the plant journal

SEB Society for Experimental Biology

The Plant Journal (2015) **83,** 38–51 doi: 10.1111/tpj.12847

SI CHROMATIN AND DEVELOPMENT

Links between genome replication and chromatin landscapes

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Received 19 February 2015; revised 27 March 2015; accepted 1 April 2015; published online 6 April 2015.
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SUMMARY

Post-embryonic organogenesis in plants requires the continuous production of cells in the organ primordia, their expansion and a coordinated exit to differentiation. Genome replication is one of the most important processes that occur during the cell cycle, as the maintenance of genomic integrity is of primary relevance for development. As it is chromatin that must be duplicated, a strict coordination occurs between DNA replication, the deposition of new histones, and the introduction of histone modifications and variants. In turn, the chromatin landscape affects several stages during genome replication. Thus, chromatin accessibility is crucial for the initial stages and to specify the location of DNA replication origins with different chromatin signatures. The chromatin landscape also determines the timing of activation during the S phase. Genome replication must occur fully, but only once during each cell cycle. The re-replication avoidance mechanisms rely primarily on restricting the availability of certain replication factors; however, the presence of specific histone modifications are also revealed as contributing to the mechanisms that avoid re-replication, in particular for heterochromatin replication. We provide here an update of genome replication mostly focused on data from Arabidopsis, and the advances that genomic approaches are likely to provide in the coming years. The data available, both in plants and animals, point to the relevance of the chromatin landscape in genome replication, and require a critical evaluation of the existing views about the nature of replication origins, the mechanisms of origin specification and the relevance of epigenetic modifications for genome replication.

Keywords: DNA replication, epigenetics, chromatin, *Arabidopsis thaliana*, plant.

INTRODUCTION

The growth and development of a multicellular organism requires thousands of cell divisions to transform the unicellular zygote into the adult body. This is particularly relevant in the case of plants, in which organogenesis occurs entirely in a post-embryonic manner. Remarkably, the integrity of the genetic material of every cell must be maintained throughout all these mitotic divisions. At the same time, a strict requirement is that the genome must be replicated fully and faithfully in order to deliver one exact copy to each daughter cell. Genome duplication occurs during the S phase of the cell cycle, and it is indeed a very risky process. There are numerous sources of potential alterations that need to be prevented or, if they occur, repaired before chromosome segregation at mitosis. Given the fundamental relevance of genome integrity for cell and organismal viability, the process of genome duplication is strictly controlled and coordinated with other cell-cycle events.

It is worth mentioning here that the first demonstration of the semi-conservative nature of DNA and chromosome replication in eukaryotes came with the pioneering work of Taylor *et al.* (1957) that was published soon after the discovery of the molecular structure of the DNA double helix. Taylor's studies are a must, and provide enlightening reading for anyone coming to the DNA replication field.

One typical feature of all eukaryotic cells is that they possess relatively large genomes of 16 000 Mb or more. Replicative DNA polymerases move along their templates at a limited speed, which has been estimated at ~1.5 kbp min^{-1} (DePamphilis and Bell, 2011). Assuming this, full replication of a genome starting at a single site would take about a month for Arabidopsis and over 13 years for onion cells. Obviously, this is not the case as the S phase normally takes a few hours to be completed in all eukaryotic cells. The reason is the presence of multiple sites across the genome, called DNA replication origins

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(ORIs), where replication starts to complete the duplication of each replicon unit. Their number seems to be closely and linearly correlated with genome size (Gilbert, 2010; Costas *et al.*, 2011a; DePamphilis and Bell, 2011).

The activation of all ORIs is highly coordinated in time, but most likely also in space, within the S phase; however, the specification of genomic sites where ORIs are located, their coordinated timing of activation and the coupling with other DNA-mediated processes (transcription, repair, recombination) are still very poorly understood in molecular terms. An idea is emerging that to understand the process not only the DNA but also the chromatin must be considered. This makes sense, as it is chromatin that is actually duplicated during the S phase. As a consequence, in addition to DNA, all the associated histones that form the nucleosomes and, very importantly, the post-translational modification patterns of histones must be transferred to the daughter chromatids. Thus, many aspects of genome replication control appear to be intimately linked to chromatin events and, in turn, the chromatin landscape also participates in genome replication (Jasencakova and Groth, 2010; Alabert and Groth, 2012). The interaction between the replication machinery and chromatin components is likely the molecular basis of such coordination. In fact, this is only part of an upstream coordination network between chromatin and cell-cycle events throughout G1, S, G2, and mitosis (Desvoyes *et al.*, 2014).

Before entering the description of each stage of genome replication it is worth noting that, as a general rule, most proteins and complexes that participate in the initiation of DNA replication and in replication fork progression are highly conserved in all eukaryotes. The reader is directed to more specialized articles and reviews for details about features of the DNA replication proteins and protein complexes involved in genome replication (for details, see Sanchez *et al.*, 2012). Here we will focus on key aspects of genome replication, with a special emphasis on links with genome-wide chromatin landscapes.

CHROMATIN LICENSING: GAINING ACCESS TO POTENTIAL ORIS

The first step in setting potential ORIs is the assembly of pre-replication complexes (pre-RC). This must take place once chromosomal segregation has terminated in mitosis and during G1. The accessibility of pre-RC to potential ORI sites is highly dependent on chromatin and nuclear organization, a process known as 'chromatin licensing' that was introduced conceptually almost 30 years ago (Blow and Laskey, 1986, 1988). Over the years it has received full biochemical, molecular and genetic support (Arias and Walter, 2007). It has been reported that pre-RCs in all systems studied associate with chromatin as early as in the telophase, although this has not been formally demonstrated in plant cells (Sanchez *et al.*, 2012; MacAlpine and Almouzni, 2013). Chromatin decondensation right after mitosis allows pre-RC assembly (Gilbert, 2010), although detailed measurements of how long pre-RC loading is allowed during G1 are not available (Figure 1). Pre-RC components are present in Arabidopsis as well as in the rest of plants for which genome sequences are available (Shultz *et al.*, 2007; Sanchez *et al.*, 2012), and are highly conserved with yeast and mammalian proteins (Table 1). Thus, pre-RCs contain the six subunits ORC1–6 (ORIGIN RECOGNITION COMPLEX 1–6), CDC6 (CELL DIVISION CYCLE 6), CDT1 (CDC10-DEPENDENT TRANSCRIPTION) and the six subunits MCM2–7 (MINICHROMOSOME MAINTENANCE 2–7) complex. A typical feature of Arabidopsis pre-RC proteins is that ORC1, CDC6 and CDT1 are encoded by two separate genes in each case. The respective proteins are highly conserved in amino acid sequence, and it is not yet known whether each of them plays distinct roles or if they act redundantly. In fact, the conservation between plants and animals in pre-RC components can be extended to the rest of proteins that participate in the steps of DNA replication following pre-RC assembly, e.g. replication fork assembly [MCM8, MCM9, MCM10, CDC45 (CELL DIVISION CYCLE 45), and the GINS (GO-ICHI-NI-SAN; 5-1-2-3 in Japanese) complex] and replication fork progression and maturation of the newly-synthesized DNA [DNA polymerases A (α), D (δ) and E (ε) complexes, PCNA (PROLIFERATING CELL NUCLEAR ANTIGEN), RFC (REPLICATION FACTOR C), RPA (REPLICATION PROTEIN A), FEN1 (FLAP STRUCTURE-SPECIFIC ENDONUCLEASE 1) and LIG1 (LIGASE 1)] (Sanchez *et al.*, 2012).

Given the evolutionary conservation of domain structure of pre-RC and DNA replication proteins in all eukaryotes, it is likely that the molecular steps for pre-

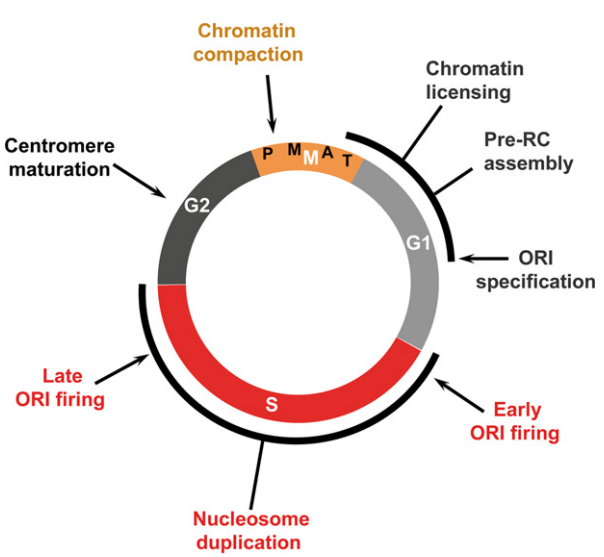

Figure 1. Chromatin-related processes that occur associated with genome replication and segregation during the cell cycle.

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Table 1 A summary of proteins involved in the major stages of chromosomal DNA replication in yeast, *Arabidopsis thaliana* and human cells

Saccharomyces cerevisiae	*Saccharomyce s pombe*	*Arabidopsis thaliana*	Homo sapiens
Pre-replication compex (pre-RC)			
ORC	*ORC*	*ORC*	*ORC*
Orc1	Orc1	ORC1A (At4g14700)	Orc1/p97
Orc2	Orc2	ORC1B (At4g12620)	Orc2/p82
Orc3	Orc3	ORC2 (At2g37560)	Orc3/p66
Orc4	Orc4	ORC3 (At5g16690)	Orc4/p50
Orc5	Orc5	ORC4 (At2g01120)	Orc5L/p50
Orc6	Orc6	ORC5 (At4g29910)	Orc6/p28
		ORC6 (At1g26840)	
Cdc6	Cdc18	CDC6A (At2g29680)	Cdc6
		CDc6b (At1g07270)	
Tah11/Sid2/Cdt1	Cdt1	CDT1A (At2g31270)	Cdt1/Rlf-B
		CDT1B (At3g54710)	
MCM helicase	*MCM helicase*	*MCM helicase*	*MCM helicase*
Mcm2	Mcm2/Cdc19	MCM2 (At1g44900)	Mcm2
Mcm3	Mcm3	MCM3 (At5g46280)	Mcm3
Mcm4/Cdc54	Mcm4/Cdc21	MCM4 (At2g16440)	Mcm4
Mcm5/Cdc46/Bob1	Mcm5/Nda4	MCM5 (At2g07690)	Mcm5
Mcm6	Mcm6/Mis5	MCM6 (At5g44635)	Mcm6
Mcm7/Cdc47	Mcm7	MCM7 (At4g02060)	Mcm7
			Gmnn/Geminin
		MCM9 (At2g14050)	Mcm9
		HAM1 (At5g64610)	Hbo1
		HAM2 (At5g09740)	
Replication fork assembly			
		MCM8 (At3g09660)	Mcm8
Mcm10/Dna43	Mcm10/Cdc23	MCM10 (At2g20980)	Mcm10
Cdc45	Cdc45/Goa1/	CDC45 (At3g25100)	Cdc45
GINS complex	*GINS complex*	*GINS complex*	*GINS complex*
Sld5/Cdc105	Sld5	SLD5 (At5g49010)	Gins4
Psf1/Cdc101	Psf1	PSF1 (At1g80190)	Gins1
Psf2/Cdc102	Psf2	PSF2 (At3g12530)	Gins2
Psf3/Cdc103	Psf3	PSF3 (At1g19080)	Gins3
Replication fork			
DNA Pol-α	*DNA Pol-α*	*DNA Pol-α*	*DNA Pol-α*
Pol1/Cdc17/Crt5	Pol1	POLA1 (At5g67100)	PolA/p180
Pol12	Spb70/Pol12	POLA2 (At1g67630)	PolA2/p68
DNA primase	*DNA primase*	*DNA primase*	*DNA primase*
Pri1	Spp1	POLA3/PRI1 (At1g67320)	Prim1/p48
Pri2	Spp2	POLA4/PRI2 (At5g41880)	Prim2a/p58
DNA Pol-δ	*DNA Pol-δ*	*DNA Pol-δ*	*DNA Pol-δ*
Cdc2/Pol3	Cdc6/Pol3	POLD1 (At5g63960)	Pold1/p125
Hys2/Pol31	Cdc1/Mis1	POLD2 (At2g42120)	Pold2/p50
Pol32	Cdc27	POLD3 (At1g78650)	Pold3/p66
	Cdm1	POLD4 (At1g09815)	Pold4/p12
DNA Pol-ε	*DNA Pol-ε*	*DNA Pol-ε*	*DNA Pol-ε*
Pol2/Dun2	Cdc20/Pol2	POLE1a (At1g08260)	Pole/p261
Dpb2	Dpb2	POLE1b (At2g27120)	Pole2/p59
Dpb3	Dpb3	POLE2 (At5g22110)	Pole3/p17
Dpb4	Dpb4	POLE3/DBP3 (gene family)	Pole4/p12
		POLE4 (DBP4 (gene family)	
RPA complex	*RPA complex*	*RPA complex*	*RPA complex*
Rfa1/Rpa1	Ssb1/Rpa1	RPA1a (At5g06510)	Rpa1/p70
		RPA1b (At5g08020)	
Rfa2/Buf1/Rpa2	Ssb2	RPA1c (At5g45400)	Rpa2/p32
Rfa3	Ssb3/Rpa3	RPA1d (At5g61000)	Rpa3/p14

(continued)

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Table 1. (continued)

Saccharomyces cerevisiae	*Saccharomyce s pombe*	*Arabidopsis thaliana*	Homo sapiens
		RPA2a (At2g24490)	
		RPA2b (At3g02920)	
		RPA3a (At3g52630)	
		RPA3b (At4g18590)	
Pol30	Pcn1	PCNA1 (At1g07370)	PCNA
		PCNA2 (At2g29570)	
RFC complex	*RFC complex*	*RFC complex*	*RFC complex*
Rfc1/Cdc44	Rfc1	RFC1 (At5g22010)	Rfc1/p140
Rfc2	Rfc2	RFC2 (At1g63160)	Rfc2/p40
Rfc3	Rfc3	RFC3 (At5g27740)	Rfc3/p38
Rfc4	Rfc4	RFC4 (At1g21690)	Rfc4/p37
Rfc5	Rfc5	RFC5 (At1g77470)	Rfc5/p36
Rad27/Erc11/Rth1	Rad2/Fen1	FEN1 (At5g26680)	Fen1
Cdc9/Mms8	Cdc17	LIG1 (At1g08130)	Lig1
Chromatin assembly			
CAF1 complex	*CAF1 complex*	*CAF1 complex*	*CAF1 complex*
Rlf2/Cac1	Pcf1	FAS1 (At1g65470)	p150
Cac2	Pcf2	FAS2 (At5g64630)	p60
Msi1/Cac3	Pcf3	MSI1 (At5g58230)	p48
HirA	Hip1	HIRA (At3g44530)	Hira
FACT complex	*FACT complex*	*FACT complex*	*FACT complex*
Spt16/Cdc68	Spt16	SPT16 (At4g10710)	Supt16h/Spt16
Pob3	Pob3	SSRP (At3g28730)	Ssrp1
Nhp6	Nhp6		
Asf1	Cia1	ASF1A (AT1g66740)	Asf1a/Cia
		ASF1B (At5g38110)	Asf1b/Cia2
Nap1	Nap1	NAP1;1 (At4g26110)	Nap1L1 to Nap1L5
		NAP1;2 (At2g19480)	
		NAP1;3 (At5g56950)	
		NAP1;4 (At3g13782)	
		NRP1 (At1g74560)	
		NRP2 (At1g18800)	
Hta1, Hta2	Hta1, Hta2	*Canonical H2A*	H2a (H2afb1,
		HTA1/H2A.1 (AT5g54640)	b2, b3, j, v, y, z)
		HTA2/H2A.2 (AT4g27230)	
		HTA10/H2.10 (AT1g51060)	
		HTA13/H2A.13 (At3g20670)	H2afx/H2ax
		Variant H2A	
		HTA3/H2A.X.3 (At1g54690)	
		H2A.X.5 (At1g08880)	H2afz/H2az
		HTA6/H2A.W.6 (At5g59870)	
		HTA7/H2A.W.7 (At5g27670)	
		HTA12/H2A.W.12 (At5g02560)	H2b (H2bfm, s, wt)
		HTA8/H2A.Z.8 (At2g38810)	
Htz1/H2az	Htb1	HTA9/H2A.Z.9 (At1g52740)	
		HTA11/H2A.Z.11 (At3g54560)	
		HTA4/H2A.Z.4 (At4g13570)	
Htb1, Htb2		*H2B*	
		HTB1/H2B.1 (At1g07790)	H3 (H3f3a, H3f3c)
		HTA5/H2A.X.5 (At1g08880)	
		HTB1/H2B.1 (At1g07790)	
		HTB2/H2B.2 (At5g22880)	
		HTB3/H2B.3 (At2g28720)	
		HTB4/H2B.4 (At5g59910)	
		HTB5/H2B.5 (At2g37470)	
		HTB6/H2B.6 (At3g53650)	H3.3b (H3f3b)
		HTB7/H2B.7 (At3g09480)	

(continued)

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Table 1. (continued)

Saccharomyces cerevisiae	*Saccharomyce s pombe*	*Arabidopsis thaliana*	Homo sapiens
		HTB8/H2B.8 (At1g08170)	
		HTB9/H2B.9 (At3g45980)	
		HTB10/H2B.10 (At5g02570)	
		HTB11/H2B.11 (At3g46030)	
Hht1, Hht2	Hht1, Hht2, Hht3	*Canonical H3.1*	H4 (Hist1h4a – Hist1H4l)
		HTR1/H3.1 (At5g65360)	
		HTR2/H3.1 (At1g09200)	
		HTR3/H3.1 (At3g27360)	
		HTR9/H3.1 (At5g10400)	
		HTR13/H3.1 (At5g10390)	
		Variant H3.3	
		HTR4/H3.3 (At4g40030)	
		HTR5/H3.3 (At4g40040)	
		HTR8/H3.3 (At5g10980)	
H3.3b (H3f3b)		*Centromeric H3*	
		HTR12/CENH3 (At1g01370)	
		Unusual H3	
		HTR6/H3.6 (At1g13370)	
		HTR14/H3.14 (At1g75600)	
		HTR10/H3.10 (At1g19890)	
		HTR7/H3.7 (At1g75610)	
Hhf1, Hhf2	Hhf1, Hhf2, Hhf3	*H4*	
		HFO1 (At3g46320)	
		HFO2 (At5g59690)	
		HFO3 (At2g28740)	
		HFO4 (At1g07820)	
		HFO5 (At3g53730)	
		HFO6 (At5g59970)	
		HFO7 (At3g45930)	
		HFO8 (At1g07669)	
Hho1		*H1*	H1 (H1fo, nt, oo, x)
		HON1/H1.1 (At1g06760)	
		HON2/H1.2 (At2g30620)	
		HON3/H1.3 (At2g18050)	

Codes in parenthesis in the *A. thaliana* column indicate TAIR accessions.

RC assembly are also conserved in the general rules. Likewise, the molecular interactions between the ORC, CDC6, CDT1 and the MCM2–7 complex are also conserved (Sanchez *et al.*, 2012). Nonetheless, it has been reported that in Arabidopsis the MCM complex remains bound to chromatin throughout the cell cycle, except in mitosis (Shultz *et al.*, 2009). This may reflect plant-specific features, reinforcing the importance of extending these studies to other pre-RC components. It may also be the consequence of MCM binding in non-ORI sites, a possibility that should be addressed in future.

It is important to keep in mind that chromatin licensing for replication is temporally associated with cell fate decisions that occur early in G1, because both depend on chromatin landscapes of high accessibility. A clear example is that of the homeobox *GL2* (*GLABRA 2*) gene, a master component of cell fate specification of atrichoblasts in the Arabidopsis root epidermis (Schiefelbein *et al.*, 2014). Fluorescence *in situ* hybridization (FISH) experiments revealed that chromatin accessibility at the *GL2* locus is cell-cycle regulated, and becomes high after the metaphase, when cells decide on atrichoblast–trichoblast fate (Costa and Shaw, 2006, 2007). Maintenance of an open chromatin state in atrichoblasts and the subsequent (sustained) GL2 expression does not occur in trichoblasts, where chromatin is rapidly converted into a closed, inaccessible state. Interestingly, the pre-RC component CDT1 was shown to increase GL2 expression (Caro *et al.*, 2007), suggesting an association, perhaps more than simply temporal, between genome replication and cell fate acquisition. Further studies are needed to expand these observations and define the molecular interactions, if any, between cell fate and DNA replication machineries and chromatin components (Caro and Gutierrez, 2007).

In animal cells, geminin (Gmn) is a protein that interacts with and inhibits Cdt1 and MCM loading (Wohlschlegel

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et al., 2000; Gillespie *et al.*, 2001; Tada *et al.*, 2001; Maiorano *et al.*, 2004; Xouri *et al.*, 2007; Miotto and Struhl, 2010). In addition to this DNA replication role, Gmn affects the expression of the *Hox* and *Six3* homeodomain genes involved in the expression of neurodifferentiation genes (Del Bene *et al.*, 2004; Luo *et al.*, 2004). Therefore, Gmn competes for chromatin factors to regulate gene expression and Cdt1. In this context, the identification of GEM (GL2 EXPRESSION MODULATOR) in Arabidopsis as a CDT1-interacting factor that participates in pre-RC assembly for DNA replication makes the similarity with animal Gmn unavoidable; however, it must be emphasized that GEM and Gmn are totally unrelated proteins from a structural point of view, although they seem to impinge on the same two processes (Caro and Gutierrez, 2007). This reinforces the relevance of the chromatin accessibility window very early in the cell cycle.

HOW DOES THE CHROMATIN LANDSCAPE AFFECT ORI SPECIFICATION?

One major and still unsolved question in the DNA replication field is how pre-RC assembly is directed to chromatin. This has been approached in different models, and the initial, and apparently disparate, outcome of these studies seems to be crystallizing in an emerging view pointing to the chromatin landscape as a major player in the mechanism. Early studies in budding yeast (*Saccharomyces cerevisiae*) revealed that the location of pre-RC assembly in the genome was strictly defined in a DNA sequence-specific manner. Thus, pre-RC has a strong affinity for the autonomously replicating sequence (ARS, an 11–bp sequence that is sufficient for pre-RC binding; DePamphilis and Bell, 2011; Mechali, 2010). Still, from the ~12 000 ARS consensus sequences that exist in the yeast genome, only ~400 initiate replication. This observation suggests that, although being sequence-dependent, the ORI specification mechanism in yeast contains an additional layer of regulation. Indeed, nucleosome organization was found to be a crucial factor in the origin selection step (Eaton *et al.*, 2010). The use of other models soon revealed that the budding yeast situation is rather unique in nature. In the fission yeast (*Schyzosaccharomyces pombe*), pre-RC assembly is not mediated by a short and specific DNA sequence, but by A + T richness (Segurado *et al.*, 2003; Heichinger *et al.*, 2006). The increase in genome size in multicellular organisms introduced several constraints because animal and plant cells do not have any indication of sequence specificity for pre-RC binding and assembly. Thus, genome-wide studies in *Drosophila* (MacAlpine *et al.*, 2004; Eaton *et al.*, 2011) and mammalian (Cadoret *et al.*, 2008; Sequeira-Mendes *et al.*, 2009; Mesner *et al.*, 2013) cells have confirmed that although pre-RC and ORIs are not specified by a DNA sequence, they do not locate at random in the genome. These studies have also revealed that the ORI sites show a large diversity in terms of sequence context, adding complexity to the identification of the signature contributing to ORI specification in multicellular organisms.

In vitro experiments have demonstrated that mammalian ORC binding to DNA is independent of a specific DNA sequence (Vashee *et al.*, 2001; Remus *et al.*, 2004; On *et al.*, 2014); however, pre-RC binding *in vivo* occurs preferentially at certain genomic sites, suggesting that, at least in multicellular organisms, the chromatin landscape may act as a key determinant for pre-RC assembly at specific sites (Figure 1). In support of this, the site-specific histone acetylation induced by the assembly of a transcription domain is sufficient to select a DNA replication origin (Danis *et al.*, 2004). Moreover, the histone acetylase Hbo1, an Orc1/2-, Cdc6- and Mcm2-interacting protein, acetylates these pre-RC components and is required for the final assembly of the MCM complex to complete pre-RC formation (Iizuka and Stillman, 1999; Burke *et al.*, 2001; Iizuka *et al.*, 2006; Miotto and Struhl, 2010). Interestingly, Arabidopsis encodes the HAM1/2 (HISTONE ACETYLASE WITH A MYST DOMAIN) acetylases, homologous to the human Hbo1 in their MYST domain, which may perform similar roles in pre-RC assembly.

It must be kept in mind that whatever the mechanism that functions to direct pre-RC to specific genomic sites, only a relatively small fraction of these ORC-bound genomic sites initiate replication in a given cell cycle. Besides providing robustness to the system with a backup set of ORIs in the case of replicative stress, this can be also related to the observation that several ORC subunits have non-replicative functions (reviewed in Sanchez *et al.*, 2012; Scholefield *et al.*, 2011).

The challenge to identify the signature of ORIs in the genome, which seems to involve both DNA sequence and chromatin signals, is a fundamental aspect of DNA replication control. Learning the combination of molecular features in DNA and chromatin that confer a given genomic site the capacity to function as an ORI would be a major advance. Genome-wide studies have served to demonstrate the association of ORIs with their chromatin landscape. An Arabidopsis 'originome', which is the collection of all possible ORIs detectable in a given experimental setting, e.g. Arabidopsis cultured cells, has recently been obtained (Costas *et al.*, 2011b). This study identified ~1500 ORIs that, based on the protocol used (pulse-labeling with BrdU of cells synchronized at the G1/S boundary), are likely to be enriched in ORIs active in early S phase. The meta-analysis revealed that the putative initiation sites, i.e. the midpoint of ORI regions (±100 nt), tend to colocalize with the 5′ end of genic regions, are slightly enriched in G + C content, and show a relatively low cytosine methylation (5mC) level. H3K4me2 and H3K4me3 are histone modifications also associated with Arabidopsis ORIs, whereas H3K4me1 is not. Furthermore, as expected from

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the anticorrelation between 5mC and the histone H2A.Z variant (Zilberman *et al.*, 2008), Arabidopsis ORIs also appear to be enriched in H2A.Z. Further studies also revealed that Arabidopsis ORIs colocalize with regions of high nucleosome density, histone H3.1 and H3.3 (Stroud *et al.*, 2012b). As the association of H2A.Z and H3.3 in the same nucleosome confers instability (Jin and Felsenfeld, 2007), it is conceivable that the colocalization of H2A.Z and H3.3 with ORI sites may contribute to establish regions of more accessible chromatin and favor the binding of pre-RC components. Remarkably, most if not all of the chromatin features of Arabidopsis ORIs are also typical of animal ORIs, including histone modifications (Cadoret *et al.*, 2008; Sequeira-Mendes *et al.*, 2009; Karnani *et al.*, 2010) and nucleosome enrichment (Lombrana *et al.*, 2013). This probably reveals that mechanisms emerged early in the evolution of multicellular organisms have been maintained in the more recent lineages (Table 2).

Immunofluorescence experiments have demonstrated that an increase in histone acetylation correlates with the S phase in several plants (Jasencakova *et al.*, 2001, 2003; Mayr *et al.*, 2003). This has been confirmed in genomic studies where ORIs were found to associate with sites enriched for H4K5K8K12K16ac (Sanchez and Gutierrez, 2009; Costas *et al.*, 2011b). In addition to this correlation, histone acetylation is relevant to define replication timing (Lee *et al.*, 2010; Costas *et al.*, 2011a; see below). In other eukaryotes a correlation between histone acetylation, ORI specification and replication timing has been reported (Vogelauer *et al.*, 2002; Aparicio *et al.*, 2004; Danis *et al.*, 2004; Hartl *et al.*, 2007; Schwaiger *et al.*, 2009).

Although the preferential association of ORIs with certain histone modifications is clear, it is also true that a considerable number of ORIs are located in places with very different combinations of marks. In an effort to rationalize the histone modification data along the genome, several epigenome maps of Arabidopsis chromosome 4 were analyzed to classify the entire length into four main chromatin states: active, repressed, silent, and intergenic domains (Roudier *et al.*, 2011).

A subsequent study comprehensively analyzed the Arabidopsis genome landscape using 15 epigenetic modifications, the histone H3.1 and H3.3 content, and DNA features, e.g. skew (Sequeira-Mendes *et al.*, 2014). This allowed the identification of prevalent combinations of chromatin features or signatures that define nine distinct chromatin states. The different patterns also characterized functional elements in the genome, such as promoters and transcriptional start sites (TSSs), potential enhancers, active and repressed transcriptional units, polycomb group (PcG)-regulated regions, intergenic domains, and two classes of heterochromatin (based on A + T content; Sequeira-Mendes *et al.*, 2014).

This information on relatively few chromatin states that cover the entire genome, as well as their topographical relationships, should be instrumental to determine the precise signature of different types of ORIs. Before full details in this direction are available, what seems to be

Table 2 A summary of features associated with DNA replication origins in yeast, *Drosophila melanogaster*, *Arabidopsis thaliana* and mammalian cells[a]

	Saccharomyces cerevisiae	*Saccharomyces pombe*	*Drosophila melanogaster*	*Arabidopsis thaliana*	Mammals
DNA consensus sequence	Yes	No	No	No	No
ORC sequence specificity	Yes	No	No	No	No
GC content	Low	Low	High	High	High
G quadruplexes	No	No	Yes	N/A	Yes
Genomic location	Mostly intergenic	Mostly intergenic	Mostly transcription units	Mostly transcription units	Mostly transcription units
Chromatin marks	H3K4me2				
	H3K36me1	N/A	H3.3	H3K4me2	H4K20me1
	H3ac		H3ac	H3K4me3	H4ac
	H4ac		H4ac	H4K5ac	
				H2A.Z	
Association with nucleosome-depleted regions	Yes	Yes (Lantermann *et al.*, 2010) No (Soriano *et al.*, 2013)	Yes	N/A	Yes

N/A – no available data.

[a]References for this table: general (Sequeira-Mendes and Gomez, 2012; Leonard and Mechali, 2013; MacAlpine and Almouzni, 2013); *S. cerevisiae* (Field *et al.*, 2008; Pryde *et al.*, 2009; Eaton *et al.*, 2010; Unnikrishnan *et al.*, 2010; Rizzardi *et al.*, 2012); *S. pombe* (Lantermann *et al.*, 2010; Givens *et al.*, 2012; Xu *et al.*, 2012; Mojardin *et al.*, 2013; Soriano *et al.*, 2013); *D. melanogaster* (Aggarwal and Calvi, 2004; Macalpine *et al.*, 2010; Cayrou *et al.*, 2011, 2012; Liu *et al.*, 2012); *A. thaliana* (Costas *et al.*, 2011b); mammals (Tardat *et al.*, 2007; Cadoret *et al.*, 2008; Sequeira-Mendes *et al.*, 2009; Miotto and Struhl, 2010; Cayrou *et al.*, 2011, 2012; Besnard *et al.*, 2012).

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clear is that ORIs located in different genomic regions possess different signatures. In other words, ORIs do not have a unique chromatin signature but several chromatin signatures, although they may tend to prefer regions of more open chromatin within the range of each chromatin state.

REPLICATION TIMING: WHEN TO FIRE ORIS?

The existence of thousands of ORIs working in a cell in each S phase to replicate the large eukaryotic genomes is probably at the basis of the need for temporal control of ORI activation (Bryant, 2010; Mechali, 2010; Jackson *et al.*, 2012; Sanchez *et al.*, 2012). Earlier data using DNA fiber autoradiography revealed that the Arabidopsis genome is organized into two major families, separated according to their activation time during the S phase: early- and late-replicating domains (Van't Hof *et al.*, 1978), a situation similar to other eukaryotes (Jasny and Tamm, 1979; Van't Hof and Bjerknes, 1981; Quelo and Verbelen, 2004; Hiratani *et al.*, 2009; Bryant, 2010; Aparicio, 2013). Studies at the cytological level have been confirmed by genomic approaches that additionally served to link replication timing control with chromatin organization (Lee *et al.*, 2010). The temporal profile of Arabidopsis chromosome 4 was obtained after sorting BrdU-labeled nuclei in the early, mid and late S phase. Most euchromatin is replicated in early/mid S phase, whereas heterochromatin (and the remaining euchromatin) is replicated later. As in the case of ORI specification, the control of replication timing seems to be also closely associated with a particular chromatin landscape. Early/mid replicating regions correspond to open chromatin states enriched in H3K56ac and, as expected, depleted in H3K9me2 and 5mC, typical marks of heterochromatin, which is enriched in late-replicating regions.

H3K56ac is also associated with initiation zones within patches of heterochromatin; however, a direct link with or requirement for H3K56ac in ORI activation is still missing. It must be noted that this histone modification plays a crucial role in nucleosome deposition past the sites of DNA synthesis, both during replication and repair (Han *et al.*, 2007; Chen *et al.*, 2008; Li *et al.*, 2008). An involvement of this mark in histone turnover at promoters has also been reported (Rufiange *et al.*, 2007).

THE RESTRICTION TO REPLICATE ONCE PER CELL CYCLE

The common situation is that an activation event of a given ORI results in the replication of a genomic region, ~10–200 kb in length, called a replicon unit. It is of primary importance for genome integrity that each genomic region is duplicated once in every cell cycle (only once and not less than or more than once). Otherwise, should multiple initiation events occur at the same ORI in the same S phase, a genomic region would become over-replicated. On the contrary, failure in activating some ORIs could lead to under-replicated regions. In both cases, they would have deleterious consequences during chromosome segregation. It is worth noting that endoreplication is not a case of altered function of the re-replication control, since during the endocycle, full genome replication occurs in each cycle, instead of an abnormal re-replicated genome part (Edgar *et al.*, 2014). The major mechanism evolved to prevent re-replication consists of the regulation of CDK (CYCLIN DEPENDENT KINASE)/cyclin activity during the cell cycle. As CDK/cyclin activity is low, the pre-RCs can be loaded and selected for activation, processes that are prevented once CDK/cyclin activity increases (Arias and Walter, 2007), ensuring the firing of ORIs once per cell cycle. Therefore, the strategies used to avoid genome re-replication rely on changing the subcellular localization of some pre-RC components, targeting them for proteasome degradation, or inhibiting their function by interaction with other proteins, with geminin being the more highly characterized protein. In the case of *Drosophila*, geminin has been implicated in controlling heterochromatin re-replication (Ding and MacAlpine, 2010).

These mechanisms have not been yet identified in plants. As it occurs in animal cells, where the mechanisms of re-replication control are species-specific, it is also likely that a variety of mechanisms have evolved in plants. Furthermore, based on the conservation of pre-RC components and of the cyclic changes in CDK/cyclin activity in the plant cell cycle, it is also likely that the mechanisms controlling re-replication in plants are similar, suggesting that they are very ancient in evolution. In any case, animal geminin seems to be an animal invention, as plants lack a geminin homologous protein (Caro and Gutierrez, 2007); however, a role of the plant CDT1-interacting protein GEM in re-replication control has not yet been explored.

More recently, different regulatory layers that rely on chromatin landscapes have been reported. Pre-RC assembly at ORIs in animal cells needs H4K20me1, a modification introduced by the Set7 histone methyltransferase and removed by PHF8 (PHD FINGER PROTEIN 8) demethylase (Yang and Mizzen, 2009; Tardat *et al.*, 2010). In this case, soon after ORI activation, Set7 is degraded by the proteasome in a PCNA (PROLIFERATING CELL NUCLEAR ANTIGEN)- and Cul (CULLIN)-Ddb1 (DNA DAMAGE BINDING PROTEIN 1)-dependent manner and, as a consequence, the levels of H4K20me1 fall sharply (Oda *et al.*, 2009; Liu *et al.*, 2010; Tardat *et al.*, 2010). Immunofluorescence experiments in Arabidopsis have detected H4K20me3 in euchromatin, whereas H4K20me1 is restricted to heterochromatin (reviewed in Desvoyes *et al.*, 2010; Fuchs *et al.*, 2006; Sanchez *et al.*, 2008); however, the presence of any form of H4K20 methylation has been questioned in Arabidopsis (Zhang *et al.*, 2007). Therefore, a potential role of H4K20 methylation in modulating re-replication needs to be carefully reevaluated in the future.

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Clearer and more direct evidence involving a histone modification in the avoidance of re-replication has been recently reported. H3K27me1, a typical mark of heterochromatin, is deposited by the methyltransferases ATXR5 and ATXR6 (ARABIDOPSIS TRITHORAX RELATED PROTEIN 5 and 6), which are transcriptional E2F targets and interactors of PCNA (Raynaud *et al.*, 2006; Jacob *et al.*, 2009). Leaf cells of the *atxr5 atxr6* double mutant show a significant increase in the content of pericentromeric sequences, as revealed by deep sequencing of genomic DNA extracted from nuclei of various ploidy levels (Jacob *et al.*, 2010). As a large fraction of extra DNA is located at specific loci within heterochromatic regions it seems that a certain level of H3K27me1 is needed to prevent heterochromatin re-replication. It is interesting that the re-replication phenotype of the *atxr5 atxr6* mutant is partially suppressed by mutations that reduce 5mC levels, such as *ddm1*, *met1* or *cmt3* (Stroud *et al.*, 2012a). A step for the future will be to determine whether the loci enriched in re-replicated DNA actually correspond to active ORIs, an approach that will require solving the challenge of identifying ORIs in whole organisms. Also, an unanswered question is how the presence of H3K27me1 is sensed and transmitted to the re-replication control pathway. One possibility is that an H3K27me1-specific protein reader binds and signals downstream to prevent further association of the pre-RC and/or its activation. Alternatively, the mechanism may depend on protein degradation of enzymes involved in H3K27me1 deposition, as has been demonstrated for the Set7 [SET domain: Su(var)3–3, Enhancer of Zeste, Trithorax] methylase. Indirect results point to a complex pathway responsible for the function of H3K27me1 in re-replication control. This mark occurs selectively on the canonical histone H3.1 by ATXR5 because H3.3 cannot accommodate it into the catalytic pocket because of steric hindrance provoked by a threonine in H3.3 (alanine in H3.1; Jacob *et al.*, 2014). This is consistent with the enrichment in H3.1 observed in heterochromatin (Stroud *et al.*, 2012b; Wollmann *et al.*, 2012). Some CDK/cyclin activity may also participate in the signaling cascade, as indirectly suggested by the enhanced phenotype of the *atxr5 atxr6* mutant when the CDK inhibitor CDK5 is overexpressed (Jegu *et al.*, 2013).

CHROMATIN REPLICATION

The process of genome replication requires the complex macromolecular organization of chromatin to be disrupted, either completely or partially, every time that the DNA replication advances. Then, chromatin must be reassembled again past the fork. This is a consequence of the specificity of replicative DNA polymerases to use naked DNA as a substrate. Thus, nucleosomes must be removed ahead of the replication fork and reassembled once the fork passes. The deposition of histone onto newly synthesized DNA does not occur in a passive association manner but is actively carried out by highly specialized histone chaperones, which are highly conserved in yeast, plants and animals (Table 1).

Two major classes of chaperones, NAP and NRP (Galichet and Gruissem, 2006; Zhu *et al.*, 2006), as well as CAF1 (CHROMATIN ASSEMBLY FACTOR 1; Das *et al.*, 2010; Polo and Almouzni, 2006; Ramirez-Parra and Gutierrez, 2007b), are in charge of depositing either histone H2A-H2B or H3-H4 dimers, respectively (Figure 1). In fact, CAF1 specifically transfers dimers containing the canonical H3.1 protein in a replication-dependent manner. Once it is introduced into the newly assembled nucleosome, histones H2A and H3 can be replaced by histone variants, a process that normally has consequences in gene expression and chromatin regulation. Thus, H3.1 is exchanged for the H3.3 variant by the HIRA (HISTONE REPRESSOR A) complex (Tagami *et al.*, 2004; Duc *et al.*, 2015) through a mechanism that is independent of DNA replication. There are other H3 chaperones identified in mammalian cells that exchange H3.3 in certain chromosomal locations. Some of them, e.g. putative ATRX (Alpha thalasemia/mental retardation/syndrome X–linked) and DEK proteins (Otero *et al.*, 2014; Waidmann *et al.*, 2014), are present in Arabidopsis. Recently, experimental data on DEK3 was reported showing that it is important to modulate the expression of downstream target genes relevant for the stress response (Waidmann *et al.*, 2014). Although it is considered within the chaperone class, ASF1 (ANTI-SILENCING FACTOR 1) actually functions as a stock of H3–H4 dimers (Zhu + 2006) before transferring them to either CAF1 or HIRA, depending on whether it is formed with H3.1 or H3.3, respectively. The H2A protein is also frequently replaced by H2A.Z, with the help of the SWR1 (SWI2/SNF2 RELATED 1) chaperone complex (March-Diaz and Reyes, 2009). The H2A.Z variant has been implicated in immunity (Lazaro *et al.*, 2008), repair and recombination (Rosa *et al.*, 2013), and temperature sensing (Kumar and Wigge, 2010).

The biochemistry of chromatin replication at the fork has been largely delineated in mammalian cells (Alabert and Groth, 2012; Alabert *et al.*, 2014). Given the similarities between most of the replication proteins in plants and animals, it is likely that the same general trends apply in all eukaryotes; however, it is at the regulatory level that differences may occur, as well as the phenotypic consequences of altered chromatin replication resulting from mutations in histone and chaperone genes. These disparities may be explained by the distinct developmental strategies. Studies in this direction are very scarce in animals because most mutations are embryonic-lethal, thus preventing easy phenotypic analysis in the adult. On the contrary, plants are very versatile and several hypomorphic mutations in chaperone-encoding genes have been described that are compatible with plant development and viability.

The relevance of histone chaperone beyond the step of nucleosome assembly or histone exchange is revealed by

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the pleiotropic phenotypes observed in chaperone mutants. Thus chaperones impact on cell division and organogenesis, and, in turn, in many cases their expression is cell-cycle regulated.

Arabidopsis contains two *ASF1* genes (*A* and *B*), which are E2F targets and act redundantly during development (Zhu *et al.*, 2011; Lario *et al.*, 2013). Vegetative growth is severely impaired because of cell division arrest; however, this is compensated by an increase in cell enlargement that, in this case, does not depend on an increase in nuclear ploidy by endoreplication (Zhu *et al.*, 2011). The phenotypic effects of the loss of function of ASF1 must be related to its biochemical activities of maintaining a stock of H3–H4 dimers, which are likely to be degraded when they are not bound to any chaperone.

Mutants in the three subunits of CAF1 are available. Still, only the two larger subunits (FASCIATA 1 and FASCIATA 2; FAS1 and FAS2) are directly related to chaperone function, because the smaller subunit MSI 1 (MULTICOPY SUPPRESSOR 1) is also found in other protein complexes (Ach *et al.*, 1997; Ausin *et al.*, 2004). Interaction with H3–H4 dimers is mediated by the large subunit FAS1 (Ridgway and Almouzni, 2000). Similar to that, the *ASF1* genes, *FAS1*, *FAS2* and *MSI1*, are all E2F targets (Ramirez-Parra and Gutierrez, 2007a), although only *FAS1* expression is cell-cycle regulated. Loss of CAF1 activity has various consequences at the cellular and organ level. Heterochromatin is less compact, its structure is lost and transposon elements are reactivated (Ono *et al.*, 2006; Schonrock *et al.*, 2006). This disrupted heterochromatin structure and function is surprisingly independent of the extent of H3K9me2 modification, which is maintained at wild-type levels (Ono *et al.*, 2006; Schonrock *et al.*, 2006). Ribosomal rDNA copies in *fas1* mutants are highly reduced and the telomeres are shortened with severe consequences for vegetative growth (Mozgova *et al.*, 2010; Muchova *et al.*, 2015). Perhaps the most striking phenotype at the cellular level is the early and systemic switch of the *fas1* cells to the endoreplication program, reaching ploidy levels much higher than those of wild-type cells (Exner *et al.*, 2006; Kirik *et al.*, 2006; Ono *et al.*, 2006; Ramirez-Parra and Gutierrez, 2007a). As a consequence, organs contain a reduced number of cells, leading to reduced organ size; however, this is very striking in roots but not in leaves, where a clear compensatory mechanism makes cells significantly larger than wild type cells, whereas the leaf size is only about half of the normal size (Ramirez-Parra and Gutierrez, 2007a).

The HIR complex consists of HIRA, UBN (UBINUCLEIN), and CABIN1 (CALCINEURIN BINDING PROTEIN 1), and has been recently identified in Arabidopsis (Duc *et al.*, 2015). Although HIRA functions independently of DNA replication, altered H3.3 deposition in *hira* mutants leads to defects in DNA replication at very early stages of mouse development (Lin *et al.*, 2014). This might be a consequence of lower chromatin accessibility as a result of reduced H3.3 levels. In plants, the phenotypes described for *hira* mutants are not directly related to genome instability; however, they exhibit defects in silencing of pericentromeric heterochromatin and lower nucleosome occupancy (Duc *et al.*, 2015). Interestingly, in a *fas1* mutant background, HIRA seems to have the ability to deposit new histones to restore nucleosome density. The consequences of this altered location of histone H3 along the genome, as well as the impact on development, should be evaluated in the future.

OUTLOOK

The high evolutionary conservation of replication proteins suggests that their biochemical relationships to form replication complexes, e.g. pre-RC, and to carry out their functions would be very similar, if not identical, across the eukaryotic scale; however, detailed analysis in different eukaryotic model systems, including yeast, animals, and plants, have revealed that major differences exist in the availability of replication components in different cell-cycle phases or cell types. Therefore, regulatory mechanisms have diversified considerably throughout evolution. Thus, learning the different mechanisms of controlling the availability of replication proteins could help understanding their relevance during the replication process. In this context, as chromatin dynamics has many cell cycle- and cell type-specific features during development, it is also expected that studies on the mechanisms of producing and degrading replication proteins should tell us details about chromatin organization, as a result of the interaction between replication proteins and chromatin components.

One of the major aims in the DNA replication field has been the search for a signature that can be associated with the presence of an active ORI. The data obtained in the early days in human oncoviruses and yeasts suggested that a single signature could determine ORI activity. This view has now changed considerably, and increasing evidence points to the existence of multiple signatures that specify ORIs and that are directly related to the location of ORIs in the genome. The identification of the landscape of chromatin states, which reduces the complexity of the eukaryotic genome, now available for Arabidopsis, *Drosophila* and human cells, is helpful to define different ORI types, depending on their genome location and chromatin landscape. A major challenge will be to identify ORI activity in cells of whole organisms, ideally in different proliferating cell types, and correlate them with the chromatin states present in that particular cell system.

In fact, this will be a necessary step towards determining whether the specific set of histone modifications around a given set of ORIs is cause or consequence of their activity as ORIs. A future step would be to manipulate the chromatin landscape and analyze the activity of individual ORIs in

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different genomic locations to infer from these data the causal relationship between chromatin modifications and ORI activity. Although this is not trivial, an additional complication to these studies is that normally it is a set of histone modifications, instead of a single one, that defines a particular chromatin state where ORIs can be ascribed.

In human cells, the presence of H4K20me is crucial to control re-replication and eventually genome stability. In Arabidopsis, high levels of H3K27me1 are necessary to avoid re-replication in pericentromeric heterochromatin. The question is whether other histone marks typical of certain genome regions are also relevant for re-replication control, either alone or as a consequence of chromatin proteins specifically bound to these marks. In any case, given the post-embryonic nature of plant growth and development, avoiding genome instability through the many successive cell divisions required to generate an adult plant is of primary importance. Directly related to plant development is the transition from the cell cycle to the endoreplication cycle. An unsolved question is whether the chromatin landscape of endoreplicating and proliferating cells is the same. And as a consequence, whether the ORIs used in endoreplicating cells occupy similar genome locations in proliferating cells and have the same chromatin signatures. The use of genomic approaches in combination with cell biology, biochemical, and molecular techniques should produce a significant synergy in future studies of chromatin replication.

ACKNOWLEDGEMENTS

We thank members of the CG laboratory for insightful discussions. This research has been supported by grant BFU2012-34821 from MINECO and by an institutional grant from Fundacion Ramon Areces to the Centro de Biologia Molecular Severo Ochoa.

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