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REVIEW ARTICLE

Defining the replication program through the chromatin landscape

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Abstract

DNA replication is an essential cell cycle event required for the accurate and timely duplication of the chromosomes. It is essential that the genome is replicated accurately and completely within the confines of S-phase. Failure to completely copy the genome has the potential to result in catastrophic genomic instability. Replication initiates in a coordinated manner from multiple locations, termed origins of replication, distributed across each of the chromosomes. The selection of these origins of replication is a dynamic process responding to both developmental and tissue-specific signals. In this review, we explore the role of the local chromatin environment in regulating the DNA replication program at the level of origin selection and activation. Finally, there is increasing molecular evidence that the DNA replication program itself affects the chromatin landscape, suggesting that DNA replication is critical for both genetic and epigenetic inheritance.

Keywords: DNA replication; ORC; chromatin; euchromatin; heterochromatin; transcription; epigenetics; genomics

Replication overview

Every time a cell divides, it must make an accurate and complete copy of the genome. Given the size of eukaryotic genomes and the duration of S-phase, this is a remarkable feat. In order to completely copy the genome within the temporal confines of S-phase, the cell must coordinate the initiation of DNA replication from hundreds and even thousands of start sites distributed throughout each of the chromosomes. These start sites of DNA replication, called *origins of replication*, are coordinately activated to ensure the rapid and precise duplication of the genome. The selection and activation of origins of replication are regulated by a complex code consisting of primary sequence, multiple activating kinases, transcriptional activity and local chromatin environment. In this review, we explore the role of the chromatin landscape in defining the DNA replication program.

In the budding yeast, *S. cerevisiae*, origins of replication were first identified as short autonomously replicating sequences (ARS) which were essential for the inheritance of an extrachromosomal plasmid (Stinchcomb et al., 1979). Genetic dissection of the sequence elements necessary for plasmid maintenance identified multiple short sequence elements (10–12 bp) that contributed to origin function (Marahrens and Stillman, 1992; Huang and Kowalski, 1996). One of these sequence elements was conserved between different origins and was termed the *ARS consensus sequence* (ACS; Van Houten and Newlon, 1990). The ACS is a degenerate T-rich motif that is necessary, but not sufficient, for DNA replication (Celniker et al., 1984; Breier et al., 2004).

The identification and analysis of metazoan origins has been hampered, in part, by genome size and lack of robust plasmid-based assays for screening potential origin sequences (Gilbert and Cohen, 1989). Unlike the conserved ACS sequence observed in *S. cerevisiae*, replication initiation in *S. pombe* (Clyne and Kelly, 1995; Dubey et al., 1996; Kim and Huberman, 1998; Segurado et al., 2003; Dai et al., 2005) and metazoans (Mechali and Kearsey, 1984; Krysan and Calos, 1991; MacAlpine et al., 2010) does not appear to require a defined consensus sequence. Despite this apparent lack of sequence specificity, several origins of replication have been identified from higher eukaryotes and two classes of origins have

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emerged. The first class initiates replication from specific locations in the genome and includes the *Drosophila* chorion locus (Orr-Weaver et al., 1989; Lu and Tower, 1997), human lamin B2 origin (Abdurashidova et al., 2000; Paixão et al., 2004) and the human β-globin locus (Kitsberg et al., 1993; Wang et al., 2004). In contrast, the second class of origins exhibit multiple initiation events distributed over broad initiation zones as observed in the Chinese hamster dihydrofolate reductase (*DHFR*) locus (Vaughn et al., 1990). The lack of a clear consensus sequence and the presence of relatively large initiation zones in metazoans compared to the sequence-specific replication origins in *S. cerevisiae* suggest that metazoan origin selection may be influenced by other chromosomal features besides primary sequence.

Despite the differences in sequence elements that define replication origins in yeast and metazoans, the protein factors required for the initiation of DNA replication are conserved in all eukaryotes. The origin recognition complex (ORC) is an essential heterohexameric protein complex required for the initiation of DNA replication (Bell and Dutta, 2002). ORC was initially identified and purified from *S. cerevisiae* as a biochemical activity that bound and protected the ACS from digestion with DNase I (Bell and Stillman, 1992). In contrast, ORC purified from higher eukaryotes lacks sequence specificity *in vitro* (Vashee et al., 2003; Remus et al., 2004). Despite this apparent lack of sequence specificity, ORC does localize to specific loci in the genomes of higher eukaryotes (Austin et al., 1999; Bielinsky et al., 2001; Ladenburger et al., 2002; Zellner et al., 2007; Karnani et al., 2010; MacAlpine et al., 2010). It is unclear what additional factors or chromosomal features target ORC to specific locations in the genome.

ORC marks potential origins of DNA replication in the genome and, in cooperation with Cdc6 and Cdt1, loads the replicative Mcm2-7 helicase complex onto the DNA through reiterative rounds of ATP-hydrolysis (Bowers et al., 2004) to form the pre-replicative complex (pre-RC; Figure 1). The assembly of the pre-RC at potential origins of replication is strictly limited to the G1 phase of the cell cycle. Origins of replication are subsequently activated in S-phase by cyclin and Dbf4-dependent kinase (CDK and DDK) activities (Sclafani and Holzen, 2007; Labib, 2010). Multiple phosphorylation events and at least six additional factors and complexes, including Dpb11, GINS, Cdc45, Sld2, Sld3 and Mcm10, lead to helicase activation, DNA unwinding and polymerase recruitment (Sclafani and Holzen, 2007; Labib, 2010). The separation of pre-RC assembly and the subsequent activation of replication origins into discrete phases of the cell cycle provides an exquisite solution to replicating the genome once and only once per cell cycle. By strictly limiting pre-RC assembly to G1, it is impossible for sequences undergoing DNA synthesis to reassemble the pre-RC onto recently synthesized nascent DNA.

In higher eukaryotes, DNA replication is a dynamic process responding to developmental and tissue-specific requirements. For example, during early embryogenesis, S-phase is only a matter of minutes and requires a higher density of replication origins than in differentiated cells (Blumenthal et al., 1974; Hyrien et al., 1995). There are also specific changes in the replication program that occur during differentiation. These changes include a change in origin activity during ES cell differentiation (Hiratani et al., 2008; Desprat et al., 2009), activation of developmentally regulated origins in the immunoglobulin heavy-chain locus during B-cell development (Zhou et al., 2002; Norio et al., 2005) and a change in replication timing at the human β-globin locus during erythroid differentiation (Epner et al., 1988). The ability to remodel the selection and activation of replication origins in response to developmental cues is likely critical to ensure that developmental and tissue-specific transcription programs are maintained while faithfully duplicating the genome. This coordination is undoubtedly regulated at the level of the local chromatin environment.

Chromatin overview

The basic organizing unit of the genome is the nucleosome –147 bp of DNA wrapped 1.7 times around a core histone octamer containing two copies of each of the core histones—H2A, H2B, H3 and H4. Incorporation of the DNA

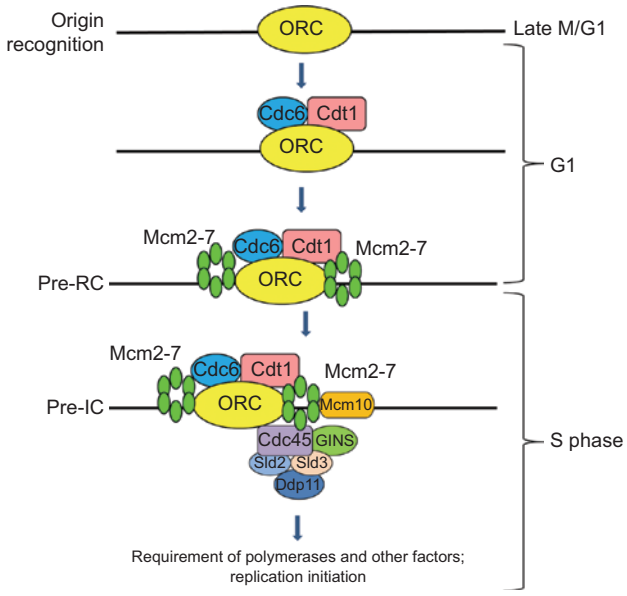

Figure 1. The selection of potential replication origins begins with the binding of the origin recognition complex (ORC), which occurs between late M phase and early G1 phase. ORC, together with Cdc6 and Cdt1 recruit the minichromosome maintenance proteins (Mcm2-7) to the origin during G1 phase, forming the pre-replicative complex (pre-RC). This recruitment of Mcm2-7 in effect licenses the origin for subsequent activation. When the cell enters S phase, cyclin and Dbf4-dependent kinase activities (CDK and DDK) facilitate the assembly of additional factors to the pre-RC, forming the pre-initiation complex (pre-IC) and ultimately leading to the recruitment of DNA polymerases and replication initiation.

into nucleosomes facilitates the packaging and compaction of the genome into the confines of the nucleus. At the most accessible level, the DNA is organized into a 10 nm fiber often referred to as "beads on a string" with the nucleosomes (beads) interspersed by linker DNA (string) and is thought to be enriched for sequences undergoing active transcription. The remainder of the genome is packaged into 30 nm fibers representing higher order structures of nucleosome compaction facilitated by histone H1. Finally, the 30 nm chromatin fibers are organized into domains and loops which are further compacted during metaphase (Razin et al., 2007).

The covalent modification of histone tails provides for a massively combinatorial "histone code" that regulates accessibility to the DNA and recruitment of trans-acting factors (Jenuwein and Allis, 2001). The numerous lysine residues on the nonconserved histone tails may be acetylated, methylated, ubiquitinylated, sumoylated and ribosylated, in addition to the phosphorylation on serine and threonine resdiues (Kouzarides, 2007). Adding to the complexity, there are also noncanonical histone variants such as H2A.x, H3.3, CENP-A, which are specific to chromatin undergoing repair, nucleosome exchange (outside of S-phase) and the centromere, respectively (Malik and Henikoff, 2003).

Chromatin exists in two distinct states: the euchromatin and heterochromatin (Woodcock and Ghosh, 2010). The majority of genes reside in euchromatin which is decondensed during interphase. Regulatory elements and gene bodies are often marked by "activating" chromatin marks that frequently include histone acetylation, methylation and ubiquitinylation (Kouzarides, 2007). In contrast, the heterochromatin is more compacted and has a lower density of genes, many of which often exhibit repressed transcription. "Repressive" histone modifications such as H3K9me and H3K27me are associated with heterochromatin and silenced gene expression (Nakayama et al., 2001; Kouzarides, 2007). Understanding the depth and breadth of the histone code and how it regulates biological processes is an ongoing challenge.

Histone modifications are critical for the expression and maintenance of epigenetic information. Moreover, the positioning and phasing of nucleosomes at regulatory elements is highly correlated with these elements' function. For example, transcription units typically have phased nucleosomes in the gene body and a nucleosome free region (NFR) immediately upstream of the transcription start site (TSS; Jiang and Pugh, 2009). Both sequence elements and transacting factors contribute to defining the NFR which is thought to permit assembly and recruitment of the transcription machinery at the promoter. In addition, nucleosome phasing is critical for protein binding and discriminating between numerous motif instances in the genome (Liu et al., 2006).

Currently, very little is known about the chromatin features and elements that direct and regulate the DNA replication program. The organization and positioning of nucleosomes, the multitude of histone tail modifications, incorporation of histone variants in the nucleosome and the recruitment of additional trans-acting factors impact all nuclear DNA-templated processes including recombination, repair, transcription and replication. Much of the research in the last two decades has focused on understanding the mechanisms by which chromatin affects and regulates the transcription of genetic information. Considerably less is known about how the DNA replication program is regulated by chromatin, but it is becoming increasingly clear that the DNA replication program responds to many of the same chromatin cues as does the transcription program.

Origin selection

The selection of potential origins of replication in S-phase begins in G1 with the assembly of the pre-RC complex on the DNA. Most of our understanding of how potential origins of replication are selected is derived from studies in *S. cerevisiae* where ORC binding and pre-RC assembly occurs at well-defined locations in the genome. Genetic and biochemical experiments have identified the cis-acting ACS (ARS consensus sequence) which is necessary for ORC binding and origin function (Newlon, 1988). However, the ACS is clearly not sufficient as there are potentially tens of thousands of matches to this degenerate T-rich sequence in the yeast genome (Breier et al., 2004). Yet, despite thousands of potential sequence matches, only 300–350 sites in the genome are occupied by ORC and function as origins of replication (Wyrick et al., 2001; Xu et al., 2006). In higher eukaryotes, ORC lacks apparent sequence specificity *in vitro* (Vashee et al., 2003; Remus et al., 2004), yet still localizes to specific genomic sites *in vivo* (Austin et al., 1999; Bielinsky et al., 2001; Ladenburger et al., 2002; Zellner et al., 2007; Karnani et al., 2010; MacAlpine et al., 2010), suggesting that origins of replication are not specified by sequences alone. Recent studies have implicated nucleosome positioning and histone modification as specificity determinants of origin selection.

Nucleosome positioning

A simple hypothesis to explain the observed specificity in ORC localization is that not all sequences are equally accessible and that chromatin organization (nucleosome positioning) is a key determinant of ORC localization. This hypothesis has been vigorously pursued in *S. cerevisiae*. Early nucleosome mapping experiments found that the ACS of the ARS1 origin is flanked by well-positioned nucleosomes (Thoma et al., 1984). The positioning of these flanking nucleosomes is critical for origin function. If the nucleosomes are experimentally forced to encroach into the ACS of ARS1, origin function on an episome is compromised (Simpson, 1990), presumably due to the loss of ORC binding. The nucleosome positioning observed on an ARS1-containing plasmid has also been confirmed in the chromosomal context at the endogenous ARS1 locus (Lipford and Bell, 2001). Thus, the location and position

of nucleosomes can influence ORC binding and origin function.

Recent advances in genomic technologies have made it possible to determine the location of nucleosomes throughout the genome in a variety of organisms (Yuan et al., 2005; Albert et al., 2007; Lee et al., 2007; Mavrich et al., 2008). In *S. cerevisiae* (as well as metazoans), promoters have a characteristic pattern of nucleosome occupancy. This nucleosome occupancy pattern consists of an array of well-positioned nucleosomes at the TSS progressing into the gene body and a region of low nucleosome occupancy immediately upstream of the TSS (Yuan et al., 2005; Segal et al., 2006; Jiang and Pugh, 2009). This NFR is associated with actively transcribed genes and likely contains the cis-acting binding sites for regulatory factors. Examination of nucleosome positioning at broadly mapped ARS elements in the yeast genome revealed that origins of replication were typically devoid of nucleosomes with weakly positioned nucleosomes on either side of the ARS element (Albert et al., 2007; Field et al., 2008). Close examination of the positioning of nucleosomes around a more precisely identified ACS sequence within the ARS revealed a distinct and conserved pattern of nucleosome organization at almost all origins of replication (Berbenetz et al., 2010; Eaton et al., 2010). Therefore, the well-defined nucleosome organization first described at a single locus (ARS1) is a feature common to the large majority of yeast replication origins.

The inevitable question—"which came first, the chicken or the egg", immediately arises—do origins of replication have an NFR because ORC is bound or does ORC bind because the region is nucleosome free? Although trans-acting factors clearly contribute to the distribution of nucleosomes throughout the genome (Liu et al., 2006), there are also sequence determinants which influence where a nucleosome will reside (Segal et al., 2006; Kaplan et al., 2009). The Segal group used high-throughput sequencing to map the preferred nucleosomal locations in the yeast genome in the absence of any trans-acting factors (Kaplan et al., 2009). Specifically, they reconstituted nucleosomes *in vitro* by assembling histone octamers onto purified yeast genomic DNA to identify the preferred nucleosomal positions encoded by primary sequence. In this *in vitro* derived catalog of nucleosome locations, the ACS matches, which were *bona fide* ORC binding sites, were indeed nucleosome free and those ACS matches which did not appear to be functional had an increased level of nucleosomal occupancy (Eaton et al., 2010). These data suggest that primary sequence contributes to the nucleosome occupancy and organization at ORC binding sites and is likely a determinant of ORC association. Clearly, the ACS sequence itself is not sufficient to position nucleosomes or recruit ORC *in vivo*; instead, structural cues in the primary sequence surrounding the ACS (e.g., helical stability, nucleosome positioning signals) are likely important for nucleosome positioning and ORC binding. These structural properties of the DNA are also conserved between related fungi (Nieduszynski et al., 2006).

It is not clear how ORC facilitates the precise nucleosome positioning observed at *S. cerevisiae* origins of replication. The precise positioning can be reconstituted *in vitro* with recombinant histones, ORC and an ATP-dependent chromatin remodeling activity (Eaton et al., 2010). Precisely positioned nucleosomes not only are a signature of yeast replication origins but also are required for origin activity. Displacement of the ACS proximal nucleosome further upstream results in loss of pre-RC assembly and origin activity (Lipford and Bell, 2001). Orc1 contains a conserved bromo-adjacent homology (BAH) domain (Callebaut et al., 1999) which, in the case of the Sir3 BAH domain (Onishi et al., 2007), is known to interact with histones and facilitate chromatin silencing (Zhang et al., 2002b). Direct interactions between ORC and flanking nucleosomes mediated by the BAH domain of ORC may be involved in establishing the local chromatin organization at a subset of replication origins. Consistent with this hypothesis, the deletion of the Orc1 BAH domain decreases the affinity of ORC for a subset of DNA replication origins, resulting in a disorganization of the surrounding nucleosomes (Müller et al., 2010).

The chromatin-flanking ORC binding sites is dynamic and undergoes active nucleosome turnover and replacement outside of S-phase (Rufiange et al., 2007; Kaplan et al., 2008). Newly synthesized histone H3 is acetylated on an internal lysine (K56) by the histone transferase Rtt109 prior to assembly into a nucleosome (Driscoll et al., 2007). H3K56Ac is incorporated into chromatin by both replication-dependent and independent mechanisms and serves as a marker for newly assembled nucleosomes. In the absence of the histone chaperone Asf1, which is required for assembling histone H3K56Ac into nucleosomes, the incorporation of histone H3 is decreased at origins of replication (Rufiange et al., 2007). Similarly, the analysis of H3K56Ac through the cell cycle revealed that nucleosomes flanking early origins of replication were being dynamically replaced even during G1 (Kaplan et al., 2008). It is unclear if ORC acts directly on the surrounding nucleosomes or indirectly by recruiting a chromatin remodeler or nucleosome positioning factor. In this regard, it is noteworthy that genome-wide analysis revealed an enrichment of the chromatin remodeler, Ino80, at origins and stalled replication forks (Shimada et al., 2008), suggesting that perhaps ORC and Ino80 together facilitate the precise nucleosome positioning and rapid histone exchange observed at origins of replication.

Mechanistically, origins of replication in *S. cerevisiae* appear to be established in the context of chromatin in two distinct steps (Figure 2). First, ORC binds accessible ACS motifs that are not occupied by a nucleosome. A-rich sequences downstream from the ACS may also facilitate the targeting of ORC to specific sequences or may function to keep the downstream region nucleosome

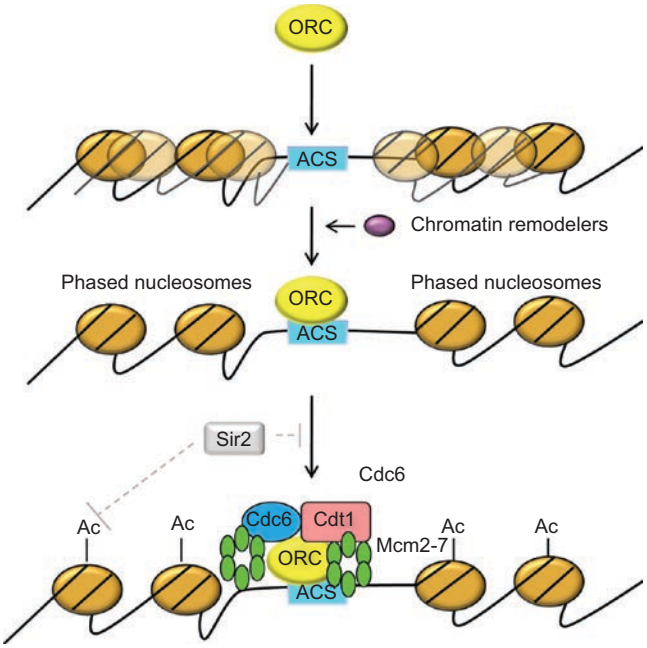

Figure 2. Chromatin organization and origin selection in S. cerevisiae. In the budding yeast, S. cerevisiae, origins of replication are defined, in part, by the ACS consensus sequence which is necessary, but not sufficient for origin selection. Primary sequences surrounding the ACS maintain a nucleosome free environment that is permissive to ORC recruitment. Upon ORC recruitment, the local nucleosomes are precisely positioned. The precise positioning of nucleosomes is dependent on both ORC and a chromatin remodelling activity and is critical for origin function. The precisely positioned nucleosomes may permit the loading of multiple Mcm2-7 complexes in G1 and contribute to origin unwinding. A subset of yeast replication origins are sensitive to the local deacetylation of chromatin mediated by Sir2.

free (Eaton et al., 2010). Following ORC binding, there is a subsequent ORC-dependent remodeling of the nucleosome organization that results in precisely positioned nucleosomes flanking the ACS (Berbenetz et al., 2010; Eaton et al., 2010). The nucleosome remodeling is presumably mediated by an ATP-dependent chromatin remodeling activity, although the specific chromatin remodeling activity utilized *in vivo* is currently unknown. The resulting precise nucleosome organization at yeast origins of replication may be critical for the subsequent loading of multiple Mcm2-7 complexes or for downstream initiation events.

Nucleosome occupancy also appears to be a determinant for ORC binding in higher eukaryotes. Studies in *Drosophila* reveal that almost two-thirds of ORC binding sites occur near the TSSs of actively expressed genes (MacAlpine et al., 2010). As in yeast, the region immediately upstream of promoters in *Drosophila* is typically free of nucleosomes (Mavrich et al., 2008; Henikoff et al., 2009). Importantly, those ORC binding sites not at promoters are also nucleosome free (MacAlpine et al., 2010), suggesting that in the absence of sequence specificity, ORC may be localizing to highly accessible regions of the genome. Recent studies in Chinese hamster cells also indicate that ORC localizes to NFRs within the *DHFR* initiation zone (Lubelsky et al., 2010).

In *Drosophila,* ORC-associated sequences reside in dynamic chromatin. ORC binding sites are enriched for the replication-independent histone variant H3.3 (MacAlpine et al., 2010). Histone H3.3 is assembled into nucleosomes outside of S-phase and marks regions of the genome undergoing active chromatin remodelling (actively expressed gene bodies, promoter regions and enhancers; Ahmad and Henikoff, 2002; Schwartz and Ahmad, 2005). Regions enriched for the histone variant H3.3 are also typically depleted for bulk nucleosomes, consistent with the dynamic nature of their occupancy (Wirbelauer et al., 2005). The Henikoff group has directly examined the genome-wide kinetics of nucleosome turnover in the fruit fly using an approach, termed CATCH-IT, to biotin label nascent histones (Deal et al., 2010). Strikingly, they observed that nucleosomes within gene bodies, surrounding regulatory elements, and ORC binding sites are undergoing active nucleosome turnover and exchange. Further investigation will be required to determine if regions of dynamic chromatin facilitate ORC binding or, alternatively, if ORC actively participates in the local remodeling of chromatin and nucleosome exchange.

Chromatin modifications and other factors

In both yeast and higher eukaryotes, DNA accessibility in the context of chromatin is important for the recruitment of ORC to specific locations in the genome. Less clear is the role of chromatin modifications and additional specificity factors (e.g. transcription factors) in recruiting ORC to the DNA or in facilitating pre-RC assembly. In metazoans, ORC frequently colocalizes near the TSSs of actively transcribed genes (Ladenburger et al., 2002; MacAlpine et al., 2004) and multiple transcription factors have been implicated in interacting with ORC and components of the pre-RC including Myc (Dominguez-Sola et al., 2007), E2F1, Rb (Bosco et al., 2001) and Myb (Beall et al., 2002). Together, these results suggest that transcription factors may act as specificity factors in recruiting ORC to the DNA.

During *Drosophila* oogenesis, ORC localizes to specific clusters of chorion genes in the polytene follicle cells surrounding the oocyte. Following the endocycles, which are regulated transitions between S and G1 without an intervening mitosis, the chorion loci undergo a developmentally programmed amplification event that is dependent on ORC and the cis-acting DNA elements, *ACE3* and *Oriβ* (Austin et al., 1999). Thus, outside of the confines of the cell cycle, the chorion loci are specifically amplified up to 60-fold over the surrounding sequence (Claycomb and Orr-Weaver, 2005). This amplification serves to increase the number of chorion gene templates and augments transcription of these loci. The transcription factors E2F1, Rb (Bosco et al., 2001) and Myb (Beall et al., 2002), all of which colocalize with ORC at

the chorion locus on chromosome 3L, are required for the proper developmentally regulated amplification. However, the role of transcription factors as specificity factors for ORC localization is still unclear as mutants in Myb still exhibit proper ORC localization at the chorion locus (Beall et al., 2002), suggesting that transcription factors may act redundantly to recruit ORC to the DNA. Alternatively, transcription factors, as part of larger complexes with transcriptional activating and repressive properties (Georlette et al., 2007), may alter the local chromatin environment and regulate downstream replication initiation events.

Genome-wide analysis of the distribution of multiple site-specific transcription factors have revealed surprising overlap in the locations occupied by these transcription factors (MacArthur et al., 2009; modENCODE Consortium, 2010). These high-occupancy (HOT) regions of the genome may either reside in a distinct chromatin environment that is promiscuous for DNA binding or there might be unexpected cooperativity in the recruitment of transcription factors to HOT regions. HOT regions share several hallmarks which may facilitate ORC binding. For example, they are frequently found near promoters of active genes, depleted for bulk nucleosomes, exhibit high nucleosome turnover and are enriched for specific activating marks. Not surprisingly, the likelihood of finding an ORC binding site increases with the number of transcription factors (or complexity) of the HOT regions (modENCODE Consortium, 2010). HOT regions may simply represent extremely accessible DNA or, alternatively, the different transcription factors may act redundantly to recruit ORC to the DNA.

Recent genome-wide origin mapping experiments in mouse and human cells have shown that origins are enriched near active promoter elements most commonly at CpG islands (Cadoret et al., 2008; Sequeira-Mendes et al., 2009). Transcription may affect not only the local chromatin environment but also the local topology of the DNA. *In vitro*, ORC binds preferentially to negatively supercoiled DNA (Remus et al., 2004) which would also be found immediately upstream of actively transcribed genes. Finally, coupling ORC localization to promoter elements may facilitate coordinating the replication program and transcription programs. For example, an origin at the TSS ensures that replication and transcription forks proceed in same direction and will minimize head-on collisions between DNA polymerase and RNA polymerase.

Actively transcribed genes often have distinct local chromatin environments. For example, promoter regions are often marked by H3K4me2 and H3K9Ac (Kouzarides, 2007). Activating chromatin marks such as histone acetylation have been clearly linked with the regulation of replication origins (see below); however, their direct role in recruiting ORC to the DNA is less clear. Perhaps, the best evidence for the role of histone acetylation in recruiting ORC to the DNA comes from experiments at the *Drosophila* chorion locus (Aggarwal and Calvi, 2004). Not surprisingly, the increased transcription of the amplified chorion locus coincides with increased histone acetylation (Aggarwal and Calvi, 2004; Hartl et al., 2007). Conversely, depletion of Rpd3, a histone deacetylase, results in a global increase in histone acetylation levels, the initiation of nonspecific replication throughout the genome and a loss of ORC localization at the chorion locus (Aggarwal and Calvi, 2004). Presumably, in the absence of Rpd3, many more sites become acetylated and can compete for ORC localization.

Segmentation of the *Drosophila* genome into distinct chromatin states based on either chromatin-binding proteins (Filion et al., 2010) or histone modifications (Kharchenko et al., 2010; modENCODE Consortium, 2010) revealed that ORC is enriched in distinct chromatin environments. Filion and colleagues identified five chromatin states, which they named after five colors. ORC was found to be enriched in "RED" chromatin, one of the two active euchromatin states. The enrichment of ORC in active "RED" chromatin is perhaps not surprising, given that ORC is enriched in dynamic and accessible chromatin (Deal et al., 2010) and that the hallmarks of "RED" chromatin include the chomatin remodelling factor Caf-1 and GAGA factor, both of which facilitate nucleosome dynamics and turnover (Bulger et al., 1995; Petesch and Lis, 2008). Kharchenko and colleagues (Kharchenko et al., 2010; modENCODE Consortium, 2010) segmented the genome into 9- and 30-state chromatin models using histone modification data and found ORC enriched in a subset of states associated with TSSs and highly enriched for nucleosome turnover. Future experiments will be required to demonstrate a causal role for these chromatin environments in the selection and regulation of replication origins.

Surprisingly, there is little direct data demonstrating a role for specific chromatin modifications in recruiting ORC to potential origins of replication. However, recent experiments in both yeast (Pappas et al., 2004) and mammalian (Miotto and Struhl, 2010; Tardat et al., 2010) systems have shown that the local chromatin environment at origins of replication can impact the assembly of the pre-RC complex. These experiments are exciting because they indicate an additional control mechanism for establishing replication origins downstream of ORC binding. Specifically, these experiments suggest that the recruitment of ORC may not be sufficient for pre-RC assembly and instead, the local chromatin environment may be critical for helicase loading.

The NAD+ dependent histone deacetylase, Sir2, is required for the establishment and maintenance of silenced heterochromatin in the yeast genome (Rusche and Lynch, 2009). Unexpectedly, Sir2 also has a negative role in regulating pre-RC formation at select origins of replication (Pappas et al., 2004). Mutations that inactivate the deacetylase activity of Sir2 rescue a temperature-sensitive mutant allele of CDC6, cdc6-4. Crampton and colleagues individually screened origins from two yeast chromosomes for sensitivity to a Sir2

mutant (Crampton et al., 2008). They found that ~20% of the origins examined were sensitive to Sir2. This sensitivity to Sir2 was not dependent on chromatin silencing as Sir3 and Sir4 mutants did not affect the origins of replication. Sir2 activity appears to directly inhibit pre-RC formation at sensitive origins. For example, Mcm2-7 loading is blocked in cdc6-4 mutants at the nonpermissive temperature, but in combination with a Sir2 mutant, Mcm2-7 loading is restored only at those sensitive origins. This effect is mediated by histone acetlyation as a histone H4 tail mutant that mimics acetylation on K16 via a glutamine residue (H4K16Q) is also able to rescue the cdc6-4 mutant. Thus, pre-RC assembly at a subset of yeast origins depends on the local acetylation of H4K16, which is negatively regulated by Sir2 activity. Only a subset of origins are sensitive to Sir2, suggesting that the local chromatin acetylation state is critical for pre-RC formation. Perhaps, the hypoacetylation of local nucleosomes impairs the precise nucleosome positioning required for helicase loading (Lipford and Bell, 2001) and that due to the complexity of nucleosomal positioning cues (sequences, trans-acting factors, topology, etc.) different origins are differentially affected.

The histone acetyltransferase Hbo1 (histone acetylase binding to Orc1) was initially identified as a histone acetyltransferase (HAT) activity that interacts with multiple components of the pre-RC including Orc1, Mcm2 and Cdt1 (Burke et al., 2001; Iizuka et al., 2006). Hbo1 is the predominant HAT responsible for the bulk of histone H4 acetylation in the genome (Doyon et al., 2006). Although Hbo1 interacts with Orc1 and Mcm2, it is recruited to origins of replication in a cell-cycle dependent manner via its interaction with Cdt1 (Miotto and Struhl, 2010).

Recent studies suggest the involvement of Hbo1 in pre-RC formation at origins of replication. Depletion of Hbo1 results in an impaired S-phase (Doyon et al., 2006; presumably due to decreased origin function), and in *Xenopus* extracts, Hbo1 is required for pre-RC formation (Iizuka et al., 2006). Although in a more reconstituted *Xenopus* system, Hbo1 was not necessary for pre-RC formation (Gillespie et al., 2001). Recent experiments by Miotto and colleagues demonstrate that recruitment of a catalytically inactive Hbo1 mutant to an origin of replication impairs Mcm2-7 loading (Miotto and Struhl, 2010). *In vitro*, Hbo1 can acetylate a variety of substrates including histone H4 as well as multiple members of the pre-RC including Orc2, Mcm2 and Cdc6 (Iizuka et al., 2006); thus, it is unclear whether histone H4 acetylation or perhaps protein acetylation promotes pre-RC formation. In support of Hbo1's acetyltransferase activity being directed at histones, the relative abundance of H4 acetylation at origins of replication is specifically decreased at a few select origins. Also, the tethering of Hbo1 (Chameaue) to *Drosophila* origins stimulates origin activity at the chorion locus (Aggarwal and Calvi, 2004); however, it is unclear if this stimulation of replication activity is mediated by increased pre-RC assembly or increased activation of the origin.

It will be interesting to see if Hbo1 facilitates origin selection throughout the genome or if its effects are limited to specific origins of replication as is the case with Sir2 which impacts less than 20% of the origins (Crampton et al., 2008). The ability to modulate histone acetylation levels at specific origins may represent a rheostat that is used to fine tune origin function in cooperation with the transcription program. The acetylation of neighboring histones likely facilitates histone exchange (a common property at origins) and perhaps the unwinding of the DNA in preparation for initiation.

Set8 (PR-Set7) is a cell-cycle regulated histone methyltransferase that catalyzes the monomethylation of histone H4 on lysine 20 (H4K20me1; Fang et al., 2002; Nishioka et al., 2002). The degradation of Set8 during S-phase is dependent on PCNA and mediated by the E3 ubiqutitin ligase $CRL4^{cdt2}$ (Abbas et al., 2010; Centore et al., 2010; Oda et al., 2010). Loss of Set8 has myriad of phenotypes including chromosome decondensation, delayed S-phase progression, G2 arrest, centrosome amplification and DNA damage (Karachentsev et al., 2005; Jorgensen et al., 2007; Houston et al., 2008; Oda et al., 2009; Tardat et al., 2010). Interestingly, stabilization of Set8 results in premature chromatin compaction and a checkpoint-mediated G2 arrest (Abbas et al., 2010; Centore et al., 2010). Tardat and colleagues further demonstrated that stabilization of Set8 results in re-replication which is presumably mediated by the reassembly of the pre-RC at H4K20me1 marked origins of replication (Tardat et al., 2010). Tethering of Set8 to a specific genomic locus resulted in an increase in H4K20me1 and recruitment of pre-RC components. These results suggest that the monomethylation of H4K20 is conducive to pre-RC loading and origin function and imply that the tight cell-cycle regulation of Set8 may represent an additional control mechanism to ensure genomic stability.

Despite the differences in ORC specificity for conserved sequence elements between yeast and higher eukaryotes, the chromatin organization is remarkably similar (Figure 3). ORC frequently colocalizes in the open and accessible chromatin upstream of TSSs. Transcription factors, chromatin remodeling activities and histone acetylation likely contribute to maintaining open chromatin. A signature of ORC-associated sequences is the dynamic turnover of nucleosomes at the ORC binding sites (Henikoff et al., 2009; Deal et al., 2010), although it is unclear whether this turnover is dependent on or facilitates ORC binding. Finally, the assembly of the pre-RC at specific origins may be mediated by the HAT, Hbo1, which interacts with multiple pre-RC components (Burke et al., 2001; Iizuka et al., 2006).

Origin activation and regulation

Classic experiments examining the patterns of tritiated thymidine incorporation on autoradiograms from metaphase chromosome spreads revealed that DNA synthesis is not uniform throughout the genome during S-phase.

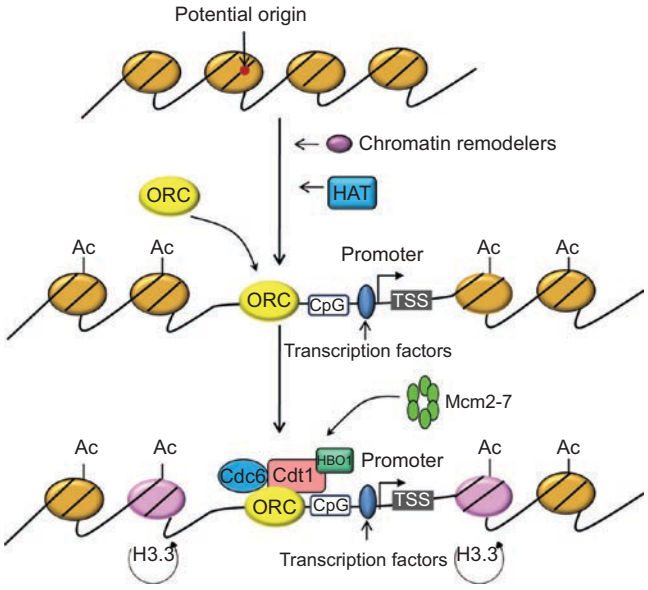

Figure 3. Origin selection in higher eukaryotes. Replication origins in higher eukaryotes lack an apparent consensus sequence, yet still map to specific locations in the genome. Despite the lack of sequence specificity, metazoan origins share several common characteristics, including: open chromatin, local histone acetylation, and proximity to transcription start sites and CpG islands. The recruitment of transcription factors, chromatin remodeling activities (to create a nucleosome free region) and the local hyperacetylation of surrounding nucleosomes likely promote ORC localization and pre-RC assembly. The nucleosomes flanking origins of replication (in pink) are dynamic and undergo nucleosome exchange throughout the cell cycle. The histone acetyltransferase Hbo1 interacts with Cdt1 and promotes recruitment of the Mcm2-7 complex to form the pre-RC, presumably via the local acetylation of histone H4.

Instead, discrete chromosomal domains were replicated at specific times (Goldman et al., 1984). Condensed chromatin, associated with a lack of gene expression, replicates late during S-phase, whereas the transcriptionally active euchromatin replicates early (Hsu et al., 1964; Lyon, 1968; Stambrook and Flickinger, 1970). These results suggest that the molecular properties of heterochromatin that are sufficient to repress transcription also impact the synthesis of DNA.

Replication timing and transcription

Recent technological advances and genome-wide approaches have refined the broad brush strokes of labeling mitotic chromosomes with tritium and provide a much higher resolution view of when a specific sequence is copied during S-phase. These genome-wide surveys of replication timing and origin usage in higher eukaryotes have demonstrated a clear correlation between transcriptional activity and replication timing in *Drosophila* (Schübeler et al., 2002; MacAlpine et al., 2004; Schwaiger et al., 2009), human (Ryba et al., 2010) and mouse cells (Farkash-Amar et al., 2008; Hiratani et al., 2008, 2010).

These studies have found that transcriptionally active regions of the genome are typically replicated before regions that are devoid of active genes. However, the correlation between replication timing and transcription is not at the level of individual genes but rather integrated over the transcriptional status of many genes in large domains. For example, in mammalian cells, the genome is partitioned into early- and late-replicating domains ranging from hundreds of kilobases to megabases (Hiratani et al., 2008). Although each early-replicating domain may be enriched for actively transcribed genes, there will also undoubtedly be inactive genes. Thus, it is impossible to predict the replication timing of a single gene based on its transcriptional status. However, if the replication timing for a chromosomal domain is known, it is possible to predict the likely expression status of genes in that domain. It should also be noted that the transcriptional activity of large genes such as the mouse β-globin locus (~3 Mb) can clearly influence the chromatin state of large domains and promote replication initiation (Norio et al., 2005). These results suggest that replication timing is more related to local chromatin structure and accessibility rather than the transcription of a specific gene. Finally, given the large size of the early- and late-replicating domains, it would suggest that multiple origins of replication are responding to, in a coordinated manner, the local chromatin environment.

Unlike in metazoans, no clear correlation has emerged linking time of replication with transcriptional activity in *S. cerevisiae* (Raghuraman et al., 2001). Although recent mathematical studies have derived a link between replication and transcription that appears to be significant at least at few loci (Omberg et al., 2007; Omberg et al., 2009), the link between replication and transcriptional activity is not clear at the genome-wide level. The compact genomes and the presence of well-defined origins in yeast may not require the developmental plasticity of mammalian origins of replication and hence there exists less cross talk with the transcription program.

Chromatin regulation of origin activation

The correlations between early replication in S-phase and transcriptional activity suggest that chromatin environments permissible for transcription also promote the activation of replication origins. Presumably, an "active" chromatin environment promotes origin activation early in S-phase. The encyclopedia of DNA elements (ENCODE) project analyzed replication timing data generated from HeLa cells in the context of multiple activating and repressing chromatin marks for 1% (30 Mb) of the human genome (ENCODE Project Consortium, 2007). Dutta and colleagues found that there was a positive correlation between the time a sequence replicates in S-phase and the activating chromatin marks H3K4me2 and H3K4me3 and H3/H4Ac, whereas H3K27me3 which marks facultative heterochromatin was anticorrelated with replication timing and enriched only in late-replicating regions analyzed by the ENCODE project (ENCODE Project Consortium, 2007; Karnani et al., 2007, 2010). Although

the replication and chromatin data sets were derived from different cell lines, the broad correlations have been confirmed by numerous independent studies in a variety of experimental systems (Hiratani et al., 2008; Schwaiger et al., 2009; Lee et al., 2010).

Additional evidence that permissive chromatin environments promote both transcription and DNA replication come from experiments analyzing the replication timing of *Drosophila* chromosomes. Early *Drosophila* studies observed that the single male X-chromosome replicated prior to the autosomes (Chatterjee and Mukherjee, 1977). In *Drosophila*, dosage compensation results in the approximate twofold up-regulation of transcription along the single copy of the male X-chromsome (Gorman and Baker, 1994).This up-regulation of X-specific transcripts ensures an equal dosage of gene products between the autosomes and the single male sex chromosome. Dosage compensation is mediated by the male-specific lethal (MSL) complex which is a ribonucleoprotein complex composed of two noncoding RNAs and five proteins including the histone acetyltransferase Mof which targets H4K16 (Akhtar and Becker, 2000). Genomic studies have demonstrated the Mof-dependent hyperacetylation of the X-chromosome in male cells (Kind et al., 2008) and an advancement of replication timing specific to the male X-chromosome (Schwaiger et al., 2009). These results strongly suggest that the transcription and replication programs are responding to the same chromosomal cues which, in the case of the X-chromosome, are likely dependent on H4K16Ac.

In yeast, origins in the vicinity of the telomere are often activated in late S-phase and the genes in these regions are typically silenced by the local chromatin environment (Ferguson and Fangman, 1992). The late activation of the telomere proximal origins was not due to the specific sequence of the origin but rather the surrounding chromatin environment (Stevenson and Gottschling, 1999). Transposition of a late-activating telomeric origin of replication ARS501 to a different region of the genome resulted in an earlier activating origin of replication (Ferguson and Fangman, 1992). Similarly, the ectopic localization of the early-activating origin ARS1 proximal to a late-replicating origin on chromosome XIV, which is nontelomeric, significantly delayed the activation of the ARS1 origin (Friedman et al., 1996). These experiments directly demonstrate that the local chromatin environment influences origin activity.

Modulation of the local chromatin environment can affect origin function. Multiple groups have demonstrated that increased histone H3 acetylation promotes origin activation. For example, tethering the histone acetylase, Gcn5, to the late-activating origin, ARS1412, results in that origin activating substantially earlier in S-phase (Vogelauer et al., 2002). Similarily, changes in global histone H3 acetylation levels mediated by the deletion of the histone deacetylase, Rpd3, results in a global speed up of S-phase which is correlated with the earlier loading of Cdc45 at replication origins (Vogelauer et al., 2002). Rpd3 is the primary histone deacetylase in yeast and exists in two complexes, Rpd3S and Rpd3L (Carrozza et al., 2005). Rpd3L is responsible for deacetylation at gene promoters (Carrozza et al., 2005) and loss of Rpd3L activity results in the earlier activation of ~100 late origins (Knott et al., 2009). In contrast, Rpd3S, which is involved in suppression of spurious intragenic transcription (Carrozza et al., 2005), has a more subtle effect specific to only a few replication origins. Together with the Gcn5 tethering experiments, these data are consistent with increased histone acetylation promoting origin activity. However, in the case of global changes in histone acetylation, mediated by loss of Rpd3, it is difficult to completely rule out the possibility of secondary effects such as the altered expression of a key replication factor.

Histone acetylation is also linked to origin activity in higher eukaryotes. In the fly, replication initiation during the developmentally programmed amplification of the chorion locus is also sensitive to changes in local histone acetylation, as artificial recruitment of Hbo1 (Chameaue) to the chorion locus stimulates origin activity (Aggarwal and Calvi, 2004). Similarly, the replication timing of the β-globin locus can be shifted both from late to early and from early to late by a tethered acetylase or targeted deacetylation, without a change in transcription (Goren et al., 2008). Finally, experiments utilizing *Xenopus* extracts revealed that the local chromatin environment serves to specify origin function (Danis et al., 2004). The loading of a transcriptional activator, GAL4-VP16, on a plasmid is sufficient to localize origin activity *in vitro*. This effect is not dependent on active transcription but instead is correlated with the local acetylation of histones at the site of initiation. Finally, in addition to histone acetylation, histone deacetylation might also facilitate origin activity at specific loci. For example, the histone deacetylase complex, Sum1/Rfm1/Hst1, has been shown to facilitate the initiation of several origins in *S. cerevisiae* (Irlbacher et al., 2005; Weber et al., 2008). These observations suggest that histone acetylation state, along with other identified mechanisms, may tune origin activity and determine when a given origin is activated during S-phase.

Replication and chromatin structure

It is clear that local chromatin modifications impact the selection and regulation of DNA replication start sites. There is also increasing evidence that the replication program may contribute to the establishment and maintenance of the epigenetic environment and impact gene expression. DNA replication proteins are involved in the establishment of heterochromatin (Bell et al., 1993; Foss et al., 1993; Micklem et al., 1993; Pak et al., 1997); and the time at which a sequence is replicated during S-phase can influence the local deposition of chromatin marks (Zhang et al., 2002a; Lande-Diner et al., 2009). Thus, DNA replication impacts the inheritance of both genetic and epigenetic information.

ORC and heterochromatin

In *S. cerevisiae*, the essential replication initiator, ORC, is involved in the establishment of silent chromatin at the cryptic mating type loci (*HML* and *HMR*), by binding at the silencer sequence elements of these loci. Mutations in the Orc2 subunit result in a loss of silencing (Bell et al., 1993; Foss et al., 1993; Micklem et al., 1993). ORC facilitates the targeting of Sir1 via the BAH domain of Orc1 to the silent loci which functions to recruit the remaining Sir proteins, Sir2, Sir3 and Sir4, to the chromatin (Triolo and Sternglanz, 1996; Zhang et al., 2002b). Thus, Sir1 and ORC serve to nucleate the remaining Sir proteins which spread across the locus, resulting in hypoacetylation and structural changes in the chromatin leading to the loss of transcription. Recent high-resolution ChIP studies from the Rine laboratory suggest that ORC may have a more extensive role in silencing and perhaps contribute to the local chromatin structure and organization of silenced sequences (Özaydın and Rine, 2010).

In higher eukaryotes, ORC also participates in the formation of heterochromatin. Multiple *Drosophila* ORC subunits (1, 3 and 4) interact with the heterochromatin protein HP1 (Pak et al., 1997). Hypomorphic mutations in Orc2 suppress position effect variegation (Pak et al., 1997) and disrupt HP1 localization (Huang et al., 1998), suggesting a role for ORC in recuiting HP1 and maintaining a heterochromatic environment. Similarly, human ORC subunits (1, 2 and 3) also interact with HP1 and localize to the heterochromatin (Prasanth et al., 2004, 2010). RNAi depletion of human HP1 results in a loss of ORC localization to the heterochromatin and similarly depletion of specific ORC subunits results in a loss of HP1 at heterochromatin (Prasanth et al., 2010), suggesting that both HP1 and ORC are codependent on one another for localization to the heterohcromatin.

Aside from heterochromatin formation, ORC facilitates several other aspects of chromosome maintenance including chromosome condensation (Pflumm and Botchan, 2001) and the establishment of cohesion, presumably via pre-RC formation (Gillespie and Hirano, 2004; Takahashi et al., 2004; Takahashi et al., 2008). It is unclear if these additional roles for ORC are also related to the initiation of DNA replication or simply represent a structural role for ORC in chromosome organization. For example, despite the role of ORC in heterochromatin formation, we know very little about how heterochromatic sequences are replicated during S-phase. Heterochromatic regions of the genome are typically the last sequences to be replicated during S-phase (Stambrook and Flickinger, 1970; Goldman et al., 1984). The compaction and repressive nature of the heterochromatin presents numerous challenges for replication initiation and fork progression. Indeed, the complex and repetitive nature of the heterochromatin has proven recalcitrant to systematic origin-mapping studies. Identifying and characterizing the differences in how euchromatic and heterochromatic origins of replication are selected, regulated and coordinated will yield important insights into how the replication program is regulated in distinct chromatin environments.

Replication and epigenetic memory

Studies of replication timing and transcription have identified a connection between transcriptional potential and the time at which a sequence is replicated during S-phase. Although it is clear that both the transcription and replication program respond to the same chromatin cues (activating chromatin marks, etc.; Schwaiger et al., 2009), there is also increasing evidence that DNA replication may play a central role in maintaining the local chromatin environment. For example, induction of HoxB gene expression in mouse P19 cells requires one round of DNA replication (Fisher and Méchali, 2003), suggesting that replication may expose the locus to additional transcriptional regulatory signals resulting in activation of transcription; alternatively, passage of the replication fork may erase repressive chromatin modifications, leading to activation of transcription. Indeed, it is staggering to consider that not only does the genome need to be copied faithfully but also the entire epigenetic program needs to be reestablished following DNA replication.

Coupling the differential deposition of activating and repressive chromatin marks with the temporal replication program provides a potential mechanism to ensure epigenetic memory (Figure 4). For example, the acetylation of nascent nucleosomes during early S-phase may provide a mechanism to ensure that early replicating and actively transcribed regions are reassembled into a similar chromatin state following DNA replication.

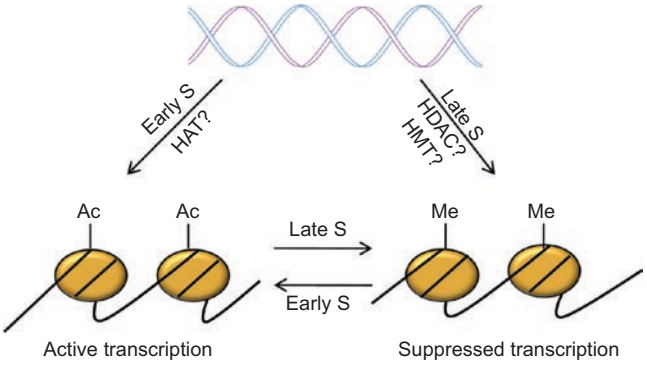

Figure 4. Replication timing and epigenetic memory. Replication timing may establish and maintain epigenetic memory. Broad domains of 'active' chromatin marked by hyperacetylated histones are replicated early, presumably due to increased origin activity. Similarly, sequences marked by hypoacetylated and methylated (H3K9, H3K27) chromatin are typically late replicating. Newly replicated sequences during early S-phase are assembled into hyperacetylated chromatin as opposed to late replicating sequences. Thus, sequences are differentially assembled into active and inactive chromatin depending on their time of replication during S-phase. The transition from early to late replication or from late to early replication can reverse the epigenetic memory and transcriptional competence.

In support of this hypothesis, the Cedar group has performed a number of elegant experiments in which they have microinjected episomal DNA into nuclei at different times during S-phase (Zhang et al., 2002a; Lande-Diner et al., 2009). They found that sequences replicated early in S-phase were more likely to be packaged into acetylated nucleosomes and exhibit active transcription (Zhang et al., 2002a). Furthermore, if a sequence shifts from early to late or late to early replication between subsequent cell cycles, there is a concomitant change in chromatin status of the locus (Lande-Diner et al., 2009). Specifically, sequences transitioning from late to early exhibit hyperactelyation on nascent histones H3 and H4; conversely, those sequences that transition from early to late replication exhibited hypoacetylation on histone H4. These changes in histone acetylation levels are likely mediated by specific HAT and HDAC activities that are associated with the replication foci (Hasan et al., 2001; Rountree et al., 2000). Coupling replication timing with histone acetylation levels provides a simple mechanism to copy the underlying or base epigenetic state which can be further tuned by the recruitment of additional chromatin-modifying enzymes to achieve the exquisite local control of transcription that is required for tissue and developmental specific gene expression.

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Declaration of interest

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