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# Origins of DNA Replication in Eukaryotes

**Yixin Hu**[1,2], **Bruce Stillman**[1,*]

[1]Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724, USA.

[2]Program in Molecular and Cell Biology, Stony Brook University, Stony Brook, NY 11794, USA.

## Abstract

Errors occurring during DNA replication can result in inaccurate, incomplete or re-replication, resulting in genome instability that can lead to diseases such as cancer or disorders such as autism. A great deal of progress has been made toward understanding the entire process of DNA replication in eukaryotes, including the mechanism of initiation and its control. This review focuses on the current understanding how the Origin Recognition Complex (ORC) contributes to determining the location of replication initiation in the multiple chromosomes within eukaryotic cells, as well as methods for mapping the location and temporal patterning of DNA replication. Origin specification and configuration varies substantially between eukaryotic species and in some cases co-evolved with gene silencing mechanisms. The possibility that centromeres and origins of DNA replication were originally derived from a common element and later separated during evolution is discussed.

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The initiation of DNA replication occurs at multiple sites along eukaryotic cell chromosomes. The mechanism that defines the location of these replication origins varies considerably, ranging from DNA sequence-specific to epigenetically determined and inherited. It is possible that origins of DNA replication and centromeres evolved from a common ancestral element.

## 1. General DNA Replication Strategy

DNA replication is an essential process in all life forms for faithfully transmitting genetic information encoded within the nuclear and mitochondrial DNA to daughter cells during somatic cell division and to gametes for inheritance of “the chemistry of life” to the next generation. Bacteria generally have circular single or multiple chromosomes with a small genome size[1]. Typically, a single replication origin exists per bacterial chromosome[2]. Archaea have bacteria-like circular chromosomes[3] and can have single or multiple clustered replication origins per archaeal chromosome[4]. In stark contrast, the polymer of DNA in each

*Corresponding Author stillman@cshl.edu.



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chromosome of a eukaryotic cell genome is much larger than bacterial and archaeal DNA[5] and the entire genome is divided into multiple chromosomes (Table 1), creating a problem for ensuring that all the DNA molecules on separate chromosomes are replicated only once per cell division cycle and are then evenly segregated during mitosis. The size of eukaryotic genomes and the rate of DNA synthesis at each replication fork necessitates coordinated initiation of DNA replication from multiple origins in each chromosome so that the genome is duplicated in a timely manner during the cell division cycle. Furthermore, mechanisms exist to ensure that each replicon is duplicated once and only once per S phase.

DNA replication is known to be bi-directional and temporally regulated in large domains (near-megabase-sized domains in mammals) (Figure 1a) with different parts of the genome replicating at different times (Figure 1b)[6]. Temporal patterning of DNA replication across the genome is influenced by different mechanisms, such as rate-limiting DNA replication factors, transcription, epigenetic control, chromosome structure and 3D chromatin organization in the nucleus[7–10]. Euchromatic regions tend to fill the nucleoplasm and replicate early during S phase, while the heterochromatic regions are more likely to be found at the nuclear and nucleolar peripheries and replicate late in S phase[11]. Chromatin is spatially folded into loops with the help of architectural proteins, such as CTCF and cohesins, and are further organized into large Topological Associated Domains (TADs) which serve as structural platforms for long-range chromatin interactions and *cis*-regulator contacts[11]. Remarkably TADs are highly correlated with DNA replication timing, with large megabase-sized A and B domains replicating at different times during S phase[12–14]. Replication timing is also dynamic and developmentally controlled[15–17].

Early embryonic cells are highly proliferative and divide very rapidly and synchronously. DNA replication could occur in less than 4 mins in early-stage *Drosophila* embryos[18], and less than 30 mins in early *Xenopus* cleavage embryos – 20-times faster than in somatic cells[19]. Replication origins are spaced much closer to each other during these rapid division stages, which can be 10-fold closer than the inter-origin distance in somatic cells and all origins become activated at roughly the same time. This density of replication origins declines when the embryo gets closer to the Mid Blastula Transition (MBT), during which zygotic transcription levels increase and cell division rates slow down[15]. In mammals, the replication fork rate is slower in the first cycle (2-cell-stage) with active replication origins closer to each other, compared to the second and the third cell division cycles (4- and 8-cell-stage) and replication fork rate increases as development proceeds. In addition, replication timing patterning, especially the early S-phase replication timing, is different between embryonic and 2-cell stage cells, which correlate with the transcription level of adjacent genes[20]. In *Caenorhabditis elegans* the temporal patterning of DNA replication during early embryogenesis precedes the onset of zygotic transcription suggesting that the temporal patterning of DNA replication is not dependent on cell type gene expression, but the mechanisms that determine replication timing may in turn influence developmental patterning of gene transcription[21].

## 2. DNA Replication Initiation Mechanism

Because eukaryotic cells have multiple and often large chromosomes, it is necessary to establish starts sites for DNA replication across the entire genome with sufficient spacing so as to ensure complete replication within the S phase. Remarkably, this is achieved by the assembly of a large, multi-protein complex at every potential origin before any DNA synthesis occurs. Once the cell enters into S phase these complexes act as sites for assembly of the replisome that occurs in a sequential manner throughout S phase, creating the temporal patterns of DNA replication mentioned above. In the budding yeast *Saccharomyces cerevisiae*, the best characterized system to date, initiation of DNA replication occurs by assembly of a pre-Replicative Complex (pre-RC) at each potential origin of DNA replication by the sequential binding to DNA of the Origin Recognition Complex (ORC) and Cell Division Cycle 6 (Cdc6) that then recruit Chromatin Licensing and DNA Replication Factor 1 (Cdt1) that chaperones the DNA helicase Mini-chromosome Maintenance Proteins 2–7 (Mcm2-7), forming ORC/Cdc6/Cdt1/Mcm2-7 (OCCM) complex (Figure 2, a–e) [reviewed in[22–24]]. Complete assembly of the pre-RC involves the recruitment of a second Mcm2-7 hexamer to form head-to-head Mcm2-7 hexamers (Figure 2, f–h) that are destined to become the core components of the DNA helicases at the two replication forks that emanate from each origin[25–28].

ORC was identified 30 years ago in budding yeast *S. cerevisiae* (herein referred to as ScORC) and is composed of six distinct subunits (Orc1-6) that binds in a sequence-specific manner to replication origins[29]. In some yeast, but not all, origins of DNA replication correspond to genetic elements called Autonomously Replicating Sequences (*ARS*) that confer upon a plasmid the ability to replicate as a mini chromosome once per cell division cycle like the rest of the genome[29]. *ARSs* may be DNA sequence specific, as in *S. cerevisiae*, but as discussed below, this is not a universal feature. Cdc6, which is related in amino acid sequence to the Orc1 subunit of ORC, was first identified in a screen for mutations that caused a cell division cycle arrest in *S. cerevisiae*[30] and later found to bind to ORC and be essential for pre-RC assembly[31,32]. The *Schizosaccharomyces pombe* homolog Cdc18 was shown to be necessary for entry into DNA replication[33]. Cdt1 was named Cdc10-dependent transcript 1 since it was initially identified as a gene regulated by the Cdc10 transcription factor in fission yeast *S. pombe*[34]. Later, it was shown to be equivalent to the Replication Licensing Factor -B (RLF-B) that is required for loading of Mcm2-7 onto DNA in a *Xenopus* egg extract and thus for assembly of the pre-RC[35–37] and has conserved functions throughout eukaryotes[38]. Thus, Cdt1 was renamed as chromatin licensing and DNA replication factor 1.

Genes encoding some of the six subunits of the Mcm2-7 helicase were originally identified in different genetic screens *in S. cerevisiae*, either as genes required for cell cycle progression[39] or for selective mini-chromosome plasmid maintenance[40]. Subsequently, these Mini Chromosome Maintenance proteins were grouped together due to their sequence similarity[41]. Further studies indicated that they are involved in the initiation of DNA replication and for replication fork progression[42–44]. Factors that were identified and characterized in one species guided its homologue identification in other species. For example, Replication Licensing Factor-M that was required for licensing of DNA replication

in *Xenopus* egg extracts consisted of Mcm2-7 proteins[45–47]. Likewise, the identification of ORC homologues in *Xenopus* and mammals was guided by ScORC[48–50].

A key feature of DNA replication in eukaryotes is that the entire genome must be duplicated, but importantly, every chromosome or replicon must be copied only once per cell division cycle. A model for cell cycle-control of origin licensing and once per cell cycle replication was introduced by Blow and Laskey in which fully replicated DNA could not be re-replicated in a single cell cycle due to exclusion of origin licensing factors from the DNA by the nuclear envelope[51]. Although cell cycle-dependent exclusion of replication licensing factors does exist, this mechanism turned out not to control once per cell cycle replication. Instead, cell cycle-dependent Deoxyribonuclease I (DNase I) hypersensitive site changes at *S. cerevisiae* replication origins *in vivo* led to the idea that origin licensing occurred by pre-RC assembly at replication origins only during G1 phase of the cell cycle[52]. Furthermore, activation of pre-RCs during S phase also resulted in their destruction and hence, re-initiation could not occur on replicated DNA. Origin licensing is restricted to the G1 phase of the cell cycle when S phase cyclin-dependent kinase (CDK) activity is absent (Figure 3). Active CDK phosphorylates pre-RC components and inhibits pre-RC assembly and hence pre-RC assembly cannot occur once cells enter into S phase and can only re-occur after cells pass through mitosis when CDKs are destroyed[53,54]. Thus, low CDK activity is necessary for pre-RC assembly at replication origins[55]. A reduction of CDK activity[56,57] or over-expression of a CDK inhibitor protein[58] was found to lead to DNA re-replication. Phosphorylation of ORC, Cdc6 and Mcm2-7 protein by CDK control pre-RC assembly[59]. Paradoxically, the same CDK activity that prevents pre-RC assembly is activated just before S phase and is required for the initiation of DNA synthesis at each origin. Active CDK (S-CDK) phosphorylates two initiation proteins Sld2 and Sld3 to drive pre-Initiation Complex (pre-IC) formation, during which the active helicase is assembled prior to the start of actual DNA synthesis at the origin[23,24,53,60–62]. Another kinase essential for cell cycle regulation of DNA replication is Dbf4-dependent Cdc7 kinase (DDK), which phosphorylates Mcm2-7 and drives the recruitment of Cdc45, a component of the pre-Initiation Complex (pre-IC) to form an active helicase that contains the Cdc45-Mcm2-7-GINS tetramer complex, called the CMG[23,24,63–65]. Thus each pre-RC is destroyed when the Mcm2-7 double hexamer is converted into the two active helicases. Pre-IC formation is a subsequent stage following the pre-RC assembly, in which licensed origins initiate replication in a temporally regulated manner throughout S phase[60,66]. The pre-IC is formed by recruiting additional factors including Cdc45, GINS, Sld7, Sld3, Sld2, Dpb11, Pol ε, RPA and Mcm10 to the Mcm2-7 double hexamer[23,24,67], thereby activating the 3’ to 5’ helicase activity[22,23,68]. Then DNA Polymerase α-primase is recruited to prime the initiation of replication and DNA polymerases drive the bi-directional replication forks, where Pol ε is dedicated to the replication of the leading strand, while Pol δ is dedicated to the replication of the lagging strand[69–74]. Pre-RC assembly with purified proteins was achieved first[75,76]. Later complete replication of DNA or chromatin templates was reconstituted with purified proteins that primarily were identified either from studies of Simian Virus 40 DNA replication *in vitro* or yeast biochemistry and genetics[23,24,70–72,77,78].

# 3. Origin Specification

## 4.1 Plasticity of Replication Origins

The location of origins of DNA replication can be as defined as 100~200 base pairs (bp) in budding yeast to as broad as ~150 kilo base pair (kb) initiation zones within megabase-sized replication domains in human chromosomes[79–82] (Table 2). Despite the large diversity in configuration of replication origins, components of the pre-RC are conserved throughout eukaryotes[83,84]. However, the mechanism of how ORC recognizes and makes contacts with DNA varies considerably in cells across eukaryotic species.

During DNA replication in *Drosophila* and *Xenopus* early embryos, plasmid or chromosomal DNA replication initiation was reported to be random or with greatly relaxed sequence specificity during the very rapid early cleavage stages[85–87]. The initiation of DNA replication in these early replication cycles occurs about every 3 kb along chromosomes with little temporal variation, but during the mid-blastula transition (MBT), when zygotic transcription begins, replication initiates from spaced out, specific sites in intergenic regions in a temporal order of replication throughout S phase[88,89]. The same pre-RC proteins are required for both the rapid embryonic cycles and the slower, temporally regulated somatic cell modes of DNA replication[84,90]. Thus, replication origin location has considerable plasticity during development. Such flexibility has also been apparent in yeasts where origins of replication can be DNA sequence-specific. In *S. cerevisiae* for example, when some or all the canonical origins on Chromosome III were deleted, the strains seem to grow relatively fine[91,92] and DNA replication could initiate from non-canonical sites[93]. A similar phenomenon occurs in Archaea where Orc1/Cdc6 related proteins normally bind DNA to sequence-specific DNA replication origins, but deletion of all origins resulted in replication initiation at random sites[94]. Therefore, any stretch of DNA seems to have the potential for initiating replication and these observation raised the question as to whether DNA replication initiation required any sequence specificity at all[51,86,95,96]. To complicate mapping of origins even further, even in *S. cerevisiae* where ORC determines the location of pre-RC assembly at specific DNA sequences, the Mcm2-7 double hexamer can translocate along the chromosome driven by transcribing RNA polymerases so that CMG helicase activation and thus initiation of DNA replication could occur at a distance from the pre-RC assembly site[97]. Therefore, mapping DNA replication start sites (Table 3) may not be the equivalent of mapping the location of origin specification and pre-RC assembly.

Further evidence for plasticity in the location of origins of DNA replication stems from the redundancy of initiation sites. Even in the *S. cerevisiae* genome, where origin location is sequence specific, there are ~12,000 potential matches to the core *ARS* consensus sequence (ACS), however there are only ~400 functional origins[98]. Within a given cell division cycle, not all of these ~400 origins become active, and within a single cell, the origin activity is known to be stochastic and vary extensively between cells[99–104]. These single molecule studies revealed more active origins than were found in bulk cell populations, likely due to some origins being used at a very low frequency that are not detected in the analysis of origin activity in a population of cells[100,101]. In metazoan cells and fission yeast, using single molecule methods (Table 3), DNA replication origin usage was also shown

to be stochastic[105–109]. Even though different single cells could have different cohorts of active origins, the data from population studies show reproducible spatial, temporal and "non-random" replication timing profiles, indicating that origin redundancy provides the diversity and plasticity for a "controlled-stochastic" origin selection process. The existence of an excess of licensed origins over the actual number used to replicate the genome in any given cell cycle resulted in the idea of dormant origins that can become active if necessary to complete genome replication, particularly in situations when cells experience DNA damage or replication stress[110–114].

### 4.2 Replication Origin Configurations

The simplest eukaryotic replication origin configuration lies in a small clade of budding yeasts that include *S. cerevisiae*. Autonomously replicating sequences (*ARSs*) were identified based on the ability of some cloned, genomic fragments from the *S. cerevisiae* genome to confer high-frequency transformation of yeast cells with a circular plasmid DNA and thence stable inheritance of the resulting mini-chromosomes[115,116]. Later, 2D agarose gel electrophoretic analysis showed that DNA replication initiated at the *ARS* sequence and nowhere else on *ARS* containing plasmids[117,118]. DNA sequence analysis demonstrated that *ARSs* contained a short, 11-base pair conserved ACS DNA sequence (now called the A element) but this was not sufficient for origin activity[119,120]. Multiple elements within *ARS1* (*ARS416*) were identified to be critical for origin activity[121]. The essential A element contains the ACS, and together with three important B elements, are conserved and provide binding sites for ORC and accessory proteins[121–123]. The B2 element was previously thought to be an instability region, called duplex unwinding element (DUE), where the initial unwinding of DNA occurs[124]. However, later studies found that B2 is unlikely to play this role but instead a role for pre-RC assembly[66,125]. Interestingly, the B2 element contains a partial, inverted ACS sequence motif to which ORC can bind[29,125,126]. Whether one[127–130] or two[126,131] ORCs are needed for loading the Mcm2-7 double hexamers has been investigated.[133]. Most studies to date have used the *ARS1* (*ARS416*) origin that has the A and B1 elements as the primary ORC binding site and the B2 element as a weak, inverted ORC binding site with a short space between the two ORC binding sites[122,126,132,133]. Biochemical and structural studies of a pre-RC assembly intermediate Mcm2-7-ORC (MO) on *ARS1* DNA and separate, single molecule analysis of pre-RC assembly show that ORC first binds to the A and B1 elements and loads the first Mcm2-7 hexamer by interacting with the Mcm2-7 C-terminal winged-helix domains and then flips to the amino-terminal side of the loaded Mcm2-7 to load a second Mcm2-7 hexamer (Figure 2, f–g), thereby creating the pre-RC with a single ORC[134–137]. It is possible that other origins with a different spacing between the A/B1 and B2 elements would employ two ORC molecules to load the two hexamers of Mcm2-7[138], and this needs further investigation.

Precisely how ORC binds to origin DNA was not understood until structural studies of ORC bound to DNA suggested that an α-helix in the Orc4 subunit that binds to a major grove in the DNA and a loop in Orc2 subunit that contacts DNA contribute to DNA sequence specific ORC binding[139,140]. The Orc2 loop is essential and subtle mutations in the Orc4 α-helix, more specifically F485 and Y486 on the α-helix, bind to a different ACS recognition motif[141]. Furthermore, deletion of this α-helix within Orc4 has been shown to

have more plasticity in its origin selection[142]. Strikingly, the Orc4 α-helix and the Orc2 loop are not found in ORC from most eukaryotes, including most fungi, plants and animals, raising the issue of how ORC from these species interacts with DNA and determines the location of origins of DNA replication. One possibility is that ORC from *S. cerevisiae* has lysine-rich sequence in an intrinsically disordered region (IDR) of the Orc1 subunit that binds to a minor grove in the origin DNA and this amino acid sequence is conserved in other eukaryotes and has been shown in ORC from human cells to bind DNA, possibly a short sequence the ORC1 IDR[139,143].

In some fission yeasts, such as *Schizosaccharomyces pombe* (*S. pombe*), *S. octosporus*, and *S. japonicus*, replication origins lack consensus sequences[144,145] but are found to be AT-rich, which are called AT islands and can be as short as ~100bp in length to the size of >500bp[146,147]. All these *Schizosaccharomyces* species contain an AT-hook domain at the N-terminus of the Orc4 protein that preferentially binds to AT-rich DNA sequences and is necessary for origin recognition[148,149] (Table 2). There are nine AT-hook subdomains in *S. pombe*, while *S. octosporus* has four and *S. japonicus* has five[146], and each subdomain is capable of binding 6–8 nucleotides of DNA[150]. Interestingly, bioinformatic analysis of some other fungi, such as *Neurospora crassa* and *Aspergillus nidulans*, suggest that AT-hooks at their Orc4 N-terminus might be used to specify replication origin location at AT-rich sequences in their genomes (N. Zali and B. Stillman, unpublished). But again, like the DNA sequence specific origins that bind the Orc4 α-helix, the A-T-hook mechanism of origin specification is restricted to a small fraction of eukaryotic species, raising the question of how the location of origins of DNA replication are specified in most eukaryotes.

In metazoan species, it has been more difficult to locate replication origins and independent studies with different methods do not show high concordance of origin features and locations[90,151], likely due to the large genome sizes, the existence of large replication initiation zones, analysis of different cell types, movement of Mcm2-7 double hexamers and technical limitations. In the late 1960s, visualization of replication tracts in mammalian cells revealed that replication initiates from multiple sites that can be simultaneously active at adjacent sites in the chromosomes[152]. Following the release of the first draft of the human genome, techniques such as quantitative mapping of RNA-capped short nascent strands (SNS) and 2-dimensional gel electrophoresis were employed to map replication origins[84,153–157]. In the past few years, many methods have been developed to map replication origins in metazoan genomes (Table 3).

Data from these newer mapping approaches show that replication origins can be kilobase-sized zones in metazoans and were reported to fire stochastically with no sequence specificity[82,109,158]. They are organized into spatially and temporally regulated replication timing domains[6]. Consistently, metazoan ORC was shown to bind DNA with no apparent sequence specificity *in vitro*[159–161]. Nevertheless, a recent study of replication origins in mouse embryonic stem cells using a CRISPR-mediated deletion and inversion of TAD boundaries suggest that *cis*-acting DNA elements are required for DNA replication initiation[162]. How these *cis*-acting sequences contribute to DNA replication is not known. Despite the lack of sequence specificity, G-rich sequences which have the potential to form G-quadruplex secondary structure have been found to be associated with sites of efficient

metazoan replication initiation and may play a role in defining replication origins, although this is not certain[163–165]. ORC from human cells was reported to preferentially bind to G4 motifs on single-stranded DNA while it binds to dsDNA randomly[166,167], although how this relates to establishing the location of replication origins is not clear. The lack of defining DNA features at metazoan origins suggest that the dispersive initiation zones could be composed of multiple discrete replication origins that fire stochastically. Consistent with this line of reasoning, a recent modified Ini-seq method (Table 3), which shows precise mapping of 23,905 replication origins with a replication initiation efficiency score assigned to each origin indicates that origin firing within the dispersive initiation zones are not randomly distributed but are arranged hierarchically, with a set of highly efficient origins near defined zone boundaries[107]. Based on this study, the concept has emerged that dispersive replication initiation zones contain within them discrete, highly efficient replication origins. The question remains is how are these clustered sites and efficient origins determined?

For the bulk of eukaryotes, including animals, plants and most yeasts, the replication origins lack consensus DNA sequence motifs, with the notable exception sequence-specific *ARSs* in the small *S. cerevisiae* related clade of budding yeasts. Interestingly, *ARSs* are not strictly required for the initiation of DNA replication *in vitro*[168,169] and can initiate from non-canonical sites *in vivo* when consensus sites are deleted from a chromosome[93]. Moreover, not all origins contain a clear consensus sequence[170,171], and ORC can bind to non-origin DNA and load Mcm2-7[76]. But ORC can direct sequence-specific replication in animal cells since fusion of a DNA binding protein such as Gal4 to ORC subunits in *Drosophila* can promote pre-RC assembly and initiation of DNA replication at a chromosomal Gal4 DNA binding site, even though replication in *Drosophila* is normally not sequence specific[172]. This raises the possibility that ORC may interact with sequence-specific DNA binding proteins to locate origins in the genome.

### 4.3 Local Environments Regulate Replication Origin Selection

Origin licensing and activation steps are critical and must be tightly regulated to ensure the accurate and complete duplication of the genome. In higher eukaryotes licensing in G1 phase is known to load an excess of Mcm2-7 protein onto the chromosomes over what is needed for genome replication, and some of this excess Mcm2-7 can be employed to activate dormant origins that protect the cells from replicative stress and rescue of stalled forks[110–112]. Even with random replication initiation, origins are spatially separated to ensure completion of a normal S phase[173,174]. What are the regulators that determine origin location and activity? Previous studies suggested that the chromatin-associated complexes may restrict pre-RC formation and the use of naked DNA for pre-RC assembly can bypass the restrictions imposed by chromatin[168], implying the chromatin or local environments could play roles in regulating replication origin licensing and activation[175,176]. Multiple regulatory factors in local environments could play critical roles in specifying the location of origins of DNA replication, such as ORC associated proteins, epigenetic replication timing patterning, rate limiting replication factors, gene transcription, nucleosome occupancy, histone modifications, and chromatin structures and 3D organization of chromosomes.

For example, it is possible that within local chromatin environments, ORC-interacting proteins such as HMGA1[177], the ORC-associated protein ORCA[178–180] or noncoding RNAs[181–183] could recruit ORC to specific chromatin sites and contribute to location-specific replication origins in metazoans. ORC, more specifically ORC1, has been shown to act like a pioneer factor by binding to the condensed chromosomes during mitosis, immediately after nuclear envelope breakdown[184,185], similar to the transcription factor FoxA1 that also binds mitotic chromosomes and promotes site specific gene transcription in G1 phase[186]. When ORC1 is inherited into the newly formed daughter nuclei, it binds the other ORC subunits and forms spatiotemporal patterns throughout G1 phase that resemble the spatiotemporal patterns of DNA replication sites in S phase, suggesting an epigenetically inherited distribution of replication patterning that is determined in G1 phase by ORC[187]. How this temporal patterning of ORC1 in human cells occurs is not known. Since the activation of origins of DNA replication throughout S phase competes for rate limiting replication factors[7,10,188], rate limiting factors that function in G1 phase may influence the nuclear patterns of ORC1. Alternatively, replication timing in S phase is known to be controlled by factors such as Rif1 that binds Protein Phosphatase 1[189] and regulates DDK activity, so it is possible that these factors also play a role in G1 phase to influence ORC1 spatiotemporal nuclear localization.

The location of transcription start sites (TSSs)[190] and CpG islands[191] are known to correlate with the location of replication origins or pre-RC binding sites, suggesting that the mechanisms that specify TSSs may also determine the location of origins of replication. For example, the forkhead transcription factors (Fkh1 and Fkh2) were found to be necessary for replication from 30% of early-firing origins[192]. However, the relationship between transcription and origin localization is complicated because gene transcription can inhibit pre-RC assembly or restrict the location of where pre-RC assembly can occur, and even after the Mcm2-7 double-hexamers are loaded onto DNA, transcription can move them[97,193–195]. Furthermore, site-specific replication initiation can be artificially achieved by manipulation of the local chromatin environment such as DNA methylation[196] or transcription factor targeting[197], but clearly both methylation and transcription factors also affect transcription, raising cause and effect issues. Nevertheless, DNA methylation has been shown to be critical and required for maintaining replication timing and normal mammalian embryo development[198,199]. But whether this is a direct effect on the mechanism of origin licensing is not known.

Nucleosome depleted regions at replication origins have been shown to be critical for ORC binding and origin efficiency[190,200–203], and ATP-dependent chromatin remodelers are required for establishing precisely positioned nucleosomes flanking an ACS on a template DNA *in vitro*[77,204,205]. Thus, at the very local level, chromatin structure can influence pre-RC assembly, perhaps because open chromatin would provide more accessibility to replication factors. But the chromatin structure over larger distances can influence the location or activation of origins of DNA replication. For example, the activation of one origin may increase the probability of firing of neighboring origins[206–208], as long as the origins are not too close to each other, in which case suppression of origin firing can occur[209,210]. Chromatin context also influences the timing of initiation of DNA replication since ectopically positioning an early-firing origin to the late-replicating telomeric region led

to the delayed firing of the translocated origin, and conversely, a late-firing origin can be replicated early when placed onto a small replicating plasmid[211]. Thus temporal patterning of DNA replication is not sequence specific, even in a cell where origin specification utilizes specific DNA sequences.

Replication efficiency in metazoan cells has been shown to correlate with DNA fragment size[212]. Furthermore, both *cis*-acting DNA elements and epigenetic factors can influence replication timing[162,213]. Histone modifications that define open chromatin have been linked to early replication origins, such as H2A.Z[214], H3 and H4 acetylation, all three methylation states of H3K4 and all three methylation states of H4K20[215–218]. A very convincing correlation between replication origins and histone modifications has been reported in *C. elegans*[21]. In this study, the inter-origin distance was on average 40kb across the entire genome and histone modifications H3K4me2, H3K4me3 and H3K27Ac, all associated with active chromatin, co-localized with the location of origins in the genome. This is consistent with open chromatin influencing both transcription and replication start sites in the genome. In contrast, repressive chromatin modifications, such as H3K9me2/3 and H3K27me3, are linked to late replication origins in human cells[219]. Consistently, tethering a histone acetylase (HAT) or a histone deacetylase (HDAC) to the replication origin regions influenced replication timing[9]. Indeed, ORC can directly or indirectly interact with HAT or HDAC, such as HBO1(KAT7)[220] and Sir2[221] and directly with modified histones such as H4K20me2 and H3K9me3[216,222]. These enzymes could either recruit ORC or be recruited by ORC to specific genomic sites and have the potential to assemble pre-RCs and contribute to regulating replication timing.

However, the histone modifications correlated to certain groups of replication origins are not universal for all origins. There are also more modifications across the genome than replication origins and these modifications are involved in diverse processes, such as transcription and response to DNA damage, etc., indicating more factors are needed to determine replication origin locations. But the open chromatin idea raised another question: how does ORC localize to the heterochromatic regions of the genome? It is possible that the 3D chromosome organization and nuclear localization play a role in giving accessibility to replication factors in different spatiotemporal domains[6]. CTCFs and cohesins together with other proteins facilitate and shape loop extrusions in chromosomes[223], and it has been proposed that the replication origins or initiation zones are colocalized with cohesins at the base of the loop extrusions and adjacent origins within these loops could have similar replication timing[224–227]. Pre-RC assembly occurs on euchromatin in early G1 phase and on heterochromatin in mid G1 guided by the ORC binding protein ORCA[180] and this differential assembly may reflect the dynamic intranuclear changes in the localization of ORC1 observed during G1 phase[187].

Taken together, all the regulatory factors display dynamic influences on individual origins and the integration of these multiple controls at each origin could determine differential origin activation probability and timing in single cells. These combinatorial factors could also contribute to the “non-random” optimal origin usage distribution profiles in cell populations, explaining the “controlled-stochastic” origin firing regulatory mechanism. Even so, replication timing is stochastic and can vary between cells and even between

homologs, suggesting that origin activation can be independent of factors that determine global replication timing[108].

In some special circumstances, DNA replication or replication origin firing is developmentally regulated by *cis*-acting factors that result in overriding the once-per-cycle replication rule. Egg shell genes in *Drosophila* nurse cells surrounding the egg are amplified by up to 16 to 32-fold by re-initiation of DNA replication[228]. ORC and the sequence-specific transcription factor E2F are required for re-amplifying the egg shell genes[229]. Interestingly, another exception occurs within stretched skin cells in zebrafish larvae where the cell can divide without DNA replication[230]. But these examples are in terminally differentiated cells, therefore, they no longer further differentiate and as a result, genome instability does not matter.

## 4. The evolutionary transitions into sequence specific origins

Origins of DNA replication in bacteria are DNA sequence-specific and thus the finding of sequence-specific origins in *S. cerevisiae* was not a surprise, but these two sequence-specific origin systems are functionally very different from each other. Furthermore, the arrangement of *S. cerevisiae* sequence-specific origins that include a combination of essential and non-essential DNA sequence elements, reminiscent of gene promoters, was an unexpected and unprecedented organization of an origin of DNA replication[121]. Importantly, the presence of a conserved origin DNA sequence facilitated in the discovery of ORC that then accelerated both genetic and biochemical studies to understand pre-RC assembly and the initiation of DNA replication and its control. Thus, *S. cerevisiae* was a fortuitous choice to study the initiation of DNA replication because its origin sequence specificity gave confidence that ORC was *the* initiator, which then resulted in the identification of ORC in other eukaryotic cells. The existence of highly conserved ORC and other pre-RC proteins raises the question of why there is significant variation in mechanisms of origin structure and specification?

It is apparent that in most eukaryotes, origins of DNA replication lack DNA sequence-specificity and how ORC is localized to these chromosomes and how it determines the location of origins of DNA replication is not completely understood. Strict DNA sequence specific origins exist in a small clade of *S. cerevisiae*-like budding yeasts, whereas in some other fungi, like *S. pombe*, ORC localizes origins to AT-rich locations in the genome. Unlike other *cis*-acting elements, such as the centromeres and transcription factor binding sites, the loss of function of any one replication origin is unlikely to have a severe effect or apparent fitness loss for the cell[231]. These observations raise the question of why sequence-specific origins exist and are rare?

In the transition budding yeasts that are distant from the *S. cerevisiae* clade of yeasts and lack the Orc4 α-helix, such as *Candida albicans* and *Pichia pastoris, ARS* sequences that are independent of a centromere have been identified[232,233]. In the case of *C. albicans*, proposed origins (*pro*ORis) on chromosome arms were predicted based on the localization of ORC chromatin immunoprecipitation results and nucleosome free regions in the genome and a loose AC-rich DNA sequence was observed[232]. However multiple mutations in this sequence only reduced *ARS* activity by 3-fold. Some of these *ARS* sequences were shown

by 2D gel analysis to have weak origin activity, whereas other *pro*ORis that functioned as *ARSs* lacked origin function. In the case of *P. pastoris*, analysis of *ARS* function on plasmids identified two separate *ARS* sequences, one class was a GC-rich sequence that when mutated eliminated *ARS* activity and a separate class with AT-rich sequences[233]. A representative of both classes was shown to colocalize with origin activity in the chromosomal context. In both the studies with *C. albicans* and *P. pastoris*, the biochemical role of the conserved sequences was not addressed. DNA sequences associated with *ARS* activity have been shown to either bind the initiator protein ORC, exclude nucleosomes or bind to other proteins that mediate *ARS* plasmid stability, such as proteins that tether plasmids to the nuclear periphery[234]. It is also possible that the GC-rich *ARSs* in *P. pastoris* overlap with gene promoters and these may contribute to origin activity. Thus, future studies of *ARS* activity in yeasts must address the biochemical function of any proposed conserved sequences to reveal how they contribute to *ARS* or origin activity, which may not be equivalent.

### 5.1 Co-evolutionary transitions of origin specificity, gene silencing mechanisms and centromeres

In species that lack DNA sequence-specific origins of replication, inherited transcriptional gene silencing can occur by RNA interference (RNAi)[141]. These species also have epigenetically defined centromeres (*CEN*)[235]. A group of intermediate branching yeasts seem to be in the transition of losing RNAi (e.g., *C. albicans*[236]; Figure 4), since they still carry some but not all genes that encode RNAi proteins (or non-canonical Dicer gene). The most recently branching budding yeasts, including *S. cerevisiae* (Figure 4), have completely lost RNAi and instead, they have gained ORC-Sir4 mediated gene silencing. This has been accompanied by the acquisition of the Orc4 α-helix, the Orc2 DNA interacting loop and sequence specific origins[141] and a DNA sequence-specific, point *CEN* configuration[235].

### 5.2 Evolutionary driving forces

Previously, it has been suggested that acquiring and maintaining a beneficial killer virus could explain the loss of RNAi in budding yeast[236]. In addition, the loss of RNAi and the acquisition of sequence-specific point centromeres were suggested to co-evolve with maintenance of the circular 2-micron plasmid, which is known as a selfish DNA element that uses the host segregation apparatus components for plasmid stability[235]. It was proposed that DNA sequence-defined, point centromeres were acquired by integration of the selfish circular 2-micron plasmid into the genome, bringing with it DNA sequences that eventually functioned as centromeres[235]. RNAi contributes to the silencing and stability of repetitive DNA sequences like heterochromatin satellite repeated sequences at centromeres and remnants of transposable elements, as well as organization of rDNA repeats[237]. The *S. cerevisiae* and *K. lactis* (Figure 4) clade of budding yeasts have lost centromere-associated repeated sequences and have replaced RNAi with Silent Information Regulator (SIR) proteins that silence gene transcription in a DNA sequence-specific manner at the silent mating type loci, as well as maintaining rDNA and telomere repeats by preventing recombination[238]. Interestingly, the acquisition of the Sir4 protein that binds either directly to ORC in *K. lactis* or indirectly via Sir1 to ORC in *S. cerevisiae*, occurred with the acquisition of the Orc4 α-helix and the Orc2 loop, both of which contribute to the DNA

sequence-specific DNA binding by ORC[141]. ORC binds in a DNA sequence-specific manner to the *cis*-acting silencer elements that flank the silent mating type loci, thereby establishing precise localization of transcriptionally silent regions in the genome by recruiting SIR proteins to these loci. The *S. cerevisiae* and *K. lactis* clade of budding yeasts have a dense genome with ~70 percent of the genome encoding protein and little gene spacing and repetitive DNA. To avoid conflicts between replication and transcription, it makes sense to place origins of DNA replication in intergenic regions and this may well be the reason why the acquisition of DNA sequence-specific origins of DNA replication in these yeasts evolved. But how were the sequence-specific origins acquired?

Interestingly, the naturally occurring replication origin from the 2-micron plasmid has similar properties to current *S. cerevisiae* chromosomal replication origins[239,240]. Like the previously proposed point centromere acquisition model[235], it is appealing to propose that the DNA sequence specific replication origins may also have been acquired from the 2-micron plasmid. Unlike the centromere where one per chromosome is required, DNA sequence specific origins must have spread throughout the genome, particularly in intergenic regions. Hence, *CEN* and replication origin *ARS* sequence may originally have coincided with the same sequence that later evolved to become physically and functionally separated to ensure that the genetic information from the large sized eukaryotic genome can be faithfully replicated in time from multiple origins and the segregation into daughter cells during cell division to be well-coordinated by a single *CEN*. Indeed, one example of intermediate branching budding yeasts, *Yarrowia lipolytica*, has *ARSs* that contain physically separatable *CEN* and replication origin (*ori*) sequences[241]. But unlike *S. cerevisiae ARS* plasmids, *Y. lipolytica ARSs* require both *CEN* and *ori* to maintain replicating plasmids[241]. Only a few replication origin sequences that are associated with *CEN* sequences have been characterized in *Y. lipolytica* and they lack sequence similarity. *Y. lipolytica* also lacks RNAi, the SIR proteins and the Orc4 α-helix and the Orc2 loop[141] (Figure 4). The nature of origins of DNA replication in *Y. lipolytica* remain enigmatic, but they may be specified by epigenetic means as proposed for animal cell replication.

Moreover, the *Y. lipolytica* genome is very GC-rich and is 1.6 times larger than the *S. cerevisiae* genome but has roughly the same number of protein coding genes which may provide a greater opportunity for DNA sequence-independent initiation in the larger intergenic regions, while still avoiding conflicts between DNA replication and transcription. Thus, it seems that *CEN* and replication origins may have started out as epigenetically defined and GC-rich and then evolved to become sequence specific in small clade of budding yeasts. One observation that supports this idea is that some of the basal branching yeasts, such as *Pichia pastoris* (Figure 4), have GC-rich replication origins, hence it was speculated that GC-rich is perhaps an ancestral trait of replication origins[242].

In ancestral eukaryotes, *CEN* and *ori* elements may have been the same or tightly linked, but then evolved into separate functional elements. If this were the case, it may explain why ORC not only plays a role in the initiation of DNA replication, but why ORC subunits localize to centromeres in human cells and maintain the integrity of *CEN* associated α-satellite sequences[243]. Another intriguing potential link between origins of DNA replication and *CEN* sequences comes from the role of histone H3K4 methylation

at origins and *CEN* sequences. As noted above, origins of DNA replication in *C. elegans* are associated with histone H4K4me2 and H3K4me3 modifications[21]. On the other hand, histone H3K4me2 modification at the α-satellite sequences of an artificial centromere is required to recruit the HJURP protein, the *CEN*-specific CENP-A histone[244]. *C. elegans* has holocentric centromeres which are multiple point centromeres located along the length of each of the chromosomes. Interestingly, CENP-A location in the genome has been determined[245] and they co-localize with origins of DNA replication (Iestyn Whitehouse, personal communication). This observation lends strong support for the notion that *CEN* and *ori* have a common ancestor.

### 5.3 Perspectives for evolutionary driving forces

So, what advantages or disadvantages do these co-evolutionary transitions provide? We suggest that the loss of the RNAi system in the intermediate branching yeasts, by driving forces such as the beneficial killer virus infection[236], would increase the transcription and replication conflicts and hence genome instability, thereby creating a higher mutation rate at fragile sites. A new gene silencing system was needed and meanwhile the possible integration of features from 2-micron plasmid into the genome could create the possibility of evolving to become new sequence-dependent systems for both *CEN* and chromosomal replication origins. Moreover, a paucity of replication origins could delay the chromosomal duplication completion and lead to the expression of fragile sites and elevate the rate of gross chromosomal rearrangements. Thus, the evolutionary transitions could be selected by limiting the fragile sites and decreasing genome instability by increasing the number of active and dormant, sequence-specific origins in the intergenic regions.

There are multiple essential questions that remain to be addressed: how does ORC localize to chromosomes in many different species? By which mechanisms does ORC contact DNA in different species? Independent studies suggested that any metazoan DNA sequence contained potential initiation sites and replication origins are epigenetically controlled in coordination with transcriptional activity. It raises the question whether metazoan origins have specific DNA elements and/or epigenetic markers or do not require such determinants. Whether or to what extent origins stochastically fire at spatially random sites or at multiple more discrete sites within the dispersive initiation zones remains a matter of debate and needs more precise metazoan replication origin mapping methods. On top of that, what role does transcription play in defining where replication initiates? In the species that have lost RNAi and have not yet gained sequence specificity, can ORC binding to DNA at random sites with lower affinity be removed by transcription, thereby placing origins of DNA replication in intergenic regions?

Intriguingly, species like *Carpediemonas membranifera* and *Carpediemonas frisia* seem to have lost canonical DNA replication proteins, such as ORC and Cdc6, and most structural kinetochore proteins, such as NDC80, during evolution[246]. What would be the mechanisms for replicating DNA in these species? Do they depend upon break induced replication (BIR) mechanisms[247]? How are their replication origins specified? Indeed, how ORC and CDC6 specify the location of origins of DNA replication remains an issue for most eukaryotic cells.

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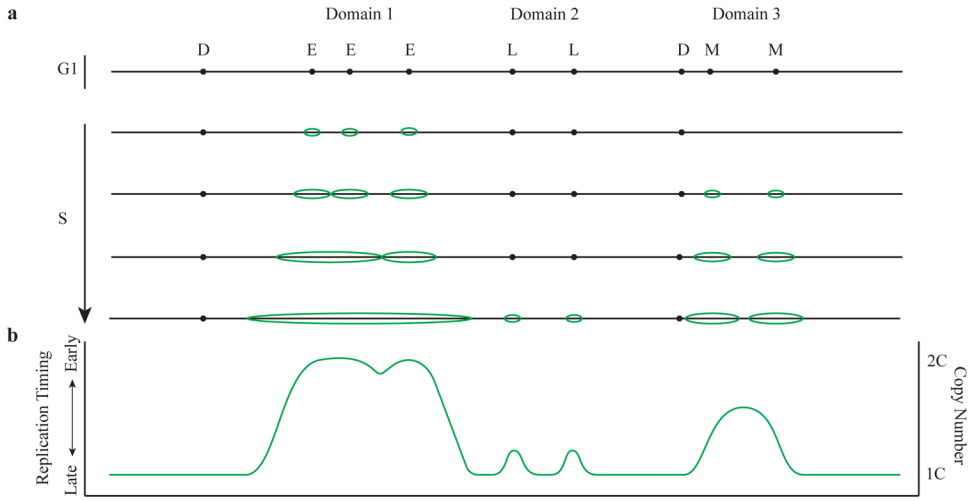



**Figure 1 |. Replication Timing.**

**a** shows the DNA replication process with various replication timing domains. Early-firing replication origins are indicated as E. Mid-firing replication origins are indicated as M. Late-firing replication origins are indicated as L. Dormant replication origins are indicated as D. Replication bubbles are indicated in green color. **b** shows the replication profile correspond to **a** measured in a population of cells with 1C and 2C genome copy number indicated. C equals to the genome copy number.

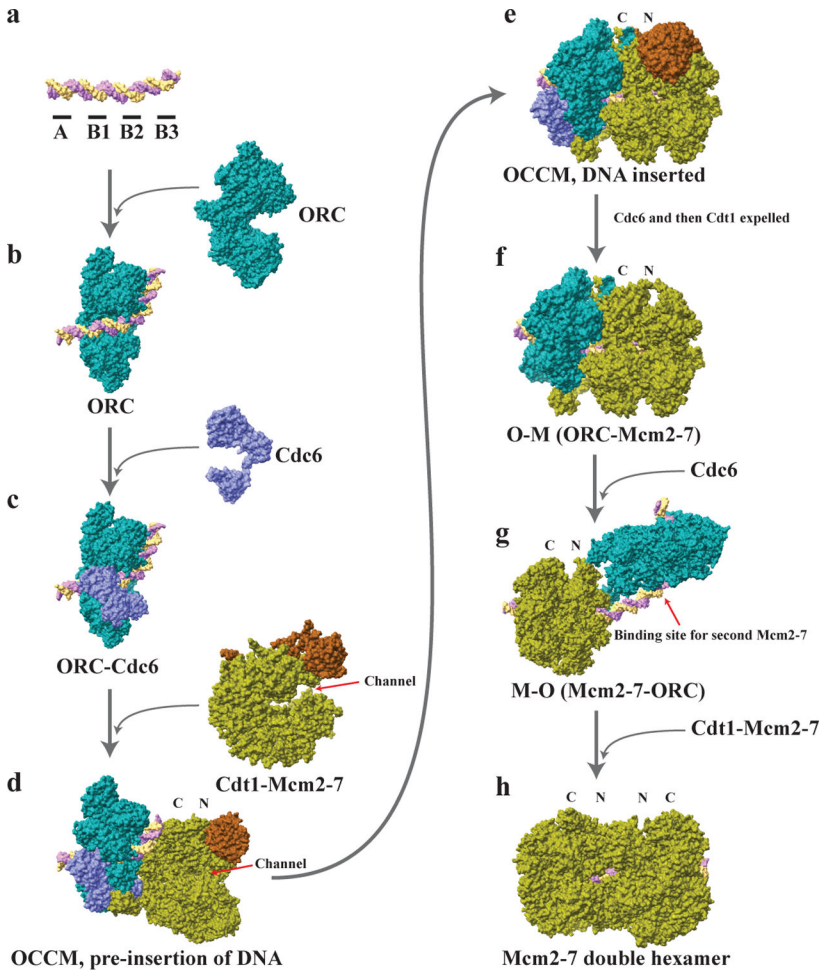



**Figure 2 |. Pre-Replicative Complex Assembly Model.**

[Adapted from[248] with image credit: molecular structures taken and adapted from the RCSB protein database, deposits 3JA8, 5ZR1[139] 5BK4[26], 5V8F[249], 5XF8[250], 6RQC[251], 6F0L[252], 6WGG and 6WGF[253] and 7MCA[254].) **a** shows the replication origin DNA (+ strand in cantaloupe color, - strand in lavender color), which in *S. cerevisiae* contains four elements (indicated as black segments) with A and B2 elements binding ORC in opposite orientations. **b** shows that ORC (in teal color) first binds to the A and B2 elements. **c** shows that ORC recruits Cdc6 (in orchid color). **d** shows that Cdt1-Mcm2-7 complex in open ring conformation (Cdt1 in Mocha color, Mcm2-7 in Asparagus color, a channel between Mcm2 and Mcm5 subunits is indicated) is recruited by ORC-Cdc6. DNA is aligned to the channel in the Mcm2-7 hexamer. The Mcm2-7 complex is oriented as the hexamer C-terminus binding to ORC-Cdc6. **e** shows the intermediate known as OCCM with the double stranded DNA inserted into the channel between Mcm2 and Mcm5 subunits in the Mcm2-7 hexamer. The hexamer is partially closed. **f** shows that the ATP hydrolysis by the Mcm2-7 expels the first Cdc6 and then Cdt1, creating the OM complex. **g** shows that ORC flips over to the N-terminus side of Mcm2-7 and presumably binds to the B2 element on DNA, creating the MO complex. The structure of the MO complex was modeled by real-space-refining docked coordinates of MCM (PDB 6EYC[255]), ORC (PDB 5ZR1[139]) and an N-terminal

Orc6 homology[134]. **h** shows that ORC can now recruit a second Cdc6, creating a binding site for a second Cdt1-Mcm2-7 complex that can be loaded in an opposite orientation to the first Mcm2-7. The Mcm2-7 double hexamer, possibly with ORC still bound to the DNA, is then ready to be activated and can unwind the double stranded DNA when entering S phase.

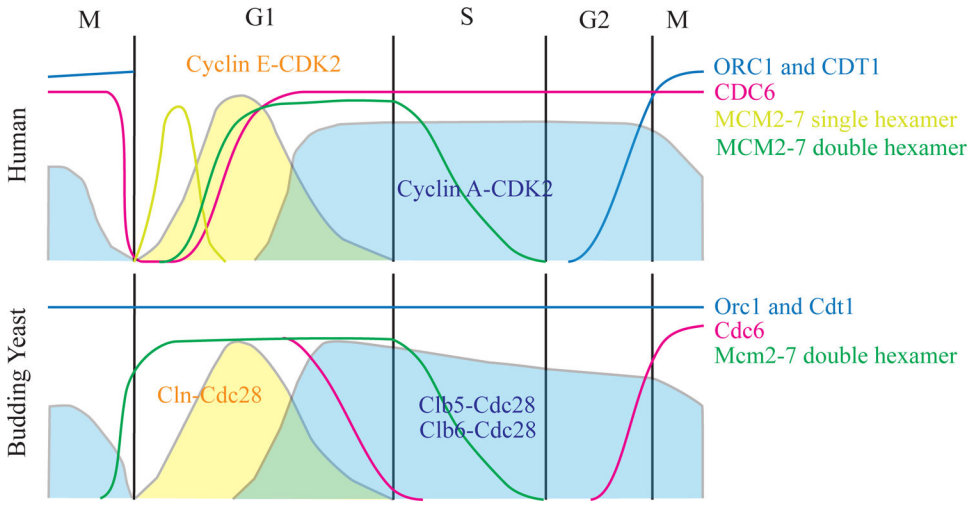



**Figure 3 |. Waves of cyclins and DNA replication proteins in human and budding yeast during cell cycle progression.**

Human and budding yeast replication proteins ORC1/Orc1 and CDT1/Cdt1 (in blue) and CDC6/Cdc6 (in pink) proteins levels as well as Mcm2-7/Mcm2-7 single hexamer (in lime green) and Mcm2-7/Mcm2-7 double hexamer (in dark green) loading levels are shown as lines. Pre-RC assembly corresponds to Mcm2-7/Mcm2-7 double hexamer formation. Cyclin-dependent kinases activities are shown as solid areas with Cyclin E-CDK2 in human and Cln-Cdc28 in budding yeast are shown in yellow, while Cyclin A-CDK2 in human and Clb5-Cdc28 and Clb6-Cdc28 in budding yeast are shown in dark blue. G1, S, G2, M phases in cell cycle are indicated.

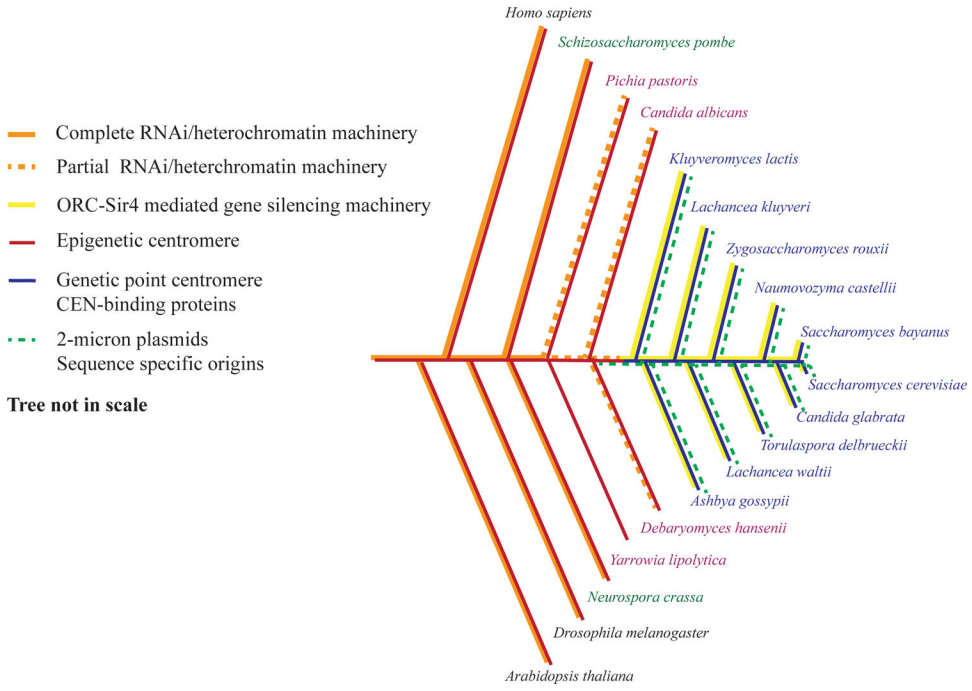



**Figure 4 |. Co-evolution of gene silencing mechanisms, centromeres and replication origin sequence specificity.**

[Adapted from[235] and[141]]. The phylogenetic tree is not drawn to scale. Most eukaryotes (font in black), including basal branching yeasts (font in green), have complete RNAi machinery or full complements of heterochromatin. Intermediate branching yeasts (font in purple) harbor partial components of RNAi or heterochromatin machinery, whereas the *Saccharomycetaceae* yeast family, the most recently branching yeasts, (font in blue) have completely lost RNAi/heterochromatin machinery and acquired ORC-Sir4 mediated gene silencing[141]. Meanwhile, the selfishly propagating 2-micron plasmids exist in *Saccharomycetaceae* lineage, where the strains have DNA sequence-defined point centromeres as well as sequence specific replication origins. *Y. lipolytica* lacks RNAi as well as the SIR proteins[141], so it is not clear what gene silencing mechanism it uses.

**Table 1 |**

**Origin recognition proteins in three domains of life.**

Table shows the comparison of proteins that bind to origins of DNA replication in bacteria, archaea and eukaryotes.

| Domains of Life | Replication origin number | Chromosome number | Genome Size | Replication Initiator Proteins |
|---|---|---|---|---|
| Bacteria | Single origin per chromosome | Single or multiple circular chromosomes | ~0.6 to ~8.0 Mb | DnaA |
| Archaea | Single or multiple origins per chromosome | Single circular chromosome (typically) | ~0.5 to ~5.8 Mb | Orc1/Cdc6 |
| Eukaryote | Multiple origins per chromosome | Multiple linear chromosomes | ~10 to >100,000 Mb | ORC Cdc6 |

**Table 2 |**

**Replication origins configurations.**

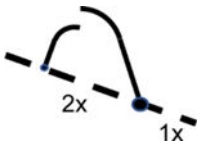
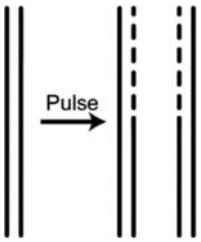
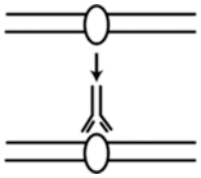
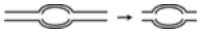
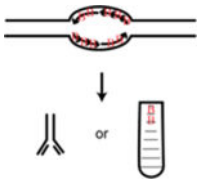
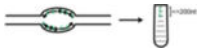
Table shows the comparison of three typical replication origins configurations.

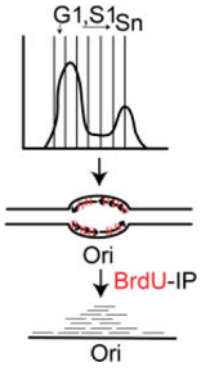
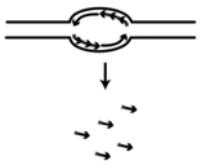
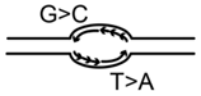
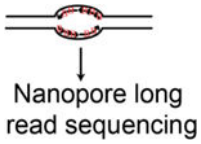
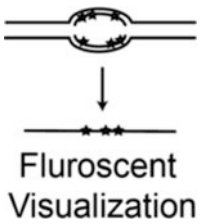
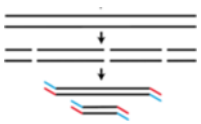
| Species | Primary DNA Sequence | Sequence specificity | Replication origins defined by | Precession |
|---|---|---|---|---|
| Metazoan | GC-rich | Nonspecific | Epigenetically defined | ~1Mb replication domains with clustered initiation zones within. No obvious DNA sequence specificity. |
| *S. pombe* related fission yeast | AT-rich | Nonspecific | Orc4 AT-hook in ORC binds A.T rich DNA, not specific DNA sequences | ~500 to ~1500bp (can be as precise as ~100bp) |
| *S. cerevisiae* (most recently branching yeast) | AT-rich | Sequence specific, ACS identified | ORC subunits interact with defined ACS DNA sequence, both DNA sequence specific and non-specific | ~100 to ~200bp |

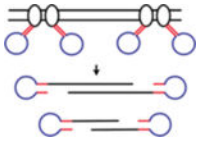
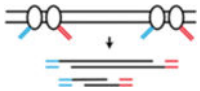
**Table 3.**

**Origin mapping methods.**

Table and figure motifs show many methods to map origins of DNA replication in cell populations and in single cells or molecules[96].

| Method | Description | Cite | Comments | Illustration |
|---|---|---|---|---|
| 2D gel assays and fork direction analysis | Uses distinct gel migration patterns to characterize replication intermediates and detect origin firing efficiency | Brewer and Fangman[117] Huberman et al., [118] | Pooled data using population cells |   |
| Density transfer | Uses density isotypes to distinguish newly synthesized DNA form parental DNA followed by DNA sequencing and quantitative copy number profiling to map general patterns of replication timing | Gilbert,[84]; Pope et al., [14]; Rhind and Gilbert[6]. | |   |
| ChIP-seq (chromatin immunoprecipitation followed by DNA sequencing) | Identify locations of ORC and MCM binding sites to predict the location of replication origins | Belsky et al., [256]; Long et al., [214]; Lucas and Raghuraman,[257]; MacAlpine et al[190,258] | |  |
| Bubble-seq | Trap replication bubbles and sequence DNA | Mesner et al., [259] | |  |
| Ini-seq (initiation site sequencing) | Label nascent DNA with DNA analogs and either immuno-precipitate the labeled nascent DNA or separate the labeled nascent DNA from unreplicated DNA using density difference | Guilbaud et al., [107]; Langley et al., [260]. | |   |
| OK-seq (Okazaki-fragment sequencing) | Isolate and sequence Okazaki fragments to map replication fork direction in asynchronous population of cells | Petryk et al., [82]. | |  |

| Method | Description | Cite | Comments | Illustration |
|---|---|---|---|---|
| Repli-seq | Label nascent DNA with BrdU; look for the enrichment of BrdU-immunoprecipitated DNA in late S phase cells compared to early S phase cells | Zhao et al., [158]. | |   |
| SNS-seq (Short Nascent Strand-sequencing) | Identify nascent RNA-primed DNA synthesized at origins by the primase DNA polymerase a (Pol a) | Besnard et al., [261]; Cadoret et al., [157]; Cayrou et al., [262]; Picard et al., [263] | |  |
| S-jump based computational origin prediction | S-jump based method which computes the excess G over C and T over A along one DNA strand where abrupt changes correlate with the location of replication origins (N-domains and U-shaped domains of skew jumps), however the location of actual origins needs experimental validation | Brodie et al., [264]. | Computational prediction data using population cells |   |
| Electron microscopy and fiber autoradiography | Visually observation of replication origins on radiolabeled DNA | | Single molecule data using population cells |  |
| DNA combing | Fluorescently label nascent DNA. DNA molecules stretched and aligned along a slide and visualized by various fluorescence microscopy techniques | Bensimon et al., [265]; Michalet et al., [266]; Pasero et al.[267]. | |  |
| DNAscent; FORK-seq etc. | Nanopore sequencing based methods that detect DNA analog labeled nascent DxwNA | Hennion et al., [268]; Muller et al., [269]; Georgieva et al., [270]; Boemo[271] | |   |
| ORM (optical replication mapping) | Fluorescent nucleotide analog pulse-labeled nascent DNA in combination with the optical mapping method (Bionano Genomics) to map long individual DNA molecules | Wang et al., [109] | |   |
| scRepli-seq | Uses a commercial whole genome amplification kit, SeqPlex from Sigma, to prepare sequencing libraries | Miura et al., [272]; Takahashi et al., [273]. | Single cell data |  |

| Method | Description | Cite | Comments | Illustration |
|---|---|---|---|---|
| LIANTI (Linear Amplification via Transposon Insertion) | Combines the T7 in vitro transcription to the Tn5 transposition to avoid the intrinsic limitations of Tn5 transposition being symmetric and reduce biases and errors during the amplification steps | Chen et al., [274] | |  |
| DLP (direct library preparation) | Uses nanoliter-volume reactions to adapt the conventional transposase-based library construction to single cells | Laks et al., [275]; Zahn et al., [276]. | |  |