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Epigenetic control of DNA replication dynamics in mammals

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Every time a cell divides it must ensure that its genetic information is accurately duplicated and distributed equally to the two daughter cells. This fundamental biological process is conserved throughout all kingdoms of life and relies on the correct and complete duplication of the DNA before a cell can divide and give rise to other cells or to multicellular organisms. Any mistakes in this process can result in genetic mutations or karyotype aberrations, which may lead to disease or even death. Whereas in prokaryotes the entire genome is replicated from a single origin, the increased genome size and complexity in mammals requires the spatio-temporal coordination of thousands of replication origins. Furthermore, this spatio-temporal order of genome replication changes throughout development and cellular differentiation. Here we present and discuss current knowledge on the control of DNA replication dynamics in mammals and the role of chromatin modifications in this basic biological process.

In prokaryotes, a smaller genome size allows a whole genome to be replicated in a timely fashion from a single replication origin. As genome size and complexity increase, a fast enough replication of the whole genome becomes more difficult to achieve, so that eukaryotic chromosomes possess a large number of origins of replication. In human cells, for instance, the number of origins that fire each cell cycle was estimated to reach a number of 50,000.[1] While in some single cell eukaryotes with less complex genomes, such as *Saccharomyces cerevisiae* (budding yeast), replication origins are defined genetically and their time of firing is constant, already in *Schizosaccharomyces pombe* (fission yeast) replication origins are defined far less stringently. When it comes to metazoan, the search for genetically defined origins has proven elusive and, in spite of great efforts, no consensus sequence defining origins of replication has been identified.[2]

With the increase in the number of sites of replication initiation, also the regulation of their firing becomes an issue. The cell needs not only to ensure the error-free duplication of the DNA strand in a timely fashion, but it needs as well to avoid the re-replication of any region, that might result from uncoordinated origin firing. To make matters yet more complex, the process of replication cannot be taken out of the nuclear context and thus needs to be coordinated with other chromatin-based processes, particularly that of transcription and the concomitant chromatin remodeling.[3] Hence, control and organization of origin firing are of critical importance.

At a global scale, the eukaryotic genome replicates in an organized, non-random manner, meaning that defined genomic regions replicate at distinct S-phase stages, as first described over 50 y ago.[4] In other words: first, not all origins fire at the same time and second, synchronously firing origins are not homogenously distributed throughout the genome. These dynamics result in the appearance of replication patterns that change in a well-conserved manner as S phase progresses and can be visualized at the light microscopy level.[5] On the other hand, at a single origin level, the firing process is believed to be a stochastic one: not every potential origin fires in every cell cycle,[6] but can have a rather higher or lower firing efficiency.[7,8] What is more, the regulation of DNA replication is not a static process. On the contrary, it is a flexible undertaking that changes throughout development and with differentiation.[9-13] This observation, as well as the unsuccessful search for an origin defining sequence in metazoan, has led to the proposal that the regulation of DNA replication cannot be explained at a genetic level alone. Plausible candidates to control replication are epigenetic factors that influence the chromatin state of different genomic regions. Already shown to influence processes such as transcription, epigenetic modifications including DNA methylation, histone modifications, non-coding RNAs, among others, intrinsically define chromatin structure and potentially play a role in any chromatin-based event. Indeed, over the last decade, correlations between certain epigenetic modifications and the particular replication timing of a region have been demonstrated in different organisms.[14-17] Furthermore, manipulation of the chromatin state at different regions has been shown to result in changes in replication timing (see **Table 1**).

Still, the influence of epigenetics on the process of origin firing and its regulation, particularly in mammals, is far from being completely elucidated and therefore the role of distinct epigenetic modifications therein is a matter of fervent research. This quest has, however, been complicated by the fact that epigenetic mechanisms seldom exert their influence on chromatin independently from each other, but are characterized by a strong crosstalk. This

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Table 1. Summary of most relevant studies on the control of replication timing in lower eukaryotes

Organism	Factor	Publication	Outcome
Yeast	Transcription	Raghuraman et al. 2001	No correlation between transcription activity and origin selection.
	Histone acetylation	Vogelauer et al. 2002	HDAC Rpd3$^{-/-}$ or HAT Gcn5 recruitment to a late origin result in earlier origin firing and concurrent Cdc45 binding.
		Zappulla et al. 2002	Sir HDACs are sufficient to reprogram an origin from early to late.
		Knott et al. 2009	Over 100 late origins are regulated by HDAC Rpd3L.
		Crampton et al. 2008	HDAC Sir2 (H4K16 deacetylation) inhibits preRC assembly at late origins by promoting unfavorable structures and inhibiting MCM binding.
		Unnikrishnan et al. 2010	Multiple acetylated residues are required for efficient origin activation.
	Histone methylation	Pryde et al. 2009	H3K36m1 together with histone acetylation advance binding time of Cdc45 to replication origins, while H3K36m3 and histone deacetylation delay it.
Drosophila	Histone acetylation	Aggarwal and Calvi, 2004	Histone hyperacetylation at replication origins affects ORC binding.
	HP1	Schwaiger et al. 2010	HP1 knockdown results in 5–10% of genome affected in replication timing, advanced replication of centromeric repeats, BUT delayed replication of unique sequences embedded in repeats (targets of HP1).
	Chromatin modifiers/modifications	Eaton et al. 2011	Chromatin environment does not regulate replication origins as a binary switch, but rather acts as a tunable rheostat to regulate replication initiation events.

fact represents a particularly high hurdle in the case of higher eukaryotes with a more complex epigenetic constitution than unicellular eukaryotes or lower metazoans. Moreover, the flexibility epigenetic marks offer as regulatory mechanisms to the cell and their concomitant variability within a population further increase the experimental difficulty in studying their effects on any process. It is, therefore, of extreme importance to combine high-throughput and in vitro biochemical methods with single cell in vivo studies. While the former are powerful tools and have extensively elucidated the molecular players involved and their interactions, they intrinsically average out the cell-to-cell variability in a population and lack any information on nuclear context, the latter permit a detailed view into cellular processes in high time resolution without losing the biological variability inherent to them. To date, however, live cell imaging methods are to a great extent limited, partly at the acquisition step, partly at the data analysis step, to rather low to medium throughput. Therefore, the development of tools to increase the efficiency of live cell analyses is of paramount importance. Furthermore, the application of super-resolution light microscopy approaches will increase the spatial resolution of the whole cell in situ analysis and help closing the gap to the in vitro biochemical approaches.

The Molecular Process of DNA Replication

From determining replication origins to completing a replication round. The first steps toward the elucidation of the molecular factors and processes involved in DNA replication were performed in vitro, using bacteriophage T4 and, later, SV40 as models of prokaryotic and eukaryotic replication, respectively.[18,19] We owe to these pioneer studies the basis of our understanding on the essential factors, their interactions and the enzymatic processes involved in DNA replication. A further milestone in the DNA replication field was the transition to an in vivo eukaryotic system with the establishment of yeast genetics as a tool to study DNA replication.[20,21] Studies in yeast have, without any doubt, been an extremely powerful tool in dissecting the different biochemical components of the replication process in the vast sense, which includes setting up the replication program, the origin firing process as well as DNA synthesis itself. However, it is important to mention that, when it comes to the spatio-temporal regulation of the replication of the genome, intrinsic differences between *S. cerevisiae* and metazoans make it dangerous to generalize conclusions arising from studies performed in yeast. Nevertheless, the biochemical players of the processes of initiation and replication are well conserved from yeast to mammals and, in general terms, seem to act following the same principles (**Fig. 1**).

Pre-replicative complex assembly: it all starts without a nucleus. Before the actual DNA synthesis process can start during S-phase, the cell has to determine where the next round of replication will, or more precisely, can potentially start. The process of origin determination starts even before G_1, at the end of mitosis and, thus, in metazoan organisms, in the absence of a nuclear membrane. The first well known step is the binding of the origin recognition complex (ORC), six related proteins, conserved from yeast to humans, which bind to potential replication origins during telophase. Their targeting mechanism, however, varies between different eukaryotes.[2] Whether the ORC subunits form a complex before their binding to chromatin or whether they actually change their association to chromatin throughout the cell cycle or, in turn, are constitutively bound to chromatin is a

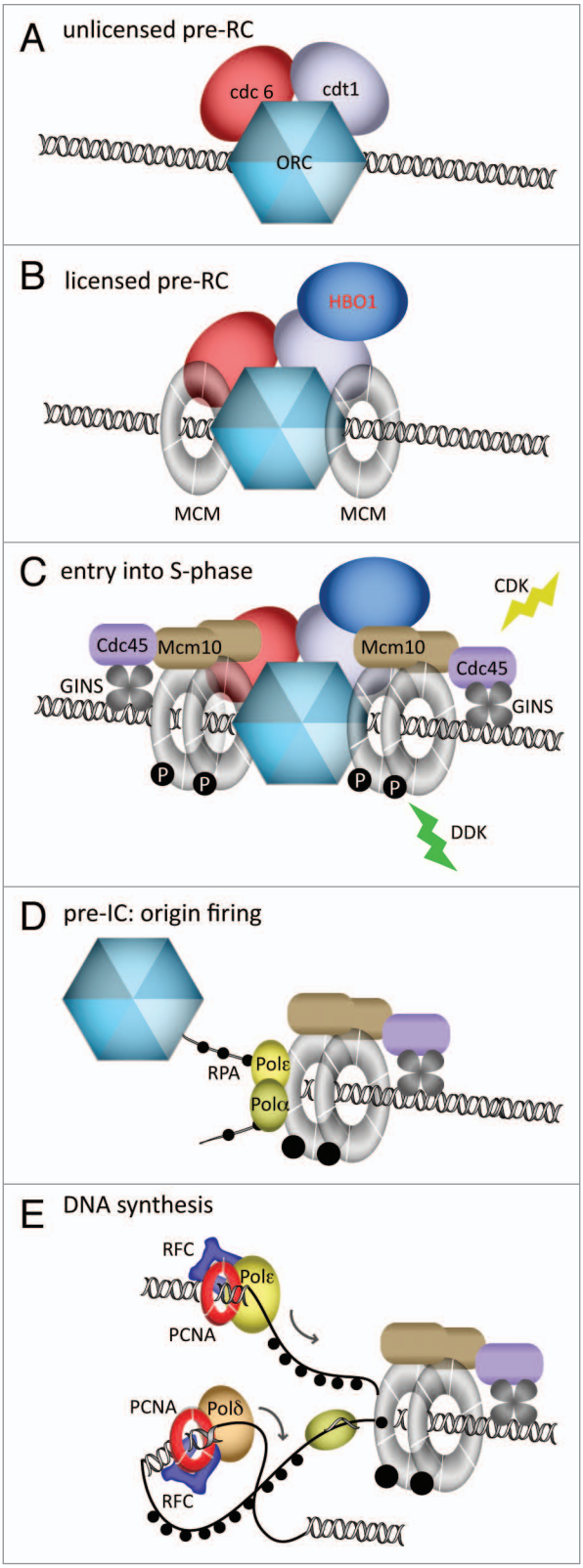

Figure 1. Schematic representation of the molecular factors involved in (A) replication origin determination, (B) licensing, (C and D) activation and (E) the actual DNA synthesis. The origin recognition complex (ORC) is either constitutively bound to chromatin or binds during late mitosis. ORC recruits Cdc6 and Cdt1. (B) In metazoan, Cdt1 binds to the histone acetyltransferase HBO1, necessary for pre-RC licensing via MCM loading. (C) DDK and CDK activities result in pre-IC (pre-initiation complex) assembly, including binding of Mcm10, Cdc45 and GINS. (D) Origin activation results in unwinding of the double helix. Single stranded DNA is stabilized by the replication protein A (RPA) and DNA polymerases α and ε are recruited. DNA pol α has also a primase activity and is thus able to synthesize RNA primers on single stranded DNA. (E) Processive DNA synthesis is achieved after a polymerase switch to DNA pol δ and the loading of the proliferating cell nuclear antigen (PCNA) sliding clamp by the replication factor C (RFC). Duplication of the lagging strand happens in a discontinuous manner, with several rounds of Okazaki fragments, starting each with the synthesis of a new RNA primer.

matter of debate. Most likely there are variations in the diverse organisms concerning the binding behavior of the different ORC subunits.[22-24] Conserved throughout eukaryotes is the fact that during the transition from mitosis to G_1 the ORC recruits, independently from each other, the initiation factors Cdc6 and Cdt1 (**Fig. 1**). Cdc6, an AAA$^+$ ATPase, might thereby modulate ORC binding to chromatin[25] and inhibits ORC binding to non-specific DNA.[26] The main function of the ORC, Cdc6 and Cdt1 is to load the Mcm2–7 complex (MCM) onto chromatin and to thereby complete the licensed pre-replication complex (pre-RC, **Fig. 1**). This is demonstrated by the observation that, after MCM loading, the ORC, Cdc6 and Cdt1 become dispensable for origin firing.[25,27,28] In fact, structural studies have shown that ORC and Cdc6 may function together as a clamp loader complex for opening and closing MCM around DNA at origins.[29] Recent studies have shown that Cdt1 recruits HBO1 (human acetylase binding to Orc1), a histone H4 acetyltransferase (HAT) to origins and that the HAT activity of HBO1 is required for MCM loading.[30] Interestingly, no yeast homolog for HBO1 has been identified so far. Moreover, HBO1 is inhibited by Cdt1 interaction with Geminin,[30,31] another specific factor of metazoan replication. These mechanisms represent an additional origin regulatory step and emphasize the differences between metazoan and unicellular organisms. The MCM renders an origin licensed for replication in the subsequent S-phase. In vitro, the MCM has a helicase activity and is therefore considered the putative replicative helicase,[32,33] able to unwind origins[34] and has furthermore even been proposed to stay bound to the replication fork.[35] However, there are several observations that do not immediately fit to this hypothesis. The so-called MCM paradox refers to the seemingly contradictory observations of the excess of nuclear MCM and its accumulation far from active replication foci and its proposed role as the replicative helicase.[36,37] Nevertheless, a fraction of MCM was recently shown to colocalize with sites of active DNA replication,[38] supporting their role as replicative helicases. All in all, while the overall principles of the many initiation steps are supported by observations in different organisms, the recruitment of the different factors to origins still needs to be elucidated in their molecular details.

Origin firing: transition from the pre-RC to the replication fork. Before the actual process of synthesizing DNA can start, the pre-RC complex must be activated by cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK) activities.[39-42] Phosphorylation of the pre-RC leads to the recruitment

of additional factors to the replication origin, such as Cdc45, Mcm10, Sld3 and GINS (**Fig. 1**, reviewed in refs. 43–45). These initiation factors are necessary for the unwinding of the replication origins and recruitment of the replicative DNA polymerases. The result is an open replication bubble containing two replication forks that will progress in opposite directions as both leading and lagging strands are replicated.

In detail, the transition from the pre-RC to the elongating state is initiated by interaction of Mcm10 with Orc2 and various Mcm2–7 subunits.[46,47] Cdc45 is then recruited to this complex[35,48] and stimulates the helicase activity of the Mcm2–7 complex.[49,50] Single-stranded DNA configuration is stabilized by replication protein A (RPA), which further stimulates origin unwinding.[34] Cdc45 and RPA binding results in the recruitment of the actual DNA synthesis machinery, including the replicative DNA polymerases ε and α to the now open origins,[51] forming the pre-initiation complex (pre-IC, **Fig. 1**). DNA polymerase α (pol α), also a primase, is the only DNA polymerase that can start de novo synthesis on single-stranded DNA and is recruited to origins to synthesize short RNA primers for leading and lagging strand. After primer synthesis, DNA polymerases are exchanged and DNA pol α is replaced by DNA pol δ or DNA pol ε, which have a higher processivity and proofreading exonuclease activity.[52] This enhanced processivity however requires association to the proliferating cell nuclear antigen (PCNA), a homotrimeric ring that serves as a loading platform for various elongation factors.[53] PCNA is loaded by the replication factor C (RFC) and moves with the replication fork at active sites of replicating DNA. Additionally, the MCM, Cdc45 and GINS also move away from replication origins as part of the replication fork machinery,[43] arguing for a role in elongation besides their initiation function.

Thus, DNA at licensed pre-RC is unwound upon activation resulting in the assembly of two replication forks that will progress in opposite directions, normally until they collide with a replication fork originating from a neighboring replication origin.[54] The replicon, or unit of DNA that is replicated from one single replication origin, is generally a symmetric structure with the origin lying in the middle and has an average size of approximately 100–200 kb in somatic cells.[55] The molecular replication machinery, in charge of duplicating a replicon, is termed the replisome.

How replication propagates: the molecular dynamics of the replisome and the domino model. Once replication has initiated at a limited number of particular loci, it needs to expand throughout the genome, so that replication of the entire genome can be achieved in a timely fashion. To reach this goal, further origins need to be activated. Analyses of the molecular dynamics of the replisome have shown that neighboring chromatin foci are not replicated by the same machinery, but rather a new replisome is assembled, preferentially close to already active replication sites.[56] The observation that new sites of DNA replication almost always appear in close proximity to active sites[57] has led to the proposal of a domino model, with a "next-in-line" mechanism determining the temporal order of origin activation.[56] Fitting to this proposal, it has been shown in human cells that the spatial continuity of replication foci correlates with their genomic continuity along chromosomes.[58] This model is further supported by the fact that neighboring replication domains tend to initiate replication at similar time points on human chromosomes,[59] as well as by the observation that the temporal order of replication of some regions corresponds to their linear order in the genome.[60] It has been proposed that this "replication wave" is most likely transmitted by local destabilization or changes in chromatin structure resulting from replication activity itself. Such structural changes, caused by active replication, would render neighboring regions more prone to replication initiation and result in the self-propagation of replication (**Fig. 2**). What the exact mechanism is by which initiation of DNA replication is propagated along the genome, is a matter of current research.

Strikingly, this spatio-temporal propagation of replication sites does not happen in a random manner throughout the nucleus. As discussed in the following section, the continuous assembly and disassembly of active replication sites throughout S-phase results in highly conserved nuclear patterns of replication that clearly differ between early, mid and late S-phase.

Organization of DNA replication: a 4D-matter

DNA replication dynamics. In metazoan cells, in situ visualization of sites of active replication results in distinct replication patterns that change as S-phase progresses and are typically divided into early, mid and late patterns based on their distinct morphological and topological features.[61] These patterns are formed by replication foci, structures of approximately 120 nm in size that are stably maintained throughout the cell cycle.[62] Each replication focus is thought to represent a series of coordinately activated sites of replication that are in close spatial proximity. As replication begins, in early S-phase (Se) many small foci can be observed throughout the nucleus, with exception of the nucle(ol)ar periphery. These regions become populated by somewhat better defined foci during mid S-phase (Sm). Finally, during the second half of S-phase (Sl), larger clusters of active replication foci accumulate into fewer, but bigger structures (**Fig. 3**).[61,63]

The replication dynamics is conserved from hydra to mammals[64,65] and reflect the higher order 3D organization of chromatin in the nucleus:[66,67] early foci correspond to euchromatic regions or R bands, characterized by a high gene density and mostly found in the nuclear interior, mid foci represent facultative heterochromatin accommodated in the nucle(ol)ar periphery and late foci mark constitutive heterochromatin.[68] The existence of such patterns demonstrates that clusters of replication origins are activated in a highly coordinated manner, with some nuclear regions being specifically activated earlier than others. Furthermore, this conserved replication dynamics raise the question of how specific replication origins are selected to fire at a particular S-phase stage.

Replication origin definition: from yeast to man. In *S. cerevisiae*, replication origins were identified as sequences that are able to replicate autonomously when inserted into a plasmid (Autonomous Replicating Sequences, ARS), all sharing an ~11 bp long conserved sequence, the autonomous consensus sequence (ACS, reviewed in refs. 69 and 70). The ACS alone is, however, not sufficient to predict a functional origin. In fact, a region of

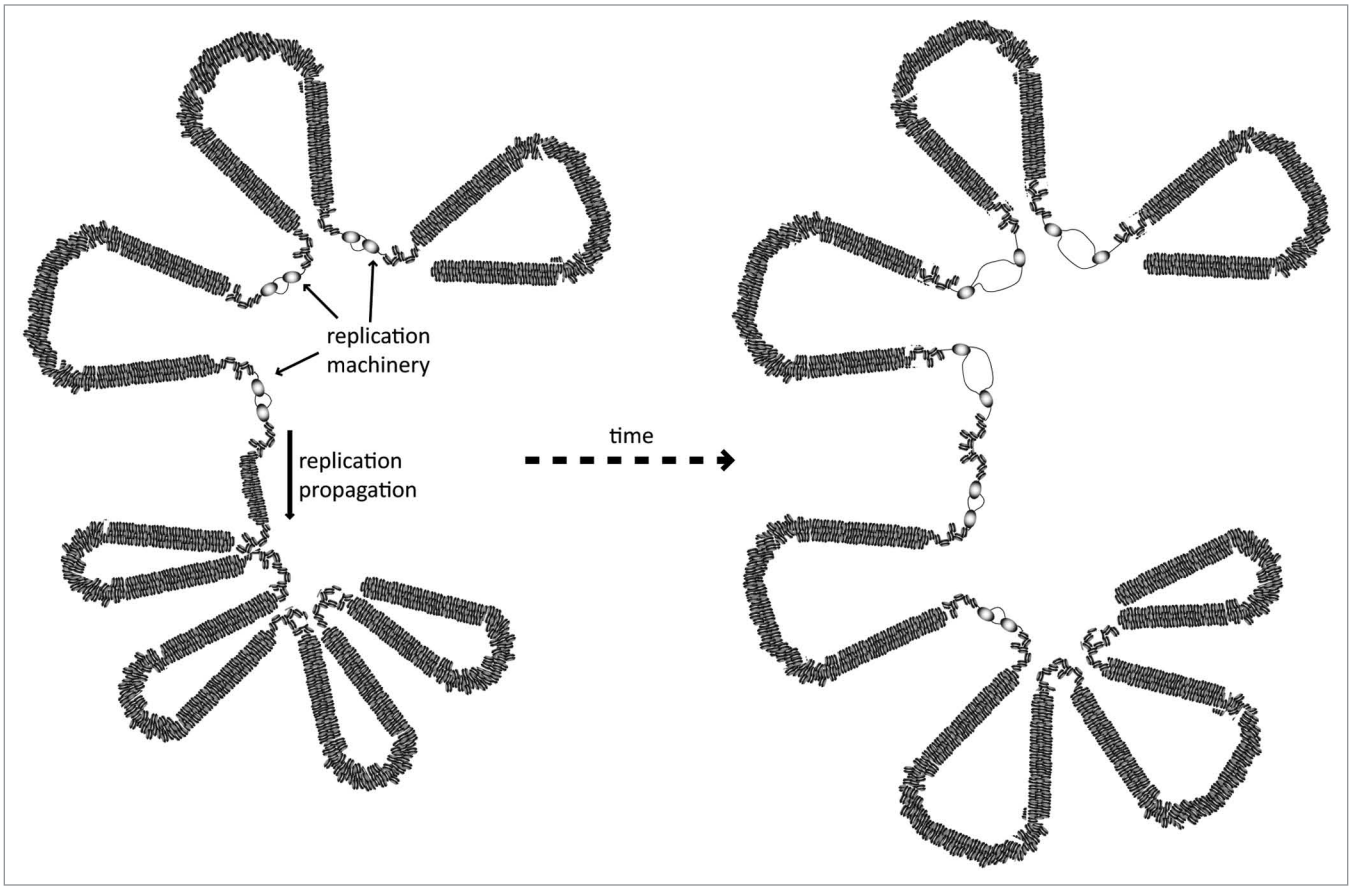

Figure 2. Spreading of active replication along the genome. Schematic drawing of how DNA replication might spread along chromatin according to the domino model. Active replication might destabilize higher order structures, rendering replication origins in neighboring chromatin regions more prone to fire. This spreading feature might be an intrinsic effect of the replication machinery, possibly of the replicative helicases or, alternatively, a replication-independent factor might precede the replication machinery and change chromatin conformation as a preparation and, thereby, positively influence origin firing.

helical instability close to the ACS is also necessary for origin activity,[71] demonstrating that sequence is, even in yeast, not the only determinant of active replication origins. In fact, yeast replication origins have a binding site for the transcription factor Abf1, which might promote origin activation[72] and Gcn5, a histone acetyltransferase (HAT) associated with transcriptional activity,[73] increases DNA replication when tethered to an origin.[74] The transcription factor binding motif is an important determinant of nucleosome depletion,[75,76] a fact that might be relevant to origin determination, since positioning nucleosomes at origins inhibits their firing.[77,78] Indeed, it has been postulated that ORC may facilitate pre-RC formation by influencing nucleosome positioning.[79]

In higher eukaryotes the sequence elements defining replication origins are much weaker, suggesting that the role of DNA structure and chromatin is crucial.[80] Indeed, in metazoan no consensus sequence defining origins has been found. In fact, any DNA injected into Xenopus egg extracts replicates well[81] and replications origins seem to be selected every cell cycle anew.[82]

Probably one of the most striking examples of the flexibility of DNA replication dynamics in metazoan is observed in Xenopus and Drosophila embryos. In these organisms, during early embryogenesis, replication starts at the same time, randomly all over the nucleus. This replication mode results in a very short inter-origin distance of approximately 15 kb.[12,83] During the mid blastula transition (MBT), there is a re-arrangement in replication dynamics that correlates with the onset of transcription and global changes in chromatin structure and results in much longer inter-origin distances. In mammals, re-programming of mouse somatic cells to a pluripotent state is accompanied by the reduced size of replication domains.[84] These developmental changes clearly illustrate that replication origins are not defined at a sequence level and its selection can adapt throughout cell differentiation.

Nevertheless, there are some sequence elements that seem to be involved in the determination of origins, such as AT-rich regions and CpG islands. This has been suggested by the fact that there is some preference for AT-rich regions in DNA injected into Xenopus extracts[81] as well as by the correlation in the location of origins and CpG islands.[85] However, these elements alone are not sufficient to predict origins and moreover, being genetic elements, they cannot compel the described developmental flexibility.

Structural elements, such as DNA loops and matrix attachment regions (MARs) have also been related to origin selection.

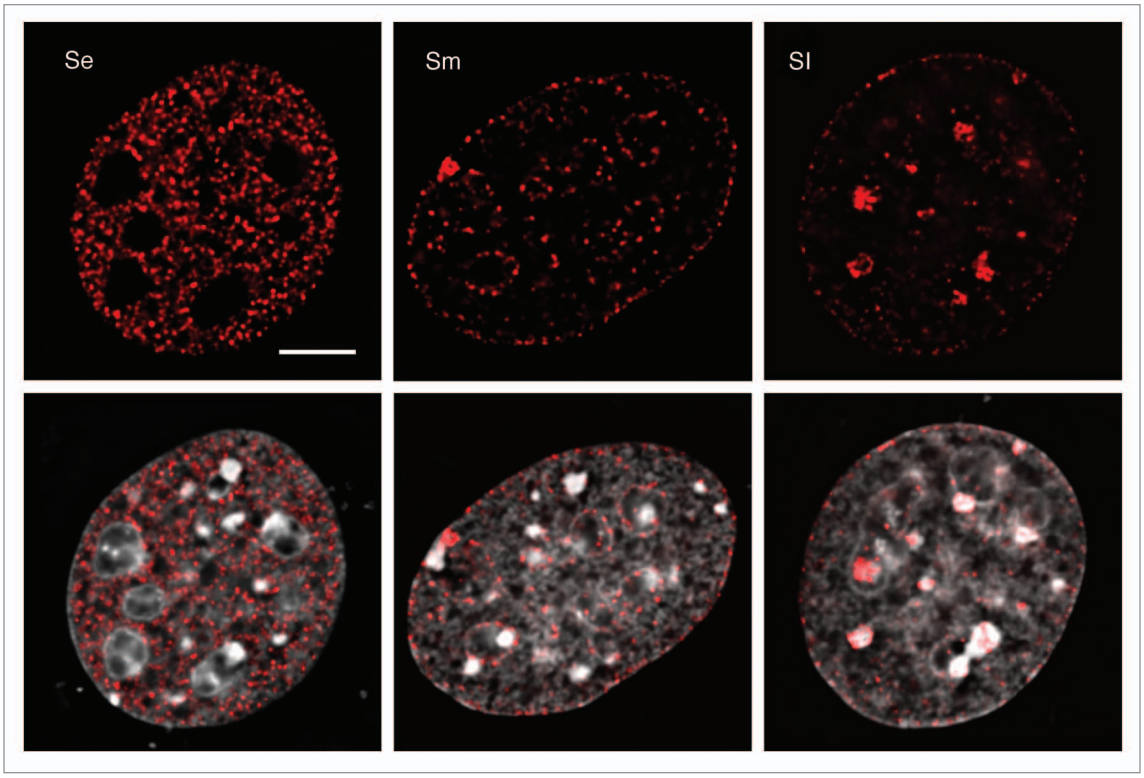

Figure 3. DNA replication follows at a global level well-conserved spatio-temporal dynamics. Here, super resolution light microscopy images of three cells exhibiting the characteristic early (Se), mid (Sm) and late (Sl) S-phase patterns are presented. Sites of nascent DNA were visualized by short pulse labeling with modified nucleotides and chemical detection of incorporated nucleotides after fixation (red). The bottom row shows an overlay of the replication staining (red) and DNA staining by DAPI (gray). Scale bar: 5 μm.

In fact, some studies propose that the so-called MARs correspond to replication origins that can cluster to form replication foci and that the size of the inter-MAR loops reflect the replicons size,[86,87] described to range between 20 and 200 kb.[88] MARs are often found at the 5'-end from genes[89] and are binding sites for topoisomerase II.[90] Studies showing that MAR-associated sequences are enriched in replication intermediates,[91] that the addition of MAR sequences to plasmids enhances episomal replication,[92,93] as well as the fact that MARs are AT-rich[94] and have therefore unique DNA unwinding properties, all lead to the proposal that indeed a link between MARs and replication origin is possible. MARs could link several replication origins together, promoting their clustering and facilitating their coordinated firing.[95]

Interestingly, the longer inter-origin distances in differentiated mammalian nuclei (around 100 kb) can be reprogrammed in Xenopus egg extract to shorter, embryonic-like, inter-origin distances (15 kb) when conditioned in mitotic egg extracts.[96] This redistribution of replication origins is concomitant to a remodeling of loop size, both of which are topoisomerase II dependent events and correlate with a redistribution of ORC.[97] These observations point to the strong relation between chromatin architecture and the regulation of DNA replication.

A different set of studies led to the proposal that there is a correlation between transcription sites and replication origins. For instance, the presence of a promoter or transcription factors can affect replication origin localization and activation in different systems.[98-102] This might happen by recruiting chromatin remodeling complexes or histone modifying complexes, or it might be caused by direct interactions between transcription factors and pre-RC components.[103] For instance, in Drosophila the ORC is associated with RNA Pol II binding sites,[104] although so far no direct interaction between pre-RC components and transcription factors has been shown convincingly.

Nevertheless, the correlation between sites of active transcription and replication origins is not always a positive one: active transcription in a gene silences origins inside that gene[105-107] or reduces the size of the initiation zone and abolishing transcription by deletions in the promoter region allows the body of the gene to become a template for initiation.[99,108,109] While in some organisms transcriptionally active genes have more efficient origins,[13,85,110] this is not always the case.[84,111] Studies showing a clear segregation between sites of active replication and active transcription[112] have made it clear that transcription activity per se is not a requisite for replication. It has, therefore, been suggested that transcription permissiveness or the chromatin structure that corresponds to it, rather than transcription itself, might facilitate origin activity.[99,108]

In general, open chromatin is considered to be a better substrate for both transcription and replication initiation: replication origins are usually enriched in open chromatin structures.[113-115] Transcriptionally active promoters are usually histone H3/H4 hyperacetylated,[116] resulting in an open chromatin conformation

and making such regions into favorable substrates for DNA replication. Nucleosome positioning has also been involved in origin activity. Although only shown in yeast, ORC might facilitate pre-RC formation by influencing nucleosome positioning[79] and placing a nucleosome at DNA replication origin inhibits initiation.[77,78] In Xenopus development, the massive rearrangement of replication dynamics that takes place during the MBT is accompanied by histone H1 incorporation, which results in inhibition of pre-RC formation.[117] A recent study in human cells proposes H4K20m1 to regulate replication origin firing and shows that deficient degradation of PR-Set7, the enzyme responsible for H4K20m1, and the resulting abnormally high levels of H4K20m1 at origins, caused significant re-replication.[118]

In both, Drosophila as well as in Xenopus, histone acetylation seems to play a role in defining origins of replication.[99,119] Supporting the positive role of histone acetylation levels on replication in human cells, Cdt1-mediated recruitment of the human HBO1, before the onset of S-phase, plays a role in replication by increasing H4 acetylation, chromatin decondensation and subsequently enhancing MCM recruitment.[120] Indeed, HBO1 knockdown results in a decrease in DNA synthesis and affects progression through S-phase. Importantly, it is the acetylating activity of HBO1 that was necessary for MCM recruitment.[30,121] This effect is counteracted by HDAC11, another partner of Cdt1, which is active during S-phase, prevents MCM recruitment and thereby avoids re-replication.[120]

Nevertheless, transcription and the associated open chromatin have been proposed to correlate better with early replication timing rather than replication activity itself. In fact, early origins correlate with actively transcribed genes, while late origins are located in non-transcribed regions.[3,122,123]

Determining DNA replication timing. In *S. cerevisiae*, the time of origin firing is, just like origins themselves, determined to a great extend genetically: the order in which the pre-RCs are activated is determined by proximal cis-acting chromosomal elements, telomeres and other DNA sequences for subtelomeric and non-telomeric late-firing origins.[124,125] In *S. pombe*, the definition of early and late replication origins is less clear. However, some genetic elements have been identified that seem to enforce late replication[126] and relocation of an inefficient origin to the early replicating segment leads to earlier replication timing.[127]

Considering the extent of the differences in the epigenetic constitution of unicellular and higher eukaryotes, already obvious in the definition itself of replication origins, observations on replication timing made in yeast cannot be directly transferred to higher organisms. The same is true for lower metazoan, such as Drosophila, an important model organism, however lacking major epigenetic modifications found in mammals. Therefore, the (epi-)genetic elements proposed to play a role in yeast and Drosophila replication timing are summarized in **Table 1**, but will not be further discussed here.

Just like the process of origin selection itself, the determination of replication timing in multicellular organisms is a dynamic process, regulated by developmental and tissue-specific signals.[13,128,129] The rearrangement of replication dynamics undergone by Xenopus embryos during the MBT (see above) is a clear example of this flexibility: before the MBT all origins fire synchronously, so there is no temporal discrimination. After the MBT, with the increase in S-phase length, some origins start firing later than others. In mammals, at least 20% of the genome undergoes changes in replication timing during directed differentiation of ES cells to neural progenitor cells.[84] These examples of developmental regulation demonstrate, similarly as for origin selection, that replication dynamics cannot be defined at a sequence level and suggests chromatin structure to play a role in the regulation of replication timing.

The notion of replication timing being controlled by a mechanism beyond the level of DNA sequence first appeared in the 1960s, when it was observed that in female mammalian cells, one of the X chromosomes is randomly inactivated and replicates with drastically different dynamics than its active homolog, clearly showing that genetics alone cannot determine replication timing.

Unlike in yeast, in metazoan there is some correlation between transcription, early replication and open chromatin structure. Already decades ago, the correlation between replication and Giemsa banding patterns was recognized: the usually actively transcribed R-bands replicate early, while gene-poor G-bands are late replicating.[68,130] But early replication is not a straightforward consequence of transcriptional activity.[11] Changes in replication timing are not directly influenced by transcription or influence transcription but rather result from a level of higher-order organization of the genome, which in turn affects transcription competence.[11,131] For instance, in human erythrocytes, the 100 kb β-globin gene cluster is active, early replicating and histone hyperacetylated. In non-erythrocytes, β-globin is inactive, late replicating and histone hypoacetylated. Tethering a histone deacetylase (HDAC) to the active promoter causes a shift to late replication. On the other hand, targeting a HAT to the inactive promoter results in advanced replication timing. Importantly, this happens without affecting transcription.[132]

As a result of these observations, it has been proposed that the open chromatin structure that permits active transcription is most likely involved in determining replication timing by turning specific chromatin domains into favorable substrates for DNA replication. This might be a consequence of increased chromatin accessibility to initiation factors resulting, for instance, in preferential ORC binding,[104] or other downstream initiation factors. Consequently, reasonable candidates to control replication timing are epigenetic modifications defining chromatin constitution.

Epigenetics and DNA Replication Timing in Mammals

Orchestrating genome duplication. Even though epigenetic mechanisms are generally accepted to play a role in determining replication timing, it has proven a difficult task to elucidate which of the many candidates really play a direct role in this process. Especially in higher eukaryotes, which exhibit a more complex epigenetic constitution, such studies have been complicated by the fact that epigenetic modifications have a high cross-talk and often act synergistically. The search for the epigenetic

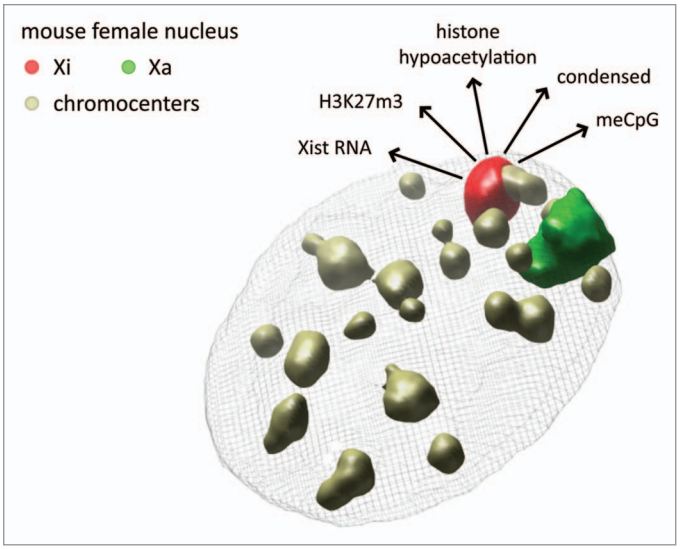

Figure 4. Epigenetics of heterochromatin. 3D-reconstruction of a female mouse fibroblast nucleus showing the active and inactive X chromosomes (Xa in green and Xi in red, respectively) and the clusters of pericentric heterochromatin (chromocenters, beige). The epigenetic markers characteristic for the prominent facultative (Xi) heterochromatic regions are annotated.

determinant of replication timing has yielded a series of results that clearly show that chromatin structure plays a major role in determining the spatio-temporal organization of DNA replication. However, the actual direct mechanism responsible for such regulation is not clear.

In principle, any epigenetic modification that discriminates between euchromatic and heterochromatin regions is a potential candidate responsible for differential replication timing, ranging from DNA methylation, to histone modifications and higher order chromatin structure.

DNA methylation refers to the covalent addition of a methyl group to the C5 position of the cytosine pyrimidine ring, a modification that, in mammals, takes place preferentially at CpG dinucleotides. CpG islands at promoter regions are usually demethylated. On the other hand, CpGs in constitutive heterochromatin are characterized by high levels of DNA methylation. The same is true for promoter regions of the inactive X chromosome in female mammalian cells, as well as of imprinted genes. All of these regions have a characteristic replication pattern, differing from that of demethylated euchromatic regions. Therefore, DNA methylation would be a reasonable candidate to influence replication timing.

Nevertheless, studies on differential replication at imprinted regions have shown that treating cells with the demethylating agent 5-azacytidine did not change replication timing of imprinted foci in relation to the earlier replicating homologous regions.[133] Moreover, it has been postulated that asynchronous replication of imprinted loci is independent of DNA methylation but consistent with differential subnuclear localization.[134] Additionally, DNA methylation is not enough to promote late replication, as shown by in vitro methylated DNA inserted into specific genomic sites. Interestingly, these sequences remained early replicating even though transcription was blocked.[135]

In contrast, in mouse F9 teratocarcinoma cells the heterochromatic major satellite repeats are abnormally demethylated and replicate earlier. Moreover, treating RAG fibroblasts with 5-azacytidine causes demethylation of major satellites and subsequently earlier replication.[136] Furthermore, a study on the replication of the inactive X chromosome has shown that its methylated CpG islands replicated later than the unmethylated ones on the active homolog.[137] However, the mechanisms maintaining the silenced state of the Xi is composed of many layers of partially redundant epigenetic mechanisms[138] and it is therefore difficult to ascertain whether the delayed replication timing is actually a consequence of higher DNA methylation. All in all, these studies make it difficult to clearly state the role of DNA methylation in replication timing.

Hyperacetylation by trichostatin A (TSA) is, unlike DNA demethylation by 5-azacytidine, able to change replication timing of imprinted regions, causing the late replicating loci to replicate earlier when compared with the homologous active regions.[133] Similarly, late replicating constitutive heterochromatin was recently shown to become earlier replicating upon hyperacetylation, independently of the level of DNA methylation.[139] Indeed, histone acetylation has been proposed to possibly be the best candidate to determine replication timing[122] since high levels of histone acetylation correlate with euchromatin, transcriptional activity and an open chromatin structure.

A study on the tandem ribosomal genes in mouse cells (rDNA) has brought about interesting results regarding chromatin structure and replication timing:[140] 60% of rDNA is actively transcribed, located in the nucleolar interior and early replicating; 40% are inactive, prefer the nuclear periphery and replicate late. The promoter region of active rDNA is demethylated and histone hyperacetylated, while in inactive rDNA of the same region is methylated and histone deacetylated.[141] The ATP-dependent chromatin remodeling complex NoRC recruits histone modifying and DNA methylating enzymes to the rDNA promoter and functions as a scaffold, coordinating the activity of various macromolecular complexes and resulting in the formation of heterochromatin structures. Overexpression of NoRC results in silencing of active rDNA and shift from early to late, suggesting that the heterochromatinization of rDNA delays its replication timing. These results illustrate the extensive crosstalk between different mechanisms defining chromatin structure and the concomitant difficulty in discerning which one is directly responsible for the temporal regulation of DNA replication.

Altogether, these studies clearly indicate that epigenetic modifications are somehow involved in determining the replication timing of specific genomic regions. However, their apparently contradictory results show that we have but scratched the surface when it comes to the intricacy of the mammalian epigenetic network regulating replication timing.

Certain regions of the genome are particularly suited to study the effects of different epigenetic mechanisms on replication timing because of their well defined epigenetic composition (**Fig. 4**), their prominent size, allowing their clear visualization and their distinctive replication timing. The inactive X chromosome (Xi) in female mammalian cells is the most prominent example of facultative heterochromatin. In the post-implantational female mammalian

embryo, random X chromosome inactivation takes place in the inner cell mass to achieve dosage compensation between male and female cells. This process results in an almost entirely silenced chromosome.[142] The first known step of this process is the expression of the Xi-specific transcript (*Xist*), which results in accumulation of the non-coding Xist RNA on what is to become the inactive territory.[143] Strikingly, the Xi is genetically equivalent to the active homolog (Xa) and therefore, it is clear that the inactivated state has to be established and maintained by epigenetic mechanisms. The hallmark epigenetic modifications that accumulate on the Xi following Xist RNA coating include accumulation of histone H3K27m3, H4K20m1, histone hypoacetylation, incorporation of the macroH2A histone variant and DNA methylation at promoter regions, as well as structural and topological changes.[144-146] Additionally, the Xi has been described, decades ago, to exhibit particular replication dynamics that clearly differ from those of the Xa and the autosomes.[147,148] Hence, the Xi is a particularly interesting example of epigenetic regulation of replication timing. Interesting, the process of facultative heterochromatinization undergone by the Xi and the concomitant changes in replication dynamics, have been proposed to be the most prominent example of what actually happens in many, less prominent, regions of the genome, making the study of the Xi replication dynamics even more relevant. Recently, extensive time lapse analyses indicated that the inactive chromosome replicates in a synchronous mode in early-mid S-phase,[149] and not late as commonly assumed. Making use of a combination of drug treatment and genetic manipulations targeting all the epigenetic hallmarks of the inactive X, it was shown that the level of histone acetylation controls the replication dynamics of the Xi.[149] Interestingly, this is reminiscent of the replication mode of the genome of flies and frogs, before the onset of embryonic transcription.[12,83,150] Fast genome replication is achieved by the simultaneous firing of all available origins, which is likely feasible by the absence of competing transcriptional processes.

Studying DNA replication in vivo and in real time. In the last years, high-throughput and whole-genome methods based on the analysis of bulk DNA, such as ChIPseq (high throughput sequencing of immuno-precipitated chromatin) and genomic tiling arrays, have increased our insights into the organization of the eukaryotic genome replication immensely.[59,151,152] Thanks to these studies we have a much more complete view on the distribution of replication origins throughout the genome, as well as on their correlation with transcriptional activity, promoter regions, histone modifications and more. Such studies have also provided valuable insights on how these parameters change in development, as well as on the degree of conservation/difference between species and even cell lines.[11,111] On the other hand, single cell based ex vivo methods, such as single DNA/chromatin fiber analysis,[153,154] which do not average out biological variation in a population, have provided important information on the one dimensional distribution of replication sites, as well as on the dynamics of the replication fork.

However, key aspects of the replication program still remain unresolved, in part because most of these methods are intrinsically based on the analysis of populations of cells and on fixed cell or ex situ work. The observations and interpretations of such results therefore represent what the bulk of the cells are doing and for the most part do not show what individual cells may be doing.[155] For instance, a peak in a replication profile shows the location of an origin initiation at that chromosomal location, but it does not give any information on whether the origin fired in all cells or only a subset of them. Another important shortcoming of high throughput sequencing methods is their limitation when it comes to the analysis of repetitive sequences. Other parameters extracted from bulk DNA analysis, such as the replication half time (the time at which an origin has fired in half of the cells in a population), extracted from 2D gel analyses, is a composite value reflecting those cells in the population that actually fire the origin and those in which the origin was replicated passively by an incoming fork. This kind of data cannot differentiate whether an origin fires early but inefficiently or late but efficiently. Such methods are further limited in the extent to which they allow conclusions on the dynamics of a process that is regulated both spatially and temporally. For instance, in the case of DNA or chromatin fiber analyses, while cells can be synchronized and a particular sequence identified, any information on the 3D higher chromatin organization is intrinsically lost.

For these reasons, in vivo studies based on single cell experiments are extremely valuable as a complement to high throughput bulk and ex vivo data. In the recent years, time-lapse microscopy has already been presented as a very promising tool in the field of DNA replication.[56,57,156] Developments in this area over the last decade have provided exciting new insights into the dynamics of DNA replication and its regulation. To really take advantage of the technical improvements that allow the observation of living cells and the visualization of the processes taking place in them over periods of up to days, the available biological tools, as well as the computational resources for data analysis, have been challenged to redefine themselves continuously. And so, advances in the field of live cell microscopy, either by improved imaging techniques, by new molecular ways of visualizing live cell processes, or by better automated and more robust ways of data analysis, are directly related to advances in our knowledge of cell biology in general and of DNA replication dynamics, in particular.

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