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Regulating the licensing of DNA replication origins in metazoa

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Eukaryotic DNA replication is a highly conserved process; the proteins and sequence of events that replicate animal genomes are remarkably similar to those that replicate yeast genomes. Moreover, the assembly of prereplication complexes at DNA replication origins ('DNA licensing') is regulated in all eukaryotes so that no origin fires more than once in a single cell cycle. And yet there are significant differences between species both in the selection of replication origins and in the way in which these origins are licensed to operate. Moreover, these differences impart advantages to multicellular animals and plants that facilitate their development, such as better control over endoreduplication, flexibility in origin selection, and discrimination between quiescent and proliferative states.

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Introduction

Human development begins when an egg is fertilized by a sperm to produce a single cell containing a genome of 3.3 billion base pairs of DNA packaged into 46 individual chromosomes. Remarkably, some 5 trillion cell divisions later, an adult human appears that contains more than 20 trillion meters of DNA, the equivalent of 100 times the distance from the earth to the sun! And yet, with the exception of trophoblast giant cells and megakaryocytes, the human genome is replicated once and only once each time a cell divides. How is this amazing feat accomplished? Although much remains unclear, four concepts have emerged that provide a framework for understanding the linkage between DNA replication and cell division in multicellular animals and plants (the metazoa). First, the protein kinases and ubiquitin ligases that regulate cell division also regulate DNA replication. Second, the rate-limiting step in initiating DNA replication is the unwinding of the two DNA template strands at replication origins by the DNA helicase activity of the minichromosome maintenance (MCM) complex, an event that is regulated through the activity of the MCM loading factor, Cdt1/RLF-B. Third, cell-cycle-dependent changes occur in the origin recognition complex (ORC) that not only help to prevent premature DNA licensing, but may serve to regulate origin selection. Finally, the transition from a quiescent state to a proliferative one appears to be regulated through stabilization of Cdc6, one of two proteins required for loading the MCM helicase.

Here we describe mechanisms common to all metazoa that prevent relicensing of DNA replication origins before cell division has been completed. Specific differences between these mechanisms and those found in single cell organisms, such as yeast, are noted, and the biological significance of these differences is discussed.

DNA replication licensing

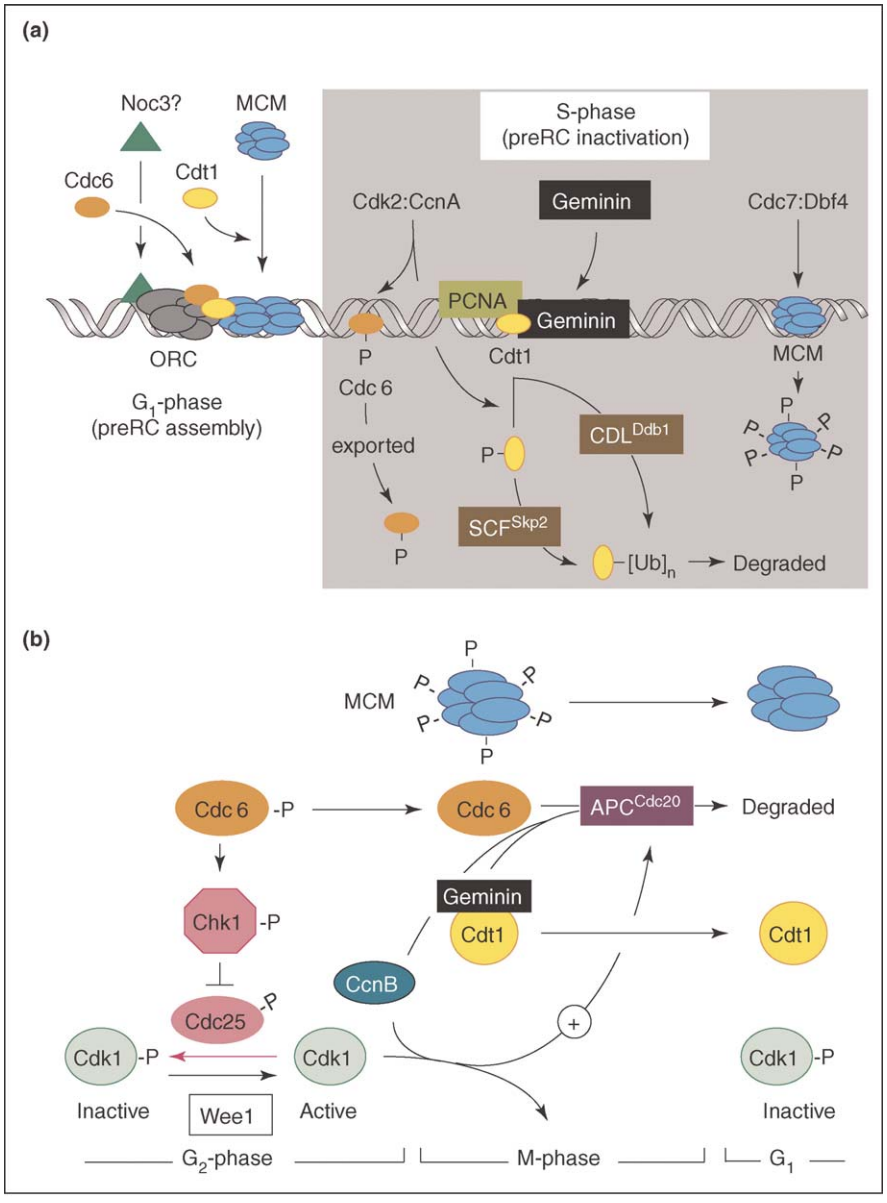
DNA replication begins with the assembly of a prereplication complex (preRC) consisting of at least 14 different proteins [1]. First, ORC binds to DNA in newly replicated chromatin at some time during the S- to early G_1-phases of the cell division cycle, a process that may be facilitated by Noc3 (Figure 1a). Cdc6 then binds to these ORC:chromatin sites during early G_1-phase, an event that is followed quickly by binding of Cdt1/RLF-B. MCM helicases are then chaperoned in by Cdt1 to complete the preRC. DNA synthesis (S-phase) begins when preRCs are acted upon by at least 22 additional proteins that activate the MCM helicase and assemble two divergent replication forks at each replication origin [2].

Loading of the MCM helicase onto DNA is referred to as DNA replication licensing, because only these replication origins can initiate DNA synthesis. Regulation of licensing refers to those mechanisms that restrict DNA replication to one and only one duplication of the genome prior to cell division, and therefore prevent endoreduplication (multiple rounds of DNA replication without an intervening mitosis). This restriction is brought about by inactivating existing preRCs during S, G_2 and early M-phase (Figure 1a), and by preventing the assembly of new preRCs until mitosis is complete and a nuclear membrane is present (Figure 1b) [3,4].

Regulating cell division

The same events that regulate eukaryotic cell division also regulate licensing of DNA replication origins, thereby

Figure 1



Assembly and inactivation of prereplication complexes during mammalian cell division. **(a)** PreRCs are assembled during the M-to-G_1-phase transition in both yeast and multicellular organisms by the sequential addition of Cdc6, Cdt1 and the MCM DNA helicase (comprised of six different subunits) to an ORC:DNA:chromatin complex that may also contain Noc3. Upon entry into S-phase, Cdc6 and Cdt1 are phosphorylated by CcnA–Cdk2. Most of the Cdc6 remains bound to chromatin, although some may be exported to the cytoplasm. Geminin binds to and inhibits Cdt1 on chromatin. Some of the Cdt1-P is also degraded by either SCF^{Skp2} or CDL^{Ddb1}. MCM is phosphorylated during S- and G_2–phase by Cdc7–Dbf4. MCM is released from DNA, presumably when two replication forks merge (replication termination). **(b)** The G_2 to M-phase transition is driven by activation of CcnB-Cdk1. This step can be inhibited by Cdc6-P activation of Chk1 protein kinase which, in turn, phosphorylates Cdc25 protein phosphatase, thereby preventing it from activating Cdk1. Cdc6 is presumably dephosphorylated during G_2-phase. Cdc6 not bound to chromatin is degraded as cells exit mitosis, but Cdc6 bound to chromatin is available for preRC assembly in early G_1-phase. During either G_2- or early M-phase, Cdt1 degradation ceases, in part due to stabilization by its association with geminin. In early M-phase, ORC is inactive, so soluble geminin:Cdt1 complexes accumulate. Geminin is then ubiquitinated by the APC as cells exit M-phase, and degraded in cells undergoing normal cell division or inactivated in early embryonic cells undergoing rapid cleavage. Since APC activity depends upon its phosphorylation by CcnB–Cdk1, inhibition of CDK activity during mitosis will prevent preRC assembly.

coordinating initiation of DNA replication with mitosis. Cell division is regulated by cyclin-dependent protein kinase (CDK), anaphase promoting complex (APC) ubiquitin ligase, and Skp1–Cullin–F-Box (SCF) ubiquitin ligase activities [5]. In metazoan cells, licensing occurs during the transition from late mitosis to late G_1-phase, when the absence of cyclins and the presence of the CDK-specific inhibitors such as p27/Kip1 suppress CDK activities (Figures 1b, 2). DNA synthesis does not begin until preRCs are activated by cyclin E(CcnE)–Cdk2 and cyclin A(CcnA)–Cdk2, S-phase CDKs whose activity is inhibited during G_1-phase by inhibitors such as p27 [6]. p27 is ubiquitinated by SCF^{Skp2} and then degraded by the 26S proteasome. DNA synthesis also depends on the appearance of the Dbf4-dependent protein kinase Cdc7, which, together with S-phase CDKs, phosphorylates several components of the preRC and DNA replication machinery [7]. The G_2-to-M-phase transition requires Cdc25 protein phosphatase to activate Cdk1, which then forms CcnA–Cdk1 and CcnB–Cdk1, both of which are required for mitosis [8]. During mitosis, Cdk1 is inactivated when CcnA and then CcnB are ubiquitinated by APC^{Cdc20} and degraded, and Cdk1 is phosphorylated by Wee1 (Figure 1b). Concurrently, Skp2 degradation is mediated by APC^{Cdh1} [9], and the cell enters G_1-phase.

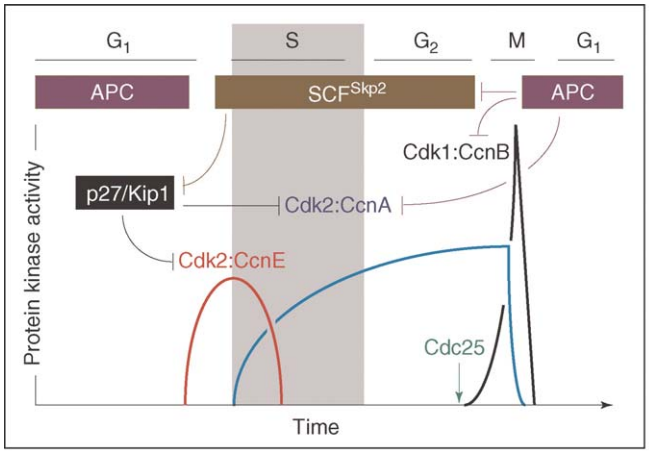
Regulating loading of the MCM DNA helicase

DNA unwinding at replication origins requires the MCM DNA helicase, and loading this enzyme onto chromatin requires Cdt1, which targets ORC:Cdc6:chromatin sites. Yeast express Cdt1 constitutively, and no Cdt1-specific inhibitors have been reported, but in metazoa Cdt1 is the primary target for regulating the licensing of DNA replication origins. Over-expression of Cdt1 can cause re-licensing and re-replication of DNA in the cells of mammals [10•,11], flies [12], frogs [13•,14,15,16•] and plants [17], revealing that intracellular levels of Cdt1 are rate-limiting for initiation of DNA replication in metazoa.

Several intriguing strategies link down-regulation of Cdt1 activity with progression into S-phase. Cdt1 protein levels are low in S- and G_2-phase, but high during M- and G_1-phase. These levels are controlled largely through cell-cycle-dependent degradation following ubiquitination of Cdt1 by three different ubiquitin ligases. Ubiquitination by SCF^{Skp2} [10•,18] is facilitated by CDK-dependent phosphorylation of Cdt1 (Figure 1a) [10•,19,20]. Thus, the same E3 ubiquitin ligase that inactivates p27 also inactivates Orc1 and Cdt1, thereby initiating DNA synthesis at preRCs that were assembled during G_1-phase while preventing reassembly of new preRCs during S-phase. However, in *Drosophila* and in mammalian cells, Cdt1 mutants that are not phosphorylated by CDK and do not interact with Skp2 are still degraded at the onset of S-phase [12,21]. Therefore, alternative proteolysis pathways exist. One pathway involves Cdt1 ubiquitination by Ddb1–Cul4a–Roc1 (DCR), a cullin-dependent E3 ubiquitin ligase. This pathway is engaged by the recruitment of Cdt1 to chromatin by PCNA, a processivity factor for replicative DNA polymerases (Figure 1a) [10•,22,23,24•]. Like the SCF pathway, it links degradation of Cdt1 with progression through S-phase. A third, APC-dependent, pathway exists in *Xenopus* egg extracts [16•].

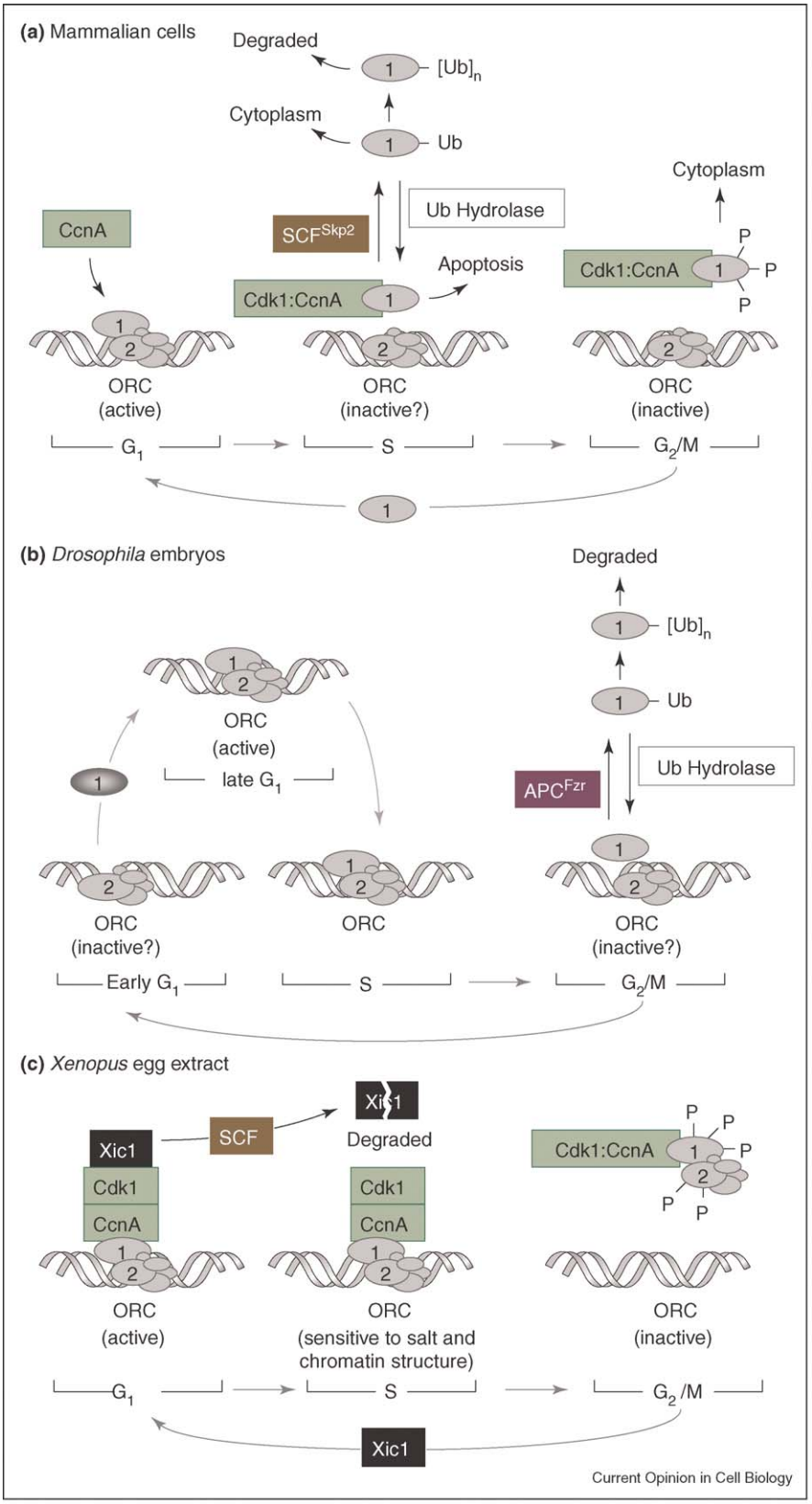
Cdt1 activity also is repressed during the S-to-M-phase transition by binding to geminin, a specific inhibitor of Cdt1 that is unique to the metazoa. Even in the absence of Cdt1 stabilization, the loss of geminin leads to rereplication of DNA in flies [25,26], human cells [27,28] and frog eggs [16•]. Geminin is active during S- to early M-phase, where it is the major inhibitor of DNA replication licensing, and then is inactivated by APC-dependent ubiquitination during late M- to early G_1-phase. Thus, since polyubiquitination usually leads to rapid proteolysis, geminin is absent during late M- and G_1-phase in mammalian cells. In the early embryos of *Xenopus* and *Drosophila*, however, geminin levels are fairly constant throughout the cell cycle [25,29,30]. Instead, transient APC-mediated ubiquitination results in the inactivation of geminin, thereby preventing it from inhibiting Cdt1 [29,31]. This inactive geminin is reactivated once it has been re-imported into nuclei just prior to the onset of S-phase [15,16•]. Geminin does not prevent Cdt1 from binding to DNA; instead, Cdt1 recruits geminin to DNA [1,30]. Cdt1 is stabilized during G_2- and M-phase by binding to geminin, which appears to protect Cdt1 from ubiquitin-mediated degradation [32•]. Thus, the net result is that metazoan cells reduce the amount of Cdt1 available during S-phase, but do not eliminate it entirely. Instead, they use geminin to stabilize the remaining Cdt1 in an inactive form until the next G_1-phase, when

Figure 2



Changes in cyclin dependent protein kinase (CDK) activities during the mammalian cell division cycle. Relative levels of Cdk2–CcnE (red line), Cdk2–CcnA (blue line), and Cdk1–CcnB (black line) activities are indicated (data taken from [8]). SCF and APC activities are also indicated.

Figure 3


(a) Mammalian cells
Degraded
[Ub]n
Cytoplasm
Ub
Cytoplasm
CcnA
SCFSkp2
Ub Hydrolase
Apoptosis
Cdk1:CcnA
Cdk1:CcnA
P
P
P
ORC
(active)
ORC
(inactive?)
ORC
(inactive)
G1
S
G2/M
(b) Drosophila embryos
Degraded
[Ub]n
Ub
ORC
(active)
late G1
APCFzr
Ub Hydrolase
ORC
(inactive?)
ORC
ORC
(inactive?)
Early G1
S
G2/M
(c) Xenopus egg extract
Xic1
SCF
Degraded
Cdk1:CcnA
Xic1
Cdk1
CcnA
Cdk1
CcnA
P
P
P
P
P
ORC
(active)
ORC
(sensitive to salt and
chromatin structure)
ORC
(inactive)
G1
S
G2/M
Xic1
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degradation of geminin allows Cdt1 to rapidly complete the assembly of preRCs.

Regulating ORC activity

In yeast, DNA replication origins are determined primarily by DNA sequences that bind ORC. ORC binds to these sequences in newly replicated DNA and then remains there throughout subsequent cell divisions. ORC activity is down-regulated during S-phase by binding to the yeast equivalent of CcnB–Cdk1 and by phosphorylation of specific ORC subunits. These interactions are not essential for initiation of DNA replication, but cells with defective ORC–CDK interactions are more prone to re-replicate their genome before mitosis is completed [33].

DNA replication origins in metazoan cells are determined both by DNA sequence and by epigenetic factors such as chromatin organization and modification [34,35]. But even more striking is the fact that one or more ORC subunits in metazoan cells undergo cell-cycle-specific modifications that alter ORC's chromatin affinity and stability (reviewed in [4]). The net result is a demonstrable loss of ORC activity during mitosis in both mammalian cells (Figure 3a) and frog eggs (Figure 3c), revealing that regulation of ORC activity can also contribute to preventing premature DNA licensing.

Mammalian ORC consists of a stable core complex of subunits Orc2 through Orc5 that interacts weakly with Orc1 and Orc6. Orc1 is essential for assembly of preRCs; Orc6 is not [36,37]. ORC proteins are localized in the nucleus where the steady-state concentrations of Orc2 through Orc6 and the amount bound to chromatin remain essentially the same throughout cell division [4]. ORC activity is regulated by cell-cycle-dependent changes in Orc1. Orc1 is tightly bound to G_1-phase chromatin but can be selectively eluted from metaphase chromatin [38•], suggesting the existence of an 'ORC cycle' in which the affinity of one or more ORC subunits for chromatin is cell-cycle-dependent. Moreover, the association of Orc1 with chromatin in some mammalian cell lines is selectively diminished during S-phase [37,39,40], and this change is reflected in the fact that Orc1 can no longer be cross-linked to replication origins in S-phase cells, whereas Orc2 can [41,42]. Orc1 reassociates tightly with chromatin during the M-to-G_1 transition, and this is followed closely by the appearance of pre-replication complexes at specific genomic sites [4]. Thus, the selective release of Orc1 from chromatin during mitosis can account for the fact that chromatin from metaphase hamster cells does not replicate in an ORC-depleted *Xenopus* egg extract, whereas chromatin from G_1-phase cells does replicate [43–45].

In HeLa and other transformed human cell lines, Orc1 is selectively degraded during S-phase by ubiquitin-dependent proteolysis, and then resynthesized during the M-to-G_1-phase transition (Figure 3a; S Ghosh and A Vassilev, unpublished) [46–48]. The mechanism by which this occurs requires binding of CcnA to Orc1, which then recruits Cdk1 as cells enter S-phase (A Vassilev, unpublished). Since HsOrc1 mutants that cannot bind CcnA are not selectively degraded during the cell cycle, assembly of an Orc1–CcnA–Cdk1 complex apparently targets Orc1 for ubiquitination by SCF^{Skp2} [46], thereby preventing reassembly of preRCs during S-phase.

In contrast to HeLa cells, the steady-state level of Orc1 remains unchanged throughout cell division in hamster CHO cells [40,44,49]. Nevertheless, a monoubiquitinated Orc1 has been detected during S-phase [40], and transiently expressed CgOrc1, like HsOrc1, can be ubiquitinated *in vivo* (S Ghosh, unpublished). Monoubiquitination is a reversible reaction that does not result in protein degradation, but can result in redistribution of proteins to other cellular compartments. For example, Orc1 with a single ubiquitin linked to its C terminus accumulates in the cytoplasm where it cannot participate in DNA replication [50]. Thus, HeLa and CHO cells may simply differ in their ability to polyubiquitinate and then degrade Orc1.

CcnA–Cdk1 is also bound to Orc1 during G_2/M-phase in CHO cells, where it is responsible for hyperphosphorylating Orc1 [38•]. Hamster Orc1 mutants that mimic phosphorylation at CDK-consensus sites accumulate in the cytoplasm instead of the nucleus [50], and phosphorylation of *Drosophila* Orc1 by CDK inhibits its ability to bind DNA [51], demonstrating that phosphorylation of Orc1 can interfere with its ability to initiate preRC assembly. Conversely, inhibition of CDK activity in metaphase mammalian cells results in rapid and stable binding of Orc1, Cdt1 and MCM to chromatin [32•,38•],

(Figure legend 3) Regulation of origin recognition complex (ORC) activity in the metazoa. **(a)** In mammalian cells, all six ORC subunits (shaded spheres) are bound tightly to chromatin during G_1-phase to provide active ORC:DNA:chromatin sites for preRC assembly. When CcnA is resynthesized during the M-to-G_1 transition, it binds to Orc1. With the onset of S-phase, CcnA–Orc1 recruits Cdk1. This allows Orc1 to be selectively degraded in some cells by SCF^{Skp2}, a ubiquitin-dependent mechanism. However, even a single ubiquitin adduct is sufficient to inactivate Orc1 by transporting it to the cytoplasm. During mitosis, Orc1 is bound to CcnA–Cdk1, hyperphosphorylated, and associated only weakly, if at all, with chromatin. When CcnA and CcnB are degraded during the M-to-G_1 transition, Cdk1 is inactivated, and Orc1 is dephosphorylated and bound to chromatin. If Orc1 is not associated with ORC, and if it is not ubiquitinated or hyperphosphorylated, then it can induce apoptosis. **(b)** In *D. melanogaster* embryos, Orc1 is selectively degraded during the M-to-G_1-transition and then resynthesized during late G_1-phase. **(c)** In *X. laevis* activated egg extract, some ORC is bound to CcnA–Cdk1, presumably via the Orc1 subunit, but Cdk1 activity is inhibited by the CDK-specific inhibitor Xic1. Following licensing of DNA replication origins, the affinity of ORC for chromatin is reduced. Upon entry into S-phase, Xic1 is degraded by an SCF ubiquitin-dependent process that may allow subsequent inactivation of ORC by hyperphosphorylation, which prevents its binding to chromatin.

demonstrating that dephosphorylation of Orc1 can facilitate preRC assembly. Thus, the same CDK activity that initiates mitosis also appears to suppress ORC activity during the S-to-M-phase transition. Furthermore, the same events that modify Orc1 behavior during cell proliferation — ubiquitination, phosphorylation and association with ORC[2–5] — also prevent it from inducing apoptosis [50], suggesting that aberrant DNA replication during mammalian development could result in apoptosis by inducing the appearance of 'unmodified' Orc1.

Analogous mechanisms regulate ORC activity in other metazoa. *Drosophila melanogaster* Orc1 is ubiquitinated by APC^{Fzr} during mitosis and then degraded as cells exit mitosis [52]. DmOrc1 is resynthesized and bound to chromatin during late G_1-phase (Figure 3b). In *Xenopus laevis* cleavage stage embryos (Figure 3c), ORC[1–5] exists as a stable complex that disengages from chromatin during S-phase and dissociates from chromatin during M-phase [4]. CDK inhibitors rapidly convert metaphase-arrested egg extracts into interphase extracts (G_1-phase) that can assemble DNA into normal nuclei and, with the exception of Cdt1/RLF-B, contain all the activities required for complete chromosome replication [31]. This suggests that phosphorylation of XlORC by mitotic CDKs directly reduces its affinity for DNA, consistent with studies on mammalian and *Drosophila* ORC. Moreover, XlORC interacts directly with CcnA–Cdk1 [53]. Upon exit from metaphase, XlORC is rapidly dephosphorylated and associates with chromatin to promote origin licensing. Some of the XlORC in interphase extracts remains associated with CcnA–Cdk1 [53] where it is presumably inhibited by Xic1, a CDK-specific inhibitor that is recruited to ORC:chromatin sites during G_1-phase and then degraded via a SCF-dependent pathway following pre-RC assembly and possibly activation [54,55]. Once licensing is complete, the affinity of XlORC for chromatin is reduced throughout interphase by a mechanism that is not yet known, but that may involve chromatin structure [56,57].

Special roles for regulating Cdc6 activity

PreRC assembly in yeast is regulated primarily by regulating the activity of Cdc6/Cdc18, an unstable protein that accumulates only during the M-to-G_1 transition. When cells enter S-phase, Cdc6/Cdc18 is rapidly phosphorylated by CDK, ubiquitinated by SCF and degraded by the 26S proteasome [5]. In metazoa, however, regulation of Cdc6 activity does not appear to be involved in preventing re-replication in proliferating cells, although it does appear to play a role in regulating exit from quiescence and checkpoint activation.

Cdc6 is phosphorylated when mammalian cells enter S-phase, but the importance of this event in regulating preRC assembly during cell division is unclear. First, human Cdc6 mutants that lack Cdk phosphorylation sites block initiation of DNA replication in some studies [58,59] but not in others [60,61]. Moreover, although such mutants can support initiation of DNA replication in *Xenopus* egg extracts, they do not induce rereplication [60,61]. However, this may simply reflect the fact that Cdt1 is rate-limiting in these extracts [13•,14,15,16•], as well as reflecting the existence of other regulatory pathways. Second, Cdc6 is stable in frog eggs and in some rodent cell lines, where the level of Cdc6 remains constant throughout the cell cycle [49,62]. However, in some human cell lines, Cdc6 is degraded during the M-to-G_1 transition by APC-dependent ubiquitination, and then resynthesized during late G_1- and S-phase before diminishing in late G_2/M-phase [63–65]. Third, whereas ectopically introduced Cdc6 is exported to the cytoplasm during S-phase by a mechanism that is dependent on CcnA–Cdk2 phosphorylation of Cdc6, this mechanism does not apply to the bulk of the endogenous Cdc6 ([66] and references therein). The simplest view is that some basal level of Cdc6 remains associated with chromatin throughout the cell cycle, while Cdc6 in excess of this level is exported from the nucleus when phosphorylated by CcnA–Cdk2.

Regulation of Cdc6, however, does play an important role in regulating the transition from a quiescent state (G_0) to G_1-phase of the cell division cycle. CDK phosphorylation of Cdc6 stabilizes it by preventing its association with APC [67•]. This appears to be necessary for Cdc6 to participate in licensing replication origins before Cdt1 activity is down-regulated in preparation for S-phase. In addition, Cdc6 phosphorylation may delay mitosis until DNA replication is completed [68,69]. Cdc6-P can induce phosphorylation of Chk1, which in turn inhibits Cdc25, the phosphatase responsible for activating Cdk1 (Figure 1b). Presumably, Cdc6 is dephosphorylated following completion of DNA replication in order to allow mitosis to proceed.

Is MCM activity regulated?

MCM activity would seem a natural target for regulation of cell proliferation. In fact, although its activity is regulated in the budding yeast, *S. cerevisiae*, this seems to be the exception rather than the rule. In *S. cerevisiae*, MCM accumulates in the nucleus during G_1-phase, but then MCM that is not bound to chromatin is exported from the nucleus during the remainder of the cell cycle [70]. Nuclear export requires CDK-dependent phosphorylation of MCM subunits. MCM is also phosphorylated by Cdk2 and Dbf4–Cdc7 during S-phase in frogs, flies and mammals as well as in the fission yeast, *S. pombe*, but its nuclear localization is not altered [71]. Phosphorylation is essential to activate the MCM for initiating DNA replication, but whether it prevents reassembly of preRCs during the S-to-M-phase transition remains to be determined. The inability to load MCM onto replicated DNA late in the cell cycle probably results from the absence of Cdt1 and ORC activities.

Conclusions

Preventing rereplication and endoreduplication

Every time a cell divides, there is danger that it may either under-replicate or over-replicate its genome. Either event would leave replication forks scattered among the genome, which could allow non-disjunction of sister chromatids during mitosis and activation of DNA damage repair pathways that could alter the cell's genetic composition. Under-replication is prevented by the presence of multiple replication origins in a single DNA molecule. Over-replication is prevented by restricting the activation of these replication origins to once per cell division, and the evidence is compelling that this is accomplished primarily by regulating the activities of Cdt1 and ORC. The fact that artificially enhancing the activity of Cdt1 can induce re-replication of DNA in metazoan cells not only demonstrates that licensing DNA replication origins in these organisms is limited by Cdt1 activity, but also implies the presence of functional ORC:Cdc6:chromatin sites during S-phase. However, the number of these sites appears to be limited, because depletion of CcnA alone can induce endoreduplication [26], consistent with the role proposed for CcnA in suppressing Cdc6, Cdt1 and Orc1 activities during S-phase (Figures 1a, 3a).

Changes in Cdt1 activity alone can induce re-replication, but they do not appear sufficient to induce endoreduplication. Re-replication is an aberrant event in which the genome is partially reduplicated, and the cell generally undergoes apoptosis. Endoreduplication is a rare but natural component of animal and plant development in which the genome is duplicated more than once without an intervening mitosis. It occurs in specific cell types and does not induce apoptosis. Therefore, endoreduplication requires more than just relaxation of the restrictions on origin licensing; it requires changes in the way cell division is regulated, changes that involve CDK, SCF and APC activities (Figure 2). This may account for the fact that Cdt1 regulation in the metazoa appears excessive, involving as it does the activities of CDK, SCF, DDB1(CDL) and geminin. Reversible mechanisms must exist, such as expression of a Cdt1-specific inhibitor, that allow deregulation of Cdt1 activity without placing in jeopardy other cellular events that depend on the activities of CDKs and ubiquitin ligases.

Although suppression of ORC activity during mitosis either by CDK-dependent phosphorylation (Figure 3a, c) or by ubiquitin-dependent degradation (Figure 3b) plays a role in preventing premature licensing, the role of ORC regulation during interphase is less clear. Selective release of Orc1 during S-phase has not been detected in some mammalian cell lines [49], and may therefore be a feature of physiological stress, cell transformation or even differences in experimental protocol (see [38•]). In addition, regulation of ORC, as well as of Cdc6, may regulate other functions as well, such as chromatin assembly [72], checkpoint activation [68,69], the transition from quiescence to proliferation [67•] and origin usage.

Selecting DNA replication origins

The selection of DNA replication origins appears to follow the Jesuit dictum, ''Many are called, but few are chosen'' (Matthew 22:14). Metazoan genomes contain many potential initiation sites for DNA replication, but during animal development, some of these sites are selectively activated while others are suppressed [73,74]. Regulation of ORC activity extends this concept by providing metazoa with the ability to select different replication origins as cells divide and differentiate. ORC[2–5] remains bound to chromatin throughout the cell cycle, while Orc1 determines which of these sites will assemble a preRC each time the cell enters G_1-phase. Moreover, these ORC[2–5]:chromatin sites appear to translocate along the chromatin. Orc2 is bound to heterochromatin in G_1 and early S-phase, but during late S, G_2 and M phases it is restricted to centromeres [72]. Thus, Orc1 may stabilize binding of ORC[2–5] to specific replication origins. In this way, regulation of ORC activity would allow initiation site selection to be reprogrammed after each cell division, and whenever quiescent cells (which lack Orc1) are stimulated to proliferate.

What next?

For practical reasons, most studies on mammalian cells have used cells cultured in vitro that are either immortalized or cancerous, while other studies have relied heavily on rapidly cleaving, unfertilized frog eggs or egg extracts. However, these experimental paradigms can differ significantly from events in mammalian embryos and adult animal tissues. Therefore, one major question for future research concerns the biological significance of these regulatory pathways during animal and plant development. A second major question arises from the fact that assembly and activation of preRCs does not require specific, autonomously replicating DNA sequences such as those found in animal virus and yeast genomes. Therefore, how do cells determine where and when to assemble preRCs, and how do they ensure that sufficient initiation sites are distributed throughout the genome to allow its complete duplication within a reasonable period of time? These and other issues should occupy our attention for some years to come.

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- •• of outstanding interest

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