Eukaryotic DNA replication origins: many choices for appropriate answers

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Abstract | At each cell division in humans, 30,000–50,000 DNA replication origins are activated, and it remains unclear how they are selected and recognized by replication factors. DNA replication in multicellular organisms must accommodate variations in growth conditions and DNA damage. It must also adapt to changes in chromatin organization associated with cell differentiation and development. The selection of replication origins in metazoans seems to involve multiple choices, with the appropriate answers depending on the identity of the cell or the conditions of growth. This suggests that during evolution, the use of replication origins became more controlled by epigenetic mechanisms affecting chromosome dynamics and expression than by DNA synthesis *per se*.

Replication fork
When replication starts, the opened DNA forms two branched structures on both sides of the replication origin that resemble forks. Fork progression is mediated by the action of DNA helicases that unwind the DNA and facilitate the movement of the DNA synthesis machinery.

Checkpoint
One of many points in the cell cycle at which the cell checks whether the cycle can progress normally or should be delayed or stopped to allow time for the current phase to be completed properly.

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doi:10.1038/nrm2976

DNA replication is required for faithful inheritance of the genome at each cell division. To what extent this process uses similar mechanisms in bacteria and complex organisms is still debated. Multicellular organisms require additional layers of complexity to adapt to larger genomes and various cell fates, and the mechanism of DNA replication initiation is an example of such adaptation.

To be duplicated, a DNA double helix must open to allow the DNA synthesis machinery to copy each DNA strand. These opening sites, called replication origins, are recognized by specific proteins, and DNA synthesis progresses from these sites in a bidirectional manner (FIG. 1a). In *Escherichia coli*, DNA replication starts from a single, sequence-specific element, and the speed of the two replication forks (60 kb min^{-1}) keeps pace with a rapid cell cycle (less than 30 min). The human genome is 700-fold larger than the *E. coli* genome, but the replication fork speed is 20-fold slower (2–3 kb min^{-1}). Thus, it would take at least 20 days to achieve a single division if there was one origin per chromosome.

Pioneering work by Huberman and Riggs[1] showed that in mammals, 30,000–50,000 origins are active at each cell cycle. Not all origins are activated at the same time; their activation follows the specific timing of DNA replication during the cell cycle (FIG. 1b). No consensus sequence has been identified to predict the localization of DNA replication origins in metazoans, and the degree of similarity between origin recognition in metazoans and the replicon model in *E. coli*[2] (whereby sequence-specific proteins recognize a sequence-specific DNA element) is also questioned. Moreover, it is now recognized that potential replication origins are in excess and only a subset are activated at each cell cycle. This leads to the notion of flexible origins, which are used stochastically at each cell cycle or with an increased use in stress conditions or in specific cell cycles.

Here, I describe replication origin features and the specificity of origin selection. I discuss the use of origins, the notion of flexible or dormant origins and the relationship of origins with cell cycle checkpoint controls. The contribution of chromosomes and chromatin structure to origin selection and firing and the influence of transcription will also be discussed, as well as how origin selection is linked with differentiation and development. Other aspects of DNA replication, particularly the function of the proteins involved, have been reviewed elsewhere[3].

Assembly of the replication complex

In metazoans, replication origins are set by a three-step process: recognition of origins, assembly of a pre-replication complex (preRC) during G1 phase and activation of the preRC (BOX 1). This process should be tightly controlled as any origin that has been activated once should not be activated a second time in the same cell cycle. Proteins involved in origin recognition are relatively well conserved but the main origin binding factor, origin-recognition complex (ORC), varies in its sequence specificity.

Saccharomyces cerevisiae ORCs specifically recognize a 12 bp consensus sequence[4], but *Schizosaccharomyces pombe* and metazoan ORCs do not exhibit sequence specificity[5,6]. In metazoans, ORC1 seems to be more involved than the other ORC subunits in the selection of the active DNA replication origin (reviewed in REF. 7). *S. pombe* Orc4 has an AT-hook domain that

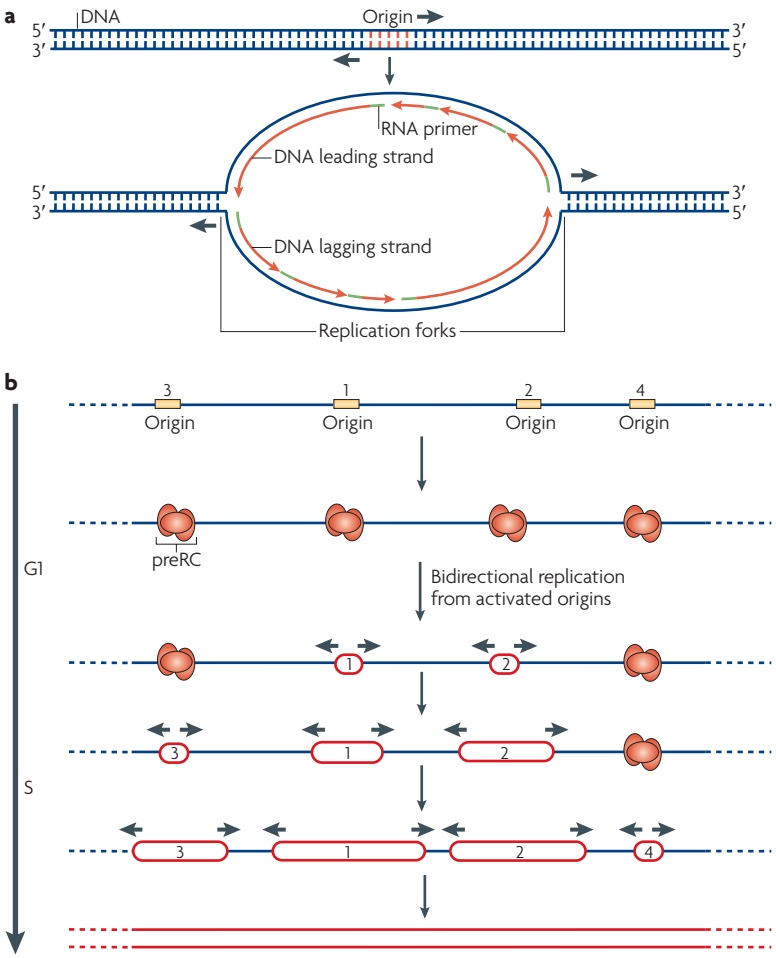


Figure 1 | **Replication origins. a** | At each replication origin, DNA synthesis starts with short RNA primers that are synthesized by DNA polymerase-α. As DNA synthesis always occurs in the 5′–3′ direction, one strand of the DNA (the leading strand) will be synthesized continuously, whereas the other strand (the lagging strand) will be synthesized discontinuously by short RNA-primed DNA fragments. Two other DNA polymerases (δ and ε) are recruited for the elongation of lagging and leading strands, respectively. **b** | Activation of replication origins during S phase. Pre-replication complexes (preRCs) are assembled at replication origins during G1 phase. Activation of replication origins occurs throughout S phase, some during early (1 and 2), and some in mid (3) or late (4) S phase.

recognizes the AT-rich elements of the region[8], and a putative AT-hook is also present in *S. cerevisiae* Orc2 (REF. 9.) *Drosophila melanogaster* ORC does not exhibit sequence specificity but has higher affinity for negatively supercoiled DNA[5] and its binding sequences are more AT-rich than the average genome[10]. Although AT-richness alone is not sufficient to define an ORC-binding site[10], it may influence replication origin activity[11,12]. Similarly, the well-characterized human lamin B2 origin has an AT-rich region and a CpG island that participate in its activity[13].

Analysis in *S. cerevisiae* and *D. melanogaster* showed interesting similarities of ORC proteins with *E. coli* DnaA[14]. DnaA and ORC are both AAA proteins that use ATP to bind DNA. DnaA proteins bind to repeated elements at the *E. Coli* origin (DnaA boxes) and open the adjacent AT-rich element. ATP binding induces the DnaA transition from a monomeric state to a right-handed helical oligomer (which remodels replication origins for preRC assembly[15]) that is a similar conformation to the *D. melanogaster* ORC[16].

All the other known factors that participate in the assembly of the replication initiation complex do not show much sequence specificity and depend on ORC for their binding to replication origins. Therefore, although the architectural features necessary for the assembly of the replication initiation complex are similar, none of the known preRC factors, including ORC, explain how specific replication origins are selected in multicellular eukaryotes. This is in contrast to transcription control, in which several transcription activators are sequence-specific.

DNA replication origin features

Several questions regarding replication origins in metazoans remain unanswered. Does the sequence of origins contribute to their features? Is there a different replication origin code in different cell lineages? To what extent is their activation stochastic during the cell cycle? *S. cerevisiae*, in which the mechanism seems simpler, has been valuable for unravelling preRC protein functions.

Origins in the yeast genome. In *S. cerevisiae*, autonomous replication sequence (ARS) elements have a common, specific 12 bp consensus sequence (the autonomous consensus sequence (ACS)) that behaves as a replicator when inserted into plasmids[17] and to which ORCs bind[4]. However, the presence of an ACS is not sufficient to predict a functional DNA replication origin — of the 12,000 ACS sites in *S. cerevisiae* genomes, only 400 are functional[18].

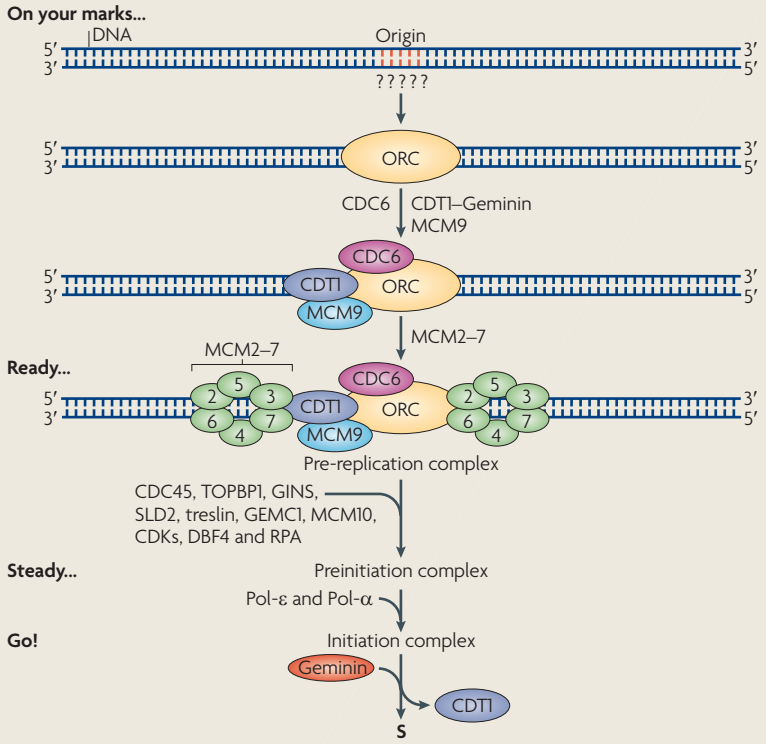
Genome-wide analyses to study replication timing[19] and map replication origins by chromatin immunoprecipitation (ChIP) using anti-ORC and anti-minichromosome maintenance protein (MCM) antibodies[20] did not uncover the known ACS consensus sequence element, unless ORC- and Mcm2-binding sites were considered together[21]. However, recent ChIP studies with anti-ORC antibodies coupled to high-throughput sequencing have identified the ACS motif[22]. Comparative genomics using different *S. cerevisiae* strains[18] confirmed that the ACS element was essential but not sufficient for origin activity and that a region of helical instability close to the ACS was also important. They also showed that *S. cerevisiae* origins are mainly located in intergenic regions. Thus, sequence specificity is essential but is not the only determinant of origin selection, even in *S. cerevisiae*. Other features, not obligatorily shared by all origins, may influence their selection, including transcription and/or the chromatin status (see below).

In *S. pombe*, ARS elements that allow autonomous replication of plasmids do not share a specific consensus sequence as in *S. cerevisiae*. Origins are characterized by AT-rich islands[23–25], and poly-dA–poly-dT tracks can replace important regions in *S. pombe* ARS elements[26]. *S. pombe* origins are also found in intergenic regions, and genome-wide mapping of Orc1- and Mcm6-binding sites and the identification of early 5-bromodeoxyuridine (BrdU)-labelled origins confirmed that preRCs are formed at long AT-rich intergenic regions[27]. Their main

CpG island
A genomic region of at least 200 bp with a high frequency of CpG sites. CpG islands are often found in the transcription promoter regions of mammalian genomes and are unmethylated when the gene is expressed.

Chromatin immunoprecipitation
A method used to localize the DNA-binding site of a protein using an antibody that specifically recognizes the protein on chromatin, which is previously broken into small pieces.

Box 1 | **Assembly of the pre-replication complex at DNA replication origins**



In eukaryotes, the origin recognition complex (ORC) — a heterohexamer with DNA-dependant ATPase activity — is the only initiation protein complex thought to directly recognize origins. After ORC binds to an origin, two factors, cell division cycle 6 (CDC6) and CDT1, are recruited and have the role of loading the minichromosome maintenance protein (MCM) complex MCM2–7 onto the replication origin (see the figure). The MCM2–7 complex is a heterohexamer that has ATPase-dependent DNA helicase activity and forms a ring around the DNA at replication origins[159]. After this helicase has bound DNA, the origins are licensed, marking the end of pre-replication complex (preRC) assembly. CDT1 is a major regulator of this reaction, as it is negatively regulated by Geminin to restrict licensing to only once per cell cycle. In *Xenopus laevis*, Geminin is already present as a subcomplex with CDT1 (REF. 160), and MCM9, a recently discovered new protein from the MCM family, cooperates with CDT1 in the binding of MCM2–7 (REF. 161). At least two MCM complexes assemble at replication origins[162,163]. The preRC is further activated by a growing list of several other factors, including CDC45 and the GINS complex, CDC7–DBF4 and cyclin E–cyclin-dependent kinase 2 (CDK2). This reaction enables the association of the DNA polymerase (Pol) machinery and MCM2–7 to travel ahead of the replication fork to open the double-stranded DNA and allow the synthesis of the complementary strand. As cells enter S phase, CDT1 is inactivated by both its release from origins by Geminin and by degradation. RPA, replication protein A; TOPBP1, DNA topoisomerase 2-binding protein 1.

characteristic is the presence of AT-rich islands[23] that can be targeted by the *S. pombe* Orc4 subunit[8], which contains an AT-hook domain not found in ORC proteins from other species.

Metazoan origins. In multicellular organisms, ARS activity assays were unsuccessful and it was difficult to identify common features of replication origins. FIGURE 2 summarizes the elements that have been found at origins, but none of these alone is predictive of a replication origin.

The use of ChIP methods to localize replication origins in higher eukaryotes was not as efficient as it was for transcription factors or chromatin proteins[28]. This might be because of the abundance of some of the preRC proteins (such as MCMs) or because they are also involved in functions other than origin localization (such as ORCs). In addition, only a small fraction of assembled preRCs is activated at each cell cycle, and, therefore, ORC-binding sites are expected to outnumber active initiation sites (see below). Recent data in *D. melanogaster* show that only 30% of ORC-binding sites are associated with early replication origins[29]. Two-thirds of ORC-binding sites are at promoter regions, and no clear sequence specificity but preferential localization at open chromatin and sites of cohesin loading were found[29].

In *Xenopus laevis* eggs, which contain replication proteins in excess, any injected DNA replicates well[30,31], with an efficiency that increases with DNA size[31]. Initiation occurs at any site, with some preference for AT-rich sequences, but this does not increase the overall efficiency of plasmid replication[32]. The same applies to endogenous chromosomes in *X. laevis* and *D. melanogaster* early embryos[33,34]. During early development, transcription is off and replication origins are set randomly at short, regular intervals. A transition from random to site-specific DNA replication occurs when transcription starts later in development[35], and, in somatic cells, origins are located at specific sites in chromosomes, although no consensus sequence is discernible.

Recent genome-wide studies to map origins in mouse and human cells[36,37] confirmed that there is a correlation with unmethylated CpG islands and promoter regions[38] (C. Carou, P. Coulombe and M.M., unpublished observations). CpG islands could be a mark of replication origins or of the associated transcription promoters, although some known replication origins do not have strict CpG islands, such as those at the β-globin and dihydrofolate reductase (DHFR) loci. CpG island methylation might regulate the timing of replication as origins at non-methylated CpG islands replicate earlier than those at methylated CpG islands[39]. However, origin activity does not seem to be altered by CpG methylation as origins are similarly used on both methylated, inactive and unmethylated, active X chromosomes[40]. Sequence asymmetries were also proposed as identifiers of replication origins[41], but this remains debated as only a few origins have this property[42].

In conclusion, CpG islands and AT-rich stretches seem to characterize metazoan DNA replication origins, but no consensus sequence has yet been revealed. A reason could be that metazoan origins are modular and are identified and regulated by defined combinations of sequence elements, similarly to transcription promoters. In early *D. melanogaster* and *X. laevis* embryos, where all components necessary for DNA replication are stored in excess, sequence specificity might be more relaxed, alleviating the need for cooperative effects of different modules.

It is therefore possible that metazoans have several classes of replication origins that use different modules recognized by different subsets of proteins. Higher eukaryote genomes are packaged in chromosomes that contain longer regions of non-coding sequences than yeast genomes. Heterochromatic regions may also require specific factors to be recognized and opened. Thus, a

Minichromosome maintenance protein (MCM). One of a group of proteins that belong to the AAA+ ATPase family and have a conserved MCM box motif. The main eukaryotic DNA-dependent and ATPase-dependent DNA helicase is MCM2–7, a complex of six different MCM subunits.

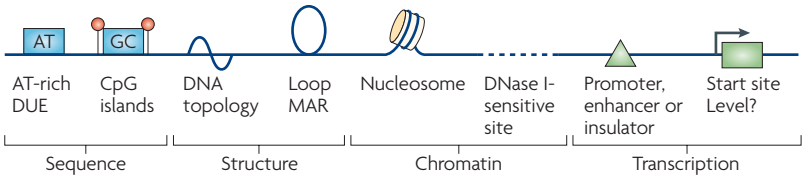


Figure 2 | **Features of DNA replication origins.** Summary of the features seen in DNA replication origins in eukaryotes. Several characteristics have been described at metazoan replication origins, but they are not present at all origins. Rather, they represent different marks or modules that can contribute to the selection of a given origin. At the sequence level, AT-rich elements and CpG islands have been reported as well as DNA regions that easily unwind (DNA unwinding elements (DUEs)), but their importance or role remains elusive. At the DNA structure level, bent DNA (or cruciform DNA) and the formation of loops has been described. At the chomatin level, nucleosome-free regions, histone acetylation and DNase-sensitive sites have been seen, but their direct participation in origin recognition as opposed to being a consequence of chromatin organization for transcription is sometimes difficult to estimate. The possible links of transcription features with replication origin recognition have been described but evidence for direct interactions between replication origin factors and transcription factors remains scarce. MAR, matrix attachment region.

universal origin consensus sequence might not be compatible with genome structure and expression plasticity of multicellular organisms.

Replication origins: a multiple choice

At each cell cycle, only a subset of replication origins are used to replicate eukaryotic genomes.

Different classes of origins. During G1 phase of the cell cycle, inactive MCM helicases are loaded on the preRCs. Activation of preRCs is regulated at two levels. First, origins are activated at different times throughout S phase and are classified as early-, mid- and late-activated origins. Second, only a fraction of all potential origins is used at each cell cycle, whereas others are 'woken' and used only in conditions that affect S phase, such as DNA damage or changes in growth conditions[43]. At first, DNA replication origin firing seems to be a relatively inefficient process in metazoans and yeast. In *S. cerevisiae* (where origins have a consensus sequence) and in *S. pombe*, the overall efficiency of origin firing is less than 50%[25,44] but varies considerably across individual origins — some are used at almost every cell cycle and others are nearly inactive. In metazoans, the efficiency measured in specific replication domains is 5–20%[45,46]. Analysis of yeast ribosomal DNA (rDNA) replication origins in the rDNA cluster by DNA combing showed that not all origins in the 100–200 identical copies of rDNA are activated. Instead, origins are activated by clusters of two or three consecutive units separated by large regions in which origins are silenced[47]. A similar situation is seen in human cells[45].

According to their use, DNA replication origins can fall into three classes: flexible, dormant (or inactive) and constitutive (FIG. 3). Constitutive origins, which are a minority of the eukaryotic origins, are used all the time in any cell cycle or cell type. Flexible origins are potential origins that can be used stochastically in different cells and explain the low origin use seen in eukaryotic cells. They also elucidate the notion of an initiation zone, in which several origins are found at relatively close intervals in a domain, such as the *DHFR* locus. Here, multiple origins are used indifferently over a 50 kb region[48]. In reality, if individual cells activate a single origin in a locus but at different places along the domain, the analysis of the whole cell population will score all the origins. The resulting pattern will reflect the sum of all individual situations and the stochastic nature of origin activation in this locus. If some origins are deleted, others nearby become more active or more efficient, reflecting a large choice of origins[48,49]. Flexible origins follow the 'Jesuit Model' proposed 17 years ago[50] — "For many are called, but few are chosen" (Matthew 22:14, The Bible) — but flexibility could be lost in two ways. First, by increasing origin use in cases of poor growth conditions or DNA damage. Second, by decreasing the choice of origins in specific domains in cells engaged in specific differentiation programmes (FIG. 3). Inactive or dormant origins are potential origins that are never used in the cell cycle in normal conditions but that can be woken in specific cell programmes or in stress conditions.

An important question is whether origin selection in a given cell is transmitted through cell divisions. In this case, flexibility would be linked to cell-to-cell variation rather than to a complete stochastic use of origins at each cell division. Chromatin fibre analysis showed that 70–90% of active origins were the same during two consecutive cell cycles[51], suggesting that there is a memory for the selection of origins in a given cell. However, this memory effect seems to apply to replication clusters and replication foci[52] rather than to individual origins inside the clusters[53].

Why are there so many potential replication origins? First, flexible origins could be abortive origins that on activation failed to elongate. Over-replication of short DNA regions (less than 200 bp) was seen during S phase in human cells, close to origin sites[54], suggesting that all potential origins are activated but only a few succeed in elongating. Second, the efficiency of DNA replication origin firing could be regulated by a limiting factor, or factors, that is not present in sufficient amount to be recruited to all origins. Such factors may include the HSK1 and homologous cell division cycle 7 (Cdc7) kinases[55], cyclin-dependent kinase (CDK) activities and CDC45 (REFS 56,57). These factors become available for late origins after being released from early replicated DNA. Third, several potential origins could provide a choice for the most suitable origin to be activated in a given chromatin context, which might vary in different cells or tissues (see below). This could explain why highly clustered origins are found in heterochromatic regions, such as subtelomeric areas[27]. The apparently stochastic nature of origin activation may therefore reflect the necessity to adapt DNA replication to the chromosomal context, developmental stage or growth conditions.

A link with the cell cycle and its checkpoints. When in the cell cycle are origin sites chosen? A series of elegant experiments showed that during G1 there is a specific point — the origin decision point (ODP) — at which replication origins are selected[58]. Mitosis also seems to be a crucial stage for the reorganization of the nucleus that is needed for the selection of origins. Differentiated

GINS complex
A complex comprising four subunits — SLD5, PSF1, PSF2 and PSF3 — that are ubiquitous and evolutionarily conserved in eukaryotes. It interacts with the MCM2–7 complex and CDC45 to activate the MCM2–7 helicase activity.

DNA combing
A method used to produce arrays of uniformly stretched DNA molecules on silanized glass. The *in vivo* incorporation of fluorescent deoxynucleotides during replication permits, after combing, the localization of DNA replication origins and the progression of the replication forks on the combed DNA molecules.

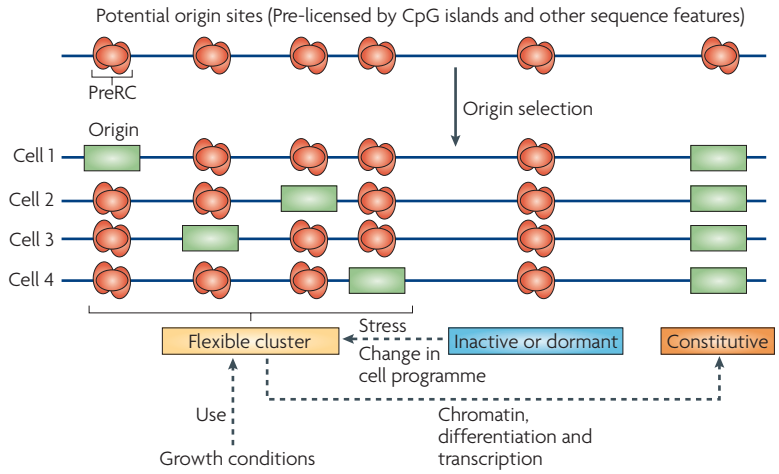


Figure 3 | **Different types of DNA replication origins.** Potential DNA replication origins are set during mitosis–G1 phase by the assembly of pre-replication complex (preRC) proteins. The selection of the origins that will be activated at the next S phase occurs at G1 phase and may vary according to the cell fate or environmental conditions. Four examples of DNA replication origin positions are shown in different cells in a growing cell population. A cluster of flexible origins contains origins that can be used differently in different cells. Their use could increase or decrease according to physiological or abnormal growth conditions. Inactive or dormant origins are rarely used or are not used at all. Constitutive origins are fixed origins that are always set at the same position by chromatin or transcriptional constraints. Replication stress can activate dormant origins or increase the use of flexible origins, resulting in an increased number of origins per replication cluster.

nuclei adopt short inter-origin spacing characteristics of early development only after exposure to a mitotic context[59]. This phenomenon is controlled by topoisomerase II and correlates with the size of chromatin loops. Topoisomerase II also interacts with the human replication origin located on the lamin B gene[60]. Similarly, mitosis is important to reset inter-origin spacing in Chinese hamster ovary cells[61].

Origin selection seems to be relatively independent from the timing of replication, which is defined earlier in the cell cycle than origin selection[58,62]. In addition, the timing of replication seems to be determined at the level of clusters of origins or even larger replication domains rather than at the level of individual origins[63,64].

The relationship between cell cycle checkpoints and increased use or activation of dormant origins has been the focus of recent investigations. Dormant origins might be spare origins that rescue DNA replication when fork progression is perturbed during replication stress[65]. Usually, inhibitors of DNA damage or DNA synthesis activate the S phase checkpoint, which prevents the firing of late origins, allowing DNA repair to take place before further S phase progression[65–67]. In conditions of reduced nucleotide precursor concentration, late origins are inhibited by the checkpoint activity, whereas dormant origins are activated[68,69]. Similarly, a double-strand DNA break enhances the use of origins proximal to the break[70]. Therefore, the checkpoint response to poor cell growth conditions prevents S phase progression but could also increase the use of flexible origins or activate dormant origins to rescue under-replicated domains. This suggests that the excess of potential origins in G1 represents an efficient safeguard against possible S phase problems. Spare origins must be 'put on stand-by' before S phase because during S phase, new preRC formation (by the licensing reaction) is not possible in order to strictly avoid re-replication. All potential origins are therefore licensed at the end of G1 phase, and a few of them are used during the cell cycle. According to this model, the excess of MCM and ORC proteins in a cell allows the licensing of all potential origins. Indeed, it was found that lowering the MCM concentration by small interfering RNA suppresses the use of dormant origins[69]. In agreement, a hypomorphic allele of MCM4 causes chromosomal instability in mice[68,71].

These observations led to the question of whether checkpoint kinases are involved in origin activation in the absence of DNA damage. The ataxia telangiectasia mutated (*ATM*) and the ATM-related (*ATR*) genes[72] encode kinases that are involved in the checkpoint response to DNA damage. ATR is activated by the stalling of replication forks[73], and two other checkpoint kinases — CHK1 (also known as CHEK1) and CHK2 (also known as CHEK2) — are downstream targets that are involved in the inhibition of late-origin activation[74]. Replication stress activates ATR, leading to phosphorylation of CHK1, the inhibition of late-origin firing and the stabilization of stalled replication forks.

The movement of replication forks can also be hindered by physiological constraints of the genome. First, some DNA sequence elements may be more difficult to duplicate than others. Second, chromatin-associated factors, insulator elements and specific nuclear structures may slow down or stall a replication fork in the absence of replication stress. Indeed, in *X. laevis*, treatment with caffeine, an inhibitor of the ATM and ATR checkpoint, slows down replication forks and increases the number of replication origins[75,76]. Similarly, in CHK1-depleted cells, the speed of replication forks is decreased and more origins are activated[77–79]. So why are late origins inhibited and why does the use of early origins increase following replication stress? I suggest the existence of two pathways (FIG. 4). The first is a general ATR-dependant pathway that inhibits late-origin firing and limits the origin usage in clusters of early origins (lateral inhibition), and the activity of which is increased by DNA damage. The second pathway is specifically induced during replication stress and overrides ATR inhibition of dormant origins. So, when ATR is inhibited by caffeine, both late and dormant origins are activated. When ATR levels are increased by replication stress, late origins are suppressed, whereas early-origin use is increased by overriding the ATR inhibitory effect on early origins. It is also possible that in some regions, a passive mechanism is at play, whereby slowing down of a replication fork gives time for a potential origin to become activated before the fork passes through it (see also REFS 69,74).

ATR and CHK1 might regulate the activation of replication origins by controlling CDC45 assembly at replication origins. Activation of the S phase checkpoints inhibits the association of CDC45 with chromatin[80–82] and therefore inhibits the transition of the preRC to the preinitiation complex. Two newly described factors are involved in this

Topoisomerase II
An enzyme that cuts and reseals both strands of DNA to remove DNA supercoiling.

Hypomorphic allele
An allele with a mutation that decreases gene expression.

Insulator element
A regulatory DNA sequence that serves as a genetic barrier to protect a gene against positional effects and the spreading of condensed chromatin or to block enhancer activity.

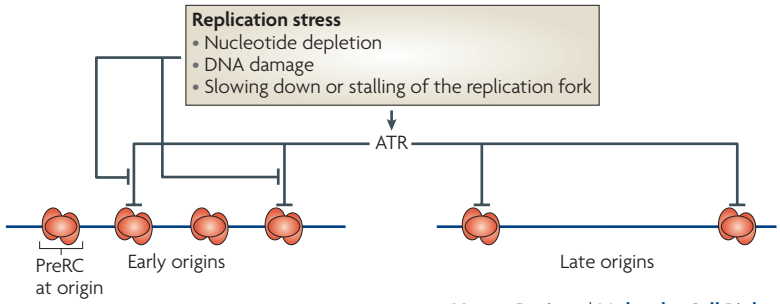


Figure 4 | **Checkpoint control of replication origins firing.** During DNA replication, a DNA lesion caused by stresses such as ultraviolet damage, oxidative damage, genotoxic drugs or growth medium deprivation may slown down or even arrest progression of the replication fork. Replication stress can induce or increase a signal transduction cascade, called the checkpoint response, which tries to maintain the integrity of the replication forks and facilitate DNA repair in coordination with the cell cycle. The checkpoint signalling through ataxia telangiectasia mutated-related (ATR) results in the inhibition of late origins. The frequency of origin usage in clusters of early origins might also be negatively regulated by the ATR pathway, through a lateral inhibition of activated origins on the potential neighbouring origins. However, replication stress might induce a second pathway that cancels this inhibitory effect on early origins, resulting in activation of dormant early origins.

transition: treslin (also known as TICRR)[83,84] and Geminin coiled-coil domain-containing protein 1 (GEMC1)[85]. Both factors interact with DNA topoisomerase 2-binding protein 1 (TOPBP1; homologous to Cut5 (also known as Rad4)), a general activator of ATR involved in the checkpoint response that regulates the association of CDC45 with replication origins[86–89].

Epigenetic features and nuclear organization

The selection of a replication origin amid the many potential ones is predominantly regulated by local chromatin structure and epigenetics (FIG. 5).

Chromatin structure. Generally, it is thought that to be efficient a replication origin should be located in a relatively open chromatin domain. In *S. cerevisiae*, positioning of a single nucleosome on the ACS consensus sequence is sufficient to block the use of the corresponding origin[90]. Similarly, nucleosome positioning by ORC facilitates DNA replication initiation[91] by promoting preRC assembly. A recent study on the nucleosome patterns at replication origins in *S. cerevisiae* shows that ACS is sufficient to exclude nucleosomes, whereas ORC is necessary for ordered nucleosome positioning around the origin[22]. Therefore, chromatin arrangement at origins seems to be crucial for a two-step process of origin selection and function.

Chromatin remodelling complexes also facilitate the formation of the origin complex in *S. cerevisiae* and higher organisms[92]. In yeast, a genome-wide scan showed that origins are more active at open chromatin structures[93]. Similarly, histone acetylation, which is associated with higher chromatin accessibility, correlates with origin activation[94,95]. In agreement, Sir2 histone deacetylase inhibits the activity of five replication origins in *S. cerevisiae* by favouring an unsuitable positioning of nucleosomes at replication origins[96]. Recently, it was shown that multiple acetylation of histones H3 and H4 enhances DNA synthesis in a replicating plasmid[97]. Histone acetyltransferase binding to ORC1 (HBO1; also known as MYST2), a major H4 histone acetylase, is required for DNA replication licensing of origins[98–100]. However, acetylation is not a universal feature of replication origins[36,101,102]. It can be used to select some origins, but generally it seems to be linked to the timing of origin activation rather than to their selection[64,103–105]. It should also be stressed that histone modifications might only be transient at replication origins and that only highly synchronized systems will unravel them.

An unexpected finding was the link between heterochromatin and replication origins. In *S. cerevisiae*, silencer elements contain replication origins[106], which assemble the ORC[107]. The ORC subunit Orc1 is more related to Sir3, which is involved in heterochromatin silencing, than to any other ORC subunits of other species[108]. Orc1 interacts with Sir1 (REF. 109) to establish a silenced state independently from its role in DNA replication. In higher eukaryotes, Sir1 is not present and the ORC1 domain that interacts with Sir1 is lost. However, this interplay is replaced by the interaction of ORC with heterochromatin protein 1 (HP1)[110]. These conserved links between heterochromatin and ORC are intriguing in view of the general idea that an open chromatin structure is more suitable for DNA replication-origin activity. Three explanations could be envisaged. Because heterochromatin is less accessible to preRC proteins, a direct interaction between HP1 and the main origin-recognition protein, ORC, would help heterochromatin to assemble the preRC. This would constitute an important sequence-independent recruitment of ORC to origins at less accessible chromatin domains. In agreement with this hypothesis, ISWI, a chromatin remodelling factor, is required for replication of heterochromatin in mammalian cells[92] but not for the less compacted chromatin of early *X. laevis* embryos[111]. Another possibility would be that HP1 is used not only at heterochromatin but also at subnuclear structures implicated in preRC assembly at euchromatin regions. Finally, in *S. pombe*, Swi6 (the HP1 homologue) activates origins by recruiting Dbf4-dependent kinase (DDK) and Cdc7 to heterochromatin foci[112] and by allowing Sld3 assembly at origins. Sld3 is needed to recruit DNA polymerase and to establish a replication fork at replication origins in yeast[113]. No Sld3 homologue has been identified in higher eukaryotes, but functional homologues have recently been detected[83,84].

Non-coding RNAs. At present, evidence of non-coding RNAs regulating DNA replication initiation is scarce. However, as non-coding RNAs play an essential part in the epigenetic control of transcription, a function in DNA replication would be predicted. A specific class of RNAs called Y RNAs has been proposed to be involved in replication initiation in vertebrates, although no interaction of such RNAs with replication proteins has been detected so far[114]. In *Tetrahymena thermophila*, rDNA amplification, which occurs during macronucleus development, is regulated by the binding of ORC to a non-coding RNA that corresponds to the 3′ end of the 26S rDNA[115]. However, this reaction is specific for the amplification of rDNA and not for other replication origins[116]. The replication

Y RNA
A small non-coding RNA component of the Ro ribonucleoprotein particle that is frequently recognized by antibodies present in autoimmune sera. There are four Y RNAs in humans.

Macronucleus
The larger of the two nuclei present in ciliate protozoans.

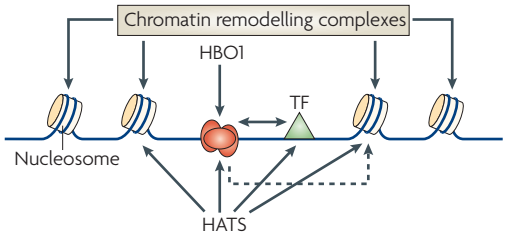


Figure 5 | **Chromatin and replication origins.** Binding of the origin recognition complex (ORC) to replication origins may be favoured by the chromatin remodelling complexes of nucleosomes that can also facilitate the access of histone acetylases (HATs) and thus the acetylation of nucleosomes. This, in turn, would facilitate pre-replication complex (preRC) assembly at origins. Proteins from the preRC, such as the minichromosome maintenance protein (MCM) complex, might also be modified by histone acetyltransferase binding to ORC1 (HBO1; also known as MYST2), a histone H4 acetylase and coactivator of CDT1. Associated factors, such as transcription factors (TFs) and heterochromatin protein 1 (HP1), can cooperate. The assembly of the preRC also results in an ordered positioning of the nucleosome around the region, leaving the origin free of nucleosomes.

of the Epstein-Barr virus (EBV) requires EBV nuclear antigen 1 (EBNA1), a virus-encoded protein that allows the recruitment of ORC to the viral replication origin by an RNA-dependent interaction[117].

Organization of DNA replication origins in the nucleus. Nakamura *et al.* were the first to show that DNA replication takes place at discrete nuclear structures[118] that form up to 1,000 foci per nucleus[52,119]. Each focus can contain 10–100 replicons, constituting a replication domain[52,120,121]. These replication factories comprise various proteins involved in the elongation of DNA replication. It was proposed that replication foci are stable chromatin territories[120,121] but recent data suggest that they are characterized by small replicon clusters that associate in large replication domains[63]. It is thought that each focus is formed by several synchronously activated replicons. These replication clusters are often thought to form chromatin loops that are anchored to a matrix structure[122]. Although this long-standing hypothesis remains controversial, the correlation between replicons, chromatin loops and replication foci deserves to be experimentally revisited using genome-wide approaches for origin mapping and new imaging technologies.

The excess of potential origins in the genome might be explained by additional roles of ORC. Several studies now point to a function of ORC in cohesin recruitment and chromosome structure during S phase. ORC-binding sites seem to correlate with cohesin-binding sites in *D. melanogaster*[29,123,124], and ORC depletion inhibits cohesin loading in *X. laevis*[129]. Sister chromatid cohesion is also impaired in Orc2-depleted yeast cells, although cohesin seems to be normally associated with chromatin[130]. Therefore, all ORC-bound origins, including those that are inactive, might play a part in sister chromatid cohesion during S phase.

Cohesin
A protein complex that is responsible for the association of the two sister chromatids during S phase.

It is important to know whether preRCs are already assembled in preformed nuclear foci before DNA synthesis is initiated. Studies in *X. laevis* suggest that this could be the case[125,126] as replication protein A (RPA) foci can be clearly detected before S phase initiation. However, it is still debated whether these foci form only when initiation of DNA synthesis is engaged. By definition, replication foci are detected with BrdU pulses or by immunofluorescence using antibodies against elongation proteins, therefore only after initiation of DNA synthesis. As they are not clearly seen with antibodies against preRC proteins, it remains questionable whether replication origins already assemble in replication foci at the preRC stage.

Is the mechanism for selecting clusters of replication origins independent from the mechanism for selecting individual origins? Firing at a given DNA replication origin may inhibit firing at nearby origins by an origin interference mechanism[45,127] that leads to the 100–120 kb average size of a replicon. A specific origin among several flexible origins would be chosen by this mechanism and a local checkpoint response might inhibit this mechanism (FIG. 4). Interestingly, although individual replication origins are flexible, large replication domains[128] and replication clusters are not, and the same clusters of replication origins are activated in subsequent cell cycles[52,53]. Control elements, either distant from the origin cluster or in the domain, could control the firing of the whole cluster.

At the level of global chromosome organization, replication domains are not uniformly distributed along chromosomes. Instead, early and late replication domains are interspersed; in mammalian cells they have a size ranging from 200 kb up to 2 megabases[63,129] (C. Carou, P. Coulombe and M.M., unpublished results), whereas they are much shorter in *D. melanogaster*[10,64] and *S. pombe*[27]. Our recent data show that early replication domains are origin-rich, whereas late replication domains are origin-poor. In yeast and mammalian cells, late-activated origins preferentially localize at the nuclear periphery, suggesting that the localization of the domains in the nucleus correlates with the timing of their replication[130]. Some sequence determinants control the timing of replication origin firing[130,131] but do not correlate with the localization of origins at the nuclear periphery.

Transcription and DNA replication origins

Links between transcription and replication result in either negative or positive regulation. Transcription may affect the activity or the choice of replication origins or may influence chromatin structure at replication origins. DNA synthesis itself may provide a window of opportunity to assemble or erase transcription factors as chromatin is opened and reassembled during the passage of the replication fork. A clear connection exists in viral genomes, in which the replication initiation protein can be both a transcription factor and a replication factor[132].

The interplay between metazoan replication origin selection and transcription correlates more with the timing of replication. First, in animal cells, there is a well-known relationship between replication origins that are activated early and actively transcribed genes, whereas origins activated late in S phase are associated with non-transcribed

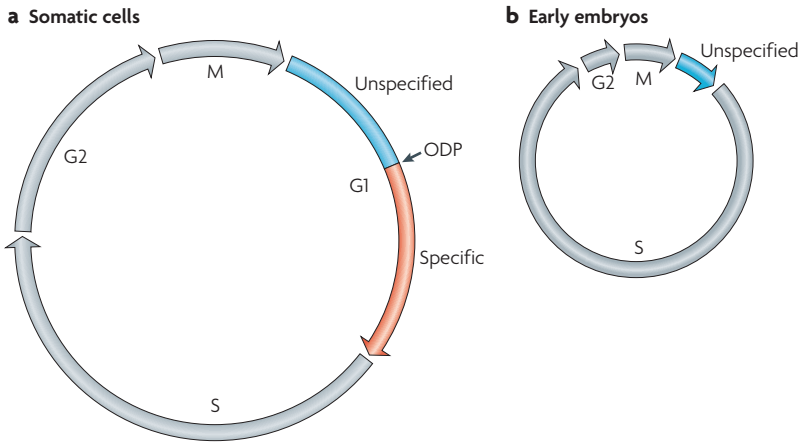


Figure 6 | **Selection of specific origins of DNA replication during the cell cycle.** **a** | In somatic cells, the events occurring from mid-G1 phase (the origin decision point (ODP)) in the cell cycle select the DNA replication origins to be used from the many potential origins set between the end of mitosis (M) and early G1 phase (blue),[58]. After the ODP, replication origin sites are set (red). **b** | Early *Drosophila melanogaster* and *Xenopus laevis* embryos go through a series of cell divisions that consist of overlapping S and M phases without clear G1 and G2 phases. DNA replication origins, which have been deprogrammed in mitosis[59], are not reprogrammed and remain unspecified because of the absence of G1 phase. Embryonic stem cells, which are characterized by a very short G1 period, may also have a larger choice of DNA replication origins.

regions as heterochromatin[133–135]. Transcriptionally active genes have more efficient origins, and this has been shown for individual genes[72,136] and in genome-wide studies[10,36,37]. However, this correlation is not seen in *S. cerevisiae*[19,20], and a recent genome-wide analysis showed that it is not always the case in *D. melanogaster*[64] or mouse cells[63]. In several cases, the assembly of a chromatin domain devoted to transcription rather than transcription *per se* affects origin activity[95,137].

Second, active transcription in a gene silences replication origins inside that gene[138–140] or reduces the size of the initiation zone[137,141]. This implies that origins activated in non-transcribed genes might be erased in transcribed alleles, further complicating genome-wide analysis. In addition, an origin is activated on a gene only during a specific time in the cell cycle; if transcription of the corresponding domain does not occur at the same time, the origin might not be affected.

Third, although active transcription may prevent firing of a potential replication origin (negative regulation), the presence of a promoter may help the selection of an active origin (positive regulation)[49,142]. This might be due to increased chromatin accessibility at the promoter region and to crosstalk between replication initiation proteins and transcription factors. In *D. melanogaster*, ORC is often associated with RNA polymerase II-binding sites[10] but direct interactions between preRC proteins and transcription factors have not been convincingly shown. Promising results were obtained in *D. melanogaster*, in which MYB, E2F1 and retinoblastoma (RB) were found to be associated with elements that control the amplification of the Chorion locus and to interact with ORC[143,144]. Such or similar associations have not yet been described for standard origins. The clustering of origins in promoter regions may also eliminate possible head-to-head collision between the DNA polymerase and RNA polymerase machineries, as seen in bacteria[145].

As well as being associated with promoters[36,37], many origins are also found inside genes. The enrichment of origins in intergenic regions is more convincing in yeast than in metazoans. However, as most replication origins are obviously present in non-coding regions, other mechanisms of origin selection must be at work.

Development and DNA replication origins

The often discussed relationship between transcription and DNA replication may reflect a link between DNA replication and cell identity[146]. Indeed, during *X. laevis* or *D. melanogaster* early embryonic development, when transcription is off, replication origins are activated at very short intervals, every 10–20 kb[33,35,147]. When transcription resumes in the embryos, origins are set at specific sites and at larger intervals[35,148], and the experimental assembly of a transcription-competent promoter is sufficient to drive site-specific replication origins[95]. The explanation might be that early *X. laevis* or *D. melanogaster* embryos use all potential origins to accelerate S phase as they have no transcription constraints. In somatic cells or in late embryos, transcription would restrain origin use and contribute to the establishment of a flexible origin usage, adapted to the cell fate. Another possibility is that a specific organization of the chromosomes during early development imposes a regular setting of origins at short intervals. A link between chromatin loops and replicon size, seen in different organisms[149] and in reprogramming experiments[59], may contribute to the organization of replication origins. Changes in the choice of origins were observed in other developmental or differentiation processes, such as during cell differentiation in *Physarum polycephalum*[136], neural differentiation of mouse cells[101], embryonic development of the fly *Sciara coprophila*[137], B cell development[72], and at the chicken β-globin locus during erythroid differentiation[102]. Links between replication and development are also established at the level of the timing of replication rather than at the level of origin localization and selection. A series of extensive analyses in mouse and human embryonic stem cells have emphasized the evidence for changes in replication timing during differentiation[63,150], which were also seen during *D. melanogaster* cell differentiation[64]. A more recent analysis carried out using cells from different germ layers during mouse development confirms these results and the interplay between replication timing and transcription[151].

A link between origins and differentiation programmes is also suggested by the unexpected involvement of preRC proteins in differentiation. For instance, in *D. melanogaster,* Latheo, a protein that interacts with ORC, is involved in neural differentiation[152]. Furthermore, Geminin plays a crucial part in inactivating the preRC complex after DNA synthesis has started and also has a function in neural differentiation that is related to the transcriptional control of the Hox genes[153–155] and chromatin remodelling[153]. In *Arabidopsis thaliana*, GEM (which, like Geminin, interacts with CDT1), is involved in root epidermis patterning[156]. More recently, the homeotic protein HOXC13 was found at three human replication origins[157].

Perspectives

Recent work emphasizes that we have many more replication origins than needed at each cell cycle and that their use is highly flexible. Flexibility is probably needed to adapt to environmental cues, obstacles encountered by the replication machinery and developmental features that require reorganization of the genome or nuclear structures. This might explain the difficulties in finding a common rule that specifies replication origins. Origins are probably multi-modular and their use might depend on the combination of modules and the concentration and affinity of the proteins that bind them. An interesting question is to what extent genome-wide studies can define these modules and predict their usage according to the combination.

Flexibility, however, does not mean that initiation is totally random. Recent genome-wide studies indicate that this is not the case[36,37]. Origins are reproducibly found at the same sites in a given cell population and flexibility refers to the choice of the activated origins among these sites in a given cell or tissue. A remaining issue is how constitutive or master origins and secondary origins that are used only in specific cell contexts are established. Origins that are activated by the checkpoint response following a replication stress may fall into this second category. A more open chromatin structure, owing to the presence of a transcription promoter region, might be suitable for setting a replication origin, but other chromatin features, not obligatorily linked to transcriptional permissiveness, might be important as well.

The flexible use of replication origins in somatic cells and the extreme use of origins in transcriptionally quiescent embryos strongly suggest that a strict positioning of origins in the metazoan genome is not required for a regulated, once-per-cell-cycle type of DNA synthesis. The role of transcription promoters and the associated chromatin features that are present in some specific cases does not seem to be a general determinant for origins. Inversely, setting a replication origin in a given chromatin domain does not necessarily favour transcriptional activity. It might be that neither replication origins control transcription nor transcription controls replication, but that an underlying structure of a chromosome fixes the regulation of both transcription and the replication origins of a domain. It might be argued that S phase rules are in fact set during the formation of mitotic chromosomes at the previous cell cycle. Indeed, in *X. laevis*[59], mammalian cells[61] and yeast[57], origin use during a given cell cycle is regulated by events that occur during previous mitoses. In this case, the selection of replication origins among several potential origins might be achieved in two steps. Mitosis might be an important first step to reset the origin code of a given cell and might be used to deprogramme its corresponding genome. The replication origins to be activated might be further fixed in G1 phase (FIG. 6) and adapted to the gene expression programme. Interestingly, pluripotent stem cells have a short G1 period and replication origin choice might be more flexible. In agreement, nuclear transfer experiments showed that adult somatic chromosomes are efficiently reprogrammed in mitotic zygotes but not in interphase zygotes[158]. The reprogramming of replication origins in mitosis might therefore be crucial for somatic cell reprogramming, even in the induction of pluripotent stem cells.

The possibility of multicellular organisms reprogramming their origins at each cell cycle might contribute to the segmentation of the genome into autonomous units of replication and transcription. This might help to organize chromosomes for different transcription programmes in different cell lineages during development and differentiation. Therefore, DNA replication origins might be much more than just entry sites for replication factories; they might be essential regulatory elements that organize the genome according to cell fate.

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Acknowledgements
I would like to thank E. Andermarcher for critical reading of this manuscript. The M.M laboratory is supported by the European Research Council (ERC advanced grant FP7/2007-2013, Grant Agreement no. 233339), the ANR, the 'Association pour la Recherche contre le Cancer', and the 'Ligue Nationale contre le Cancer'.

Competing interests statement
The authors declare no competing financial interests.

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