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The many faces of the origin recognition complex

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The hetero-hexameric origin recognition complex (ORC) is well known for its separable roles in DNA replication and heterochromatin assembly. However, ORC and its individual subunits have been implicated in diverse cellular activities in both the nucleus and the cytoplasm. Some of ORC's implied functions, such as cell cycle checkpoint control and mitotic chromosome assembly, may be indirectly related to its roles in replication control and/or heterochromatin assembly. Other suggested roles in ribosomal biogenesis and in centrosome and kinetochore function are based on localization/interaction data and are as yet inconclusive. However, recent findings directly link ORC to sister chromatin cohesion, cytokinesis and neural dendritic branching.

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Introduction

The hetero-hexameric origin recognition complex (ORC) was originally identified as an origin binding protein complex in budding yeast, and homologues of all six subunits (ORC1–6) have since been identified in all eukaryotic species examined, including humans [1,2]. The best studied aspect of ORC is its function in binding directly to replication origins and recruiting Cdc6, Cdt1 and Mcm2–7 proteins to form pre-replication complexes (pre-RCs) upon exit from mitosis [3]. However, ORC also plays a role in transcriptional silencing of the mating type loci in budding yeast, and an analogous role in heterochromatin formation in mammalian cells. More recently, additional roles for ORC and its subunits have emerged (Figure 1).

Here, we summarize recent findings on the non-replication roles of ORC. In particular, we address whether these roles represent independent functions for ORC or can be accounted for indirectly by ORC's role in replication. For those roles that do appear to be independent, we consider reasons why they might be mediated by the same protein complex. We will not discuss the roles of ORC in regulation of DNA replication or programmed DNA amplification, as they have been the focus of several excellent prior reviews [1,2,4]).

Silencing and transcriptional regulation

The first non-replication function for ORC to be recognized was its participation in the establishment of transcriptionally silent chromatin domains at the budding yeast silent mating type loci (HMR and HML). Genetic studies identified mutants of ORC that were active for either replication or silencing, demonstrating that separate protein domains mediate these independent functions of ORC [1]. The N-terminus of the largest ORC subunit, ORC1, recruits the silent chromatin protein Sir1 [5•,6•]; this appears to be the primary role of ORC in silencing, since targeting Sir1 directly to an altered silencer via a DNA binding domain can bypass the need for ORC. This appears to be a conserved eukaryotic role for ORC, as both *Drosophila* [7] and mammalian [8–11] ORC interact with HP1 through the bromo-adjacent homology (BAH) domain on the N-terminus of ORC1 [8]. Furthermore, specific ORC2 mutations in *Drosophila* [7] result in delocalization of HP1 and reduced spreading of heterochromatin (i.e. act as suppressors of position-effect variegation) and siRNA knockdown of ORC2 in human cells results in de-localization of HP1 [9].

Silencing refers to the assembly of a region of repressive chromatin that self-propagates to spread across many nucleosomes. However, ORC may also mediate localized promoter-specific gene repression. Genome-wide transcriptome analyses in budding yeast have revealed a class of genes that are induced by the orc2-1 mutation [12•,13•]. These genes are also induced when cells withdraw from the cell cycle during starvation. Moreover, starvation-induced genes cluster near ORC binding sites, and the ORC binding site at one of these genes was shown to be required for repression [12•,13•]. Since ORC is central to proliferation, it will be interesting to determine whether ORC down-regulation in metazoans induces a related set of genes that facilitate withdrawal from the cell cycle during terminal differentiation.

How did the replication and transcriptional repression functions of ORC come to be carried out by the same protein complex? One possibility is that this situation is the result of co-evolution of ORC with repressor proteins. The fact that origins preferentially localize to intergenic regions, which in streamlined genomes such as budding yeast puts them in close proximity to promoters, would

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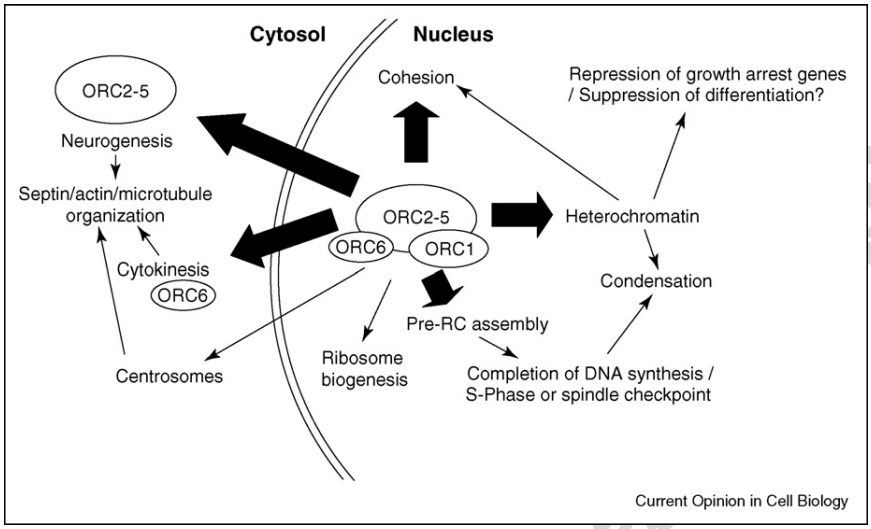
Figure 1



The many faces of the origin recognition complex (ORC). In this diagram, roles for ORC that are supported by functional evidence are indicated with a thick arrow, while roles that can be explained indirectly or that are supported primarily by localization or physical association of ORC are indicated with a thin arrow.

create a breeding ground for such co-evolution. Indeed, transcription factors such as Abf1 [14], Myb [15] and E2F [16] influence the activity of replication origins, possibly through modifications of the local chromatin environment [14]. Since the components mediating stable chromatin states are frequently self-reinforcing, cells may have become dependent upon ORC for the recruitment of chromatin factors and vice versa. In fact, Sir4 and ORC cooperate to recruit Sir1 [17] and origins that contain binding sites for the transcriptional repressor Sum1 require Sum1 for their replication activity [18•]. In mammalian cells as well, chromatin factors recruit ORC to replication origins [19,20].

Co-evolution of replication and silencing functions may have been driven by the need to replicate different segments of large eukaryotic chromosomes at different times during S-phase. In budding yeast, the regions of tightest ORC binding promote the assembly of late-replicating heterochromatin. Although ORC seeds the assembly of initiation-competent pre-RCs at ORC-dependent silencers, they only function as replication origins under conditions where local replication has been delayed [21]. In fact, replication origins with the strongest ORC binding activity function most poorly as replication origins and most strongly as silencers [22]. Careful dissection of elements required for silencing versus origin activity at HMR reveals that, while origin activity is not incompatible with a certain level of Sir1-dependent silencing, silencing in the vicinity of active origins is incomplete [23•]. Hence, it is possible that ORC's role in the assembly of silent chromatin may contribute to the distribution of early and late replicating segments of the genome. The reverse is also possible: the highly condensed state of heterochromatin may make it more difficult to replicate, making it necessary to recruit ORC more tightly or more densely within heterochromatin to ensure duplication takes place (reviewed in [24]).

S-phase checkpoint regulation: a reflection of pre-RC assembly

Whenever replication forks stall, either by DNA damage or conditions that reduce fork processivity, cell cycle checkpoint pathways are triggered that lead to inhibition of later origin firing and cell cycle arrest during S-phase. Checkpoint responses are complex and, in different contexts, mutations in ORC subunits can either abrogate or activate a checkpoint response. However, most of these results can be traced to defects in pre-RC assembly that result in a reduction in the number of functional origins. On the one hand, severe ORC mutations significantly increase the distances that forks must travel to complete replication and the time that arrested forks must wait to be rescued by a merging fork. Even in the absence of fork arresting agents, this can trigger a Rad9-dependent DNA damage checkpoint response, arresting cells in late S-phase [25•,26•]. On the other hand, massive numbers of arrested forks that accumulate when cells are exposed to mutagens or fork arresting agents trigger an Mrc1/Claspin-dependent S-phase checkpoint response, and ORC mutations can cause the number of arrested forks to drop below the threshold necessary to trigger this checkpoint [27].

There are a few reports that deregulation of ORC can cause apoptosis. In one of these reports, overexpression of

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the ORC1 subunit in mammalian cells leads to its accumulation at the nuclear envelope and to subsequent cell death [28]. However, this phenotype may be a general misfolded protein response, resulting in the formation of aggresomes (L Archibold and DM Gilbert, unpublished) [29] rather than an ORC-specific cellular mechanism. Another report suggests that a mammalian-like apoptosis response to the orc2-1 mutation may be triggered by excessive checkpoint activation [30]. Hence, the majority of papers describing roles for ORC in S-phase checkpoint activation and apoptosis can be traced to its primary role in the assembly of pre-RCs.

Mitotic chromosome assembly: condensation and spindle attachment

Defects in passage through mitosis are a common phenotype of ORC mutants. In budding yeast, many ORC mutations activate the mitotic spindle assembly checkpoint that monitors bipolar attachment of microtubules to kinetochores, and disabling this checkpoint can abrogate mitotic arrest [25•]. It is not clear how ORC could influence spindle assembly, but chromatin immuno-precipitation in fission yeast localizes ORC1 and ORC4 to the kinetochore in the absence of Mcm6 [31], suggesting a non-replication role at the kinetochore. In human cell lines, immuno-fluorescence studies found ORC2 to be released from all chromatin except kinetochores from late S-phase onwards, and to localize with tubulin at centrosomes throughout the cell cycle [9], and also found ORC6 localized to kinetochores specifically during mitosis [32]. Following siRNA knockdown of human ORC2, many cells arrested in mitosis and were found to have multiple centrosomes and abnormally condensed chromosomes that were poorly aligned on the mitotic spindle [9]. This suggests that ORC might coordinate the completion of replication with centrosome duplication and/or proper spindle attachment to kinetochores. However, centrosome amplification can be induced by DNA damage [33], so this effect of ORC2 knockdown may result from incomplete replication. It will be important to identify mutations in ORC2 that affect centrosome binding and to determine their effect on centrosome regulation.

In *Drosophila*, defects in ORC2, ORC4 or ORC5 mutations lead to abnormally condensed chromosomes [34–36]. Since similar phenotypes were observed with mutants in other replication proteins (Mcm10, Cdc45, Mcm2, Mcm4, Mcm5, Rfc4 and PCNA) [35,37], and the condensation defects coincide with regions in which replication timing is disrupted and resemble what is seen when chromosome condensation is induced before the completion of DNA synthesis (premature chromosome condensation; PCC), it has been suggested that these mitotic defects reflect an as-yet-uncharacterized mechanism coordinating DNA replication with chromosome condensation and kinetochore function.

Recent observations have suggested a more direct role for ORC in chromosome condensation. When isolated *Xenopus* sperm chromatin is introduced into a *Xenopus* egg extract, focal centers of the single-strand binding protein RPA form on chromatin independent of DNA synthesis. These centers persist throughout a cycle of DNA replication and are not removed until phosphorylated by Cdk1 during mitosis. Non-phosphorylatable mutants of RPA cannot be removed during mitosis and in their presence chromosomes cannot properly condense. Interestingly, ORC2 — apparently even in the absence of other ORC subunits — is necessary to recruit Cdk1 to chromatin, which facilitates RPA removal and proper chromosome condensation [38•]. However, it should be kept in mind that the function of these RPA foci is not clear and they have not been observed in other systems [39].

In summary, ORC has been implicated in the assembly of mitotic chromosomes and the function of centromeres, but no experiment has made a direct functional link between ORC and these chromosomal functions. Hence, it remains possible that many of the mitotic defects seen with ORC mutations result from indirect effects of the roles of ORC in replication or heterochromatin assembly.

Sister chromatid cohesion

After replication, sister chromatids must be held together to facilitate their segregation during mitosis. This cohesion is also essential to create the tension sensed by the spindle assembly checkpoint [40]. It has recently become apparent that there are two partially redundant mechanisms to achieve sister chromatin cohesion (SCC) and ORC has been linked to both of these mechanisms, albeit so far in different systems. The first mechanism is well characterized and is mediated by cohesin proteins [40]. In vertebrates, cohesin loads onto chromatin during telophase, coincident with pre-RC assembly and, in *Xenopus* egg extracts, pre-RCs are required to recruit cohesin onto chromosomes and to establish cohesion. Hence, in this system ORC is indirectly required for SCC via its role in pre-RC assembly [41,42].

A novel mechanism for SCC that has been identified in budding yeast is mediated directly by ORC, independent of cohesin [43••]. When budding yeast were rapidly depleted of ORC2 during G_1-phase, they continued through S-phase uninterrupted, as ORC is dispensable for pre-RC maintenance, but were blocked in mitosis through activation of the spindle checkpoint. Arrested cells displayed precocious separation of sister chromatids at most sites examined, a phenotype that could even be induced by depleting ORC in cells after they were arrested in mitosis with normal SCC. Cohesin was recruited normally in these cells and the degree of precocious separation of chromatids in the absence of ORC combined additively with the effects of cohesin mutations, consistent with a prior study showing that

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ORC mutants are synthetic-lethal with mutations in the cohesin complex [44], and strongly suggesting that ORC directly mediates SCC by a mechanism independent of cohesin. Importantly, whereas cohesin mutations could not be restored by re-expression of cohesin in G_2/M, re-expression of ORC during G_2/M completely restored cell cycle progression, viability and re-joining of sister chromatids. Moreover, an array of ORC binding sites could promote cohesion at a specific site without recruiting cohesin, condensin, Sir2 or Sum1. Together, this report provides direct evidence that ORC can promote SCC by a mechanism independent of replication or cohesin. It is now of great interest to determine whether parallel mechanisms exist in other eukaryotes. It is interesting to note that *Drosophila* ORC1 is degraded by the anaphase promoting complex [45], an enigma that could be partially explained if in this system ORC degradation is necessary to separate chromosomes.

It is as yet difficult to conclude whether ORC's role in cohesion is indirectly related to its role in heterochromatin assembly. Heterochromatin is essential for SCC in many systems [46,47], but in these cases SCC is mediated through cohesin. Moreover, cohesion by ORC does not appear to require the presence of Sir2,3,4 [43••] suggesting that if ORC mediates cohesion by promoting a specific chromatin structure, it is probably not the same silent chromatin structure that ORC promotes at HMR/HML.

Coordinating cytokinesis with chromosome segregation

In both *Drosophila* and mammalian cells, the smallest subunit of ORC, ORC6, has been implicated in coordinating cytokinesis with pre-RC formation and chromosome segregation, a role that it performs independently of the rest of the complex. ORC6 localizes on its own to the cellular membrane in *Drosophila* [48], which at the end of mitosis is exaggerated at the cytokinetic furrow. A search for ORC6 interacting partners identified the septin protein *pnut*, and an ORC6 protein with a deletion of its *pnut* interaction domain was shown to act in a dominant-negative fashion to inhibit cytokinesis [49]. ORC6 also localizes to the cytokinetic furrow in mammalian cells, co-localizing with midbody proteins that coordinate segregation and cytokinesis [32]. RNAi/siRNA knockdown in either *Drosophila* or human cells results in reduction of DNA synthesis, but also in the prominent appearance of cells that have completed mitosis without cytokinesis (multinucleate cells), a phenotype that is not seen following knockdown of other ORC subunits. Together, these results provide compelling evidence for a role for ORC6 in cytokinesis, and suggest that ORC6 may coordinate cytokinesis with replication.

While this is an intriguing model, ORC6 has presented something of an enigma in that its role in DNA binding and even pre-RC assembly has been questioned. ORC6 is dispensable for sequence-specific DNA binding of budding yeast ORC, although it is an essential protein *in vivo* [1,50]. Human Orc6 is loosely bound to ORC and is also dispensable for DNA binding [51]. Moreover, human ORC1–5 is sufficient to restore replication activity in an ORC2-depleted *Xenopus* egg extract [51]. By contrast, ORC6 is tightly associated with the *Drosophila* ORC and is essential for both DNA binding and replication activity in a *Drosophila* embryo extract [44]. Recently, *Drosophila* ORC6 was shown to have DNA binding activity on its own, with a specificity that can account for the poly(dA) preference of ORC binding [52]). DNA binding is mediated by a helix-turn-helix motif that is conserved in vertebrate Orc6, and point mutations in this DNA binding domain abolished DNA binding and replication activity and had a dominant-negative effect on DNA synthesis. This provides compelling evidence that Orc6 is critical for ORC function. The possibility that ORC6 was not depleted in the *Xenopus* egg extract experiments needs to be investigated and is critical for interpreting the role of Orc6 in coordinating replication and cytokinesis.

Ribosome biogenesis

In budding yeast, the essential protein Yph1, which is required for 60S ribosomal subunit biogenesis, was found to co-immunoprecipitate and co-purify with ORC in a complex that also contained Mcm proteins [53]. While a functional role for ORC in this complex still remains to be demonstrated, it will be interesting to determine whether this interaction reflects the existence of a pathway that coordinates DNA replication and ribosome biogenesis with cellular growth rates.

Tissue-specific roles for ORC: a glimpse of what's to come

Since ORC is a cell cycle regulatory protein, it makes sense to speculate that downregulation of ORC might accompany or even drive withdrawal from the cell cycle during terminal differentiation of tissues, possibly through de-repression of genes regulated by ORC. However, unique subsets of ORC subunits are expressed in different mammalian tissues, including terminally differentiated and non-proliferating tissues [54]. Are these subunits being usurped for ancillary roles in tissue-specific functions? Recent evidence argues that this is the case.

In *Drosophila*, ORC2 and 3 localize to the pre-synaptic terminals of terminally differentiated motor neurons, and ORC3 mutations result in defects in learning and synaptic plasticity [55,56]. Recently, *Drosophila* ORC2–5 (which are thought to form a core complex that probably cannot bind DNA without ORC1 and 6) were found to be highly expressed in adult brains and localized to dendrites [57••]. Little or no ORC1, ORC6, Mcms 2,4,6 or Cdc6 protein could be detected. ORC2–5 were associated with synap-

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tosomic membranes. In cultured hippocampal cells, siRNA knockdown of ORC3 or ORC5 or overexpression of a dominant negative orc3 severely impaired an early step in the formation of dendritic branchpoints, and overexpression of an Orc4 point mutation in the ATPase domain resulted in hyper-branching. Since branching involves reorganization of the actin cytoskeleton, and since septins such as *pnut* are involved in aspects of actin filament organization during cytokinesis [58], it is very tempting to think of ORC as a participant in the process of cellular morphogenesis, operating outside the nucleus and independently of the cell cycle and potentially coordinating dynamic events at the interface between cytoskeleton and membrane biology.

Conclusions

At present, the literature on non-replication roles for ORC is scattered and there are still many discrepancies between different model systems that need to be addressed. ORC's roles in replication and heterochromatin assembly are clearly highly conserved in eukaryotes but the question of why these two separable functions are carried out by the same protein complex is still an unresolved and very interesting issue. Many of the other suggested roles for ORC are likely to be derived from these two primary functions, but its roles outside the nucleus are clearly independent. If the newly identified role of ORC in cohesion in budding yeast is conserved in higher eukaryotes, it will have to be reconciled with the popular model that metazoan ORC is removed from chromatin before mitosis [2]. Likewise, a unified model for cross talk between cytokinesis and pre-RC assembly mediated by ORC6 will require reconciliation with results suggesting that vertebrate ORC6 is dispensable for pre-RC assembly. Suggested roles for ORC in centrosomes and centromere function are intriguing but will require more rigorous demonstration of function beyond localization. Finally, the role of ORC in neurogenesis and the selective presence of different ORC subunits in various differentiated tissues suggest that there will be many surprises in the future.

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