multifaceted. Modifications at different sites could be independent of each other, with each being sufficient to achieve the maximal output. In this case, each modification event serves as a simple "on/off " switch. Alternatively, modifications at two or more sites synergize with each other to impart an exponential effect, thereby generating a combined switch. This is reminiscent of the countdown mechanism that has been proposed for multisite phosphorylation of the CDK inhibitor Sic1 (Orlicky et al., 2003). Modifications events at different sites could also have additive effects, thereby producing a linear output and regulating protein function in a quantitative manner. Such a "gauge-like" mode of action has been documented for histone acetylation and PDGF receptor phosphorylation (Schreiber and Bernstein, 2002). Therefore, multisite modification is important for coordinating the qualitative and quantitative control of protein function *in vivo*.

4.3 Protein acetylation and phosphorylation

The first commonality between acetylation and phosphorylation is the diversity of substrates. Acetylation can occur on histones, DNA-binding transcription factors, acetylases, nuclear import factors and α-tubulin. As in the case of phosphorylation, substrates can be nuclear or cytoplasmic. Although clearly not as prevalent as phosphorylation at the moment, the list of acetylated proteins is increasing rapidly. Acetylation can regulate different functions, also a feature of kinases. It can modify the recognition of DNA, the stability of proteins and the interaction between proteins. Our limited knowledge of acetylated targets also suggests that they may regulate different cellular processes, such as microtubule function or nuclear import (Kouzarides, 2000).

Thus, the analogy between acetylation and phosphorylation does hold true when we consider that both modifications affect multiple different proteins and regulate them in a variety of different ways. There are of course differences. For

example, kinases do not necessarily associate with their substrates as avidly as acetylases. There are also far fewer acetylases than kinases, but this discrepancy may well reflect our current bias in defining these enzymes as histone acetylases. Perhaps if substrates other than histones were used in the screening for new acetylases, new families of enzymes might be identified that recognize only non-histone proteins. It is also true to say that homology within the catalytic domain of acetylases is not as high as, for example, between kinases, so we may be missing some obvious candidates.

Where the analogy with kinases breaks down is when we consider the signalling aspects of phosphorylation. There is no evidence as yet for an acetylation cascade, i.e. an acetylase modifying the enzimatic activity of a second acetylase in order to transmit a biological signal. However, the elements for the implementation of an acetylation cascade have been identified. The bromodomain, which recognizes acetyl-lysines, may be analogous to the SH2 domain, which recognizes phosphotyrosine and transmits the phosphorylation signal. A similar signalling function may be attributed to bromodomains, although this could be limited to nuclear events, given that bromodomains have not been identified in cytoplasmic proteins.

Acetylation as a regulatory modification has come a long way since the identification of histone acetylases and deacetylases. Perhaps it is time to drop the "histone" prefix for these enzymes given the multiplicity of other targets. It is this diversity of substrates that makes acetylation comparable to phosphorylation. However, acetylation lags behind phosphorylation in many ways, not least at the level of knowing which acetylase is the true *in vivo* enzyme for a given substrate.

Specific inhibitors of acetylases would be very useful for dissecting different *in vivo* pathways. Such inhibitors have been invaluable to the kinase field.

Despite this discrepancy in knowledge and even given the eventuality that acetylation may not exactly parallel phosphorylation, the fact remains that both phosphorylation and acetylation can regulate key cellular processes in response

to extracellular signals. So this evidence alone is sufficient to propose that we are witnessing the birth of a new biologically relevant regulatory modification to rival phosphorylation (Kouzarides, 2000).

4.3.1 Examples of coordinated post-translational protein modification by phosphorylation and acetylation

p53 protein.

The function of the p53 protein has proven to be much more intricate than anticipated by most scientists. The p53 tumour suppressor is a tightly regulated protein that acts by stopping cell-cycle progression or promoting apoptosis when cells encounter stress stimuli such as oncogene activation or DNA damage (Bode and Dong, 2004). Having a short half-life, p53 is normally maintained at low levels in unstressed mammalian cells by continuous ubiquitylation and subsequent degradation by the 26S proteasome. This is primarily due to the interaction of p53 with the RING-finger ubiquitin E3 ligase MDM2. When the cell is confronted with stress, however, p53 ubiquitylation is suppressed and p53 is stabilized and accumulates in the nucleus, where it forms a homotetrameric complex (Friedman et al., 1993). Only tetrameric p53 seems to be fully active as a transcriptional activator or repressor of distinct target genes that contain p53 sequence-specific DNA binding sites (Davison et al., 1998). Of the >150 genes targeted by p53, most are associated with regulation of cell-cycle arrest, apoptosis and/or DNA repair processes (Fig. 16) that function to prevent proliferation of damaged cells.

The importance of p53 in cancer development is illustrated by the fact that p53 is highly mutated (>18,000 mutations) in many different cancers (Olivier et al., 2004) and is probably rendered inactive by a range of indirect mechanisms (for example, MDM2 amplification or loss of ARF) in most other cancer types. The mutations are found mainly in the specific DNA-binding core domain of p53 — residues 98–292. The result of these mutations is the generation of a full-length p53 mutant protein that can be accumulated at high levels in many cancer cell

types. Active p53 is subject to a complex and diverse array of covalent post-translational modifications, which markedly influence the expression of p53 target genes. The most commonly reported post-translational modifications of p53 include phosphorylation of serines and/or threonines and acetylation, ubiquitylation and sumoylation of lysine residues.

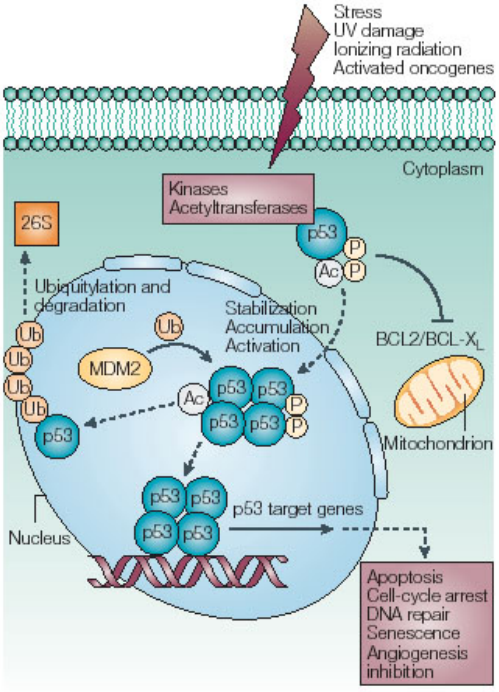

Figure 16. Activation of p53 and cellular response. Stress signals converge on p53 and activate various protein kinases and/or acetyltransferases, which phosphorylate or acetylate p53, respectively. These posttranslational modifications generally result in stabilization and activation of p53 in the nucleus, where p53 interacts with sequence-specific DNA binding sites of its target genes. The transcriptional activation leads to diverse cellular responses such as apoptosis, cell-cycle arrest or DNA repair. When p53 is no longer needed, it is targeted for ubiquitylation by MDM2 and moved out of the nucleus to be degraded by the 26S proteasome. p53 can also act outside of the nucleus to induce apoptosis by binding with anti-apoptotic proteins such as BCL2 (Bode and Dong, 2004).

Although phosphorylation of proteins associated with cell survival and proliferation is commonly regulated by tyrosine kinases (Craven et al., 2003), as

yet none of the nine tyrosines in p53 are known to be phosphorylated. Other reported modifications of p53 include glycosylation (Shaw et al., 1996) and ribosylation (Wesierska-Gadek et al., 1996).The ensuing cellular response is dependent on the particular post-translational modifications conferred on the p53 protein, which are themselves dependent on cell type and the nature of the external stimuli, making the overall picture of p53 regulation highly complicated.

Post-translational phosphorylation and acetylation usually drive p53 transcriptional activation because these modifications generally result in p53 stabilization, accumulation and activation in the nucleus.

Over the past few years, innumerable research reports have provided a clearer understanding of the function of phosphorylation and acetylation in the regulation of p53. However, mapping this knowledge onto differences between normal and cancerous cells is a daunting task that is still in its infancy. Although the frequency and importance of acetylation might actually rival phosphorylation as a crucial posttranslational modification, phosphorylation is so far the most commonly reported protein modification that occurs in mammalian cells. Phosphorylation is a reversible mechanism that is crucial for the regulation of the biological activity of hundreds of proteins. Protein kinases and phosphatases usually activate or inactivate a particular protein. Phosphorylation of p53 generally results in its stabilization. In some cases, previous phosphorylation might be required for subsequent acetylation of p53, and almost every type of cellular stress increases acetylation levels of p53 in a range of cell types (Ito et al., 2001). Several proteins act as histone acetyltransferases, which add one or more acetyl groups to p53. Acetylation seems to be important for p53 stability and transcriptional activation (Barlev et al., 2001), although this is controversial (Espinosa and Emerson, 2001; Nakamura et al., 2000). Some of the present controversy surrounding the importance of p53 acetylation might reflect differences in the experimental protocols used and the mutants analysed, or might be a consequence of cell-type-specific differences. Other, as yet unidentified, post-translational modifications might also be involved. However, most published papers generally show that the deacetylation of p53 results in its

ubiquitylation and degradation (Juan et al., 2000; Luo et al., 2001; Luo et al., 2000; Vaziri et al., 2001). The carboxyterminal residues acetylated in response to DNA damage are also ubiquitylated by MDM2; therefore, acetylation could prevent ubiquitylation, resulting in stabilization of p53 (Ito et al., 2001; Rodriguez et al., 2000). Similarly, because MDM2 is overexpressed in many cancers, competition with ubiquitylation, resulting in aberrant acetylation required for activating p53 could be compromised by destabilization and degradation of p53. The picture is further complicated by the recent finding that p53 transactivation might also be repressed by neddylation in a process promoted by MDM2.

Even though observations of post-translational modification of p53 have been substantiated many times under diverse experimental conditions, the specific functional consequence or exact temporal sequence of each specific modification event is not clear. The likelihood that one isolated phosphorylation or acetylation event will have an observable impact on cellular function is small. On the other hand, one might expect that p53-mediated cellular function is linked to a highly orchestrated cascade of phosphorylation/dephosphorylation events that are intermingled with acetylation/deacetylation, ubiquitylation/deubiquitylation and other modification events that are yet to be determined.

Many groups have examined the regulation of p53 by post-translational modifications, but only a few have attempted to compare the phosphorylation or acetylation pattern of the p53 protein in normal cells with stressed or cancer cells.

Buschmann and colleagues (Buschmann et al., 2001) examined the status of p53 phosphorylation in normal human fibroblasts during cell-cycle progression. Results indicated that in unstimulated cells, phosphorylation of serines 9, 15, 20 and 372 peaked during G1, whereas phosphorylation of Ser37 and Ser392 peaked during G2/M phase. Ser37 was the only site phosphorylated during S phase and acetylation of p53 was highest at G0. These data support the concept that phosphorylation of p53 is a dynamic and transient event, which might be

predictable under controlled conditions and might also involve a tightly coordinated cascade of activation and deactivation events.

Hyperphosphorylation or hyperacetylation have been observed in some cancer cells lines. For example, Ser9 and Ser15 of human p53 were reported to be hyperphosphorylated in cells derived from the human glioblastoma line T98G (Ullrich et al., 1993). Higashimoto and colleagues showed that untreated human pulmonary epithelial type II (A549) cells, which express wild-type p53, showed a background of constitutive phosphorylation at Ser6 and Ser9 that increased approximately tenfold after exposure to either ionizing radiation or UV light. Kao and colleagues (Kao et al., 2004) recently reported that expression of the hepatitis C virus (HCV) core protein induced hyperacetylation of Lys373 and Lys382 of p53 and, depending on the level of HCV expression, either increased (low expression level) or suppressed (high expression level) phosphorylation of p53 at Ser15.

The HCV core protein is extensively involved in the pathogenesis of HCV infection and related carcinogenesis, and is known to interfere with normal functions of many cellular proteins (Ray and Ray, 2001). The significance of this excessive post-translational phosphorylation or acetylation in some cancer cell lines is not totally clear yet, but might affect the stability of p53 or its interactions with other proteins in a manner similar to what is observed for mutant p53.

The retinoblastoma protein.

In tumour cells, the retinoblastoma tumour suppressor protein (pRb) is sequestered by viral oncoproteins (E1A) and regulated by phosphorylation through G1 cyclin-dependent kinases (CDKs), primarily cyclin D/ CDK4 and cyclin E/Cdk2, which sequentially phosphorylate pRb as the cells move towards S-phase (Smink, 2001).

Chan and colleagues (Chan et al., 2001) have identified acetylation as a new type of regulator of pRb function and assessed whether acetylation might

influence pRb phosphorylation. pRb acetylation is mediated by p300 through adenovirus E1A recruitment of pRb and p300/CREB binding protein (CBP) in a multimeric complex.

Acetylated pRb was less efficiently phosphorylated by CDKs. An implication of these results is that acetylation can alter the ability of CDKs to modulate pRb-dependent growth control. Lysines 873/874 have been implicated as part of CDK-docking site (Adams et al., 1996). If the cyclin E/Cdk2 docking activity of lysines 873/874 were to be modulated by acetylation, then this process might provide a regulatory mechanism to explain the influence of the acetylation of these lysine residues on the reduction in pRb phosphorylation by cyclin E/Cdk2. Indeed, Chan and colleagues presented a general mechanism that may contribute to this role. Specifically, they have proposed that the targeted acetylation of pRb by p300, together with the resulting obstruction to cyclin/CDK phosphorylation and with the retention of cells in a growth-arrested state, is likely to favour the ability of cells to respond to differentiation-inducing signals. These recent advances suggest acetylation as a new control mechanism in regulating pRb activity and a new mechanism through which viral oncoproteins can affect tumour-suppressor activity.

SECTION II

PRELIMINARY REMARKS

The Ph-D thesis contains three manuscripts as follows:

1) Subnuclear distribution of the largest subunit of the human origin recognition complex during the cell cycle (Lidonnici et al., 2004).

2) Specific interaction of the retinoblastoma protein with Orc1 and its recruitment to human origins of DNA replication (Mendoza et al., *submitted*).

3) Acetylation of human Cdc6 by GCN5 acetyltransferase regulates site-specific, CDK-mediated protein phosphorylation in the S-phase of the cell cycle (Paolinelli et al., *submitted*).

Subnuclear distribution of the largest subunit of the human origin complex during the cell cycle

Specific contribution of Roberta Paolinelli to the work described in this manuscript: I have collaborated with Lidonnici and colleagues by analysing protein/protein interactions (GST-Pull Down assays and FRET experiments) and cell cycle distribution upon ectopic protein expression (flow cytometry analysis).

1 Summary

In eukaryotes, initiation of DNA replication requires the activity of the origin recognition complex (ORC). The largest subunit of this complex, Orc1p, has a critical role in this activity. Here we have studied the subnuclear distribution of the overexpressed human Orc1p during the cell cycle. Orc1p is progressively degraded during S-phase according to a spatio-temporal program and it never colocalizes with replication factories. Orc1p is resynthesized in G1. In early G1, the protein is distributed throughout the cell nucleus, but successively it preferentially associates with heterochromatin. This association requires a functional ATP binding site and a protein region partially overlapping the bromo-adjacent homology domain at the N-terminus of Orc1p. The same N-terminal region mediates the *in vitro* interaction with heterochromatin protein 1 (HP1). Fluorescence resonance energy transfer (FRET) experiments demonstrate the interaction of human Orc1p and HP1α *in vivo*. Our data suggest a role of HP1 in the recruitment but not in the stable association of Orc1p with heterochromatin. Indeed, the subnuclear distribution of Orc1p is not affected by treatments that trigger the dispersal of HP1.

2 Results

The subnuclear distribution of Orc1p changes during the cell cycle

The subnuclear distribution of Orc1p has been poorly investigated. We decided to study this aspect using the overexpressed human protein (Orc1p*). As a starting point we verified the ability of Orc1p* to behave as the endogenous protein. In particular, Orc1p* (fused to the Flag epitope) associated with chromatin, interacted with the endogenous Orc2p subunit of ORC and its level oscillated during the cell cycle being minimal in S and G2/M phases (Fig. 1). More importantly, Orc1p* was able to bind *in vivo* to the lamin B2 origin of DNA replication, one of the few human DNA sequences shown to interact with ORC *in vivo* (Abdurashidova et al., 2003; Ladenburger et al., 2002; Paixao et al., 2004). This was assessed by chromatin immunoprecipitation (ChIP) analysis of HeLa cells transiently transfected with Orc1-Flag. After *in vivo* crosslinking, chromatin was immunoprecipitated with the anti-Flag antibody and the immunopurified DNA was used in competitive PCR to determine the abundance of the lamin B2 origin (B48) relative to that of a non-origin sequence which is not bound by ORC (B13) (Fig. 2). Competitive PCR is based on the coamplification of a fixed amount of immunopurified genomic DNA, with unknown concentration, and decreasing amounts of a competitor template. The genomic and the competitor sequences are recognized by the same primer set. Because the competitor is 35 bp longer than the genomic sequence, coamplification results in the production of two DNA fragments that can be resolved by gel electrophoresis (Fig. 2 B). The 1:1 ratio between the competitor and genomic bands provides a measure of the amount of the genomic sequence in the immunopurified material. As shown in Fig. 2 B, the 1:1 ratio was obtained at a 10^{-8} dilution of the competitor for B48 sequence and at $3x10^{-9}$ for B13, indicating a threefold enrichment of the origin over the B13 sequence in the immunopurified DNA (Fig. 2 C). As this value is comparable to that measured for the same region after immunoprecipitation of the endogenous Orc proteins (Ladenburger et al., 2002; Paixao et al., 2004) the

results in Fig. 2 C support the conclusion that Orc1p* associates with pre-replication complexes assembled on chromosomal DNA replication origins.

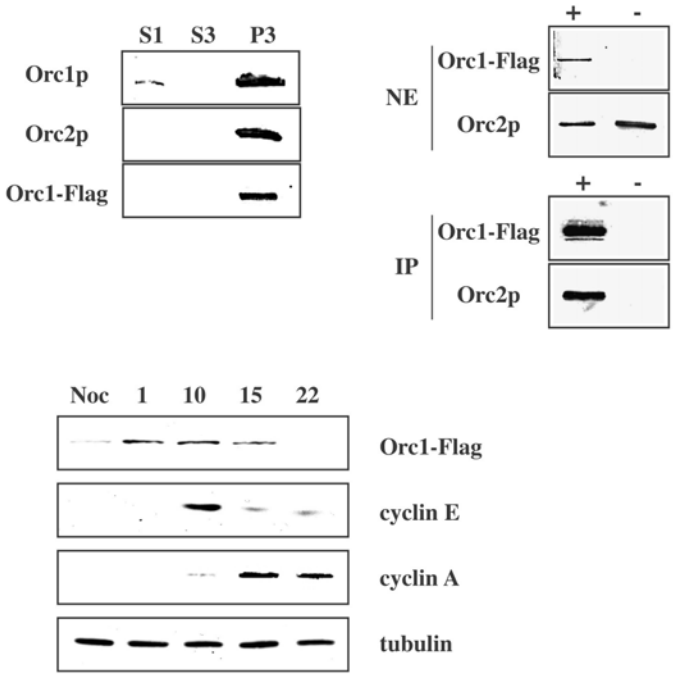

Figure 1. Orc1p* binds to chromatin, co-immunopurifies with Orc2p and its level is cell cycle-regulated. (A) Western blot analysis of fractionated HeLa cells. S1, cytoplasm; S3, nucleoplasm; P3, chromatin-enriched fraction. Endogenous Orc1p and Orc2p were revealed with specific polyclonal antibodies, Orc1p* was localized with the anti-Flag polyclonal antibody. (B) Western blot analysis of nuclear extracts (NE) prepared from non-transfected HeLa cells (–) or from HeLa cells expressing Orc1-Flag (+). Extracts were probed with the anti-Flag polyclonal antibody and the anti-Orc2 polyclonal antibody. The same extracts were used in co-immunoprecipitation experiment with the monoclonal anti-Flag M2 affinity gel (IP). The presence of Orc1-Flag and of Orc2p in the immunoprecipitate was revealed in western blotting with anti-Flag and anti-Orc2 polyclonal antibodies. (C) HeLa cells expressing Orc1-Flag were arrested in mitosis with nocodazole (Noc) and allowed to recover from the block for 1, 10, 15 and 22 hours. At each time point, aliquots of the whole cell extract were analyzed by western blotting with the anti-Flag antibody. Synchronization was verified following the expression of cyclin A and E. The level of α-tubulin in each sample is also shown.

Based on these results we investigated the distribution of Orc1p*, tagged either at the amino- or at the carboxyl-terminus with Flag or GFP, in human HeLa, monkey COS7 and mouse NIH-3T3 cells.

In all cell lines, the protein showed diffuse nuclear staining with a few sites of preferential accumulation that overlapped pericentric heterochromatin intensely decorated with DAPI (Fig. 3 A). This pattern was observed 24 hours and 48 hours after transfection (not shown). The association with heterochromatin was confirmed by the colocalization with heterochromatin protein 1 (HP1) (Jones et al., 2000; Maison et al., 2002) (Fig. 3 B). Thus, the association with heterochromatin of Orc1p* occurs in cell lines of different mammalian species and is not influenced by the position and type of the tag (Flag compared to GFP).We next investigated the distribution of Orc1p* during the cell cycle. HeLa cells expressing Orc1-GFP were stained with antibodies against the replicative factor PCNA (proliferating cell nuclear antigen) that associates with replication factories in S phase but is almost undetectable in G1 nuclei. Most Orc1-GFP positive cells were in G1 phase as indicated by the lack of PCNA staining (Fig. 4 A) and by FACS analysis (Fig. 4 B). This result is consistent with the degradation of the protein during S phase (Fig. 1C) (Mendez et al., 2002; Tatsumi et al., 2003). To investigate this aspect in more detail, we cloned Orc1-Flag into pIRES-hrGFP-2a, a vector that directs the production of a dicistronic transcript in which the gene of interest and the GFP sequence are separated by an internal ribosomal entry site (IRES) (Fig. 5 A).Exponentially growing HeLa cells were transiently transfected with pOrc1-Flag/GFP and analyzed by confocal laser microscopy to reveal the distribution of Orc1-Flag, the reporter GFP protein and the endogenous PCNA. In this assay GFP visualized transfected cells, and in fact cells expressing Orc1-Flag were always GFP-positive. In contrast, as shown in Fig. 5 B, a fraction of GFP-positive cells was not stained by the anti-Flag antibody. As indicated by the PCNA subnuclear distribution, these cells were in mid/late S and G2 phases. A similar pattern was observed in mouse NIH-3T3 cells (Fig. 5). About 14% of Flag-positive cells displayed PCNA in the typical early S-phase pattern (Fig. 6 C). In these cells Orc1p* and PCNA occupied

mutually exclusive nuclear areas (Fig. 4 C). Collectively these results are consistent with a model whereby origin firing is accompanied by the displacement of Orc1p from chromatin and its successive degradation. As Orc1p* displays a similar distribution in human and mouse cells (Fig. 3 A, Fig. 6 B and Fig. 5) we decided to investigate its subnuclear localization during G1 phase in NIH-3T3 cells that are more suitable for visualization of heterochromatic foci. Transfected cells were synchronized in mitosis with nocodazole and then released for increasing time intervals in G1.

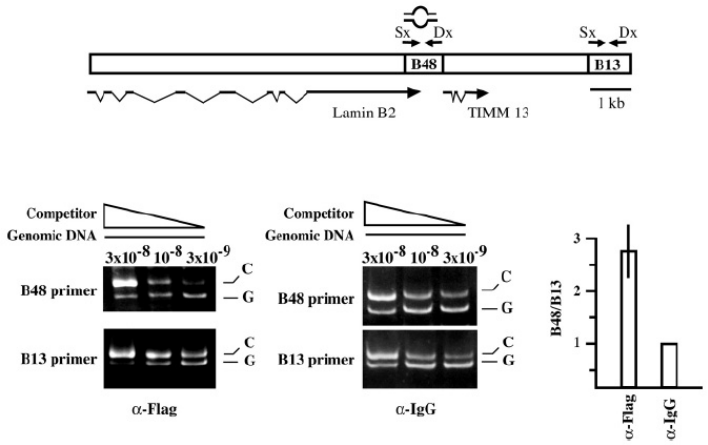

Figure 2. Chromatin immunoprecipitation (ChIP) analysis of HeLa cells transiently transfected with Orc1-Flag. (A) Schematic representation of the genomic region comprising the origin of DNA replication associated to the lamin B2 and TIMM 13 genes. The origin sequence is recognized by B48 primers. The control B13 region is also indicated. (B) Crosslinked chromatin was immunopurified with anti-Flag (a-Flag) or with a control non-specific antibody (a-IgG). After reversion of crosslinking, immunopurified DNA was subjected to competitive PCR with B48 and B13 primer sets. Dilutions of the competitor DNA (see Materials and Methods) are indicated above each lane. The identity of the amplification bands is indicated on the right of each panel. The relative abundance of B48 and B13 sequences in the immunopurified DNA is determined by the ratio of genomic (G) to competitor (C) bands. (C) Histogram showing the enrichment of B48 over B13 sequences in the immunopurified DNA obtained with a-Flag and a-IgG antibodies. Bar indicates the variation seen in three independent experiments.

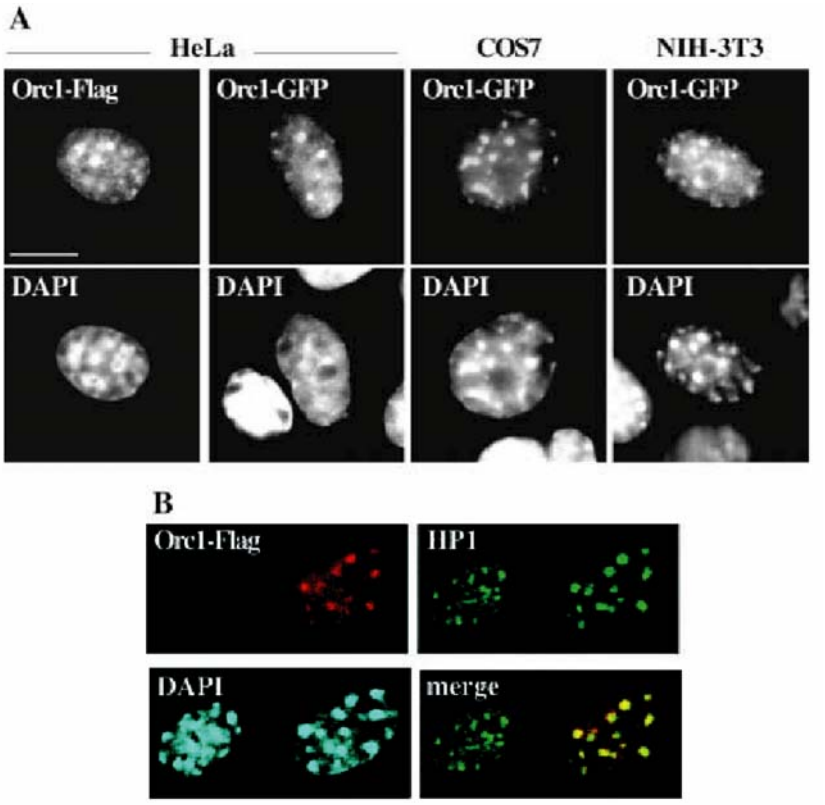

Figure 3. Distribution of Orc1p* in different mammalian cell lines. (A) Exponentially growing human HeLa cells were transfected with either Orc1-Flag or Orc1-GFP. After 48 hours, cells were fixed and processed for conventional microscopy. The distribution of Orc1-Flag was revealed with the rabbit anti-Flag antibody and a TRITC-conjugated anti-rabbit secondary antibody. The localization of Orc1-GFP was revealed by GFP fluorescence. Cells were counterstained with DAPI. The distribution of Orc1-GFP was also determined in transfected monkey COS7 and mouse NIH-3T3 cells. (B) Asynchronous NIH-3T3 cells were transfected with Orc1-Flag and after 48 hours were fixed and stained with the rabbit anti-Flag and the rat anti-HP1β antibodies. Antigen-antibody complexes were revealed with a TRITC-conjugated anti-rabbit secondary antibody and with a Cy5- conjugated anti-rat secondary antibody (visualized in green). Nuclei were stained with DAPI. Confocal images of the same field were taken. Bar, 10 mm.

As expected from the degradation of Orc1p* during S phase (Fig. 1, Fig. 5 and Fig 6), most of the cells transfected with pOrc1-Flag/GFP (more than 95%) were not stained by anti-Flag antibodies when blocked at the mitotic spindle

checkpoint with nocodazole (not shown). As shown in Fig. 7, in early G1 (2 hours after release from nocodazole block) Orc1-Flag was not associated with heterochromatic foci stained by DAPI and by the anti-HP1 antibody. Instead, colocalization with heterochromatin was detectable in 35% of mid G1 (6 hours) and in 65% of late G1 (9 hours, not shown).

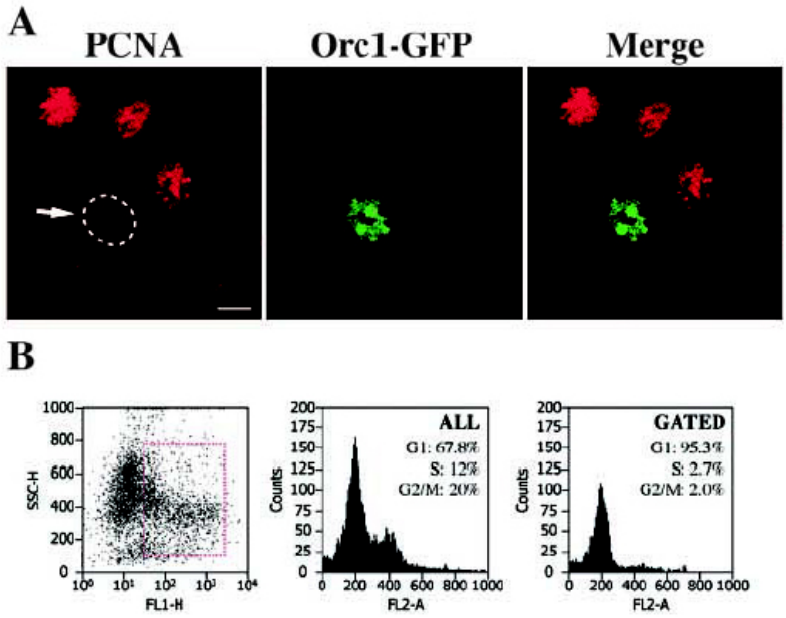

Figure 4. Orc1p* labels G1 cells. (A) HeLa cells expressing Orc1-GFP were stained with the anti-PCNA PC10 monoclonal antibody and with the anti-mouse Cy5-conjugated secondary antibody (red). The fluorescent signal of the Orc1-GFP fusion (green) is also shown. Confocal laser images of the same field were taken and merged. The arrow indicates a PCNA-negative nucleus expressing Orc1-GFP. Bar, 10 mm. (B) FACS analysis. The FACS profile in the left side panel shows the fluorescence of the total cell population 48 hours after transfection. The gate (dotted line) shows the sub-population of transfected cells expressing Orc1-GFP (29% of total). The other two panels show the DNA content of the total cell population (ALL) and of cells expressing Orc1-GFP (GATED). The distribution of the cells in the cell cycle according to the DNA content is indicated on the right side of each panel.

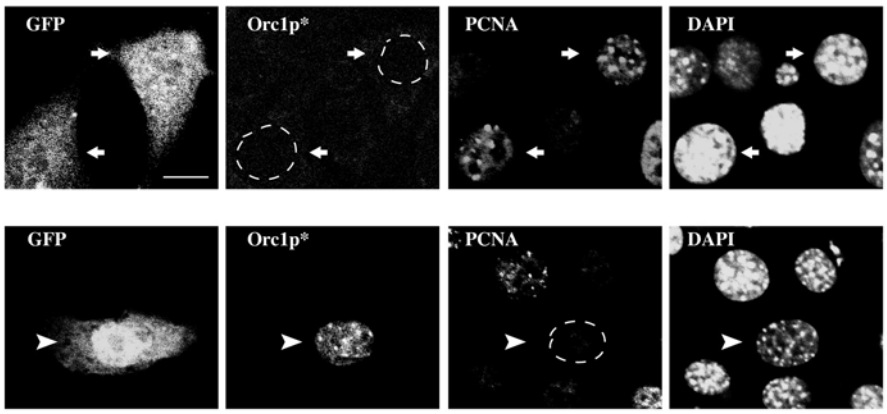

Figure 5. Immunolocalization of Orc1p* in NIH-3T3 cells. Exponentially growing NIH-3T3 cells were transfected with pOrc1-Flag/GFP plasmid described in Fig. 4. After 48 hours, cells were fixed and stained with anti-Flag polyclonal and with anti-PCNA monoclonal antibodies. Antibodies were revealed with the TRITC-conjugated anti-rabbit and with the Cy5-conjugated anti-mouse secondary antibodies. Nuclei were stained with DAPI. Transfected cells were revealed by GFP fluorescence. Confocal laser images were taken. (upper panels) GFP-positive, PCNA-positive S-phase nuclei in which Orc1-Flag is not detectable (arrows). (lower panels) GFP-positive, PCNA negative G1 nucleus that expresses Orc1-Flag (arrowhead). To preserve the GFP signal, we skipped Triton extraction before fixation. Under these conditions PCNA staining was detectable not only in S-phase replicative patterns but also in G2-phase cells characterized by intense homogenous staining.

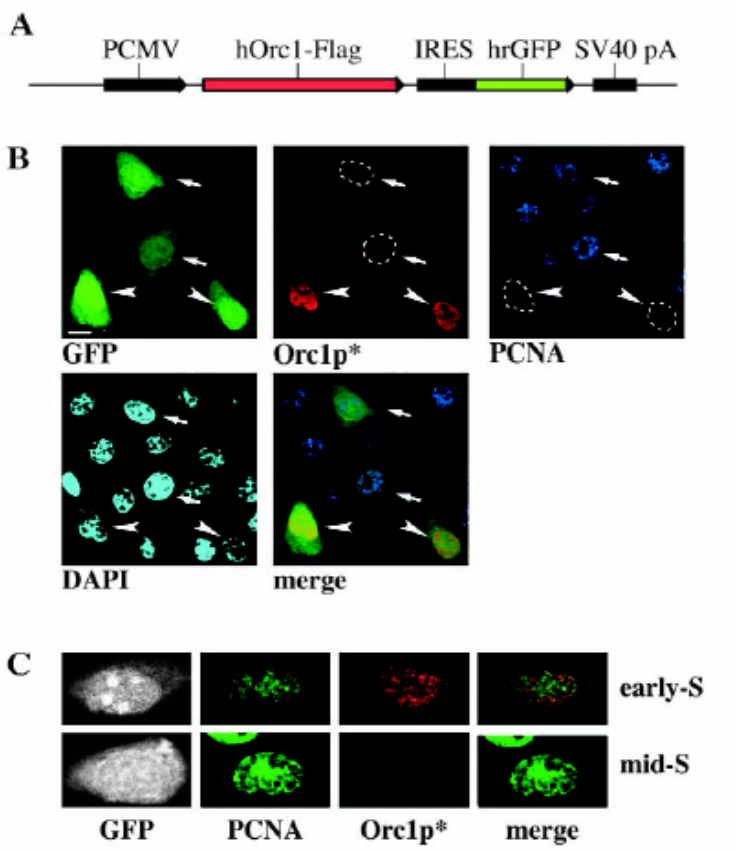

Figure 6. Orc1p* in S-phase nuclei. HeLa cells were transfected with a plasmid that directs the expression of a dicistronic mRNA in which the sequence of Orc1-Flag is upstream of the GFP. (A) Schematic diagram of the pOrc1-Flag/GFP plasmid. PCMV, cytomegalovirus promoter; IRES, internal ribosomal entry site; SV40 pA, SV40-derived RNA processing site. (B) Cells were co-stained with the anti-Flag polyclonal and anti-PCNA monoclonal antibodies. Antibodies were revealed with the TRITC-conjugated anti-rabbit and Cy5-conjugated anti-mouse secondary antibodies. Nuclei were stained with DAPI. Confocal laser images were taken. The overlay of GFP (visualized in green), Orc1-Flag (red) and PCNA (blue) is shown (merge). The arrows indicate PCNA-positive GFP-positive nuclei in which Orc1- Flag is not detectable. Arrowheads show transfected nuclei expressing Orc1-Flag that are PCNA-negative. To preserve the GFP signal, we skipped Triton extraction before fixation. Under these conditions PCNA staining was detectable not only in Sphase replicative patterns but also in G2-phase cells characterized by intense homogenous staining. Bar, 10 mm. (C) Shows a PCNA-positive, Orc1p*-positive nucleus in early S phase (early-S) and a PCNA-positive, Orc1p*-negative nucleus in mid S phase (mid-S). The merged images show that in early S phase, Orc1p* does not colocalize with PCNA.

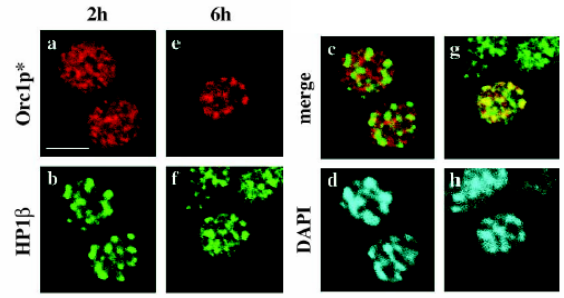

Figure 7. Orc1p* in G1-phase nuclei. (a-h) Transiently transfected NIH-3T3 cells were blocked at the mitotic spindle checkpoint with nocodazole and allowed to recover for 2 hours (a-d) and 6 hours (e-h). Fixed cells were incubated with the rabbit anti-Flag polyclonal antibody (a,e), and with the anti-HP1β antibody (b,f). Antibodies were revealed with the anti-rabbit TRITC-conjugated (red) and with the anti-rat Cy5-conjugated (green) antibodies. Confocal laser images are shown. The overlay of Orc1p* (red) and HP1β (green) is also shown (merge). Chromatin was stained with DAPI (d,h). Bar, 10 mm.

Protein determinants involved in Orc1p* focalization

Based on the results in the previous section we sought to identify the determinants responsible for the association of Orc1p* with heterochromatin. In all species, Orc1p has a modular structure with a BAH and an ATPase domain located at the N- and C-terminal regions of the protein, respectively. We expressed different portions of Orc1p, tagged with either GFP or Flag, in NIH-3T3 cells. This analysis showed that the region from position 151 to 269, partially overlapping the BAH domain, was necessary for Orc1p focalization (see Δ151-269 in Fig. 8 A, B). The same result was obtained in HeLa cells (not shown). However, a mutant lacking the first 150 amino acids [(151-861)-GFP] was still recruited to heterochromatin ruling out the involvement of the BAH domain (Fig. 8 A). We produced further internal deletion mutants to map more precisely the determinant directing the association with heterochromatin. Surprisingly, two reciprocal mutants (Δ151-231 and Δ232-269) both colocalized with DAPI foci suggesting the existence of functionally redundant sequences

involved in Orc1p* focalization. Although necessary, the 151-269 region was unable to direct the reporter GFP to heterochromatin [see (151-269)-GFP and (1-269)-GFP in Fig. 8 A], indicating the involvement of additional motifs. As stated above, the C-terminal part Orc1p contains an ATPase domain comprising Walker A and Walker B motifs.

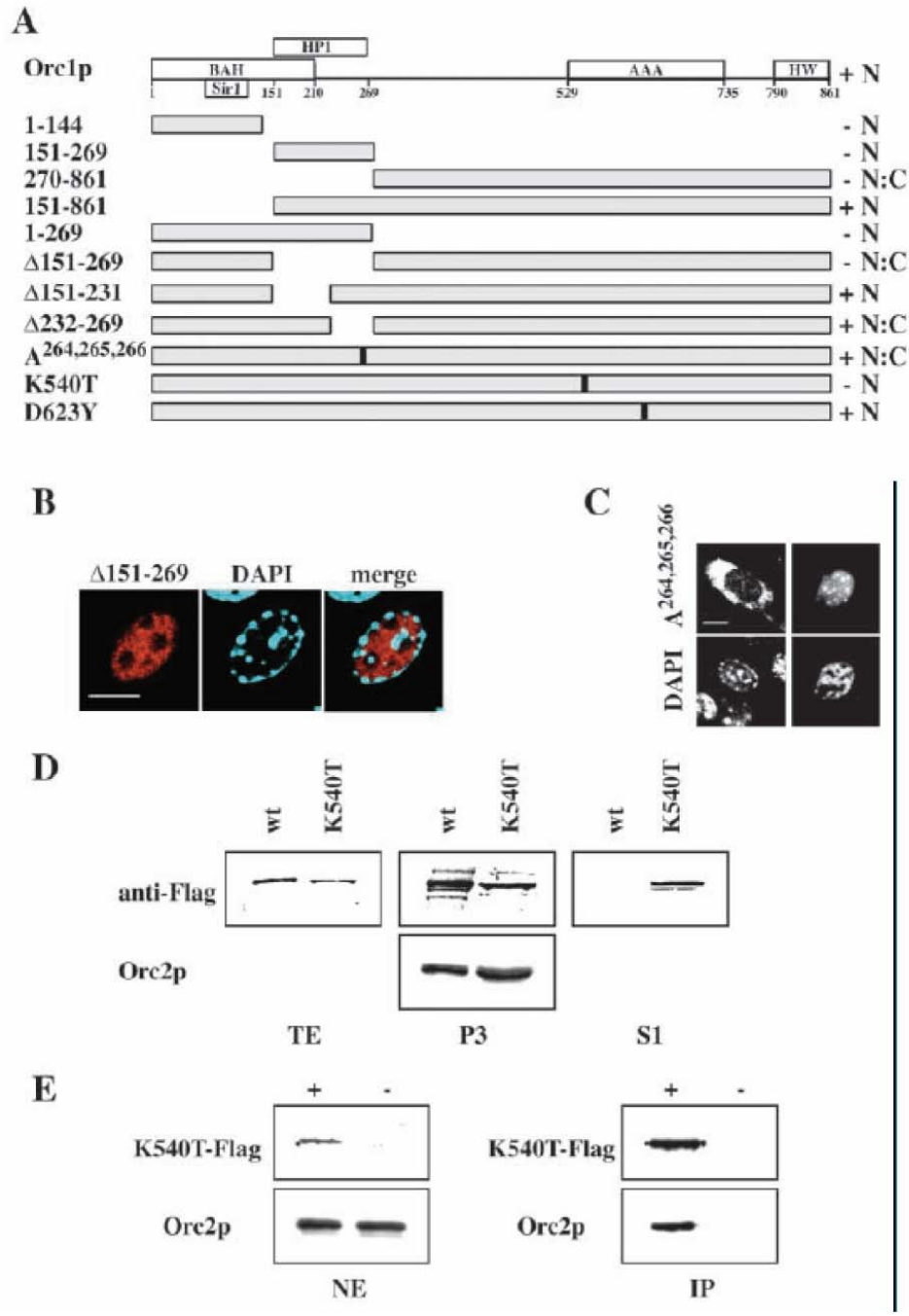

Figure 8. *(For figure legend see next page).*

Figure 8. Analysis of Orc1p mutants. (A) All mutants were fused to either GFP or Flag epitope and their subcellular distribution was assessed in transiently transfected NIH-3T3 cells. The region of human Orc1p included in each fusion is indicated by the corresponding amino acids on the left hand side. Internal deletion mutants are indicated with a Δ symbol. Substitution mutants are indicated by the corresponding single-code amino acid letter. Black bars indicate substituted residues. The ability of each mutant to accumulate at DAPI foci was scored as + (targeting proficient) or – (targeting deficient). The localization in cell nucleus (N) or in the cytoplasm (C) is indicated. A diagram including the main functional domains of the protein is shown at the top. BAH, bromo-adjacent-homology domain; HP1, HP1-binding site (Pak et al., 1997); Sir1, Sir1-binding site (Zhang et al., 2002c); AAA, ATPase domain; HW, putative DNA binding site (Liu et al., 2000). (B) Immunolocalization of the Δ151-269 mutant fused to the Flag epitope in NIH-3T3 cells. Chromatin was stained with DAPI. Confocal laser images of the same field were taken and merged. (C) Immunolocalization of the A264,265,266 mutant fused to the Flag epitope in NIH-3T3 cells. Images show cells with cytoplasmic (left) or nuclear (right) distribution of the overexpressed protein. Chromatin was stained with DAPI. Confocal laser images were taken. Bar, 10 mm. (D) Chromatin-binding of Orc1p* (wt) and Orc1-K540T-Flag (K540T) mutant was analyzed by western blotting with the anti-Flag antibody. The amount of protein in the chromatin (P3) and soluble fractions (S1) was compared for each construct. The amount of P3 fraction analyzed for each sample was corrected for the efficiency of transfection determined by measuring in parallel the fraction of Orc1p* expressing cells. The level of endogenous Orc2p in the loaded P3 fractions is also shown. TE, total cell extracts. (E) Nuclear extracts (NE) of HeLa cells expressing K540T-Flag epitope (+) and of non-transfected cells (–) were probed with the anti-Flag polyclonal antibody and the anti- Orc2 polyclonal antibody. The same extracts were used in co-immunoprecipitation experiments with the monoclonal anti-Flag M2 affinity gel (IP). The presence of Orc1-Flag and of Orc2p in the immunoprecipitate was revealed by western blotting with anti-Flag and anti-Orc2 polyclonal antibodies.

In *S. cerevisiae,* substitution of lysine 485 in the Walker A motif with threonine abrogates ATP binding, whereas replacement of aspartic acid 569 in the Walker B motif with tyrosine reduces the rate of ATP hydrolysis (Klemm and Bell, 2001). Taking advantage of the fact that both residues are evolutionary conserved (NCBI, Conserved Domain Database), we produced the human homologues of the Walker A (K540T) and Walker B (D623Y) mutants. As indicated in Fig. 8 A, mutation of K540 affected the subnuclear distribution of Orc1p* suggesting the importance of ATP-binding. No effect was observed in the case of the D623Y mutant. The relevance of the Walker A domain was also verified in HeLa cells by both immunofluorescence (not shown) and biochemical cell fractionation. As

shown in Fig. 8 D, although the wild-type Orc1p* was exclusively found in the chromatin (P3) fraction, half of the mutated protein was detectable in the soluble (S1) material indicating that the K540T substitution reduced the interaction of Orc1p* with chromatin to approximately 50%. No effect was detectable by coimmunoprecipitation in the nuclear cell extract on the ability of Orc1p* to interact with Orc2p (Fig. 8 E).

During the characterization of these mutants we realized that, as indicated in Fig. 8 A, removal of residues 151-269 affected the nuclear accumulation of the protein. The analysis of this sequence with the PSORT II program identified a putative nuclear localization signal (NLS) starting at residue 259 (PGRIKRKV). Replacement of amino acids RKV with three alanine residues (mutant ^{A264,265,266}) was sufficient to hamper nuclear accumulation and the mutated protein was cytoplasmic (Fig. 8 C) or distributed to the whole cell body (not shown) in more than 60% of the transfected cells. The failure to completely abrogate nuclear localization (Fig. 8 C, right side) however, suggests that additional motifs can mediate nuclear import of ^{A264,265,266}. Although it showed an altered distribution, this mutant as well as Δ151-269, was still degraded in S phase similar to the wild-type protein (not shown). This indicates that degradation of Orc1p during the cell cycle does not require nuclear accumulation.

In vitro interaction between human Orc1p and HP1

The results in the previous section identified two regions involved in the association of Orc1p with heterochromatin, one of which is located at the N-terminus of the protein. In both *S. cerevisiae* and in *Drosophila* the N-terminal region of Orc1p is involved in the interaction with Sir1/ HP1 proteins (Pak et al., 1997; Zhang et al., 2002c). We thus asked whether the 151-269 region of human Orc1p could play the same role. *In vitro* translated [^{35}S]-labeled human Orc1p and Orc2p were challenged for their ability to bind a GST-HP1α fusion. As shown in Fig. 9 A, Orc1p (upper panel, left side) but not Orc2p (upper panel, right side) was specifically retained on agarose beads loaded with GST-HP1α but

not on beads loaded with GST alone (see quantification of the radioactive signals in Fig. 9 B). Next, we performed a similar GST pull-down experiment by using a series of *in vitro* translated proteins corresponding to different portions of human Orc1p.

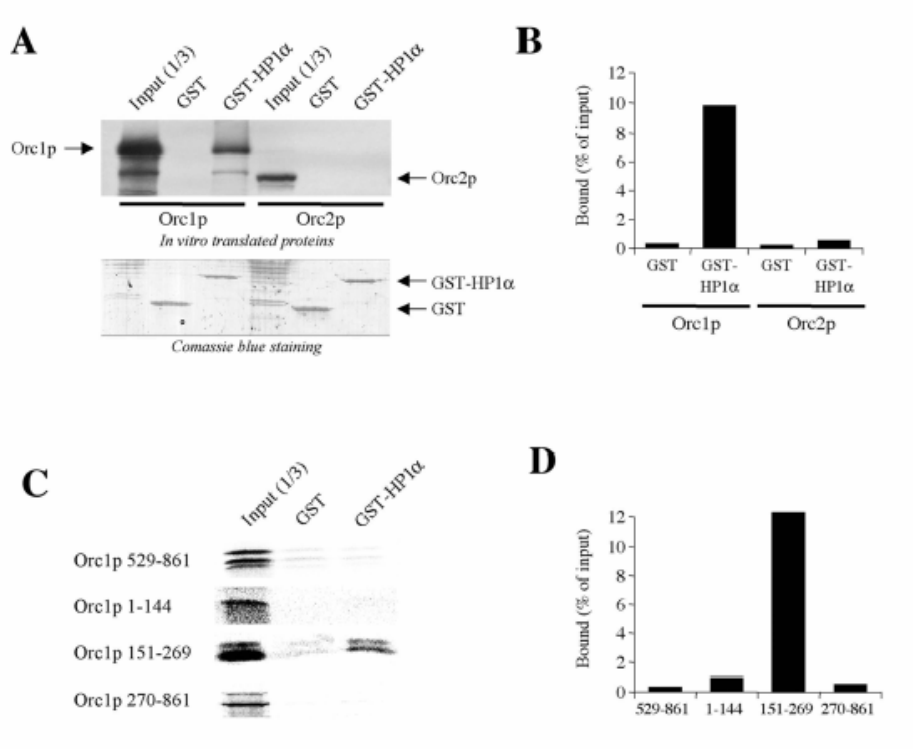

Figure 9. Orc1p but not Orc2p binds to HP1α *in vitro*. (A) GST pull-down experiment performed by incubating 2 mg of either GST or GST HP1α immobilized on glutathione-agarose beads with in vitro translated [35S]-labeled Orc1p or Orc2p. After binding at 4°C, the beads were extensively washed and bound proteins were loaded onto a 10% acrylamide-SDS gel. The upper panel shows the autoradiograph; the lower panel shows the gel after staining with Coomassie blue. The input lanes contain the labeled proteins prior to binding. (B) Quantification of the GST pulldown experiment from A. The amount of radioactivity bound to the beads is indicated as a percentage of the input material. (C) HP1α binds to a region of Orc1p encompassing amino acids 151-269. The indicated [35S]-labeled deletion mutants of Orc1p were incubated with either GST or GSTHP1α and processed as described in A. (D) Quantification of the GST pulldown experiment of panel C.

As shown in Fig. 9 C and quantified in Fig. 9 D, binding to HP1α required amino acids 151-269 of Orc1p. This conclusion is consistent with the *in vitro* analysis of the interaction between *Drosophila* Orc1p and HP1 proteins (Pak et al., 1997) and raises the possibility that HP1 might have a role in Orc1p focalization.

Visualization of direct Orc1p-HP1α interaction in human cells by Fluorescence Energy Transfer (FRET)

A powerful technique to study the formation of specific protein complexes inside the cell is based on fluorescence resonance energy transfer (FRET) between two interacting proteins tagged with optically matched pairs of fluorophores; the presence of FRET indicates protein-protein interaction at distances in the order of a nanometer (Marcello et al., 2001).

FRET exploits radiationless energy transfer driven by dipole-dipole interaction occurring from a fluorophore (the donor) in the excited state to another fluorophore (the acceptor) when in close proximity; energy transfer is followed by acceptor fluorescence. The presence of FRET indicates actual protein-protein interaction at distances in the range of the FRET length scale, the Förster radius (R_0), defined as the distance at which FRET efficiency (E_T) is 50%. E_T is defined as the ratio between the sixth power of R_0 and the sum of the sixth power of R_0 and the sixth power of R.

R is the actual distance among the donor and the acceptor fluorophores. E_T dramatically decreases when R increases by a fraction of the nanometer (nm) around R_0, which is commonly of the order of the nm for many pairs of matched fluorophores (18–20). In particular, E_T reaches 98 and 1.5% for donor-acceptor separations lower than 0.5 R_0 and higher than 2 R_0, respectively. This implies that simple co-localization of two proteins is not sufficient to yield energy transfer; thus, the presence of FRET is a powerful indicator of physical protein-protein interaction.

FRET experiments were performed by transfection of human HeLa cells with plasmids expressing the pair of proteins under investigation bearing the EGFP and BFP proteins fused to their N-terminus.

We decided to apply this methodology to investigate the *in vivo* interaction between Orc1p and HP1α. FRET experiments were performed by transfection of human HeLa cells with plasmids expressing HP1α and Orc1p fused at their N-terminus with EGFP and BFP respectively. FRET image analysis of individual transfected cells are shown in Fig. 10 A. Upper panels show the intracellular distribution of fluorescence at 520 nm (the peak wavelength of EGFP emission) under excitation at 480 nm; lower panels show the fluorescence of the same fields after excitation of BFP at 350 nm. Under these conditions, only cells expressing EGFP-HP1α together with BFP-Orc1, but not with BFP alone or BFP fused to Orc2, could be visualized. Detailed quantitative analysis of several cells transfected with the different protein pairs are presented in Fig. 10 B, which shows the experimental FRET signal and its distribution. All the cells transfected with EGFP-HP1α and BFP-Orc1 showed FRET values higher than those detected in the control transfections (P <0.001), indicating direct interaction between the two proteins. In the same set of experiments, FRET was also found positive between EGFP-Orc1 and BFP-Orc2, further supporting the direct binding of the two proteins *in vivo*.

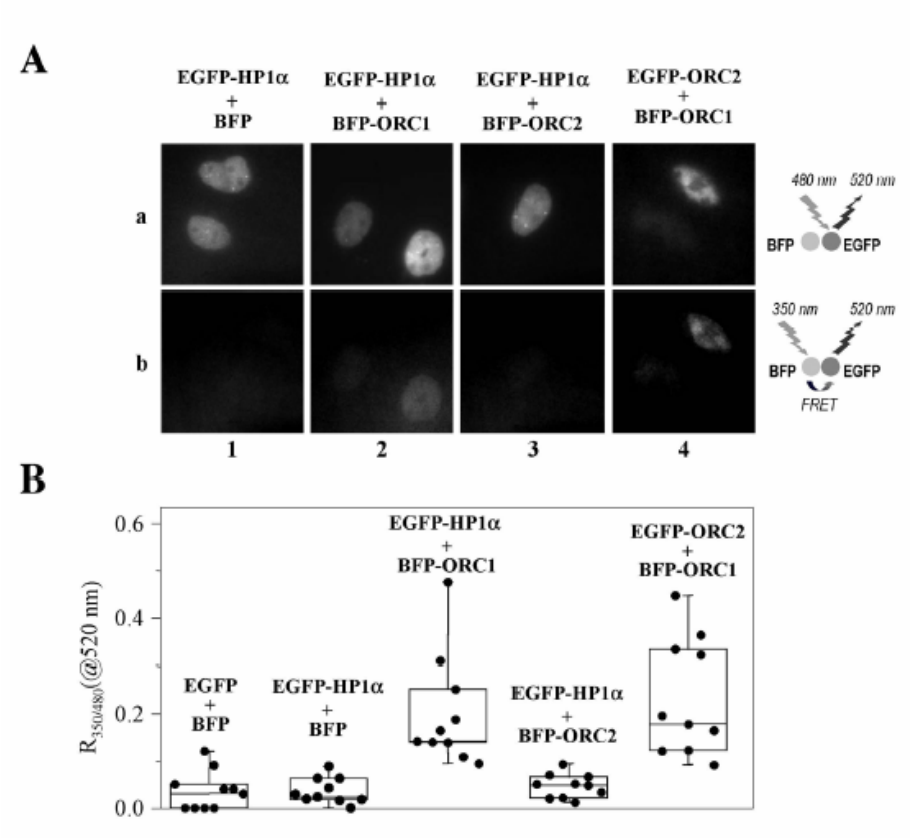

Figure 10. Orc1p but not Orc2p binds to HP1α inside the cells. (A) Visualization of FRET in human HeLa cells. The plasmids indicated on top of each column were transfected in asynchronous HeLa cells; individual transfected cells were visualized by excitation at 480 nm and collection at 520 nm, showing EGFP fluorescence after direct EGFP excitation (panels in row a), and by excitation at 350 nm and collection at 520 nm, showing EGFP fluorescence after BFP excitation, indicating FRET (panels in row b). (B) Quantification of FRET between EGFP-HP1α and BFP-Orc1. Fluorescent emission at 520 nm from individual cells transfected with the indicated constructs was recorded after excitation at 350 or 480 nm, and integrated intensities over the whole cell were evaluated. The plotted values (indicated by dots) represent the ratio between these two measurements: higher values indicate more efficient resonant energy transfer between BFP and EGFP. Ten consecutively analyzed cells were considered for each transfection; both their individual fluorescence ratios and their percentile box plot distributions are shown. In each box, the horizontal lines from top to bottom mark the 10th, 25th, 75th and 90th percentiles. Cells transfected with pEGFP-HP1α and pBFP-Orc1 plasmids showed FRET between the two fluorescent proteins that was dependent on the presence of both the HP1α and Orc1p moieties, thus indicating binding between these two proteins *in vivo*.

Orc1p focalization survives TSA and RNase A treatments

The results in the previous sections indicate that the association of Orc1p with heterochromatin requires a protein region that mediates the interaction with HP1. We asked whether HP1 was necessary for the stable association of Orc1p with heterochromatin.

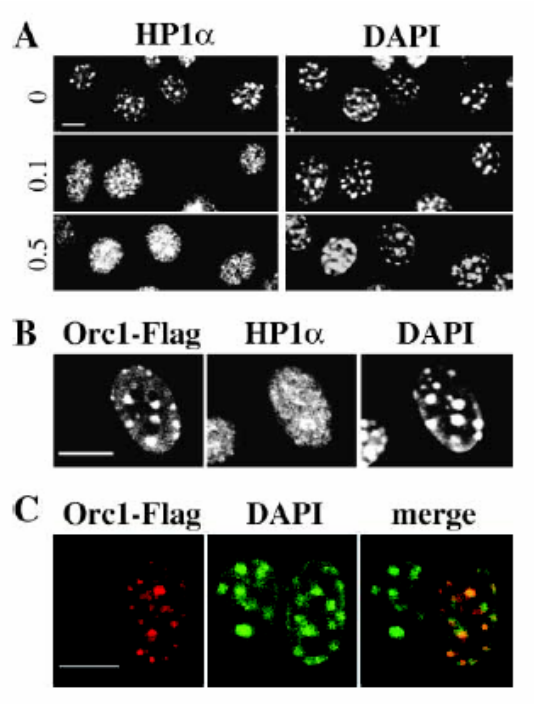

Figure 11. Orc1p* focalization survives TSA and RNase treatment. (A) NIH-3T3 cells were treated with 0, 0.1 and 0.5 mg/ml TSA for 6 hours. Fixed cells were stained with the anti-HP1a antibody. Heterochromatin was stained with DAPI. Dispersal of HP1 from DAPI foci is already detectable at 0.1 mg/ml TSA. (B) Cells expressing Orc1-Flag were incubated in the presence of 0.5 mg/ml TSA as in panel A. Fixed cells were co-stained with the anti-Flag and anti-HP1α antibodies. Heterochromatin was stained with DAPI. In contrast to HP1, Orc1-Flag still colocalizes with DAPI foci. (C) Cells expressing Orc1-Flag were incubated with RNase A as described in Materials and Methods. Orc1-Flag was visualized with the anti-Flag antibody; heterochromatin was stained with DAPI visualized in green for a better resolution of the merged image. Confocal laser images are shown. Bar, 10 mm.

It has recently been described that treatment of mouse cells with trichostatin A (TSA), an inhibitor of histone-deacetylase, induces redistribution of HP1 owing to a loss of HP1- binding sites in acetylated pericentric regions (Maison et al., 2002). We analyzed the localization of Orc1-Flag and of the endogenous HP1q in transiently transfected NIH-3T3 cells after TSA treatment. As shown in Fig. 11, TSA treatment (6 hours of growth in 0.5 µg/ml) triggered the redistribution of HP1 (Fig. 11 A) without affecting the association of Orc1p* with DAPI bright spots (Fig. 11 B). This was also confirmed by biochemical cell fractionation (not shown). An RNA component has recently been described as a crucial parameter involved in the maintenance of higher-order chromatin structure at pericentric heterochromatin (Maison et al., 2002; Muchardt et al., 2002). Cell treatment with RNase A induced the displacement of HP1 from heterochromatic regions whereas DAPI staining corresponding to pericentric regions was essentially preserved (not shown). The same treatment, however, had no effect on the distribution of Orc1-Flag and the protein was still accumulated at pericentric heterochromatin (Fig. 11 C). Thus, the association of Orc1p with DAPI bright spots survives treatments that displace HP1. Collectively these results suggest a model whereby HP1 is involved in the recruitment of Orc1p* to heterochromatin. Thereafter, binding of Orc1p* to AT-rich sequences in DAPI stained DNA would make the association with heterochromatin HP1-independent.

3 Discussion

Orc1p seems to have a regulatory role in the assembly of the ORC in human cells. However, the characterization of its function is still limited. This is partially due to the lack of suitable antibodies, which has so far hampered the analysis of the subnuclear distribution. Moreover, a few contradictory reports have been published in the last few years about the protein stability in mammals. According to some authors, the protein is permanently bound to the origins and its level remains constant throughout the cell cycle (Okuno et al., 2001).

On the other hand, others suggest that Orc1p is displaced from chromatin after origin firing, but it is still controversial whether the displaced protein is eventually degraded by the proteasome (Araki et al., 2003; Kreitz et al., 2001; Mendez et al., 2002; Sun et al., 2002; Tatsumi et al., 2003). Here we have investigated the behavior of epitope-tagged human Orc1p overexpressed in mammalian cells (Orc1p*). Orc1p* shows important features of the endogenous protein: it interacts with Orc2p, another subunit of ORC, and binds to a well characterized origin of DNA replication.

By applying an approach that does not entail cell synchronization we have shown that the level of Orc1p* is regulated during the cell cycle. This is in line with that previously reported for the endogenous protein (Mendez et al., 2002) and rules out the possibility that Orc1p degradation takes place during the preparation of cell extracts. Moreover, our analysis indicates that the level of Orc1p* is regulated both in human and mouse cells. This raises the possibility that the different regulation of Orc1p observed in different mammalian species could be caused by the divergence of the protein sequence (65.8% identity between the human and mouse protein, EMBL-EBI ClustalW program). Finally, our results suggest that degradation of Orc1p during S phase follows a precise program. The level of Orc1p* decreases during the S phase and the protein is no longer detectable in mid- and late-S-phase nuclei (Fig. 6). Moreover, in early S phase, Orc1p* does not colocalize with replication factories stained by PCNA. This is in agreement with a model whereby origin firing is accompanied by the displacement and degradation of Orc1p to prevent re-replication during S phase.

Orc1p and heterochromatin

Another interesting observation concerns the association of Orc1p* with heterochromatin in mid and late G1. The association of ORC with transcriptionally silenced, late replicating portions of the genome has been observed in other organisms, where ORC seems to play an active role in the assembly of these chromatin conformations. In the yeast *S. cerevisiae*, ORC

binding to an ARS element is required for the recruitment of Sir factors and hence for the transcriptional silencing of the HML locus (Vujcic et al., 1999). In *Drosophila,* mutations of Orc2p were shown to perturb HP1 localization (Huang et al., 1998; Pak et al., 1997). Finally, it has been reported that human Orc2p binds *in vivo* to α-satellite sequences that compose pericentric heterochromatin (Keller et al., 2002). We have added a few molecular details to the characterization of the association of Orc1p with heterochromatin and we have identified two protein domains that are involved in this phenomenon. The first one (aminoacids 151-269) mediates the *in vitro* interaction with HP1, a component of heterochromatin. This finding extends to the human protein the ability to interact with heterochromatic proteins previously demonstrated in budding yeast and *Drosophila* (Pak et al., 1997; Zhang et al., 2002c). In these systems, ORC acts as the recruiter whereas the opposite apparently occurs in humans. Indeed, the association of Orc1p* with heterochromatin is temporally delayed with respect to HP1 (Fig. 8) and requires the HP1 binding domain.

Surprisingly, the displacement of HP1 from heterochromatin induced by deacetylase inhibitors or by RNase A (Maison et al., 2002; Muchardt et al., 2002) does not affect Orc1p localization (Fig. 12). We hypothesize that HP1 plays a role in the recruitment, but not in the stable association of Orc1p with heterochromatin, which might be mediated by interactions with other components of these nuclear districts, one possible candidate being the AT-rich repeated DNA elements. This is also suggested by the identification of the Walker A motif as the second domain involved in the subnuclear localization of Orc1p. Indeed, a mutation in this motif that abrogates ATP binding reduces both the affinity of ORC for DNA (Chesnokov et al., 2001) and the association of Orc1p with heterochromatin. The amount of endogenous Orc1p is tightly controlled and the number of Orc1p molecules approximately corresponds to the estimated number of replication origins (Kreitz et al., 2001). Thus, Orc1p* focalization could reflect the behavior of excess protein that does not participate to replication competent ORCs. However, the nature of the two domains involved in the association with heterochromatin suggests that this distribution

could be functionally relevant. Human ORC displays limited, if any, sequence specificity of binding to DNA. This implies that the selection of specific DNA replication origins must rely on other factors such as chromatin proteins or transcription factors that might help the association of ORC with DNA (Vashee et al., 2003). In this perspective, HP1 could have a role in targeting ORC to heterochromatin thus improving the replication of highly compacted DNA regions. On the other hand, heterochromatin could represent a sort of buffer to sequester the surplus of Orc1p* until degradation in S phase. This could be part of a mechanism that finely tunes the selection of DNA replication origins operating during the cell cycle, and which becomes more evident under our experimental conditions. The meaning of the association of Orc1p with heterochromatin is still obscure. Heterochromatin has evolved as a nuclear domain to silence expressed genes by sequestering them in compartments not accessible to transcription factors. It is possible that ORC could play a major role in coordinating DNA replication with the chromatin organization and the expression pattern of the genome. This possibility is consistent with the capacity of Orc1p to interact with proteins such as Noc3 and histone acetyl-transferase HBO1, enabling the modulation of chromatin structure (Iizuka and Stillman, 1999; Zhang et al., 2002b). Growing experimental evidence indicates that initiation of DNA replication in higher eukaryotes does not simply depend on sequence elements but also depends on important parameters such as the packaging status and the nuclear position of chromatin. ORC is a good candidate to link all these different aspects acting as a chromatin remodeling factor.

4 ADDENDUM: Fluorescence resonance energy transfer (FRET) microscopy imaging of live cell protein localizations

Fluorescence resonance energy transfer (FRET) is a distance-dependent physical process by which energy is transferred nonradiatively from an excited molecular fluorophore (the donor) to another fluorophore (the acceptor) by means of intermolecular long-rage dipole-dipole coupling (Sekar and Periasamy, 2003). FRET can be an accurate measurement of molecular proximity at angstrom distances (10-100 Å) and highly efficient if the donor and acceptor are positioned within the Förster radius (the distance at which half the excitation energy of the donor is transferred to the acceptor, typically 3-6 nm). The efficiency of FRET is dependent on the inverse sixth power of intermolecular separation (Förster T., 1965. Delocalised excitation and excitation transfer. In Modern Quantum Chemistry. Vol.3, O. Sinanoglu, editor. Academic Press Inc., New York, 93-137; Clegg R. M., 1996. *Fluorescence energy transfer. In Fluorescence Imaging Spectroscopy and Microscopy.* Vol. 137. X.F. Wang and B. Herman, editors. John Wiley & sons Inc., New York, 179-251; Lakowicz J.R.,, 1999. *Principles of Fluorescence Spectroscopy*. 2nd ed. Plenum Publishing Corp., New York, 692 pagg), making it a sensitive technique for investigating a variety of biological phenomena that produce changes in molecular proximity (dos Remedios et al., 1987). Technological advances in light microscopy imaging, combined with the availability of genetically encoded fluorescent proteins provide the tools necessary to obtain spatial and temporal distribution of protein associations inside living cells (Day, 1998; Elangovan et al., 2002; Heim and Tsien, 1996). The widely used donor and acceptor fluorophores for FRET studies come from a class of autofluorescent proteins, called GFPs. The spectroscopic properties that are carefully considered in selecting GFPs as workable FRET pairs include sufficient separation in excitation spectra for selective stimulation of the donor GFP, an overlap (>30%) between the emission spectrum of the donor and

the excitation spectrum of the acceptor to obtain efficient energy transfer, and reasonable separation in emission spectra between donor and acceptor GFPs to allow independent measurement of the fluorescence of each fluorophore (Pollok and Heim, 1999). GFP-based FRET imaging methods have been instrumental in determining the compartmentalization and functional organization of living cells and for tracing the movement of proteins inside cells (Hanson and Kohler, 2001).

Whereas light microscopy initiated our understanding of cellular structure and the associated function, molecular biological studies over the past few decades have shown that cellular events, such as signal transduction and gene transcription, require the assembly of proteins into specific macromolecular complexes. Traditional biophysical or biochemical methods did not provide direct access to the interactions of these protein partners in their natural environment. Intensity-based imaging techniques applying the method of FRET microscopy (wide field, confocal, and multiphoton [MP]) were subsequently developed, facilitating the study of these interactions inside intact living cells (Periasamy A., 2001. *Methods in Cellular Imaging*. Oxford University Press, New York, 434 pagg.). New imaging technologies, coupled with the development of new genetically encoded fluorescent labels and sensors and the increasing capability of computer software for image acquisition and analysis, have enabled more sophisticated studies of protein functions and processes ranging from gene expression to second-messenger cascades and intercellular signaling (van Roessel and Brand, 2002). FRET microscopy relies on the ability to capture fluorescent signals from the interactions of labeled molecules in single living or fixed cells. If FRET occurs, the donor channel signal will be quenched and the acceptor channel signal will be sensitized or increased (Herman B., 1998 *Fluorescence Microscopy*. 2nd ed. Springer-Verlag New York Inc., New York 170 pagg). With FRET microscopic imaging, not only colocalization of the donor and acceptor-labeled probes within ~0.09 μm^2 can be seen, but molecular associations at close distances can be verified. Several FRET microscopy techniques exist, each with its own advantages and disadvantages. They are

used for various biological applications, including studies of organelle structure, conjugated antibodies, cytochemical identification, and oxidative metabolism. Wide-field microscopy is the simplest and most widely used technique. It is used for quantitative comparisons of cellular compartments and time-lapse studies for cell motility, intracellular mechanics, and molecular movement. A confocal FRET image, with improved lateral resolution, yields a wealth of spectral information with several advantages over a wide-field image, including controllable depth of field and the ability to collect serial optical sections from thick specimens. All of these intensity-based FRET techniques require processing software to remove the unwanted bleedthrough components in the FRET image.

4.1 FRET applications in Cell Biology

FRET imaging using GFP spectral mutants provides the ability to localize and monitor ion binding and molecular protein–protein interactions in living cells, but FRET is also used to study the structure, conformation, hybridization, and automated sequencing of nucleic acids (Sekar and Periasamy, 2003). Chromosome FISH, based on hybridization of a nucleic acid fragment to its complement, has become extremely important for gene mapping, identification of mutations, clinical diagnostics, and studies of chromosomal and nuclear architecture. A homogeneous DNA diagnostic assay based on template-directed primer extension detected by FRET, named the template-directed dye-terminator incorporation assay, has been developed for mutation detection and high throughput genome analysis (Chen et al., 1997b).

A more recent approach to the characterization of gene expression involves the use of a "fluorescent timer," a mutant of the dsRed fluorescent protein that shifts color from green to red over time. Green fluorescence indicates recently translated protein, which over the course of hours undergoes an oxygen-dependent autocatalytic reaction to generate a red fluorescence, denoting matured protein. As the timer protein switches fluorescence over time, it can be used as a timer for gene expression. A tissue thus indicates its fluorescent timer production history by its ratio of green to red fluorescence; tissues that have

recently initiated gene expression appear green, those with continuous expression appear yellow to orange, and those that have ceased expression appear entirely red.

FRET also finds significant application in membrane fusion assays and real-time PCR assays. In the lipid-mixing assays based on NBD–rhodamine energy transfer (Struck et al., 1981), membranes labeled with a combination of FRET donor and acceptor lipid probes are mixed with unlabeled membranes. FRET decreases when the average spatial separation of the probes is increased upon fusion of labeled membranes with unlabeled membranes. In real-time PCR, the amount of fluorescence emission at each cycle is monitored as an indicator of amplicon production. Fluorescence monitoring of PCR for detection and quantification has become a standard method with many applications, including expression analysis and pathogen detection.

FRET immuno-assays, comprising a Cy5 NH_2-terminally labeled phosphopeptide, which is recognized by an antiphosphotyrosine primary mouse antibody, followed by a Cy3-labeled secondary antibody, are useful in measuring specific antibody–antigen interactions. FRET occurs when the components are sequentially bound together and excitation at Cy3 wavelengths produces emission at Cy5 wavelengths.

Disruption of the interaction between the phosphopeptide and primary antibody will result in a reduction of the FRET signal observed. FRET is also used in the design and synthesis of FRET-based fluorogenic enzyme substrates, useful in monitoring the enzymatic activity.

4.2 Fluorescence Resonance Energy Transfer from Cyan to Yellow

There is now widespread interest in the ability to detect protein-protein interactions in fixed or living cells by using fluorescence resonance energy transfer (FRET) microscopy.

Although FRET has been used in both spectrophotometry and microscopy for a number of years (Herman, 1989; Tsien et al., 1993), it has yet to become a routine technique in microscopy studies. Part of this relates to the difficulty in labelling cells with donor-acceptor fluorophores (Karpova et al., 2003).

In recent years, this obstacle has been overcome with the introduction of fluorescent proteins, namely BFP and GFP or CFP and YFP, which form reasonable donor-acceptor FRET pairs (Cubitt et al., 1995; Heim and Tsien, 1996; Ormo et al., 1996; Patterson et al., 2000; Periasamy and Day, 1999). Using genetic engineering techniques, these fluorophores are easily fused to proteins of interest. As a consequence, there has been intense interest in detecting protein-protein interactions using FRET, particularly with CFP and YFP fusion proteins, which are more photostable than BFP. Some of the notable successes with CFP/YFP FRET include (Damelin and Silver, 2000; Elangovan et al., 2002; Janetopoulos et al., 2001; Miyawaki et al., 1997; Sorkin et al., 2000; Vanderklish et al., 2000; Xia et al., 2001).

Reports of CFP-YFP FRET detected by microscopy are rather limited in number considering the intense, widespread interest in the approach. This reflects the fact that at present FRET is far from a routine techniques. One reason for this may be the difficulty in achieving FRET in the first place, given the requirements for proximity and orientation of the fluorophores (Clegg R. M., 1996. *Fluorescence energy transfer. In Fluorescence Imaging Spectroscopy and Microscopy.* Vol. 137. X.F. Wang and B. Herman, editors. John Wiley & sons Inc., New York, 179-251). An additional contributing factor, however, is the difficulty of analysing FRET reliably. The most widely used approach requires excitation of the donor and then detection of acceptor emission. This approach suffers from complications caused by bleed-through of CFP fluorescence into the YFP channel as well as inadvertent excitation of YFP during CFP excitation. These effects can be mitigated by different algebraic correction schemes, which vary in their sophistication and complexity (Gordon et al., 1998; Xia and Liu, 2001). Corrections for spectral overlap must be performed first, and therefore FRET cannot be immediately analysed.

An alternative and apparently appealing approach to detect FRET is acceptor photobleaching (Bastiaens and Jovin, 1996; Bastiaens et al., 1996; Kenworthy, 2001; Kenworthy and Edidin, 1999; Wouters et al., 1998). Its principle is that energy transfer is reduced or eliminated when the acceptor is bleached, thereby yielding an increase in donor fluorescence. Such an increase in fluorescence following bleaching is particularly diagnostic of FRET, because in most circumstances fluorescence normally decreases following a bleach. Another advantage of acceptor photobleaching is that correction issues are mitigated. Increases in donor fluorescence cannot be related to acceptor bleed-through, because the acceptor was bleached.

The acceptor photobleaching method has found application in several recent studies (Kenworthy et al., 2000; Wouters et al., 1998), including some using CFP and YFP fusion proteins (Day et al., 2001; Llopis et al., 2000; Siegel et al., 2000).

4.3 HP1α/Orc1 *in vivo* interaction by acceptor photobleaching

HP1α localization inside the cells is normally detectable as nuclear dots corresponding to heterochromatic regions (Fig. 12). Applying the FRET technique using GFP and BFP as fluorophores pairs, as shown previously, we found a specific interaction between HP1α and Orc1 proteins inside the nucleus of HeLa cells, but failed to visualize a stronger FRET signal in the heterochromatic regions.

We decided therefore to apply a different approach using the method of Karpova and colleagues (Karpova et al., 2003).

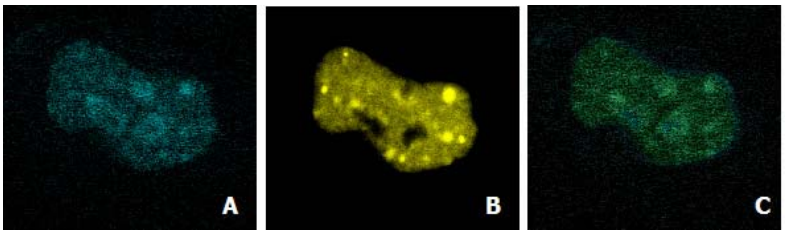

Figure 12. Expression of YFP-HP1α (A) and CFP-Orc1 (B) fusion proteins in the nucleus of HeLa cells. HP1 α is particularly localized in nuclear dots corresponding to heterochromatic regions and extranucleolarly. Orc1 colocalizes with HP1α in some nuclear dots and intranucleolarly (note the perinucleolar ring-shaped localization of Orc1) (C) merge image of images shown in A and B.

To optimize the imaging of CFP and YFP in our system and to eliminate cross-talk between the channels, we modified the exiosting filter set on our LSM51o Zeiss confocal microscope and fine-tuned the conditions of imaging accordingly to Karpova and colleagues. Based on the excitation spectra for CFP and YFP (Tsien, 1998), the 458 argon laser line should primarily excite CFP, the 514 line should primarily excite YFP and the 488 line should excite neither fluorophore efficiently (Fig. 13 A and B). Accordingly, we used the 458 line to excite CFP and the 514 line to excite YFP. To permit this dual excitation, the microscope was configured with a 458/514 nm double dichroic in the excitation path. In the emission path, we inserted a 505 nm beam-splitter to help separate fluorescence from CFP and YFP. In our confocal system, the beam-splitter transmits longer wavelengths to PMT1.

To improve specific detection of YFP emission at PMT1, we used a 530 nm long-pass filter (LP530). The 505 nm beam-splitter reflects shorter wavelengths corresponding to CFP emission to either PMT2 or PMT3. Because the light path to PMT3 is more direct, we used it for detection of CFP fluorescence. To improve specific detection of CFP emission at PMT3, we inserted a 470-500 nm band pass filter into the light path (BP470-500).

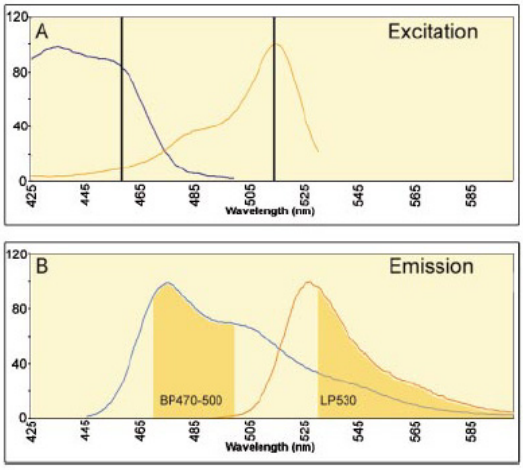

Figure 13. Spectral characteristics of CFP and YFP and the microscope configuration used to distinguish the dyes. (A) Excitation spectra for CFP (blue curve) and YFP (orange curve). The two argon laser lines used for excitation (458 and 514) are shown as vertical black lines. (B) Emission spectra for CFP (blue curve) and YFP (orange curve). Shaded yellow regions indicate the transmission bandpasses of the emission filters used.

Henceforth, we use the term "CFP channel" to refer to the combination of the 458 laser-line excitation with the BP470-500 emission filter and PMT3. We used the term "YFP channel" to refer to the combination of the 514 laser-line excitation with the LP530 emission filter and PMT1. With this preceding configuration, the cross-talk between CFP and YFP was significantly restricted (Karpova et al., 2003).

To distinguish CFP from YFP, we transfected HeLa cells with constructs encoding either CFP or YFP (not shown), or a CFP-YFP fusion containing equal amounts of CFP and YFP (Fig. 14 and 15). In this chimeric molecule, the two fluorescent proteins are separated by a two amino acid linker, and therefore FRET should occur. For example, an analogous CFP-YFP fusion separated by a short linker (19 aminoacids) has been reported to produce FRET (Vanderklish et al., 2000). Indeed, we provided evidence for FRET in the CFP-YFP fusion, as shown in the graph (Fig. 16).

Figure 14. Acceptor photobleaching of cells transfected with a CFP-YFP fusion. On the left, CFP (donor) and YFP (acceptor, smaller panels) channels before the bleach (pre bleach). On the right, the same channels after the bleach (post bleach). Note the increase in CFP fluorescence.

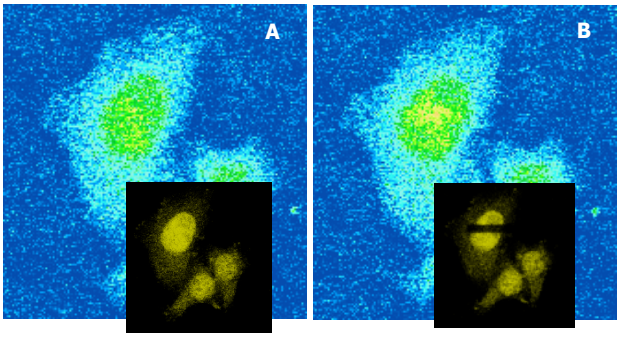

Figure 15. Acceptor photobleaching of cells transfected with a CFP-YFP fusion. (A) CFP (donor) and YFP (acceptor, smaller panels) channels before the bleach. (B) The same channels after the bleach. The postbleach CFP channel image reveals a concentrated yellow fluorescence (Leica pseudocolor) that indicates a quite high level of CFP-YFP fusion protein.

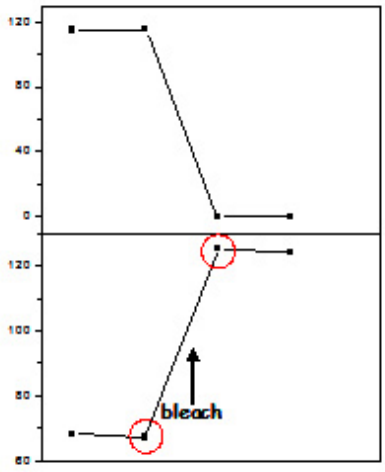

Figure 16. Acceptor photobleaching of cells transfected with a CFP-YFP fusion. Quantification of fluorescent intensity.

This result is according to what shown by Karpova and colleagues and proves that *bona fide* FRET can be detected using acceptor photobleaching on the confocal microscope operating with different lines from a single argon laser.To evaluate HP1α/Orc1 interaction, HeLa cells were transfected with CFP-Orc1 and YFP-HP1α constructs or CFP-Orc1 alone (not shown). Following transfection, Orc1 and HP1α express inside the nucleus and partially co-localise. In these colocalized areas, probably corresponding to heterochromatic regions, we performed acceptor photobleaching. As shown in Fig. 17, we observed a strong increase in CFP fluorescence in some nuclear dots. The graphs in Figg. 18 and 19 show the quantification of fluorescence intensity and the measured E_f over a set of 10 different cells.

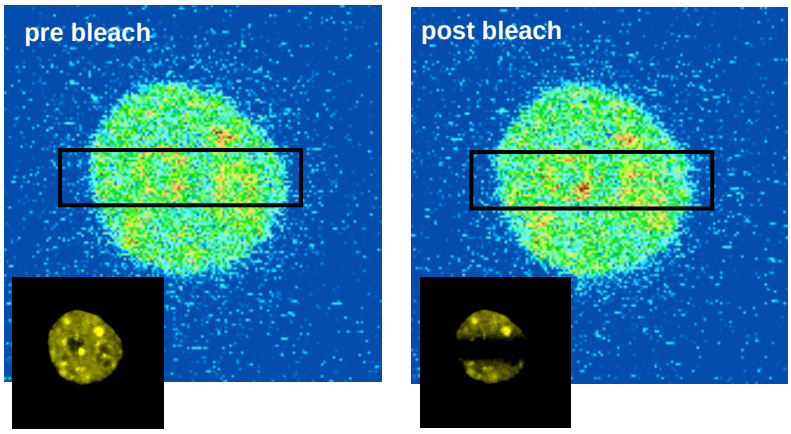

Figure 17. Acceptor photobleaching of cells transfected with CFP-Orc1 and YFP HP1α. CFP (donor) and YFP (acceptor, smaller panels) channels before the bleach. The same channels after the bleach. The postbleach CFP channel image reveals a concentrated red fluorescence (Leica pseudocolor) that indicates a high level of CFP-Orc1. Note the increase in CFP fluorescence in some nuclear dots (heterochromatic region).

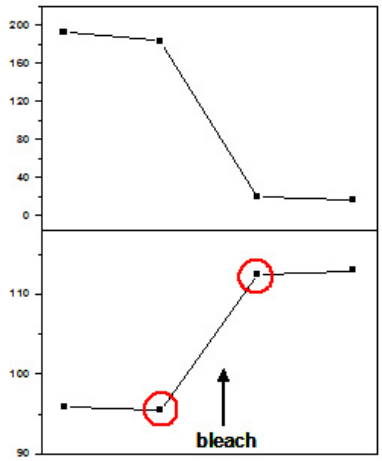

Figure 18. Acceptor photobleaching of cells transfected with CFP-Orc1 and YFP HP1α. Quantification of fluorescent intensity.

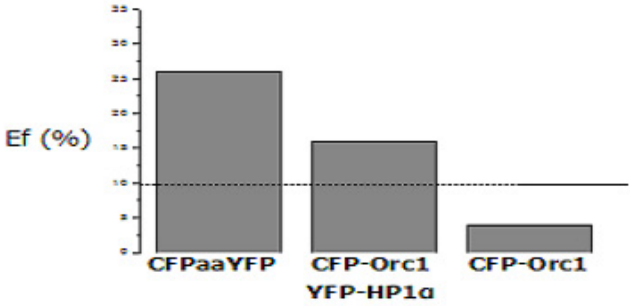

Figure 19. Quantification of fluorescent intensity (Ef) as reported (Karpova et al., 2003). FRET is positive when Ef>10%.

Finally, accordingly to Wilson and colleagues, we adopted another method to detect FRET between protein pairs applying the fluorescence emission spectra (Wilson et al., 2002). Cells were imaged using the 458 and 514 nm laser lines to excite CFP and YFP, respectively. The decrease in 480 nm emission and increase in 530 nm emission implies that FRET is occurring (Fig. 20).

Wilson and colleagues used the following formula to calculate FRET:

$$R = I@480nm / I@530nm$$

When R<0,5, the authors considered the FRET value optimal. We found indeed that:

CFP-aa-YFP	R=0.308 +/- 0.092
CFP-ORC1 + YFP-HP1α	R=0.075 +/- 0.035
CFP-PML*	R=2.459 +/- 1.109
CFP-PML + YFP-SUMO*	R=0.106 +/- 0.045

(*) fluorescent proteins from our laboratory used as positive and negative control examples (PML/SUMO interaction in (Duprez et al., 1999)).

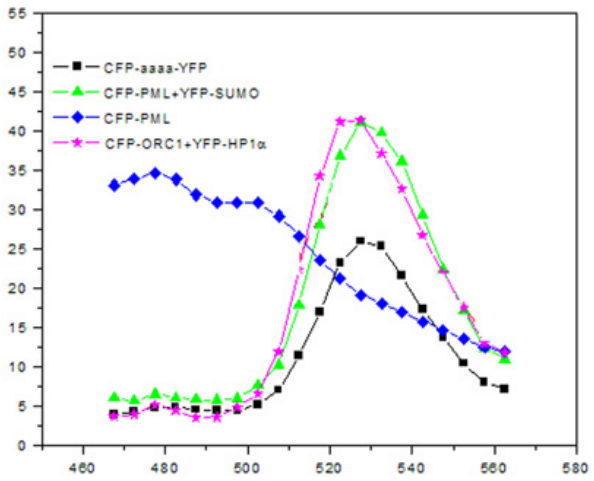

Figure 20. Fluorescence emission spectra of HeLa cells expressing the CFP-YFP fusion protein, CFP-Orc1 and YFP-HP1α proteins and other fluorescent proteins as controls. As described in the text, the decrease in 480 nm emission and the increase in 530 nm emission demonstrate that FRET is occurring for the CFP-YFP fusion protein and for Orc1/ HP1α interaction.

Specific interaction of the Retinoblastoma protein with Orc1 and its recruitment to human origins of DNA replication

Specific contribution of Roberta Paolinelli to the work described in this manuscript: I have collaborated in several aspects of the work, including protein/protein interactions (GST-Pull Down assays, co-immunoprecipitation and FRET experiments) and cell cycle distribution (flow cytometry analysis).

1 Summary

The retinoblastoma protein (Rb) is a crucial regulator of cell cycle progression by binding with E2F transcription factor and repressing the expression of a variety of genes required for the G1-S phase transition. Here we show that Rb and E2F1 directly participate in the control of initiation of DNA replication in human cells by specifically binding to origins of DNA replication in a cell cycle regulated manner. We show that, both *in vitro* and inside the cells, the largest subunit of the origin recognition complex (Orc1) specifically binds hypo-phosphorylated Rb and that this interaction is competitive with the binding of Rb to E2F1. Thus, the displacement of Rb-bound Orc1 by E2F1 at origins of DNA replication marks the progression of the G1 phase of the cell cycle toward the G1/S border.

2 Results

Rb and E2F1 proteins are recruited to human origins of DNA replication

To explore whether human E2F-1 and Rb might be also recruited to human origins of DNA replication, we took advantage of the availability of three origins that our laboratory has described in molecular detail over the last several years.

One of these origins encompasses the 3' end of the lamin B2 gene and the promoter of the mitochondrial inner membrane translocase gene (*TIMM13*) in chromosome 19q – Lamin B2 origin (Abdurashidova et al., 2003; Giacca et al., 1994); the other two origins (GM-CSF1 and GM-CSF2 origins) are located downstream of the GM-CSF gene in the human chromosome 5q (Todorovic et al., 2005). Using a high resolution chromatin immunoprecipitation (ChIP) procedure, we have recently mapped the regions of binding of several components of the pre-replication complex, including Orc1, Orc2, Mcm5 and Cdc6 at these origins in close correspondence to the sites of nascent DNA synthesis (Todorovic et al., 2005) .

Cross-linked chromatin from asynchronous HeLa cells was immunoprecipitated with antibodies against Rb, E2F1, Orc1 and Orc2 proteins, as well with a control antibody. Sequence-specific primer and probe sets for real time PCR analysis were designed to amplify and detect origin (region B48 in lamin B2 and regions #17 and #23 in GM-CSF; Figures 1 and 2 respectively) as well as non-origin areas (B10 and for the Lamin B2 and #21 for the GM-CSF origins). These ChIP experiments revealed that, along with Orc1 and Orc2, Rb and E2F1 were also enriched 2-5 times on all three human origins as compared to the non-origin areas (Fig. 1 and 2 for Lamin B2 and GS-CSF origins respectively).

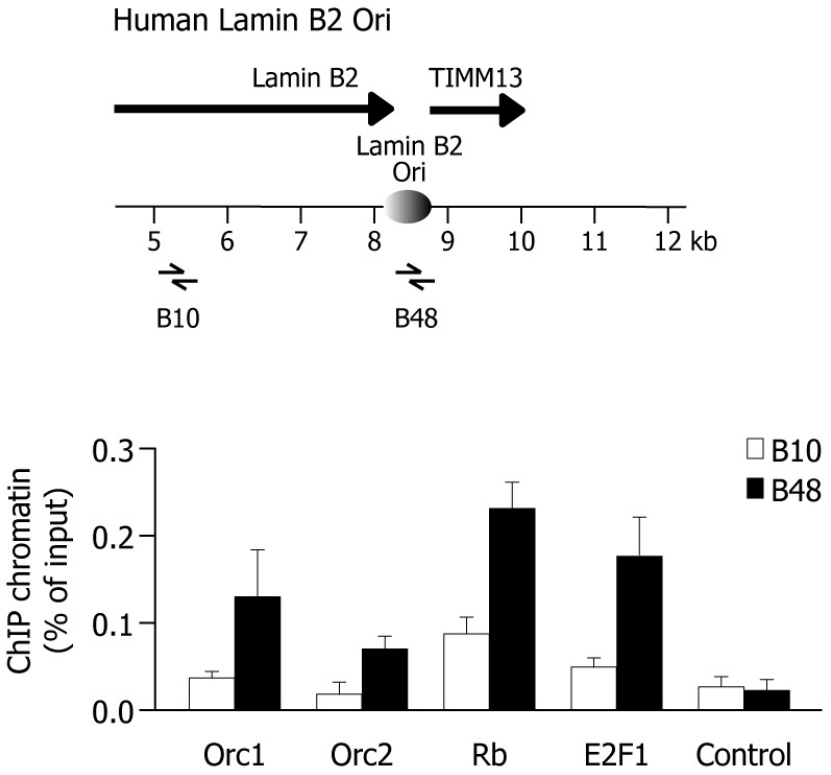

Figure 1. Rb and E2F1 proteins are recruited to human origins of DNA replication. The scheme shows the genomic regions containing the Lamin B2 origin. Converging arrows indicate sets of primers. The histogram shows the quantification of crosslinked DNA immunoprecipitated by ChIP on the Lamin B2 origin. Each graph shows the specific amplified genomic regions from the origin and the antibodies used for ChiP experiments. The bars indicated as Control show the results obtained by using an irrelevant antibody. The histogram reports the results (mean and standard error of the mean, indicated by error bars) of at least three different experiments. The results are presented as percentage of the amounts of precipitated chromatin over input DNA.

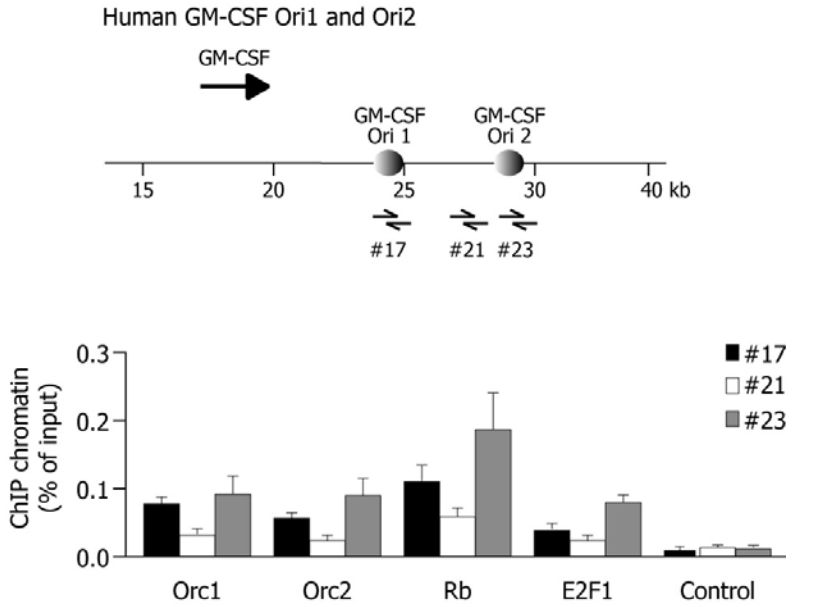

Figure 2. Rb and E2F1 proteins are recruited to human origins of DNA replication. The scheme shows the genomic regions containing the two GM-CSF origins. Converging arrows indicate sets of primers. The histogram shows the quantification of crosslinked DNA immunoprecipitated by ChIP on the two GM-CSF origins. Each graph shows the specific amplified genomic regions from the origins and the antibodies used for ChiP experiments. The bars indicated as Control show the results obtained by using an irrelevant antibody. The histogram reports the results (mean and standard error of the mean, indicated by error bars) of at least three different experiments. The results are presented as percentage of the amounts of precipitated chromatin over input DNA.

Orc1 specifically interacts with Rb in vitro

To understand the possible mechanisms of recruitment of Rb and E2F1 to origin DNA, we started investigating the interactions between the two proteins and different components of the origin recognition complex by a series of GST-pulldown experiments. We found that [^{35}S]-labeled human Orc1 was specifically retained on GST-Rb immobilized on glutathione agarose beads (Fig. 3). In the same experiment, both binding of E2F1 to Orc1 or either E2F1 or Rb to Orc2 were all negative.

In mammalian cells, Rb is known to be a member of the family of pocket proteins, which are characterized by the presence of conserved sequence domains at their C-terminus that are involved in binding to various cellular protein partners. Within the family, Rb, p107, and p130 all contain the A and B pocket domains.

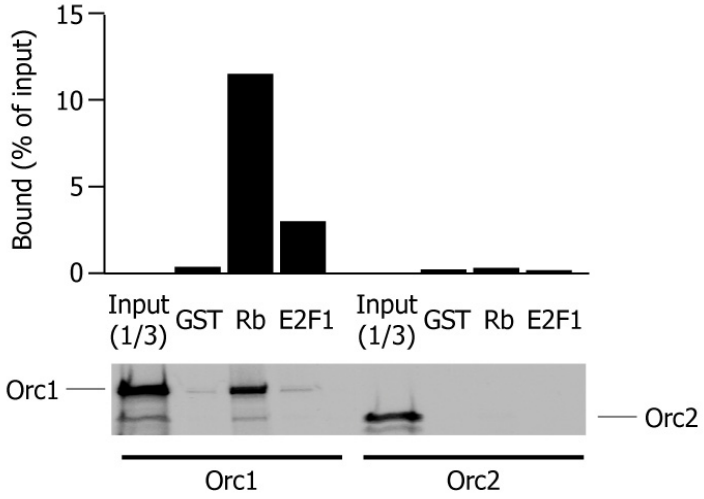

Figure 3. Orc1 specifically interacts with Rb *in vitro*. GST pull-down experiment performed by incubating GST, GST-Rb or GST-E2F1 fusion proteins immobilized on gluthatione-agarose beads with in vitro translated [^{35}S]-labelled Orc1 or Orc2 proteins. The upper panel shows the quantification of the [^{35}S]-labelled protein after *in vitro* binding. The amount of radioactivity bound to the beads is indicated as a percentage of the input material. The lower panel shows the autoradiography. The Input lanes contain the labelled proteins prior to binding.

In addition, Rb also shows an additional C domain that is specific to this factor (C domain) (Fig. 4 a). To assess whether binding to Orc1 might also extend to other pocket protein family members, we performed GST pull-down experiments using GST fusion proteins corresponding to the pocket domains of p107 and p130.

Orc1 was found to only bind Rb, but not to the other proteins; in the same experiment, Orc2 binding was negative to all proteins (Fig. 4 b). These results clearly indicate that the interaction with Orc1 is specific for Rb.

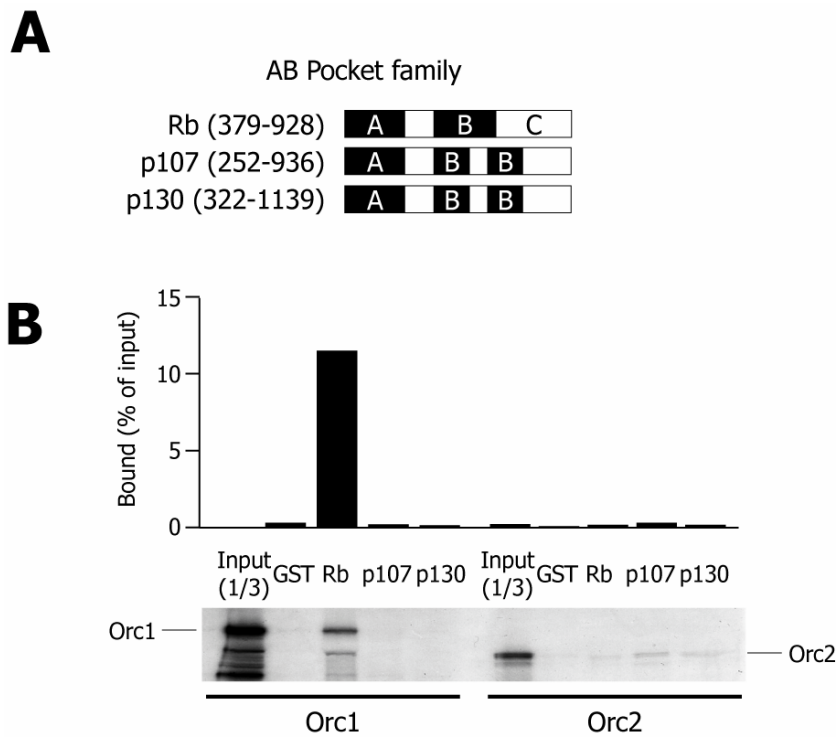

Figure 4. Binding to Orc1 is specific for Rb. (A) Scheme of the conserved A/B pocket domains in the three members of the RB family, which were used as GST fusion proteins. (B) Result of a GST pull-down experiment performed with these proteins and in vitro translated Orc1 and Orc2.

To better characterize the domains in the C-terminal portion of Rb that are responsible for Orc1 binding, we obtained a series of GST fusion proteins carrying the whole Rb C-terminus (fragment AE in Fig. 5 A), the A and B pocket domains (fragment AB) or only the C-terminus (fragment SE). These proteins were tested for binding to radiolabeled Orc1. The integrity of the C-terminal domain of Rb was found to be essential for binding Orc1 (Fig. 5 B). Indeed, the C-terminus alone also retained partial capacity to bind to Orc1. These results are in agreement with the observation that binding to Orc1 is specific to Rb but not to other members the pocket family of proteins.

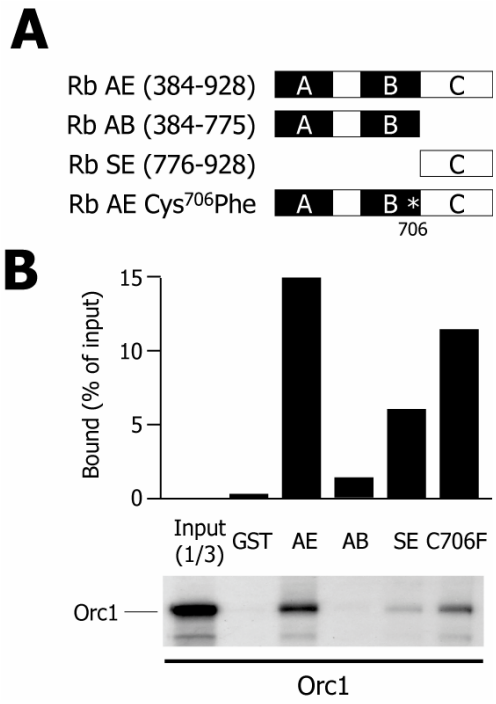

Figure 5. Binding to Orc1 requires the C-terminal region of Rb. (A) Scheme of the Rb truncated or mutated proteins used for mapping the domains required for binding to Orc1 (AE, containing the A, B and C pockets; AB, A and B pockets only; SE, C pocket only; AE Cys706Phe, which does not bind LxCxE proteins). These proteins were obtained as GST fusions and used for the GST pulldown experiment shown on the right side of the panel (B).

Next we sought to identify the Orc1 protein motifs responsible for the association with Rb. Orc1 is known to have a modular structure, conserved in most species, that includes a BAH and an ATPase domains located at the N- and C-terminal regions of the protein, respectively; a region that we have recently observed to specifically interact with the HP1 heterochromatin protein is partially overlapping with the BAH motif (Lidonnici et al., 2004). We obtained a series of Orc1 mutants carrying various deletions in these domains, as schematically shown in Fig. 6 a.

These mutants were *in vitro* transcribed and translated, and used in GST-

pulldown experiment for binding to GST-Rb. Neither the N-terminus alone (aa 1-144) nor the C-terminus alone (aa 529-861) was sufficient for binding, thus restricting the Rb-interacting area to aa 145-529. Fragment 270-861 is capable of binding to Rb at the same extent as the full length protein, suggesting that the interacting domain is between aa 270 and 529. However, fragment 151-269 still shows residual binding, thus suggesting that binding is also contributed by residues before aa 270 (Fig. 6 B). With the limitations of this type of analysis, these results collectively suggest that the Orc1 binds Rb *in vitro* through a central region of the protein, which does not involved neither its BAH nor its ATPase domains. Different cellular proteins are known to bind Rb through a conserved **L**x**C**x**E** motif (Chan et al., 2001). Intriguingly, Orc1 displays such a motif starting at position 504 (**L**P**C**R**E**).

This sequence is only found in human Orc1 and not in other Orc subunits, and is absolutely conserved in mammals (Fig. 7). To test the involvement of this amino acid stretch in Rb binding, we obtained a mutant Orc1 protein in which the sequence LPCRE was mutated to **L**P**G**R**K**. Binding of this protein to the Rb pocket region was tested in GST pulldown experiments. As shown in Fig. 7, this mutated protein was still capable to bind the Rb C-terminal domain, thus indicating that its integrity was not essential for the interaction. Consistent with this finding, we also observed that the mutation of cysteine 706 of Rb to alanine, which is known to interfere with Rb binding to the LxCxE motif (Pennaneach et al., 2001), did not significantly interfere with binding of Rb to Orc1.

Collectively, these results indicate that binding of Orc1 i) is specific for Rb and not for other pocket family members; ii) requires the integrity of the Rb C-terminal domain; iii) involves the central portion of Orc1; iv) occurs in an LxCxE motif-independent manner.

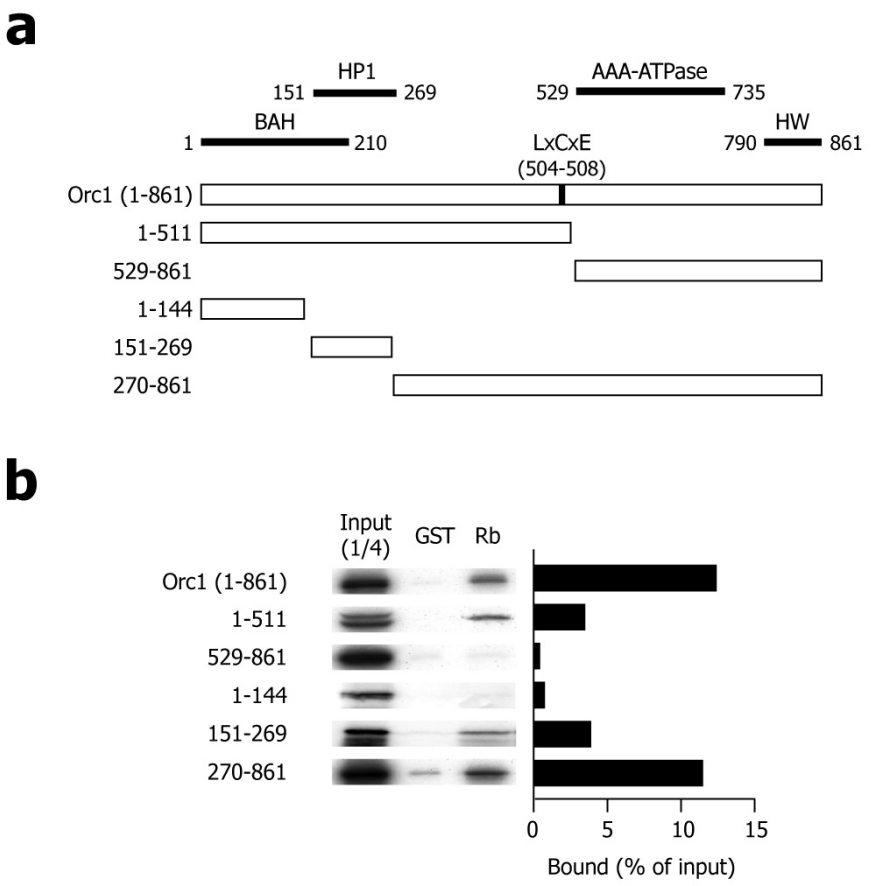

Figure 6. Orc1 specifically interacts with Rb *in vitro*. (a) Schematic representation of the main functional domains of the Orc1 protein. BAH, bromo-adjacent homology domain; HP1, HP1 binding domain; AAA, ATPase domain; HW, putative DNA binding site. The fragments of Orc1 subsequently tested by in vitro GST pull-down are indicated by the corresponding amino acids on the left side. (b) GST pulldown experiment performed with the Orc1 fragments indicated in panel above and labeled by *in vitro* translation, and challenged to GST or GST-Rb proteins.

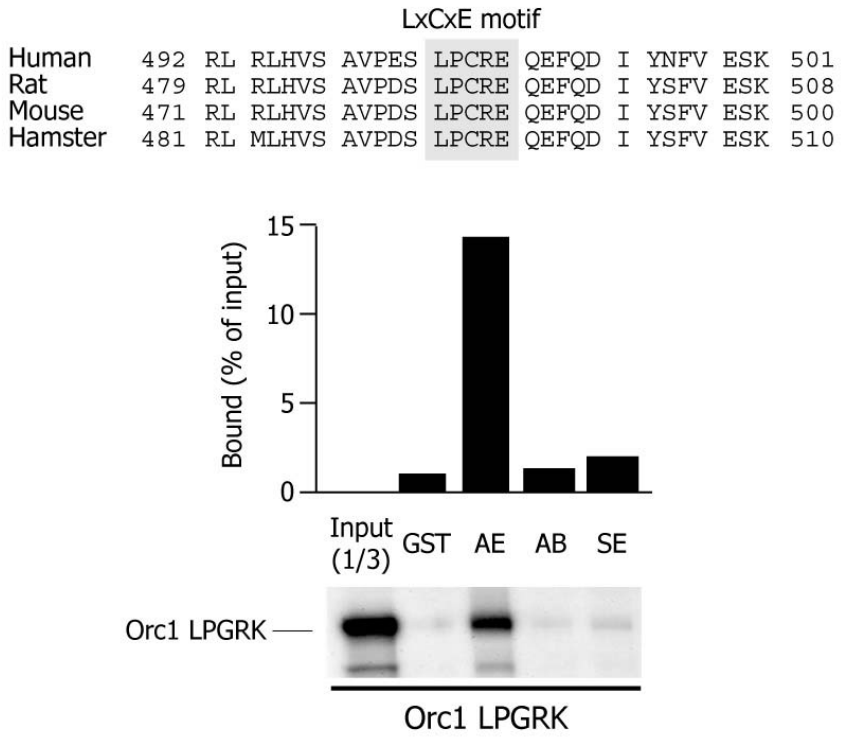

Figure 7. Sequence alignment showing the conserved LxCxE motif found in the Orc1 subunit of human, rat, mouse and hamster (upper part) and GST pull-down experiment performed with the *in vitro* translated Orc1 LPGRK protein (mutated in the LxCxE motif of Orc1) and the GST-fusion proteins AE, AB and SE (lower part).

E2F1 competes with Orc1 for Rb-binding

The notion that the integrity of the whole Rb C-terminus was required for Orc1 binding, and the observation the isolated Rb C domain alone was also able to bind Orc1, raised the intriguing possibility that the Rb/Orc1 interaction might be mutually exclusive with the formation of an Rb/E2F complex. Indeed, Rb contains two distinct E2F binding sites, one (the large ABC pocket) important for stable association with E2Fs on DNA and a second one (the C pocket) specific for E2F1 (Dick and Dyson, 2003) (Fig. 8).

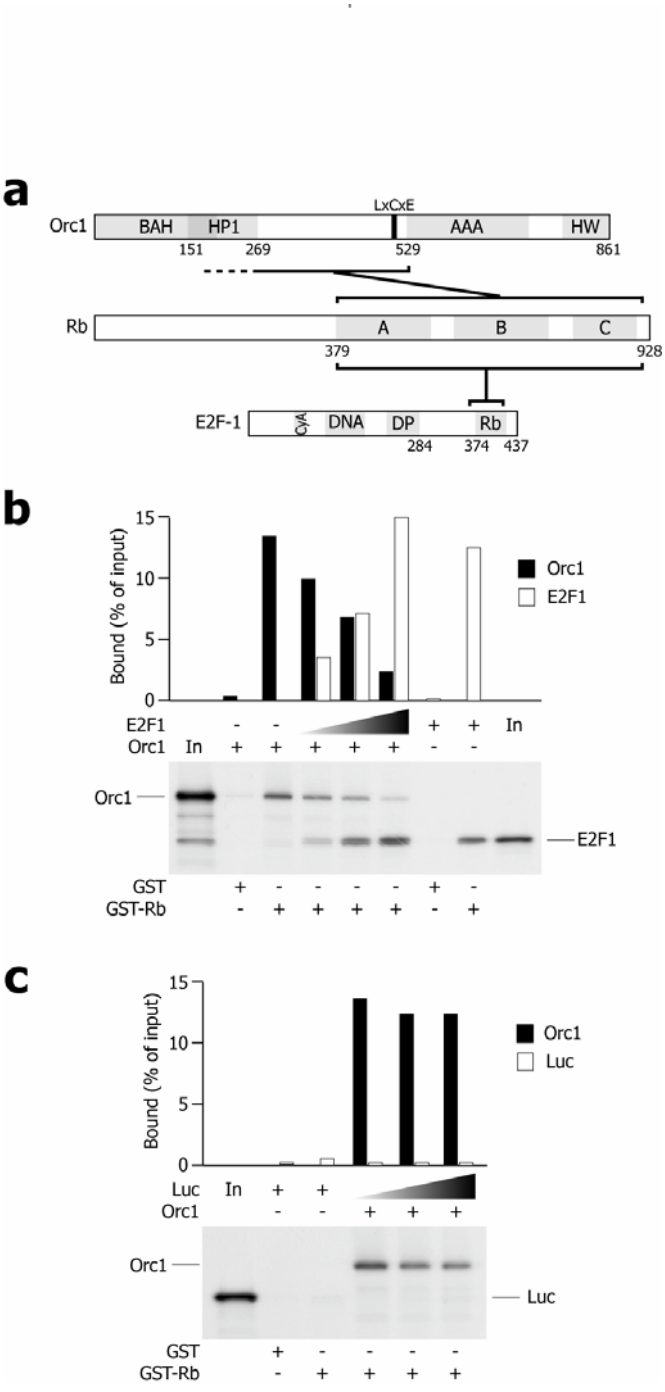

Figure 8. *(for figure legend see next page)*

Figure 8. E2F1 competes with Orc1 for binding to Rb. (a) Schematic representation of the regions involved in the association of Rb to Orc1 and of those that that are necessary for the stable association with E2F1. (b) GST pull-down experiment performed by incubating a fixed amount of *in vitro* translated Orc1 together with scalar amounts of *in vitro* translated E2F1 with an immobilized GST fusion protein containing the ABC pocket of Rb. The graph shows the quantification of bound E2F1 and Orc1 radioactivity; the input lanes (In) contain the labelled proteins prior to binding. (**c**) Competitive GST pull-down control experiment performed with *in vitro* translated Orc1 and luciferase (Luc) proteins using identical experimental conditions as in (b).

Competitive GST pulldown experiments were performed by incubating the recombinant GST-Rb fusion protein with *in vitro* translated Orc1 in the presence of increasing amounts of *in vitro* translated E2F1. As shown in Fig. 8 b, E2F1 was found to compete with Orc1 for binding to GST-Rb. In contrast, when a control luciferase protein was used to substitute E2F1, binding of Orc1 to Rb was unaffected (Fig 8 c).

This result clearly indicates that the binding of Orc1 to Rb is mutually exclusive with binding of Rb to E2F1.

Endogenous Orc1 forms a stable complex with hypo-phosphorylated Rb in human cells

To determine whether Orc1 protein forms a complex with Rb *in vivo*, we performed a series of co-immunoprecipitations experiments with specific antibodies against Orc1, Rb and E2F1 using whole cell lysates from HeLa cells. Western blot analysis of Rb and E2F1 proteins after immunoprecipitation with anti-Orc1 antibodies revealed the co-immunoprecipitation of endogenous Rb (but not of E2F1). Consistent with the *in vitro* results, both endogenous E2F1 and Orc1 co-immunoprecipitated with Rb, andonly Rb co-immunoprecipitated with E2F1 (Fig. 9). These results were further confirmed by transfecting a HA-tagged version of Orc1 in HeLa cells, followed by immunoprecipitation with an anti-HA antibody. In these conditions, endogenous Rb was found to specifically co-immunoprecipitate with HA-Orc1, in addition to endogenous E2F1 (Fig. 10).

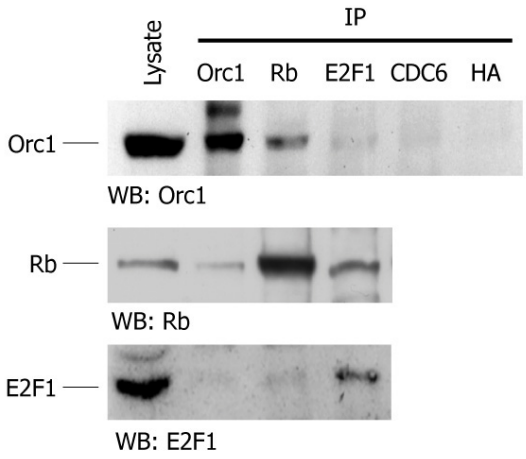

Figure 9. Co-immunoprecipitation experiments performed with lysates from asynchronous HeLa cells using the indicated antibodies for immunoprecipitation and western blottings.

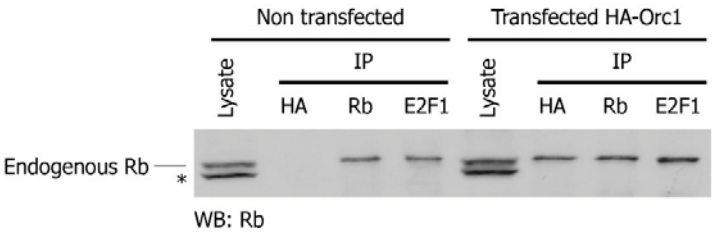

Figure 10. Immunodetection of endogenous Rb after co-immunoprecitation with exogenous HA-tagged Orc1 in transiently transfected asynchronous HeLa cells. Additional co-immunoprecipitations with Rb and E2F1 proteins in non-transfected and HA-Orc1-transfected HeLa cells were performed as controls. The band marked by an asterisk (*) represents an unspecific band detected with the mouse anti-Rb antibody IF8.

The same result was also obtained in U2OS cells (Fig. 11). In addition, in these cells, we also tested the interaction of Rb with a HA-tagged protein corresponding to Orc1 mutated in the LxCxE domain (HA-Orc1LPGRK). Consistent with the GST pulldown experiments, which showed that this mutation did not alter binding of Orc1 to Rb, we found that endogenous Rb effectively co-immunoprecipitated with the transfected HA-Orc1LPGRK protein (Fig. 11).

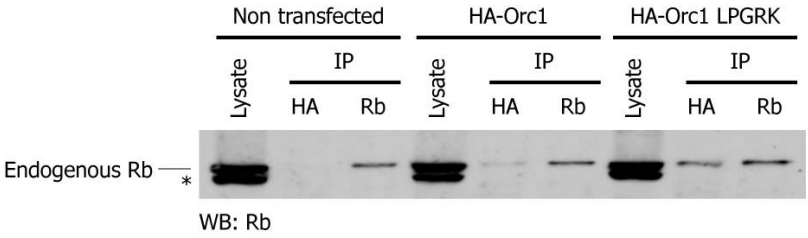

Figure 11. Endogenous Rb detected by western blotting after immunoprecipitation with anti-HA peptide antibody in non-transfected, wt HA-Orc1-, and mutant HA-Orc1 LPGRK-transfected U2OS cells. Additional immunoprecipitations for Rb were performed as controls on the same lysates. The band marked by an asterisk (*) represents an unspecific band detected with the mouse anti-Rb antibody IF8.

When performing these immunoprecipitations experiments, we observed that, while the starting whole cell lysates and the Rb immunoprecipitation generated a smear when analyzed by western blotting using anti-Rb antibody, the Orc1 immunoprecipitates always gave rise to a sharper band corresponding to the lower molecular weight forms of Rb (Fig. 6). This observation suggested that Orc1 preferentially associated with the hypophosphorylated forms of Rb. To confirm this possibility, we treated the Rb immunoprecipitates with the PP2A phosphatases, and obtained bands of the same molecular weight as those that co-immunoprecipitated with Orc1 (Fig. 12).

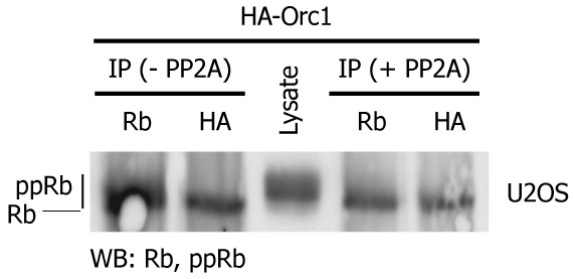

Figure 12. Immunoblotting to visualize the phosphorylated forms of endogenous Rb, after immunoprecipitation with anti-Rb and anti-HA peptide antibodies in U2OS cells transfected with wt HA-Orc1 (first two lanes from the left). The same immunocomplexes were also treated with the PP2A phosphatase before blotting (last two lanes on the right side).

Collectively, these results indicate that both endogenous and transfected Orc1 form a complex with Rb in different cell types independently of the LxCxE motif, that the formation of this complex is mutually exclusive with binding of Rb to Orc1, and that it involves the hypophosphorylated forms of Rb.

Visualization of Orc1-Rb interaction inside the cells by Fluorescence Resonance Energy Transfer (FRET)

FRET image analysis of individual asynchronous cells is shown in Figg. 13, 15 and 17. For each protein pair, the upper panels show the intracellular distribution of fluorescence at 520 nm (the peak wavelength of EGFP emission) under excitation at 480 nm; the lower panels show the fluorescence of the same fields at 520 nm after excitation of BFP at 350 nm. Under these conditions, when a cell expresses two non-interacting proteins, fluorescence at 520 nm is only detected after excitation of EGFP at 480 nm (see, for example, the EGFP-Orc1 and BFP protein pair). However, if the two proteins interact closely (<100), FRET occurs between the two fluorophores and fluorescence at 520 nm is also detectable after excitation of BFP at 350 nm. For each analyzed protein pair, quantitative analysis of the intensity of fluorescence of at least 10 cells was performed under the two illumination conditions. These results are presented in the box plots (Fig. 14, 16 and 18), indicating the percentile distributions of the FRET efficiency, measured as the ratio between emission at 520 nm after excitation at 350 nm and 480 nm (Figg. 14, 16 and 18).

These experiments clearly revealed that Orc1 and Rb physically interacted inside the cell's nucleus. Other positive interactions were detected between Rb and E2F1, but not between Rb and Orc2, between Orc1 and E2F, or between Orc1 and either MCM2 or MCM3. Of interest, Orc1 was found to bind Orc2 (as already reported (Kneissl et al., 2003; Lidonnici et al., 2004), as well as MCM2 to MCM3.

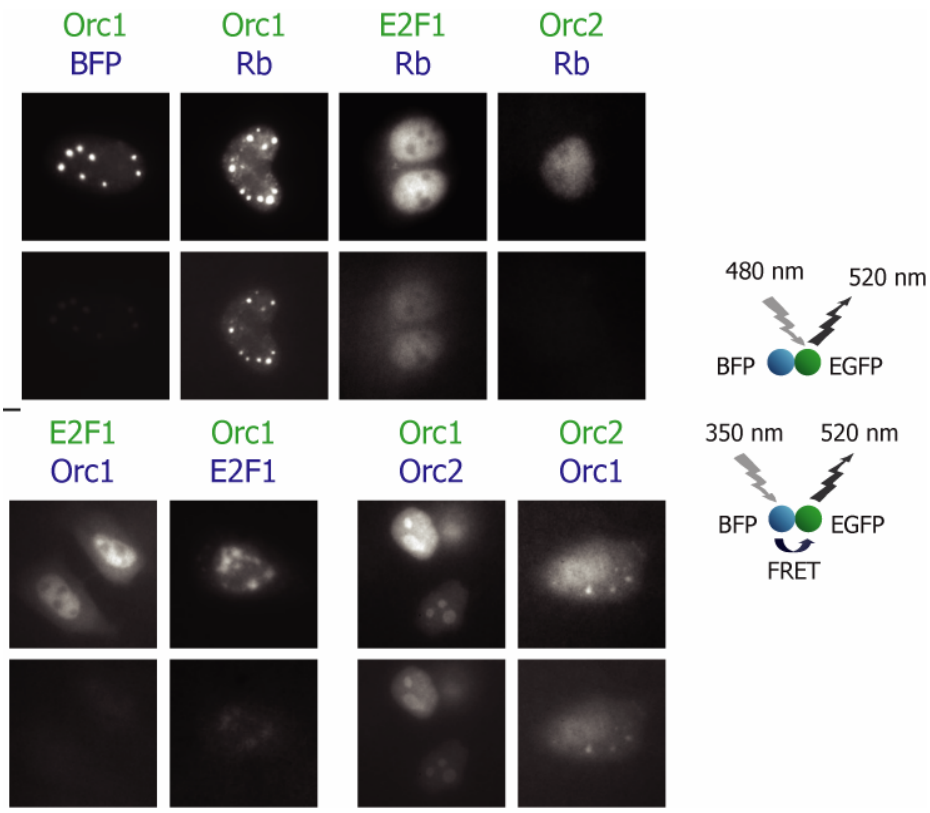

Figure 13. FRET analysis. HeLa cells were transiently transfected with expression vectors coding for the proteins indicated on top of each panel fused to either EGFP (green color) or BFP (blue color). Individual transfected cells were visualized by excitation at 480 nm and collection at 520 nm, showing EGFP fluorescence after direct EGFP excitation (panels in the upper row), and by excitation at 350 nm and collection at 520 nm, showing EGFP fluorescence after BFP excitation, indicating FRET (panels in the lower row).

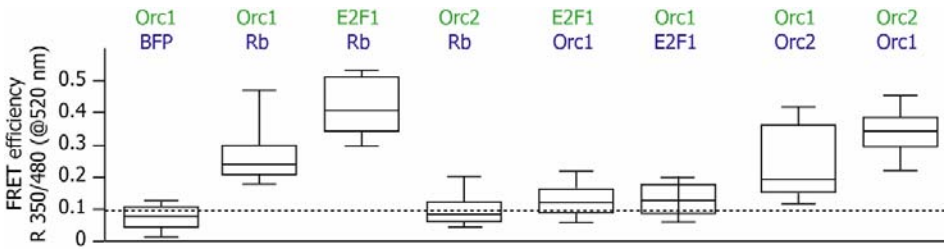

Figure 14. The box plot below for each image pair shows the quantification of FRET. Fluorescent emission at 520 nm from individual cells was recorded after excitation at 350 or 480 nm, and integrated intensities over the whole cell were evaluated. The percentile box-plot distribution of the ratio between these two measurements is shown by considering at least 10 consecutively analyzed cells for each protein pair. Horizontal lines of the percentile box plot distribution, from top to bottom, mark the 10th, 25th, 50th, 75th, and 90th percentile respectively.

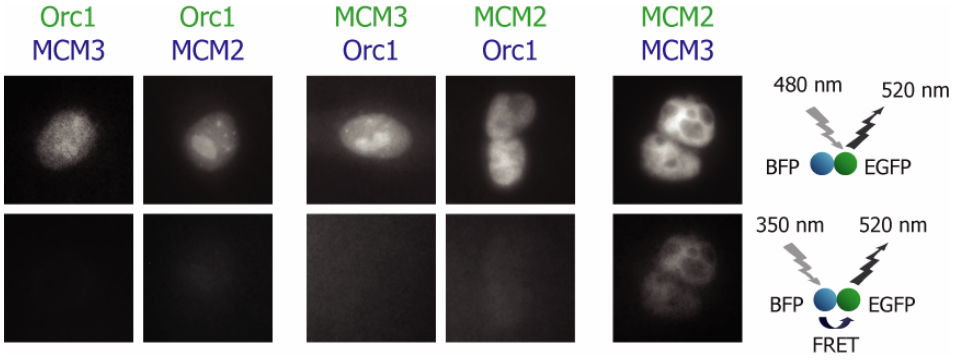

Figure 15. FRET analysis.

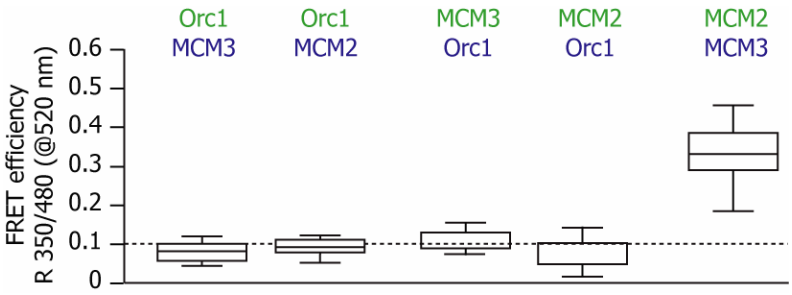

Figure 16. Quantification of FRET analysis.

We also exploited FRET to visualize and quantify the binding of Rb with the same set of Orc1 truncation mutants used in the GST pulldown interaction mapping experiments. We found that the EGFP-Orc1 (1-144) protein was negative for FRET with BFP-Rb; in contrast, clear positivity was detected for both the C-terminal fragment of Orc1 (270-861) and the intermediate fragment (151- 269) (Figg. 17 and 18).

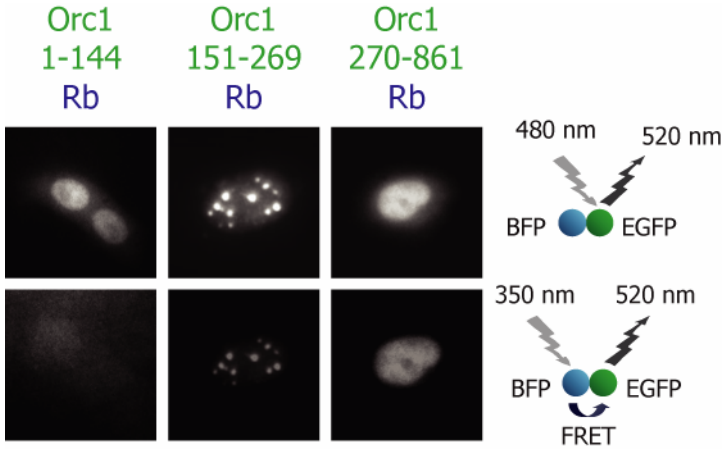

Figure 17. FRET between Rb, tagged with BFP, and the same set of Orc1 truncation mutants considered for the GST pulldown experiments, tagged with EGFP.

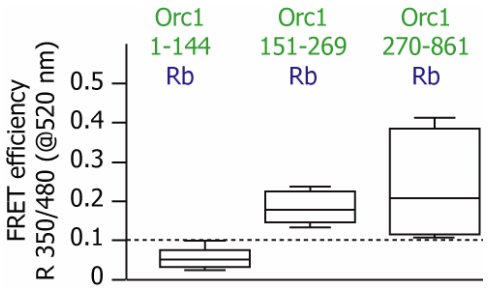

Figure 18. Quantification of FRET analysis.

These results provide an *in vivo* confirmation that the Orc1 fragment 270-861 is capable of binding to Rb at the same extent as the full length protein, and that binding is also contributed by residues extending before amino acid 270.

Orc1 and E2F1 are recruited to the lamin B2 origin at different temporal windows of G1 phase

The finding that Rb and E2F associate with origins of DNA replication raises the intriguing possibility that these proteins might directly regulate some aspects of origin function. To start addressing this issue, we analyzed whether the recruitment of Rb and E2F at origins might vary during the cell cycle.

HeLa cells were synchronized in mitosis by sequential treatment with thymidine and nocodazole, and then released from the block and harvested at different times (Fig. 19).

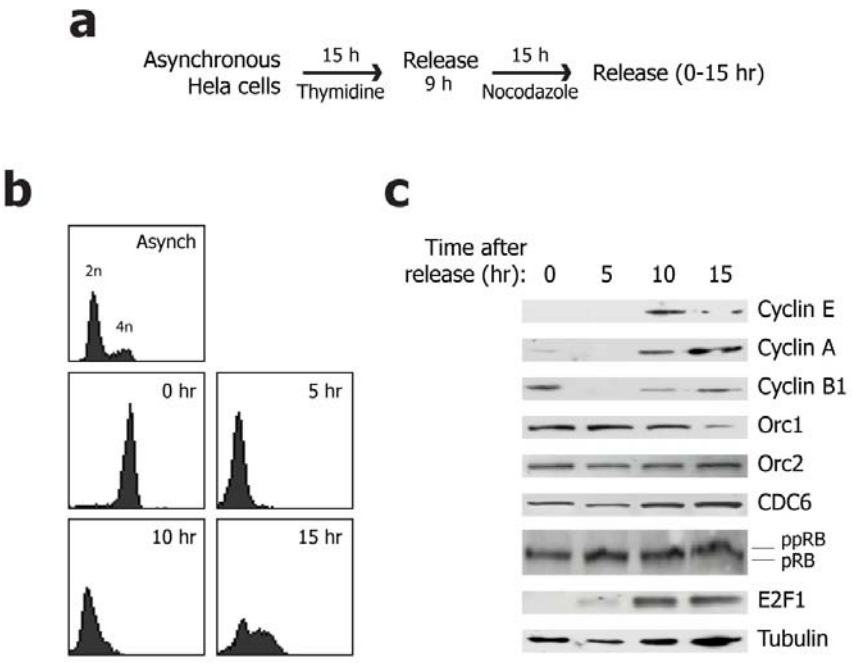

Figure 19. (*For figure legend see next page)*

Figure 9. HeLa cells synchronization**.** (a) Experimental scheme for HeLa cell synchronization. HeLa cells were synchronized in mitosis by a double thymidine/nocodazole block, and then followed G1 after release from the block (b) Flow cytometry profiles of asynchronous cells (Asynch), cells blocked in mitosis (0 hr) or cells at different times after release. (**c**) Western blot analysis of whole-cell extracts obtained from cells at different times points during synchronization.

The effectiveness of the synchronization treatment to arrest cells in the M phase (0 h) was confirmed by both their 4n DNA content and by the expression of cyclin B1 (Fig. 19 b and c respectively). At 5 h from release, cells had an early G1 profile, characterized by a 2n DNA content. At 10 h, cells were in proximity to the G1/S border, expressing both cyclin E and A. Finally, at 15 h most cells had entered S phase and were characterized by high levels expression of cyclin A. Orc1 was found expressed at high levels at 0, 5, and 10 h from mitotic block release; E2F1 started to be present at 5 h and its levels increased at 10 and 15 hours; hypophoshorylated Rb was detected at 0 and 5 h, while its phosphorylated forms were mainly apparent at 10 and 15 hours; Orc2 and Cdc6 were present throughout all time points (Fig. 19 c). The chromatin of cells synchronized at the different time points was *in vivo* croosslinked with formaldehyde and the association of Rb, E2F1, Orc1 and Orc2 and HDAC1 proteins with the lamin B2 origin was analyzed by ChIP (Fig. 20).

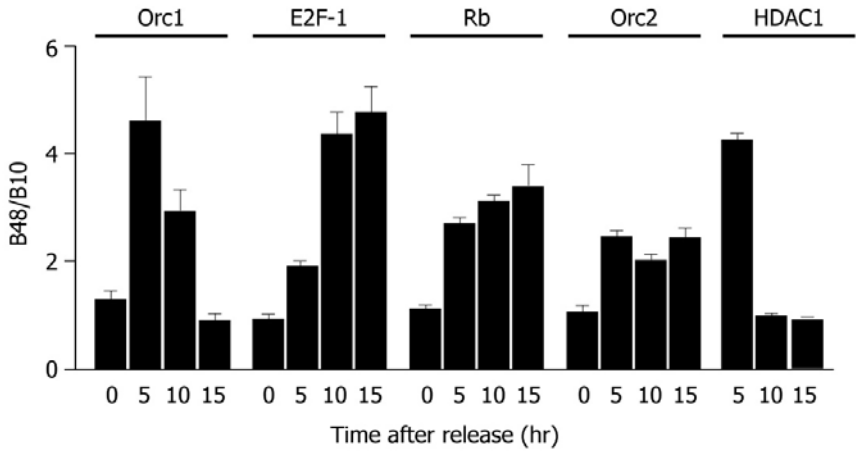

Figure 20. Cell cycle-dependent association of Rb and E2F1 with the Lamin B2 origin (HeLa cells). Quantification of cross-linked lamin B2 origin DNA immunoprecipitated by ChIP. On top of the graph, the antibodies used for ChIP are shown. The histogram reports the results (mean±sem) of at least three independent experiments. The results are presented as the fold enrichment of the lamin B2 origin region (B48) over the irrelevant B10 region, after normalization for the levels of immunoprecipitated chromatin using an unrelated antibody as control.

In M-phase cells (0 h), no significant enrichment was found for any of these factors compared to chromatin immunoprecipitated with irrelevant antibody. Strikingly, Orc1 was found to bind the origin at 5 hr (~5-fold increase over control), and then to be progressively released from it at 10 h (G1/S; ~3 fold increase), to return to background levels at 15 h (S phase). In contrast, E2F1 showed a reciprocal behavior, namely started to be associated with the origin as soon as it was expressed (5 h; ~2-fold enrichment over background), while its binding to the origin increased at 10 and 15 h (G1/S and S respectively; ~4-5 fold enrichment). Orc2 and Rb were constantly found associated with the origin DNA at all time points after nocodazole block release (~2-3 fold enrichment). The histone deacetylases HDAC1 was found associated with the origin at 5 h, while it appeared to have left the origin at later time points, concomitant with E2F1 recruitment. Orc2 and Rb were constantly found associated with the origin DNA at all time points after nocodazole block release (~2-3 fold enrichment).

To better address the study of the kinetics of E2F/Rb recruitment to the lamin B2 origin during the G1 phase of the cell cycle, as well as to investigate factor binding in the G0 phase, we took advantage of the possibility of synchronizing human T98G cells in G0 by serum starvation for 72 h (Galbiati et al., 2005) (Fig. 21 a). Upon re-addition of serum, cells synchronously progressed throughout G1 (Fig. 21 b).

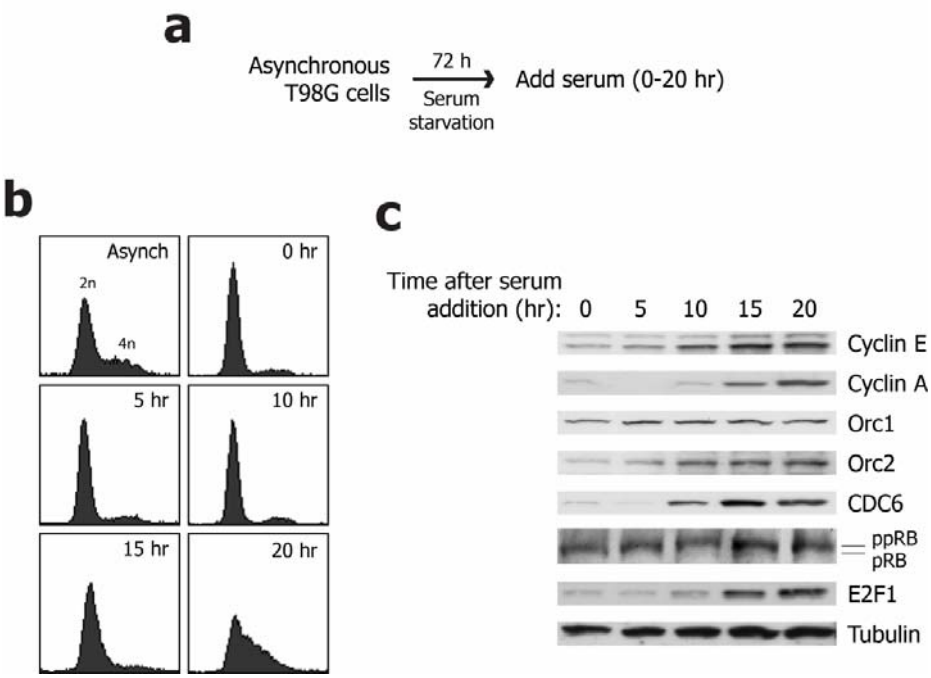

Figure 21. T98G cells synchronization. (a) Experimental scheme for T98G cell synchronization. Cells were cultured without serum for 72 hours and then followed for 20 hours after addition of serum. (b) Flow cytometry profiles of asynchronous cells (Asynch), cells blocked in G0 by serum starvation (0 hr) or cells at different times after serum stimulation. (c) Western blot analysis of whole-cell extracts obtained from cells at different times points during synchronization.

In accordance with recently published data (Mailand and Diffley, 2005), cyclin E and cyclin A levels started to raise in middle G1 (10 h) and late G1 (15 hr) respectively, with both cyclins being present at G1/S (20 h). Cdc6 started to appear middle G1; Orc1 and Orc2 were present all throughout the

synchronization process, even if the levels of the latter protein increased after middle G1. E2F1 progressively increased after middle G1, concomitant with the appearance of hyperphosphorylated pRb (Fig. 21 c). Immunoprecipitations were performed with chromatin crosslinked at the different time points (Fig. 22). In G0 cells, the only antibody that gave significant enrichment on the lamin B2 origin DNA was the one against Rb (~4 fold over background); binding of Rb to the origin remained constant at all the subsequent time points. Similarly, Orc2 started to be detected (~3-fold enrichment) in early G1 and remained rather constantly bound onto the origin. Of interest, Orc1 binding to the origin was not detectable in G0, despite the protein was expressed; in early G1, the protein started to associate with the origin and its enrichment peaked in mid G1 (4-5 fold). At the later time point (15 h) it progressively decreased to become unapparent in cells at G1/S. In contrast, E2F1 showed an opposite behavior, since it started to associate to the origin as early as its levels rose in G1 (~2 fold enrichment over background in middle G1, ~4 fold in late G1, over 5 fold in late G1).

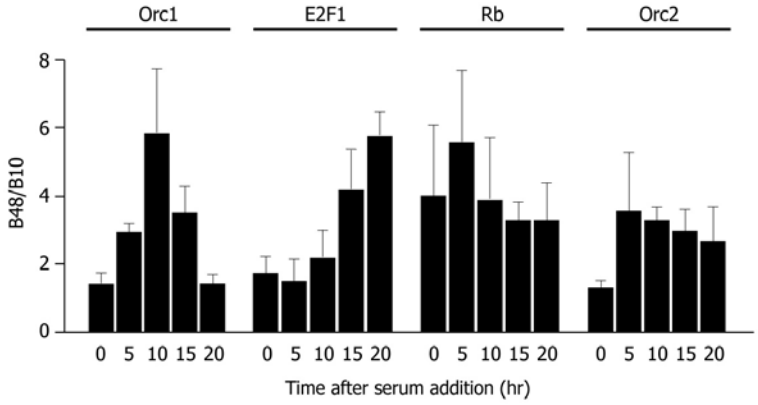

Figure 22. Cell cycle-dependent association of Rb and E2F1 with the Lamin B2 origin (T98G cells). Results of ChIP experiments for the lamin B2 origin, using the antibodies indicated on top of the panel. The results are presented as in Fig. 20.

Collectively, the data obtained in HeLa cells synchronized by a double thymidine/nocodazole block and those observed in T98G cells after release from serum starvation are concordant in showing a reciprocal behavior of Orc1 and E2F1 recruitment onto the origin DNA. In early G1 cells, the origin is engaged in binding Rb, Orc2 and Orc1; in correspondence to the G1/S boundary, Orc1 appears to leave the complex and to be replaced by E2F1.

Downregulation of Orc1 blocks cells in G1 and increase binding of E2F-1 to origin DNA

In eukaryotic cells, pre-RC formation is restricted to the G1 phase of the cell cycle. Origin selection is determined by the Orc core complex, which is formed by the Orc 2, 3, 4, and 5 subunit, the interaction of Orc1 and Orc6 with this core being more labile (Vashee et al., 2001). In this *scenario*, we sought to determine whether depletion of Orc1 protein could influence the recruitment of Orc2 and Rb/E2F1 complex at origins of DNA replication.

Orc1 depletion was achieved by RNA interference on U2OS cells. Cells were transfected with the siRNA duplex for 72 h and then harvested for western blotting and FACS analysis. Fig. 23 shows that, despite a marked reduction (>80%) of Orc1 protein, Orc2, Cdc6, E2F1, Rb and cyclin A and E protein levels did not vary significantly.

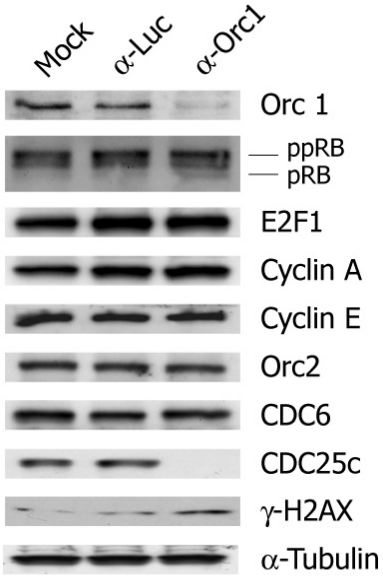

Figure 23. Down-regulation of Orc1. Western blotting using the indicated antibodies at 72 h after treatment of U2OS cells with siRNAs against Orc1 or Luciferase (Luc) control.

Interestingly, no CDC25c protein was detectable in the Orc1 siRNA-treated cells, and expression of the phosphorylated form of histone H2AX was increased; both observations are consistent with the induction of a damage checkpoint as a consequence of the Orc1 knock down.

As shown by the flow cytometry profiles in Fig. 24, Orc1 depletion determined a remarkable reduction of the number of cells in the S phase of the cell cycle (from 24% to 15% of total), and a consequent increase in the number of cells in G1 (from 61% to 68%). Treatment with an anti-Luciferase siRNA control did not modify the cell cycle profiles. To better document the reduction in the number of S-phase cells upon treatment with the anti-Orc1 siRNA, we analyzed DNA synthesis after a 1 h pulse of the siRNA-treated cells with BrdU, followed by flow cytometry using an anti-BrdU antibody.

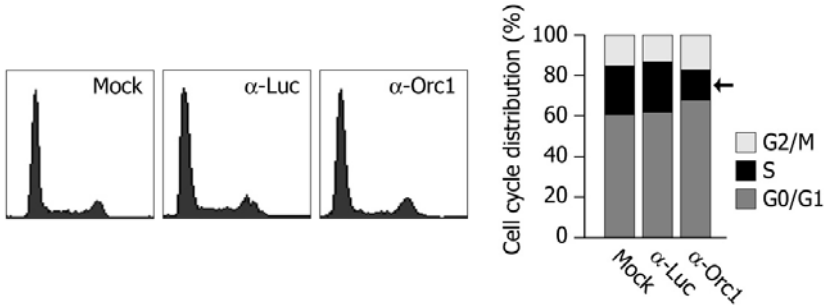

Figure 24. Down-regulation of Orc1 determined a remarkable reduction of the number of cells in the S phase of the cell cycle. Flow cytometry profiles of U2OS cells treated for 72 h with the indicated siRNAs. The histogram on the right side shows the distribution of the cells in the different phases of the cell cycle; the reduction in the number of S-phase cells after Orc1 silencing is indicated by an arrow.

As shown in Fig. 25, the number of cells with a DNA content between 2n and 4n that incorporated BrdU (S-phase cells) was reduced from 21.5% to 14.7%, thus indicating that the consequence of the Orc1 knock down is a marked inhibition of DNA synthesis. This inhibition was even more pronounced in the Rb-null Saos-2 cells (from 18.1% to 7.9% cells in S-phase after Orc-1 siRNA treatment; data not shown). In the siRNA treated cells, we studied the recruitment of Orc1, Orc2, Rb and E2F1 to the lamin B2 origin, as well as to the two origins in the GM-CSF gene domain. We found that binding of Orc2 and Rb was not significantly affected by the Orc1 knock down (Fig. 26).

In contrast, Orc1 depletion induced a marked increase in E2F-1 binding, a result which is consistent with the ChIP binding data in the cell cycle, which clearly showed a mutually exclusive recruitment of Orc1 and E2F-1 over the origin region. No significant differences were detected among the three analyzed origins.

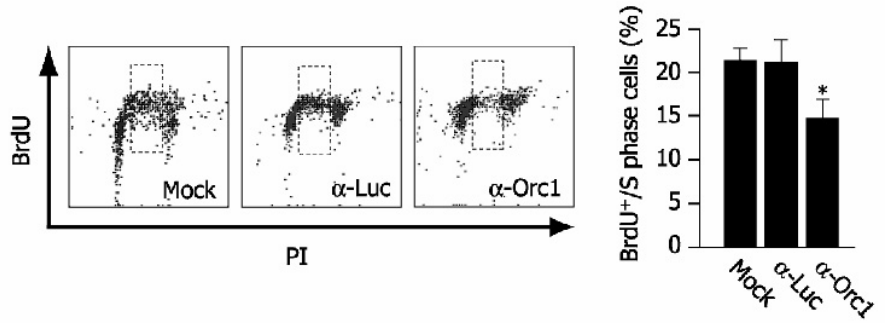

Figure 25. Down-regulation of Orc1 inhibits DNA replication. Flow cytometry profiles showing simultaneously detection of DNA content (propidium iodide staining) and BrdU incorporation (anti-BrdU antibody) at 72 h after RNAi. The dashed boxes indicate BrdU positive, S-phase cells. The histogram on the right side reports the percentage of S-phase/BrdU positive cells (mean±sem, indicated by error bars) of three different experiments. The asteric (*) indicates significant statistically difference between ORC depletion experiments and luciferase control experiments.

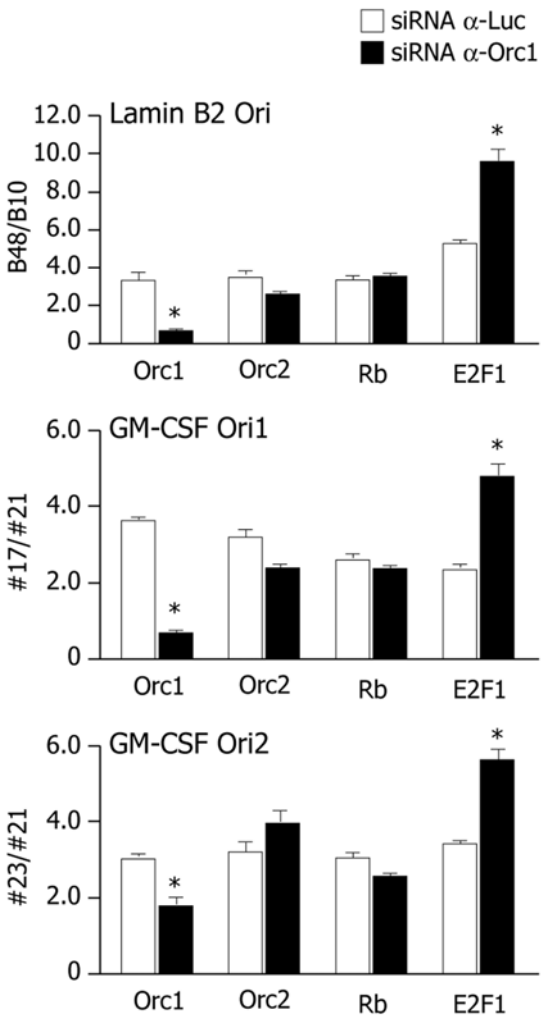

Figure 26. Down-regulation of Orc1 enhances E2F1 recruitment to origins of DNA replication. Results of ChIP experiments performed in U2OS cells at 72 h after siRNA silencing of Orc1. The histograms show the quantification of origin-specific, cross-linked and immunoprecipitated DNA for the Lamin B2 (upper graph), GM-CSF Ori1 (middle graph) and GM-CSF Ori2 (lower graph) origins after immunoprecipitation using the antibodies shown below each bar pair. The results are expressed as fold of enrichment of the specific origin sequences over a neighboring control sequence, as shown in Figs 1a and 1c. The means±sem of at least three different experiments are shown. The asterisk (*) indicates statistically significant difference between ORC depletion experiments and control Luc experiments.

3 Discussion

The work presented in this manuscript presents clear-cut evidence that both Rb and E2F are part of the protein complex that is recruited to origins of DNA replication at the G1 phase of the cell cycle. This observation raises series obvious issues, including the identification of the molecular determinants responsible for their recruitment, the understanding of the relationship of these proteins with the other components of the pre-RC and, most notably, the definition of their actual function in regulating origin activity.

As shown by the immunoprecipitation experiments, Rb and E2F1 are recruited to the lamin B2 origin as well as to the two investigated origins downstream of the human GM-CSF gene; enrichment for the two factors is detected at the same location where other components of the pre-RC are found and in close correspondence to the sites of nascent strand DNA synthesis (Todorovic et al., 2005). These three origins are rather dissimilar in both primary sequence and chromatin context. The lamin B2 origin encompasses the 3' end of the lamin B2 gene and the promoter of the TIMM13 gene, which is located downstream. This region is transcribed at high levels and in a constitutive manner (Biamonti et al., 1992). In contrast, GM-CSF Ori1 and GM-CSF Ori2 are located ~7 Kb downstream of the GM-CSF gene, in a region that shows no apparent transcription or canonical marks of the presence of cis-acting transcriptional regulation elements. In addition, the three origins show neither obvious primary sequence similarity nor the presence of canonical E2F binding sites. Given these considerations, our first speculation has been that binding of Rb/E2F to the origin region might be mediated through the interaction of either protein with known components of the pre-RC. Indeed, our results support the model in which Rb is bound near ORC at origins of DNA replication by forming a specific complex with the largest ORC subunit, Orc1. In particular, the in vitro data that collectively support the specificity of this novel interaction indicate that binding of Orc1) is specific for Rb and not for other pocket family members, ii) requires the integrity of the C-terminus of Rb, iii) involves the central portion of Orc1;

and iv) occurs in an LxCxE motif-independent manner. In addition, the in vivo data show physical interaction between Orc1 and Rb inside the nucleus (FRET) and preferential binding of Orc1 to the hypophosphorylated form of Rb (co-immunoprecipitations). However, it should be clearly pointed out that the recruitment of Rb to origins during the normal cell cycle occurs at a time point at which Orc1 is not yet bound (such as at 5 hr after entry into G1 after stimulation of serum-starved T98G cells) or in conditions in which Orc1 is knocked down (as in the siRNA experiments). Thus, binding of Rb to the origin area definitely precedes, and thus is independent of, binding of Orc1, and therefore, the assembly of the pre-RC; what might be the determinants of Rb recruitment, therefore, still remain elusive. In this respect, it is worth mentioning that Rb has been shown to localize to multiple discrete DNA foci during S phase (Barbie et al., 2005) and that its docking to chromatin might thus be dependent on its interaction with other cellular factors. Among the over 100 factors other than E2F that specifically bind Rb (Morris and Dyson, 2001) there are other proteins that participate in the DNA replication process, including MCM7, MCM4, DNA polymerase α and replication factor C (Gladden and Diehl, 2003; Pennaneach et al., 2001; Schmitz et al., 2004; Sterner et al., 1998; Takemura et al., 1997). Binding to these factors, however, is likely to occur at a later stage during the origin activation process.

As far as E2F1 is concerned, our *in vitro* competition GST-pulldown assays and our in vivo ChIP data along G1 phase progression and after Orc1 knock down indicate a mutually exclusive binding of Rb to either Orc1 (early G1) or E2F1 (late G1 and S phase). Indeed, the integrity of the whole Rb C-terminal region, and, in particular, of the C domain, is required for both binding to Orc1 (our findings) as well as to E2F1 (Dick and Dyson, 2003). The finding that E2F1 displaces Orc1 from origin-bound Rb during late G1 are consistent with the consolidated notion that Orc1 leaves chromatin in late G1 and during S-phase (Li and DePamphilis, 2002). Intriguingly, both Rb and E2F1 have been found associated with DNA replication foci in primary cells during early S-phase, a time point at which the canonical regulation of Rb by G1-phase cyclin-CDK

phosphorylation would instead predict its dissociation from the transcription factor (Weinberg, 1995). Indeed, these results are perfectly consistent with our ChIP data on origin DNA. On this ground, we propose that the primary determinant of E2F binding to the origin regions might not be primary DNA sequence recognition by E2F itself, but its interaction with chromatin-bound Rb.

The finding that Rb and E2F1 associate with origins of DNA replication raises the obvious possibility that these proteins might directly regulate some aspects of origin function. The kinetics of recruitment of the different factors onto the origin region (Rb and Orc1 during early G1 phase, Rb and E2F1 during late G1, along with other pre-RC components (Zannis-Hadjopoulos et al., 2004); the preferential binding of Orc1 to the hypophosphorylated form of Rb; and, finally, the presence of HDAC1 onto the origin region in early G1 all suggest that the Rb/Orc1 complex negatively regulate origin function. Indeed, this conclusion is consistent with the observation that mutations in the *Drosophila* Rb (as well as E2F) homologues fail to limit DNA replication through their interactions with DmORC (Bosco et al., 2001). This negative function might be exerted by a variety of mechanisms, which recapitulate the known properties of hypophosphorylated Rb, including the modulation of chromatin conformation, or the direct negative regulation on components of the replication licensing machinery.

In conclusion, our results show that Rb participates in the formation of the protein complex that regulates DNA replication origins during the normal cell cycle in mammalian cells, in addition of being a major component of the intra-S-phase checkpoint response after γ-irradiation (Avni et al., 2003). The observations that Rb is essential for a proper spatial organization of DNA replication in mammalian cells (Barbie et al., 2004) that primary cells approaching senescence undergo pRB-dependent, large-scale changes in chromatin structure (Narita et al., 2003) and that cell cycle exit and terminal differentiation are mediated by Rb (Sidle et al., 1996) all raise the important question of whether the actual mechanisms mediating some of these effects

might be the specific suppression of DNA replication initiation by origin-bound Rb.

Acetylation of human Cdc6 by GCN5 acetyltransferase regulates site-specific, CDK-mediated protein phosphorylation in the S phase of the cell cycle.

Specific contribution of Roberta Paolinelli to the work described in this manuscript: I have carried out most of the work described in this manuscript as my main PhD research project.

1 Summary

In eukaryotic cells, the Cdc6 protein is essential to promote the assembly of pre-replicative (pre-RC) complexes in the early G1 phase of the cell cycle. In budding yeast, the protein is then degraded as a mechanism to ensure proper origin licensing once-per-cell cycle. In metazoans, however, Cdc6 remains stable through the entire S phase and mitosis, and other mechanisms have evolved to prevent inappropriate origin activation. Here we show that, in late G1 and early S phase, Cdc6 is found in a complex also containing Cyclin A/Cdk2 and the acetyltransferase GCN5. We discovered that GCN5 specifically acetylates Cdc6 at three residues flanking its cyclin-docking motif and that this modification is crucial for the subsequent phosphorylation of the protein by Cyclin A-CDKs at a specific residue close to the acetylation site. GCN5-mediated acetylation and site-specific phosphorylation of Cdc6 are both necessary for the relocalization of the protein to the cell cytoplasm, as well as regulating its stability. We propose that this two-step intramolecular signaling regulatory program by sequential modification of Cdc6 is essential to ensure proper S-phase progression.

2 Results

Cdc6 associates with a nuclear HAT and is acetylated *in vivo*

While searching for cellular proteins that interact with known members of the pre-RC and are capable to modify chromatin at DNA replication origins, we incubated HeLa cell nuclear extracts with immobilized recombinant GST-Cdc6 and purified histones in the presence of [^{14}C]-acetyl-CoA. We indeed found that Cdc6 associated with a nuclear factor(s) possessing HAT activity. Strikingly, we also observed that GST-Cdc6, but not GST control, was itself a substrate for acetylation (Fig. 1).

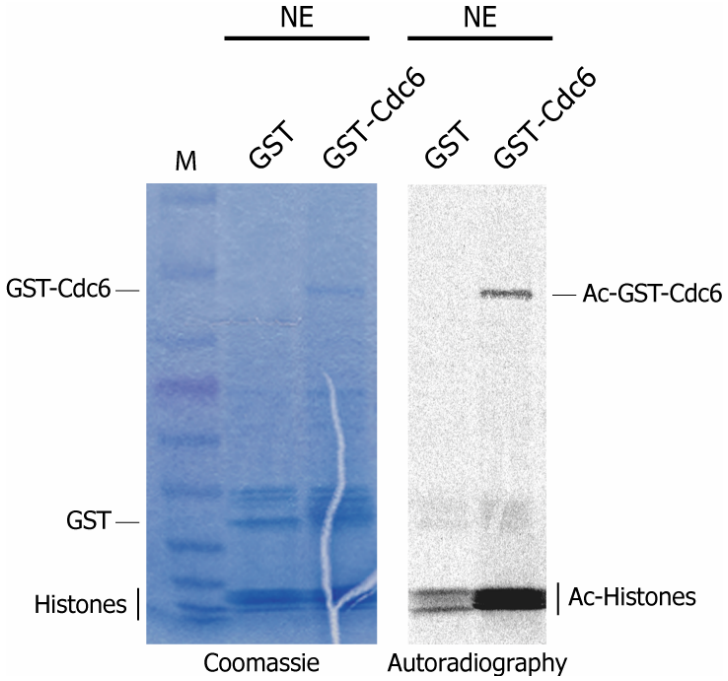

Figure 1. Cdc6 protein is acetylated. Cdc6 is acetylated by nuclear HATs *in vitro.* Hela cells nuclear extracts were incubated with bacterially-purified GST-Cdc6 or GST and purified histones on agarose beads in the presence of ^{14}C acetyl-CoA; after incubation, the reaction mixture was resolved by SDS-PAGE and the gel exposed to Cyclone screen (right panel). Left panel: Coomassie-stained gel.

We performed a similar assay incubating the nuclear extracts with GST-Rb ABC, a known substrate of acetylation (Chan et al., 2001), obtaining a similar result (Fig. 2).

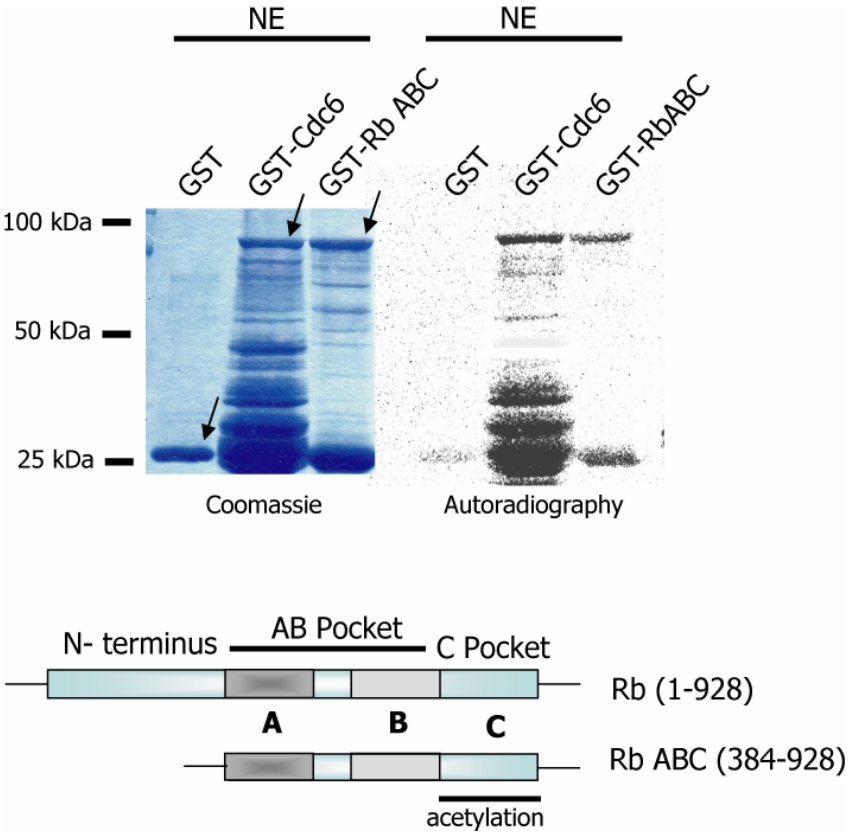

Figure 2. Cdc6 protein is acetylated. Cdc6 is acetylated by nuclear HATs *in vitro.* Hela cells nuclear extracts were incubated with bacterially-purified GST-Cdc6, GST-Rb ABC or GST on agarose beads in the presence of ^{14}C acetyl-CoA; after incubation, the reaction mixture was resolved by SDS-PAGE and the gel exposed to Cyclone screen (right panel). Left panel: Coomassie-stained gel. Below: a representative scheme shows the deleted form of Retinoblastoma protein, used in the assay as positive control, containing the domain critical for acetylation (Chan et al., 2001).

To detect acetylation of endogenous Cdc6, extracts from HeLa cells were immunoprecipitated using an anti-acetyl-lysines antibody and immunoblotted using an anti-Cdc6 antibody and *viceversa* in two different cell lines (Fig. 3). The

immunoprecipitation specifically detected an acetylated band of 63 kDa corresponding to endogenous Cdc6 protein; analogous findings were also obtained in 293T and U2OS human cell lines (not shown).

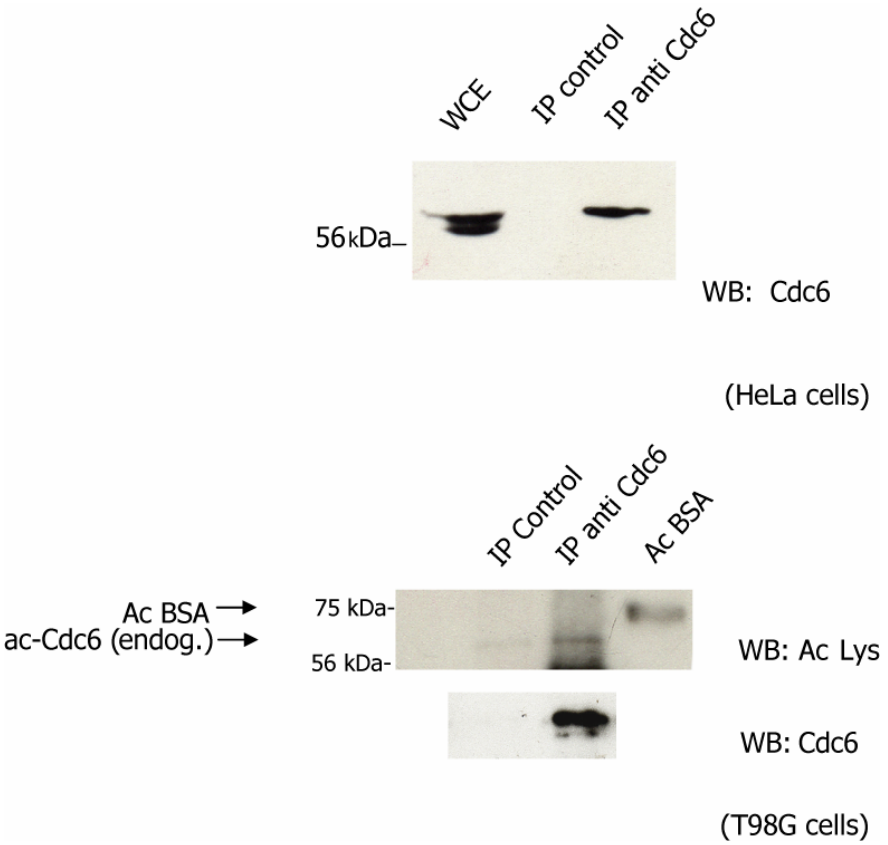

Figure 3. *In vivo* acetylation of endogenous Cdc6. Extracts of HeLa cells were immunoprecipitated with an anti Ac-Lys antibody and immunoblotted using an anti-Cdc6 antibody (IP anti-Ac-Lys) or a control antibody (IP control). The lower band visible in the Cdc6 immunoblot marks probably a background band as other authors have previously described for the same antibody (Duursma and Agami, 2005a). Extracts of T98G cells were immunoprecipitated with an anti-Cdc6 antibody (IP anti-Cdc6) or with a control antibody (IP control) and immunoblotted with an anti Ac-Lys antibody. 10 ng of acetylated BSA (Ac BSA, Sigma) was loaded as hybridization control.

To confirm the.*in vivo* acetylation of Cdc6 further, 293T cells were transfected with Flag-tagged Cdc6, treated with trychostatin A (TSA), an inhibitor of cellular

deacetylases, and followed by immunoprecipitation with an anti-Flag antibody and immunoblotting with an anti-acetyl-lysine antibody. Acetylated Flag-Cdc6 could be readily detected in the anti-Flag immunoprecipitates (Fig. 4). Taken together, these results indicate that Cdc6 associates with a nuclear HAT (HATs) and is modified by acetylation inside the cells.

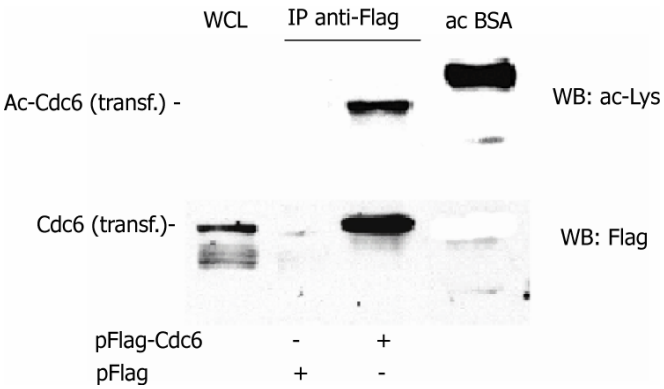

Figure 4. *In vivo* Flag-Cdc6 acetylation. Extracts of 293T cells transfected as indicated were immunoprecipitated with an anti-Flag antibody and immunoblotted using an anti Ac-Lys antibody (upper panel). The same filter was incubated with an anti-Flag antibody (lower panel). Cells were treated with 250 ng/mL of trychostatin A, an histone deacetylase inhibitor (TSA), for 16 hours.

GCN5 acetyltransferase binds and acetylates Cdc6 *in vitro* and *in vivo*.

By performing a series of *in vitro* HAT assays, we observed that Cdc6 was a specific substrate of recombinant GCN5 acetytransferase. GST-Cdc6 or GST alone were incubated with recombinant GCN5 in the presence of [^{14}C]-acetyl-CoA, the reaction products were separated by SDS-PAGE and detected by autoradiography. As shown in Fig. 5 A, GST-Cdc6, but not GST, scored clearly positive for acetylation. In addition to Cdc6, GCN5 and its major degradation product were also positive for acetylation, due to the autocatalytic activity of the enzyme (Col et al., 2001).

Moreover, GST-Cdc6 is a substrate of p300 acetyltransferase too, but with a lesser extent (Fig. 5 B). On the contrary, in this assay, we found that the

heterochromatin binding protein HP1α (see previous paragraphs for details) is strongly positive for p300-mediated acetylation.

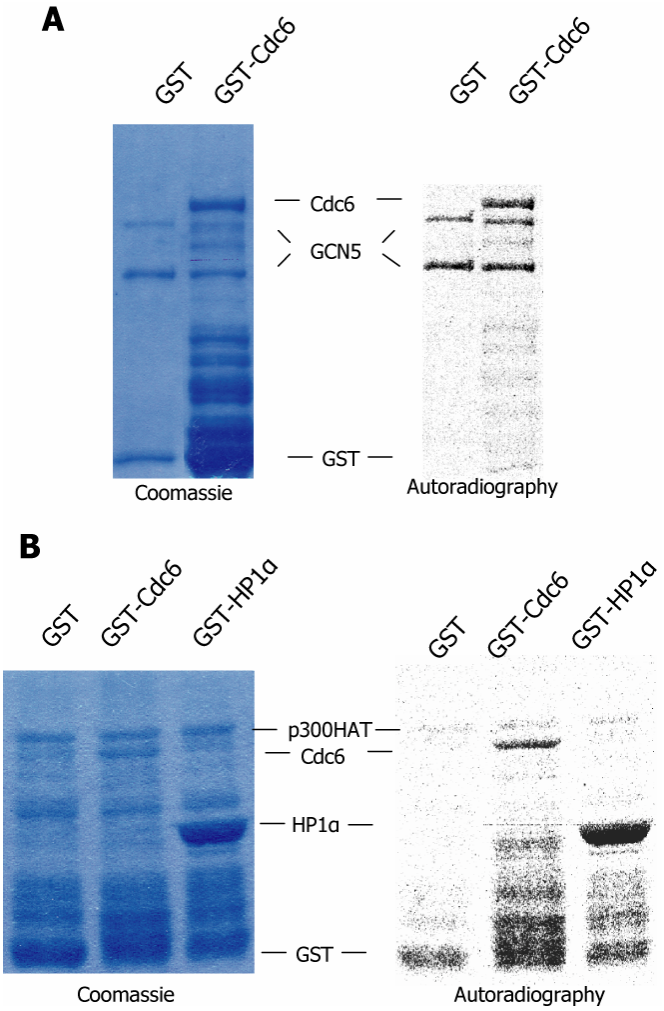

Figure 5. GST-Cdc6 is acetylated by GCN5 and p300 acetyltransferases *in vitro*. (A) Bacterially-purified and dyalised GST-GCN5 was incubated with GST-Cdc6 or GST on agarose beads in the presence of ^{14}C acetyl-CoA; after incubation, the reaction mixture was resolved by SDS-PAGE and the gel exposed to Cyclone screen. Right panel: gel exposed to Cyclone screen. The autoacetylated bands of GCN5 show the presence of HAT activity. Left panel: Coomassie-stained gel. (B) A bacterially purified truncated form of p300 containing the catalytic domain (Cereseto et al., 2005) of the protein was incubated with GST-Cdc6, GST-HP1α or GST. The autoacetylated band of p300 HAT show the presence of HAT activity.

To determine which portion of Cdc6 was acetylated by GCN5, a series of GST-Cdc6 fragments was generated, carrying C- or N-terminal deletions (Fig. 6); these mutants were then tested as GCN5 substrates by the *in vitro* HAT assay.

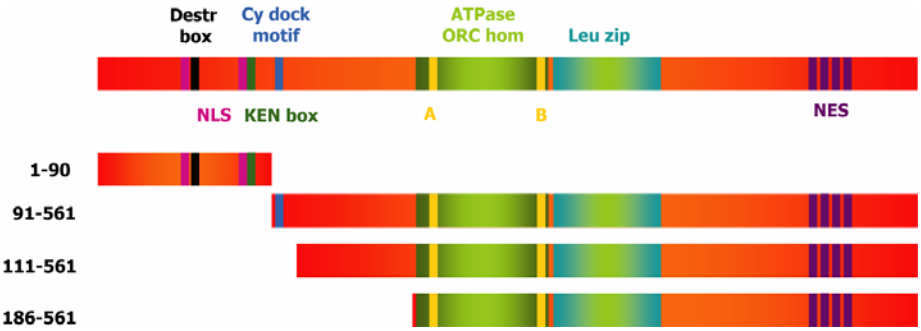

Figure 6. Representative scheme of Cdc6 aminoacidic domains used in the HAT assay shown in Fig. 7.

Acetylation was very low or undetectable for the GST-Cdc6 fragments encompassing either the C-terminus (aa 186-561) or the N-terminus (aa 1-90) of the protein (Fig. 7). Fragment 91-561 was acetylated at a similar level as wild type Cdc6, while acetylation dropped to background levels in fragment 111-561. Thus, the target region for GCN5 acetylation lies between amino acids 91 and 110.

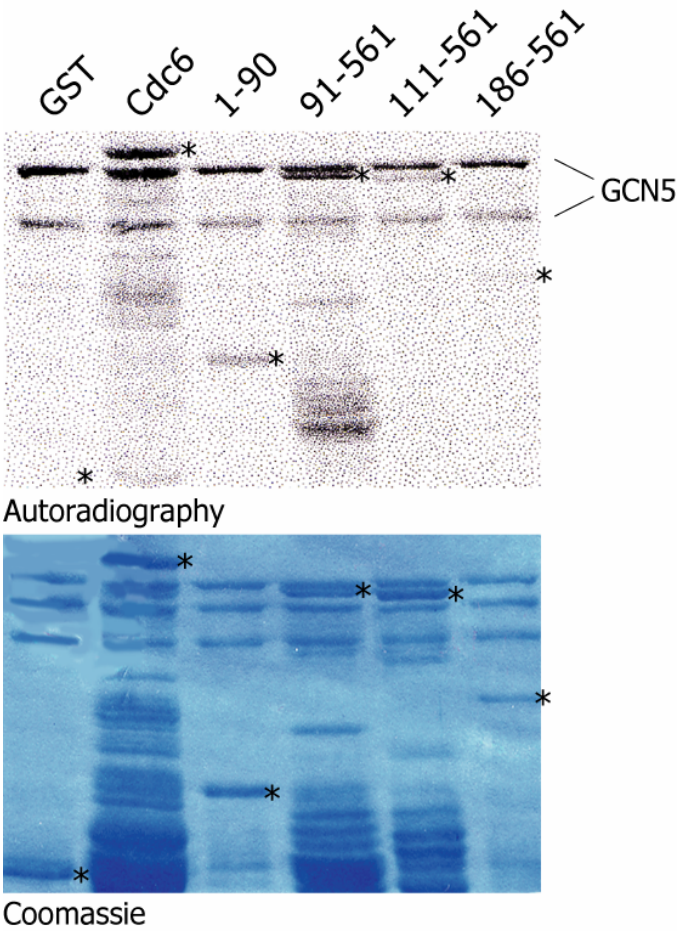

Figure 7. A 11 aminoacids-in length region of Cdc6 is critical for acetylation. GST-GCN5 was incubated with GST-Cdc6, deleted proteins or GST on agarose beads in the presence of ^{14}C acetyl-CoA; after incubation, the reaction mixture was resolved by SDS-PAGE and the gels exposed to Cyclone screen. Upper panel: gel exposed to Cyclone screen. Lower panel: Coomassie-stained gel.

Since this region contains three lysines at positions 92, 105, and 109, these amino acids were mutated, either one at a time or in combinations, into arginines, which bear the same positive charge as lysines, but cannot be modified by acetylation (Fig. 8). As shown in Fig. 9, all the singly mutated recombinant proteins scored positive for acetylation by GCN5. Acetylation of the

double mutants was significantly reduced, while the triple mutant (K3R) was not acetylated at all. From this set of experiments, we conclude that the lysines at positions 92, 105 and 109 are all critical for GCN5-mediated acetylation.

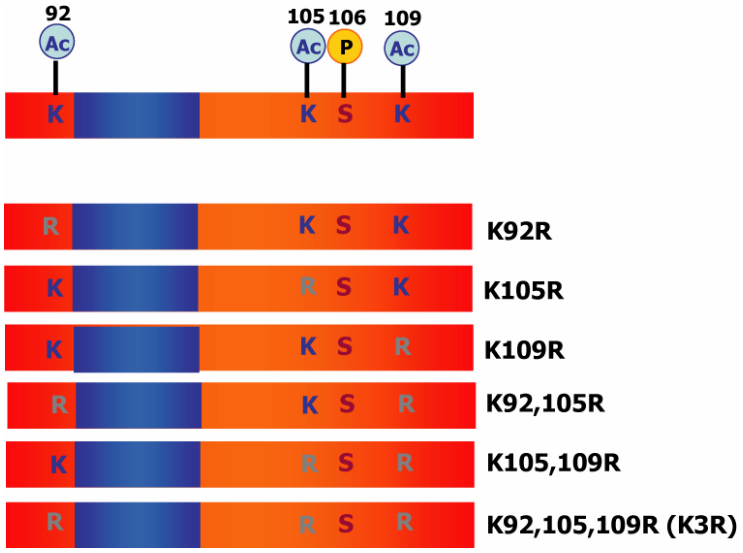

Figure 8. Representative scheme of the Cdc6 KR point mutants assayed for acetylation by GCN5 as shown in Fig. 9.

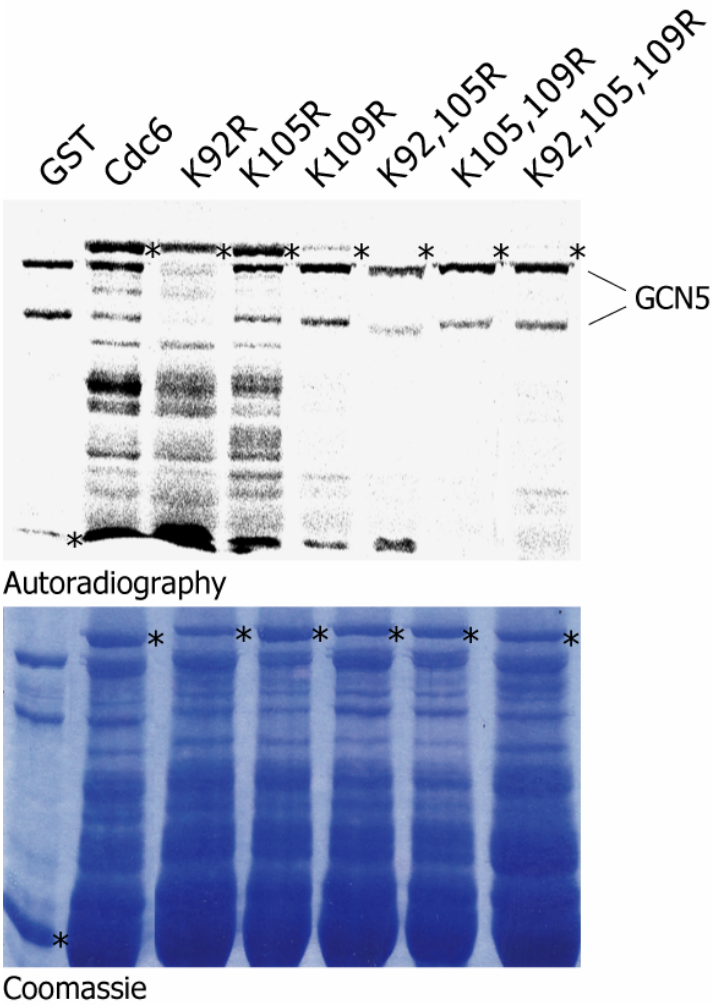

Figure 9. GCN5 acetylates 92, 105 and 109 lysine residues of Cdc6. The Cdc6 KR point mutants were assayed for acetylation by GST-GCN5. Upper panel: gel exposed to Cyclone screen. Lower panel: Coomassie-stained gel.

Acetylation of several cellular factors by HATs, including other pre-RC components (Burke et al., 2001; Iizuka and Stillman, 1999; Takei et al., 2002; Takei et al., 2001) is concomitant with the specific binding of the enzyme to its substrate. We therefore tested whether GCN5 might directly bind Cdc6. In a series of GST-pull down experiments, we indeed found that the two proteins specifically interacted *in vitro*. We found that the interaction involved the N-terminal region of Cdc6, since a fragment encompassing Cdc6 aa 1-60 was still capable of binding GCN5 (Figg. 10 and 11), and the C-terminus of GCN5, since fragments 384-476 (corresponding to the bromodomain) (Yang, 2004b) and 271-383 (which separates the bromodomain from the HAT domain) of GCN5 were both capable to independently bind Cdc6 (Figg. 12 and 13).

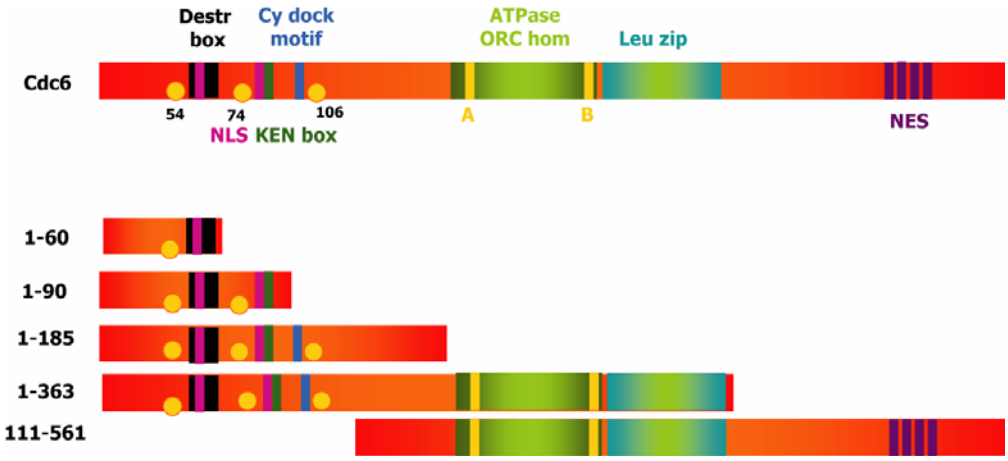

Figure 10. Representative scheme of Cdc6 aminoacidic domains used in the GST Pull down assay shown in Fig. 11.

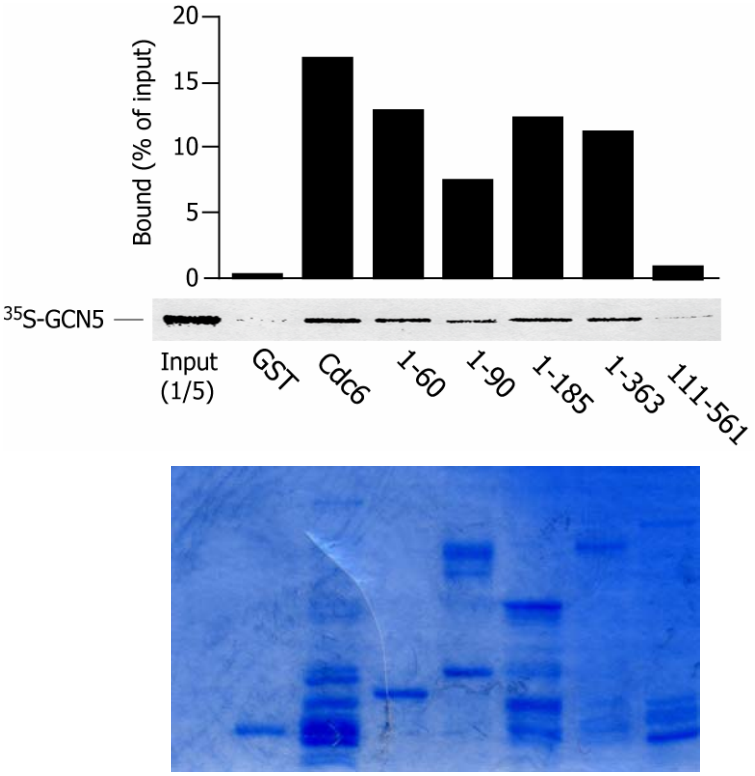

Figure 11. GCN5 binds the N-terminal region of Cdc6 *in vitro*. Bacterially-purified GST-Cdc6, GST or Cdc6 deleted mutants (1-90, 1-185, 1-363, 11-561) on agarose beads were incubated with fixed amount of ^{35}S-GCN5 and analysed on an SDS-PAGE gel. The graphs express the amounts of bound proteins as percentages of the input of radiolabeled proteins after exposition of the gel to Cyclone screen. Lower panel: Coomassie-stained gel.

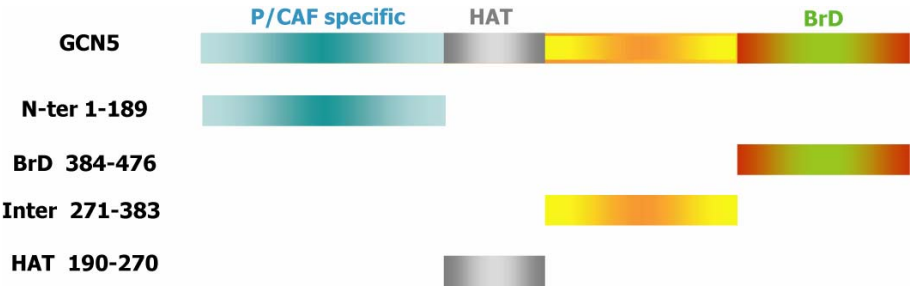

Figure 12. Representative scheme of GCN5 aminoacidic domains used in the GST Pull down assay shown in Fig.13.

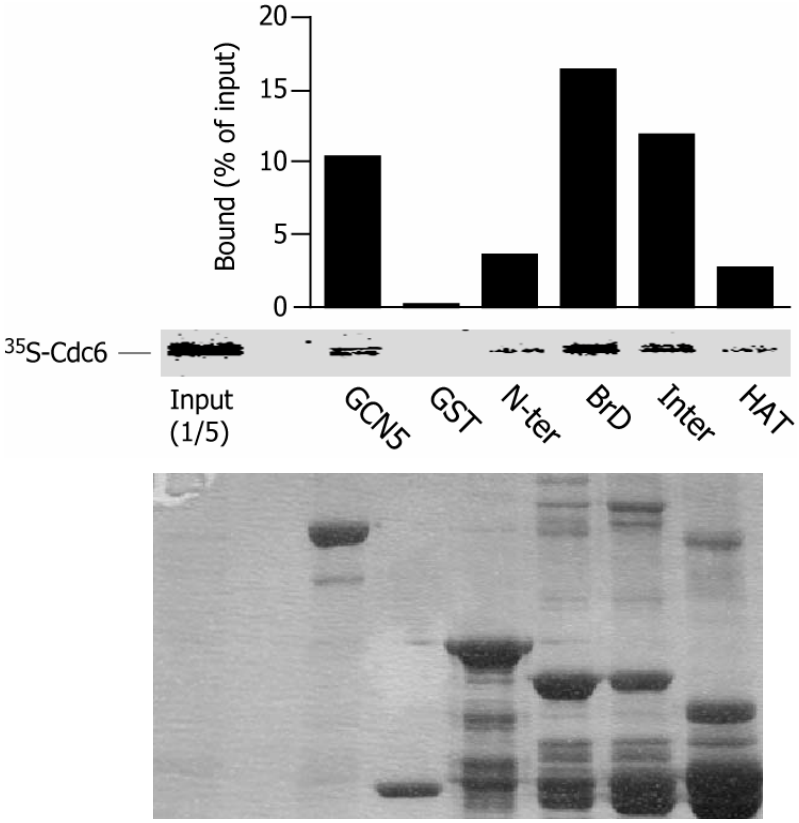

Figure 13. Cdc6 binds the C-terminal region of GCN5 containing the BROMO domain *in vitro* . Bacterially-purified GST-GCN5, GST or GCN5 deleted mutants (1-189, 190-270, 271-383, 384-476) on agarose beads were incubated with fixed amount of ^{35}S-Cdc6 and analysed on an SDS-PAGE gel. The graphs express the amounts of bound proteins as percentages of the input of radiolabeled proteins after exposition of the gel to Cyclone screen. Lower panel: Coomassie-stained gel.The HAT domain of GCN5 scored negative.

Binding between the two proteins also occurred inside the cells, since endogenous Cdc6 co-immunoprecipitated with endogenous GCN5 when using anti-GCN5 or *viceversa* (Fig. 14). Overexpressed GCN5 also behaved similar to endogenous GCN5 in binding to cellular Cdc6 (Fig. 15). Of interest, a catalytically inactive GCN5 mutant, which bears two amino acid substitutions (Y260A and

F261A) at critical residues within the enzyme's catalytic site (GCN5mut) (Paulson et al., 2002) was also capable of binding Cdc6, further supporting the notion that the interaction between the two proteins does not involve the HAT domain of GCN5.

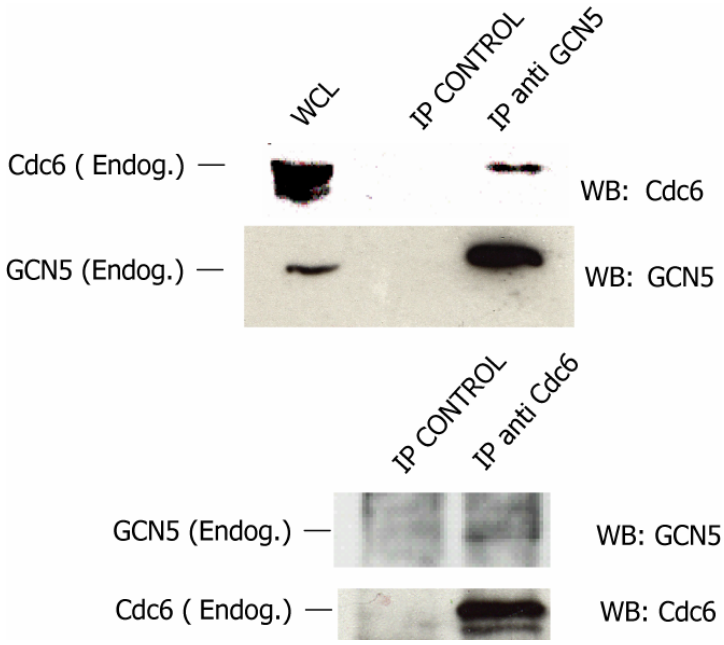

Figure 14. GCN5 binds Cdc6 *in vivo*. Extracts of HeLa cells were immunoprecipitated with an anti-GCN5 antibody (IP anti-GCN5) or a control antibody (IP CONTROL) and immunoblotted using an anti-Cdc6 antibody. The same filter was incubated with an anti-GCN5 antibody (upper panels). Extracts of T98G cells were immunoprecipitated with an anti Cdc6 antibody (IP anti-Cdc6) or a control antibody (IP CONTROL) and immunoblotted using an anti-GCN5 antibody. The same filter was incubated with an anti-Cdc6 antibody (lower panels).

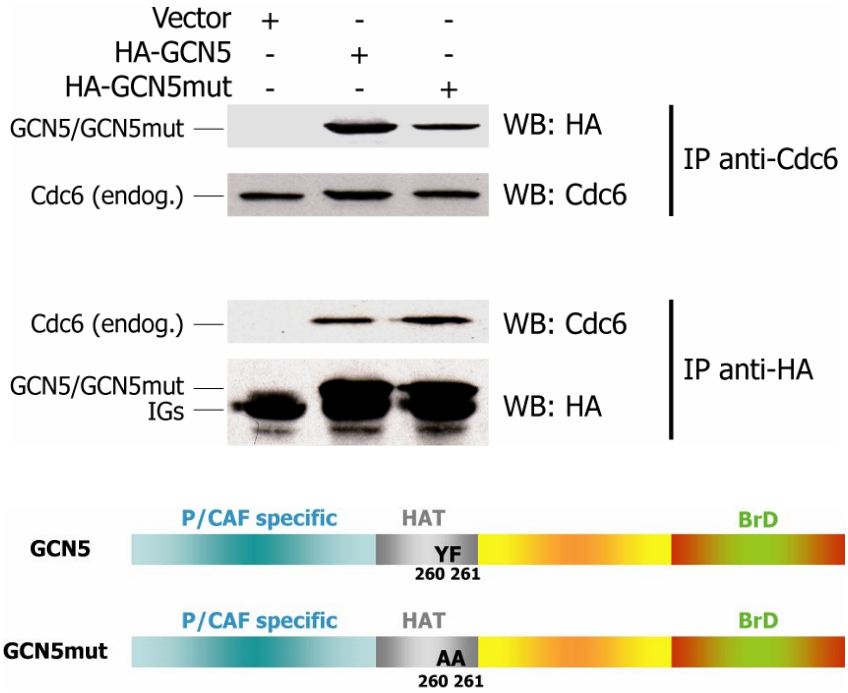

Figure 15. Endogenous Cdc6 binds HA-GCN5/GCN5mut *in vivo*. Extracts of HeLa cells transfected as indicated were immunoprecipitated using an anti Cdc6 antibody and immunoblotted using anti-HA antibody. The same filters were incubated with an anti-Cdc6 antibody (upper panels). The same lysates were immunoprecipitated using an anti-HA antibody and immunoblotted using an anti-Cdc6 antibody and the same filters incubated with an anti-HA antibody (lower panels). The scheme below shows the two point mutations on the catalytic domain of GCN5 that disrupt its catalytic activity (GCN5mut).

Moreover, we have demonstrated that Flag-Cdc6 co-immunoprecipitates with HA-GCN5 (Fig. 17).

To start exploring the effects of GCN5-mediated Cdc6 acetylation inside the cells, we transfected HeLa cells with vectors expressing either wt GCN5 or GCN5mut. The levels of endogenous acetylated Cdc6 (detected by an anti-acetyl-lysine antibody) were significantly enhanced by the expression of enzymatically active GCN5, but not of its inactive mutant, despite the two proteins were expressed at similar levels (Fig. 16).

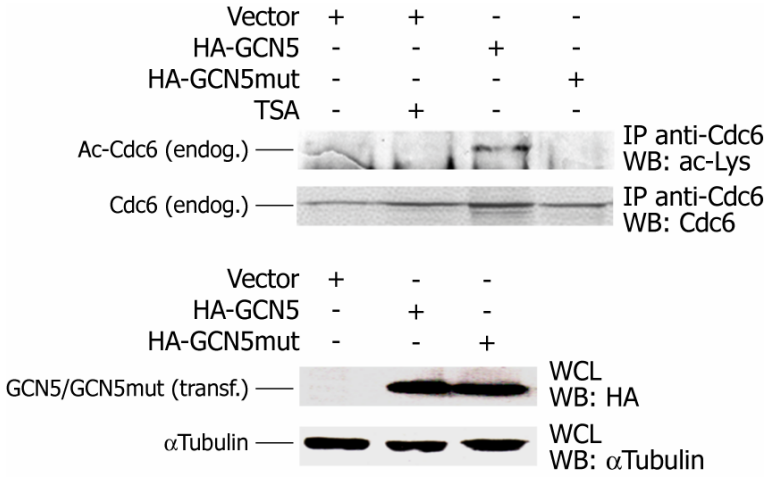

Figure 16. *In vivo* HA-GCN5-dependent Cdc6 acetylation. Extracts of HeLa cells transfected as indicated were immunoprecipitated with an anti-Cdc6 antibody and immunoblotted using an anti Ac-Lys antibody. The same filter was incubated with an anti-Cdc6 antibody (upper panels). The same lysates (lower panels) to show the expression level of HA-GCN5 and HA-GCN5mut. αTubulin is shown as loading control.

Enhancement of Cdc6 acetylation by GCN5 expression was higher than that obtained by cell treatment with TSA. We also tested the effects of the expression of GCN5 and GCN5mut on transfected wt Cdc6 or Cdc6 (K3R) in 293T cells. Similar to endogenous Cdc6, acetylation of transfected wt Cdc6 was also markedly increased in response to wt GCN5 but not to GCN5mut expression.Consistent with the *in vitro* acetylation data, the Cdc6 (K3R) mutant was not sensitive to GCN5 overexpression (Fig. 17). Of interest, however, this protein was still found acetylated inside the cells, indicating that other HATs might also acetylate Cdc6 *in vivo* at different lysine residues. In addition, this mutant, while not a substrate for GCN5 acetylation, still co-immunoprecipitated with the enzyme. This finding further reinforces the notion that binding between the two proteins does not involve the region of Cdc6 which is acetylated by GCN5.

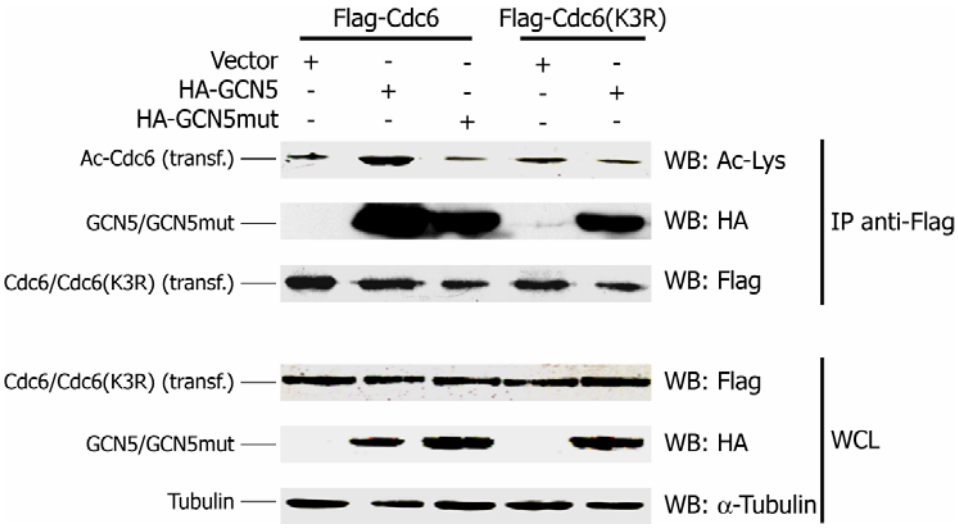

Figure 17. GCN5 acetylates Cdc6 *in vivo*. Ha-GCN5 specifically acetylates 92, 105 and 109 lysines *in vivo*. Extracts of 293T cells transfected as indicated were immunoprecipitated using an anti Flag antibody and immunoblotted using an anti Ac-Lys, an anti-HA or an anti-Flag antibodies. The unacetylable FLAG-Cdc6 mutant is still able to bind GCN5. Below, the same lysates were immunoblotted with anti-HA, anti-FLAG and anti-α Tubulin antibodies.

GCN5-mediated acetylation of Cdc6 affects Ser106 phosphorylation

Taken together, the results reported above indicate that Cdc6 associates with and is specifically acetylated by GCN5 at lysines 92, 105 and 109 both *in vitro* and inside the cells. Of interest, the acetylated lysine residues frame the Cyclin-docking motif of Cdc6 (Delmolino et al., 2001), and, in particular, lysine 105 is adjacent to serine 106, one of the three Cdc6 serine residues (54, 74 and 106) that are specifically phosphorylated by the CDKs that control the cell cycle (Jiang et al., 1999; Petersen et al., 1999). We therefore questioned whether GCN5-dependent acetylation might affect Cdc6 phosphorylation. We initially observed that the overexpression of GCN5 determined a selective increase in the levels of Cdc6 phosphorylated at S106, as shown by immunoprecipitation using an anti-phospho-S106 antibody followed by western blotting using an anti-Cdc6 antibody (Fig. 18).

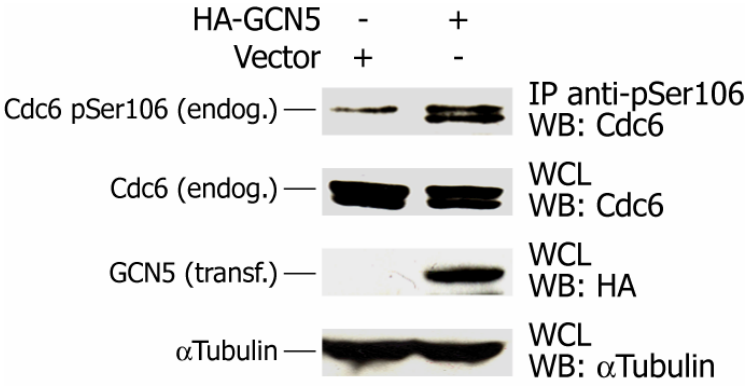

Figure 18. pSer106 Cdc6 expression increases upon GCN5 overexpression in human cells. Extracts of HeLa cells transfected as indicated were immunoprecipitated with an anti-pSer106 Cdc6 antibody and immunoblotted using an anti-Cdc6 antibody. The same lysates were immunoblotted using anti-Cdc6, anti-HA or anti-α Tubulin antibodies.

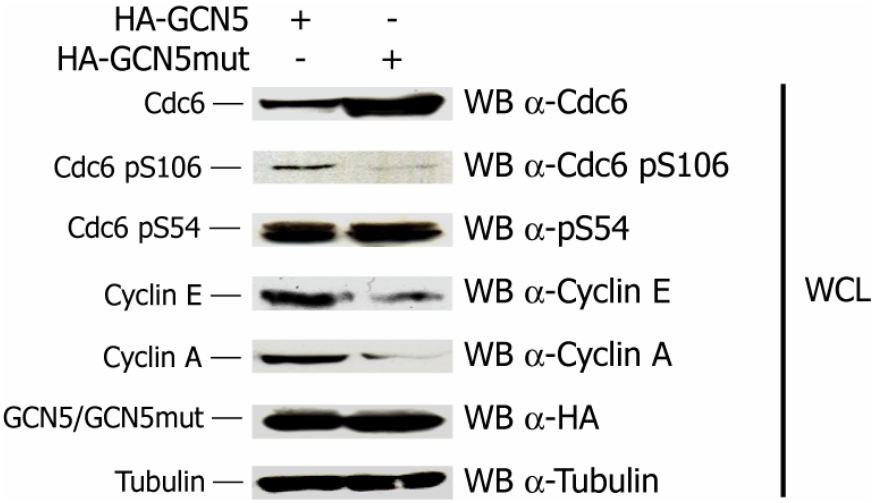

Figure 19. GCN5 specifically enhances pSer106 Cdc6 expression. Extracts of HeLa cells transfected as indicated were incubated with anti–Cdc6, anti-pSer106 Cdc6, anti-pSer54 Cdc6, anti-Cyclin E, anti-Cyclin A, anti-HA or anti-α Tubulin antibodies.

The same result was also evident by the direct visualization of the levels of phosphorylated Cdc6 in total cell lysates from transfected cells (Fig. 19). In the same lysates, no modification was observed for the levels of phospho-S54 Cdc6.

In contrast to wt GCN5, the expression of the HAT-inactive version of the protein did not affect Cdc6 S106 phosphorylation, but led to a significant accumulation of protein inside the cells, and to the decrease in the levels of Cyclin E and, most notably, of Cyclin A, suggestive of a perturbation of cell cycle progression.

These results initially disclosed an unexpected link between GCN5-mediated Cdc6 acetylation and the specific phosphorylation of the protein on S106. To further explore this issue, we analyzed the levels of phosphorylation of transfected wt Cdc6 of the Cdc6(K3R) mutant, and of an additional mutant we constructed bearing an alanine to serine substitution at position 106 (Cdc6 (S106A; Fig. 20). Strikingly, the K3R mutant resulted to be not phosphorylated on S106, similar to the S106A mutant and unlike wt Cdc6. Both mutants, however, were still phosphorylated on S54 (Fig. 21). The anti-Cdc6 pS106 antibody was still able to recognize the K3R mutant when phosphorylated *in vitro* by Cyclin A/CDK, thus indicating that the K3R mutation *per se* did not impair epitope recognition (data not shown). Moreover, both the K3R and S106A mutants are still able to co-immunoprecipitate with endogenous Cyclin A (see Fig. 48), thus confirming that the Cyclin-docking motif remains functional. Moreover, phosphorylation of wt Flag-Cdc6 on S106 is not enhanced upon GCN5mut overexpression (not shown). Similar results were obtained with YFP-tagged constructs (Fig. 22).

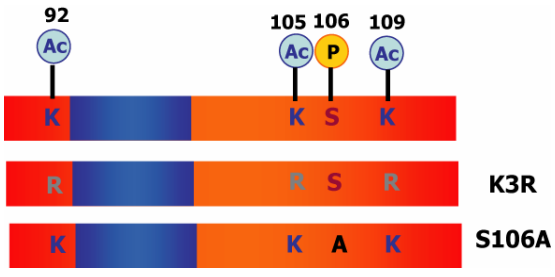

Figure 20. Schematic representations of the FLAG/YFP-tagged Cdc6 mutated proteins used in the *in vivo* assays shown in Figg. 21 and 22.

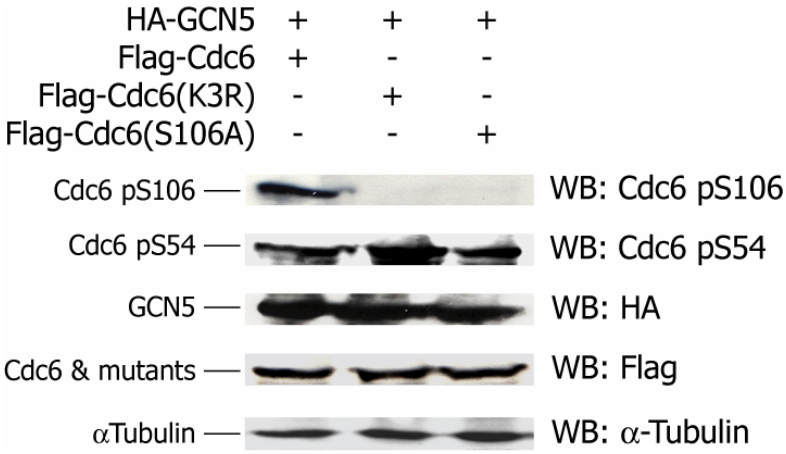

Figure 21. 92, 105 and 109 lysine residues are critical for Cdc6 phosphorylation on the single 106 serine residue. Extracts of 293T cells transfected as indicated were incubated using anti-pSer106 Cdc6, anti-pSer54 Cdc6, anti-HA, anti-Flag or anti-α-Tubulin antibodies.

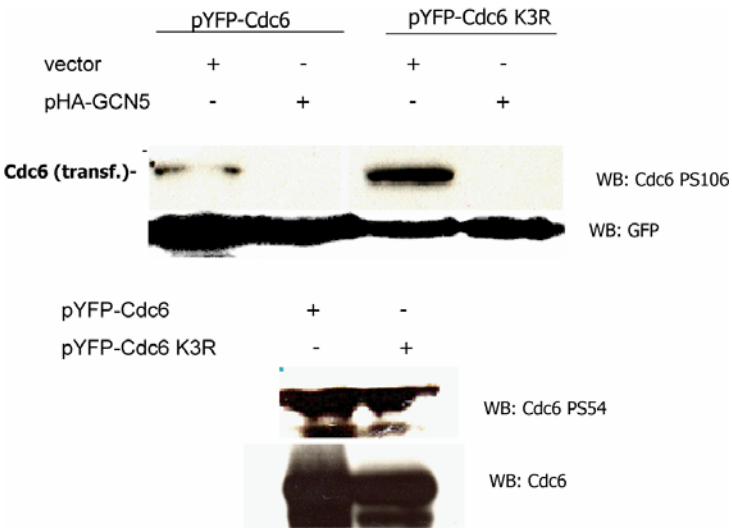

Figure 22. 92, 105 and 109 lysine residues are critical for Cdc6 phosphorylation on the single 106 serine residue. Extracts of 293T cells transfected as indicated were incubated using anti-pSer106 Cdc6 and anti-GFP antibodies. Below, the expression of pSer54 Cdc6 and Cdc6 has been assayed with specific antibodies.

Finally, to better define the role of GCN5 in mediating endogenous Cdc6 S106 phosphorylation, we knocked down GCN5 by RNA interference (Palhan et al., 2005). We observed that GCN5 depletion markedly increased the levels of total Cdc6 (Fig. 23) and selectively inhibited Cdc6 S106 phosphorylation, while leaving S54 phosphorylation unaltered (Fig. 24). In the same siRNA-treated cell lysates, the levels of MCM3 protein, a factor that that is also modified by acetylation (Takei et al., 2001), resulted unaltered. Taken all these results together, we conclude that GCN5-mediated acetylation perturbs the levels of Cdc6 protein by specifically affecting its phosphorylation on S106.

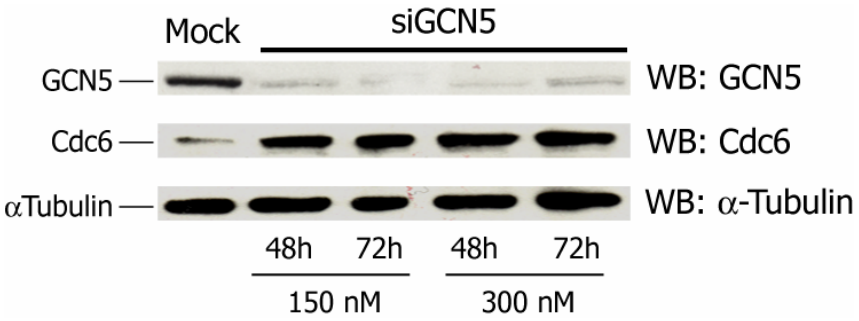

Figure 23. GCN5 depletion leads to endogenous Cdc6 accumulation. HeLa cells lysates transfected with water (mock) or different concentrations of a siRNA for GCN5 for 48 or 72 hours were immunoblotted using anti-GCN5, anti-Cdc6 or anti-Tubulin antibodies.

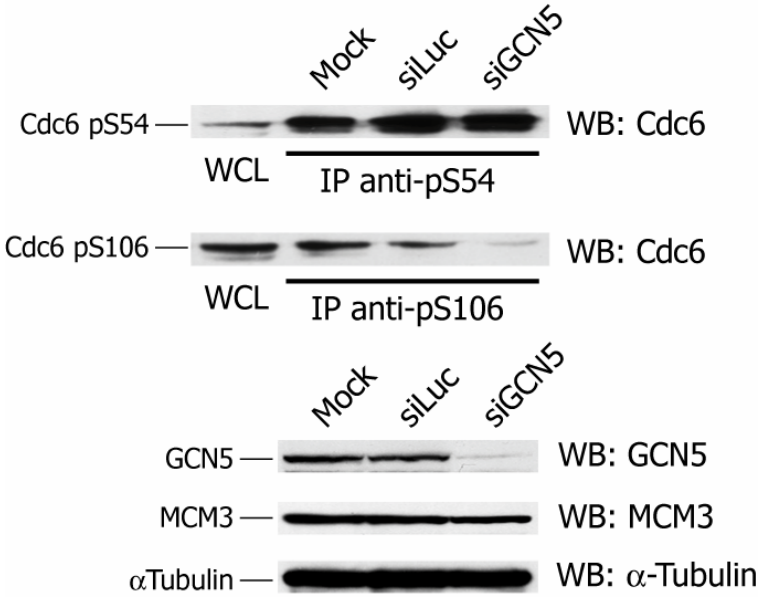

Figure 24. pSer106 Cdc6 level drammatically drops upon GCN5 knock down. HeLa cells lysates transfected with water (mock) or 100 nM GCN5- or Luciferase-siRNA for 72 hours were immmunoprecipitated using anti-pSer54 (upper panel) or anti-pSer106 (lower panel) antibodies and immunoblotted using an anti-Cdc6 antibody. Below, the same lysates were immunoblotted using anti-GCN5, anti-MCM3 or anti-α Tubulin antibodies.

GCN5 acetylates Cdc6 in early S-phase

Since the Cdc6 protein is a key regulator of replication competence, we analyzed the levels of Cdc6 acetylation in human glioblastoma T98G cells, which accumulate in G0 upon serum starvation and then synchronously enter G1 after re-addition of serum (Galbiati et al., 2005; Takahashi et al., 2000) (Fig. 25). The levels of GCN5 were reduced in cell lysates from serum-starved cells as compared to asynchronous cells, while they started to increase 6 h after serum addition (early G1), peaked at 20 h (early S), and decreased at 24 h (mid S-phase) (Fig. 27 A). Cdc6 were almost undetectable under serum starvation and started to progressively increase from 16 h after serum re-addition onward.

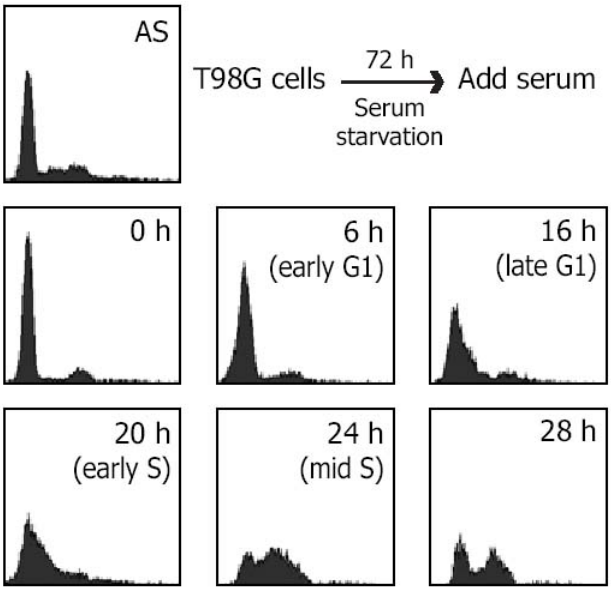

Figure 25. T98G cells synchronization. The flow cytometry profiles show the cellular DNA content after propidium iodide staining at different times (0, 6, 16, 20, 24, 28 h) after serum addition.

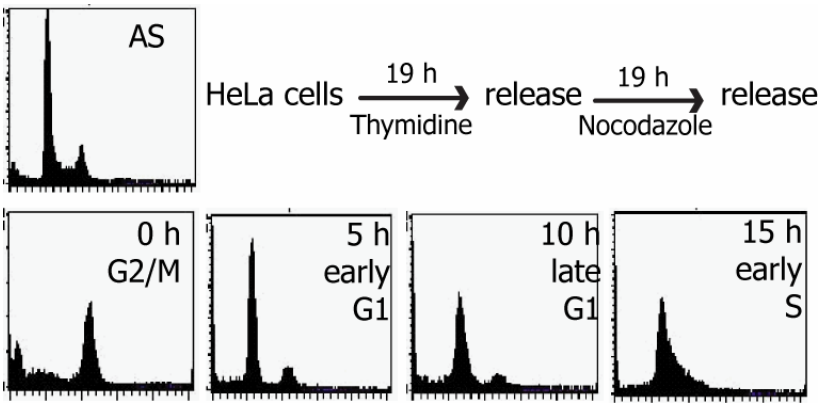

Figure 26. HeLa cells synchronization. The flow cytometry profiles show the cellular DNA content after propidium iodide staining at different times (0, 5, 15, 20 h) after nocodazole release.

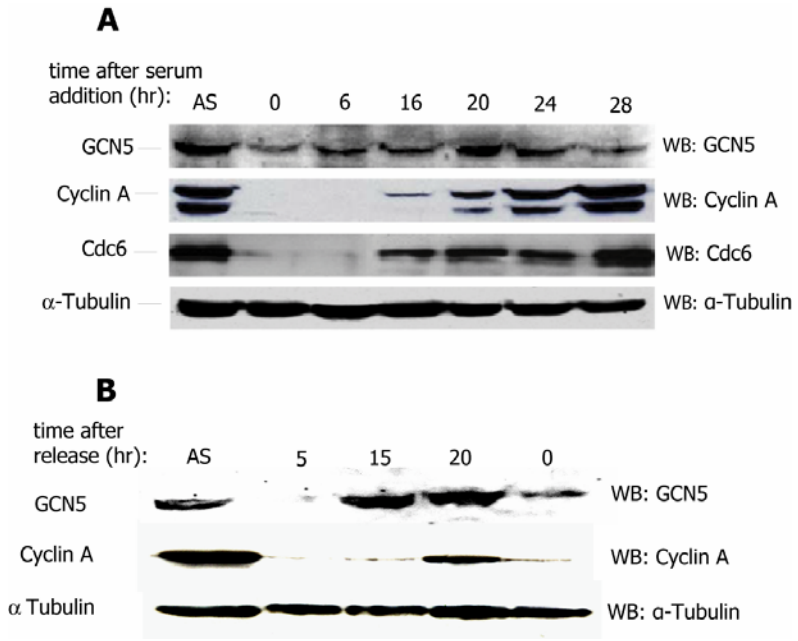

Figure 27. GCN5 expression level peaks in early S phase. A) Extracts of T98G cells harvested at the indicated time points were immunoblotted using anti-GCN5, anti-Cyclin A, anti-Cdc6 or anti-Tubulin antibodies. B) Extracts of HeLa cells harvested at the indicated time points were immunoblotted using anti-GCN5, anti-Cyclin A or anti-Tubulin antibodies.

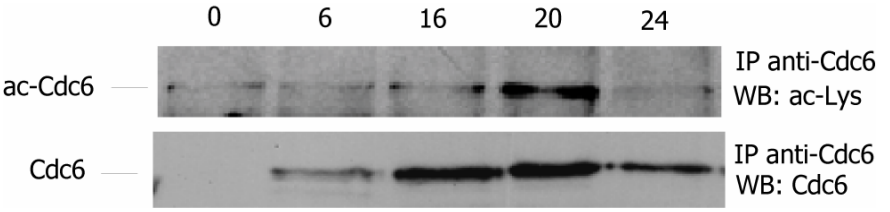

Figure 28. GCN5 acetylates Cdc6 in early S-phase. Extracts of T98G cells harvested at the indicated time points were immunoprecipitated with an anti-Cdc6 antibody and immunoblotted using anti Ac-Lys antibody; the same filter was incubated with an anti-Cdc6 antibody.

Of interest, acetylated Cdc6, as detected by immunoprecipitation with an anti-Cdc6 antibody followed by western blotting using an anti-acetylated lysine

antibody, showed a clear enrichment at 20 h (early S), to then return at basal levels at 24 h (mid S) (Fig. 28). Thus, the amounts of GCN5 and acetylated Cdc6 both peak in early S phase. Similar findings were also obtained in HeLa cells synchronized by a thymidine/nocodazole block (Figg. 26 and 27 B). Moreover, we investigated on the subcellular distribution of endogenos GCN5 and Cdc6 proteins during the cell cycle in T98G cells (Fig. 29).

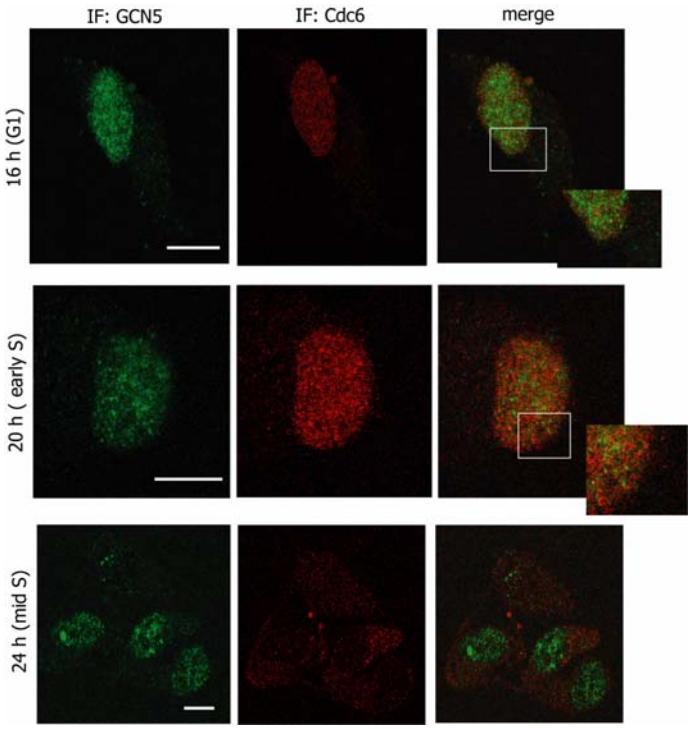

Figure 29. Cdc6 and GCN5 partially colocalize in early S phase. T98G cells harvested at the indicated time points were analysed by confocal immunofluorescence microscopy (confocal IMF) using anti-Cdc6 and anti-GCN5 antibodies followed by, respectively, an Alexa 594-conjugated anti-mouse antibody and a FITC-conjugated anti-rabbit antibody. Scale bar 10 µm.

We found that the two proteins partially co-localize at 20 hours (early S phase). Next we monitored the levels of the two Cdc6 phospho-isoforms during cell cycle progression. Phosphorylation of S54 was very low at 6 h (late G1), while it significantly increased from 16 h onward (Fig. 30). Phosphorylation of S106 (which was only detectable after immunoprecipitation using the specific anti-

phospho antibody followed by immunoblotting using the anti-Cdc6 antibody) was less pronounced at 16 h as compared to S54, and peaked at 20-24 h. Of interest, Cdc6 is known to be phosphorylated in the S phase in a cyclin A/Cdk2-dependent manner (Delmolino et al., 2001; Herbig et al., 2000; Jiang et al., 1999; Petersen et al., 1999; Saha et al., 1998).

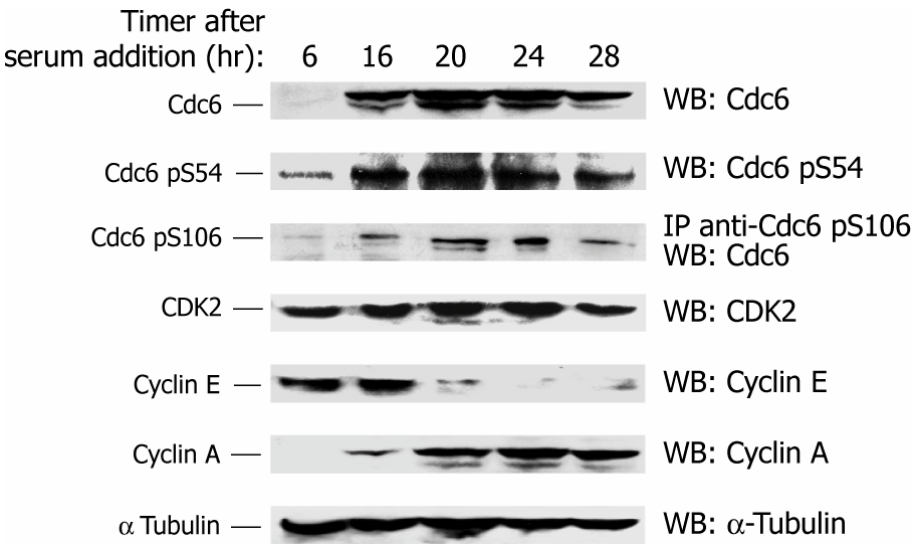

Figure 30. pS54 Cdc6 and pS106 Cdc6 are differentially expressed during cell cycle. Extracts of T98G cells harvested at the indicated time points were immunoblotted using anti-Cdc6, anti-pSer54, anti-pSer106, anti-Cdk2, anti-Cyclin A, anti-Cyclin E or anti-α-Tubulin antibodies.

Indeed, in the T98G synchronization, Cyclin E peaked at 6 (early G1) and 16 h (late G1), while Cyclin A started to appear at 16 h and its levels increased at 20 (early S) and 24 hours (mid S). These findings are thus consistent with the possibility that Cdc6 S106 phosphorylation might be attributable to Cyclin A/CDK2. To further explore this possibility, we overexpressed either Cyclin E or Cyclin A and monitored the levels of the two Cdc6 phospho-isoforms. We found that S106 phosphorylation significantly increased upon Cyclin A (but not Cyclin

E) overexpression, while the levels of phospho-S54 remained unaltered (Fig. 31).

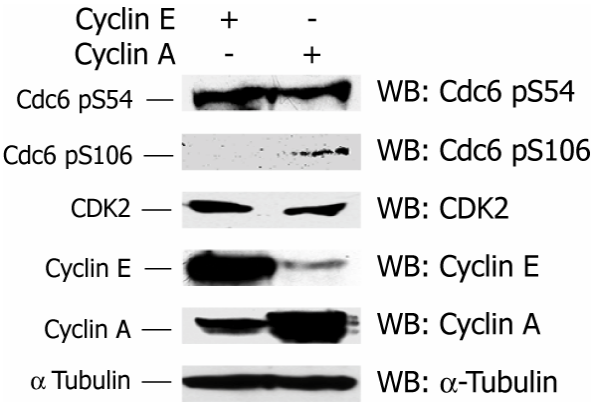

Figure 31. pS106 Cdc6 specifically increases upon Cyclin A overexpression. HeLa cells transfected as indicated were immunoblotted using anti-pSer54, anti-pSer106, anti-Cdk2, anti-Cyclin A, anti-Cyclin E or anti-α-Tubulin antibodies.

GCN5 and Cdc6 complex with Cyclin A/CKD2 in early S phase

We proceeded to investigate whether the interaction between Cdc6 and GCN5 might vary during cell cycle progression. Extracts from synchronized T98G cells were immunoprecipitated using an antibody against GCN5 or antibodies recognizing total Cdc6 as well as phospho-S54 or phopho-S106, and immunoblotted using anti-GCN5 or anti-Cdc6 antibodies. Binding between Cdc6 and GCN5 was maximal at 20 hours after serum addition, the same time point at which both GCN5 levels and Cdc6 acetylation peaked (Fig. 32). Both phospho-S54 and phospho-S106 Cdc6 bound GCN5 at this time point. However, the amount of co-immunoprecipitated GCN5 was clearly higher in the anti phospho-S106 immunoprecipitates. This was even more clear at 24 hours after serum addition (mid S), when co-immunoprecipitation was only detected using the anti-phospho-S106 but not the anti-phospho-S54 antibody.

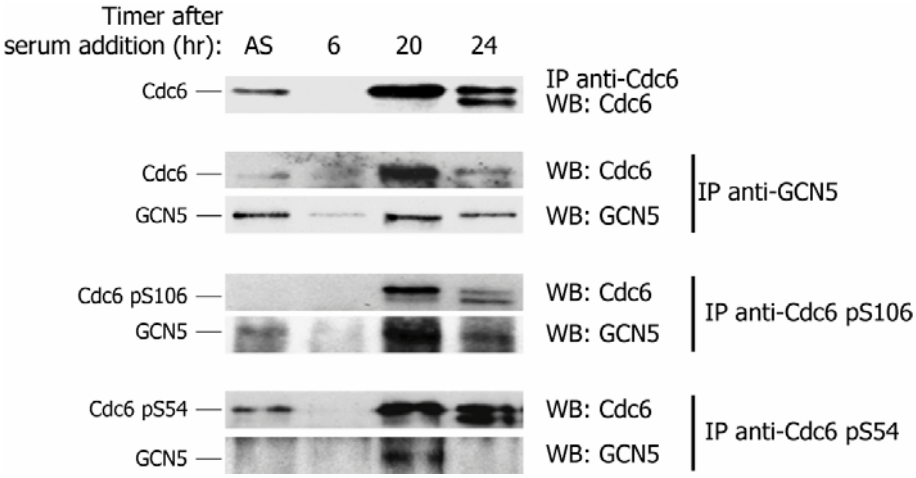

Figure 32. GCN5 and Cdc6 co-immunoprecipitate in early S-phase. Extracts of T98G cells harvested at the indicated time points were immunoprecipitated using an anti-GCN5 antibody and immunoblotted using anti-Cdc6 and anti-GCN5 antibodies. The same exctracts were immunoprecipitated using an anti-Cdc6 antibody and immunoblotted using anti-GCN5 and anti-Cdc6 antibodies (upper panels) and immunoprecipitated using an anti-pSer54 or anti-pSer106 antibodies and immunoblotted using anti-GCN5 and anti-Cdc6 antibodies (lower panels).

Taken together, the cell cycle experiments showed that: i) Cdc6 associated with GCN5 in early S-phase; ii) at the same time point, the protein became acetylated; iii) binding between GCN5 and Cdc6 was preferential for the S106-phosphorylated form of Cdc6; iv) acetylation, phosphorylation and GCN5-binding were concomitant with the expression of Cyclin A; v) the overexpression of Cyclin A selectively increased phosphorylation of Cdc6 on S106.

Since GCN5 often participates in the formation of multi-component protein complexes (Guelman et al., 2006; Muratoglu et al., 2003; Timmers and Tora, 2005), we wondered whether it might also associate with Cyclin A/Cdk2. Indeed, both GCN5 and Cdc6 were co-immunoprecipitated together with Cdk2 by an antibody against Cyclin A, as well as CDK2 by an antibody against GCN5 (Fig. 33). Of interest, this complex formed at 20 and 24 h after serum release (S phase), while, at 16 h (late G1), Cyclin A only immunoprecipitated Cdk2, despite both Cdc6 and GCN5 were abundantly expressed inside the cells.

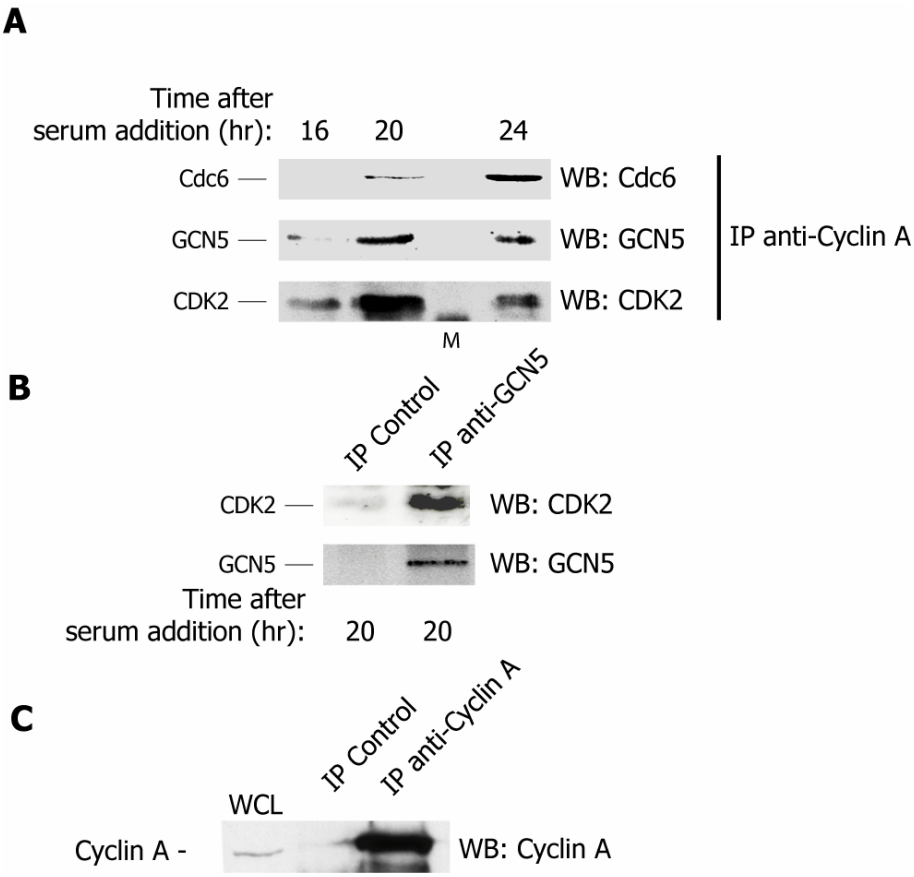

Figure 33. Cdc6 and GCN5 interact with Cyclin A/Cdk2 in early S-phase. (A) Extracts of T98G cells harvested at the indicated time points were immunoprecipitated using an anti-Cyclin A antibody and immunoblotted with anti-Cdc6, anti-GCN5 or anti-Cdk2 antibodies. (B) Anti-GCN5 antibody immunoprecipitates CDK2 from lysates of T98G cells collected at 20 hours after serum addition. (C) Extracts of T98G cells were immunoprecipitated with an anti-Cyclin A antibody and immunoblotted with an anti-Cyclin A antibody to detect the total amount of immunoprecipitated Cyclin A used in (A).

Taken together, the observations above are consistent with the possibility that Cdc6 phosphorylation on S106 might preferentially occur on acetylated Cdc6. Therefore, we immunoprecipitated either total Cdc6 or phospho-S106 Cdc6 and assessed the levels of Cdc6 acetylation in the two immunoprecipitates. As shown in Fig. 34 A, the amount of acetylated Cdc6 was remarkably enriched in the

phospho-S106 immunoprecipitate (ratio acetylated Cdc6:total immunoprecipitated Cdc6 = 0.45 using the anti-Cdc6 antibody; = 1.90 using the anti-phospho-S106 antibody; Fig. 34 B). Notably, the immunoprecipitated phospho-S106 Cdc6 is phosphorylated on serine 54.

By using the same approach, we also wondered whether the acetylation state of the two phosphorylated forms of Cdc6 might be different. We found that the anti-phospho-S106 antibody immunoprecipitated a remarkably higher amount of acetylated Cdc6, as compared to the anti-phospho-S54 antibody, despite being the levels of total Cdc6 immunoprecipitated by the former antibody lower (Fig. 35 A).

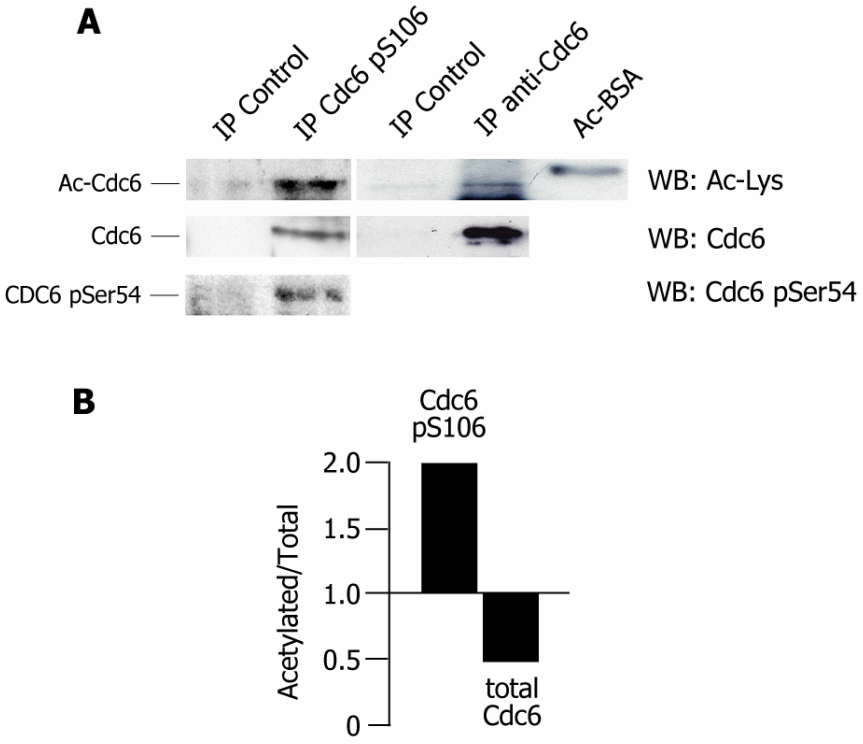

Figure 34. Cdc6 acetylation is strictly related to phosphorylation on serine 106. A) Extracts of non-synchronous T98G cells were immunoprecipitated using anti-Cdc6 and anti-pSer106 antibodies and immunoblotted using anti-Ac Lys or anti-Cdc6 antibodies. (B) The graphs express the ratio acetylated Cdc6/total immunoprecipitated Cdc6.

Upon quantification, the ratio between acetylated Cdc6 and total immunoprecipitated Cdc6 was 2.10 for the anti-phospho-S106 antibody (Fig. 35 B similar to the experiments shown in Fig. 34 B) and 0.35 for the anti-phospho-S54 antibody.

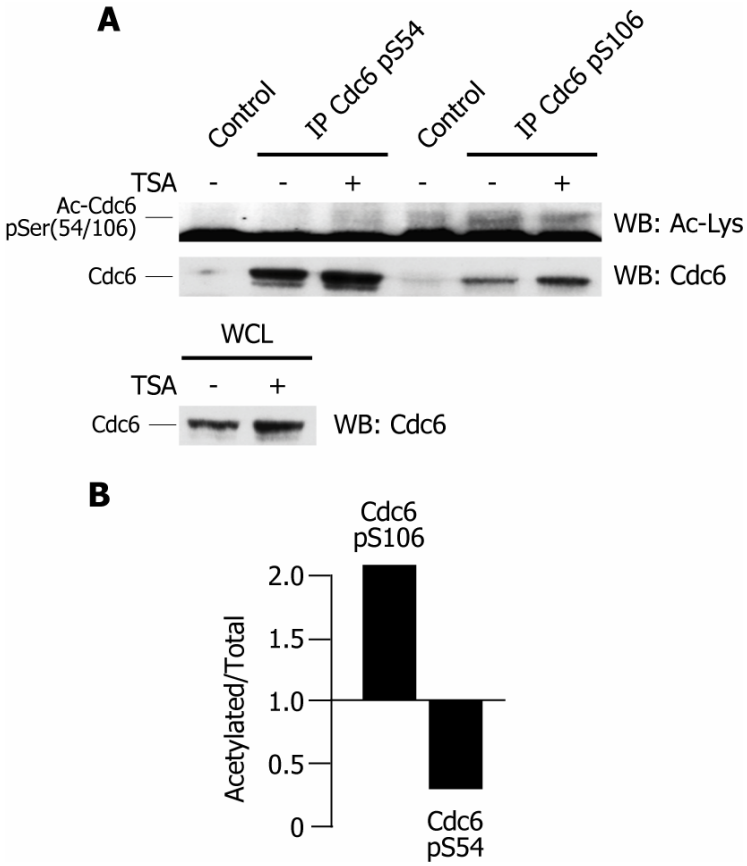

Figure 35. Cdc6 acetylation is strictly related to phosphorylation on serine 106. (A) Extracts of non-synchronous HeLa cells treated as indicated were immunoprecipitated using anti-pSer54 or anti-pSer106 antibodies and immunoblotted using anti-Ac Lys or anti-Cdc6 antibodies. (B) The graphs express the ratio acetylated Cdc6/total immunoprecipitated pSer54 Cdc6 or pSer106 Cdc6.

The only detectable effect of TSA was to slightly increase the levels of total Cdc6 in the cells. Thus, acetylation of Cdc6 appears to be strictly linked to S106 phosphorylation.

To directly demonstrate that GCN5-mediated acetylation of Cdc6 affects Ser 106 phosphorylation, we have attempted to see whether a Cdc6 peptide encompassing the acetylated region might become a preferential Cyc/CDK substrate once acetylated *in vitro*. The experiment was performed by incubating peptides from Cdc6 region 88-111, either acetylated on lysines 92, 105 and 109 or not, with immunoprecipitates from T98G cells in early S phase, obtained with an antibody against Cyclin A (Fig. 36). The results obtained indicate that the acetylated peptide is preferentially phosphorylated by the Cyclin A/CDK immunocomplex as compared to the non-acetylated one.

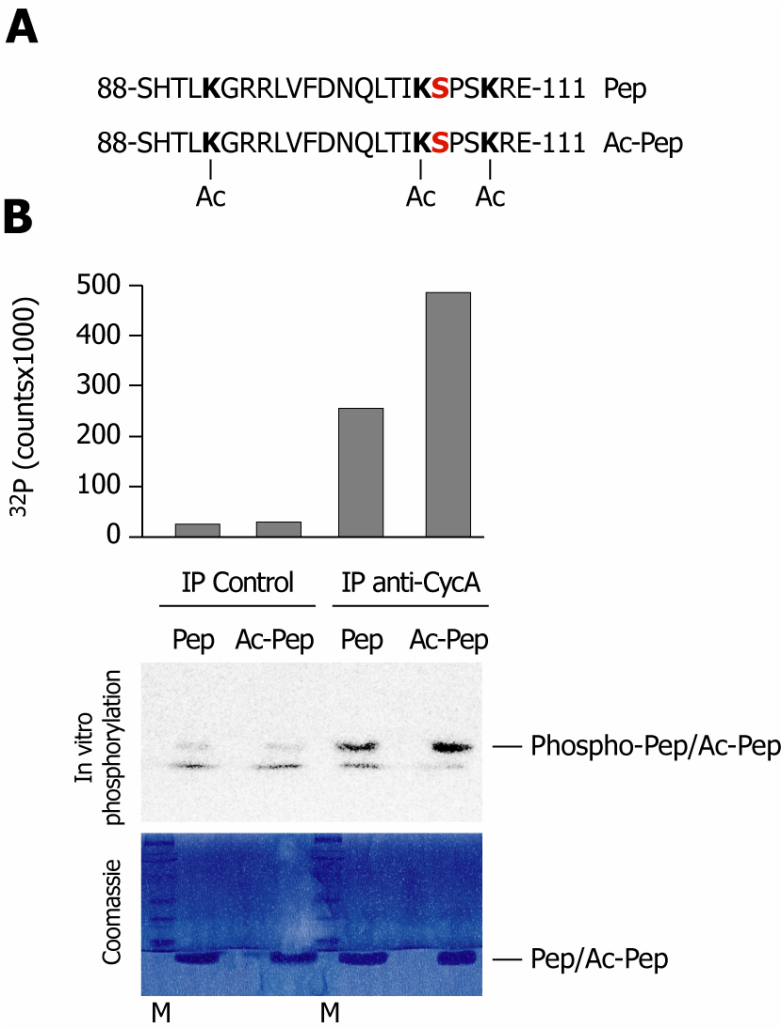

Figure 36. Increased phosphorylation of an acetylated Cdc6 peptide by Cyclin A/CDK immunocomplex. (A) Two peptides (Pep and Ac-Pep) were synthesized, corresponding to the human Cdc6 sequence from aa 88 to 111, containing the Cyc motif, lysines 92, 105 and 109 (in bold) and serine 106 (in red); the three lysines were acetylated in Ac-Pep. (B) The two peptides were incubated, in the presence of radioactive ATP, with an immunoprecipitate obtained from a lysate of T98G cells in early S-phase (20 h after serum addition, see Fig. 5) using anti-cyclin A (IP α-CycA) or anti-GFP (IP control) antibodies, resolved by SDS-PAGE and analyzed by autoradiography. The acetylated peptide shows significantly higher phosphorylation.

Acetylation and S106-phosphorylation of Cdc6 regulate its subcellular localization

In human cells, Cdc6 is known to be phosphorylated in a Cdk2/Cyclin A-dependent manner in the S phase and then translocated to the cytosol and subsequently degraded (Delmolino et al., 2001; Diffley, 2004; Herbig et al., 2000; Jiang et al., 1999; Petersen et al., 1999). Confocal immunofluorescence analysis performed in non-synchronized HeLa cells using a monoclonal anti-Cdc6 antibody revealed that in about 55% of the cells endogenous Cdc6 was nuclear, while in about 45% of cells the protein had a cytoplasmic localization (Fig. 37). However, most (>90%) of the asynchronous cells that expressed Cyclin A, an S phase-marker, belonged to the subset of cells with exclusive cytoplasmic localization of Cdc6 (Fig. 38).

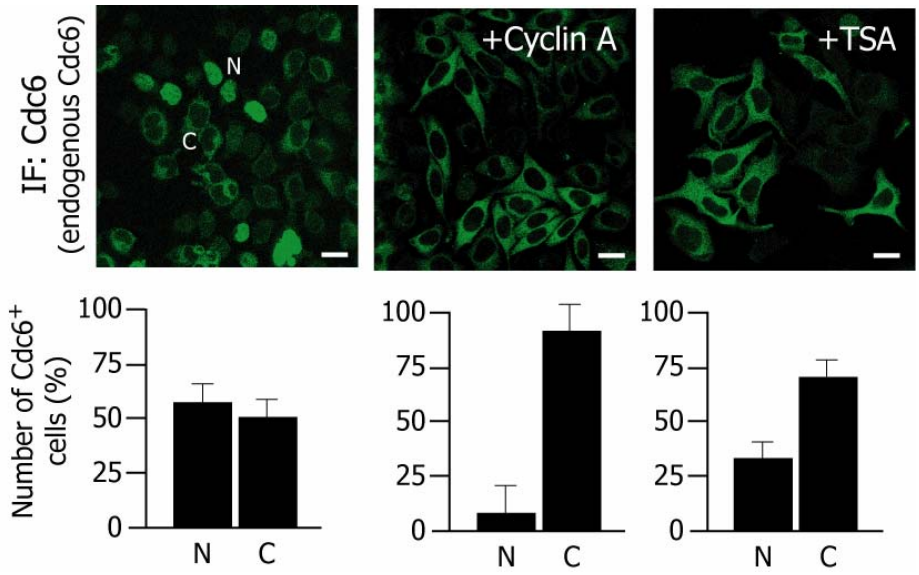

Figure 37. Cdc6 cytosolic localization increases when Cyclin A is overexpressed or upon TSA treatment. Subcellular distribution of endogenous Cdc6 (left panel). Non-synchronous HeLa cells transfected (mid panel) or treated as indicated (right panel), were analysed by confocal IMF using an anti-Cdc6 antibody followed by a FITC-conjugated anti-mouse antibody. Cdc6 cytosolic localization increases when Cyclin A is overexpressed or upon TSA treatment. The graphs below express the amount of analysed cells showing a nuclear (N) or cytoplasmic (C) distribution of the protein. Scale bar:10 μm.

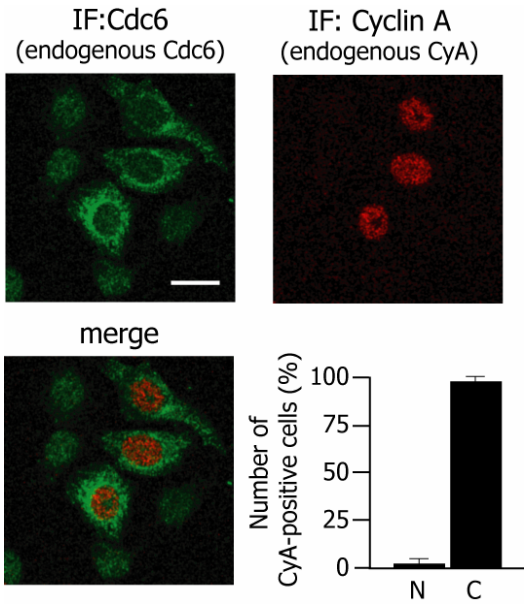

Figure 38. Cdc6 is cytosolic when endogenous Cyclin A is expressed inside the cells. Non-synchronous HeLa cells were analysed by confocal IMF using anti-Cdc6 or anti-Cyclin A antibodies followed, respectively, by FITC-conjugated anti-mouse or Alexa594-conjugated anti-rabbit antibodies. The graph below expresses the amount of Cyclin A-expressing cells showing a nuclear (N) or cytoplasmic (C) distribution of the protein. Scale bar 10 μm.

In keeping with these findings, Cdc6 was localized in the cytoplasm in over 90% of the cells in which Cyclin A had been transfected. Given the link we disclosed between GCN5-dependent Cdc6 acetylation and its phosphorylation by cell cycle CDKs, we wondered whether the acetylation of the factor might influence its subcellular localization. In a first set of experiments we treated cells with TSA or overexpressed GCN5, followed by the visualization of endogenous Cdc6 distribution in the nuclear and cytoplasmic compartments. We found that cell treatment with TSA increased the number of cells with exclusive cytoplasmic localization (68% vs. 45% of the untreated control; $P<0.01$; Fig. 36). Analogous results were obtained by the overexpression of active GCN5 (71% of cell with cytoplasmic localization among those positive for the expression of the transfected protein; $p<0.01$), but not of its catalytically inactive mutant (Fig. 39).

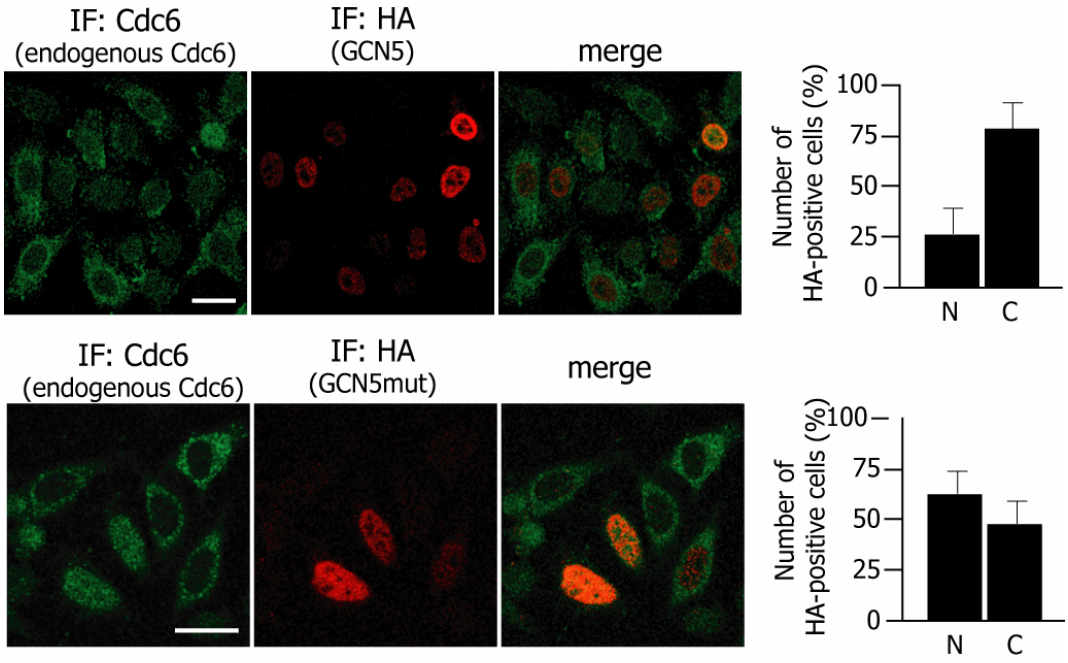

Figure 39. GCN5 overexpression drives endogenous Cdc6 into the cytoplasm. Asynchronous HeLa cells transfected as indicated were analysed by confocal IMF using anti-Cdc6 and anti-HA antibodies followed by, respectively, FITC-conjugated anti-mouse and Alexa 594-conjugated anti-rabbit antibodies. The graphs below express the amount of analysed HA-cells showing a nuclear (N) or cytoplasmic (C) distribution of Cdc6 protein. Scale bar 10 μm.

Flow cytometry experiments on the cells transfected with wt GCN5 showed a significant increase in the number of S-phase cells, similar to what obtained by overexpression of Cyclin A (Fig.40).

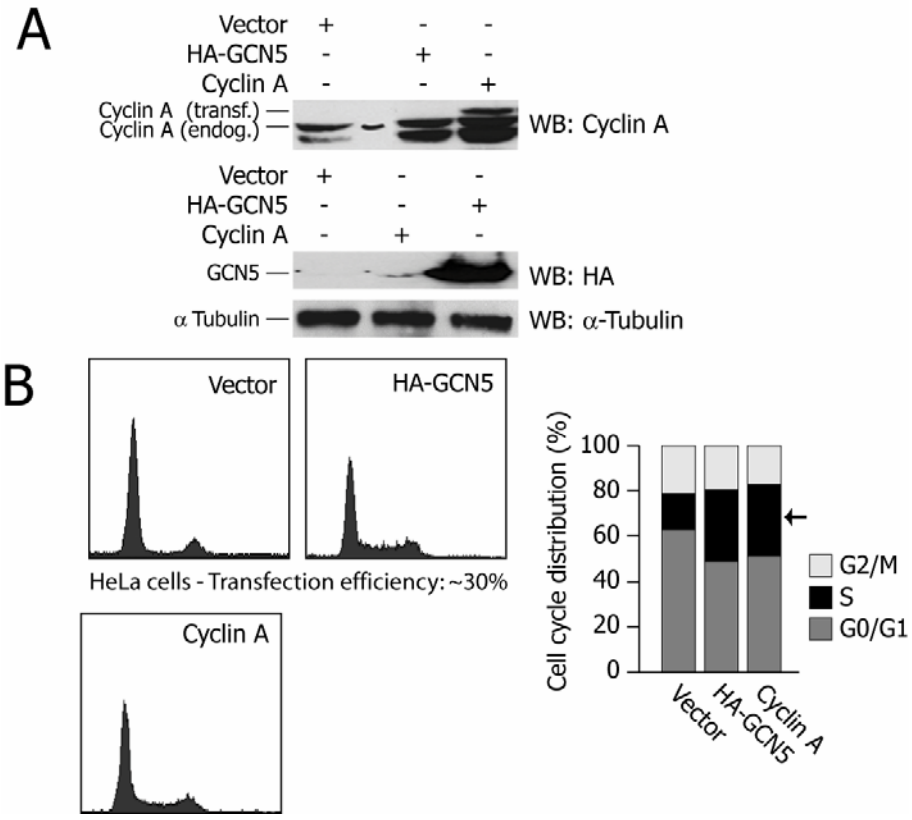

Figure 40. GCN5 overexpression drives cells to S phase. (A) Transfected HeLa cells extracts were run on an SDS-PAGE gel and immunoblotted with anti-Cyclin A, anti-HA or anti-αTubulin antibodies to verify protein expression levels. (B) Flow cytometry profiles after transfection. The histogram on the right side shows the distribution of the cells in the different phases of the cell cycle; the increase and the number of S-phase cells after GCN5 and Cyclin A overexspression is indicated by an arrow.

Finally, we knocked down GCN5 expression by RNA interference (>90% reduction in endogenous GCN5 levels). This treatment determined a marked increase in the number of cells in the G1 phase (Fig. 41 A). Moreover, we pulsed H1299 cells with bromodehoxyuridine (BrdU) to detect cells in active DNA synthesis, and we observed that the anti-GCN5 siRNA causes a significant reduction in the number of BrdU-positive cells in the S-phase (Fig 41 B).

In keeping with this finding, we observed that, in the cells in which endogenous GCN5 had been silenced, the localization of endogenous Cdc6 was prevalently nuclear (>75% of cells with nuclear staining; Fig. 42).

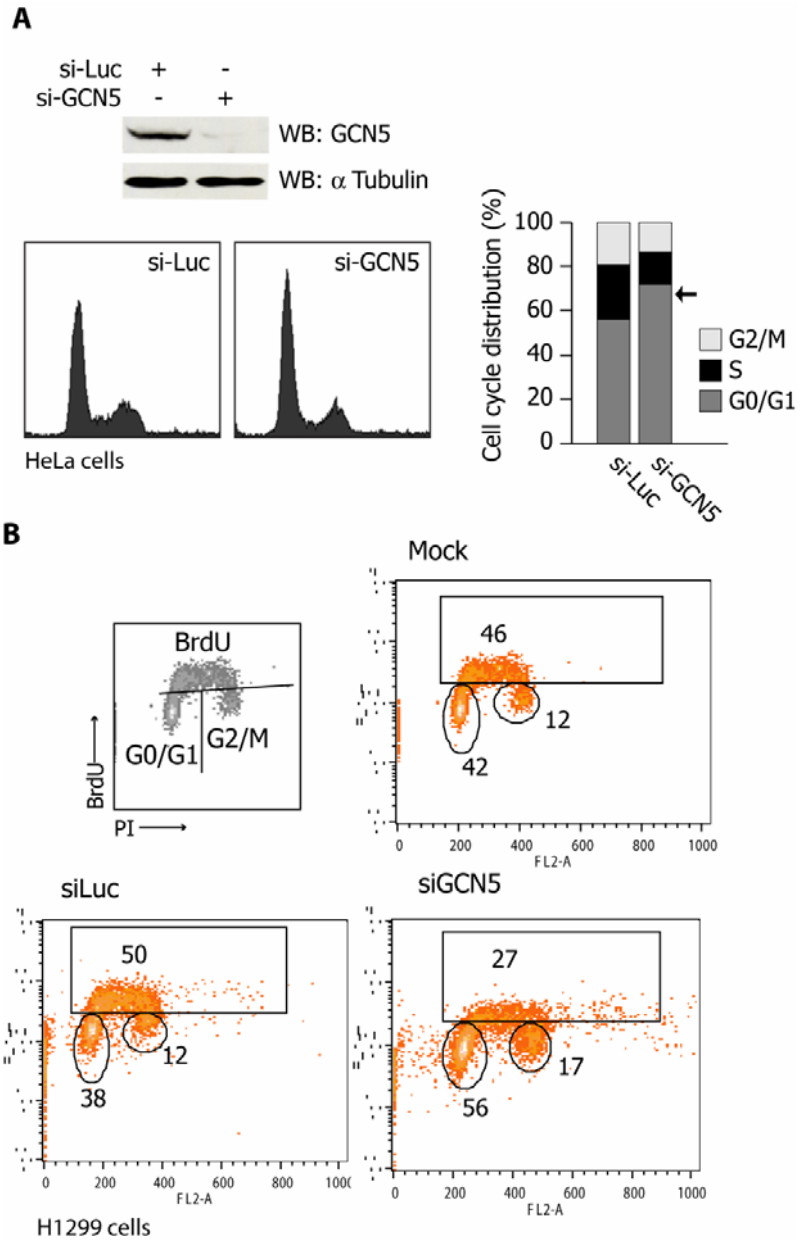

Figure 41. (For figure legend see next page).

Figure 41. GCN5 depletion triggers cells to accumulate in G1 phase. (A) Western blot analysis of GCN5 after RNAi. Flow cytometry profiles of treated HeLa cells after RNAi with siGCN5 and siLuciferase duplex. The histogram on the right side shows the distribution of the cells in the different phases of the cell cycle; the increase and the number of G1-phase cells after GCN5 silencing is indicated by an arrow. (B) Flow cytometry profiles of treated H1299 cells after RNAi with siGCN5 and siLuciferase duplex. The cells were pulsed with BrdU and stained with an anti-BrdU antibody and with Propidium Iodide (PI) and analysed by flow citometry. The percentage of cells in the different phases of the cell cycle are indicated.

Cells treated with a control siRNA against luciferase showed a subcellular distribution of Cdc6 indistinguishable from untreated cells. Notably, when endogenous GCN5 expression level peaks, endogenous Cdc6 is cytosolic, thus confirming our immunoblot data on synchronised T98G and HeLa cells.

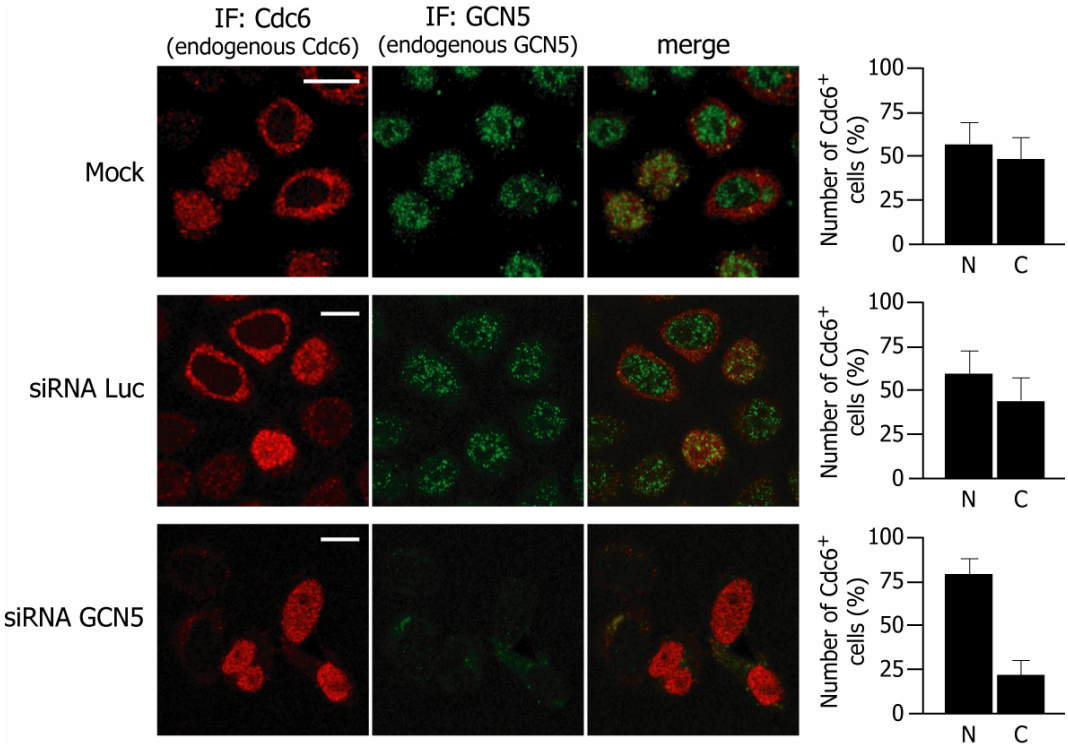

Figure 42. GCN5 depletion leads to the nuclear accumulation of endogenous Cdc6. Non-synchronous HeLa cells transfected with water (mock) or 100 nM GCN5- or Luciferase-siRNA for 72 h were analysed by confocal IMF using anti-Cdc6 and anti-GCN5 antibodies followed by, respectively, Alexa 594-conjugated anti-mouse and FITC-conjugated anti-rabbit antibodies. The graphs express the total amount of analysed cells in Fig. 45 showing a nuclear (N) or cytoplasmic (C) distribution of the protein. Scale bar 10 µm.

Next we explored the subcellular localization of transfected Flag-Cdc6, of the Cdc6(S106A) mutant, which cannot be phosphorylated on S106, and of the Cdc6(K3R) mutant, which cannot be acetylated and is thus equally not phosphorylated on S106. We observed that transfected Cdc6 had a similar distribution as endogenous Cdc6 (about 55% and 45% of the cells with nuclear and cytoplasmic localization respectively; Fig. 43). With analogy to endogenous Cdc6, this distribution was modified by cell treatment with TSA, upon which Cdc6 became cytoplasmic in over 65% of the cells ($p<0.01$). Strikingly, both the S106A and the K3R mutants were strictly nuclear, and their localization was

unaltered by TSA treatment (Figg. 44 and 45). Notably, both mutants were still able to co-immunoprecipitate with Cyclin A similar to the wt protein (Fig. 48).

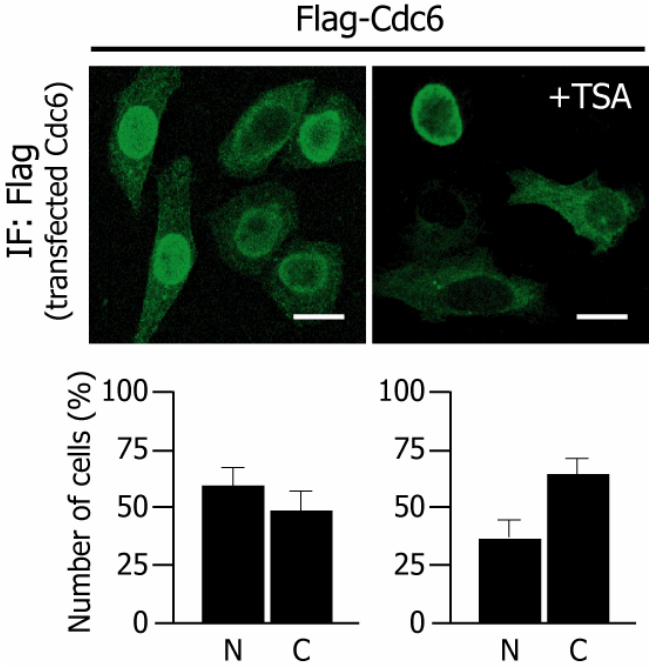

Figure 43. Subcellular distribution of Flag-Cdc6. Non-synchronous HeLa cells transfected and treated as indicated were analysed by confocal IMF using an anti-Flag antibody followed by a FITC-conjugated anti-mouse antibody. The graphs below express the total amount of cells showing a nuclear (N) or cytoplasmic (C) distribution of the protein. Scale bar 10 μm.

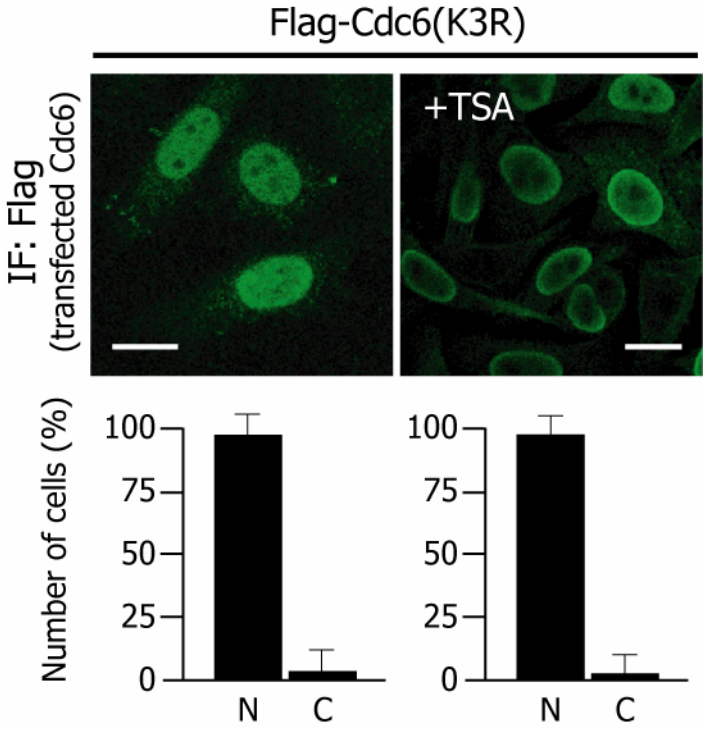

Figure 44. The Flag-Cdc6(K3R) mutant does not enter the cytoplasm. Non-synchronous HeLa cells transfected and treated as indicated were analysed by confocal IMF using an anti-Flag antibody followed by a FITC-conjugated anti-mouse antibody. The graphs below express the total amount of cells showing a nuclear (N) or cytoplasmic (C) distribution of the protein. Scale bar 10 μm.

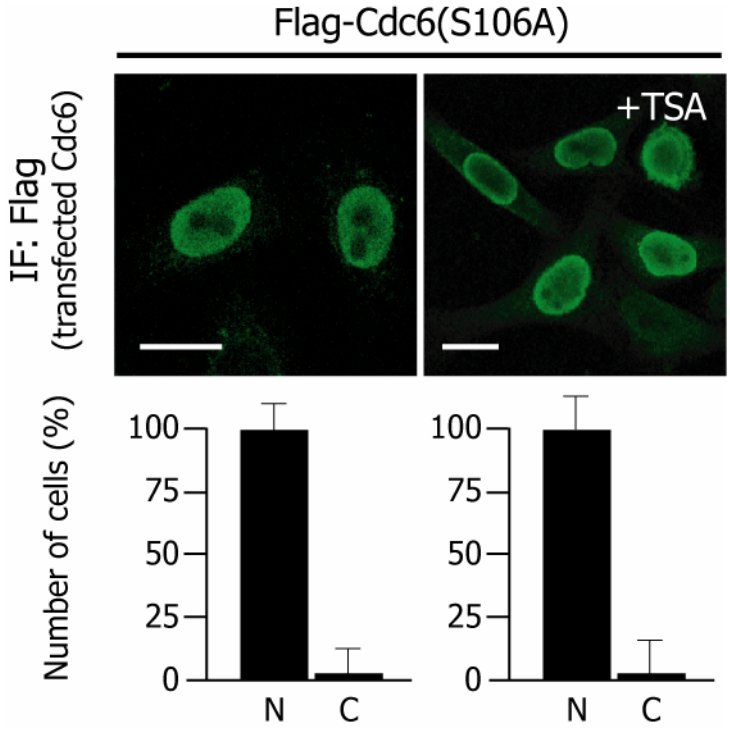

Figure 45. The Flag-Cdc6(S106A) mutant does not enter the cytoplasm. Non-synchronous HeLa cells transfected and treated as indicated were analysed by confocal IMF using an anti-Flag antibody followed by a FITC-conjugated anti-mouse antibody. The graphs below express the total amount of cells showing a nuclear (N) or cytoplasmic (C) distribution of the protein. Scale bar 10 μm.

We proceeded to transfect cells with the different Cdc6 mutants together with catalytically active GCN5, and subsequently visualise the transfected proteins by anti-tag specific antibodies. We observed that, similar to endogenous Cdc6, the overexpression of GCN5 determined the redistribution of transfected Cdc6 in the cytoplasm (75% of cells with cytoplasmic localization; $p<0.01$ over control; Figg. 46 and 47). In contrast, the localization of both the S106A and K3R Cdc6 mutants remained strictly nuclear also in the GCN5-expressing cells. Both mutants were still able to co-immunoprecipitate with HA-GCN5 similar to the wt protein (Fig. 48).

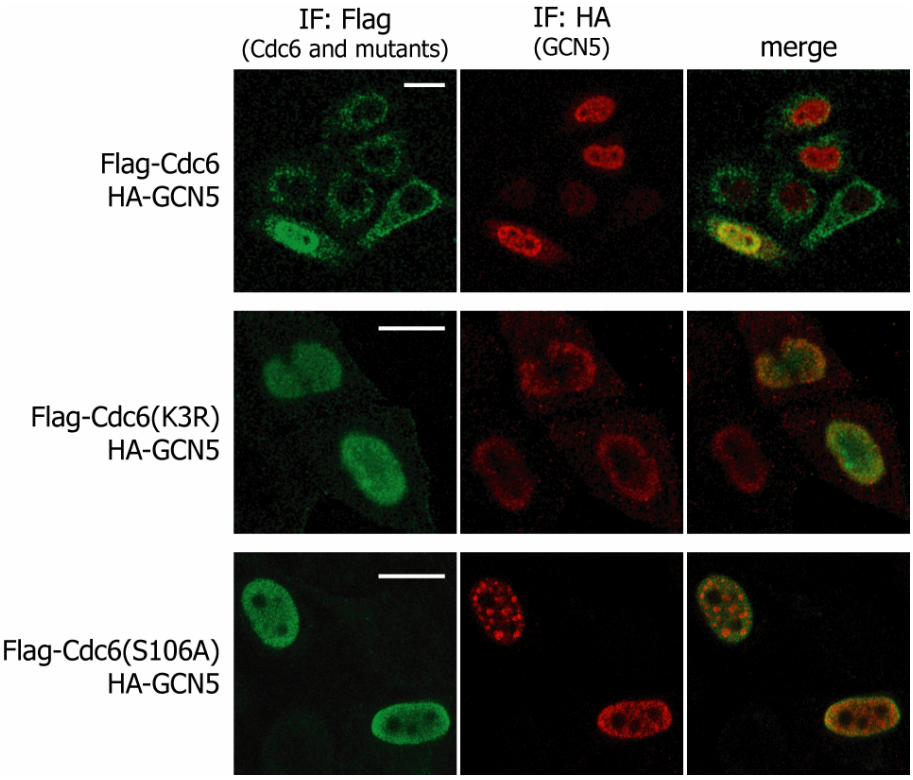

Figure 46. The Flag-Cdc6(K3R) and Flag-Cdc6(S106A) mutants do not enter the cytoplasm upon GCN5 overexpression. Non-synchronous HeLa cells transfected and treated as indicated were analysed by confocal IMF using anti-Flag and anti-HA antibodies followed, respectively, by a FITC-conjugated anti-mouse and a Alexa 594-conjugated anti-rabbit antibodies. Scale bar 10 μm.

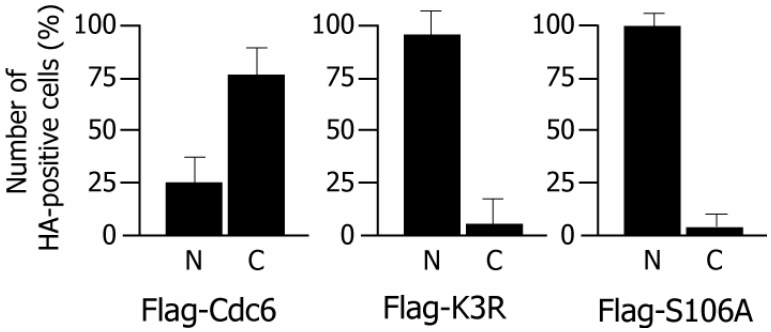

Figure 47. The Flag-Cdc6(K3R) and Flag-Cdc6(S106A) mutants do not enter the cytoplasm upon GCN5 overexpression. The graphs express the total amount of cells showing a nuclear (N) or cytoplasmic (C) distribution of the protein.

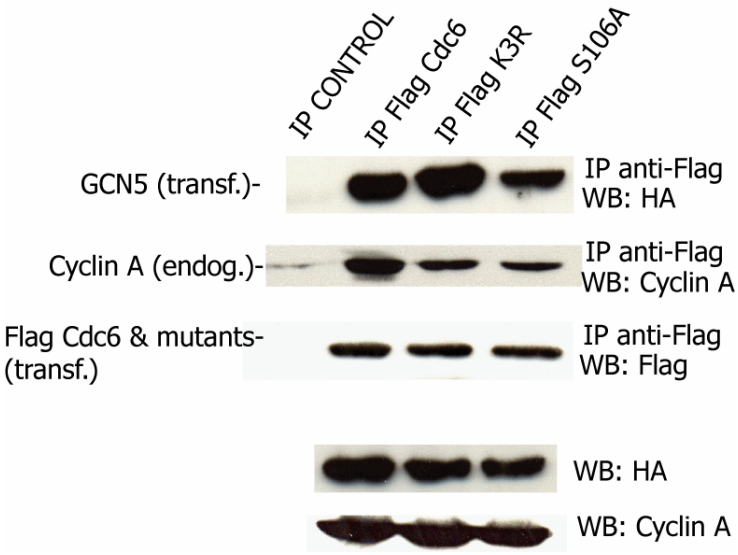

Figure 48. The Flag-Cdc6(K3R) and Flag-Cdc6(S106A) still bind HA-GCN5 and endogenous Cyclin Extracts of 293T cells transfected as indicated were immunoprecipitated using an anti-Flag antibody and immunoblotted using anti-HA, anti-Cyclin A or anti-Flag antibodies. Transfected HeLa cells extracts were run on an SDS-PAGE gel and immunoblotted with anti-Cyclin A, anti-HA or anti-αTubulin antibodies to verify protein expression levels.

We wondered whether the peculiar nuclear localization of the Cdc6 S106 and K3R mutants might reflect the association of these proteins with chromatin. Whole lysates from cells transfected with wild type Cdc6 or with the two mutants were fractionated to separate the cytosolic, nuclear soluble and nuclear insoluble compartments (Mendez and Stillman, 2000). Both mutants were indeed mostly found in the two nuclear fractions and, in particular, K3R was highly enriched in the insoluble chromatin pellet (Fig. 49). Consistent with this observation, when lysates from non-transfected cells were partitioned in the same manner, Cdc6 phosphorylated on S54 was found distributed in both the cytoplasmic and nuclear insoluble compartments, similar to total Cdc6. In contrast, Cdc6 phosphorylated on S106 was present exclusively in the cytoplasmic fraction (Fig. 50). Taken together, these observations are in keeping with the conclusion that Cdc6 acetylation promotes detachment of Cdc6 from

chromatin and that both this modification and S106 phosphorylation are essential to induce the relocalization of the protein to the cytoplasm.

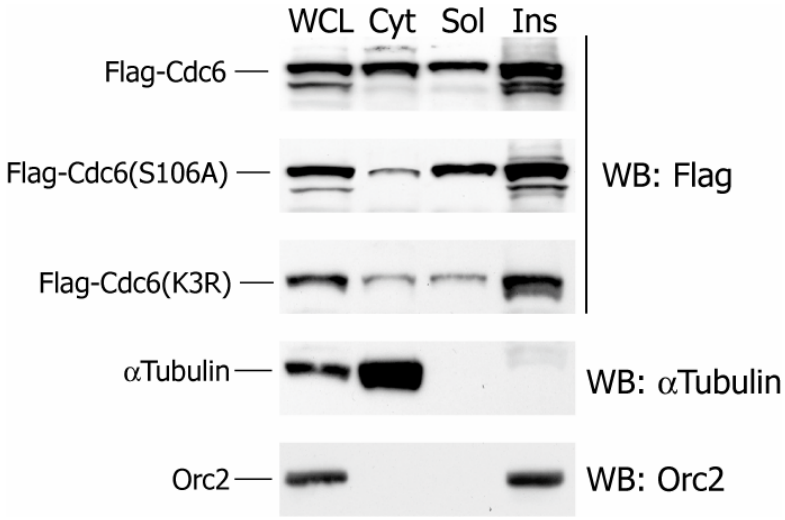

Figure 49. Cdc6(K3R) and Cdc6(S106A) localize to the insoluble nuclear compartment. Whole cell lysates (WCL) from cells untransfected (for Cdc6, Orc2 and αTubulin) or transfected with wt Cdc6, Cdc6(K3R) and Cdc6(S106A) were fractionated to generate a cytoplasmic (Cyt), a soluble nuclear (Sol) and an insoluble nuclear (Ins) fractions, in which protein levels were assessed by western blotting.

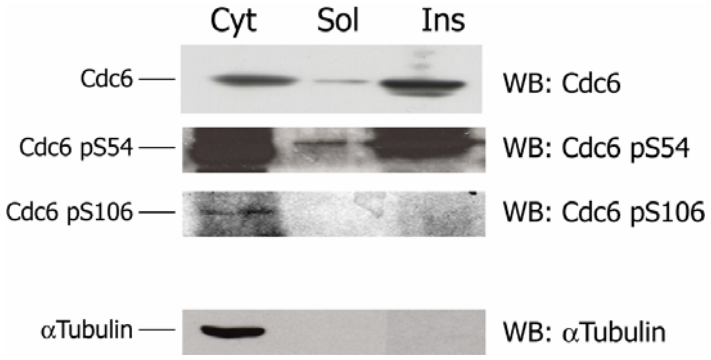

Figure 50. Cdc6 pS106 is highly enriched in the cytoplasmic compartment. Western blot analysis of cytoplasmic (Cyt), nucleoplasmic (Sol) and insoluble (Ins) fractions prepared from asynchronous HeLa cells. Endogenous proteins were revealed with specific antibodies, as indicated on the left side.

K3R mutations block cell cycle progression and stabilize Cdc6.

Next we wondered whether the change in chromatin association and subcellular localization of the two Cdc6 mutants might perturb the normal cell cycle progression. Overexpression of wild type Cdc6 or some of its other mutants does not exert a dramatic effect in primary cells, since multiple mechanisms (including Cdt1 inhibition by geminin and p53-dependent DNA damage response) prevent cellular DNA re-replication (Nishitani et al., 2004; Vaziri et al., 2003)**.** However, overexpression of Cdc6 in p53-/- cells has been shown to escape checkpoint inhibition and to induce, to some extent, DNA re-replication (Vaziri et al., 2003). We therefore analyzed the effect of our mutants in the p53-null H1299 human lung carcinoma cell line. Twenty-four hour after transfection, cells were treated with BrdU to selectively label cells in active DNA synthesis; after 1 hour, BrdU incorporation and DNA content were visualized by flow cytometry. As shown in Fig. 50, the overexpression of both wt Cdc6 and the S106A mutant determined a reduction in the number of cells incorporating BrdU; in sharp contrast, the Cdc6(K3R) mutant markedly increased the number of cells involved in DNA synthesis (from 43% to 67%), while significantly reducing the number of cells in G1 (from 44% to 30%) and, most notably, in G2/M (from 16% to 3%). No significant re-replication was however observed with any of the mutants. This striking result is consistent with the notion that the overexpression of the K3R mutant, which is tightly chromatin-bound, might specifically impair S-phase progression.

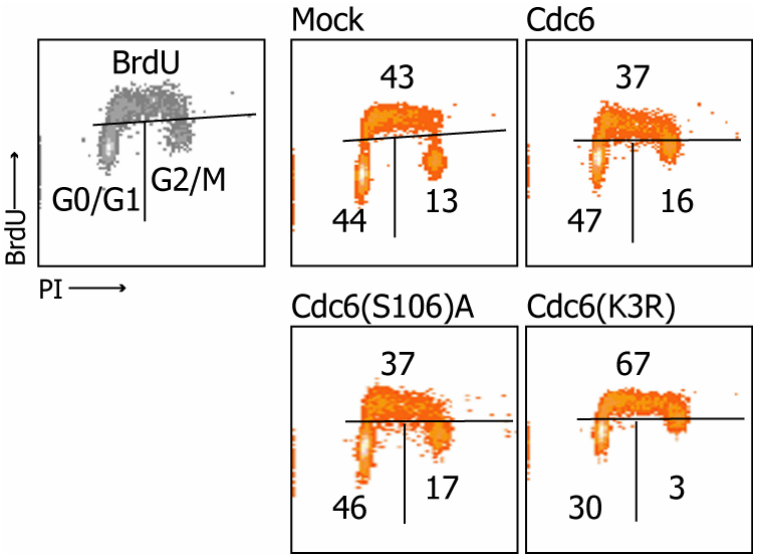

Figure 51. Cell cycle distribution and BrdU incorporation in H1299 cells expressing wt Cdc6 and Cdc6(K3R) and Cdc6(S106A) mutants. Cells, either untreated or transfected to express the indicated proteins (efficiency of transfection >90%), were pulse-labeled with BrdU for 1 hour. Cells were then fixed, stained with an anti-BrdU antibody and with propidium iodide (PI), and analyzed by flow cytometry. The percentage of cells in the different phases of the cell cycle are indicated.

Cytoplasmic transport of human Cdc6 correlates with degradation of the protein in the S-phase (Delmolino et al., 2001; Diffley, 2004; Herbig et al., 2000; Jiang et al., 1999; Petersen et al., 1999). We therefore wanted to assess the stability of the constitutively nuclear Cdc6 S106A and K3R mutants relative to wt Cdc6. After treatment of asynchronous H1299 cells with cycloheximide (CHX) to block protein synthesis, the half-life of transfected wt Cdc6 was found to be less than 1 hour, similar to endogenous Cdc6 and in analogy with other p53 null cell lines (Duursma and Agami, 2005a). In contrast, both the Cdc6(S106A) and Cdc6(K3R) mutants were remarkably more stable; in particular, after 4 hours of CHX treatment, more than 75% of Cdc6(K3R) was still present inside the cells (Figs. 52 and 53).

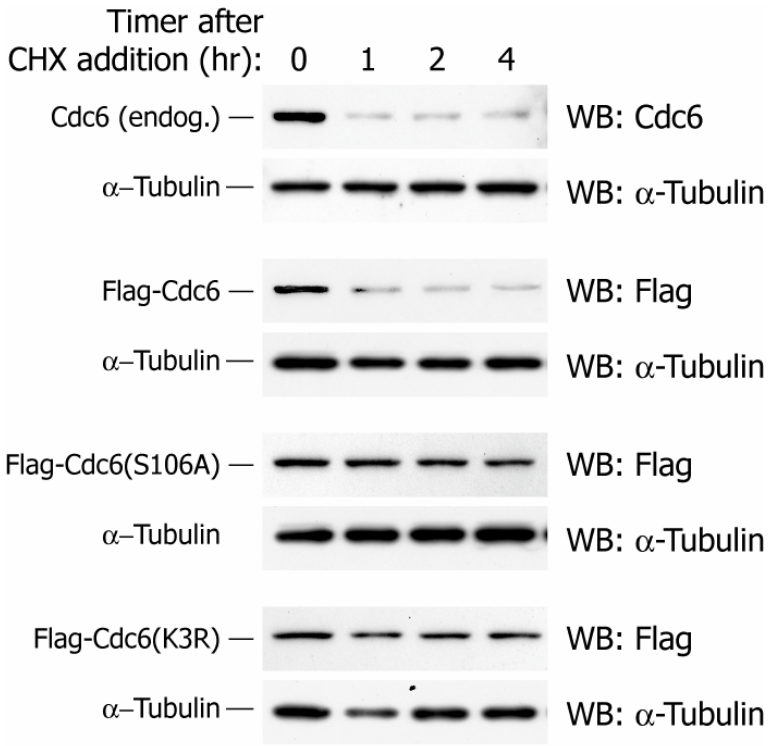

Figure 52. Stability of endogenous wild type Cdc6, transfected wt Cdc6 and the Cdc6(K3R) and Cdc6(S106A) mutants. After transfection, H1299 cells were treated with CHX for the indicated time points, and total cell lysates were analyzed by western blotting. αTubulin was used as a loading control.

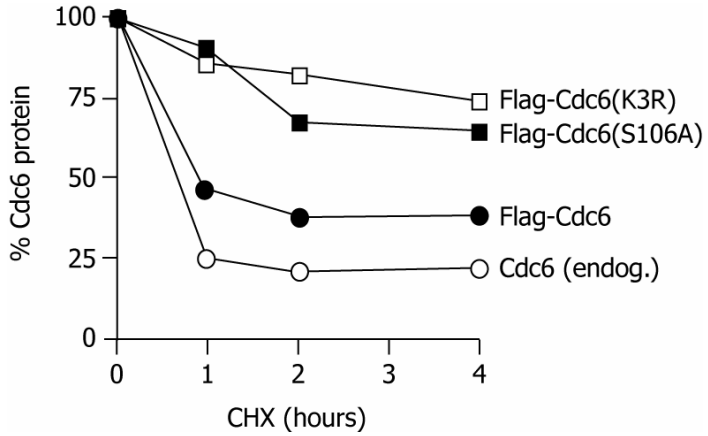

Figure 53. Quantification of the experiment shown in Fig. 51. The amount of each protein is expressed as a percentage of the initial level.

This finding highlights the relevance of Cdc6 acetylation in controlling the stability of the protein, and fully concurs with the observation that GCN5 depletion and GCN5mut overexpression both markedly increase the total amount of Cdc6 (Figs. 23 and 19 respectively).

3 Discussion

This study demonstrates that Cdc6 is regulated by a multi-site modification program, in which the coordinated acetylation and phosphorylation of the N-terminus of the protein regulate chromatin binding and subcellular protein localization.

In essence, our data show that both endogenous and overexpressed Cdc6 are acetylated inside the cells by the GCN5 HAT at three lysines that frame the Cyclin-docking motif in the N-terminus of the protein, a region that is not directly involved in pre-RC formation (Drury et al., 1997). Acetylation regulates the levels of Cdc6 phosphorylation at serine 106, since the overexpression of GCN5 increases phosphorylation at this residue, while point mutants in the three acetylated lysines are no longer phosphorylated. During the cell cycle, acetylation of Cdc6 occurs in the early S phase, when the levels of both Cyclin A and GCN5 peak. At this time point, both GCN5 and Cdc6 are found in a complex with Cyclin A and Cdk2. Cyclin A-mediated phosphorylation of Cdc6 on serine 106 requires prior acetylation of Cdc6. Finally, cell treatment with a deacetylase inhibitor or overexpression of GCN5 force cytoplasmic relocalization of both endogenous and transfected Cdc6, an effect that is also obtained by transfection of Cyclin A. In a consistent manner, Cdc6 proteins bearing mutations at either serine 106 or at the three lysines that are acetylated are exquisitely nuclear and their stability is increased.

Work originally performed in the Xenopus *in vitro* replication system has indicated that Cyclin E and A have specialized roles during the transition from G0 to S phase (Coverley et al., 2002). While Cyclin E stimulates pre-RC assembly, Cyclin A activates DNA synthesis by replication complexes that are already assembled on one hand, while it inhibits the assembly of new complexes on the other. Thus, Cyclin E opens a "window of opportunity" for pre-RC assembly that is closed by Cyclin A (Coverley et al., 2002). Recent work indicates that an

essential mechanism that permits the opening of this window is the specific phosphorylation of Cdc6 by Cyclin E, which prevents degradation of the protein by the APC/C and thus permits pre-RC assembly (Mailand and Diffley, 2005). This conclusion has been mainly reached by using an antibody against Cdc6 phosphorylated on serine 54. Our work extends these findings further by showing that, when the cells enter the S-phase, Cdc6, phosphorylated on serine 54, is found in a complex that also includes GCN5, Cyclin A and Cdk2. At this precise moment, GCN5 specifically acetylates Cdc6 and this modification allows further phosphorylation of the protein on serine 106. The finding that the 3KR mutant, which is not acetylated, is still normally phosphorylated on serine 54 but not at all on serine 106 is fully consistent with this conclusion. A model summarizing these findings is schematically drawn in Fig. 54.

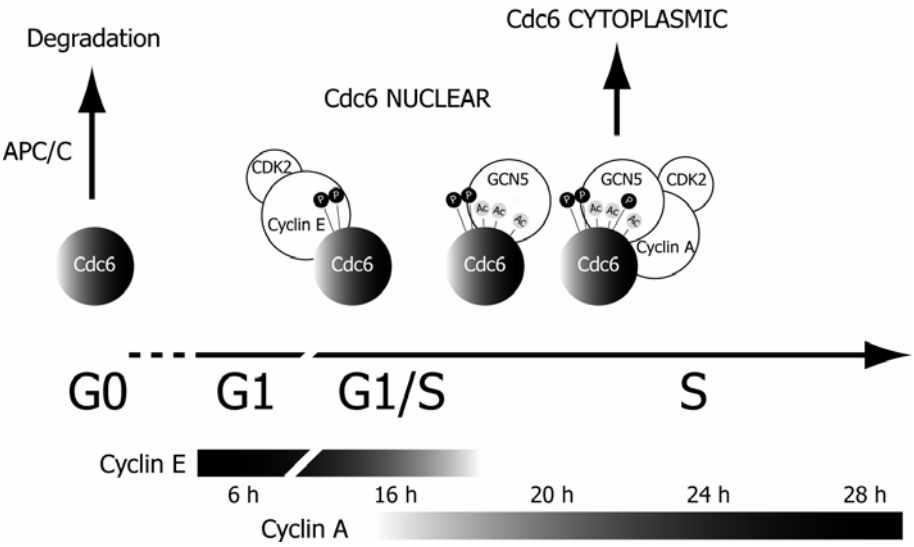

Figure 53. Model of "two-step intramolecular signaling regulatory program" for Cdc6 at the onset of S phase. Details of the model are described in the text.

Our results disclose the events that occur at a step that follows pre-RC assembly and origin firing, namely the steps that coincide with Cyclin A's appearance. Work performed in X. laevis using a XCdc6 protein mutated in the three CDK

phosphorylation sites has indeed suggested that XCdc6 phosphorylation by CDKs is not essential for either regulated binding of XCdc6 to chromatin nor for the subsequent loading of the MCMs, thus suggesting that Cdc6 phosphorylation might be required at later stages of the replication process (Coverley et al., 2000). Indeed, our work indicates that acetylation and subsequent specific phosphorylation of Cdc6 on serine 106 are essential to allow detachment of the protein from chromatin, relocalization to the cytoplasm and degradation, and that these events are essential to ensure proper cell cycle progression. Consistent with this conclusion, experimental evidence obtained in both in X. laevis and in human cells has shown that the overexpression of Cdc6 in G2 cells inhibits mitosis by inducing a checkpoint pathway involving Chk1 (Clay-Farrace et al., 2003; Oehlmann et al., 2004), and that this property is modulated by the phosphorylation of Cdc6 at selective residues (Clay-Farrace et al., 2003).

The definition of the subcellular localization of Cdc6 has roused much controversy over the last few years. While several authors have reported convincingly that the protein is exclusively nuclear in the G1 phase while most of it relocalizes to the cytoplasm during the S phase (Delmolino et al., 2001; Herbig et al., 2000; Jiang et al., 1999; Petersen et al., 1999; Saha et al., 1998), other data have suggested that this might be a peculiarity of an exogenously overexpressed protein, while the majority of the endogenously expressed one would remain essentially nuclear throughout the cell cycle (Alexandrow and Hamlin, 2004; Mendez and Stillman, 2000). Our results in fact challenge this last conclusion, and clearly indicate that human endogenous Cdc6 (stained by an anti-Cdc6 monoclonal antibody) is found in both the nucleus and the cytoplasm in non-synchronous cells. Consistent with the reported binding and kinase activity of Cyclin A/Cdk2 (Coverley et al., 2000; Delmolino et al., 2001; Petersen et al., 1999), in most of the cells that express endogenous Cyclin A, Cdc6 is only found in the cytoplasm (Fig.38), and Cyclin A overexpression forces the cytoplasmic relocalization of endogenous Cdc6 (Fig.37), as also reported by other laboratories (Mailand and Diffley, 2005; Petersen et al., 1999). Cdc6 acetylation plays an essential role in determining the cytoplasmic relocalization

of the protein. This conclusion is supported by the observation that the overexpression of GCN5 (but not that of its catalytically inactive mutant) forces the cytoplasmic relocation of both endogenous and transfected Cdc6, while the GCN5 knock down has the opposite effect (Fig. 42). In a consistent manner, both the K3R mutant (which is not acetylated) and the S106A mutant (which is not phosphorylated on serine 106) have a strictly nuclear localization, irrespective of GCN5 overexpression (Figs. 44, 45 and 46). These data reinforce the conclusion that the cytoplasmic relocalization of Cdc6 is strictly dependent on the consequential acetylation and phosphorylation of Cdc6 on serine 106.

What is the fate of acetylated Cdc6? Silencing of GCN5 expression by RNA interference and overexpression of a catalytically inactive GCN5 protein both markedly increase the total amount of Cdc6 (Figs. 23 and 19 respectively), as well as force its prevalent nuclear localization. Consistent with the notion that acetylation precedes, and is essential for, the specific serine 106 phosphorylation, GCN5 knock down and the lack of its enzymatic activity both selectively impair serine 106 phosphorylation (Figs. 24 and 19 respectively). These observations suggest that the acetylation of Cdc6 might be essentially involved in the regulation of its stability. The notion that both the Cdc6 K3R and S106A mutants are significantly more stable than the wild type protein (Figs. 52 and 53) are fully consistent with this possibility. Of interest, while the overexpression of either of the two mutants did not induce DNA re-replication, the K3R mutant determined a marked increase in the number of BrdU-positive cells and a significant decrease of both G1 and, most remarkably, G2/M cells (Fig. 51). This result indicates that Cdc6 acetylation is essential to ensure proper S-phase progression. Since most of this mutant is found in the insoluble nuclear compartment (Fig. 49), we propose that acetylation essentially controls the release of Cdc6 from chromatin during the S-phase, and that this event is essential for S-phase progression.

The results presented in this manuscript also underscore the role of GCN5 as a general cell cycle regulator (Kikuchi et al., 2005). Indeed, this HAT appears to play a key role in regulating the expression of several cell cycle-related genes,

such as Cyclin A, Cyclin D3, PCNA and CDC25B. The observation that GCN5 also regulates the function of one key regulator of pre-RC formation and controls licensing extends this concept further. The finding that Cdc6 is specifically acetylated by GCN5 and that this HAT finely tunes its function does not exclude the possibility that the protein might also be the substrate of other HATs. Indeed, recent work performed in *Xenopus* has shown that Cdc6 might also be an *in vitro* substrate for the Hbo1 acetyltransferase (Iizuka et al., 2006), the same HAT that also interacts with Orc1 and Mcm2 (Burke et al., 2001; Iizuka and Stillman, 1999).

Finally, our findings support the recently coined concept that protein acetylation and phosphorylation might occur in different proteins as two closely interconnected modifications that are part of a multi-step regulatory program (Yang, 2005). Examples of other factors in which these modifications are strictly related and, in some instances, sequential, include p53 and p73 , Rb, Foxo1 and c-Myc (Bode and Dong, 2004; Chan et al., 2001; Matsuzaki et al., 2005; Ozaki et al., 2005; Vervoorts et al., 2006). It will be interesting to understand the exact changes that the acetylation of Cdc6 determines in the molecular structure of the protein and how these changes might impinge on the subsequent site-specific phosphorylation.

EXPERIMENTAL PROCEDURES

Plasmids

(manuscript #1) The human Orc1 and Orc2 cDNAs were obtained by RT-PCR amplification from total RNA of human HeLa cells and cloned as *Bam*HI-*Xba*I and *Kpn*I-*Xho*I fragments into the pcDNA3 vector (Invitrogen, USA) respectively. The human HP1α cDNA was obtained from a Superscript normal human prostate cDNA library on the pCMV•SPORT6 vector (Life Technology). To express the Orc1-GFP fusion, human Orc1 cDNA was cloned in-frame at the N-terminus of the GFP ORF in pEGFP-N1 vector (Clontech, USA). To obtain the Orc1-Flag fusion the human Orc1 cDNA was cloned into *Sac*I-*Sal*I sites of pIRES-hrGFP-2a plasmid (Stratagene, Carlsbad, CA). This vector contains a dicistronic expression cassette in which the multicloning site is followed by the internal ribosomal entry site (IRES) of the encephalomyocarditis (EMCV) virus linked to the humanized recombinant GFP coding sequence. This allows the expression of the gene of interest fused to the Flag epitope to be monitored at the single-cell level owing to the translation of the GFP from the same transcript. GFP and Flag-tagged mutants of Orc1 were generated by two-step PCR-mediated mutagenesis with suitable primers, as previously described (Montecucco et al., 1998). Restriction enzymes were purchased from Promega, Madison, WI. Oligonucleotides were purchased from MWG-Biotech AG (Germany). Plasmids were verified by DNA sequencing (Thermo Sequenase^{TM}Cy$^{5.5}$ Amersham Bioscience). pGEX2T-HP1α was obtained from pCMV•SPORT6-HP1α by PCR amplification and cloning of a *Bam*HI-*Eco*RI fragment into pGEX-2T (Amersham Bioscience). The Orc1 deletion mutants for the GST pull-down assays (1-144, 151-269, 270-861) were obtained as *Hin*dIII-*Kpn*I fragments from the respective GFP fusions and cloned into the pcDNA3 vector for *in vitro* transcription. The Orc1 C-terminal mutant 529-861 was obtained as an *Eco*RI-*Xba*I fragment from plasmid pcDNA3-Orc1 and subcloned into the same vector. pEGFP-HP1α was obtained by PCR amplification of pCMV•SPORT6-HP1α and cloning of an *Eco*RI*Bam*HI fragment into pEGFP-C1 (Clontech) digested with *Eco*RI*Bgl*II. pBFP-Orc1 was obtained by recovering the

Orc1 segment from pEGFP-Orc1 as an *Hin*dIII-*Mlu*I fragment followed by cloning into the pBFP-C1 vector (Clontech). pEGFP-Orc2 was obtained by PCR amplification of pCDNA3-Orc2 and cloning of a *Kpn*I-*Sma*I fragment into pEGFP-C1.

(manuscript #2) The human Orc1, Orc2 and E2F1 cDNAs were obtained by RT-PCR amplification and cloned into the pcDNA3 vector (Invitrogen, USA). All the mutated and deleted versions of Orc1 were obtained by recombinant PCR and cloned into the pcDNA3 vector with the addition of an N-terminal HA tag. The vector expressing GST-E2F1 has been already described (Marzio et al., 2000). The vectors expressing the GST fusion proteins containing the AB pockets of Rb, p107, and p130 (Hauser et al., 1997) are a kind gift of Prof. D. Cress. The vectors expressing the GST fusion proteins of the deleted and point-mutated versions of the large ABC pocket region of Rb (AE, AB, SE, and $AE^{Cys706Phe}$) (Pennaneach et al., 2001) were kindly provided by Prof. A. Fotedar. For the FRET experiments, the E2F1, Orc1, Orc2, Mcm2, Mcm3 and Rb cDNAs were obtained by PCR and subcloned in frame in both the pEBFP-N1 and pEGFP-N1 vectors (Clontech); the Orc1-GFP, Orc2-BFP constructs have been already described (Lidonnici et al., 2004).

(manuscript #3) The expression vectors pFlag-Cdc6 and pGEX20T-Cdc6 were constructed by PCR amplification of the Cdc6 cDNA from the pcDNA3-Cdc6 vector (a kind gift of C. Pelizon) and subcloned into pFlagCMV 2 (Stratagene) and pGEX20T vectors, respectively. pGEX2T-GCN5 short isoform (GCN5 S) expressing vector was a kind gift of M. Benkirane, CNRS Montpellier. pGEX-2T-GCN5 deletion mutants were obtained by PCR amplification of GCN5 cDNA with primers specific for all the deleted versions. pcDNA3-HA-GCN5 was prepared by subcloning the GCN5 cDNA into the pcDNA3-HA vector (Invitrogen).

The expression vector pcDNA3-HA-GCN5mut containing the catalytically inactive GCN5 S mutant (Y260A/F261A) (Paulson et al., 2002) was constructed by recombinant PCR. Different versions of Cdc6 (1-60, 1-90, 1-185, 1-363, 91-561, 111-561, 186-561) and GCN5 S (1-189, 190-270, 271-383, 384-476) deleted mutants were obtained by PCR amplification and cloned into the pGEX vectors.

The pGEX-Cdc6 KR and the pFlag-Cdc6 KR and SA point mutants were constructed using recombinant PCR starting from each original vector. The pCMX-cyclin A and pCMX-cyclin E vectors were a kind gift of J. Pines (MRC, Cambridge). All constructs were verified by nucleotide sequencing before use.

Cell cultures, synchronization and treatments

(manuscript #1) HeLa, COS7 and NIH-3T3 cells were grown as monolayers in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 4 mM glutamine and 50mg/ml gentamicin. Exponentially growing cells were transfected using FUGENE 6 transfection reagent (Roche, Germany). Routinely we used 1 mg of plasmid DNA for $3x10^5$ cells. 24 or 48 hours after transfection cells were analyzed by western blotting and/or immunofluorescence. Synchronization of HeLa cells in mitosis was obtained by growing cells in 40 ng/ml nocodazole (Sigma, St Louis, CA) for 16 hours as described in the previous paragraphs (Rossi et al., 1999). For NIH-3T3 synchronization, cells were grown for 20 hours in 100 ng/ml nocodazole. For synchronization in G1 phase, mitotic cells were shaken off and released in fresh medium for different times. HeLa and NIH-3T3 cells were incubated for 6 hours in trichostatin A (TSA; Sigma) containing medium at the concentrations indicated in the text. RNase treatment was performed as described in (Chiodi et al., 2000). Briefly, cells grown on coverslips were incubated at 4°C for 10 minutes in extraction buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES pH 6.8, 3 mM MgCl2, 2 mM vanadylribonucleaside complexes, 0.5% Triton X-100 and the following protease inhibitors: 2 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, 0.2 mM AEBSF) and for an additional 10 minutes in the same buffer with 250 mM ammonium sulfate. After washing, cells were incubated for 20 minutes at 37°C with 25 mg/ml RNase A (Roche) and then subjected to immunofluorescence.

(manuscripts #2, 3) HeLa, T98G, U2-0S, HEK 293T and Saos-2 cell lines were maintained in Dulbecco's modified Eagle's Medium (DMEM) with Glutamax (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.). H1299 cell line was maintained in RPMI 1640 Medium with Glutamax (GIBCO) supplemented with 10% fetal bovine serum (Life Technologies, Inc.).

HeLa cells were synchronized in M phase by sequential treatment with 2.5 mM thymidine (Sigma) for 15 h, washed and released in fresh medium for 9 h and, finally, blocked with 50 ng/ml nocodazole (Sigma) for 15 h. For subsequent synchronization through the G1 to the S phase, mitotic HeLa cells were shaken-off, washed and released in fresh medium at different times.

T98G cells were synchronized by serum deprivation for 72 hr and stimulated with 20% FBS (final concentration) to allow cell cycle re-entry. (Galbiati et al., 2005; Takahashi et al., 2000).

Treatments with trichostatin A (TSA, Sigma) were performed by adding the drug (250 ng/ml) overnight.

BrdU incorporation experiments were performed (manuscript #2) on cells transiently transfected for 72 h with siRNA duplexes. Cells were pulsed for 1 h with BrdU at final concentration of 1 mM and BrdU-positive cells were detected by using a FITC conjugated anti-BrdU antibody (Abcam). BrdU incorporation experiments were performed (manuscript #3) on transiently transfected cells at 48 h after transfection. Cells were pulsed for 1 h with BrdU (final concentration 10 μM) and BrdU-positive cells were detected by using a FITC-conjugated anti-BrdU antibody (Becton Dickinson).

Cells were collected and analyzed by double-flow cytometry analysis on a FACSCalibur (Becton Dickinson) instrument, to simultaneously determine the cell cycle profile (DNA content) by incorporation of propidium iodide (SIGMA), and the S phase cell population by incorporation of BrdU. Cell cycle profile distributions were determined with the CellQuestPro and Modfit LT 3.0 softwares.

Cell cycle profile analysis of Orc1-GFP-transfected cells

HeLa cells were transiently transfected with pOrc1-EGFP plasmid by the calcium phosphate method in six-well culture plates (Corning). 48 hours after transfection, cells were collected and analyzed by double-cytofluorometric analysis on a FACSCalibur (Becton Dickinson). Cells were analyzed for cell cycle profile (DNA content) by incorporation of propidium iodide solution (Sigma), and simultaneously for Orc1-GFP expression.

Cell extracts, chromatin isolation, western blottings and co-immunoprecipitations

(manuscript #1) To prepare total cell extracts, cells were harvested by centrifugation (5 minutes, 1300 g, 4°C) resuspended in Laemmli buffer, and boiled for 10 minutes (Montecucco et al., 2001). Cytosolic (Cyt, S2), nucleosolic (S3) and chromatin-bound (P3) fractions were prepared following a biochemical fractionation method (Mendez and Stillman, 2000). To isolate the chromatin fraction, HeLa cells were resuspended ($4x10^7$ cells/ml) in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 MgCl2, 0.34 M sucrose, 10% glycerol, 1 mM dithiothreitol and protease inhibitors) plus 0.1% Triton X-100. Cells were incubated for 5 minutes on ice. Nuclei were collected in pellet 1 (P1) by low-speed centrifugation. Nuclei were washed and lysed in hypotonic buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol and protease inhibitors) that induces nuclear swelling. Insoluble chromatin (P3) was collected by centrifugation. The following primary antibodies were used for detection of protein antigens: Flag-probe (D8) polyclonal antibody (Santa Cruz Biotechnologies); anti-Orc1 and anti-Orc2 polyclonal antibodies kindly supplied by Bruce Stillman (CSH Lab, USA); anti-cyclin E monoclonal antibody (Santa Cruz Biotechnologies); anti-Cyclin A monoclonal antibody (Sigma); anti-α-Tubulin monoclonal antibody (Sigma). Primary antibodies were revealed with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies and enhanced chemiluminescence systems

(Super Signal West Dura Extended, Pierce, Rockford, IL and ECL, Amersham Bioscience).

For co-immunoprecipitation experiments, nuclear extract was prepared from G1-phase HeLa cells expressing Orc1-Flag. The extract was incubated with anti-Flag M2 affinity gel (Sigma) for 1 hour at 4°C. The immunoprecipitated material was analyzed by western blotting with the anti-Flag and anti-Orc2 polyclonal antibodies.

(manuscript #2) Polyclonal antibodies anti-Orc1 were produced and purified by immunization of rabbits with a His-tagged Orc1 250-480 Aa polypeptide (Todorovic et al., 2005). Anti-Orc2 antibodies were from MBL. Monoclonal Anti-Rb (554136) antibodies were purchased from BD Biosciences/Pharmingen. Rb (IF8), Rb (C-15), E2F1 (KH95), E2F1 (C-20), Cdc6 (108.2), Cdc6 (H-304), Cyclin E (M-20), Cyclin A (H-432), Cyclin B1 (H-433), HDAC1 (C-19) antibodies were from Santa Cruz Biotechnologies. Anti-HA (3F10) antibodies were from Roche and anti-αtubulin (B-5-1-2) antibody from Sigma. Whole cell extracts were prepared in HNNG buffer (15 mM Hepes pH 7.5, 250 mM NaCl, 1% NP-40, 5% glycerol, 1 mM PMSF) supplemented with 25mM NaF, 10 mM β-glycerophosphate, 0.2 mM sodium orthovanadate and protease inhibitors cocktail tablet (Roche) for immunoblotting. Immunoblots were carried out with 30 to 50 µg of whole-cell lysate. Immunoprecipitations were performed on 1-2 mg/ml of total protein lysate. Lysates for immunoprecipitation were incubated overnight with the appropriate amount of antibody (usually 1 to 2 µg) at 4°C. Immunocomplexes were collected with protein A/G plus agarose beads, washed in HNNG buffer, treated with DNase I (Gibco BRL) for 15 min at room temperature. Beads were sequentially washed at 4°C with HLNG buffer (as HNNG but with LiCl), TE buffer and finally resuspended in Laemmli sample buffer. Proteins were separated on 4-20% Tris-glycine gradient gel (Invitrogen) and detected by immunoblotting using the enhanced chemiluminescence systems (Super Signal West Dura Extended, Pierce, Rockford, IL and ECL, Amersham Bioscience).

(manuscript# 3) Antibodies against Cdc6 (sc-9964), Cdc6 (sc-8341), Cdc6 (sc-13136), p-Cdc6 Ser 54 (sc-12920), p-Cdc6 Ser 106 (sc-12922), Cyclin E (sc-481), Cyclin A (sc-571), GCN5 (sc-6303 and sc-20698), Anti-Mcm3 (sc-9850) and anti-HA (sc-805) were from Santa Cruz Biotechnology. Anti-Flag M2 (F1804) and anti-αTubulin (T6074) were from Sigma; anti-Cdk2 (610145) was from BD Transduction Laboratories™ and the anti-Orc2 antibody was from MBL. The anti-acetyl lysines antibodies (#9441 and #06-933) were from Cell Signaling Technology and Upstate Biotechnology, respectively.

Whole cell extracts were prepared in HNNG buffer (15 mM Hepes pH 7.5, 250 mM NaCl, 1% NP-40, 5% glycerol, 1 mM PMSF) supplemented with 20 mM sodium butyrate (Sigma), 10 mM NaF (Sigma) and protease inhibitors cocktail tablet (Roche). Immunoblots were carried out with 30 to 50 µg of whole-cell lysates. Immunoprecipitations were performed on 1-2 mg/ml of total protein. Lysates for immunoprecipitation were incubated overnight with the appropriate amount of antibody (usually 1 to 2 µg) at 4°C. Immunocomplexes were collected with protein A/G plus agarose beads (Santa Cruz Biotechnology), protein A trisacryl beads (Pierce) or anti-Flag M2-conjugated agarose beads (Sigma), washed in HNNG buffer, treated with DNAase I (Gibco BRL) for 15 min at room temperature. Beads were sequentially washed at 4°C with HLNG buffer (as HNNG but with LiCl), TE buffer and finally resuspended in Laemmli sample buffer. Proteins were separated on 10% SDS-PAGE gel (Invitrogen) and detected by immunoblotting using the enhanced chemiluminescence systems (ECL, Amersham Bioscience).

Cytosolic (S2-Cyt), nucleosolic (S3-Sol) and chromatin-bound, nuclear insoluble (P3-Ins) fractions were prepared following biochemical fractionation as described (Mendez and Stillman, 2000).

Immunofluorescence

(manuscript #1) Cells grown on coverslips were rinsed with cold PBS, fixed for 10 minutes in 2% paraformaldehyde and permeabilized in PBS-0.5% Triton X-

100 for 10 minutes at 4°C. When required, soluble proteins were extracted by incubating the cells for 2 minutes on ice in CSK buffer (10 mM HEPES-KOH, pH 7.4, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 0.5% Triton X-100 and protease inhibitors). Epitope-tagged proteins were detected with the anti-Flag polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). HP1- proteins (α, β) were revealed with the anti-mouse M31/human HP1β antibody (Serotec, UK) and mouse anti-HP1α monoclonal antibody (Chemicon International, Temecula, CA). For PCNA detection with PC10 monoclonal antibody (Santa Cruz Biotechnologies) cells were fixed with paraformaldehyde and permeabilized with cold methanol as described in (Montecucco et al., 1998). Primary antibodies were diluted at working concentration in PBS containing 2% skimmed milk (DIFCO, USA). After 1 hour at 37°C in a humid chamber, coverslips were washed three times with PBS. Secondary antibodies used were: TRITC-conjugated anti-rabbit IgG, Cy5-conjugated antirabbit IgG, Cy5-conjugated anti-rat IgG, FITC-conjugated anti-rat IgG, TRITC-conjugated anti-mouse IgG (all from Jackson ImmunoResearch Lab, UK). DNA was stained with 0.1 mg/ml 4,6'-diamino-2-phenylindole (DAPI; Sigma). Conventional epifluorescence microscopy was performed with a Leitz Orthoplan microscope equipped with a 50x objective. Photomicrographs were taken with Camedia digital camera C-3030 (Olympus). Confocal microscopy was performed with a Leica TCS SP2 confocal laser microscopy apparatus equipped with a 63x/NA=1.32 oil immersion objective. We used the 488-nm laser line for excitation of FITC and GFP (detected at 500 nm<λFITC<540 nm), the 543-nm laser line for 5223 Orc1p localization during the cell cycle TRITC fluorescence (detected at λ>570 nm) and the 633-nm laser line for excitation of Cy5 (detected at $650<\lambda_{Cy5}<700$ nm). Images were exported to Adobe Photoshop (Adobe, San Jose, CA).

(manuscript #3) Immunofluorescence analysis was conducted as described in (Marcello et al., 2003). Briefly, HeLa cells were incubated with primary and secondary antibodies at 37°C for 1 h in a humidified chamber. The secondary FITC- (sc-2010) and Alexa 594-conjugated antibodies (A11032, A11072) were from Santa Cruz Biotechnology and Invitrogen Molecular Probes,

respectively.Confocal fluorescence acquisitions were performed using a TCS-SL Leica confocal microscope. Images were acquired with the Leica software.

Analysis of protein stability

Protein stability experiments were performed on cells transiently transfected with Flag-Cdc6 expressing vectors. At 24 hours after transfection, cycloheximide (Sigma) was added at a final concentration of 30 µg/ml. Cell lysates were obtained at different time points, and protein levels were assessed by immunoblotting using the ECL system. Densitometric analysis of specific band intensities was performed on films by Kodak 1D software (Kodak).

Fluorescence resonance energy transfer (FRET)

From Green to Blue.

Cells were transiently transfected with expression plasmids expressing EGFP and BFP fusion proteins for the different proteins fused to different fluorophores by the calcium phosphate method in LabTek II four-chamber glass slides (Nalgene). Cells were fixed in 2% paraformaldehyde after 48 hours and mounted directly in 70% glycerol for FRET analysis. FRET measurements were carried out with an epifluorescence Axioskop 2 Zeiss microscope mounting a 103 W HBO lamp, a 100x/NA=1.3, oil immersion Plan-Neofluar objective and Nomarsky optics, performed as described in (Marcello et al., 2001; Marcello et al., 2003). The existence of FRET was inferred by determining the ratio between EGFP fluorescence following excitation at 350 nm to that following excitation at 480 nm. First, EGFP emission was collected by integrating the fluorescence signal around 520 nm (band width 40 nm) under EGFP excitation at 480 nm (wavelength selection was obtained by 40 nm band-pass filters, excitation power was 5 W/cm2). Second, EGFP emission in the same frequency range was measured after excitation at 350 nm (power density 2 W/cm^2 and band width 60 nm). Background was detected out of the cell under study for each frame and subtracted from the relevant fluorescent signal. Following this procedure, the

ratio between the two measured EGFP emissions (data taken following excitation at 350 nm divided by those at 480 nm) provides the FRET efficiency. Fluorescence was collected by a PentaMax 512-EFT intensified CCD camera with detection times of the order of 0.1 s (in particular, for data taken under excitation at 350 nm they were 5 times longer than for those relative to 480 nm excitation). Data acquisition and analysis were performed with Metamorph software (Universal Imaging Corp.). When evaluating FRET ratios, emission intensities were scaled to take into account the different detection times. For the quantification of subcellular FRET, the boundaries of individual subcellular compartments were first drawn on the corresponding fluorescent images collected by illuminating the same cells at 480 and 350 nm; FRET was calculated according to the ratios between the averages of the two signals within the regions defined by these boundaries (Marcello et al., 2001; Marcello et al., 2003).

From Cyan to Yellow.

The CFP-YFP fusion plasmid was constructed by inserting the *BspEI-BglII*-containing fragment of pEYFP-C1 into the appropriate sites of the pECFP-C1 plasmid (BD Biosciences Clontech) (Karpova et al., 2003). The YFP–containing fragment was obtained by PCR using the following primers: 5'-AAG TCC GGA ATG GTG AGC AAG GGC GAG GA-3' and 5'-TCG AGA TCT CTT GTA CAG CTC GTC CAT GAC-5'. In this fusion, CFP and YFP were separated by two amino acid residues. Human CFP-Orc1 was obtained by recovering the Orc1 segment from the pEGFP-Orc1 expression vector in a *Hin*dIII-*Mlu*I fragment followed by cloning into the pCFP-C1 vector (Clontech). pEYFP-HP1α was obtained by PCR amplification of the pCMV•SPORT6-HP1α expression vector and cloning of a *EcoRIBamHI* fragment into pEYFP-C1 (Clontech) digested with *EcoRI-BglII*. HeLa cells ($5x10^5$) were seeded into four-well glass chamber slides (LabTek II-Nalge Nunc) and transfected with the Effectene transfection kit (Qiagen) for 24 h with 400 ng of pEYFP-HP1α and pECFP-Orc1 at a 1 to 1 molar ratio, or with 400 ng of pCFP-EYFP. Fluorescent images of samples fixed with 2% paraformaldehyde

were acquired using a TCS-SL Leica confocal microscopy. To calculate the FRET efficiency as a percentage (Ef):

$$Ef = (F\ cfp\text{-}post - F\ cfp\text{-}pre) x\ 100 / F\ cfp\text{-}post$$

as reported (Karpova et al., 2003), where *F cfp-pre* and *F cfp-post* are, respectively, the CFP intensity before and after photobleaching.

Chromatin Immunoprecipitation (ChIP)

(manuscript # 1) $3x10^7$ exponentially growing HeLa cells were transfected with Orc1- Flag and synchronized in G1 as described above. Cells were crosslinked for 3 minutes with 1% formaldehyde (Merck). Chromatin was purified and then fractionated through a Cesium chloride density gradient as previously described (Frouin et al., 2002; Paixao et al., 2004). After dialysis against 50 mM Tris-HCl pH 8, 150 mM NaCl and 5 mM EDTA, chromatin was immunopurified with 10 mg anti- Flag M2 affinity gel (Sigma) or 10 mg anti-immunoglobulin G (IgG) (Santa Cruz Biotechnologies) antibody as negative control. The immunopurified chromatin was incubated in PK buffer (0.5% SDS, 100 mM NaCl, 300 mg/ml proteinase K) for 3 hours at 56°C. Crosslinking was reverted at 65°C for 6 hours. DNA was purified by standard phenol-chloroform extraction and ethanol precipitation and dissolved in 50 ml of TE buffer. DNA was then analyzed by competitive PCR.

(manuscript # 2) Cells were fixed by adding formaldehyde (Fluka) directly to the cell culture medium at 1% final concentration. Cross-linking was allowed to proceed for 7 min at 37°C and was stopped by the addition of glycine (Sigma) at a final concentration of 125 mM. Cells were washed and harvested in ice-cold PBS by centrifugation, the cellular pellet was resuspended in HNNG buffer and chromatin was sheared by sonication (average size of 0.5–1.5 kb fragments) on ice and centrifuged to pellet debris. Immunoprecipitations were performed as above. Protein-DNA immunocomplexes were collected with protein A/G plus agarose beads (Santa Cruz), washed sequentially in HNNG buffer and HLNG buffer, resuspended in TE buffer and treated at 100 µg/ml of RNase A (Roche)

for 30 min at 37°C. Samples were incubated for 1 h at 56°C with 0.5 mg/ml Proteinase K (Sigma), and for 15 h at 65°C to revert crosslinks. DNA was extracted with phenol:chloroform:isoamyl alcohol 25:24:1 (Invitrogen), ethanol precipitated and resuspended in 10 mM Tris HCl pH 7.5 for real time PCR.

Competitive PCR analysis

Competitive PCR was performed using primer sets B48 (B48II Dx, 5'-GACTGGAAACTTTTTTGTAC-3'; B48 Sx, 5'-TAGCTACACTAGCCAGTGACCTTTTTCC-3') and B13 (B13 Dx, 5'-GCCAGCTGGGTGGTGATAGA-3'; B13 Sx, 5'-CCTCAGAACCCAGCTGTGGA-3'). A constant volume of immunopurified DNA was coamplified with decreasing amounts of competitor template for 40 cycles. The competitor consists of a 130 bp stuffer DNA flanked by the target sequences for B13 and B48 primer sets and was obtained as described in (Giacca et al., 1994). Amplification products were resolved on 10% SDS-PAGE and stained with ethidium bromide. The intensity of the amplification band was quantified with the NIH-Image program (version 1.62).

Real Time PCR

Sequence-specific primer and probe sets for real time PCR analysis were designed by Primer Express 1.5, in order to amplify and detect origin as well as non-origin areas within the human Lamin B2 and GM-CSF origins (sequences are available from the authors upon request). Real-time PCR was carried out in triplicate using Universal Master Mix (Applied Biosystems) on an ABI Prism 7000 Sequence Detector System (Applied Biosystems). Sequence Detector software (version 1.7) was utilized for data analysis and relative fold enrichment was determined by the comparative cycle threshold (C_T) method.

In vitro Acetylation Assay

HAT assays were performed as reported in (Marzio et al., 2000). Briefly, GST fusion proteins used as substrates were incubated with HeLa nuclear extracts or recombinant purified GST-GCN5 and [^{14}C]-acetyl-CoA in HAT buffer (50 mM Tris pH 7.5, 5% glycerol, 0.1 M EDTA, 50 mM KCl, and 2 mM sodium butyrate) in a final volume of 20 µl for 45 min at 30°C. The nuclear extracts were prepared as described in (Dignam et al., 1983). Acetylated proteins were visualized by phosphoimaging (Cyclone, Packard) after separation on SDS-PAGE electrophoresis.

GST pulldown assay

[^{35}S]-labelled proteins used for *in vitro* binding assays were produced by using the TNT Reticulocyte Lysate System (Promega) according to the manufacturer's instructions, by using the corresponding pcDNA3 vectors as templates. The recombinant GST fusion proteins were produced and purified from BL21 bacteria transformed with the respective plasmids as described in (Marzio et al., 2000). Briefly, Bacterial cultures were grown in terrific broth + ampicillin and protein production was induced with IPTG 0,5 mM for 3 hours at 30°C with OD_{600} between 0,6 and 0,8. Bacteria were then resupended in lysis buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5% glycerol, 2 mM dithiothreitol) and sonicated by 4 pulses of 30 sec each. Bacterial lysates were mixed with a 50% (vol/vol) slurry of glutathione cross-linked agarose beads and the GST-fusion proteins were allowed to bind the beads at 4°C on a rotating wheel for 1 hour. The suspension was then loaded on an empty plastic column (Bio-Rad, Richmond, CA), letting the unbound proteins pass through, and the beads were washed with 400 beds volumes of lysis buffer. The purity and integrity of the proteins were routinely checked by sodium dodecyl sulfate-plyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. GST pulldown assays were performed as described in (Marcello et al., 2000). To remove contaminant bacterial nucleic acids, recombinant proteins were pretreated with nucleases (0.25 unit/µl DNase

I and 0.2 µg/µl RNase) for 1 hr at 25°C in 50 mM Tris•HCl, pH 8/5 mM $MgCl_2$/2.5 mM $CaCl_2$/100 mM NaCl/5% glycerol/1 mM DTT. Subsequently, GST fusion proteins immobilized on agarose beads were washed and resuspended in NETN buffer (20 mM Tris•HCl, pH 7.5/100 mM NaCl/1 mM EDTA/0.5% Nonidet P-40/1 mM DTT/1 mM phenylmethylsulfonyl fluoride) supplemented with 0.1 mg/ml ethidium bromide to impede the possible formation of nonspecific interactions between residual DNA and proteins. Six hundred cpm of ^{35}S-labeled p300 or hTAF32 proteins was added and incubated at 4°C on a rotating wheel. After 1 hr, bound proteins were washed five times with 1 ml of NETN buffer and separated by electrophoresis in an SDS/7% polyacrylamide gel. Dried gels were quantitated by INSTANT IMAGER (Packard) or by phosphoimaging (Cyclone) (Marcello et al., 2000; Marzio et al., 1998).

Immunoprecipitation-kinase assay

Cyclin A/Cdk cellular complexes were obtained by immunoprecipitation with an anti-Cyclin A antibody (sc-571) overnight at 4°C from 1 mg of T98G protein cell extract and then incubated with protein A trisacryl beads (Pierce) and extensively washed with HNNG supplemented with 10 mM NaF and 0,2 mM Na_3VO_4. Cyclin A/Cdk immunocomplexes were then washed and equilibrated with kinase buffer (50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 20mM NaF). Kinase assays on specific Cdc6 peptides were performed by incubating the Cyclin A/Cdk immunocomplexes with a Cdc6 peptide (5,0 µg) in 20 µl reaction buffer (kinase buffer + 10 µM ATP + 2.5 mM DTT) in the presence of 5 µCi of [^{32}P]-ATP for 30 min at 30°C. Reactions were stopped by adding SDS sample buffer followed by boiling. Phosphorylated proteins were visualized by phosphoimaging (Cyclone, Packard) after separation by 12,5% SDS-PAGE. Densitometric analysis of band's intensity was performed on Cyclone phosphor screens by the Optiquant 04.00 software. All quantifications experiments were performed at least three times.

The non acetylated (SHTLKGRRLVFDNQLTIKPSKREC) and acetylated (SHTL{Ac-K}GRRLVFDNQLTI{Ac-K}PS{Ac-K}REC) peptides corresponding to residues 88 to

111 of human Cdc6 protein were synthesized on solid phase (Fmoc/t-Bu chemistry). The synthesis was automatically performed with a PS3 Protein Technology synthesizer on a 0.05 mmol scale. After cleavage from the resin, the peptides were precipitated with diethylether, washed and freeze-dried. The reduced peptides were purified by RP-HPLC on aZorbax 300SB-C18 column (Agilent).

RNA Interference Experiments

(manuscript #2) Cells were transiently transfected with smart pool siRNAs (Dharmacon) against Orc1 for 72 h at 40 nM final concentration by oligofectamine-mediated transfection (Invitrogen) following the manufacturer instructions. RNAi control experiments were performed using a duplex siRNA against luciferase (Dharmacon).

(manuscript #3) Cells were transiently transfected with an siRNA against GCN5 for 48 or 72 hours at different final concentrations (75, 100, 150 and 300 nM) by GeneSilencer (Genlantis) following the manufacturer's instructions. RNAi control experiments were performed using a duplex siRNA against Luciferase (LUC) (Palhan et al., 2005). RNAi conditions were optimized for knockdown of the targeted sequences: GCN5 (Dharmacon-SMARTpool selected, 5'-AACCAUGGAGCUGGUCAAUGA-3'), LUC (5'-NNAUGAACGUGAAUUGCUCAA-3').

Statistical analysis

P values are from two-tailed Student's t test.

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