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REVIEW ARTICLE

THE 'ORC'HESTRAL SYMPHONY IN DNA REPLICATION ACROSS THE SPECIES

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ABSTRACT

The onset of DNA replication which is the essential step in the reproduction of any cell requires specific interactions of initiator proteins at defined loci called origins. Although the origin size varies from prokaryotes to eukaryotes, the origin recognition complex which is one of the major players initiating DNA replication across the species is conserved. The orchestrated binding of ORC facilitates DNA melting which in turn is the inception for the duplication of the DNA thus helping to emanate bidirectionally from the origins. The conserved DNA binding domains, ATP regulation and the structural properties of the DNA are the key features of the replication machinery. The ORC proteins exhibit a multifaceted role besides its fundamental role in DNA replication. The ORC mutations leads to various diseases.

INTRODUCTION

Faithful duplication of the genetic material followed by its segregation equally into two daughter cells during cell division is a fundamental process of life. This high fidelity DNA replication occurs during the S phase of the cell cycle and it is orchestrated by multiple proteins. According to the replicon model proposed by Jacob *et al.*, 1963, the DNA replication starts at a specific *cis*-acting genetic element called replicator with the help of *trans*-acting regulatory proteins, called initiators. Although the replicon model intended to explain the replication from a single origin of circular bacterial chromosomes, it was quickly adopted as the paradigm for the eukaryotic chromosomes as well.

Among the eukaryotes, replicators were first isolated in budding yeast in 1970s as small genomic DNA fragments facilitating autonomous plasmid replication and were named as Autonomously Replicating Sequence (ARS) elements (Struhl *et al.*, 1979). The hunt for initiator proteins in eukaryotes picked up pace after the discovery of the Origin Recognition Complex (ORC) comprising of six protein subunits in budding yeast in 1992 (Bell and Stillman, 1992). These proteins are conserved from yeast to humans W.R.T functions (Bell and Dutta, 2002). In this review, we focus on the ORC conservation and diversity across diverse species and their regulation.

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Initiator proteins in prokaryotes

In prokaryotes, single initiators (bacterial DnaA), the highly conserved proteins bind to recognition sites and assemble into complexes on DNA. The energy to form higher-order structures to mediate localized DNA melting at the origin is rendered by ATP that binds to DnaA (Bramhill and Kornberg, 1988; Duderstadt *et al.,* 2011; Erzberger *et al*., 2006; Katayama *et al*., 2010; Ozaki *et al*., 2008). As localized DNA unwinding is required for the DnaA to bind, ATP binds to DnaA which further recruits the DnaB helicases to the origin. In *E. coli,* replication starts at a single genomic locus called oriC which contains multiple DnaA (initiator protein) binding sites and proceeds bidirectionally. DnaA binds to these sequences and opens an adjacent AT-rich element (Kohzaki and Murakami, 2005; Hwang and Kornberg, 1992; Gilbert 2001).

Halobacterium encodes a total of 10 Orc1 / Cdc6 homologues. In *Halobacterium spp*., the presence of two origins has been proposed (Kennedy *et al.,* 2001). The *Sulfolobus* origin has sequence repeats that are related to a core inverted repeat present in the full Origin Recognition Box (ORB) elements which were capable of binding Cdc6-1 (Robinson N.P *et al.,* 2004). *M. jannaschii* and *Methanopyrus kandleri* do not have a clear homologue of Cdc6/Orc1, although a putative homologue has been reported for *M. jannaschii* (Aravind and Koonin, 1999; Liu *et al*., 2000). Recent studies in *S. islandicus* demonstrated that none of the Orc1/Cdc6 genes are essential for viability (Samson *et al.,* 2013). Although the archaeal

Aeropyrum pernix Orc1 proteins that are AAA+ (ATPases associated with various cellular activities) proteins are reported to undergo ATP hydrolysis, the functional consequences of ATP binding and hydrolysis are not yet elucidated (Singleton *et al.,* 2004). There seems to be a sequence dependent binding of initiator proteins in prokaryotes.

ORC in parasites / protozoans

In *Plasmodium falciparum* DNA pol α, DNA pol δ, PCNA, RPA1, ORC1, and MCM4 are expressed during those stages of the asexual phase where DNA replication occurs (Chavalitshewinkoon *et al.,* 1993; Horrocks *et al.,* 1996; Voss *et al.,* 2002; Gupta *et al.,* 2006 and Mehra *et al.,* 2005). PfORC1 was found to be essential for initiation of DNA replication (Mehra *et al.,* 2005) and the N-terminal PfORC1 is involved in silencing *var* gene family (Deshmukh *et al.,* 2012). The PfORC5 is functionally characterized and is found to contain ATP binding activity (Gupta *et al.,* 2008). In *Tetrahymena thermophila,* an ORC-like protein complex containing an integral RNA subunit is largely responsible for rDNA origin recognition and interaction (Mohammad *et al.,* 2007). The only ORC ortholog, ORC1 in *Leishmania major* is found to be expressed in actively dividing *Leishmania* promastigotes (Kumar *et al.,* 2008). The only annotated ORC1/CDC6 (El-Sayed *et al.,* 2005) of *Trypanosoma spp* is confirmed as a prereplication machinery component as it replaced yeast CDC6 in a yeast phenotypic complementation assay and also silencing of Trypanosome ORC1/CDC6 expression by RNA interference in *T.brucei* impaired the DNA replication (Godoy *et al.,* 2009). RNAi against TbORC1/CDC6 resulted in loss of VSG silencing in *T. brucei* (Tiengwe *et al.,* 2012 and Benmerzouga *et al.,* 2013). Both *T. cruzi* and *T. brucei* Orc1/Cdc6 exhibit ATPase activity (Calderano *et al.,* 2011).

ORC in eukaryotes

The *S.cerevisiae* ORC (ScORC) forms a stable hetero-hexameric complex binding to the specific DNA sequences throughout the cell cycle (Gibson *et al.,* 2006). ScOrc1 to ScOrc5 contain a predicted AAA+ domain which is essential for DNA binding. Of these AAA+ containing ORC subunits, only Orc1, Orc2, Orc4 and Orc5 appear to have direct contact to the origin DNA (Clarey *et al.,* 2006; Lee and Bell, 1997 and Speck *et al.,* 2005). Orc3 helps in forming the stable complex without directly binding to the DNA whereas the Orc6 does not bind to the DNA but helps in recruiting multiple Cdt1 molecules (Asano *et al.,* 2007; Chen and Bell 2011; Chen *et al.,* 2007 and Takara and Bell, 2011). The structure – function relationships of the ORC reveals various DNA binding motifs such as Walker A and B motifs and ATPase domains. The BAH module plays an important role by linking DNA methylation, replication and transcriptional regulation (Callebaut *et al.,* 1999). The repertoire of ORC and accessory proteins such as Cdc6, Cdt1 and Mcm2-7 help in the assembly of pre-replication complex at origins.

Besides replication, ScORC plays a role in silent mating type loci (HMR and HML) which is known from the genetic screens that identified mutations in *ScORC2* and *ScORC5* as defective for mating type silencing (Foss *et al.,* 1993; Micklem *et al.,* 1993). Also, they were supported by the ACS matches in all four mating-type silencers (*HMRE, HMRI, HMLE* and *HMLI*) that act as replication origins in plasmids (Dubey *et al.,* 1991). A novel mechanism for sister chromatid cohesion is mediated directly by ScORC2 that is Sir protein independent (Shimada and Gasser, 2007). Co-immunoprecipitation of Yph1 along with ScORC has demonstrated the role of ScORC in 60S ribosomal subunit biogenesis (Du and Stillman, 2002).

As a case of exception, the SpORC uses the 9 AT-hook motifs located in the N-terminal of ORC4 to bind to asymmetric AT-rich sequences. Although they often contain asymmetrically distributed clusters of A's and T's, they do not contain any specific consensus sequence analogous to the *S. cerevisiae* ACS. This pattern is required for the ARS activity (Zhu *et al.,* 1994; Clyne and Kelly, 1995; Dubey *et al.,* 1996; Kim and Huberman, 1998; Okuno *et al.,* 1999). The non-replication role of SpORC was elucidated by the ChIP experiments in fission yeast that localizes ORC1 and ORC4 to the kinetochore in the absence of Mcm6 (Hayashi *et al.,* 2007).

DmORC isolated from *Drosophila* embryo nuclear extract as a stable complex (Gossen *et al.*, 1995) is cell cycle-dependent, and is regulated by the degradation of ORC1 *via* the ubiquitin proteasome pathway at the late M phase (Araki *et al.,* 2003). DmORC1 is resynthesized during late G1-phase. DmORC1 is also an ATPase and is involved in ATP-dependent DNA binding (Chesnokov *et al.,* 2001). In contrast to ScORC6, which is not required for DNA binding, the DmORC6 is required for the DNA binding and is an integral part of the DmORC complex (Chesnokov *et al.,* 2001). The DmORC6 alone has DNA binding activity, likely due to the predicted TFIIB-like DNA binding domain in the smallest subunit (Liu *et al.* 2011). DmORC binds DNA with little sequence specificity. DmORC localizes to open chromatin regions that are depleted of nucleosomes (MacAlpine *et al.,* 2004, 2010) and therefore DmORC may target the topology rather than the sequence of the origin DNA (Remus *et al.,* 2004). Mutations in DmORC subunits causes DNA replication defects, but cells are also observed to arrest in mitosis, although this has been attributed to DNA damage as a result of incomplete DNA replication (Chesnokov *et al.,* 2001; Loupart *et al.,* 2000; Pflumm and Botchan 2001). DmORC subunits, however, localize to centromeric heterochromatin and also bind the HP1 protein that is associated with heterochromatin (Badugu *et al.,* 2005; Huang *et al.* 1998; Pak *et al.* 1997; Shareef *et al.,* 2001; 2003) and a similar interaction is also observed between XORC and *Xenopus* HP1. A mutation in DmORC suppresses heterochromatin-dependent transcriptional repression (*i.e.,* position-effect variegation) (Pak *et al.* 1997). In Drosophila, defects in ORC2, ORC4 or ORC5 mutations lead to abnormally condensed chromosomes (Loupart *et al.,* 2000, Pflumm and Botchan 2001, Page *et al.,* 2005). DmORC2 and DmORC3 localize to the pre-synaptic terminals of terminally differentiated motor neurons, and ORC3 mutations result in defects in learning and synaptic plasticity (Pinto *et al.,* 1999; Rohrbough *et al.,* 1999).

Recently Yang *et al.* 2010 cloned the cDNA sequences and genomic sequences of all six ORC subunits of the Lepidoptera

Bombyx mori (silk worm). The six BmORC subunits had evolved individually from ancestral genes in early eukaryotes. Although the six genes were co-regulated during the embryo development, the quantitative RT-PCR results shows differential expression of these genes in thirteen tissues of the 5th-instar day-6 larvae. BmORC proteins may have additional functions as they also participate in the replication of BmNucleopolyhedrovirus.

The assembly of replication competent chromatin involves the sequential binding of ORC and MCMs to DNA in Xenopus. Immunodepletion of ORC inhibited initiation of DNA replication in a *Xenopus* egg extract, suggesting that ORC is essential for initiation of DNA replication (Romanowski *et al.,* 1996; Rowles *et al.,* 1996; Carpenter *et al.,* 1996). XORC initiates DNA replication preferentially at sequences targeted in *S. pombe* (Kong *et al.,* 2003).

Recombinant human Orc4 alone was shown to bind the lamin B2 origin DNA *in vitro* and in an ATP independent manner (Stefanovic *et al.,* 2003). Human ORC binds to the latent replication origin of Epstein–Barr virus in B cells where it is required for the maintenance of the EBV plasmid (Chaudhuri *et al.,* 2001; Dhar *et al.,* 2001b ; Julien *et al*., 2004) . The purified human ORC when added to ORC-depleted *Xenopus* egg extracts was shown to be capable of promoting the initiation of DNA replication from any DNA sequence *in vitro*, with no preference for human origin sequences showing its lack of DNA sequence specificity (Vashee *et al.* 2003). The six subunits of human ORC were first identified by sequence similarity to their yeast counterparts (Dhar *et al.* 2001a; Dhar and Dutta 2000; Gavin *et al.* 1995 ; Siddiqui and Stillman 2007 ; Tugal *et al.,* 1998 ; Vashee *et al.,* 2001). The assembly of ORC and the stability of the complex are both ATP-dependent (Ranjan and Gossen 2006).

In humans, the siRNA studies gives a clear evidence of the role of ORC2 localization with tubulin at centrosomes throughout the cell cycle demonstrating its role in proper spindle attachment to the kinetochores (Prasanth *et al.,* 2004) whereas ORC6 was found to localize to kinetochores specifically during mitosis (Prasanth *et al.,* 2002). The stage specific expression of ORC in different mammalian tissues, including terminally differentiated and non-proliferating tissues (Thome *et al.,* 2000) is found to play an important role in the development of the organism.

The cloning of subunit ORC2 from *Arabidopsis thaliana* (Gavin *et al.,* 1995) and of ORC1 from rice (Kimura *et al.,* 2000) has made it likely that multisubunit ORC complexes might function in plants in ways similar to the other eukaryotes. A two-hybrid binary interaction assay in *A.thaliana* suggests that AtORC3 plays a central role in maintaining the complex associations in preRC assembly. Two homologs of ORC1 – AtORC1a and AtORC1b were identified in *A. thaliana* which is a unique feature of the Arabidopsis genome. The diverse expression profiles of the AtORC4 suggest its role in differential regulation during development. The high AtORC1 expression in non-proliferating plant tissues suggests that they might have additional role besides DNA replication licensing (Masuda *et al.,* 2004). AtORC2 is found to play a role connecting DNA replication to chromosome structure (Collinge *et al.,* 2004). The expression of AtORC genes occurs in a cell cycle-dependent manner; the two AtORC1 (a and b) genes are differently expressed in proliferating and endoreplicating cells (Trivino *et al.,* 2005). In maize, ZmORC3 is the only gene expressed in green leaves where the expression of the other genes is negligible demonstrating the tissue specific role of ORC (Witmer *et al.,* 2003).

The pre-replicative complex assembly, the ATP regulation and the DNA structural plasticity – the well orchestrated mechanism of ORC

By analogy, the ORC assembly and complex formation resembles the well orchestrated music symphony (Figure 1). The normal cell cycle that consists of four phases: G1-phase (preparation for DNA replication), S-phase (DNA replication), G2 phase (preparation for mitosis) and M-phase (mitosis) is highly regulated. The decision to undergo division or remain in quiescence is taken following mitosis. Once cells are committed to undergo DNA replication, they must complete the process of cell division or die. Thus, commitment to DNA replication is the primary point at which cells regulate their division cycle. Reinitiation of DNA replication at the same replication origins during a single cell division cycle is prevented in eukaryotic cells by regulating at least three steps in the assembly of pre-RCs – association of Cdc6, Cdt1 and Mcm(2–7) proteins with chromatin. A fourth regulatory step in which ORC activity is inhibited during the G1 to S-phase transition and not reestablished until mitosis is complete and a nuclear membrane has reassembled. Therefore, regulation of ORC activity becomes the premier step in determining when DNA replication begins. Cell cycle dependent changes in ORC activity and in some cases the affinity of one or more ORC subunits for chromatin is referred to as 'the ORC cycle' (DePamphilis, 2003).

The ordered assembly of ORC, Cdc6p, Cdt1p and Mcm2–7p and a number of replication factors is the preRC formation. The cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK) activate the helicase and load the replisome which is termed as "DNA replication licensing" is a crucial regulation step of eukaryotic DNA replication (Blow and Gillespie, 2008). In yeast cells, ORC remains bound to the replication origins throughout the cell cycle, but ORC is phosphorylated during the S to M periods, and this phosphorylation inhibits its ability to assemble a pre-RC. In *S. pombe*, ORC and Sap1 bind to the origins during G1-S phase transition in cell cycle that helps in loading CDC18, the CDC6 homolog in fission yeast. The presence of Sap1-like protein is not known in metazoans (Sun and Kong, 2010). In Xenopus egg extract, ORC binds to sperm chromatin, but the stability of ORC/chromatin sites is reduced following pre-RC assembly by its phosphorylation by Cdk1/cyclin A during G2/M. In mammalian cells, selective destabilization of the Orc1 subunit from the chromatin occurs when cells enter S-phase by mono-ubiquitination (Ub) and in some cases polyubiquitination ([Ub]n) and then degraded. Thus, the manifestations of ORC cycle in yeast, frogs and mammals suggests stable ORC/chromatin sites that can initiate assembly of a pre-RC

that are not present until mitosis is complete and a nuclear membrane is present.

The requirement of ATP by ORC proteins to interact specifically with origin DNA (except in *S.pombe* ORC (SpORC) is another important criteria. In all the species studied so far, ORC1p, ORC4p and ORC5p contain potential ATP binding sites. ATP hydrolysis by ORC to regulate DNA binding is well studied in the budding yeast (ScORC) and in *Drosophila melanogaster* ORC (DmORC) (Klemm *et al.,* 1997; Chesnokov *et al.,* 2001). Hence there is an ordered assembly of the pre-RC proteins and the replication machinery is well regulated by ATP regulation.

This orchestrated mechanism not only coordinates the ordered assembly of ORC but also alters the structural properties of the DNA locally thus making the environment thermodynamically favourable which in turn recruits other replication proteins thus making replication feasible. Stress-induced duplex destabilization (SIDD) studies in *S.cerevisiae* origins (ORC binding sites) were found to be consistent with that of the duplex unwinding element (DUE) (Prashanth Ak and Benham, 2005). Similar studies in fission yeast ORC binding sites proximal to the destabilized regions (origins) in DNA further confirms the DNA plasticity as an important aspect of DNA unwinding and replication (Yadav *et al.,* 2012). Thus the regulation is conferred by the orchestrated assembly of ORC along with the inherent superhelicity of the DNA irrespective of the sequence dependent or independent nature, ATP requirement from prokaryotes to higher eukaryotes.

Conclusion

ORC is a pivotal regulator of various cellular processes. Notably, a number of reports connect ORC to numerous human diseases (Shen, 2013), including Meier–Gorlin syndrome (Bicknell *et al.,* 2011a and b), EBV (Epstein–Barr virus)-infected diseases (Tao *et al.,* 2006), American trypanosomiasis and African trypanosomiasis (Dang and Li, 2011). However, much of the underlying molecular mechanism remains unclear. In those genetic diseases, mutations in ORC alter its function and lead to the dysregulated phenotypes; whereas in some pathogen-induced symptoms, host ORC and archaeal-like ORC are exploited by these organisms to maintain their own genomes. Moreover, ORC plays significant role in silencing and transcriptional regulation, S-phase check point regulation, mitotic chromosome assembly, sister chromatid cohesion, coordinating cytokinesis, ribosome biogenesis, tissue specific roles besides its role in replication. Evolution, speciation and development of the organisms has although caused divergence in the species, the basic ORC machinery that orchestrates DNA replication is found to be conserved across all the species studied so far. Further characterization of ORC genes in other organisms may provide new insights to its diverse functions that may or may not couple with the DNA replication process.

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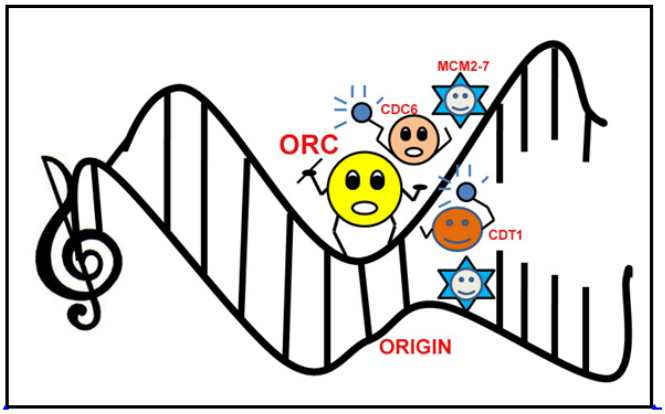

Figure 1. Cartoon depicting the orchestral assembly of preRC proteins binding to the DNA replication origin

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