[Frontiers in Bioscience 4, d805-815, December1, 1999]

THE METAZOAN ORIGIN RECOGNITION COMPLEX

David G. Quintana and Anindya Dutta

Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, 75 Francis Street, Boston, MA 02115, USA

TABLE OF CONTENTS

1. ABSTRACT

Regulated initiation of DNA replication relies on the firing of initiator proteins that bind specifically to origin DNA. The discovery of the first eukaryotic initiator, the *Saccharomyces cerevisiae* Origin Recognition Complex (ORC) has allowed us to discern some aspects of how the onset of replication is regulated. However, understanding the specifics of replication in metazoan organisms can only be achieved by directly addressing these questions in animal cells. This review deals with the current state of knowledge on the metazoan Origin Recognition Complex, its composition and regulation in higher eukaryotes, its role in the initiation of replication and beyond replication, and its possible connection with human pathology.

2. INTRODUCTION

Proliferating cells have to replicate the DNA they will confer to their progeny. For the faithful transmission of the genetic information every base pair in the genome must be copied once and only once. To achieve this, eukaryotic cells rely on thousands of origins of replication distributed throughout the chromosomes that are fired in a tightly regulated manner once per mitotic division cycle. Origins of replication from different organisms conform to the replicon model (1). According to this model, origins are defined by a specific DNA sequence, the replicator, and a protein or complex of proteins that recognizes the sequence and binds to it, the initiator. Upon firing, the initiator melts the DNA duplex in an ATP-dependent manner, allowing the loading of the DNA polymerase machinery. Additionally, the initiator may serve as an origin-specific tether for other proteins required in the replication such as the licensing factors that in eukaryotes mark unfired origins from those in already replicated regions.

The discovery of the first *bona fide* eukaryotic initiator, the *Saccharomyces cerevisiae* Origin Recognition Complex (ORC) (2), and the subsequent identification of human, *Caenorhabditis elegans*, *Arabidopsis thaliana*, *Schizosaccharomyces pombe* (3) and *Drosophila melanogaster* homologs (4), represented a critical breakthrough to address crucial questions regarding the control of replication onset: How is replication initiation coupled to the cell cycle? Which is the ultimate signal that fires the origins of replication? How is the timing of firing determined for early and late origins? How is replication from each origin licensed so as to avoid re-replication? How does the cell sense completion of replication? Which sequences define metazoan replicators? Most of our current knowledge on eukaryotic initiation of replication is based on the budding yeast *S. cerevisiae* where ORC was first identified. However, understanding the specifics of replication in animal cells is crucial for comprehending the mechanisms underlying phenomena such as the change in usage of origins during development, or the gene amplification events that occur in normal and pathological states, as well as for designing future strategies for

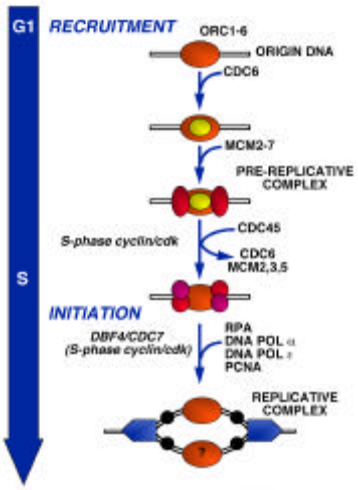

Figure 1. Assembly of initiation factors at the *S. cerevisiae* origins (see text).

intervention. An intense effort has been devoted in recent years to the identification and characterization of metazoan Origin Recognition Complexes which we review in here. For recent reviews on the topic, please see Dutta and Bell (5) and Stillman (6).

3. THE *SACCHAROMYCES CEREVISIAE* ORIGIN RECOGNITION COMPLEX

The discovery that certain genomic sequences confer autonomous replication activity (ARS) to plasmids in the yeast (7-9) and that deletion of these sequences in the genome abolishes initiation of replication in that region (10-14) allowed the identification of the protein activity that binds to these putative origin sequences, the six-subunit Origin Recognition Complex (2). Experimental evidence supports that ORC is an eukaryotic initiator. Mutations in ARS that block the binding of ORC also abolish replication (2, 15, 16). The cloning of the genes for the different ORC subunits showed that all six are independently essential for viability. Conditional mutants in the genes result in arrest of yeast at the large-budded stage and subsequent entry into an abortive S phase. In addition, ORC mutants show decreased frequency of origin firing and an increased loss of ARS-containing plasmids (17-23). In the budding yeast, ORC is assembled as a stable complex bound to DNA throughout the cell cycle (24-27), indicating that additional factors must regulate origin firing. Several proteins have been identified that sequentially bind, thus assembling the so-called pre-replicative complex (pre-RC) (see figure 1). The unstable protein CDC6 binds to ORC upon exit from mitosis (25, 28, 29). The CDC6 gene was first identified in a screen for temperature sensitive mutants that were cell division cycle (CDC) defective (30). Binding of CDC6 to ORC is required for the subsequent incorporation of the MCM complex to the pre-RC (26, 31, 32). The six MCM2-7 (mini-chromosome maintenance) proteins were originally identified in screens for mutants that result in loss of plasmids bearing the yeast origin ARS or that were cell cycle defective (33, 34). The MCM complex is part of the eukaryotic licensing system that marks replication-competent chromatin (see this issue).

Regardless of the requirement of ORC, CDC6 and MCMs for initiation of replication, pre-RC genomic footprints are detected both in active and inactive origins *in vivo* (35), indicating that additional factors must determine which pre-RCs are competent for initiation. One candidate factor is CDC45. CDC45 is essential for DNA replication (36), and CDC45 protein incorporates to the origins at the G1 ->S transition, depending on CDC6, MCM and S-phase cyclin-cdk activity (37).

For the onset of replication S-phase cyclin-cdk and Dbf4-Cdc7 protein kinase activities are required (38-41) (and this issue). The same cyclin-cdk activity that drives cells into S phase also prevents re-assembly of pre-RCs at the origins, thus avoiding re-replication (40, 42). Upon passage through mitosis, destruction of the mitotic cyclins by the APC and the proteasome results in termination of cdk activity, allowing pre-RCs to re-assemble for a new round of replication.

4. METAZOAN ORCS

4.1. Complexes homologous to *S. cerevisiae* ORC in higher eukaryotes

Metazoan complexes formed by proteins homologous to the *S. cerevisiae* Origin Recognition Complex subunits have been identified in *Drosophila* and *Xenopus*. A stable complex containing homologs to yeast ORC1-6 can be isolated from *Drosophila* embryos by co-purification with ORC2 (4, 43). In *Xenopus* egg extracts a multiprotein complex containing homologs to ORC1-5 and other unidentified proteins has been isolated (44-48). Also in the established somatic WAK *Xenopus* cell line, at least ORC1, 2 and 4 are associated as seen by coimmunoprecipitation with anti-ORC1 antibodies (48).

Human and mouse homologs for ORC1 (3, 49), ORC2 (3, 50), ORC3 (48, 51), ORC4 (48, 52) and ORC5 (48, 53, 54) have been cloned and characterized. In contrast to *Drosophila* and *Xenopus*, an endogenous complex containing all identified human ORC subunits has not been isolated. Yet, co-immunoprecipitation experiments show that in human cells, endogenous ORC2 is associated with ORC4 (52) and with ORC3 (51), and that ectopic ORC1 or ORC5 associate *in vivo*, with endogenous ORC2 (3) and ORC2-4 (51, 52, 54) respectively. Several reasons may account for the difficulty in isolating a human holocomplex. First ORC5 is poorly extracted under conditions that preserve protein-protein interactions, probably because ORC5 is associated with the nuclear matrix fraction (54). Second, no association of endogenous ORC1 with other ORC subunits in asynchronous human cell cultures has yet been detected (54, 55). It is possible that in human cells ORC1 assembles into ORC only in a limited window of time during the cell cycle. The cell cycle regulation of ORC1 in animal cells (see below) may account for the difficulty in seeing this subunit in an endogenous complex with other human ORC homologs in

somatic cell extracts. The *Drosophila* and *Xenopus* holocomplexes containing ORC1 have been isolated from embryonic extracts, where large supplies of maternal proteins required for the rapid proliferation of early development are present. Third, although human ORC2 and ORC4 co-precipitate, several of the other proteins co-precipitating with each of them are different (52). Although at least some of these proteins might be in a common complex that is dissociated during the experimental procedures, mammalian ORC might also have evolved into a more intricate system that will only be elucidated once all ORC interacting proteins are identified. One indicator of a higher degree of complexity in mammalian cells is an alternatively spliced form of ORC5, ORC5T, which has been identified and is expressed in human cells (54). The role of ORC5T is not yet known, although it interacts *in vivo* with ORC4 but not with ORC2 under conditions where ORC5 interacts with both. One such subunit might play a regulatory role in replication. Alternatively ORC5T may represent a separation of functions in higher eukaryotes and be involved in functions aside from the onset of S phase. It is worth noting that ORC5 has been suggested to play a still undefined role in mitosis in *S. cerevisiae* (56), and that *Drosophila* ORC is involved in heterochromatin formation (43) (see section 7).

4.2. ORC subunits and CDC6 belong to a super-family of proteins that bind and hydrolyze NTPs

In addition to ORC subunits, *Xenopus* (57) and human (55, 58, 59) homologs for the budding yeast CDC6 have been identified. Metazoan CDC6 is essential for initiation of replication (see below).

Protein sequence comparison reveals that CDC6, ORC1, ORC4 and ORC5 share extensive sequence similarity (21, 48, 52, 58, 60). Similarities include highly conserved Walker A and B motifs. These sequences are present in numerous DNA binding proteins that exhibit duplex unwinding or helicase activity, where they function as purine nucleotide binding and hydrolysis domains respectively (61). The function of the Walker A and B motifs in the different metazoan ORC subunits has not been investigated. In *S. cerevisiae*, ORC binds ATP and this binding is stimulated by, and required for, ORC binding to ARS DNA (62). The subunit responsible for this activity is ORC1. ORC1 mutants in the Walker A and B motifs are unable to complement a deletion in the ORC1 gene (21, 62). ORC5 has only a Walker A motif, and its role is less clear. Yeasts with mutations in theWalker A motif of ORC5 are viable (22) and complexes with mutant ORC5 can bind to ARS (62), but the mutation reduces ORC function *in vivo* (22). Interestingly, two different human clones for ORC5 have been reported whose single sequence difference is a mutation in the Walker A motif that would suppress ATP binding (54). The finding that hypomorphic ORC mutants may result in maldevelopment of specific brain structures in *Drosophila* (see 5.1) opens the intriguing possibility that the observed genetic polymorphism of ORC5 in humans may be of functional relevance.

Sequence homology extends beyond the proteins mentioned above to include both prokaryotic and eukaryotic proteins of the "clamp-loader" family and unknown archaebacterial proteins (48, 60). "Clamp-loaders" like RF-C or the *E. coli* gamma complex utilize the energy of hydrolysis of ATP to load ring-shaped sliding clamps (PCNA, beta complex) on to DNA. Therefore, the eukaryotic ORC subunits might be the result of an evolutionary specialization from a common ancestor with the "clamp-loader" family of proteins. The archebacterial proteins homologous to ORC/CDC6 proteins, probably belong to the same family of proteins and are either similarly involved in the initiation of DNA replication (like CDC6, ORC) or in the loading of a processivity factor for DNA polymerase (like RF-C).

5. FUNCTION OF METAZOAN ORC

5.1. Metazoan ORC is essential for initiation of replication

Metazoan ORC, like its *S. cerevisiae* counterpart, is essential for replication. In *Drosophila*, ORC2 is encoded by the k43 gene (63) first identified as a late-larval lethal null mutant (64, 65). Notably, the defects leading to death are limited to structures that depend on continued diploid cell division after maternal supplies of protein are depleted in late larval stages, with larvae characteristically showing small or missing imaginal discs (66, 67). k43 null mutants also show diploid cells with reduced mitotic index and fragmented chromosomes (66, 67). An ORC2 mutant, $k43^{fs293}$, has been identified that specifically results in suppression of gene amplification while diploid cell chromosome replication is not affected (63, 68). The mutants develop into viable adults but females are sterile due to underproduction of the chorion proteins required for the egg shell. The $k43^{fs293}$ mutation may selectively affect gene amplification by two mechanisms. Chorion gene amplification relies essentially on a single origin (69, 70). Because the frequency of origin firing is decreased to half in the $k43^{fs293}$ hypomorphic mutation (63), one observes a more profound defect in amplification of regions with a single origin of replication than in chromosomal replication, where a moving fork may extend from neighboring origins. Alternatively the $k43^{fs293}$ mutation disrupts a domain in *Drosophila* ORC2 essential for interaction of an amplification-specific factor. This would make this mutant a useful tool to identify the factors that determine gene amplification during oogenesis, and possibly in the identification of analogous functions involved in gene amplification in human cancer.

Similar results showing that ORC is essential for replication have been obtained for Latheo, the *Drosophila* ORC3 homolog. Flies carrying null mutations in Latheo arrest replication completely after second instar larvae, as shown by BrdU incorporation and the arrested development of the CNS. The time of the arrest correlates with depletion of the maternal supplies of proteins that support cell division through embryonic and larval stages. Null mutants die in early pupal stage lacking imaginal discs (51). Latheo was originally identified in a behavioral screen for olfactory memory mutants in fruit flies (71). The original lat^{P1} mutant with impaired learning capacity is a mild

hypomorphic mutation that affects anatomical structures of the adult brain. Homozygous lat^{P1} mutants develop normally with the exception of a significant reduction in the volume of the mushroom bodies, a brain structure involved in olfactory memory. The number of neurons in the mushroom bodies depends most critically on continued proliferation of mid-brain neuroblasts for an extended period of time during development (51). The hypomorphic mutation in Latheo/ORC3 might specifically affect the size of this adult tissue compartment, perhaps because the replication machinery is most stressed in these specific neuroblasts on account of their prolonged proliferation window. Alternatively, reduction in ORC activity might decrease (but not abolish) origin firing. As a result the chromosomes are replicated from fewer origins resulting in a subtle elongation of S phase. This slight prolongation of the cell cycle might have no effect on most tissue compartments whose size is measured during development by the number of cell divisions. However, in certain tissue compartments, inhibitory influences from neighbors might arrive after a specific time of development. In those compartments alone the number of cells is expected to diminish when the cell cycle is slightly prolonged. Whether polymorphisms in this or other ORC subunits exist in humans that may result in similar developmental deficits is an intriguing question. Interestingly, a human ORC5 clone has been identified with a mutation that removes the ATP binding Walker A motif (54), a mutation that in yeast results in reduced ORC function *in vivo* (22).

ORC is also essential for the initiation of replication in *Xenopus* egg extracts. Immunodepletion of ORC1, ORC2 or ORC3 from the extracts completely abolishes *in vitro* replication initiation of sperm DNA, but not elongation of replication on single-stranded DNA templates (44-47). Replication can be rescued in ORC-depleted extracts by addition of MCM-depleted extracts which by themselves are also unable to support replication (46). Similar immunodepletion experiments demonstrate that CDC6 is also essential for initiation in egg extracts (57). Additional support for ORC being the initiator in animal cells comes from the observation that ORC1 from egg extracts binds to demembranated sperm DNA to a saturation limit of one molecule every 16 kb (45), close to the observed 10-20 kb replicon size in early embryos (72-74). Finally, in *Xenopus* cells in culture, ORC immunostaining partially overlaps with sites of DNA replication during early, but not later, time-points in S phase (46).

No direct proof of involvement of ORC in initiation of replication has been demonstrated in mammalian cells. *In vivo* immunodepletion of CDC6 by microinjection of specific antibodies into human cell nuclei prevents entry into S phase, indicative of an essential role for at least this protein in the initiation of replication in mammals (59, 75).

5.2. ORC binding to chromatin

Does ORC remain bound to chromatin throughout the cell cycle in animal cells as in budding yeast? One would expect ORC binding to chromatin to be a more dynamic process in animal cells, where origin usage changes upon the transition from embryonic to somatic cell cycles, or upon methylation or transcription status, or upon change from endoreduplication to gene amplification in oogenesis. Most experimental evidence suggests indeed that metazoan ORC does not remain permanently bound to the chromatin throughout the entire cell cycle. At least during oogenesis in *Drosophila* follicle cells, a dramatic change in the ORC nuclear localization occurs when cells reach stage 10B of ovarian development and switch from endoreduplication cycles of the entire genome to amplification of specific genes. Both ORC1 and ORC2 change from a diffuse nuclear immunolocalization to discrete foci coincident with the loci of gene amplification, according to two independent groups (76, 77). Furthermore, at stage 11 of ovarian development, when replication ceases in follicle cells, ORC2 is no longer detectable (77). Other lines of evidence also support that metazoan ORC is not permanently bound to the chromatin. During *Drosophila* development ORC1 protein is not immunodetected in non-proliferating tissues (76), although as discussed below the presence of this subunit seems to be distinctive of proliferating cells, and therefore its absence does not necessarily reflect the absence of other ORC subunits. In *Xenopus* egg extracts and cells in culture, ORC remains bound to chromatin during S phase, but later the association seems to cease according to biochemical and microscopic studies (45, 46, 57). In human cells, the cell cycle variations of an *in vivo* footprint in the putative origin at the Lamin B2 locus have been convincingly described (78). The footprint enlarges along G1 phase, in a way compatible with the assembly of a pre-RC, shrinks during S phase, and remains unaltered through G2 phase until it disappears completely at M phase. However, it has not been demonstrated that ORC is responsible for this footprint. Presence of ORC bound to chromatin in G2 phase receives circumstancial support from the observation that permeabilized HeLa G2 nuclei are able to replicate in ORC-depleted *Xenopus* extracts, but not in MCM-depleted extracts (46).

On the other hand, ORC has been reported to remain bound to chromatin through the entire cell cycle in different *Drosophila* tissues, and loss of nuclear signal during mitosis was suggested to be due to an experimental artifact (43). While the question remains open, a structural role of ORC in the formation of heterochromatin (43) does not in itself eliminate the possibility of ORC ever leaving the chromatin in M phase. The structural requirement for ORC binding could be limited to a specific time during the cycle of proliferating cells and may not be necessary in M phase.

One mechanism has been described on how localization of ORC may be regulated. The change in ORC distribution that occurs in the switch from chromosomal endoreduplication to gene amplification requires the transcription factor E2F (77). As described above, during ovarian development in *Drosophila* follicle cells undergo several rounds of endoreduplication, with diffuse nuclear ORC staining, and then enter a phase of gene amplification at four specific foci, with ORC localizing to these foci solely. Mutants in E2F or DP with an intact transactivation domain and an inactive DNA binding domain, abolish the

focal localization of ORC. An E2F mutant with a deletion in the pRb binding and transactivation domain but an intact DNA binding domain still allows the relocalization of ORC to amplification foci, and results in earlier amplification. This observation indicates that the DNA binding domain of E2F but not the transcription activation domain is involved in assisting chromatin binding of ORC, either directly by physically interacting with ORC, or indirectly by changing the chromatin structure adjoining the E2F binding site.

5.3. ORC recruits other factors to the origins

In *Xenopus* egg extracts, loading of CDC6 to the pre-replicative complexes requires ORC (57), and CDC6 is in turn required for the loading of the MCM complex onto chromatin (45, 46, 57), whereas the loading of ORC needs neither CDC6 nor MCM protein (46), pointing to a sequential assembly of the components of the preRCs. Similarly, in quiescent human cells ORC2 is bound to the chromatin and MCM5 is present in a soluble nuclear fraction but unable to bind to chromatin when cells are induced to enter the cell cycle. Once CDC6 protein is expressed in G1, MCM5 can now bind to the chromatin. Therefore ORC appears to facilitate the loading of CDC6 which promotes the loading of MCM protein (79). There is no direct evidence about which ORC subunits are involved in recruiting other proteins to the origins. However, in *S. cerevisiae*, the electrophoretic mobility of ORC6 changes prior to CDC6 loading and changes back at the time CDC6 is lost from the chromatin (80). *S. cerevisiae* ORC6 is dispensable for *in vitro* DNA binding (81) but essential for DNA replication, suggesting the role of ORC6 in the complex may be to recruit CDC6 and other proteins to the origin.

Competence to initiate DNA replication in a mammalian *in vitro* system coincides with the time of maximum CDC6 accumulation and maximum binding of the MCM complex to the chromatin. At this point, entry into S phase can be accelerated just by recombinant CycE/cdk2 (see below). This licensing can be accelerated by adding recombinant *Xenopus* CDC6 to permeabilized G1 phase nuclei (79). However, during a specific time interval after release from quiescence, nuclei fail to respond to exogenous CDC6 (79). At this time endogenous CDC6 is already present and bound to the chromatin but MCMs remain soluble, indicating the presence of a negative factor, or the absence of a positive factor that regulates MCM binding. A *Xenopus* protein, geminin, inhibits DNA replication by preventing the incorporation of MCM complex onto chromatin (82). Geminin accumulates during S, G2, and M phases, and disappears at the time of the metaphase-anaphase transition, and could be the hypothetical negative factor that regulates MCM binding.

It is uncertain whether in the normal cell cycle CDC6 remains bound to the chromatin (57) or is released upon loading of the MCM complex (83) in *Xenopus* egg extracts. Once the binding of MCMs to the chromatin has taken place, however, replication can proceed independent of chromatin-bound ORC and CDC6, although it still depends on cdk2 (83). Whether at this point the origin DNA duplex is already unwound and stabilized by RPA, MCMs or other proteins is not known. Indeed, a weak but significant helicase activity has been identified in a subcomplex of human MCMs *in vitro* (84).

6. REGULATION OF METAZOAN ORC

6.1. Regulation of ORC expression

The mRNA expression of human and *Drosophila* ORC1 (76, 85) and human CDC6 (55, 59, 75, 86) is cell cycle regulated with the human CDC6 promoter responding to the E2F transcription factor. This dependence links initiation of replication with the pRb-E2F cell cycle control pathway, and ensures that when quiescent cells are stimulated to enter the cell cycle, ORC1 and CDC6 are both synthesized at the G1/S transition and are available for origin function.

The regulation of metazoan ORC1 by E2F confers an additional level of control of initiation of replication to animal cells as compared to *S. cerevisiae*, where the six-subunit ORC is bound to chromatin throughout the cell cycle (2, 24) and only CDC6 is regulated (87, 88). In fruit flies the abundance of ORC1 protein varies during development and the cell cycle, accumulating in proliferating cells in late G1 and S (76). Additional evidence indicates that the level of ORC1 protein determines origin utilization (76). Expression of ectopic ORC1 during *Drosophila* development alters the proliferation program. Proliferation is detected in tissues where no proliferation is expected under normal conditions, and it correlates with the presence of ORC1 protein. Ectopic ORC1 results in general DNA replication in follicle cells in stage 10B of ovarian development where otherwise replication is restricted to four foci undergoing gene amplification. It will be interesting to learn how ectopic ORC1 drives quiescent cells into replication overcoming the need of limiting factors like CDC6 (*Drosophila* CDC6 has not been reported yet, although it is reasonable to assume that a homolog for this gene, which is present in yeasts, *Xenopus* and mammals, exists in fruit flies) or S-phase cyclin-cdk activity.

Overexpression of exogenous ORC1 or CDC6 in mammalian cells does not result in the same effect as described above for *Drosophila*, whereas overexpression of E2F in starved fibroblasts does drive cells into S phase without the addition of serum (86). Although levels of ectopic ORC1 or CDC6 may not have reached a critical threshold, these results may indicate that the expression of additional genes regulated by E2F may be limiting for replication initiation in mammalian cells. In any case, mRNA expression of ORC1 and CDC6, but not other ORC subunits, correlate with proliferation in human tissues (54, 75), indicating that ORC1 and CDC6 may be limiting factors in determining which chromatin-bound ORC complexes are competent for pre-RC formation. Given the dependency of ORC1 and CDC6 expression on E2F and the fact that deregulation of the pRb/E2F pathway is a common trait in many human malignancies (89, 90), anomalous activation of E2F may induce expression of ORC1 and CDC6 and promote the driving of cells from quiescence into proliferation.

In contrast to the restricted expression of ORC1 and CDC6 to proliferating cells, expression of mammalian ORC2-5 is not cell cycle regulated (52, 54, 55). The mRNA of these subunits are detected in tissues that do not proliferate significantly such as spleen and ovary (49, 50, 54), suggesting that ORC may be involved in additional functions besides initiation of replication (see section 7).

6.2. CDC6 availability is regulated by changes in subcellular localization

In contrast to the regulation in mRNA expression, human ORC1 and CDC6 protein levels do not fluctuate in whole cell extracts throughout the cell cycle (55), indicating that the protein is not totally degraded at the G1/S transition as is CDC6 in yeasts (87, 88, 91-93). Instead, control of the availability of CDC6 appears to be exerted by regulation of subcellular localization, with the protein localized in nuclei during G1 and in cytoplasm upon onset of S phase (55). Also in *Xenopus* egg extracts CDC6 is not degraded in S phase but appears to move from the chromatin to the nuclear envelope (57).

The observed cell cycle dependent changes in subcellular localization of CDC6 in human cells might be explained either by destruction of nuclear CDC6 and cytoplasmic accumulation of newly synthesized protein, or by the relocalization of the protein within the cell. Several lines of evidence give support to the latter (94-96). Overexpression of exogenous cyclin A leads to rapid cytoplasmic localization of CDC6. Ectopic CDC6 protein in which two N-terminal cdk phosphorylation consensus sites (that are phosphorylated *in vivo*) have been removed remains always nuclear. A mutant with acidic residues instead of the cdk substrate serines to mimick the negative charge associated with phosphorylation remains cytoplasmic.

Phosphorylation of CDC6 by cyclin A-cdk2 at the onset of S phase and the subsequent removal of the protein from the nucleus suggest a mechanism that could prevent re-replication. However, overexpression of non-phosphorylatable CDC6 mutants which localize to the nucleus is not sufficient to induce re-replication (94-96) in the way it does in fission yeast (97), indicating that additional mechanisms preventing re-replication are active in animal cells. A protein, geminin, has been identified in *Xenopus* that accumulates from S to M phase and inhibits the loading of MCM onto the chromatin (82), and could be involved in preventing re-replication.

6.3. Origin firing

The ultimate signals and targets responsible for origin firing have not been demonstrated. In budding yeast, both S-phase cyclin-cdk and Dbf4-Cdc7 protein kinase activities are required for the onset and progress of replication. In human cells, once MCMs are loaded onto the chromatin, cyclin E-cdk2 in a buffered solution is enough to initiate replication in G1 nuclei *in vitro* (79). The efficiency of replication initiation is equivalent to that induced by S-phase cytosolic extracts, indicating that the targets activated are likely the same in both cases. This requirement of cyclin E couples origin firing to the pRb/E2F cell cycle control pathway at still another level (see above, regulation). Neither cyclin A-cdk2 nor cyclin B-cdc2 are able to support initiation in the same *in vitro* experiment (79). In fact cyclin B-cdc2 inhibits initiation, consistent with its role in mitosis and the need that initiation is repressed to avoid re-replication after a round of semi-conservative replication is finished and cells exit S phase. However, cyclin A-cdk2 has been localized to replication foci (98), suggesting it may play a regulatory role.

Human CDC6 can bind to (55, 94) and be phosphorylated by cycA-cdk2 *in vivo*, triggering its subcellular relocalization from nucleus to cytoplasm (94). In agreement with the above mentioned observations, this phosphorylation does not seem to be the signal that fires the origins, since ectopic non-phosphorylatable mutants do not act as dominant negative mutants that block the onset of S-phase, although it is not known whether they are otherwise functional (94-96). It has been proposed that the role of this phosphorylation is more likely to avoid re-assembly of pre-RC on already-fired origins by subcellular relocalization of the protein as described in 5.2.

Xenopus ORC1 and 2, but not 4 are hyperphosphorylated in M-phase (metaphase) extracts with respect to interphase extracts (44, 47, 48). Although the significance of this phosphorylation is unknown, the timing is compatible with a potential release of ORC from the chromatin (45, 46, 57, 83). Human ORC1 is phosphorylated *in vitro* by human cyclin A-cdk2 and to a lesser extent by human cyclin B-cdc2 (49).

7. ORC BEYOND REPLICATION

The pattern of tissue specific ORC expression in mammals reveals a surprising result (49, 50, 54). In addition to the expected expression in proliferating tissues like testis or colon mucosa, ORC subunits are also expressed in variable levels in tissues without significant proliferation, like spleen, ovary or prostate. These observations support the involvement of ORC in functions independent of cell proliferation.

One such possible function has been identified in Drosophila, implicating ORC in formation of silencing-related heterochromatin (43). This result is compatible with the involvement of yeast ORC in transcription silencing (17, 18, 20, 22, 99). Early observation showed abnormal chromosome condensation in *Drosophila* k43 mutants (67). The k43 gene encodes *Drosophila* ORC2 (63). In fruit flies, ORC colocalizes and interacts with HP1, a structural component of heterochromatin. HP1 lacks DNA binding activity, and ORC2 mutants disrupt the localization of HP1 to the heterochromatin (100), indicating that the role of ORC in heterochromatin formation in animal cells resembles that played by yeast ORC in telomeric and cryptic mating loci silencing, as a DNA-bound tether for the proteins responsible for the assembly of the silenced chromatin. Interestingly, flies heterozygous for ORC2 (+/-) show suppression of position effect variegation, indicating that the requirement for ORC in

chromatin structure is dosage-dependent. The implications of this finding are ample. For instance a copy of the ORC5 gene is found consistently deleted in some tumors (54). It is possible that as seen in *Drosophila*, hemizygosity results in haploinsufficiency and reduced silencing, allowing an increased expression of oncogenes and growth factors. The observation that uterine leiomyomas with the chromosomal deletion that includes ORC5 grow slower in culture than leiomyomas with other chromosomal aberrations supports the idea that loss of one copy of the gene may result in haploinsufficiency.

Possibly also related to a structural role for ORC in chromatin, *S. cerevisiae* ORC5 has been shown to be required in early M phase in addition to G1/S (56), although the exact role in mitosis is currently unknown. On a similar note, the fly ORC3 protein (latheo) is expressed in the adult brain, and the protein is detected in the cytoplasm in the synaptic vesicles, where it has been proposed plays a role in neuronal plasticity (101).

8. PERSPECTIVE

If ORC is the initiator in animal cells, it is expected to bind to replicator sequences. Despite substantial effort replicators in higher eukaryotes have remained elusive to date. With metazoan Origin Recognition Complexes finally at hand, it may be anticipated that the identity of the long-sought metazoan replicators will be soon uncovered.

How ORC binding to DNA is influenced by chromatin structure will help understand how origins are modified according to development and transcription activity in higher eukaryotes. A dramatic change in origin usage occurs for instance during *Xenopus* development after the mid-blastula transition (102), coincident with the commencement of gene transcription and subsequent changes in chromosome structure. Also accessory factors affect origin localization and usage. Accessory factors like transcription factors in the origins may help by bending the DNA so as to facilitate duplex unwinding or ORC binding or accessibility of the preRC to other replication factors. An interesting observation is that the binding of c-myc near the c-myc human origin of replication facilitates replication initiation at the origins (103). The potential involvement of E2F in directing ORC to specific loci (77) may be another example of this phenomenon.

How many pieces of the puzzle are still missing remains to be seen. So far, human cells seem to cope with at least an alternatively spliced form of ORC5, ORC5T, whose function is not known yet (54). The ultimate targets that are activated at initiation of replication awaits further elucidation. Which elements of the pre-RC are actually required for the firing? Is ORC dispensable in animal cells once CDC6 and MCM are loaded? Even if ORC is dispensable for origin firing in the *Xenopus* egg extract *in vitro* system (83), we cannot rule out that ORC is required *in vivo* even after the loading of MCM protein. The possible roles of ORC in chromatin structure related to silenced heterochromatin suggest a role in the condensation of mitotic chromosomes. Yeast ORC may be one target of the checkpoint kinase Rad53 that slows the rate of replication in response to DNA damage (104). A similar mechanism might be active in mammals, where CHO cells respond to ionizing radiation by inhibiting initiation (at the DHFR locus) rather than inhibiting elongation (105, 106). Although this checkpoint mechanism has been detected by using DNA-damaging agents, under normal conditions mutation of Rad53 results in the early firing of normally late S phase origins, indicating that Rad53 also delays the timing of initiation from late origins during normal yeast cell growth (104, 107, 108)

As though a role as the eukaryotic initiator was not important enough, it is exciting to see the influence of ORC extending well beyond the (replication) origins. Involvement in the formation of silenced-state heterochromatin, consistent reduction to hemizygosity in tumors, and potential involvement in learning defects are some of the ramifications that already extend the role of the metazoan Origin Recognition Complex.

9. ACKNOWLEDGEMENTS

Work in the Dutta lab was supported by grant CA60499 from the N.I.H.

10. REFERENCES

1. Jacob F, Brenner S & Cuzin F: *Cold Spring Harbor Symp Quant Biol* 28, 329-48 (1963).

2. Bell SP & Stillman B: ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. *Nature* 357, 128-34 (1992).

3. Gavin KA, Hidaka M & Stillman B: Conserved initiator proteins in eukaryotes. *Science* 270, 1667-71 (1995).

4. Gossen M, Pak DT, Hansen SK, Acharya JK & Botchan MR: A Drosophila homolog of the yeast origin recognition complex. *Science* 270, 1674-7 (1995).

5. Dutta A & Bell SP: Initiation of DNA replication in eukaryotic cells. A*nn Rev Cell Dev Biol* 13, 293-332 (1997).

6. Stillman B: Cell cycle control of DNA replication. *Science* 274, 1659-64 (1996).

7. Struhl K, Stinchcomb DT, Scherer S & Davis RW: High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. *Proc Natl Acad Sci U S A* 76, 1035-9 (1979).

8. Stinchcomb DT, Thomas M, Kelly J, Selker E & Davis RW: Eukaryotic DNA segments capable of autonomous replication in yeast. *Proc Natl Acad Sci U S A* 77, 4559-63 (1980).

9. Chan CS & Tye BK: Autonomously replicating sequences in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A* 77, 6329-33 (1980).

10. Brewer BJ & Fangman WL: The localization of replication origins on ARS plasmids in S. cerevisiae. *Cell* 51, 463-71 (1987).

11. Marahrens Y & Stillman B: A yeast chromosomal origin of DNA replication defined by multiple functional elements. *Science* 255, 817-23 (1992).

12. Newlon CS, Collins I, Dershowitz A, Deshpande AM, Greenfeder SA, Ong LY & Theis JF: Analysis of replication origin function on chromosome III of Saccharomyces cerevisiae. *Cold Spring Harb Symp Quant Biol* 58, 415-23 (1993).

13. Rao H, Marahrens Y & Stillman B: Functional conservation of multiple elements in yeast chromosomal replicators. *Mol Cell Biol* 14, 7643-51 (1994).

14. Theis JF & Newlon CS: Domain B of ARS307 contains two functional elements and contributes to chromosomal replication origin function. *Mol Cell Biol* 14, 7652-9 (1994).

15. Rao H & Stillman B: The origin recognition complex interacts with a bipartite DNA binding site within yeast replicators *Proc Natl Acad Sci U S A* 92 2224-8 (1995).

16. Rowley A, Cocker JH, Harwood J & Diffley JF: Initiation complex assembly at budding yeast replication origins begins with the recognition of a bipartite sequence by limiting amounts of the initiator, ORC. *EMBO J* 14, 2631-41 (1995).

17. Bell SP, Kobayashi R & Stillman B: Yeast origin recognition complex functions in transcription silencing and DNA replication. *Science* 262, 1844-9 (1993).

18. Foss M, McNally FJ, Laurenson P & Rine J: Origin recognition complex (ORC) in transcriptional silencing and DNA replication in S. cerevisiae. *Science* 262, 1838-44 (1993).

19. Li JJ & Herskowitz I: Isolation of ORC6, a component of the yeast origin recognition complex by a one-hybrid system. *Science* 262, 1870-4 (1993).

20. Micklem G, Rowley A, Harwood J, Nasmyth K & Diffley JF: Yeast origin recognition complex is involved in DNA replication and transcriptional silencing. *Nature* 366, 87-9 (1993).

21. Bell SP, Mitchell J, Leber J, Kobayashi R & Stillman B: The multidomain structure of Orc1p reveals similarity to regulators of DNA replication and transcriptional silencing. *Cell* 83, 563-8 (1995).

22. Loo S, Fox CA, Rine J, Kobayashi R, Stillman B & Bell S: The origin recognition complex in silencing, cell cycle progression, and DNA replication. *Mol Biol Cell* 6, 741-56 (1995).

23. Hardy CF & Pautz A: A novel role for Cdc5p in DNA replication. *Mol Cell Biol* 16, 6775-82 (1996).

24. Diffley JF & Cocker JH: Protein-DNA interactions at a yeast replication origin. *Nature* 357, 169-72 (1992).

25. Diffley JF, Cocker JH, Dowell SJ & Rowley A: Two steps in the assembly of complexes at yeast replication origins in vivo. *Cell* 78, 303-16 (1994).

26. Aparicio OM, Weinstein DM & Bell SP: Components and dynamics of DNA replication complexes in S. cerevisiae: redistribution of MCM proteins and Cdc45p during S phase. *Cell* 91, 59-69 (1997).

27. Liang C & Stillman B: Persistent initiation of DNA replication and chromatin-bound MCM proteins during the cell cycle in cdc6 mutants. *Genes Dev* 11, 3375-86 (1997).

28. Cocker JH, Piatti S, Santocanale C, Nasmyth K & Diffley JF: An essential role for the Cdc6 protein in forming the pre-replicative complexes of budding yeast. *Nature* 379, 180-2 (1996).

29. Liang C, Weinreich M & Stillman B: ORC and Cdc6p interact and determine the frequency of initiation of DNA replication in the genome. *Cell* 81, 667-76 (1995).

30. Hartwell LH: Sequential function of gene products relative to DNA synthesis in the yeast cell cycle. *J Mol Biol* 104, 803-17 (1976).

31. Donovan S, Harwood J, Drury LS & Diffley JF: Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. *Proc Natl Acad Sci U S A* 94, 5611-6 (1997).

32. Tanaka T, Knapp D & Nasmyth K: Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. *Cell* 90, 649-60 (1997).

33. Moir D, Stewart SE, Osmond BC & Botstein D: Cold-sensitive cell-division-cycle mutants of yeast: isolation, properties, and pseudoreversion studies. *Genetics* 100, 547-63 (1982).

34. Maine GT, Sinha P & Tye BK: Mutants of S. cerevisiae defective in the maintenance of minichromosomes. *Genetics* 106, 365-85 (1984).

35. Santocanale C & Diffley JF: ORC- and Cdc6-dependent complexes at active and inactive chromosomal replication origins in Saccharomyces cerevisiae. *EMBO J* 15, 6671-9 (1996).

36. Hopwood B & Dalton S: Cdc45p assembles into a complex with Cdc46p/Mcm5p, is required for minichromosome maintenance, and is essential for chromosomal DNA replication. *Proc Natl Acad Sci U S A* 93, 12309-14 (1996).

37. Zou L & Stillman B: Formation of a preinitiation complex by S-phase cyclin CDK-dependent loading of Cdc45p onto chromatin. *Science* 280, 593-6 (1998).

38. Schwob E, Bohm T, Mendenhall MD & Nasmyth K: The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in S. cerevisiae. *Cell* 79, 233-44 (1994).

39. Dahmann C, Diffley JF & Nasmyth KA: S-phase-promoting cyclin-dependent kinases prevent re-replication by inhibiting the transition of replication origins to a pre-replicative state. *Curr Biol* 5, 1257-69 (1995).

40. Piatti S, Bohm T, Cocker JH, Diffley JF & Nasmyth K: Activation of S-phase-promoting CDKs in late G1 defines a "point of no return" after which Cdc6 synthesis cannot promote DNA replication in yeast. *Genes Dev* 10, 1516-31 (1996).

41. Bousset K & Diffley JF: The Cdc7 protein kinase is required for origin firing during S phase. *Genes Dev* 12, 480-90 (1998).

42. Hua XH, Yan H & Newport J: A role for Cdk2 kinase in negatively regulating DNA replication during S phase of the cell cycle. *J Cell Biol* 137, 183-92 (1997).

43. Pak DT, Pflumm M, Chesnokov I, Huang DW, Kellum R, Marr J, Romanowski P & Botchan MR: Association of the origin recognition complex with heterochromatin and HP1 in higher eukaryotes. *Cell* 91, 311-23 (1997).

44. Carpenter PB, Mueller PR & Dunphy WG: Role for a Xenopus Orc2-related protein in controlling DNA replication. *Nature* 379, 357-60 (1996).

45. Rowles A, Chong JP, Brown L, Howell M, Evan GI & Blow JJ: Interaction between the origin recognition complex and the replication licensing system in Xenopus. *Cell* 87, 287-96 (1996).

46. Romanowski P, Madine MA, Rowles A, Blow JJ & Laskey RA: The Xenopus origin recognition complex is essential for DNA replication and MCM binding to chromatin. *Curr Biol* 6, 1416-25 (1996).

47. Carpenter PB & Dunphy WG: Identification of a novel 81-kDa component of the Xenopus origin recognition complex. *J Biol Chem* 273, 24891-7 (1998).

48. Tugal T, Zou YX, Gavin K, Pappin D, Canas B, Kobayashi R, Hunt T & Stillman B: The Orc4p and Orc5p subunits of the Xenopus and human origin recognition complex are related to Orc1p and Cdc6p. *J Biol Chem* 273, 32421-9 (1998).

49. Wolf DA, Wu D & McKeon F: Disruption of re-replication control by overexpression of human ORC1 in fission yeast. *J Biol Chem* 271, 32503-6 (1996).

50. Takahara K, Bong M, Brevard R, Eddy RL, Haley LL, Sait SJ, Shows TB, Hoffman GG & Greenspan DS: Mouse and human homologues of the yeast origin of replication recognition complex subunit ORC2 and chromosomal localization of the cognate human gene ORC2L. *Genomics* 31, 119-22 (1996).

51. Pinto S, Quintana DG, Smith P, Mihalek RM, Hou Z-H, Boynton S, Jones CJ, Hendricks M, Velinzon K, Wohlschlegel JA, Austin RJ, Lane WS, Tully T & Dutta A: Latheo Encodes a Subunit of the Origin Recognition Complex and Disrupts Neuronal Proliferation and Adult Olfactory Memory When Mutant. *Neuron* 23, 45-54 (1999).

52. Quintana DG, Hou ZH, Thome KC, Hendricks M, Saha P & Dutta A: Identification of hsorc4, a member of the human origin of replication recognition complex. *J Biol Chem* 272, 28247-28251 (1997).

53. Ishiai M, Dean FB, Okumura K, Abe M, Moon KY, Amin AA, Kagotani K, Taguchi H, Murakami Y, Hanaoka F, O'Donnell M, Hurwitz J & Eki T: Isolation of human and fission yeast homologues of the budding yeast origin recognition complex subunit ORC5: human homologue (ORC5L) maps to 7q22. *Genomics* 46, 294-8 (1997).

54. Quintana DG, Thome KC, Hou ZH, Ligon AH, Morton CC & Dutta A: ORC5L, a new member of the human origin recognition complex, is deleted in uterine leiomyomas and malignant myeloid diseases. *J Biol Chem* 273, 27137-45 (1998).

55. Saha P, Chen J, Thome KC, Lawlis SJ, Hou Z-H, Hendricks M, Parvin JD & Dutta A: Human CDC6/Cdc18 Associates with Orc1 and Cyclin-cdk and Is Selectively Eliminated from the Nucleus at the Onset of S Phase. *Mol Cell Biol* 18, 2758-2767 (1998).

56. Dillin A & Rine J: Roles for ORC in M phase and S phase. *Science* 279, 1733-7 (1998).

57. Coleman TR, Carpenter PB & Dunphy WG: The Xenopus Cdc6 protein is essential for the initiation of a single round of DNA replication in cell-free extracts. *Cell* 87, 53-63 (1996).

58. Williams RS, Shohet RV & Stillman B: A human protein related to yeast Cdc6p. *Proc Natl Acad Sci U S A* 94, 142-7 (1997).

59. Hateboer G, Wobst A, Petersen BO, Le CL, Vigo E, Sardet C & Helin K: Cell cycle-regulated expression of mammalian CDC6 is dependent on E2F. *Mol Cell Biol* 18, 6679-97 (1998).

60. Perkins G & Diffley JF: Nucleotide-dependent prereplicative complex assembly by Cdc6p, a homolog of eukaryotic and prokaryotic clamp-loaders. *Mol Cell* 2, 23-32 (1998).

61. Koonin EV: A common set of conserved motifs in a vast variety of putative nucleic acid-dependent ATPases including MCM proteins involved in the initiation of eukaryotic DNA replication. *Nucleic Acids Res* 21, 2541-7 (1993).

62. Klemm RD, Austin RJ & Bell SP: Coordinate binding of ATP and origin DNA regulates the ATPase activity of the origin recognition complex. *Cell* 88, 493-502 (1997).

63. Landis G, Kelley R, Spradling AC & Tower J: The k43 gene, required for chorion gene amplification and diploid cell chromosome replication, encodes the Drosophila

homolog of yeast origin recognition complex subunit 2. *Proc Natl Acad Sci U S A* 94, 3888-92 (1997).

64. Shearn A, Rice T, Garen A & Gehring W: Imaginal disc abnormalities in lethal mutants of Drosophila. *Proc Natl Acad Sci U S A* 68, 2594-8 (1971).

65. Shearn A & Garen A: Genetic control of imaginal disc development in Drosophila. *Proc Natl Acad Sci U S A* 71, 1393-7 (1974).

66. Szabad J & Bryant PJ: The mode of action of "discless" mutations in Drosophila melanogaster. *Dev Biol* 93, 240-56 (1982).

67. Gatti M & Baker BS: Genes controlling essential cell-cycle functions in Drosophila melanogaster. *Genes Dev* 3, 438-53 (1989).

68. Snyder PB, Galanopoulos VK & Kafatos FC: Trans-acting amplification mutants and other eggshell mutants of the third chromosome in Drosophila melanogaster. *Proc Natl Acad Sci U S A* 83, 3341-5 (1986).

69. Delidakis C & Kafatos FC: Amplification enhancers and replication origins in the autosomal chorion gene cluster of Drosophila. *EMBO J* 8, 891-901 (1989).

70. Heck MM & Spradling AC: Multiple replication origins are used during Drosophila chorion gene amplification. *J Cell Biol* 110, 903-14 (1990).

71. Boynton S & Tully T: Latheo, a new gene involved in associative learning and memory in Drosophila melanogaster, identified from P element mutagenesis. *Genetics* 131, 655-72 (1992).

72. Blow JJ & Watson JV: Nuclei act as independent and integrated units of replication in a Xenopus cell-free DNA replication system. *EMBO J* 6, 1997-2002 (1987).

73. Mahbubani HM, Paull T, Elder JK & Blow JJ: DNA replication initiates at multiple sites on plasmid DNA in Xenopus egg extracts. *Nucleic Acids Res* 20, 1457-62 (1992).

74. Hyrien O & Mechali M: Chromosomal replication initiates and terminates at random sequences but at regular intervals in the ribosomal DNA of Xenopus early embryos. *EMBO J* 12, 4511-20 (1993).

75. Yan Z, DeGregori J, Shohet R, Leone G, Stillman B, Nevins JR & Williams RS: Cdc6 is regulated by E2F and is essential for DNA replication in mammalian cells. *Proc Natl Acad Sci U S A* 95, 3603-8 (1998).

76. Asano M & Wharton RP: E2F mediates developmental and cell cycle regulation of ORC1 in Drosophila. *EMBO J* 18, 2435-2448 (1999).

77. Royzman I, Austin RJ, Bosco G, Bell SP & Orr WT: ORC localization in Drosophila follicle cells and the effects of mutations in dE2F and dDP. *Genes Dev* 13, 827-40 (1999).

78. Abdurashidova G, Riva S, Biamonti G, Giacca M & Falaschi A: Cell cycle modulation of protein-DNA interactions at a human replication origin. *EMBO J* 17, 2961-9 (1998).

79. Stoeber K, Mills AD, Kubota Y, Krude T, Romanowski P, Marheineke K, Laskey RA & Williams GH: Cdc6 protein causes premature entry into S phase in a mammalian cell- free system. *EMBO J* 17, 7219-29 (1998).

80. Weinreich M, Liang C & Stillman B: The Cdc6p nucleotide-binding motif is required for loading mcm proteins onto chromatin. *Proc Natl Acad Sci U S A* 96, 441-6 (1999).

81. Lee DG & Bell SP: Architecture of the yeast origin recognition complex bound to origins of DNA replication. *Mol Cell Biol* 17, 7159-68 (1997).

82. McGarry TJ & Kirschner MW: Geminin, an inhibitor of DNA replication, is degraded during mitosis. *Cell* 93, 1043-53 (1998).

83. Hua XH & Newport J: Identification of a preinitiation step in DNA replication that is independent of origin recognition complex and cdc6, but dependent on cdk2. *J Cell Biol* 140, 271-81 (1998).

84. Ishimi Y: A DNA helicase activity is associated with an MCM4, -6, and -7 protein complex. *J Biol Chem* 272, 24508-13 (1997).

85. Ohtani K, DeGregori J, Leone G, Herendeen DR, Kelly TJ & Nevins JR: Expression of the HsOrc1 gene, a human ORC1 homolog, is regulated by cell proliferation via the E2F transcription factor. *Mol Cell Biol* 16, 6977-84 (1996).

86. Ohtani K, Tsujimoto A, Ikeda M & Nakamura M: Regulation of cell growth-dependent expression of mammalian CDC6 gene by the cell cycle transcription factor E2F. *Oncogene* 17, 1777-85 (1998).

87. Zwerschke W, Rottjakob HW & Kuntzel H: The Saccharomyces cerevisiae CDC6 gene is transcribed at late mitosis and encodes a ATP/GTPase controlling S phase initiation. *J Biol Chem* 269, 23351-6 (1994).

88. Piatti S, Lengauer C & Nasmyth K: Cdc6 is an unstable protein whose de novo synthesis in G1 is important for the onset of S phase and for preventing a 'reductional' anaphase in the budding yeast Saccharomyces cerevisiae. *EMBO J* 14, 3788-99 (1995).

89. Johnson DG & Schneider BR: Role of E2F in cell cycle control and cancer. *Front Biosci* 3, d447-8 (1998).

90. Macleod K: pRb and E2f-1 in mouse development and tumorigenesis. *Curr Opin Genet Dev* 9, 31-9 (1999).

91. Kelly TJ, Martin GS, Forsburg SL, Stephen RJ, Russo A & Nurse P: The fission yeast cdc18+ gene product

couples S phase to START and mitosis. *Cell* 74, 371-82 (1993).

92. Nishitani H & Nurse P: p65cdc18 plays a major role controlling the initiation of DNA replication in fission yeast. *Cell* 83, 397-405 (1995).

93. Muzi-Falconi M, Brown GW & Kelly TJ: cdc18+ regulates initiation of DNA replication in Schizosaccharomyces pombe. *Proc Natl Acad Sci U S A* 93, 1566-70 (1996).

94. Petersen BO, Lukas J, Sorensen CS, Bartek J & Helin K: Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization. *EMBO J* 18, 396-410 (1999).

95. Jiang W, Wells NJ & Hunter T: Multistep regulation of DNA replication by Cdk phosphorylation of HsCdc6. *Proc Natl Acad Sci USA* 96, 6193-8 (1999).

96. Delmolino L & Dutta A: *manuscript in preparation*

97. Jallepalli PV, Brown GW, Muzi FM, Tien D & Kelly TJ: Regulation of the replication initiator protein p65cdc18 by CDK phosphorylation. *Genes Dev* 11, 2767-79 (1997).

98. Cardoso MC, Leonhardt H & Nadal GB: Reversal of terminal differentiation and control of DNA replication: cyclin A and Cdk2 specifically localize at subnuclear sites of DNA replication. *Cell* 74, 979-92 (1993).

99. Fox CA, Loo S, Dillin A & Rine J: The origin recognition complex has essential functions in transcriptional silencing and chromosomal replication. *Genes Dev* 9, 911-24 (1995).

100. Huang DW, Fanti L, Pak DT, Botchan MR, Pimpinelli S & Kellum R: Distinct cytoplasmic and nuclear fractions of Drosophila heterochromatin protein 1: their phosphorylation levels and associations with origin recognition complex proteins. *J Cell Biol* 142, 307-18 (1998).

101. Rohrbough J, Pinto S, Mihalek RM, Tully T & Broadie K: Latheo, a Drosophila Gene Involved in Learning, Regulates Functional Synaptic Plasticity. *Neuron* 23, 55-70 (1999).

102. Hyrien O, Maric C & Mechali M: Transition in specification of embryonic metazoan DNA replication origins. *Science* 270, 994-7 (1995).

103. McWhinney C & Leffak M: Autonomous replication of a DNA fragment containing the chromosomal replication origin of the human c-myc gene. *Nucleic Acids Res* 18, 1233-42 (1990).

104. Shirahige K, Hori Y, Shiraishi K, Yamashita M, Takahashi K, Obuse C, Tsurimoto T & Yoshikawa H: Regulation of DNA-replication origins during cell-cycle progression. *Nature* 395, 618-21 (1998).

105. Larner JM, Lee H & Hamlin JL: Radiation effects on DNA synthesis in a defined chromosomal replicon. *Mol Cell Biol* 14, 1901-8 (1994).

106. Lee H, Larner JM & Hamlin JL: A p53-independent damage-sensing mechanism that functions as a checkpoint at the G1/S transition in Chinese hamster ovary cells. *Proc Natl Acad Sci U S A* 94, 526-31 (1997).

107. Santocanale C & Diffley JF: A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication. *Nature* 395, 615-8 (1998).

108. Tanaka T & Nasmyth K: Association of RPA with chromosomal replication origins requires an Mcm protein, and is regulated by Rad53, and cyclin- and Dbf4-dependent kinases. *EMBO J* 17, 5182-91 (1998).

Keywords: ORC, Origin Recognition Complex, Initiation of DNA Replication, Cell Cycle Control.

Send correspondence to: Dr David G. Quintana or Dr Anindya Dutta, Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, 75 Francis Street, Boston, MA 02115, USA Tel: +1 617 732 6581, Fax: +1 617 732 7449, E-mail:dquintana@rics.bwh.harvard.edu, adutta@rics.bwh.harvard.edu

Received 9/15/99 Accepted 10/25/99