ROLE OF REPLICATION PROTEINS IN THE MAINTENANCE OF GENOME INTEGRITY

BY

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DISSERTATION

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ABSTRACT

Precise duplication of our genome is critical for cell survival and human health. Meanwhile, appropriate cellular response to genotoxic stress during DNA replication prevents genome instability and ensures the fidelity of replication. In this thesis, I first review and discuss the current understanding of how DNA replication is regulated from G1 origin licensing to S phase replication elongation, as well as the coordination between replication and DNA repair/DNA damage response. I then describe in Chapters 2 and 3 the studies of two important replication proteins, Orc6 and RFWD3, in the maintenance of genome integrity.

In Chapter 2, I investigate the functional significance of the smallest origin recognition complex subunit, Orc6. In eukaryotes, the Origin Recognition Complex (ORC) is required for the initiation of DNA replication. As part of the ORC, it has been reported that Orc6 is essential for pre-replication complex (pre-RC) assembly and cell viability in yeast and *Drosophila*. However, the role of human Orc6 in replication remains unclear. I characterize human Orc6 function for G1 origin licensing versus S phase progression. I demonstrate that in human cells, Orc6 is dispensable for G1 licensing, opposing to its suggested role for MCM loading observed in other species. Instead, I identify an unexpected role for human Orc6, which is to promote S-phase progression post pre-RC assembly and regulate DNA damage response. Moreover, Orc6 localizes at the replication fork and is an accessory factor of the mismatch repair (MMR) complex. In response to oxidative damage during S-phase, Orc6 facilitates MMR complex assembly and activity, without which the checkpoint signaling is abrogated. Mechanistically, Orc6 directly binds to MutSα and enhances the association of MutLα to MutSα, thus enabling efficient mismatch repair. This study here reveals that Orc6 plays a fundamental role in genome surveillance during S-phase.

In Chapter 3, I focus on RFWD3, an E3 ligase known to facilitate homologous recombination by removing RPA and RAD51 from DNA damage sites. The role of RFWD3 in DNA damage conditions has been extensively studied. However, the role of RFWD3 in unperturbed cell cycle progression remains to be solved. Preliminary data from our lab have shown that RFWD3 interacts with Proliferating Cell Nuclear Antigen (PCNA) via its PCNA-interacting (PIP) motif. Further, knockdown of RFWD3 causes slower replication fork progression. Here, I demonstrate that in unperturbed human cells, the interaction with PCNA

stabilizes RFWD3 at the replication fork. Cells lacking RFWD3 show a prolonged S-phase and replication stress phenotype with increased frequency of fork stalling. The S-phase defect is rescued by WT-RFWD3, but not by the PIP-mutant, suggesting that the interaction of RFWD3 with PCNA is critical for DNA replication. Finally, I observe reduced ubiquitination of RPA in cells lacking RFWD3, suggesting an inefficient RPA removal during replication progression. Together, I propose that the stabilization of RFWD3 by PCNA at the replication fork enables the polyubiquitination of RPA and its subsequent degradation for proper DNA replication.

In Chapter 4, I summarize the significance of my findings of these two critical replication proteins, Orc6 and RFWD3, in regulating genome integrity. I also discuss several interesting questions that have emerged from my research, as well as future directions for their research.

To my family, for their unconditional love and support.

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CHAPTER 1. INTRODUCTION

1.1 DNA replication, cell cycle and genomic integrity

In every cell cycle, billions of nucleotides need to be duplicated within hours, with extraordinary precision and accuracy. The molecular mechanism by which cells regulate the replication event is very complicated, and the entire process begins way before the onset of S phase. During G1 phase of the cell cycle, cells prepare for essential replication factors and determine origin sites where replication initiates. During S phase, the replication process is tightly coupled with the DNA repair system to ensure the fidelity of replication. Meanwhile, defects in replication and any damage occurred must be recognized by DNA damage response and the cell cycle must be stopped before cells are allowed to divide. The coordination of these processes throughout the cell cycle is therefore critical to achieve genomic integrity and prevent diseases. In this chapter, I describe the current understanding of these regulatory events.

1.2 Origin licensing

Accurate DNA replication is critical for achieving genome integrity and cell survival. The assembly of pre-replication complex (pre-RC), which is the very first step required for DNA replication, is a stepwise event that starts from the binding of ORC (Origin Recognition Complex) to the origins on the DNA during G1 phase (Bell, 2002; Bell and Dutta, 2002). ORC then recruits Cdc6, Cdt1, and finally the MCM (minichromosome maintenance) 2-7 complex, component of the DNA replicative helicase, to the origin. An origin is considered "licensed" once the MCM2-7 double hexamer is loaded, which can later fire to initiate replication during S phase (Bleichert, 2019; Limas and Cook, 2019; Parker et al., 2017).

1.2.1 ORC and pre-replication complex

Origin Recognition Complex (ORC) is a heterohexameric protein complex that recognizes DNA replication origins and serves as the initiator of DNA replication (Bell and Stillman, 1992). Six subunits of ORC were named Orc1 through Orc6 in descending order of their molecular mass in *S. cerevisiae*, where they were first identified. After being discovered in

S. cerevisiae, the orthologs of all six subunits have been identified subsequently in many other eukaryotic organisms, and ORC's function as the replication initiating factor is highly conserved across species (Carpenter et al., 1996; Dhar and Dutta, 2000; Gavin et al., 1995; Gossen et al., 1995; Quintana et al., 1997; Quintana et al., 1998; Takahara et al., 1996; Tugal et al., 1998; Yu et al., 1998). Despite being conserved, certain species-specific differences have evolved that are functionally important.

The assembly of ORC has been extensively examined using *in vitro* reconstitution system as well as cell biological methods. For human ORC, Orc2 to 5 form the core ORC complex, while Orc1 associates with core ORC in a cell cycle-dependent manner and Orc6 interacts with core ORC in a dynamic and transient manner (Dhar et al., 2001; Ghosh et al., 2011; Siddiqui and Stillman, 2007; Vashee et al., 2001). By electron microscopy and X-ray crystallography approaches, structures of ORC have also been reported from *S. cerevisiae*, *Drosophila* to human (Bleichert et al., 2013; Bleichert et al., 2015; Chen et al., 2008; Clarey et al., 2008; Tocilj et al., 2017). High-resolution crystal structures using trimmed *Drosophila* ORCs suggested that subunits within ORC are arranged in the order of Orc1-Orc4-Orc5-Orc3-Orc2 as ring-shaped (Bleichert et al., 2015). The pentameric ORC ring has a gap between Orc1 and Orc2, allowing the DNA entry into its central channel. Orc6, which is not part of the pentameric ring, attaches peripherally to Orc3 through its C terminus.

Early identification and characterization of ORC in *S. cerevisiae* also showed that ORC binds to origin DNA in an ATP-dependent manner (Bell and Stillman, 1992). Indeed, protein sequences information revealed that Orc1-5 are AAA+ (ATPases Associated with diverse cellular Activities) family proteins with conserved AAA+ fold, including walker A and Walker B motifs, suggesting their ATP binding ability and ring-shaped protein complex formation (Bell et al., 1995; Giordano-Coltart et al., 2005; Speck et al., 2005). Orc1-5 also contain winged-helix (WH) domain at their C terminus. Orc6, albeit a member of ORC, does not contain such structural features. Moreover, the primary sequence of Orc6 is only weakly conserved between yeast to human (Dhar and Dutta, 2000). Although most of the AAA+ family proteins function as hexameric assemblies, ORC has only five AAA+ fold proteins. In the five ORCs with AAA+ fold, Orc1, Orc4 and Orc5 bind ATP (Bleichert et al., 2015; Klemm et al., 1997; Makise et al., 2003; Ranjan and Gossen, 2006; Siddiqui and Stillman, 2007). However, only Orc1 has ATPase activity and its binding to ATP is required for the DNA binding activity of ORC as shown in *S.*

cerevisiae and *Drosophila* (Chesnokov et al., 2001; Klemm et al., 1997). Interestingly, in *S. pombe*, it was shown that ORC's DNA binding is ATP-independent (Kong and DePamphilis, 2001). As Orc1-5 associate with DNA as a complex, the requirement of Orc6 in ORC's DNA binding varies between different species. It has been reported that Orc6 is required for DNA binding of ORC only in *Drosophila* (Balasov et al., 2007), but not in other species. Recently, a high-resolution crystal structure of *S. cerevisiae* ORC binding to origin DNA was reported (Li et al., 2018). It demonstrated for the first time that *S. cerevisiae* Orc6 participates in the ORC binding to origin DNA. The latest structural study then elucidated the requirement of Orc6 in MCM loading (Miller et al., 2019). However, it remains unknown that if these hold true for other species. Significant progress has also been made in solving the structure of human ORC in recent years, but all the currently reported human ORC structures are lacking Orc6 (Jaremko et al., 2020; Tocilj et al., 2017).

In budding yeast, all six ORC subunits remain chromatin-associated throughout the cell cycle (Liang and Stillman, 1997). However, in mammalian cells, Orc1 is released from chromatin upon S phase entry in a CDK-dependent manner, gets ubiquitinated by SCF^{Skp2} and degraded via ubiquitin-mediated proteasomal degradation (Kreitz et al., 2001; Li and DePamphilis, 2002; Li et al., 2004; Méndez et al., 2002). Only at M phase to G1 transition is Orc1 able to re-associate with chromatin and enable origin licensing (Abdurashidova et al., 2003; Fujita et al., 2002; Kara et al., 2015; Ohta et al., 2003). The cell cycle dependent regulation of Orc1 proves to be one of many mechanisms that allow replication to occur once and only once per cell cycle (DePamphilis, 2005). In addition, a recent report suggested that budding yeast ORC dimerizes in a cell cycle dependent manner to control licensing (Amin et al., 2020). It remains to be seen if human ORC is regulated in a similar way.

Recent years, a debate about whether ORC is an essential factor for origin licensing has been brought up by a study showing that human cells remain viable with no dramatic defect in replication after the depletion of Orc1 or Orc2 by CRISPR-mediated knockout (Shibata et al., 2016). This study challenged the long-standing view that ORC is the essential initiator of DNA replication. It is possible that partial ORC rings are still functional and able to load MCMs for proper replication; it is also possible that there exists an ORC-independent licensing system, but there is no evidence supporting this idea (Bell, 2017). A follow-up study depleting another ORC

subunit Orc5 or co-depleting Orc2/Orc5 showed that ORC is dispensable for replication (Shibata and Dutta, 2020). Meanwhile, other studies continue to suggest the essentiality of ORC using genome-wide or ORC-specific CRISPR screens (Chou et al., 2020; Meyers et al., 2017; Tsherniak et al., 2017). It is also important to note that these different studies used different approaches and experimental systems to deplete and examine ORC's role. Future studies are necessary to address this critical question.

After ORC binds to the origin, Cdc6 is the next factor to associate to the ORC. Cdc6 was one of the genes identified in the temperature-sensitive cell division cycle (cdc) mutant strains (Hartwell, 1974). It was later found that it is part of the pre-RC, downstream of ORC and upstream of MCM chromatin loading (Cocker et al., 1996; Donovan et al., 1997; Liang et al., 1995; Tanaka et al., 1997). Like Orc1-5, Cdc6 belongs to the AAA+ family and has ATPase activity (Liu et al., 2000; Speck et al., 2005). Evidence from yeast to human all point that Cdc6's ATPase activity is critical for the regulation of its pre-RC function (Herbig et al., 1999; Perkins and Diffley, 1998; Wang et al., 1999; Weinreich et al., 1999). It has the most extensive sequence similarity with Orc1 and they are suggested to be paralogs (Bell et al., 1995; Giraldo, 2003). Cdc6 directly interacts with ORC *in vivo* and *in vitro* (Mizushima et al., 2000; Saha et al., 1998). Cdc6 associates with chromatin-bound ORC in an ATP-dependent manner (Evrin et al., 2013; Perkins and Diffley, 1998; Ticau et al., 2015). After ORC binds to the DNA, Cdc6 is recruited and docks into the Orc1/Orc2 gap, forming ORC-Cdc6 complex and closing the ring to encircle DNA (Sun et al., 2013; Sun et al., 2012; Yuan et al., 2017). Therefore, Cdc6 provides the sixth AAA+ fold protein to the pentameric ORC, establishing the classic hexameric AAA+ complex toroid configuration.

S. cerevisiae Cdc6 and its ortholog, Cdc18 in *S. pombe*, are rapidly degraded when cells enter S phase via ubiquitin-mediated proteasomal degradation, after being phosphorylated by S and M phase CDKs (Drury et al., 1997; Luo et al., 2003; Mimura et al., 2004; Perkins et al., 2001; Piatti et al., 1995). Human Cdc6 is also regulated by CDK but through a different mechanism. Human Cdc6 is targeted for degradation by APC/C-dependent proteolysis (Petersen et al., 2000). While during G1 phase where licensing takes place, Cdc6 is protected from destruction by cyclinE/Cdk2-dependent phosphorylation (Mailand and Diffley, 2005). In S phase, it was proposed that Cdc6 is phosphorylated by cyclinA/Cdk2 and exported from nucleus (Jiang et al., 1999; Petersen et al., 1999; Saha et al., 1998). However, this model was challenged

by other studies showing that a fraction of Cdc6 remains chromatin-bound even in S phase (Alexandrow and Hamlin, 2004; Coverley et al., 2000; Fujita et al., 1999). In addition, a more recent study showed that Cdc6 is also targeted for degradation by $SCF^{cyclinF}$ complex late in the cell cycle (Walter et al., 2016). Therefore, the detailed mechanism regulating Cdc6 during cell cycle remains to be delineated.

After the formation of ORC-Cdc6 complex on origin DNA, MCM2-7 hexamer can then be loaded with the help of Cdt1 (Cdc10-dependent transcript 1), which acts as a molecular chaperone for MCM (Pozo and Cook, 2016). Cdt1 was first identified in *S. pombe* as a gene regulated by the cell cycle transcription factor Cdc10 (Hofmann and Beach, 1994), and recognized as an initiator of replication due to its ability to induce re-replication (Nishitani et al., 2000). Cdt1 is conserved in eukaryotes from yeast to human and is required for MCM2-7 loading in all eukaryotes tested (Devault et al., 2002; Maiorano et al., 2000; Whittaker et al., 2000; Wohlschlegel et al., 2000). Cdt1 forms a stable complex with MCM2-7, primarily by interacting with MCM6 subunits (Ferenbach et al., 2005; Liu et al., 2012a; Wei et al., 2010; Wu et al., 2012), and helps maintain the integrity of MCM2-7 (Frigola et al., 2013; Kawasaki et al., 2006; Remus et al., 2009; Takara and Bell, 2011; Wu et al., 2012). Moreover, structural studies in *S. cerevisiae* suggested that the binding of Cdt1 to MCM2-7 prevents MCM2/5 gate closure (Frigola et al., 2017; Ticau et al., 2017; Zhai et al., 2017), which in turn allows the DNA to pass and enter the central channel. On the other hand, in *S. cerevisiae*, Cdt1 has also been reported to be critical for MCM loading to ORC-Cdc6 through its interaction with Orc6 (Asano et al., 2007; Chen and Bell, 2011; Chen et al., 2007; Semple et al., 2006). However, it is unknown if this interaction occurs in metazoans. There are other reports suggesting that in *S. cerevisiae* MCM2-7 itself directly engages with ORC-Cdc6 (Fernández-Cid et al., 2013; Frigola et al., 2013), but Cdt1 helps to overcome an MCM6-dependent autoinhibitory mechanism which would otherwise sterically hinder the MCM docking to ORC-Cdc6 (Fernández-Cid et al., 2013). Together, these studies demonstrated the critical role of Cdt1 in origin licensing.

The cell cycle-dependent regulation of Cdt1 is the major regulatory point for preventing re-replication. *S. cerevisiae* Cdt1 is controlled by CDK phosphorylation that inhibits its interaction with Orc6 (Chen and Bell, 2011), and subject to nuclear export during S phase (Tanaka and Diffley, 2002). In other eukaryotes including human, however, Cdt1 protein is degraded upon S phase entry (Nishitani et al., 2004; Nishitani et al., 2001; Zhong et al., 2003).

Cdt1 is controlled by two independent E3 ligase pathways to ensure its destruction after S phase entry. First, Cdt1 is phosphorylated by CDKs, which leads to its recognition by SCF^{Skp2} E3 ligase for ubiquitination and degradation (Li et al., 2003; Liu et al., 2004; Nishitani et al., 2006; Sugimoto et al., 2004). Second, degradation of Cdt1 is further restricted to S phase by "replication-coupled destruction" through interacting with chromatin-bound PCNA (Proliferating Cell Nuclear Antigen), which is the processivity factor for DNA polymerase at replication fork (Arias and Walter, 2006; Senga et al., 2006). Cdt1 contains a conserved PCNA-interacting protein (PIP) box that mediates its interaction with PCNA; the interaction in turn triggers Cdt1 ubiquitination and degradation by $CRL4^{Cdt2}$ E3 ligase (Havens and Walter, 2009). In addition, metazoan Cdt1 is inhibited by direct binding of another cell cycle-oscillating protein, Geminin (Cook et al., 2004; De Marco et al., 2009; Lutzmann et al., 2006; Quinn et al., 2001; Tada et al., 2001). Geminin level is elevated in S phase and degraded by APC/C upon M phase (McGarry and Kirschner, 1998), thus allowing Cdt1 to be active only during G1.

MCM2-7 hexamer loading marks the final step of the pre-RC assembly on the chromatin. MCM2-7 loading requires the coordinated function of ORC, Cdc6 and Cdt1. MCM (minichromosome maintenance) proteins were first identified in genetic screens that are designed to uncover genes that are required for replication in *S. cerevisiae* (Maine et al., 1984). The orthologs were subsequently identified in other eukaryotes, and shown to have an essential function in replication (Madine et al., 1995; Todorov et al., 1995; Treisman et al., 1995). Biochemical characterization soon revealed that MCM2-7 assemble into a hexameric complex *in vivo* and *in vitro* (Adachi et al., 1997; Chong et al., 1995; Thömmes et al., 1997). Similar to Orc1-5 and Cdc6, MCM2-7 belongs to AAA+ family (Koonin, 1993). MCM hexamer is formed in the order of MCM5-MCM3-MCM7-MCM4-MCM6-MCM2, with an open gate between MCM2 and 5 to allow the passage of DNA (Costa et al., 2011; Davey et al., 2003). As discussed above, Cdt1 helps to stabilize MCM integrity and prevent gate closure. The interaction of Cdc6 to C-terminus of MCM3 has also been reported to be critical for MCM loading (Frigola et al., 2013). Following the loading of MCM, both Cdc6 and Cdt1 are ejected in an ATP hydrolysis-dependent manner. An ORC-Cdc6-Cdt1-MCM (OCCM) intermediate can be stabilized by using a nonhydrolyzable ATP analog (Sun et al., 2013), in a state prior to the loading of the next MCM2-7 hexamer.

The defining step of origin licensing is the formation of stable head-to-head MCM2-7 double hexamer around DNA (Evrin et al., 2009; Remus et al., 2009). The formation of double hexamer has been extensively examined using *S. cerevisiae* proteins. However, conflicting results were observed using different methods. Biochemical studies suggested that two hexamers are loaded independently on two inverted ORC binding sites near each other (Coster and Diffley, 2017; Frigola et al., 2013). In contrast, single molecular experiments showed that two single hexamers are loaded in a sequential way (Ticau et al., 2015), and the loading of the second MCM requires a distinct Cdc6 molecule (Ticau et al., 2017). The latest cryo-EM experiments identified a key intermediate after OCCM, called MCM-ORC (MO), demonstrating that double hexamer loading is indeed coupled and sequential (Miller et al., 2019). In OCCM, the MCM C-terminal domains are in contact with ORC. In the newly observed MO structure, however, ORC binds in an inverted configuration with N-terminal sides of MCM through Orc6 N-terminus. Therefore, it was concluded that the first MCM hexamer loading, coupled with the release of first set ORC-Cdc6 and Cdt1, creates a distinct binding site and allows the second ORC binding in an inverted orientation. This configuration then recruits the second Cdc6 and allows for the loading of the second Cdt1-MCM2-7 using the same mechanism.

1.2.2 Orc6

Among all the pre-RC proteins - ORCs, Cdc6, Cdt1 and MCMs - Orc6 is the most enigmatic component. As briefly introduced in the previous section, this smallest ORC protein is the least conserved subunit in ORC and highly dynamic with respect to its association with the other ORC components. Orc6 is an integral part of ORC in yeast and *Drosophila* (Bell and Stillman, 1992; Chesnokov et al., 2001), but only weakly associates with ORC in human and *Xenopus* (Dhar et al., 2001; Dhar and Dutta, 2000; Gillespie et al., 2001; Vashee et al., 2001). In fact, early attempts of ORC isolation from human and *Xenopus* were missing Orc6. Only after an extensive examination of experimental conditions could Orc6 be co-purified with ORC from human cells (Ghosh et al., 2011). In terms of binding of ORC to origin DNA, biochemical data suggested that Orc6 is only required in *Drosophila* (Balasov et al., 2007), but not in yeast (Frigola et al., 2013; Kong and DePamphilis, 2001; Lee and Bell, 1997), *Xenopus* (Gillespie et al., 2001), or human (Giordano-Coltart et al., 2005; Vashee et al., 2003). It was shown that

human Orc6 binding to DNA is independent of Orc1-5 complex (Thomae et al., 2011). In *S. cerevisiae*, although it was suggested previously that Orc6 is not required for DNA binding, the latest structural studies suggest that yeast Orc6 is indeed involved in ORC binding to origin DNA and promoting MCM loading (Li et al., 2018; Miller et al., 2019). It is still unknown if this mechanism holds true in other species. Meanwhile, in *S. cerevisiae*, depletion of Orc6 after pre-RC formation causes destabilization of pre-RC and impairs origin firing (Semple et al., 2006), suggesting that Orc6 also functions downstream of pre-RC assembly. Nevertheless, no such role for Orc6 has been evaluated in higher eukaryotes.

Orc6 shares no structural similarity to other ORC subunits, which are all AAA+ family proteins. Structural and sequence analysis in *Drosophila* and human revealed that N-terminus of Orc6 contains two cyclin-box folds that are homologous to the DNA binding domain of transcription factor TFIIB (Balasov et al., 2007; Chesnokov et al., 2003; Liu et al., 2011a). The C-terminus contains a helix region that is required for Orc6 binding to ORC complex (Bleichert et al., 2013; Bleichert et al., 2015). Specifically, Orc6 uses its C-terminus to interact directly with Orc3. Interestingly, *S. cerevisiae* Orc6 is significantly larger (435 amino acids; ~50 kDa) than all other eukaryotic counterparts (e.g. 252 amino acids; ~28 kDa in human). It was later found that *S. cerevisiae* Orc6 contains a large insertion of 100-200 amino acids between the two TFIIB domains (Bleichert et al., 2013). This inserted region has an important "RXL" motif, which associates with S-phase cyclin (Wilmes et al., 2004), thus contributing to a regulatory mechanism that is specific to *S. cerevisiae*.

Metazoan Orc6 is also involved in cytokinesis (Bernal and Venkitaraman, 2011; Chesnokov et al., 2003; Prasanth et al., 2002). Studies in *Drosophila* elucidated that Orc6 functions in cytokinesis by interacting with Septin complex protein Pnut through its C-terminus (Balasov et al., 2009; Chesnokov et al., 2003; Huijbregts et al., 2009), and Orc6 itself forms dimers through its N-terminus, thus facilitating filaments formation of Septin complex (Akhmetova et al., 2015). However, there is currently no evidence showing that human Orc6 forms dimers. It is also worth noting that human Orc6 is present in sub-stoichiometric levels to other ORCs; the large fraction of Orc6 is not associated with ORC indicating ORC-independent functions (Dhar et al., 2001; Vashee et al., 2001). The ORC-independent Orc6 might be involved in diverse functions including regulating cytokinesis and other potential functions that remain to be explored.

Together, all the conflicting data among species highlight the non-conservative nature of Orc6. Why Orc6 has evolved faster than other ORC subunits remains a mystery. It is clear though that Orc6 plays a critical role in regulating cell cycle progression and genome integrity. Therefore, further studies are needed to address the enigmatic nature of Orc6 and identify why there is a large fraction of human Orc6 that is not in a complex with ORC. My work of elucidating human Orc6 function in replication and DNA damage response will be presented in Chapter 2.

1.2.3 Origin selection

Studies in *S. cerevisiae* for replication initiation sites revealed a conserved DNA sequence called autonomously replicating sequence (ARS) (Newlon, 1988). The ARS comprises one A element and three B elements (B1, B2, B3), which are essential for origin function (Marahrens and Stillman, 1992). The most important element among them is the 11-basepair A element that constitutes the ARS core consensus sequence (ACS), which is the primary site of ORC binding (Bell and Stillman, 1992). The cryo-EM structure of ORC binding to ARS explained sequence-specific DNA recognition of ORC (Li et al., 2018). However, *S. cerevisiae* appears to be the only eukaryote with a well-defined DNA origin consensus sequence.

In *S. pombe*, origins are AT-rich clustered stretches (Dai et al., 2005; Okuno et al., 1999; Segurado et al., 2003). Because of this feature, *S. pombe* ORC binding to origins is dependent on its Orc4, which contains an unique AT-hook domain (Chuang and Kelly, 1999; Kong and DePamphilis, 2001; Lee et al., 2001; Moon et al., 1999). This Orc4 AT-hook motif is absent in all other eukaryotes, and is necessary and sufficient for *S. pombe* origin selection and ORC binding. Other than sequence specificity, origins in both budding and fission yeast are maintained as nucleosome-free regions (Berbenetz et al., 2010; Eaton et al., 2010; Givens et al., 2012; Xu et al., 2012).

Origins in higher eukaryotes do not show any features at the level of a defined DNA sequence. Metazoan ORC binds to DNA promiscuously and replication can initiate from random sequences (Hyrien and Méchali, 1993; Méchali and Kearsey, 1984; Vashee et al., 2003), suggesting that origins may be defined by 3-dimensional structures of DNA, chromatin environments or interactions with other proteins. Before the publication of the first genome-scale

study in 2008, only about 20 origins had been identified and categorized in the entire human genome (Aladjem, 2007; Cadoret et al., 2008). Certain determinants in metazoan origin selection have been revealed by genome-wide studies in the past decade (Aladjem and Redon, 2017). Nucleosome positioning is an important factor, as metazoan origins, like in yeast, are maintained as nucleosome-free regions (Eaton et al., 2011; Karnani et al., 2010; Lubelsky et al., 2011; MacAlpine et al., 2010). In human cells, it was demonstrated that origins correlate with DNase I-hypersensitive regions (Mesner et al., 2013). Similarly, genome-wide mapping of origins also discovered that origins are located near transcription start sites (TSSs), active promoters, CpG islands and G-quadruplexes in metazoan (Bartholdy et al., 2015; Besnard et al., 2012; Cadoret et al., 2008; Cayrou et al., 2015; Martin et al., 2011; Petryk et al., 2016; Prorok et al., 2019; Sequeira-Mendes et al., 2009). However, none of these factors is strictly required for origin selection. For example, origins only associate with TSSs where transcription activity is moderate, but are depleted from highly transcribed TSSs (Martin et al., 2011).

Histone variants and modifications also play critical roles in origin specification. Methylation of H4K20 is the most well studied event regulating origin establishment. Mono-methylation of H4K20 by PR-Set7 (also known as Set8 or KMT5A) promotes loading of pre-RC (Beck et al., 2012; Tardat et al., 2010). This function could be through the later establishment of H4K20me2 from H4K20me1, as ORC can bind to H4K20me2 via direct interaction of Orc1's N-terminal bromo-adjacent homology (BAH) domain (Kuo et al., 2012). Further, nucleosomes containing histone variant H2A.Z directly recruit SUV420H1 to convert H4K20me1 to H4K20me2, thereby recruiting Orc1 and facilitating licensing (Long et al., 2020). Additionally, H3K79me2 has also been suggested to enrich at a subset of origins in human cells (Fu et al., 2013). Interestingly, methylation of H3K79 also helps to prevent re-replication, as depletion of DOT1L, the sole enzyme that catalyzes the methylation of H3K79, leads to re-replication and S phase defects. Another histone modification enzyme, histone acetyltransferase HBO1, has also been reported to facilitate replication initiation events (Iizuka et al., 2006). HBO1 interacts with several pre-RC components including Orc1, MCM2 and Cdt1 (Iizuka et al., 2006; Miotto and Struhl, 2008), and the cell cycle-regulated H4 acetylation at multiple sites by HBO1 is critical in the licensing process (Miotto and Struhl, 2010). More recently, it was shown that HBO1 also acetylates H3K14 at selected replication origins in the vicinity of TSSs (Feng et al., 2016).

Other than DNA or chromatin features discussed above, origin selection at certain chromatin regions can also be mediated by pre-RC's interacting proteins that contain their own DNA or chromatin binding ability. One well-known example is ORCA (ORC-associated, also known as LRWD1), which was identified by our lab with Orc2 IP-MS proteomic screening (Shen et al., 2010). ORCA is required for S phase entry in human cells, interacts with multiple pre-RC proteins and stabilizes them on chromatin. More importantly, tethering of ORCA to chromatin is sufficient to recruit ORC and initiate licensing (Shen et al., 2012; Shen et al., 2010). ChIP-seq and analysis of ORCA during G1 phase showed that ORCA binds to a subset of origins that is late replicating and enriched at heterochromatin with repressive marks like H3K9me3 and methyl-CpG (Wang et al., 2017). It is worth noting that ORCA also functions in regulating heterochromatin structure (Giri et al., 2015). Thus, ORCA emerges as a critical player linking chromatin organization and origin licensing. Several other ORC interacting proteins in human cells have also been reported to facilitate origin establishment. HMGA1a, an AT-hook domain containing protein, mediates the localization of ORC to AT rich heterochromatin regions (Thomae et al., 2008). Moreover, RepID, a WD-40 domain containing protein, associates with a subset of origins with a common G rich motif, and regulates the licensing process and prevention of re-replication (Jang et al., 2018; Zhang et al., 2016). Together, origin selection in human can be dictated by different mechanisms and combinations of them.

1.2.4 Role of ORC beyond origin licensing

In addition to origin licensing, ORC and different ORC subunits have been suggested to participate in various other cellular functions. Along with its identification as the replication initiator, studies in budding yeast showed that ORC is involved in transcription silencing (Bell et al., 1993; Foss et al., 1993; Micklem et al., 1993). Later it was demonstrated that ORC also regulates transcription silencing and heterochromatin organization in higher eukaryotes. Human Orc1 has been suggested to control centriole and centrosome copy number (Hemerly et al., 2009). Moreover, human Orc2 localizes to centromeres, and the depletion of Orc2 results in abnormal chromosome segregation (Prasanth et al., 2004). It was later shown that Orc2 at centromere is SUMOylated, and this SUMOylation is required for regulating centromeric histone modification (Huang et al., 2016). In budding yeast, Orc2 depletion after pre-RC assembly leads

to defects in sister-chromatid cohesion (Shimada and Gasser, 2007). In mouse cells, Orc1-4 colocalize to centrosome and Orc2 is a substrate of Plk1, suggesting their role in mitosis (Stuermer et al., 2007). On the other hand, mutations in *latheo*, the gene encodes Orc3 protein in *Drosophila*, lead to neuronal defects (Rohrbough et al., 1999). It was suggested that in *Drosophila*, LAT/Orc3 functions in regulating Ca2+- and activity-dependent synaptic plasticity (Pinto et al., 1999). In addition, localization of human Orc5 to chromatin induces large-scale chromatin decondensation (Giri et al., 2016). Metazoan Orc6 is involved in cytokinesis as described in the previous section.

Interestingly, human Orc3 and Orc6, along with ORCA, have been identified in a proteomic screen as ATM/ATR substrates, suggesting their potential involvement in DNA damage response (Matsuoka et al., 2007). Additionally, Orc6 is the only ORC subunit that enriched at nascent DNA by proteomic screens (Alabert et al., 2014; Wessel et al., 2019), indicating another potential function of Orc6 involved in replication process.

1.3 Origin activation and S phase progression

Origins are ready to fire once being licensed in G1 phase. At the onset of S phase, a wave of protein modifications and recruitments regulate the conversion of inactive MCM2-7 complex to active CMG (Cdc45, MCM2-7, GINS) helicase. The CMG helicase, together with polymerases, PCNA, RPA (Replication Protein A), and other essential proteins, initiates DNA unwinding and replication fork establishment. The activation of helicase, like pre-RC assembly, is regulated by a series of events that is highly conserved in eukaryotes.

1.3.1 CMG helicase and pre-initiation complex

MCM2-7 hexamer itself has very limited helicase activity. It was later identified that the full helicase activity requires the assembly of Cdc45 and GINS (from the Japanese go-ichi-ni-san meaning 5-1-2-3, representing Sld5, Psf1, Psf2 and Psf3 subunits) complex with MCM2-7, forming CMG helicase (Gambus et al., 2006; Ilves et al., 2010; Moyer et al., 2006; Pacek et al., 2006). CMG contains a copy of MCM2-7, a single Cdc45 and one GINS tetramer forming a stable 11 subunits helicase. The assembly begins at the G1 to S transition, where MCM double

hexamer serves as a platform to recruit DDK (Dbf4-dependent Cdc7 kinase) via interaction by MCM2 and MCM4 (Ramer et al., 2013; Sheu and Stillman, 2010). DDK is a cell cycle regulated kinase (Cheng et al., 1999; Oshiro et al., 1999). Together with S phase CDK, they are two essential kinases that regulate the assembly of other initiation factors to MCM2-7. DDK phosphorylates multiple MCM subunits including MCM2, 4 and 6 (Cho et al., 2006; Masai et al., 2006; Randell et al., 2010; Sheu and Stillman, 2006; Tsuji et al., 2006). Notably, DDK only targets chromatin bound MCM in the context of a double hexamer but not a single hexamer (Costa et al., 2014; Francis et al., 2009; Kang et al., 2014; Li et al., 2015; Sun et al., 2014), suggesting only the double hexamer configuration is allowed to initiate productive replication. The phosphorylated MCMs then recruit Cdc45 (Masai et al., 2006). The recruitment process is facilitated by Sld3 and Sld7 in *S. cerevisiae*, or their functional homologs Treslin and MTBP in human (Boos et al., 2013; Deegan et al., 2016; Tanaka et al., 2011). In addition to Cdc45, GINS is also associated with MCM2-7. The assembly of GINS complex into MCM requires CDK activity and several chaperone proteins. Sld3 and Dpb11 (TopBP1 in vertebrates) facilitate a complex containing GINS, Sld2 (RecQ4 in vertebrates) and Polε binding to MCM (Muramatsu et al., 2010; Takayama et al., 2003; Tanaka et al., 2013). In this process, CDK phosphorylates Cdc45, Sld2, Sld3 and Sld7 to facilitate their protein interaction (Heller et al., 2011; Muramatsu et al., 2010). The protein complex formed at this point is defined as pre-initiation complex (pre-IC) (Zou and Stillman, 1998). In the pre-IC, MCM double hexamer splits into two single hexamers, suggesting they are ready to form bi-directional replication forks (Miyazawa-Onami et al., 2017). MCM10 is also a critical protein required for origin firing. Although tightly coupled, the pre-IC formation can be separated from the subsequent firing event *in vivo* in cells lacking MCM10 (Miyazawa-Onami et al., 2017), or when MCM10 is omitted in the *in vitro* reconstitution reactions (Yeeles et al., 2015).

1.3.2 Origin firing and replication elongation

After the pre-IC formation, MCM10 is recruited by interacting with CMG (Douglas and Diffley, 2016; Homesley et al., 2000; Wohlschlegel et al., 2002). MCM10 is essential for origin firing not only by activating CMG but stabilizing the replisome (Im et al., 2009; Ricke and Bielinsky, 2004; van Deursen et al., 2012). Meanwhile, the single strand DNA binding ability of

budding yeast MCM10 is important for stabilizing the origin melting reaction (Perez-Arnaiz and Kaplan, 2016). A recent study further suggested that MCM10 is required for CMG to transit between dsDNA and ssDNA (Wasserman et al., 2019). Importantly, in addition to its role in helicase activation, MCM10 also travels along at the replication fork and stimulates replication elongation (Alabert et al., 2014; Lõoke et al., 2017). On the other hand, extensive structural and single-molecule studies have elucidated that active CMG translocates on DNA with its N-terminal domain in front and C-terminal motor domain pushing from behind, and ssDNA passing through the middle channel making contact with several MCM subunits (Burnham et al., 2019; Meagher et al., 2019; O'Donnell and Li, 2018; Yuan et al., 2020).

Three main polymerases, polymerase α, ε, and δ, are required for unperturbed eukaryotic replication (Burgers and Kunkel, 2017). Polymerase α complex contains RNA primase as well as DNA polymerase subunits. After MCM10 associates with the pre-IC, pol α is recruited to CMG mediated by CTF4 (AND-1 in human) (Kilkenny et al., 2017; Simon et al., 2014; Xu et al., 2009; Zhu et al., 2007). Both leading and lagging strand syntheses are initiated by pol α, which starts *de novo* generation of short RNA primers (Pellegrini, 2012). A limited number of deoxyribonucleotides are then incorporated downstream of RNA primers by pol α's DNA polymerase activity (Baranovskiy et al., 2016), before pol ε or pol δ taking over the DNA synthesis.

Extensive biochemical characterization has been done to study the polymerases at the replication fork. By analyzing the mis-incorporation bias on different strands using yeast carrying well defined error-prone mutations on pol ε or pol δ, it was shown that pol ε is the leading strand polymerase, and pol δ is responsible for the lagging strand replication (Nick McElhinny et al., 2008; Pursell et al., 2007). Similar results were obtained by measuring the strand-specific ribonucleotide incorporation in pol ε or pol δ mutant strands (Clausen et al., 2015; Koh et al., 2015; Reijns et al., 2015). Moreover, lagging strand DNA polymerase requires the capacity of strand displacement for the maturation of Okazaki fragments. Pol δ exhibits efficient strand displacement synthesis comparing to pol ε (Garg et al., 2004), which is consistent with the model that pol δ is the lagging strand polymerase. On the other hand, Pol ε has a high intrinsic processivity compared to pol δ (Hogg et al., 2014), making it suitable for the bulk DNA synthesis at the leading strand. The processivity is increased further by the interaction with

PCNA. Nevertheless, the very low processivity of pol δ can be significantly improved when interacting with PCNA (Chilkova et al., 2007).

PCNA is also a critical protein for DNA replication and essential for viability in all organisms (Choe and Moldovan, 2017; Prelich et al., 1987). PCNA forms a homo-trimer that encircles DNA, and acts as a sliding clamp for DNA polymerases. PCNA is loaded onto DNA at the primer template junction by RFC (Replication Factor C) complex (Cullmann et al., 1995; Ohashi and Tsurimoto, 2017; Waga and Stillman, 1994). Other than functioning as a processivity factor for replicative machinery, PCNA is shown to be a docking platform for a large number of proteins involved in different DNA metabolic processes (De Biasio and Blanco, 2013). These processes include DNA repair, cell cycle control, chromatin assembly, sister chromatid cohesion and so on. Most of the interacting partners of PCNA utilize a conserved peptide motif called PIP (PCNA-Interacting Peptide) box to bind PCNA. The interaction with PCNA not only localizes these binding proteins to specific chromatin environments, but also in many instances regulates their activation or stability.

1.3.3 Defects in replication initiation and elongation – Meier-Gorlin syndrome

Meier-Gorlin syndrome (MGS) is a rare inherited developmental disease. MGS patients exhibit several tissue-specific defects including primordial dwarfism, small ears, and missing patella (de Munnik et al., 2015). To date, there are nine genes where the mutations have been linked to MGS: *ORC1*, *ORC4*, *ORC6*, *CDC6*, *CDT1*, *GMNN*, *CDC45L*, *MCM5* and *DONSON* (Bicknell et al., 2011; Burrage et al., 2015; de Munnik et al., 2012; Evrony et al., 2017; Fenwick et al., 2016; Guernsey et al., 2011; Hossain and Stillman, 2012; Knapp et al., 2020; Reynolds et al., 2017; Vetro et al., 2017). All of them encode proteins involved in origin licensing, DNA replication and cell cycle function, indicating the defects in these processes cause MGS. Studying their mutations is a good way to investigate how these proteins function. DONSON is an example. The link between the mutations on *DONSON* and microcephalic dwarfism led to the identification of its protein function in stabilizing replication fork and preventing genomic instability (Reynolds et al., 2017). Another example is Orc6. The first identified MGS mutation of Orc6 is at Y232S on the C-terminus. It was shown that the mutation disrupts the interaction between Orc6 and Orc3 by using both human proteins and *Drosophila* model (Bleichert et al.,

2013). This helped to address the protein interaction of Orc6 in association with ORC, and the pathology when this interaction is abrogated. Interestingly, there is another MGS mutation K23E at N-terminus of Orc6 protein recently being identified on *ORC6* (Li et al., 2017). Since it should not disrupt Orc6's interaction with ORC, further studies are needed to understand how this mutation affects Orc6 function, and at which step during the replication process it affects.

1.3.4 Spatiotemporal control of replication

On the genome-wide scale, replication is a spatially and temporally regulated event (Marchal et al., 2019). Different segments of the genome replicate at distinct times of the S phase. Genomic studies have revealed that these segments can range from hundreds kilobase to megabase scale, referred to as replication domains (RDs) or constant timing regions (CTRs). This replication timing program is very robust and conserved, suggesting its fundamental biological importance (Agier et al., 2018; Müller and Nieduszynski, 2012; Ryba et al., 2010; Ryba et al., 2011). One critical reason to maintain a well-coordinated timing program is to ensure that the number of replication forks does not exceed the limits of available nucleotides and protein factors at any given time (Mantiero et al., 2011). However, it is still not fully understood how this temporal order is decided and why the genome should be replicated in such an order. Nevertheless, alterations of this temporal organization program are observed in cancers (Blumenfeld et al., 2017). It has also been shown that the replication timing is highly stable in the same type of cell (Dileep and Gilbert, 2018; Takahashi et al., 2019), but changes drastically during differentiation and between cell types (Hiratani et al., 2008; Rivera-Mulia et al., 2015; Ryba et al., 2011).

Spatiotemporal organization of DNA replication was observed early by cytological studies using pulse-chase experiments (O'Keefe et al., 1992). Early-replicating chromatin is localized in central regions of the nucleus while late-replicating chromatin is at the nuclear periphery and around the nucleolus. After the large-scale chromatin architecture was characterized by Hi-C (Dixon et al., 2012; Lieberman-Aiden et al., 2009), it was soon identified that early-replicating DNA strongly correlates with actively transcribed gene-rich open chromatin. On the contrary, late-replicating DNA correlates with silent heterochromatin regions (Ryba et al., 2010; Yaffe et al., 2010), which is consistent with cytological studies. Further

analysis also revealed that the boundaries of RDs align with boundaries of TADs (topologically associated domain) (Pope et al., 2014; Xiang et al., 2018). However, removing the chromatin sites at the TAD boundaries or depleting CTCF or cohesin, two important proteins involved in the regulation of TADs, does not affect replication timing (Oldach and Nieduszynski, 2019; Sima et al., 2019). Several histone marks also correlate with replication timing. Active transcription marks are correlated with early-replication, including H3K4me1/2/3, H3K9ac and H3K27ac (Yue et al., 2014). H3K9me2 showed a strong correlation with late-replicating regions (Ryba et al., 2010). However, similar to CTCF and cohesin depletion, the removal of H3K9me2 by knocking out G9a histone methyltransferase has no impact on replication timing (Yokochi et al., 2009). These results showed the robustness of the replication timing regulation. Early replication timing also correlates with higher origin and pre-RC/pre-IC protein binding density (Das et al., 2015; Dellino et al., 2013; Lubelsky et al., 2014; Mesner et al., 2013; Miotto et al., 2016). Whether these are direct effects is unclear, since it is possible that the chromatin environment affects the accessibility of the initiation proteins.

Many chromatin features are correlated with replication timing as discussed above. Nevertheless, there are some factors that have more direct roles in regulating replication timing. RIF1 is a regulator of genome-wide replication timing (Yamazaki et al., 2013). This function of RIF1 is conserved from yeast to human, and the depletion of RIF1 causes global replication timing changes (Cornacchia et al., 2012; Foti et al., 2016; Yamazaki et al., 2012). Chip-seq analysis showed that RIF1 is enriched at late-firing origins. By recruiting PP1 (Protein Phosphatase 1), RIF1 counteracts DDK-mediated phosphorylation at origins (Hiraga et al., 2014; Hiraga et al., 2017), therefore inhibiting the firing of origins until late S phase. LncRNAs (long non-coding RNAs) also play roles in modulating replication timing. One group of lncRNAs called ASARs (ASynchronous replication and Autosomal RNAs), monoallelically expressed from chromosome 6 and 15 (ASAR6 and ASAR15, respectively), coats the chromosomes from which they are expressed and delays the replication of those chromosomes (Donley et al., 2015; Donley et al., 2013; Platt et al., 2018). Taken together, the spatiotemporal organization of replication is a complex process, and more studies are needed to identify novel factors and regulatory mechanisms.

1.3.5 Dormant origins

Another important concept of DNA replication is that in each G1 phase, there are way more origins being licensed than actually fired in the subsequent S phase (Courtot et al., 2018). These origins, which are inactive in normal replication, are called “dormant origins”. It has been discovered for a long time that MCM is loaded in excess onto chromatin. Studies from yeast to human pointed out that MCM can be loaded up to 20-fold in excess over the numbers of loaded ORC or replication origins (Edwards et al., 2002; Tye, 1999; Woodward et al., 2006). Indeed, It has been shown that DNA replication can exert normally with significantly reduced MCM proteins in the cells (Cortez et al., 2004; Edwards et al., 2002; Tsao et al., 2004). Meanwhile, it was also found that though the replication efficiency is maintained in these MCM reduced cells, they showed defects in the S-phase checkpoint. Critically, the MCM-depleted cells became hypersensitive to low, otherwise nontoxic, levels of replication stress and DNA damage (Ibarra et al., 2008; Woodward et al., 2006). Thus, the role of these excess licensed dormant origins was demonstrated as a safeguard system when cells are facing replication stress, and the dormant origins only fire when other replication forks fail to finish replication. On the other hand, the selection mechanism is poorly understood regarding which subset of licensed origins should be activated while others should stay dormant. It was suggested that a fraction of pre-RC is multi-mono-ubiquitinated on Orc3/Orc5 by an E3 ligase OBI1, which in turn marking those pre-RCs out of all available pre-RC for activation (Coulombe et al., 2019). However, the mechanism of how OBI1 selects its substrate is still unknown. Recently, one study made an interesting observation that the parental MCMs inherited from previous cell cycle have a distinct function from the nascent MCMs (Sedlackova et al., 2020). It was shown that the parental MCMs are preferred for forming active CMGs even though both parental and nascent MCMs can form pre-RC on chromatin. On the other hand, nascent MCMs serve to adjust the pace of active CMGs, possibly by acting as physical resistance of replication fork progression. It was therefore proposed that the surplus of licensed MCMs not only provides a backup system upon replication stress, but also acts to manage replication fork speed to actively prevent replication stress associated DNA damage. Nevertheless, the mechanism that differentiates these two groups of MCMs remains to be studied.

1.4 Preventing genomic instability during DNA replication

DNA is continuously being challenged by exogenous or endogenous sources of damages throughout the cell cycle. During DNA replication, the genome is particularly vulnerable to these insults. DNA damage resulting from these stresses must be precisely repaired to maintain the integrity of the genome.

1.4.1 Replication stress

A significant threat to genomic and chromosomal stability comes from replication stress. Replication stress is referred to as any condition that causes the slowing or stalling of replication fork progression and perturbs the dynamics of DNA synthesis (Gaillard et al., 2015; Zeman and Cimprich, 2014). Replication stress can be induced by exogenous or endogenous sources. Exogenous causes include ionizing radiation (IR), ultraviolet (UV) irradiation and chemotherapeutic drugs that lead to DNA damage, interstrand crosslinks and DNA breaks. These DNA lesions block CMG unwinding or polymerases, causing fork to stall. Chemical compounds such as hydroxyurea (HU) that result in dNTP depletion/imbalance also induce replication stress by causing uncoupling of DNA polymerase with CMG helicase (Muñoz and Méndez, 2017). Similarly, endogenous DNA damage such as naturally occurred depurination or oxidation can lead to replication stress. On the other hand, a great fraction of endogenous causes of replication stress has resulted from sequence or chromatin features that are intrinsically difficult to replicate. These include short tandem repeats and microsatellites regions (Kim and Mirkin, 2013), secondary structures such as hairpins or G-quadruplexes (Bochman et al., 2012; Paeschke et al., 2013), centromeres and telomeres regions (Black and Giunta, 2018; Higa et al., 2017). Collisions of replication and transcription machinery and R-loop, the three-stranded structures containing a DNA-RNA duplex and the excluded ssDNA, also impede with replication progression (García-Muse and Aguilera, 2019; Gómez-González and Aguilera, 2019). Common fragile sites, the chromosomal regions prone to experience replication stress and break upon replication inhibition, usually contain one or several above features (Feng and Chakraborty, 2017; Voutsinos et al., 2018). Interestingly, common fragile sites also correlate with origin-poor and ORC-poor regions, indicating that inefficient origin activity and reduced dormant origins play roles at these sites (Miotto et al., 2016; Sugimoto et al., 2018). Meanwhile, oncogene activation or

overexpression also causes replication stress (Primo and Teixeira, 2019). Many mechanisms underlie oncogene-induced replication stress, including defects in origin licensing due to short G1/premature S phase entry, depletion of dNTPs and exhaustion of replication factors associated with increased origin firing, and increased replication-transcription collisions (Ekholm-Reed et al., 2004; Jones et al., 2013; Kotsantis et al., 2016; Macheret and Halazonetis, 2018; Toledo et al., 2013). The role of oncogene-induced replication stress in early cancer development is well established.

One direct consequence of replication stress is the formation of exposed ssDNA due to fork stalling. Thus, replication stress is tightly associated with the activation of ATR pathway of the DDR.

1.4.2 DNA damage response

The DNA damage response (DDR) constitutes an elaborate network of signal pathways that prevents genomic instability. The DDR involves DNA lesion recognition, followed by a cellular signaling cascade that serves two main purposes: to halt cell cycle progression and to facilitate DNA repair. The phosphatidylinositol 3-kinase related protein kinases (PIKKs) DNA-PK (DNA-dependent protein kinase), ATM (ataxia-telangiectasia mutated), and ATR (ATM and Rad3-related) are three central kinases that regulate the DDR when they are activated by upstream damage recognition mechanisms (Blackford and Jackson, 2017; Ciccia and Elledge, 2010). DNA-PK and ATM are activated primarily by blunt ends of double-strand breaks (DSBs), while ATR responds to a wide range of DNA damage and replication stress resulting in single-strand DNA (ssDNA) exposure (Falck et al., 2005).

DNA-PK is comprised of a catalytic subunit (DNA-PKcs) and Ku70-Ku80 heterodimer (Blunt et al., 1995; Gottlieb and Jackson, 1993; Hartley et al., 1995). DNA-PK acts as a crucial regulator sensing DSBs in the cells and repairing them by NHEJ (non-homologous end joining) (Jette and Lees-Miller, 2015). In fact, NHEJ repairs the majority of the DSBs occurring in the cells, except those that happened during DNA replication, which are repaired preferably by HR (homologous recombination) (Scully et al., 2019; Shrivastav et al., 2008). Ku70-Ku80 (also known as XRCC6-XRCC5) heterodimer binds to DSB ends (Walker et al., 2001), and recruits DNA-PKcs to form holoenzyme and promote DNA end tethering (Britton et al., 2013; Graham et

al., 2016). Several other factors are then subsequently recruited to complete NHEJ, including XRCC4, XLF, PAXX and DNA ligase IV (LIG4) (Ahnesorg et al., 2006; Nick McElhinny et al., 2000; Ochi et al., 2015). It was proposed that the kinase activity of DNA-PKcs is important (Dobbs et al., 2010; Jette and Lees-Miller, 2015; Jiang et al., 2015), as it extensively phosphorylates itself as well as other NHEJ factors. Meanwhile, ATM can also trans-phosphorylate DNA-PKcs, and they have overlapping substrates (Gapud et al., 2011; Zha et al., 2011). However, only the auto/trans-phosphorylation of DNA-PKcs regulates NHEJ activity (Cui et al., 2005; Uematsu et al., 2007). The molecular functions of phosphorylations on other NHEJ proteins remain unclear, as phosphorylation on several NHEJ factors are not required for NHEJ (Douglas et al., 2005; Yu et al., 2008; Yu et al., 2003).

Compared to DNA-PK, ATM and ATR are two master regulators controlling broader cellular signaling cascades and checkpoint activation. Moreover, there is a fair amount of cross-talk between them. ATM is rapidly recruited to DSBs by MRN (Mre11-Rad50-Nbs1) complex (Falck et al., 2005; Paull, 2015), which are the earliest factors binding to the DSB ends similar to Ku heterodimer. MRN complex then activates ATM kinase activity (Lee and Paull, 2004, 2005; Uziel et al., 2003). ATM can facilitate DSBs repair through both NHEJ and HR, and the decision is cell cycle and CDK dependent (Hustedt and Durocher, 2016). ATM promotes NHEJ during G1 by working together with DNA-PK (Riballo et al., 2004), as well as through phosphorylating 53BP1, which is a major factor promoting NHEJ (Bothmer et al., 2011; Callen et al., 2013). In contrast, ATM phosphorylates BRCA1 (Cortez et al., 1999), which functions antagonizing 53BP1 and promoting HR, during S phase (Hustedt and Durocher, 2016). HR requires the generation of ssDNA from DSB sites by end-resection at DNA blunt ends (Cejka, 2015; Chapman et al., 2012), which is conducted by several nucleases including Mre11, CtIP, Exo1 and Dna2. ATM can stimulate CtIP, an MRN-interacting protein that is the main regulatory point in end-resection (Sartori et al., 2007), to promote this process (Wang et al., 2013). On the other hand, once activated, ATM initiates a signaling cascade that regulates a broad spectrum of cellular processes. Chk2 is the main ATM downstream effector kinase, which is activated when getting phosphorylated by ATM (Matsuoka et al., 1998). Together they phosphorylate and activate pathways including cell cycle checkpoint, DNA repair, chromatin remodeling, transcription, translation and many more (Matsuoka et al., 2007; Shiloh and Ziv, 2013).

ATR is activated by a wide range of genotoxic stress that involves ssDNA accumulation. The activation of ATR starts from RPA loading onto exposed ssDNA, which can be generated by end-resection of DSBs, replication stress or intermediates during the DNA repair processes (Branzei and Foiani, 2010; Byun et al., 2005; Cejka, 2015; Chapman et al., 2012; Li et al., 2016; Marechal and Zou, 2015; Novarina et al., 2011). RPA-ssDNA then serves as a platform to recruit ATR via its partner protein ATRIP (ATR interacting protein) (Zou and Elledge, 2003). Two independent mechanisms then function to activate ATR. The binding of Rad17/RFC and Rad9-Rad1-Hus1 (9-1-1) complexes to the junction of RPA-ssDNA and dsDNA recruits TopBP1, the first ATR-activating domain (AAD) containing protein, to activate ATR kinase activity (Delacroix et al., 2007; Kumagai et al., 2006; Lee et al., 2007; Mordes et al., 2008). On the other hand, ETAA1, the second protein identified with an AAD, binds to RPA directly and activates ATR in parallel of TopBP1 (Bass et al., 2016; Haahr et al., 2016; Lee et al., 2016). Subsequently, the activated ATR phosphorylates its downstream effector kinase Chk1 (Zhao and Piwnica-Worms, 2001). Like the ATM-Chk2 axis, ATR-Chk1 controls many cellular events during DNA damage. One proteomic study identified more than 700 ATM/ATR downstream substrates, highlighting the complexity of the DDR network (Matsuoka et al., 2007). A thorough examination of these substrates and their functions remains to be elucidated.

One canonical role of activated Chk1/Chk2 is to phosphorylate CDC25 to inhibit its activity and promote its degradation (Donzelli and Draetta, 2003). CDC25 is a phosphatase required to remove inhibitory modifications on cyclin-dependent kinases (CDKs) (Dunphy and Kumagai, 1991). Its degradation thereby arrests the cell cycle progression. On the other hand, p53 is also phosphorylated by ATM/ATR pathways under different genotoxic stresses (Banin et al., 1998; Canman et al., 1998; Tibbetts et al., 1999), which results in stabilizing p53 and leads to its activation (Hafner et al., 2019). ATM and ATR also target other p53 regulating proteins including MDM2 to control p53 stability (Cheng and Chen, 2010), and eventually lead to checkpoint activation and cell cycle arrest.

Another important function of DDR is to deposit S139 phosphorylation on histone variant H2AX, which is called γH2AX (Burma et al., 2001; Rogakou et al., 1998). γH2AX not only is a cytological marker for DNA damage, but also functions as a platform that scaffolds a serial repair protein assembly event and affects chromatin compaction (Scully and Xie, 2013). Despite first being identified as an ATM target and associated with DSBs, γH2AX is also formed by

ATR under different conditions (Hanasoge and Ljungman, 2007; Katsube et al., 2014; Ward and Chen, 2001).

RPA, the primary ssDNA binding protein required in ATR activation, is also a crucial substrate in the DDR (Marechal and Zou, 2015). RPA32 subunit is hyper-phosphorylated at its N-terminus upon different kinds of DNA damage (Anantha et al., 2007). Ser33 is a direct target of ATR (Olson et al., 2006). Subsequently, S4/S8 and several other sites are phosphorylated by DNA-PK and/or ATM (Anantha et al., 2007; Wang et al., 2001). Along with several CDK-dependent phosphorylations as well as other types of modification such as ubiquitination, post-translational modification on RPA has been suggested to be critical under DNA damage condition regulating DNA repair and checkpoint activation (Ashley et al., 2014; Liu et al., 2012b; Marechal and Zou, 2015; Murphy et al., 2014; Shi et al., 2010; Vassin et al., 2009).

1.4.3 DNA repair pathways during replication

Damages occurred during replication need to be precisely repaired. These damages include base lesions such as abasic sites and DNA methylation/oxidation, misincorporation errors, DNA adducts such as protein-DNA crosslinks, interstrand crosslinks, and DNA breaks. Different repair pathways are responsible for these distinct errors (Cortez, 2019).

DNA alkylation/oxidation and bulky adducts are mainly repaired by BER (Base Excision Repair) and NER (Nucleotide Excision Repair) (Spivak, 2015; Wallace, 2014). Although they are extremely crucial, these repair systems function throughout the cell cycle in the context of dsDNA, not necessarily associated with replication. Abasic sites, generated by spontaneous base loss or removal of damaged bases by glycosylases, are one of the most common lesions in human cells (Dianov et al., 2003). Repairing of abasic sites is also mainly conducted by BER. However, a recently identified protein HMCES, functioning at replication fork by interacting with PCNA using PIP box, senses abasic sites in ssDNA and promotes their repair during replication progression (Mohni et al., 2019; Thompson et al., 2019). This provides a novel mechanism of DNA repair during S phase.

Misincorporation errors or mismatches occurring during DNA replication are repaired by mismatch repair (MMR). In eukaryotes, the MutSα/β and MutLα regulate the recognition and

incision, respectively. MMR is active throughout the cell cycle with the highest activity observed during S phase (Edelbrock et al., 2009; Schmidt and Hombauer, 2016; Schroering et al., 2007). This is due to the association of MMR complex to the replication fork by interacting with PCNA. In fact, MMR proteins are among the highest enriched proteins at replication fork (Alabert et al., 2014; Sirbu et al., 2013). This ensures that the MMR machinery is able to fix errors as they are generated (Flores-Rozas et al., 2000; Gu et al., 1998; Hombauer et al., 2011; Kleczkowska et al., 2001). Moreover, interaction with PCNA also enhances the incision activity of MutLα and downstream resection during repair (Chen et al., 2013; Liberti et al., 2011; Pluciennik et al., 2010). Interestingly, recent findings strongly suggest that the MMR is also critical for the response to both alkylating and oxidative DNA damage (Bridge et al., 2014; Gupta and Heinen, 2019), which were believed to be repaired solely by BER/NER. Defects in MMR cause errors during DNA replication and are linked to hereditary cancer syndrome, Lynch syndrome, as well as colorectal cancer (Goellner, 2020; Li and Martin, 2016). Together these suggest the essential role of MMR for genomic integrity.

As discussed previously, DNA breaks in S/G2 phase is preferably repaired by HR due to the requirement of a sister chromatin template during its repair (Prado, 2018). At DSB sites, the 5'-3' end resection process creates ssDNA coated by RPA, and then BRCA2 facilitates the loading of Rad51 to replace RPA, forming Rad51 filaments. The Rad51 filaments then search for homologies and initiate strand exchange to complete HR (Ranjha et al., 2018). Other than functioning at DSBs, HR is also a key mechanism for the tolerance of replication stress (Ait Saada et al., 2018; Lambert et al., 2005; Petermann et al., 2010). BRCA2-Rad51 axis plays a critical role in these processes. Upon fork stalling or collapse, it protects nascent ssDNA from degradation, the absence of which leads to genome instability (Hashimoto et al., 2010; Kolinjivadi et al., 2017b; Mijic et al., 2017; Schlacher et al., 2011). Many other factors have also been identified modulating BRCA2-Rad51 mediated fork protection (Bhat et al., 2018; Ray Chaudhuri et al., 2016; Rondinelli et al., 2017; Taglialatela et al., 2017). Moreover, Rad51 promotes fork restart using its strand invasion ability (Ait Saada et al., 2018). Thus, HR is involved in replication fork protection and promoting fork restart after stalling.

The association of HR with human disease is clear. *BRCA* genes are well known for their connection to breast/ovarian cancer (King et al., 2003). Fanconi anemia (FA), a genetic disorder that is characterized by developmental defects, bone marrow failure and predisposition to cancer,

is originally identified associated with defects in HR-mediated interstrand crosslink (ICL) repair (Ceccaldi et al., 2016). Many FA genes such as *FANCD1 (BRCA2), FANCS (BRCA1), FANCR (RAD51), FANCN (PALB2), FANCU (XRCC2), FANCW (RFWD3)* are HR factors, FA pathway is therefore also known as FA/BRCA pathway. Meanwhile, recent research has established the connection between replication stress with FA, and many new FA genes have been identified that function in different aspects of DNA replication and repair (Kolinjivadi et al., 2020; Kolinjivadi et al., 2017a; Niraj et al., 2019). Collectively, FA pathway is recognized as an important pathway regulating genome integrity.

1.4.4 RFWD3

RFWD3 (RING finger and WD repeat domain 3) is a ubiquitin E3 ligase functioning in the DDR. RFWD3 has emerged to be a critical player in HR and checkpoint control. It was first identified as an ATM/ATR phosphorylation substrate upon DNA damage (Matsuoka et al., 2007). Early studies showed that RFWD3 regulates p53 function under DNA damage condition by interacting MDM2 and p53 (Fu et al., 2010). The complex formation of RFWD3 with MDM2/p53 further leads to the ubiquitination of p53 and stabilizes it. RFWD3 also directly interacts with RPA to regulate DNA damage responses and checkpoint activation (Gong and Chen, 2011; Liu et al., 2011b). Later it was shown that RFWD3 ubiquitinates RPA at stalled replication fork, which promotes replication fork restart and repair through HR (Elia et al., 2015). The role of RFWD3 in HR was further supported by other studies focusing on interstrand crosslink (ICL) repair. It was shown that RPA-mediated recruitment of RFWD3 to stalled replication fork is essential for ICL repair (Feeney et al., 2017). Moreover, RPA as well as RAD51 are both targeted by RFWD3 ubiquitination, which in turn promotes their efficient removal from the DNA damage sites to allow HR (Inano et al., 2017). Other than the biochemical characterization of RFWD3, biallelic mutations in *RFWD3* were identified in one patient with FA (Knies et al., 2017). Thus, *RFWD3* has been assigned the alias *FANCW* as an FA gene. A recent study also reported that RFWD3 modulates stalled fork stability in BRCA2-deficient cells (Duan et al., 2020). While a lot of studies have been done to investigate the function of RFWD3 during DNA damage response and HR, it is still unclear if RFWD3 functions during unperturbed cell cycle and DNA replication. Preliminary data from our lab

showed that depletion of RFWD3 under normal condition causes S phase accumulation and reduced replication fork speed, suggesting the potential function of RFWD3 regulating S phase progression (Wang, 2017). Moreover, we identified that RFWD3 interacts with PCNA via a PIP box in its WD-40 domain. My work of investigating the function of RFWD3 in unperturbed DNA replication will be present in Chapter 3.

1.5 References

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CHAPTER 2. ORC6 AT REPLICATION FORK ENABLES EFFICIENT MISMATCH REPAIR

2.1 Introduction

Accurate duplication of the genetic material and faithful transmission of genomic information are critical to maintain genome stability. Errors in DNA replication and repair mechanisms are deleterious and cause genetic aberrations leading to malignant cellular transformation and tumorigenesis. Origin recognition complex (ORC) proteins are critical for the initiation of DNA replication (Bell and Stillman, 1992), and the individual subunits of ORC also play vital roles in several non-preRC functions, including heterochromatin organization, telomere maintenance, centrosome duplication and cytokinesis (Chesnokov, 2007; Sasaki and Gilbert, 2007). Mutations within several *ORC* genes, including *ORC1, ORC4 and ORC6*, have also been linked to Meier-Gorlin Syndrome, a rare genetic disorder in children characterized by primordial dwarfism (Bicknell et al., 2011; Bleichert et al., 2013; Guernsey et al., 2011; Hossain and Stillman, 2012; Kuo et al., 2012).

ORC serves as the landing pad for the assembly of the multiprotein pre-replication complex at the origins of replication during G1 (Bell and Dutta, 2002). The smallest subunit of ORC, Orc6, is highly dynamic with respect to its association with the other ORC components (Dhar and Dutta, 2000; Siddiqui and Stillman, 2007; Vashee et al., 2001). Orc6 is an integral part of ORC in yeast and *Drosophila* (Bell and Stillman, 1992; Chesnokov et al., 2001), but only weakly associates with ORC in human and *Xenopus* (Dhar et al., 2001; Dhar and Dutta, 2000; Gillespie et al., 2001; Vashee et al., 2001). Orc6 possesses DNA binding ability and is believed to be critical for DNA replication initiation in all eukaryotes (Balasov et al., 2007; Chen et al., 2007; Li et al., 2018; Semple et al., 2006; Thomae et al., 2008); however, human Orc6 and Orc1-5 can bind to DNA independently (Thomae et al., 2011). In human cells, Orc6 is present in sub-stoichiometric levels to the other ORC subunits. There is evidence that hOrc6 protein directly binds to the Orc3 subunit and integrates as a part of ORC *in vivo* in human cell lines (Siddiqui and Stillman, 2007). However, a significant fraction of hOrc6 is not associated with the ORC complex, suggesting that hOrc6 is involved in ORC-independent functions within the cell (Dhar et al., 2001; Vashee et al., 2001). In support of this, metazoan Orc6 is also required for

cytokinesis and this function of Orc6 is facilitated by its binding to septin proteins (Balasov et al., 2007, 2009; Chesnokov et al., 2003; Prasanth et al., 2002). In yeast, Orc6 is dispensable for progression through mitosis and cytokinesis, but the depletion of Orc6 after pre-RC assembly has been shown to impair replication origin firing (Semple et al., 2006). However, no such role for Orc6 has been evaluated in higher eukaryotes.

Accurate duplication of the genetic material and correction of errors during S phase are efficiently coordinated with the machinery that ensures genomic integrity (Cook, 2009; Cortez, 2019). Mismatches occurring during DNA replication are recognized and removed by the Mismatch Repair (MMR) system. The key components of the MMR system that have been identified to date are MutSα (MSH2-MSH6 complex), MutSβ (MSH2-MSH3 complex), MutLα (MLH1-PMS2 complex), RFC-loaded PCNA, and Exonuclease 1 (EXO1) (Kunkel and Erie, 2015; Modrich, 2006). The association of MMR machinery to the replication fork is facilitated by PCNA, an essential replication factor that is also required for MMR (Flores-Rozas et al., 2000; Gu et al., 1998; Hombauer et al., 2011; Kadyrov et al., 2006; Kleczkowska et al., 2001; Umar et al., 1996). In eukaryotes, MMR is active throughout the cell cycle with the highest activity observed during S phase (Edelbrock et al., 2009; Schmidt and Hombauer, 2016; Schroering et al., 2007). Several replication accessory factors, chromatin associated factors and epigenetic regulators also influence MMR (Awwad and Ayoub, 2015; Kadyrova et al., 2011; Loughery et al., 2011; Schopf et al., 2012; Yuan et al., 2004), yet the functional relevance of these interactions has not been well understood. It is also worth noting that mutations within several replication factors result in defective MMR, however, the mechanistic studies are lacking.

We report that hOrc6 associates with the replication fork and is primarily required for DNA replication progression but not for G1 licensing. During S-phase, Orc6 forms an integral component of the mismatch repair (MMR) complex, and controls MMR complex assembly and activity. Further, in response to oxidative damage, often repaired by MMR, Orc6 gets phosphorylated during S-phase, which in turn inhibits DNA replication progression. Loss of Orc6 results in defective MMR activity, resulting in loss of ATR signaling. Based on our results, we conclude that hOrc6 has a fundamental role in genome surveillance during S-phase.

2.2 Results

2.2.1 Orc6 is a component of the replication fork

Orc6 is known to display robust DNA binding ability in metazoans (Balasov et al., 2007; Xu et al., 2020). In order to understand the type of DNA structure that facilitates the binding of hOrc6, we performed Single Molecule Pull down (SiMPull) assays (Jain et al., 2011). We observed that hOrc6 bound more tightly to replication fork structures (Kd 6.34+0.49nM) compared to ssDNA (19.24+5nM) and dsDNA (15.82+1.24nM) structures (Figure 2.1A and 2.1B). In order to test if hOrc6 associates with the replication fork *in vivo*, we performed isolation of Proteins On Nascent DNA (iPOND) (Sirbu et al., 2013). Nascent DNA was labeled with EdU, conjugated with biotin, and proteins associated with biotin-EdU-labeled DNA were pulled down. Thymidine chase experiment was used as the mature chromatin control. Orc6 showed accumulation on nascent DNA, whereas it did not enrich on the mature DNA (Figure 2.2A). To confirm this data and gain quantifiable results, we performed quantitative in situ analysis of protein interactions at DNA replication forks (SIRF) (Roy et al., 2018). Nascent DNA was labeled with EdU, conjugated with biotin, then proximity ligation assay (PLA) was performed to determine the association between hOrc6 and biotin-EdU-labeled DNA. SIRF experiments also showed that hOrc6 associated with nascent DNA (Figure 2.2B). Similar to our observation, hOrc6 was found in proteomic screens of proteins enriched in nascent DNA by nascent chromatin capture (SILAC $\log_2$ ratio = 0.76+/-.45) (Alabert et al., 2014) as well as by iPOND ($\log_2$ ratio = 1.28) (Wessel et al., 2019), suggesting its association with replication forks in human cells. Furthermore, immunoprecipitation experiments (Figure 2.2C) and SiMPull (Figure 2.2D and 2.2E) demonstrated that hOrc6 interacted with several replication fork components, including the single-stranded DNA binding protein RPA, the DNA clamp PCNA, and the clamp loader RFC. These results demonstrate that hOrc6 is enriched at the replication fork and associates with the fork components, implying that Orc6 in human cells could be involved in functions downstream of pre-RC assembly.

2.2.2 Orc6 is required for accurate S phase progression

In yeast and *Drosophila*, Orc6 is involved in origin licensing as part of ORC (Bleichert et al., 2013; Miller et al., 2019); however, the function of human Orc6 has not been fully examined.

The depletion of Orc6 in human cells with an intact p53-mediated checkpoint status caused a decrease in S phase population with a concomitant reduction in the chromatin association of PCNA (Figure 2.3A and 2.3B). The inability of the hOrc6-depleted cells to progress through S phase could either be due to reduced origin firing caused by defects in preRC assembly and/or inhibition of replication elongation. It has been shown that in metazoans the C-terminus of Orc6 interacts with Orc3 and hence recruits Orc6 to the core ORC (Bleichert et al., 2013). We addressed if the association of hOrc6 to ORC and the suggested role for ORC in licensing is responsible for the S-phase defects in Orc6-depleted cells. To test this, we performed complementation experiments using either full-length Orc6 or C-terminal truncated Orc6 (a.a. 1-187), which doesn't associate with ORC. We observed that the C-terminal truncation mutant rescued the S-phase defect to a similar extent to that of the full length Orc6 (Figure 2.3C). This suggests that, in human cells, the S phase defect of Orc6 depletion might not be due to defects of ORC function in origin licensing. To evaluate this further, we used flow cytometry to measure MCM licensing status in conjugation with cell cycle analysis (Matson et al., 2017). Cells were extracted using detergent before fixing to remove soluble MCMs. The chromatin bound MCMs served as an indicator of origin licensing and were detected by immunostaining with MCM3 antibody (Figure 2.3D). Strikingly, hOrc6-depleted cells showed a similar level of chromatin bound MCMs in G1 population to control cells, suggesting that the depletion of Orc6 did not alter the MCM association to the chromatin (Figure 2.3E and 2.3F). In contrast, Orc1 knockdown showed a severe licensing defect as indicated by dramatic reduction of chromatin-bound MCMs. However, even with proper licensing, hOrc6-depleted cells were unable to efficiently enter S phase.

In *Saccharomyces cerevisiae*, when Orc6 was depleted during late G1, MCM proteins were displaced from chromatin, and cells failed to progress through S phase, suggesting that efficiency of replication origin firing was compromised (Semple et al., 2006). However, similar role has not been evaluated for Orc6 in higher eukaryotes. To further assess if hOrc6 is truly dispensable for G1 licensing and functionally separate the role of hOrc6 in G1 from post-G1, we utilized a degron system, which allows degradation of Orc6 by ubiquitin-proteasomal degradation at any specific time point during the cell cycle (Giri et al., 2015). We tagged hOrc6 with a destruction domain (DD). This DD tag is recognized by the proteasomal machinery and facilitates the rapid degradation of the DD-Orc6. In the presence of Shield1, a molecule that

masks the DD tag, DD-Orc6 is prevented from degradation (Figure 2.4A). DD-Orc6 could substitute for endogenous Orc6, as the tagged-hOrc6 rescued the cell cycle defects observed in cells depleted of endogenous hOrc6 (Figure 2.4B and 2.4C; compare samples 2 and 3). We depleted the endogenous Orc6 using siRNA targeting the 3'-UTR of Orc6 in U2OS cells stably expressing DD-Orc6 and carried out the experiment in the presence of Shield1. We then synchronized the DD-Orc6-expressing cells depleted of endogenous Orc6 into early G1 phase (Figure 2.4D), and degraded DD-Orc6 by removing Shield1 from the medium. By determining the loading of preRC components onto chromatin in G1 cells lacking Orc6, we monitored the chromatin association of preRC components (Orc2, Cdt1, MCM3). The chromatin loading of these factors remain unaffected in the cells depleted of hOrc6, again suggesting that Orc6 is dispensable for replication licensing in human cells (Figure 2.4E).

Using the DD-Orc6 stably expressing cells, we evaluated the effect of hOrc6 loss in post-G1 cells on progression through S phase (Figure 2.4F). The degradation of hOrc6 in post-G1 cells resulted in slower progression through S phase. PI flow profile at 4-8 hrs time point post thymidine release revealed that more cells progressed through S phase in control cells compared to Orc6-depleted (Orc6-si1 & -si2 lacking endogenous as well as DD-Orc6) cells (Figure 2.4G). BrdU-PI profile further corroborated these results demonstrating the defects in S phase progression of Thymidine-released post-G1 cells in the absence of hOrc6 (Figure 2.4H).

In order to determine why the loss of hOrc6 in post-G1 cells caused defects in S phase progression, we determined the status of chromatin loading of preRC and pre-IC components in these cells (Figure 2.5A). We did not observe any defect in the total and chromatin-associated fraction of ORCs and MCMs in presence or absence of hOrc6 (Figure 2.5B). Similarly, PCNA and the DNA polymerases displayed comparable chromatin loading in the presence or absence of hOrc6. However, cells depleted of hOrc6 only during G1/S and S phase showed significant reduction in the chromatin association of Cdc45, a critical component of the CMG helicase (Figure 2.5B). The WT-Orc6 was able to efficiently rescue the Cdc45 loading in Orc6-depleted cells (Figure 2.5C). We further tested the role of hOrc6 in the chromatin loading of Cdc45 using a parallel cell biological approach. Using an *in vivo* reporter assay (Shen et al., 2010), we tethered MCM2 to a heterochromatic gene locus in U2OS 2-6-3 cells and examined the recruitment of endogenous Cdc45 to that site. In control cells, we found that the tethering of MCM was sufficient to recruit endogenous Cdc45 to the locus (52.8%; n = 36) (Figure 2.5D).

However, MCM2 failed to recruit Cdc45 to the locus in cells depleted of endogenous hOrc6 (27.5%; n = 40) (Figure 2.5D). Both biochemical and cell biological approaches demonstrate that hOrc6 play vital roles in the recruitment of Cdc45 to the chromatin in post-G1 cells. All of these results imply that hOrc6 plays an essential role in DNA replication.

Next, we directly tested the function of hOrc6 in DNA replication by using DNA combing assay. Active replication forks were labeled by incorporation of 5-chloro-2'-deoxyuridine (CldU) followed by 5-iodo deoxyUridine (IdU), and the DNA fiber length was measured to determine fork movement. We did not observe any change in the fork velocity in control and hOrc6-depleted cells (Figure 2.5E). These results together imply that hOrc6 is required for Cdc45 association to MCM, and hence facilitates efficient helicase activation and S phase entry.

2.2.3 Loss of Orc6 sensitizes cells to DNA damage and Orc6-depleted cells fail to activate ATR in response to replicative stress

Cells with replication defects often show reduced tolerance toward replication stress and DNA damage. Because we observed slower progression of cells through S phase upon Orc6 depletion (Figure 2.4G and 2.4H), we examined the replication fork dynamics in hOrc6-depleted cells upon replication stress treatment. Cells were first labeled with CldU for 30 min, treated with hydroxyurea for 2 hrs to induce stalling of replication fork, and subsequently released into the fresh medium containing IdU for another 30 min. DNA combing assay revealed that the cells lacking hOrc6 showed a significant and consistent decrease in fork restart (Figure 2.5F). This result implies that the slower S phase progression observed in hOrc6-depleted cells could be attributed to defects in the fork restart.

To test if the slowed progression through S phase resulted in replication stress and/or DNA damage, we performed comet assay to assess the extent of DNA breaks in hOrc6-depleted cells. There were no double- or single-strand breaks in the absence of hOrc6 under unperturbed condition. However, hOrc6-depleted cells in the presence of DNA damaging agents, including camptothecin (CPT) and hydrogen peroxide (H_2O_2) showed increased level of DNA damage compared to WT cells, as observed by both alkaline and neutral comet assays (Figure 2.6A and 2.6B). Furthermore, hOrc6-depleted or hOrc6-knockout cells were sensitive to DNA damage, as

observed by significant nuclear fragmentation and decreased cell survival post-H_2O_2 treatment (Figure 2.6C and 2.6D). We reasoned that in the absence of hOrc6, the cells either fail to repair the damage or fail to sense DNA damage.

We treated U2OS cells (with or without Orc6) with various DNA damaging agents, including H_2O_2 (oxidative damage), Neocarzinostatin (Radiomimetic drug), and HU for 4 hrs (to induce ssDNA and replication stress due to fork stalling) or for 24 hrs (to induce fork collapse and DSBs), and monitored the activation of ATR and ATM. The cells lacking Orc6 failed to activate ATR, as evident by decreased phospho-Chk1 and phospho-RPA, in response to different DNA damaging agents (Figures 2.7A and 2.7B). However, the ATM remained active as evident by intact Chk2 phosphorylation in both control and hOrc6-depleted cells. On the other hand, depletion of the other ORC subunits (Orc1 and Orc2) did not show significantly impaired ATR activation upon DNA damage (note* pRPA32) (Figure 2.7C). DNA-damaged cells lacking hOrc6 also showed reduced chromatin-association of RPA as observed by immunofluorescence staining using RPA antibodies following pre-extraction procedures (Figure 2.7D).

We next examined if the defects in RPA phosphorylation and Chk1 phosphorylation in cells lacking hOrc6 are a result of defective replication, or if they were due to a direct role of hOrc6 in ATR pathway. We synchronized the DD-Orc6-expressing cells (control as well as endogenous Orc6-depleted) in S phase by thymidine block, and subsequently degraded DD-Orc6 by removing Shield1 from the medium after the cells were released into S phase. We then induced DNA damage in the control and hOrc6-depleted S phase-synchronized cells (Figure 2.7E) and determined the status of ATR activity. We continued to observe defective ATR activation, as evident by reduced pChk1 and pRPA in Orc6-depleted cells that had been accumulated in S phase (Figure 2.7E). In addition, we synchronized control and hOrc6 knockout cells (hypomorph) into G2 phase and treated them with DNA damaging agents. The control G2 cells showed robust Chk1 and RPA phosphorylation whereas the Orc6-KO G2 cells failed to activate ATR, suggesting that hOrc6 facilitates ATR activation and that ATR activation defects in Orc6-depleted cells are not because of cell cycle effects (Figure 2.7F). These results support our model that hOrc6 is required for ATR activation and that the defects observed in Orc6-depleted cells are not simply a reflection of cell cycle defects.

The defect in RPA32 phosphorylation in hOrc6-depleted cells indicates that Orc6 may be involved in the upstream steps of the ATR signaling pathway. Processing of different DNA damages by various repair pathways yields ssDNA as a critical repair intermediate, which also serves to activate ATR. The two following possibilities could attribute to defects in ATR signaling upon hOrc6 depletion. First, hOrc6 could function in the recruitment of ATR signaling proteins to RPA-ssDNA platform. Secondly, hOrc6 could play a role in generating ssDNA after DNA damage. To test if hOrc6 plays a direct role in ATR activation, we performed an *in vitro* assay to examine the recruitment of ATR signaling proteins onto RPA-ssDNA platform in the presence or absence of hOrc6. We pre-assembled RPA-ssDNA complex by mixing recombinant RPA with 3'-biotinylated 70-mer single-strand DNA. We captured the complex using streptavidin-coated magnetic beads. These RPA-ssDNA structures were then incubated with nuclear extracts from control and Orc6-depleted U2OS cells, and we retrieved the proteins that bound to the structure. Western blotting was used to determine the effect of ATR signaling proteins (RPA32, pRPA32, ATRIP, Chk1, pChk1) recruitment and activation with or without hOrc6 (Figure 2.8A). We did not observe any defect in the recruitment of all of the proteins in Orc6-depleted cells. We also determined if hOrc6 plays any role in the RPA association to ssDNA. We performed ssDNA *in vitro* pull down with hOrc6 first being added to ssDNA then RPA, or vice-versa (Figure 2.8B). Again, these experiments demonstrated that hOrc6 did not have a direct role in facilitating RPA association to ssDNA. Based on these experiments, we concluded that hOrc6 does not play any direct role in the recruitment of signaling proteins to ssDNA and subsequent ATR activation.

Next, we evaluated whether hOrc6-depleted cells compromised the levels of ssDNA in cells. Towards this we quantified the levels of ssDNA in control and hOrc6-depleted cells using BrdU staining under non-denaturing conditions. DD-Orc6 cell line was used to ensure that both control and Orc6 knockdown cells were synchronized in S phase and had incorporated equal amount of BrdU. We observed a significant reduction in ssDNA in cells lacking Orc6 (Figure 2.9A). Based on these results, we conclude that hOrc6 is required for the generation of ssDNA post-DNA damage.

Our results suggest that the inability to generate ssDNA in hOrc6-depleted cells contributes to the defects in ATR activation. Many repair pathways require the generation of ssDNA, such as end resection after DSBs to allow homologous recombination. During excision

repair and mismatch repair pathways, ssDNA is also generated in a regulated manner by the removal of damaged bases/strands (Yan et al., 2014). Since we observed hOrc6 at the replication fork, we set out to determine the efficiency of ssDNA generation at the fork in cells lacking Orc6 under DNA damage condition. Cells labeled sequentially with CldU and IdU (20 mins each) were immediately treated with DNA damaging agent. The resection/degradation of nascent DNA strand post-DNA damage was then calculated by measuring the length of the IdU-labelled fiber in DNA fiber assay. We observed that DNA-damaged cells lacking hOrc6 displayed significantly longer IdU tracks, indicating defects in nascent DNA degradation (Figure 2.9B). This result further confirms that the inability to generate ssDNA in hOrc6 depleted cells causes defects in ATR activation.

2.2.4 Orc6 is upregulated and phosphorylated in response to oxidative stress

In yeast, Orc6 is phosphorylated in a cell cycle-dependent manner, with increased Orc6 phosphorylation as the cells exit G1. The hyperphosphorylation of yOrc6 following START is one of the known mechanisms by which cells prevent re-replication (Nguyen et al., 2001). We have observed that a population of hOrc6 is phosphorylated during DNA damage. We successfully mapped the DNA damage-mediated phosphorylation site of human Orc6 at T229 by performing PhosTag gel electrophoresis (Chakraborty et al., 2014) (Figure 2.10A).

By generating an antibody that specifically recognized only the pT229-modified version of hOrc6, we further investigated this particular phosphorylation. Orc6-pT229 occurred prominently when the cells were treated with oxidative or alkylating stress inducing agents such as okadaic acid, H_2O_2 and methyl methanesulfonate (MMS) (Figure 2.10B). Interestingly, cells showed enhanced Orc6-pT229 in response to oxidative stress, preferentially during S phase (Figure 2.10C). Both the total and the pT229-modified levels of Orc6 were elevated upon oxidative stress (Figures 2.10D-F). Treatment of cells with phosphatase (CIP) showed a loss of signal, confirming the specificity of the phospho-antibody (Figure 2.10D and 2.10E). Strikingly, Orc6-pT229 induced within 20 minutes of H_2O_2 treatment and persisted for ~30 mins post-recovery from H_2O_2 release, following which the levels of Orc6-pT229 declined (Figure 2.10F). Meanwhile, the cells recovered from H_2O_2 continued to show enhanced levels of total Orc6.

We had previously demonstrated that cells lacking hOrc6 failed to activate ATR upon oxidative stress. Interestingly, the T229 of hOrc6 is an ATM/ATR consensus (TQ/SQ) site, implying that DNA oxidative stress-induced phosphorylation of Orc6-T229 could be mediated by ATM/ATR axis. In order to test whether the oxidative stress-induced Orc6-pT229 contributes to ATR activity, we generated HA-Orc6 U2OS cell lines stably expressing phospho-dead (T229A) or phospho-mimic (T229E). We found that ATR activity (pChk1 & pRPA32) post-oxidative stress was partially rescued by both wild type and T229E mutant of Orc6-expressing cells but not the cells that expressed the T229A mutant, further supporting the importance of pT229 of Orc6 in DDR (Figure 2.11A). It is critical to note that T229 is located adjacent to the 'YxxWK' conserved motif within the C-terminus of hOrc6, mutation of which is reported to impede the recruitment of Orc6 to ORC, and is linked to the Meier-Gorlin syndrome (Bleichert et al., 2013). We therefore tested whether the hOrc6-pT229 affected Orc6's DNA binding activity and its interaction with other ORC subunits. We observed that both T229A and T229E mutants bound to DNA more efficiently than even the WT-Orc6 (Figure 2.11B) as observed by SiMPull assays. Also, the WT and both the mutants showed comparable levels of interaction with Orc2 (Figure 2.11C). These results imply that hOrc6-pT229 does not contribute to the DNA binding activity of Orc6, and also its ability to interact with ORC.

2.2.5 Orc6 associates with the MMR complex by interacting with MutSα

To gain mechanistic insights into the role of hOrc6 in S phase progression and in DNA damage response, we identified hOrc6-interacting proteins during S phase (in presence or absence of DNA damage) by performing immunoprecipitation followed by mass spectrometry. The hOrc6 interacting-proteome revealed known interactors as well as other previously unreported interactions. Strikingly, we found that in S phase extract, hOrc6 interacted with all the components of the MutSα (MSH2 and MSH6) and MutLα (MLH1 and PMS2) complexes that are known to initiate DNA mismatch repair (MMR) (Figure 2.12A). More importantly, H_2O_2-treated cells revealed enhanced interaction between hOrc6 and members of the MMR complex. We validated the *in vivo* association between hOrc6 and MSH2 using PLA. The interaction between hOrc6 and MSH2 was enhanced in oxidative stress-induced cells (Figure 2.12B). Furthermore, the association between these two proteins became more significant in S

phase cells (Figure 2.12B). To further examine the physical interaction of Orc6 with MutSα and MutLα, we conducted GST pull-down using purified proteins. We found that Orc6 directly interacts with MutSα but not MutLα (Figure 2.12C). Moreover, GST pull down using Orc6 truncations identified that Orc6 interacts with MutSα through its middle TFIIB (TFIIB-B) domain (Figure 2.12D and 2.12E). Our results indicate that hORC6 physically interacts with MutSα complex during S phase, and also during oxidative DNA damage.

2.2.6 Orc6 promotes MutLα recruitment to MutSα and facilitates MMR activity

The MMR process initiates when MutSα recognizes a mismatch on the daughter strand and recruits MutLα (Reyes et al., 2015). To determine the role of hOrc6 in the MMR process, we addressed the association of MutSα and MutLα in cells lacking hOrc6. Using PLA approach, we found that the interaction between members of the MutSα and MutLα was severely compromised (MSH6/MLH1 or MSH6/PMS2) in Orc6-depleted cells (Figure 2.13A). However, the interaction between components of MutSα complex itself (MSH2/MSH6) remained unaltered in cells lacking Orc6 (Figure 2.13B). Next, we determined the status of the chromatin association of individual members of the MutSα and MutLα complex in control and Orc6-depleted cells. DD-Orc6 cells were collected following the same protocol as in figure 2.7E to ensure cells are in S phase. Components of MutSα (MSH2 and MSH6) loaded onto the chromatin equally efficiently in control and Orc6-depleted (untreated as well as H_2O_2-treated) cells (Figure 2.13C). However, components of MutLα (MLH1 and PMS2) showed reduced chromatin association in H_2O_2-treated cells lacking Orc6 (Figure 2.13C). These data demonstrate that hOrc6 is required for efficient MMR complex assembly on chromatin during oxidative DNA damage.

To further address the mechanism of how hOrc6 functions in MMR complex assembly, we tested if the Orc6 promotes the association between MutLα and MutSα. We performed in vitro co-immunoprecipitation assay to determine the level of MutLα recruitment to immobilized MutSα using purified proteins in presence or absence of DNA. We observed that in the presence of Orc6, MutLα binds more efficiently to MutSα (Figure 2.13D and 2.13E). It is known that the affinity between MutSα and MutLα strongly increases in the presence of mismatch-containing DNA, which we have observed in our co-IP (Figure 2.13D, lane 1 and lane 3). However, the enhanced affinity between MutSα and MutLα by Orc6 is DNA and mismatch-independent, as we

found the Orc6 facilitated the binding of MutLα to MutSα in all the experimental settings containing either heteroduplex DNA, no DNA or homoduplex DNA (Figure 2.13D and 2.13E). Moreover, the TFIIB-B domain of Orc6 is sufficient to promote MutLα binding to MutSα (Figure 2.13F). Therefore, these data suggest that Orc6 is an accessory factor which by binding to MutSα increases the affinity of MutLα to MutSα.

Having established that hOrc6 plays a role in MMR complex assembly and that in the absence of hOrc6, MutLα is recruited inefficiently, we set out to address if hOrc6 promotes MMR activity. To this end, we performed the *in vivo* MMR assay, whereby we determined the reversion of a mutated codon within EGFP and quantified the activity by measuring EGFP signal in the cells (Traver et al., 2015). We prepared a heteroduplex plasmid containing a mis-pair in the EGFP codon, where the sense strand is wild-type EGFP and the antisense strand has a mutation, which results in a premature stop codon (Figure 2.13G). Therefore, only when cells were able to repair the mismatch on the antisense strand could they express full-length wild-type EGFP and give fluorescence signal. Using this assay, we quantified the extent of MMR activity in WT U2OS cells that is MMR-proficient (Schopf et al., 2012) and compared it to Orc6-depleted U2OS cells. We observed that depletion of hOrc6 caused significant reduction in EGFP signal intensity in the T/C heteroduplex, suggesting that without Orc6, cells are less proficient in MMR (Figure 2.13H and 2.13I). Finally, to gain physiological view of hOrc6 function in the MMR, we investigated the microsatellite instability (MSI), a hallmark of defective MMR, in cells lacking hOrc6. For this purpose, Orc6 knockout U2OS cells (hypomorph) were sub-cultured to passage 16 (more than 30 divisions), and the genomic DNA was isolated for PCR analysis. By comparing Orc6 KO cells with wild-type U2OS, we observed alterations in the length of a dinucleotide-containing the microsatellite loci, D5S346, indicating that the loss of hOrc6 induces MSI, but not so for a mononucleotide-containing repeat (BAT-25) (Figure 2.13J). Our results collectively demonstrate that hOrc6 bound to MutSα facilitates the recruitment of MutLα to chromatin and thus is required for efficient MMR activity (Figure 2.14).

2.3 Discussion

Primarily, based on studies from the yeast model system, Orc6, the smallest ORC subunit, is believed to function in DNA replication origin licensing and initiation. However,

unlike other ORC subunits, function of human Orc6 is less clear due to its poorly conserved nature and conflicting biochemical data among species. In terms of replication licensing, recent structural studies have elucidated the requirement of yeast Orc6 in MCM loading (Li et al., 2018; Miller et al., 2019). However, it is still uncertain if human Orc6 functions the same way as in budding yeast. Our findings here suggest that hOrc6 is by and large dispensable for G1 origin licensing, indicating divergent roles for Orc6 in human and yeast licensing processes. Recently, using CRISPR approach to knockout human Orc1, Orc2, Orc5 and Orc2/Orc5, it was reported that human core ORC is dispensable for replication (Shibata and Dutta, 2020; Shibata et al., 2016). However, in human cells, acute depletion of Orc1 and Orc2 resulted in defects in the chromatin loading of MCMs. Nevertheless, in our experimental system, Orc6 behaved differently in the sense that its depletion did not alter MCM loading to the chromatin. Our observations of a significant S phase phenotype in the same experimental system strongly argue the important function of Orc6 after origin licensing.

Yeast Orc6 is also required after the licensing steps since depleting Orc6 after pre-RC formation has been shown to impair replication origin firing by destabilizing pre-RC (Semple et al., 2006). In human cells, we demonstrate that Orc6 associates with the replication fork and interacts with the components of the replication fork, the observation that are also supported by proteomic data (Alabert et al., 2014; Wessel et al., 2019). Using the DD-Orc6 to degrade Orc6 at specific stages of the cell cycle, we find hOrc6 to be dispensable for the licensing step, but essential for S phase progression. Our results argue that in human cells, Orc6 definitely functions after pre-RC formation. Most significantly, in cells lacking hOrc6, we find that Cdc45 loading onto chromatin was impaired. These results point to an inefficient CMG helicase activity and defective DNA replication progression in the absence of hOrc6. Without hOrc6, deficiency of Cdc45 chromatin loading results in fewer origins being activated, but the ones that are activated display normal fork progression rate.

The earliest steps of DNA replication, including the establishment of preRC are coordinated with the DNA damage network that prevents genomic instability (Cook, 2009). ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad3-related) are the key kinases that regulate DDR (DNA damage response) (Marechal and Zou, 2013). While ATM activation requires double strand DNA break bound by the MRN complex (Lee and Paull, 2005), ATR is typically activated when it senses single-stranded DNA coated by RPA (Marechal and Zou,

2013; Shiotani and Zou, 2009). Utilizing Stable Isotope Labeling by Amino acids in Cell culture (SILAC) quantitative proteomic approach, an earlier study identified over 700 ATM/ATR substrates upon IR-induced DNA damage, including hOrc6 at T229 site (Matsuoka et al., 2007). Multiple preRC factors have also been identified and studied. For example, Cdc6 physically interacts with ATR and this is thought to regulate the activation of replication checkpoint (Yoshida et al., 2010). Similarly, Mcm2 is an ATR substrate and Mcm2 associates with ATRIP and it is thought that the phosphorylation of MCM inhibits its DNA helicase activity or it may contribute to maintaining MCM at the stalled fork to prevent fork collapse (Cortez et al., 2004). Based on these data, it was proposed that a potential mechanism to block re-replication after DNA damage involves phosphorylation of preRC components by one or several of the checkpoint kinases. We observed that hOrc6 levels were elevated upon specific kinds of genotoxic stress like oxidizing agents, and it is phosphorylated in response to oxidative stress. We propose that the phosphorylated hOrc6 either acts as a brake or as a sensor of oxidative DNA damage to prevent replication progression. Future studies will test how phosphorylated hOrc6 inhibits replication progression in response to oxidative DNA damage.

Mismatch repair (MMR) is a process that recognizes and fixes errors during DNA replication. Our results indicate that hOrc6 is an accessary factor of the MMR complex, promoting MMR complex assembly and activity (Figure 7J). It is worth noting that although the structures of MutS and MutL are available, the interaction between them has been difficult to study (Friedhoff et al., 2016). During the eukaryotic MMR process, MutSα recognizes the mismatch and undergoes ATP-dependent conformational changes that allows the binding of MutLα. This MutSα-MutLα complex is very transient and dynamic. By using site-specific crosslinking, the transient *E. coli* MutS-MutL complex was successfully captured, providing valuable information about the MutS conformation when interacting with MutL (Groothuizen et al., 2015). However, the information about the human MutSα-MutLα structure is still lacking. It is also unclear if *in vivo* there is any additional factor influencing the MMR complex assembly. Our results that the association of Orc6 with MutSα increases the affinity of MutLα binding to MutSα is therefore of great importance for the MMR field. We propose that the binding of Orc6, as an accessory factor, stabilizes the MutSα at a conformation that allows MutLα to bind. Future structural studies are needed to investigate how hOrc6 influences MutSα structure.

During MMR, ssDNA is an important intermediate generated by EXO1 excision after the incision step (Constantin et al., 2005; Genschel and Modrich, 2003; Kadyrov et al., 2006; Zhang et al., 2005). More recently, it has been shown that MMR processing of a methylation-induced DNA lesion behind the replication fork causes ssDNA accumulation, interrupts fork progression, and might induce replication stress (Gupta et al., 2018). Thus, inefficient MMR activity could lead to reduced ssDNA generation. This is consistent with the defects in ssDNA generation that we observed in hOrc6-depleted cells. This is further corroborated by the fact that cells treated with oxidative or alkylating agents showed increased DNA damage without Orc6. Since there is not an efficient way to specifically induce mismatch on DNA, most studies utilize oxidative or alkylating agents. Base excision repair (BER) and nucleotide excision repair (NER) are believed to be the two main pathways that remove these lesions. However, studies suggest that MMR is also critical for the response to oxidative DNA damage (Bridge et al., 2014). Moreover, defects in MMR activity directly lead to reduced ATR signaling, as knockdown of MSH2 causes reduced Chk1 phosphorylation (Wang and Qin, 2003). Additionally, recognition of O6-meG/T mispairs by MutSα and MutLα directly recruits and activates ATR/ATRIP complex independent of RPA-ssDNA (Yoshioka et al., 2006). Thus, our finding that ATR is not fully activated in hOrc6-depleted cells is likely due to defective MMR repair pathway. On the other hand, *in vitro* studies have pointed that MMR is accomplished within 10-15 minutes (Constantin et al., 2005; Zhang et al., 2005), however *in vivo* it is likely to take longer depending on the amount and severity of the damage. We observe that phosphorylation of hOrc6 upon damage is rapid and the dephosphorylation is accomplished within 30 minutes, coinciding with the time needed for MMR. Together, our results point to a new regulatory mechanism in human cells whereby Orc6 travels along with replisome and acts as an accessory factor of MutSα upon encountering a mismatch, undergoes phosphorylation and promotes MMR complex formation to facilitate DNA damage repair and ATR activation.

It is well known that defects in MMR cause errors during DNA replication and are linked to a hereditary cancer syndrome, Lynch syndrome, often associated with microsatellite instability (Goellner, 2020). Further, colorectal tumors are often associated with defects in MMR (Li and Martin, 2016). It is worth noting that Orc6 levels are highly elevated in colorectal cancers (Xi et al., 2008), and the reduction of Orc6 sensitizes colorectal cancer cells to chemotherapeutic drugs (Gavin et al., 2008). Moreover, *ORC6* has been included as a predictor in three commonly used

prognostic multigene expression profiles for breast cancer (Koleck and Conley, 2016). It has been known for a long time that the elevated level of Orc6 correlates with genome instability, yet the molecular details had remained to be elucidated. Our present studies provide novel insights into the role of Orc6 in the maintenance of genome integrity.

2.4 Material and methods

2.4.1 Cell lines

Human cell lines U2OS and HEK293T were grown in DMEM containing high glucose and supplemented with 10% fetal bovine serum (FBS). Plasmids and siRNAs were delivered using Lipofectamine 2000 and Lipofectamine RNAiMax (Invitrogen), respectively. U2OS cells stably expressing HA-Orc6 or DD-Orc6 and Orc6 knockout cells were maintained in media containing selective antibiotics. Cell synchronization was done by nocodazole arrest for M and G1 phase samples, and by thymidine block for G1/S, S and G2 phase samples.

2.4.2 Isolation of Proteins On Nascent DNA (iPOND)

A modified version of iPOND (Sirbu et al., 2013), described as aniPOND (Leung et al., 2013), was used. In brief, 1.5 x 10^8 293T cells were pulsed with 10μM of EdU for 10 min. For mature DNA samples, cells were chased with 10μM Thymidine for 1h. Cells were lysed in NEB (20mM HEPES pH7.9, 50mM NaCl, 3mM MgCl2, 300mM sucrose, and 0.5% NP-40) on ice for 15 min and nuclei were harvested by centrifugation. After washing with PBS, nuclei were incubated in freshly prepared click reaction cocktail (2 mM copper sulfate, 10 μM biotin-azide, and 100 mM sodium ascorbate in PBS) at 4 °C for 1h. Nuclei were then washed in PBS and resuspended in B1 buffer (25mM NaCl, 2mM EDTA, 1% NP-40 in 50mM Tris-HCl pH8). Next, sonication was performed at 4 °C using a bioruptor (Diagenode). Samples were then centrifuged at max speed for 10 min at 4 °C and supernatants were collected. An equal volume of B2 buffer (150mM NaCl, 2mM EDTA, 1% NP-40 in 50mM Tris-HCl pH8) was added to bring up the NaCl concentration, and input was taken at this point. EdU labeled DNA was then pulled down with 50μl of streptavidin beads (Dynabeads MyOne Streptavidin C1) at 4 °C overnight. Beads

were washed with B2 buffer three times before boiled in laemmli sample buffer to elute captured proteins.

2.4.3 in Situ protein Interactions at Replication Forks (SIRF)

For SIRF experiments (Roy et al., 2018), cells were first pulsed with 125 μM EdU for 10 min. For Thymidine chases, cells were washed with PBS then 100 μM of Thymidine was added for 3h. Cells were then fixed in 2% paraformaldehyde (PFA) for 15 min at room temperature and permeabilized on ice with PBS containing 0.5% triton X-100. After washing with PBS, click reaction was performed with biotin-azide for 1h at room temperature. The coverslips were then blocked with blocking solution and preceded to standard PLA procedure using anti-biotin and antibodies indicated in the figures.

2.4.4 Proximity Ligation Assay (PLA)

PLA was performed using Sigma Duolink PLA as per the manufacture's protocol. In brief, cells on coverslips were fixed in 2% PFA for 15 min at room temperature and permeabilized on ice with PBS containing 0.5% triton X-100. Coverslips were then blocked at 37 °C for 1h and incubated with primary antibodies overnight at 4 °C. After washing in buffer A, coverslips were incubated with Duolink PLA probes anti–mouse MINUS and anti–rabbit PLUS for 1h at 37 °C. Coverslips were then washed in buffer A and ligation was performed using ligation reaction mixture at 37 °C for 30 min. Next, coverslips were washed in buffer A and incubated in amplification reaction mixture for 100 min at 37 °C in the dark. Coverslips were subsequently washed twice in buffer B and once in 0.01x buffer B before DAPI staining.

For PLA experiments together with EdU labeling, 10 μM EdU was added 30 min before fixation. Click reaction using AF488-azide (Invitrogen) was performed after the permeabilization step and before the blocking step mentioned above, and all steps were performed in the dark.

2.4.5 Immunostaining

Cells were pre-extracted in 0.5% triton X-100 in CSK buffer (10mM PIPES pH 6.8, 100mM NaCl, 300mM sucrose, 3mM MgCl2) for 5 min on ice before fixation if needed. Cells were then fixed in 2% PFA for 15 min. If the pre-extraction was not performed, then cells were permeabilized on ice with PBS containing 0.5% triton X-100 for 5 min. Coverslips were then blocked in 1% normal goat serum (NGS) in PBS for 30 min and incubated with primary antibodies. Next, cells were washed in NGS/PBS and incubate in fluorophore-conjugated secondary antibodies for 45 min at room temperature. Cells were then washed in PBS and stained with DAPI.

For ssDNA visualization, cells were cultured in 10 μM BrdU for 36 h before any treatment to ensure same amount of BrdU incorporation. Cells were pre-extracted in CSK buffer containing 0.5% Triton X-100 for 5 min on ice, followed by fixing in 2% PFA for 20 min. Cells were washed with PBS and treated with chilled methanol for 15 min. Next, cells were treated with chilled acetone for 30 sec, washed with PBS again and blocked in PBST containing 2% BSA for 1 h. Cells were then incubated with the FITC-conjugated BrdU antibody at 4°C overnight. After washes with PBS, cells were stained with DAPI.

2.4.6 Immunoprecipitation

Cells were washed with PBS and lysed in IP lysis buffer (50mM Tris pH7.4, 150mM NaCl, 1mM MgCl2, 10% glycerol, 0.2% NP-40) containing protease inhibitors. Lysates were then sonicated and treated with benzonase nuclease (Sigma) for 30 min at room temperature, then EDTA was added to 2mM. Centrifugation was done at max speed for 10 min to remove insoluble debris. Next, lysates were pre-cleared with Gammabind G sepharose (GE healthcare Life Science) for 30 min at 4 °C. Antibodies were then added into lysates and incubated at 4 °C overnight. Proteins bound by antibodies were pulled down by Gammabind G Sepharose for 3h at 4°C. After incubation, beads were washed in lysis buffer and captured proteins were eluted and analyzed with western blot or mass spectrometry. Mass spectrometry and data analysis were performed by the Taplin Biological Mass Spectrometry Facility.

2.4.7 Flow cytometry

For PI cell cycle profile, cells were collected and washed once in ice cold PBS, resuspended in PBS + 1% NGS, and fixed in 90% chilled ethanol overnight. Cells were then washed and resuspended in PBS + 1% NGS with 120 μg/ml propidium iodide (PI) and 10 μg/ml RNase A for 45 min at 37 °C. DNA content was measured by flow cytometry. For BrdU-PI fow, cells were pulsed with BrdU for 30 min and stained with FITC-conjugated BrdU antibody before PI staining.

MCM-PI flow was done following methods previously described (Matson et al., 2017) with modifications. Briefly, cells were collected and pre-extracted with 0.5% triton X-100 in CSK buffer for 5 min on ice to remove soluble MCMs. Cells were then fixed in 1% PFA for 15 min RT, and stained with MCM3 antibody for 1h at 37 °C in 1% BSA/PBS with 0.1% NP-40. Next, Cells were incubated with fluorophore-conjugated secondary antibody for 1h at 37 °C. Finally, after washes, cells were stained with PI as indicated above.

2.4.8 Chromatin fractionation

U2OS cells were resuspended with solution A (10mM HEPES pH7.9, 10mM KCl, 1.5mM MgCl2, 0.34M sucrose, 1mM DTT, 10% glycerol and 0.1% Triton X-100) and incubate on ice for 5min. The cytoplasmic fraction (S2) was then separated from the nuclei by centrifuging at 4°C at 1400g for 4min. Isolated nuclei were then washed with solution A without Triton X-100. The nuclei pellet was resuspended with solution B (3mM EDTA, 0.2mM EGTA, and 1mM DTT) and incubated on ice for 30min. The nuclear soluble fraction (S3) was then separated by centrifuging at 4°C at 1700g for 4min. The S2 and S3 fractions can be combined as total soluble fraction (S). The isolated chromatin pellets were then washed with buffer B. Finally, the chromatin pellets were resuspended in solution A and sonicated for 1min to get P3 fraction.

2.4.9 Comet assay

Comet assay was performed using CometAssay Kit (Trevigen) following the manufacturer's instructions. In brief, cells were collected by trypsinization, embedded in low-melting agarose and placed on CometSlides. After agarose solidifying, the slides were immersed

in lysis solution for 30 min then subjected to electrophoresis for 30 min. For alkaline comet assay, the slides were incubated in alkaline unwinding solution before electrophoresis. After washing in water and 70% ethanol for 5 min each, the slides were allowed to dry and DNA was visualized using SYBR safe staining.

2.4.10 DNA fiber assay

Cells were labeled with 50mM CldU and 200mM IdU according to schemes in figures. DNA fibers were prepared on vinyl-silane coated coverslips using the FiberComb molecular combing system (Genomic Vision) as per the manufacture's protocol. To visualize the CldU and IdU tracks, DNA fibers on coverslips were denatured in denaturation solution (0.5M NaOH, 1M NaCl) for 8min at room temperature. Coverslips were then washed with PBS and dehydrated in 70%, 90%, and 100% ethanol for 5min each. Coverslips were blocked with 1% BSA in PBST, followed by incubating in antibodies against CldU and IdU. After washing in BSA/PBST, the coverslips were incubated in FITC-conjugated goat anti-rat IgG and Texas Red-conjugated goat anti-mouse IgG. The images were captured using Zeiss Axiovision system.

2.4.11 Protein purification

Human RPA and MutSα were purified as described previously (Binz et al., 2006; Dzantiev et al., 2004). p11d-tRPA was a kind gift from Dr. Marc Wold (University of Iowa, Iowa City, IA). Human GST-Flag-Orc6 was induced for overexpression in E. coli BL21 (DE3) codon (+). The cells were collected and resuspended in GST buffer (50mM Tris pH7.5, 0.1mM EDTA, 150mM NaCl, 1mM DTT and 5% glycerol) containing lysozyme 0.5mg/ml and 0.1% Triton X-100 followed by sonication. The lysate was collected by centrifugation and the supernatant was subjected to GSTrap column (GE healthcare) using an AKTA pure system. After washing with GST buffer containing 500mM NaCl, GST-Orc6 was eluted in GST buffer containing 20mM reduced glutathione. Human Flag-MutLα was expressed in insect Sf9 cells and purified by chromatographies on α-Flag beads and MonoQ and MonoS columns.

2.4.12 GST pull down assay

GST control or GST-Flag-Orc6 was induced for overexpression in E. coli BL21 (DE3) codon (+), and lysate was prepared as described in protein purification part. The lysate was incubated with Glutathione-Agarose beads (Sigma) for 1h at 4°C. After protein binding, the beads were washed twice with GST buffer containing 500mM NaCl and once with GST buffer. For each GST pull down reaction, 2.5 µl of the packed beads containing 25 µg GST fusion protein was equilibrated in buffer A (20 mM HEPES pH 7.4, 100 mM NaCl, 0.1 mM EDTA, 0.01% Nonidet P-40, 5% glycerol, 0.1 mM DTT, and 0.2 mM PMSF). MutSα or MutLα were added as indicated in the figures to a final reaction volume of 30 µl containing buffer A. After incubated for 1h at 4°C, the beads were washed extensively with buffer A and bound proteins were eluted by boiling in laemmli sample buffer.

2.4.13 In vitro MutLα recruitment by co-immunoprecipitation

For each co-IP reaction, 5 µl packed GammaBind G sepharose beads were mixed with 5 µg of MSH2 antibody (Santa Cruz) overnight at 4°C. The beads with MSH2 antibody immobilized on were then equilibrated in MMR buffer (20 mM HEPES pH 7.4, 100 mM KCl, 5 mM MgCl2, 2 mM ATP, 4 mM DTT, 0.4 mg/ml BSA, 3–5% (v/v) glycerol), and MutSα was added in the final volume of 30 µl containing MMR buffer. The mixture was incubated for 2h at 4°C with gentle rotation. Afterward, the beads were washed, and 5′-nicked G-T or A-T DNA (Dzantiev et al., 2004) and proteins (GST control/GST-Flag-Orc6 and MutLα) were subsequently added to the reaction mixture as indicated in the figures; the final reaction volume was 30 µl containing MMR buffer. After incubating for 1.5h at 4°C, the beads were extensively washed twice in MMR buffer containing 5% skim milk and once in MMR buffer. The proteins bound were finally eluted by boiling in laemmli sample buffer.

2.4.14 Mismatch repair (MMR) assay

In vivo mismatch repair efficiency is measured using MMR assay previously described (Traver et al., 2015). pCS2-EGFP-WT (wild type) was a kind gift from Dr. Marcel Méchali (Laboratory of DNA Replication and Genome Dynamics, Institute of Human Genetics, CNRS,

Montpellier, France). pCS2-EGFP-t456g was generated from WT by Quikchange Site-Directed mutagenesis procedure. The pCS2-EGFP-WT plasmid was amplified into linear form with two primers, WT_Fwd and WT_Rev, of which only the reverse one was 5'-phosphorylated. The mutated pCS2-EGFP-t456g was amplified with another set of primers, MIS_Fwd and MIS_Rev, of which only the forward one was 5'-phosphorylated. The PCR products were purified and digested using Lambda exonuclease (NEB) that degrades only the 5'-phosphorylated strands. The remaining single strands were then mixed (WT+t456g), denatured at 97°C for 5 min and annealed by slowly cooling to room temperature, allowing them to form nicked circular plasmids with T/C mismatch. The heteroduplex products were transfected into cells to observe mismatch repair efficiency by measuring EGFP signal.

2.4.15 Microsatellite Instability (MSI) Assay

U2OS Orc6 KO cells (clone 3) were cultured to passage 16 (more than 30 divisions) and then genomic DNA was extracted. Genomic DNA of wild-type U2OS cells was used as control. Genomic PCR was carried out under following conditions: initial denature at 98 °C for 5 min, followed by 35 cycles of 98 °C for 30 sec, annealing temperatures specific to each primer pair for 30 sec and 72 °C for 30 sec, with a final extension at 72 °C for another 7 min. PCR products were analyzed by 15% non-denaturing polyacrylamide gel electrophoresis and visualized by EtBr staining.

2.4.16 Single Molecule Pull down (SiMPull)

For replication fork-like DNA substrate preparation, partial duplexes of T1/P1 and T2/P2 were generated by annealing equimolar concentrations of the oligonucleotides in a buffer containing 10 mM Tris, pH 8.0 and 50 mM NaCl at 95 °C for 3 min, followed by slow cooling to room temperature. The two duplexes were then combined together and incubated overnight at room temperature to generate the replication fork probe. The reaction mixture was subjected to PAGE purification prior to use.

For double-strand DNA substrate, a T1-int/T2 duplex was generated similarly by annealing T1-int and T2 at 95 °C for 3 min, slow cooling to ambient temperature, followed by

PAGE purification. The purified constructs were stored in buffer containing 10 mM Tris, pH 8.0 and 25 mM NaCl.

Single-molecule experiments were performed on a prism-type TIRF microscope equipped with an electron-multiplying CCD camera (EM-CCD) (Roy et al., 2008). For single-molecule pull-down experiments quartz slides and glass cover slips were passivated with 5000 MW methoxy poly-(ethylene glycol) (mPEG, Laysan Bio) doped with 2-5 % 5000 MW biotinylated PEG (Laysan Bio). Each passivated slide and cover slip was assembled into flow chambers. GST-Flag-Orc6 wild-type and its variants (T229A and T229E) were diluted to 1 nM and pulled down with biotinylated antibodies against GST (Abcam, ab87834), already immobilized on the surface via neutravidin-biotin linkage. Any unbound protein was washed off after 10 min incubation and a predefined concentration of DNA substrate (replication fork-DNA, dsDNA, i.e. T1-int/T2 or ssDNA, i.e. T1-int) was introduced in the imaging chamber. The protein-DNA complexes were imaged post 15 min incubation with the DNA substrate, in a buffer containing 20 mM HEPES (pH 8), 60 mM KCl, 5 mM MgCl2, 0.5 mM EDTA and 8% Glycerol. Cy3- and Cy5- tagged DNA were excited at 532 nm and 640 nm respectively and the emitted fluorescence signal was collected via band pass filters (HQ 570/40, Semrock for Cy3 and 665LP, Semrock for Cy5). 15 frames were recorded from each of 20 different imaging areas (5,000 μm2) and isolated single-molecule peaks were identified by fitting a Gaussian profile to the average intensity from the first ten frames. Mean spot-count per image for Cy3 and Cy5 was obtained by averaging 20 imaging areas using MATLAB scripts. All experiments were carried out at room-temperature.

For Orc6 interaction with RPA, SiMPull experiments were carried out in flow chambers prepared on quartz microscope slides, which were passivated with methoxy-polyethylene glycol (mPEG) doped with 1% biotin-PEG. Biotinylated HA antibody was immobilized on PEG passivated surfaces at approximately 20 nM concentration for 20 min after coating the flow chambers with 0.2 mg/ml NeutrAvidin for 5 min. Cells were collected 24 h after transient transfection, and lysed in high salt buffer (50 mM Tris-HCl pH 7.4, 500 mM NaCl, 10% glycerol, 0.25% Triton X-100 with protease inhibitors) at 4°C for 20 min. Equal amount of zero salt buffer were added and incubated for another 10 min. After centrifugation, the supernatants were used for SiMPull analysis. Samples were appropriately diluted with T50 buffer (10 mM

Tris-HCl pH 8.0, 50 mM NaCl, 0.1 mg/ml BSA) to obtain optimal single molecule density on the surface. Diluted Samples were incubated in the chamber for 20 min and washed with the buffer.

2.4.17 Single-strand DNA in vitro pull down

To generate ssDNA for the in vitro binding assay, synthetic 70-mer DNA oligomers with 3' biotinylation were attached to streptavidin-coated magnetic beads (Dynabeads™ MyOne™ Streptavidin C1) in binding buffer (10mM Tris pH7.5, 100mM NaCl, 10% glycerol, 0.01% NP-40, 10mg/ml BSA). Generally, 100 pmol of biotinylated DNA oligomers were incubated with 100 µl of dynabeads for 30 min at room temperature. The ssDNA-bound beads were washed with binding buffer to remove unbound DNA oligomers. For each reaction, 5 µl ssDNA-bound beads were used. For RPA coated ssDNA-beads, 25 pmol of purified RPA was incubated with every 5 µl of ssDNA-beads. The beads were used for in vitro pull down as indicated in the figures.

2.4.18 oligos used in this study

WT_Fwd: 5'-GTTGCTCTTGCCCGGCG and WT_Rev: 5'-[Phos]CGGTCGCCGCATACACTA
MIS_Fwd: 5'-[phos]GCTACAGAGTTCTTGAAGTG and MIS_Rev: 5'-CCGCCTACATACCTCGCT
D5S346_Fwd: 5'-ACTCACTCTAGTGATAAATCGGG and D5S346_Rev: 5'-AGCAGATAAGACAGTATTACTAGTT
BAT-25_Fwd: 5'-TCGCCTCCAAGAATGTAAGT and BAT-25_Rev: 5'-TCTGCATTTTAACTATGGCTC
Orc6 siRNA1: Sense 5'-AGAUAGAUAAACGGAAUUGGAGCCA and Antisense 5'-UGGCUCCAAUUCCGUUUAUCUAUCUAU
Orc6 siRNA2: Sense 5'-UGUACAGCCUAAGUUAAUAAAUGTT and Antisense 5'-AACAUUUAUUAACUUAGGCUGUACAAU
T1: 5'-CGTGACTTGATGTTAACCCTAACCCTAAGATATCGCGTTAAGTGAGTGTGAGGATACATGTAGGCAATTGCCACGTGTCTATCAGCTGAAGTTGTTCGCGACGTGCGATCGTCGCTGCGACG
T2: 5'-CGTCGCAGCGACGATCGCACGTCGCGAACAACTTCAGCTGATAGACACGTGGCAATTGCCTACATGTATCCTCACACTCTGAATACGCGATATCTTAGGGTTAGGGTTAACATCAAGTCACG
P1: 5'-CGTCGCAGCGACGATCGCACGTCGCGAACAACTTCAGCTGATAGACACGTGGCAATTGCCTACATGTATCCTCACACTCTGT
P2: 5'- CCACGTGTCTATCAGCTGAAGTTGTTCGCGACGTGCGATCGTCGCTGCGACG/Cy3
T1-int: 5'-CGTGACTTGATGTTAACCCTAACCCTAAGATATCGCGTTATCAGAGTGTGAGGATACATGTAGGCAATTGCCACGTGTCTATCAGCT/iCy5/GAAGTTGTTCGCGACGTGCGATCGTCGCTGCGACG
Biotinylated ssDNA for in vitro pull down: 5'-TGCAGCTGGCACGACAGGTTTTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCT[BtnTg]

2.5 Figures

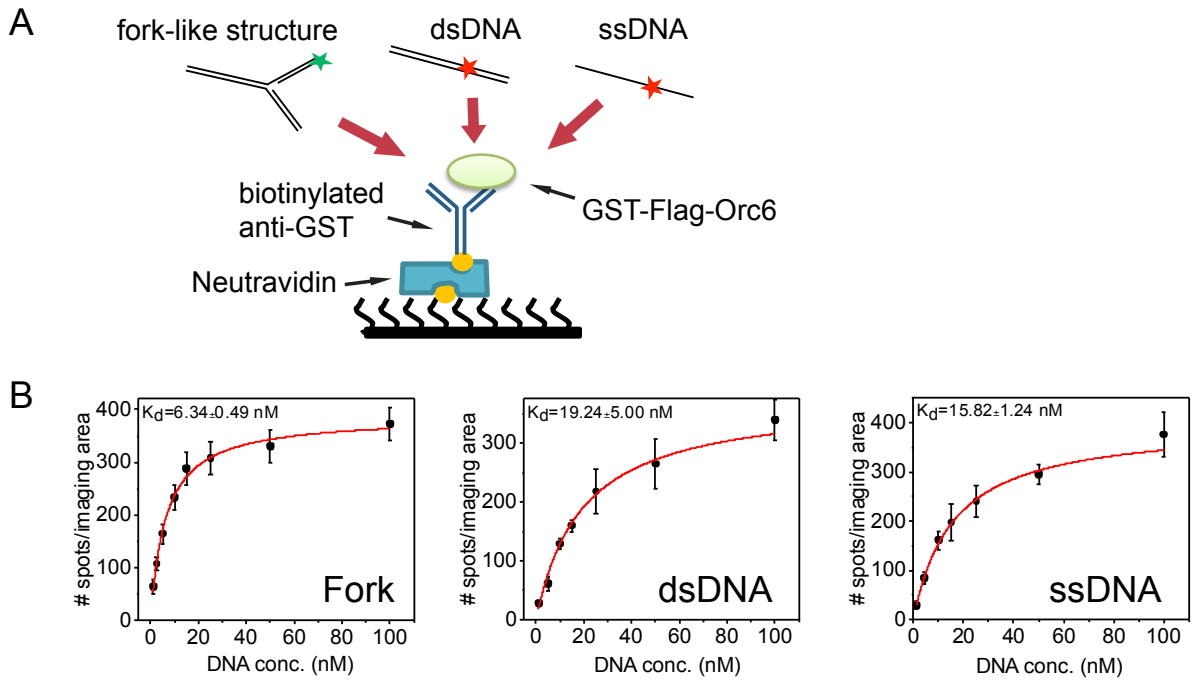

Figure 2.1 Orc6 preferably binds to fork like DNA structure

(A) Schematic illustration of SiMPull to test Orc6's DNA binding ability to different DNA substrates. (B) Binding curves showing Orc6 binding affinity to different DNA substrates.

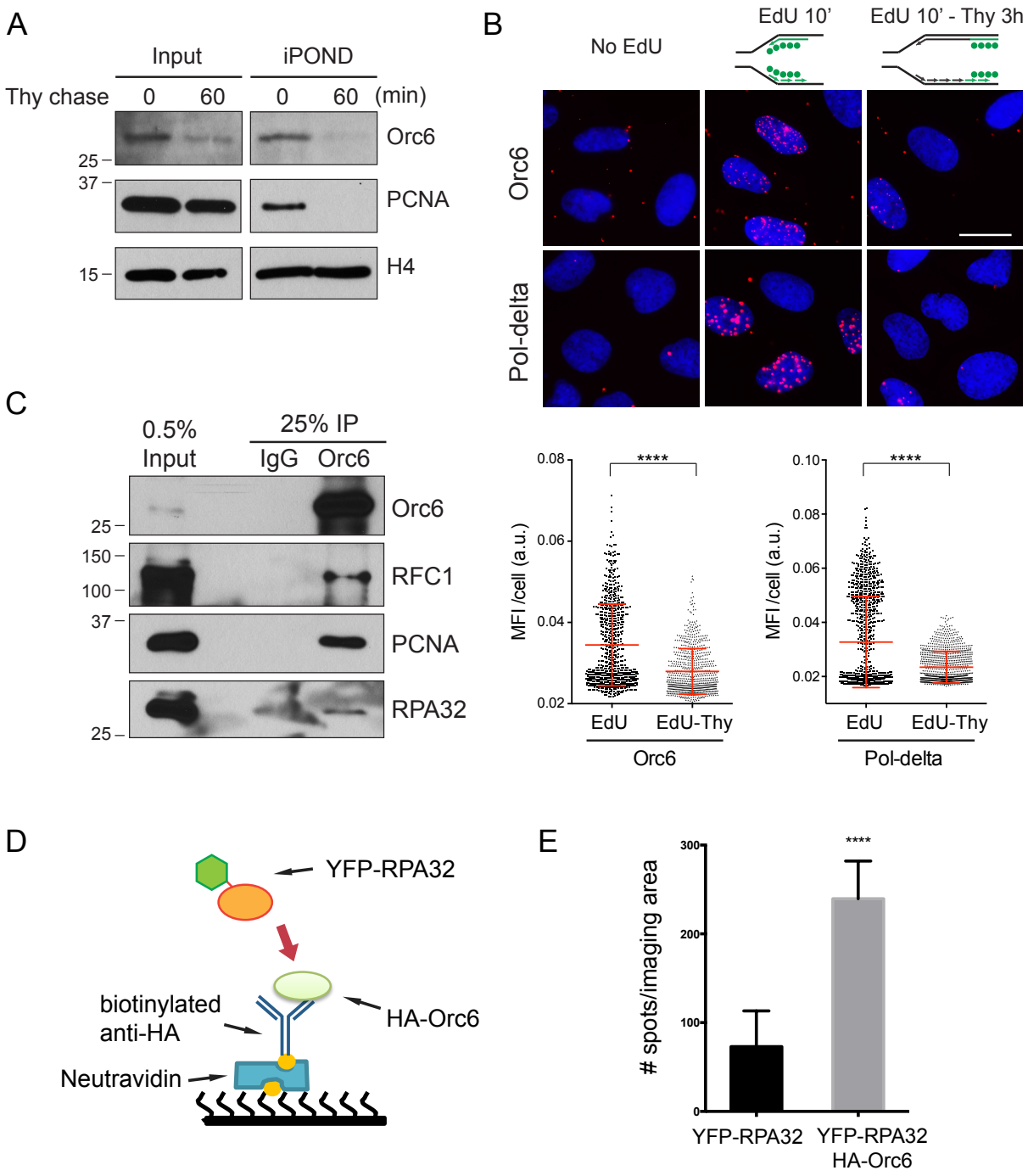

Figure 2.2 Orc6 is at replication fork and associates with fork components

(A) Western blot of iPOND. Thymidine chase 0 min for nascent DNA and 60 min for mature DNA. PCNA as a positive control for nascent DNA. (B) Upper panel: representative images for SIRF. Red foci indicate the association. PLA of Pol-delta/Edu-biotin served as a positive control.

Figure 2.2 (cont.) DAPI as counterstain. Scale bar, 25μm. Lower panel: quantification results. Experiments were performed in triplicates and one representative experiment is shown; n > 700 for each group. Mean ± SD. ****p < 0.0001 by unpaired two-tailed Student's t test. MFI: mean fluorescence intensity. a.u.: arbitrary unit. (C) Immunoprecipitation of endogenous Orc6 from U2OS cells. (D) Schematic illustration of SiMPull to examine interaction between Orc6 and RPA32. (E) Quantification of Orc6 and RPA32 interaction. One signal spot represents one RPA molecule. Mean ± SD. ****p < 0.0001 by unpaired two-tailed Student's t test.

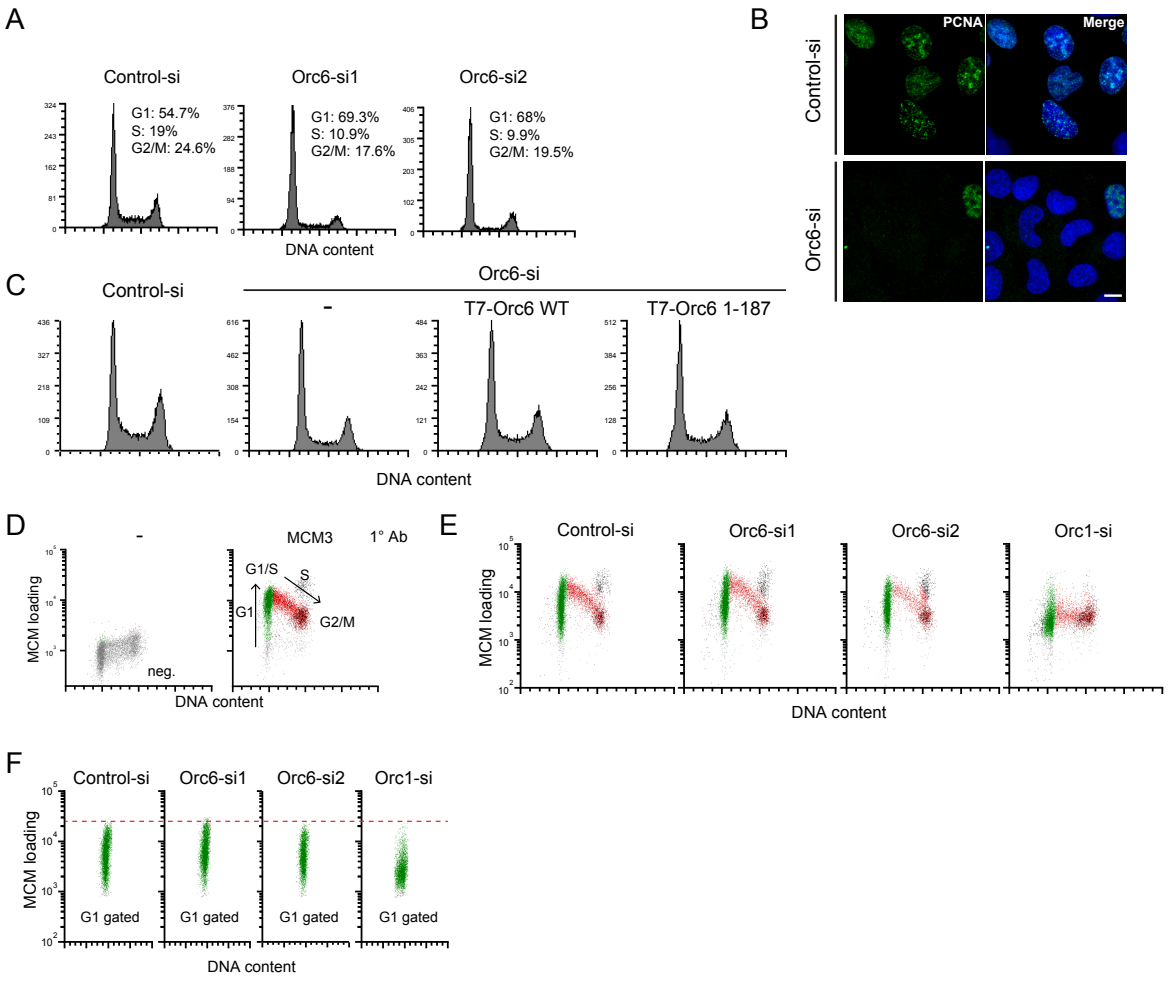

Figure 2.3 The S phase defect in Orc6-depleted cells is not due to defects in ORC association or MCM chromatin loading

(A) Cell cycle profile of Orc6 knockdown cells. (B) Immunostaining analysis of Orc6 knockdown cells. PCNA staining was used to mark S phase cells. Scale bar, 15μm. (C) Cell cycle profile of endogenous Orc6-depleted cells substituted with tagged wild-type Orc6 or C-terminal truncated Orc6 (a.a.1-187). (D) Flow cytometric analysis of asynchronized U2OS cells to measure chromatin bound MCM in conjugation with PI staining cell cycle profile. Left panel: MCM3 antibody omitted as negative gating for MCM staining. Right panel: illustration of cell cycle progression; loading of MCM on chromatin increases through G1 (green); S phase cells (red) with increasing DNA content and decreasing chromatin MCM, until reaching G2/M (maroon). (E) MCM-PI flow of Orc6 and Orc1 knockdown cells. (F) G1 populations from (E) were highlighted for comparing the MCM loading.

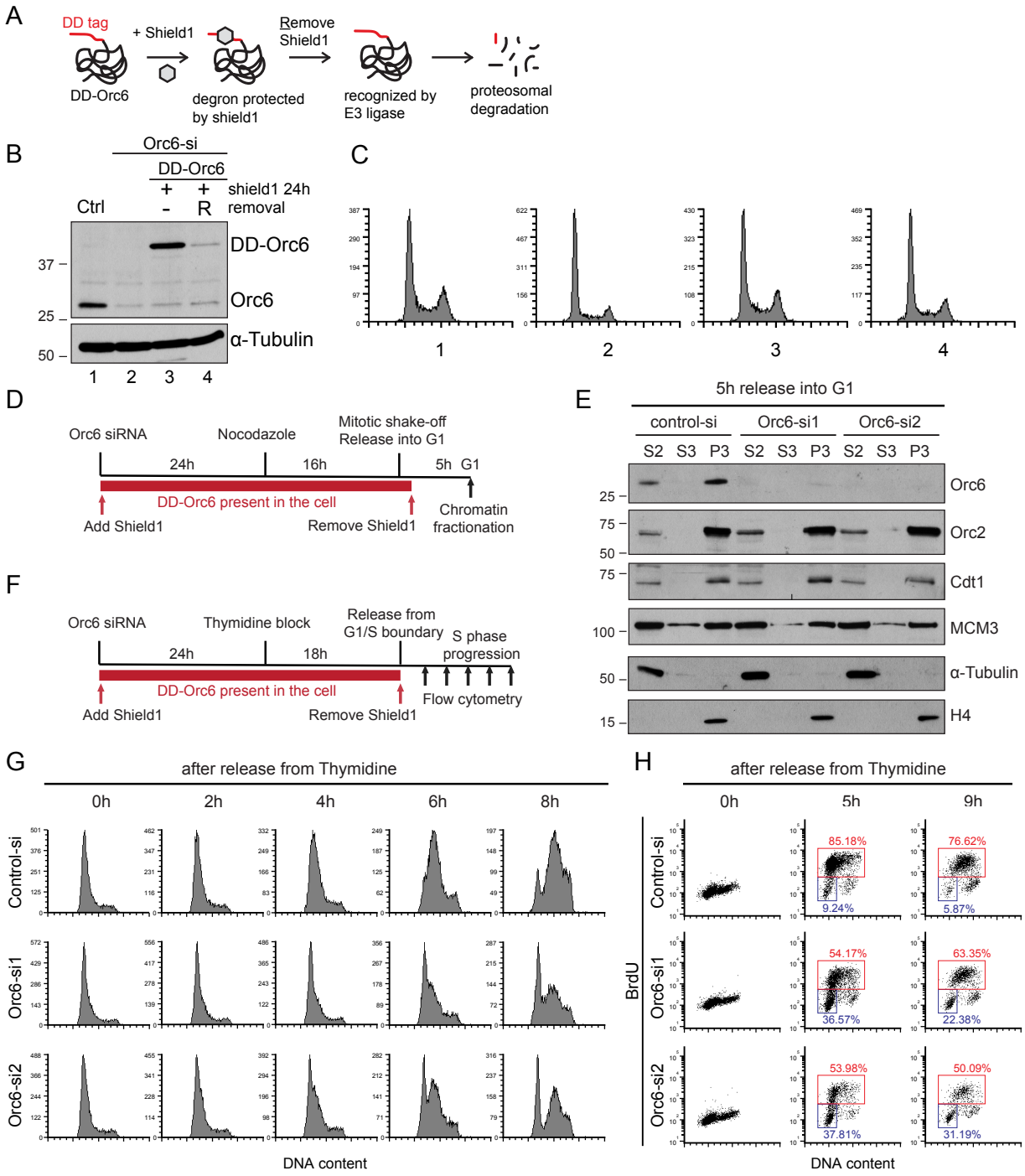

Figure 2.4 Separation of Orc6 function in G1 and S phase using DD-Orc6 degron system showing that Orc6 is dispensable for G1 licensing but required for S phase progression

(A) Schematic illustration of the DD degron system for controlling the degradation of DD-Orc6.

(B) Western blot showing the protein level of endogenous Orc6 and DD-Orc6 in the absence and

Figure 2.4 (cont.) presence of Shield1. (C) Cell cycle profile of samples from (B) by PI flow cytometry. (D) Schematic of the protocol for specifically depleting Orc6 in G1 phase. (E) Western blot analysis of the G1 phase chromatin fractionation. S2, cytosolic; S3, nuclear soluble; P3, chromatin fraction. α-tubulin and H4 serve as loading control for cytosolic and chromatin fraction, respectively. (F) Schematic of the protocol for specifically depleting Orc6 in S phase. (G) S phase progression determined by PI flow cytometry. (H) S phase progression determined by BrdU-PI flow cytometry.

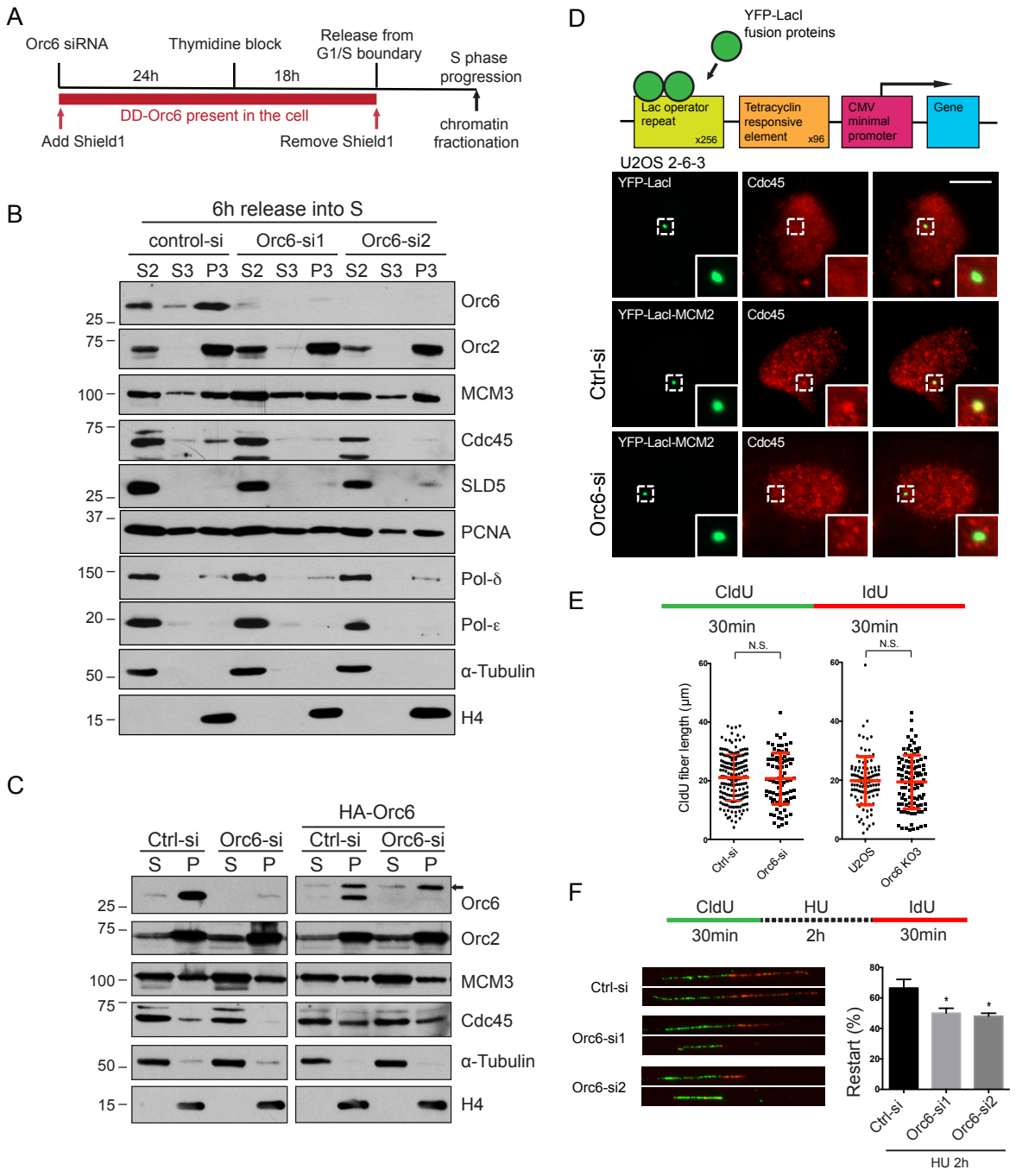

Figure 2.5 Depletion of Orc6 causes reduced cdc45 chromatin loading and defects in replication fork restart

(A) Schematic of the protocol for specifically depleting Orc6 in S phase. (B) Western blot analysis of the S phase chromatin fractionation. (C) Similar in (B), western blot showing the chromatin fractionation of Orc6-depleted samples expressing HA-Orc6. *Arrow* indicates HA-

Figure 2.5 (cont.) Orc6. (D) Upper panel: schematic of the heterochromatic locus stably integrated in U2OS 2-6-3 cells. LacI fusion proteins are forcibly tethered to the LacO repeats, and YFP is fused to LacI for visualizing the loci. Lower panel: images of cdc45 recruitment to the heterochromatin loci in YFP-LacI-MCM2 expressing control and Orc6-depleted cells. YFP-LacI as a negative control. A representative experiment (n = 2) is shown. Positive cdc45 recruitment in YFP-LacI, 5.3% (n = 19); in YFP-LacI-MCM2 control, 52.8% (n = 36); in YFP-LacI-MCM2 Orc6-si, 27.5% (n = 40). Scale bar, 15μm. (E) DNA fiber assay was used to monitor replication fork progression speed. Mean ± SD. (F) Representative images of DNA fiber (left panel). Percentages of restart tracks in total tracks counted (right panel). Mean ± SD, n = 3. *$p < 0.05$ by unpaired two-tailed Student's t test.

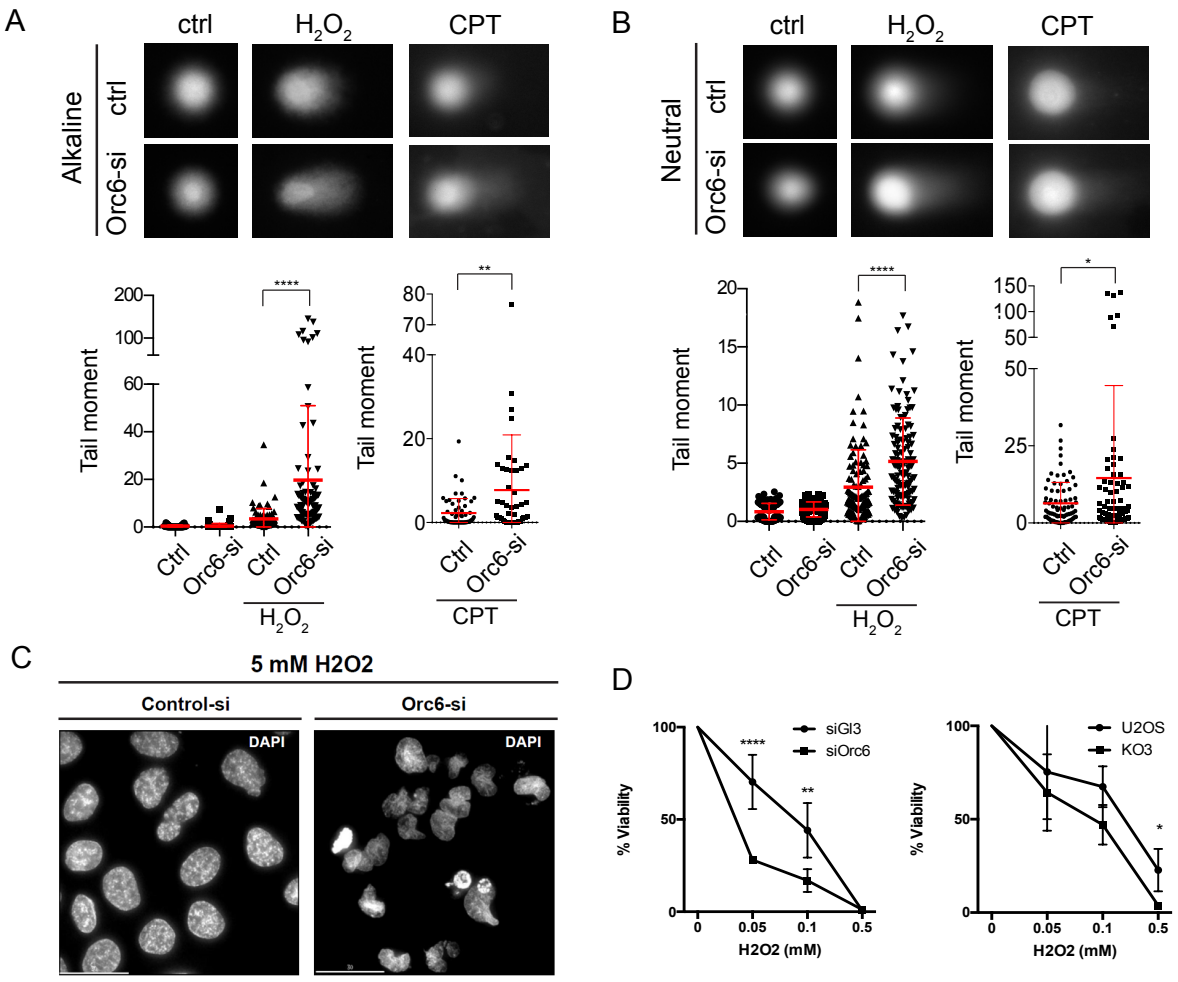

Figure 2.6 Depletion of Orc6 sensitizes cells to DNA damage

(A) Alkaline comet assay. Representative images are shown at top. One representative experiments for each is shown for H_2O_2-treated (n = 5) and CPT-treated (n = 2). Mean ± SD. **p < 0.01, and ****p < 0.0001 by unpaired two-tailed Student's t test. (B) Neutral comet assay. Representative images are shown at top. Mean ± SD. *p < 0.05, and ****p < 0.0001 by unpaired two-tailed Student's t test. (C) DAPI staining of control or Orc6 knockdown cells treated with H_2O_2. Scale bar, 30μm. (D) Clonogenic survival assay of H_2O_2-treated control and Orc6 knockdown cells (left); control and Orc6 knockout cells (right). Mean ± SD, n = 3. *p < 0.05, **p < 0.01, and ****p < 0.0001 by unpaired two-tailed Student's t test.

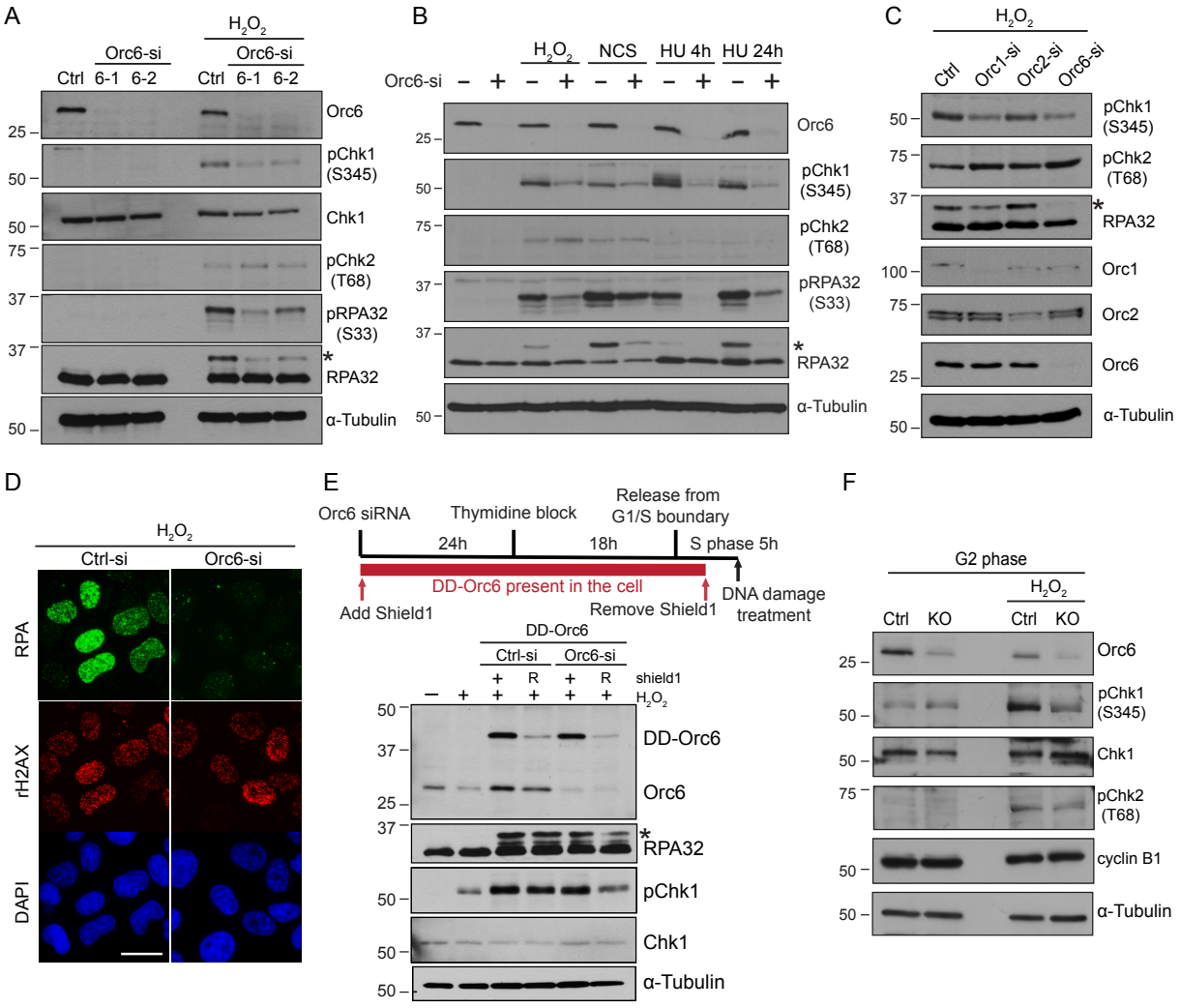

Figure 2.7 Loss of Orc6 leads to compromised ATR signaling pathway

(A) Western blot analysis of ATM/ATR pathway in control and Orc6 knockdown cells treated with hydrogen peroxide. *Asterisk* indicates hyperphosphorylation of RPA32. (B) Western blot of ATM/ATR signaling pathways for different DNA damage drug treatment in control and Orc6 knockdown cells. (C) Western blot for Orc1, Orc2 and Orc6 knockdown cells. (D) Immunostaining of chromatin-associated RPA and rH2AX in control and Orc6-depleted cells treated with H_2O_2. Scale bar, 25μm. (E) Upper panel: Schematic of the protocol for specifically depleting Orc6 in S phase and collecting DNA damage samples. Shield1 was removed 1h before H_2O_2 treatment and cells were collected 1h after treatment. Lower panel: western blot for analyzing ATR activation. R, removal of Shield1. (F) Western blot of G2 synchronized control and Orc6 knockout cells. Cyclin B1 as G2 marker.

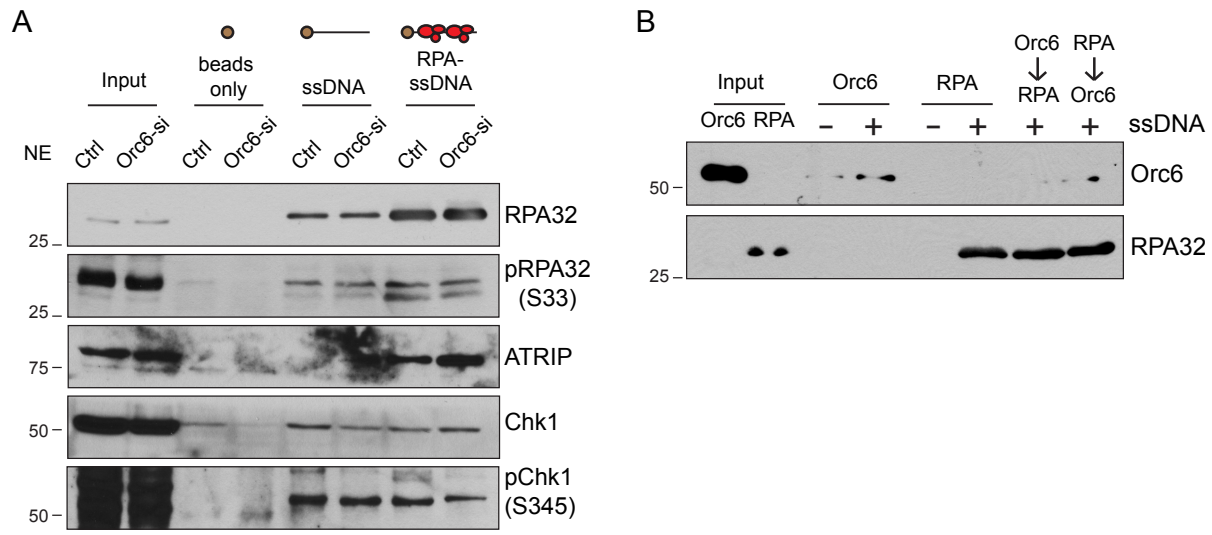

Figure 2.8 Reduced ATR activation in Orc6-depleted cells is not due to changes in ATR signaling proteins' recruitment to RPA-ssDNA

(A) In vitro ssDNA pull-down assay to determine the recruitment of ATR signaling proteins. ssDNA along or ssDNA pre-coated with purified RPA were incubated with control or Orc6-depleted nuclear extracts. Samples obtained after biotin-ssDNA pull-down were analyzed by western blotting. NE, nuclear extract. (B) In vitro ssDNA pull-down for effects of Orc6 to RPA's ssDNA binding ability. Orc6 along, RPA along, Orc6 first then RPA or RPA first then Orc6 were added to ssDNA. Proteins bound to ssDNA were analyzed using western blot.

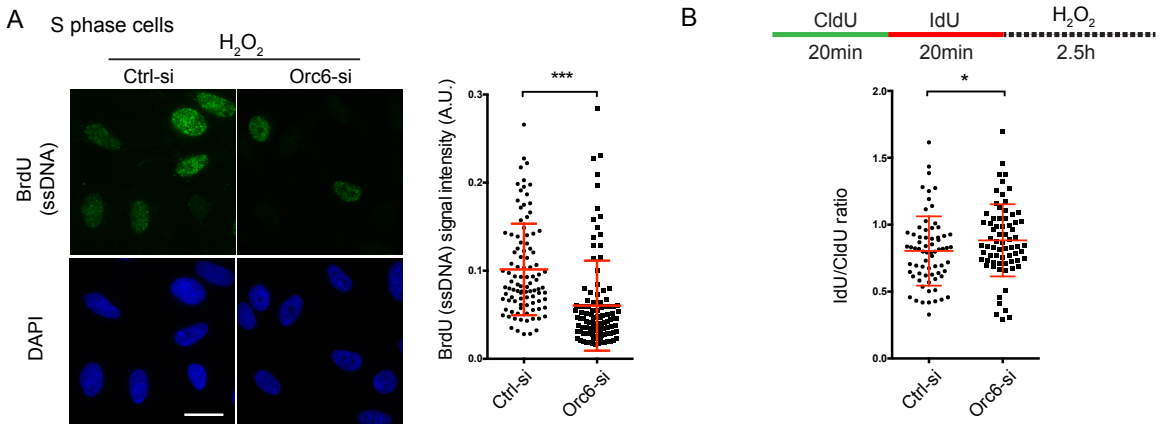

Figure 2.9 Loss of Orc6 leads to reduced ssDNA generation

(G) Native BrdU staining for visualizing ssDNA (right) and quantification results (left). Scale bar, 25μm. A representative experiment (n=3) is shown. Mean ± SD; n > 100 for each group. ***p < 0.001 by unpaired two-tailed Student's t test. (G) DNA fiber assay to determine nascent DNA resection/degradation after fork stalling. Higher IdU/CldU ratio indicates less resection, hence less ssDNA generation. A representative experiment (n = 2) is shown. Mean ± SD. ****p < 0.0001 by unpaired one-tailed Student's t test.

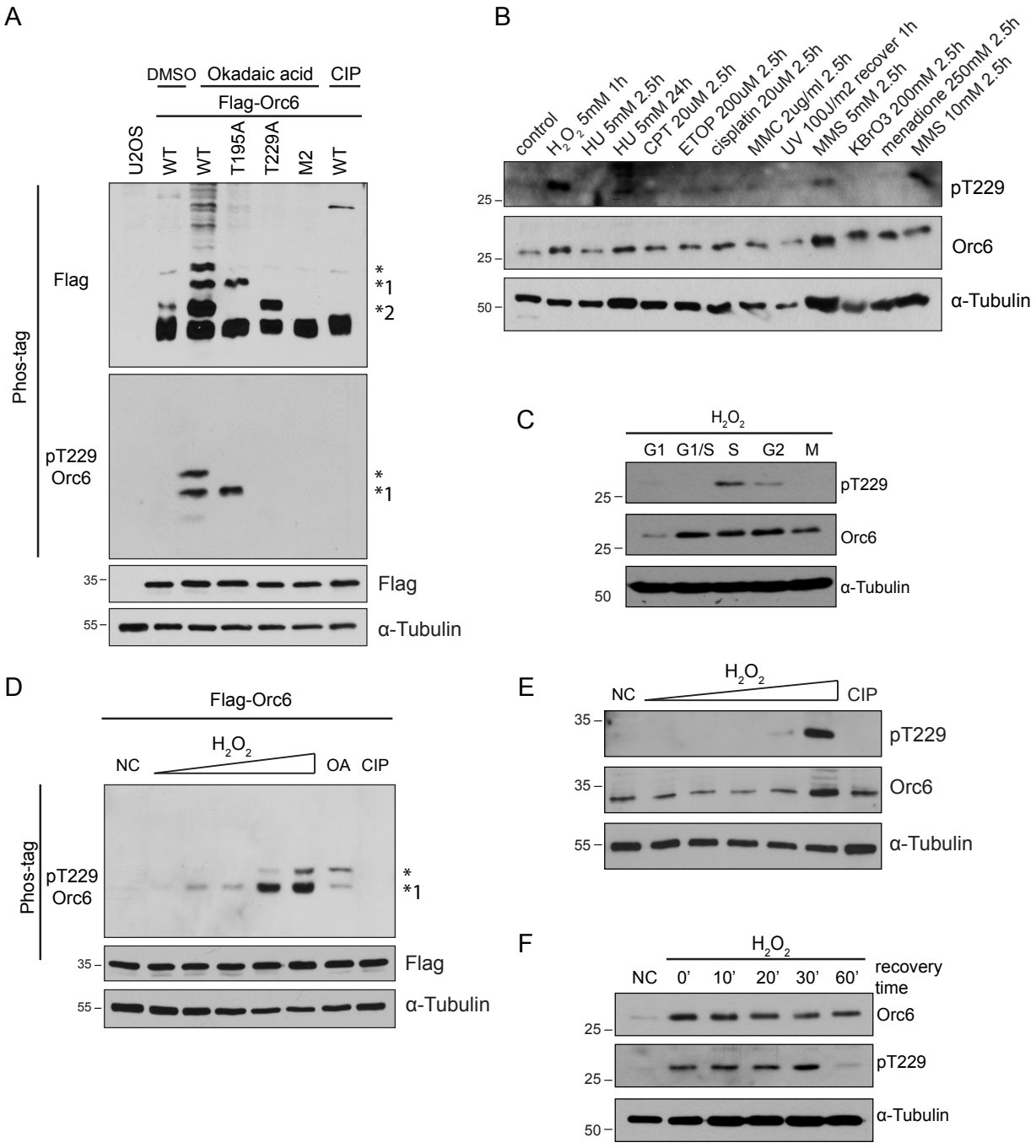

Figure 2.10 Orc6 is phosphorylated at Thr229 upon oxidative DNA damage

(A) Phos-tag gel analysis of Orc6 phosphorylation. U2OS cells were transfected with Flag-Orc6-WT, T195A, T229A or M2 (both T195 and T229 were mutated to Ala), and treated with okadaic acid to induce accumulation of phosphorylation. * corresponds to Orc6 phosphorylated on both T195 and T229; *1 indicates T229 phosphorylation. *2 indicates T195 phosphorylation. CIP,

Figure 2.10 (cont.) calf intestinal phosphatase. (B) Western blot for testing Orc6 T229 phosphorylation upon different genotoxic drug treatments. (C) Western blot showing Orc6 T229 phosphorylation pattern during cell cycle. (D) Phos-tag gel analysis to determine the dose-dependent effects of T229 phosphorylation upon H_2O_2 treatment. U2OS cells were transfected with Flag-Orc6-WT and treated with different concentration of H_2O_2 (0.25, 0.5, 1, 2, 5 mM). OA, okadaic acid. (E) Western blot for endogenous Orc6 T229 phosphorylation. (F) Western blot of T229 phosphorylation regulation. Cells were collected at indicated time point after release from 20 min H_2O_2 treatment. NC, negative control.

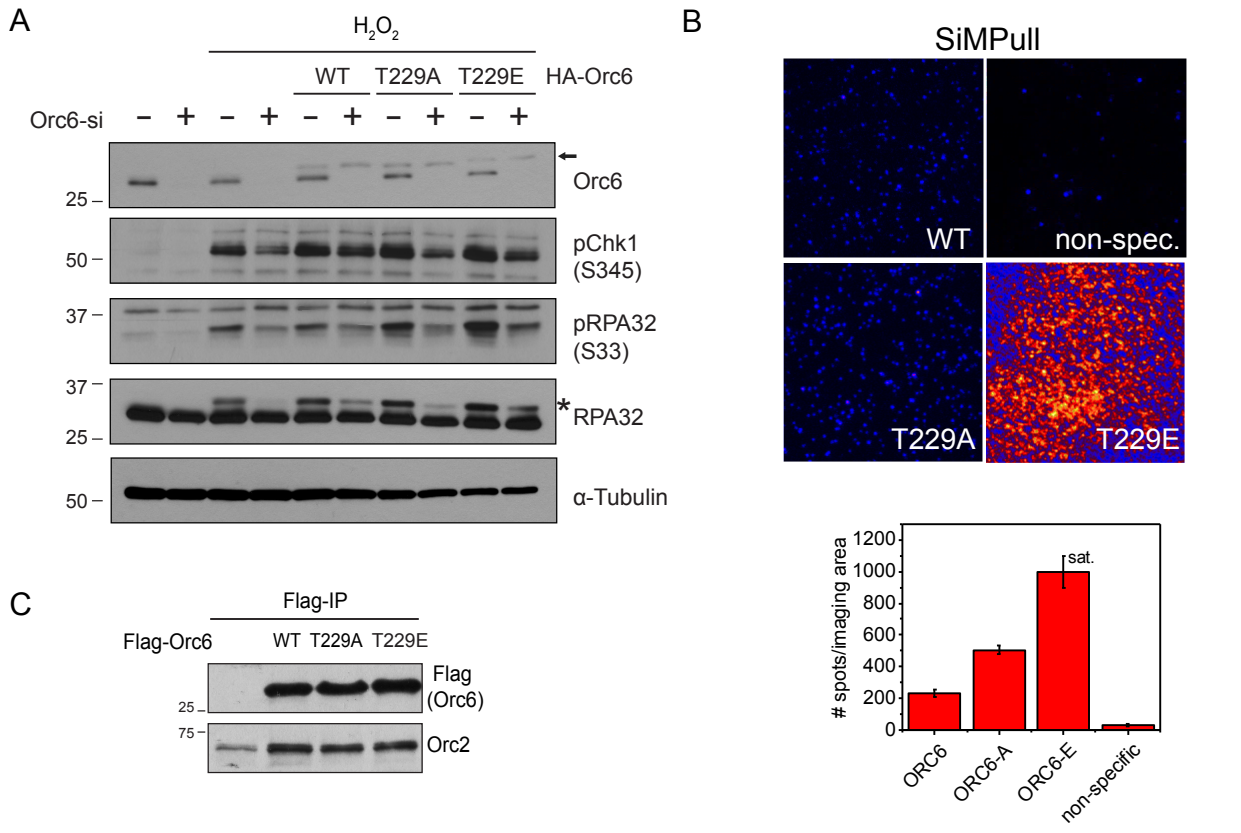

Figure 2.11 Analysis of Orc6 T229 mutants

(A) ATR activation analyzed by western blot of various U2OS cells (HA-Orc6-WT, HA-Orc6-T229A, HA-Orc6-T229E) depleted of endogenous Orc6. *Arrow* indicates HA-Orc6. *Asterisk* indicates hyperphosphorylated RPA32. (B) SiMPull for investigating the effect of Orc6 T229 phosphorylation to its DNA fork structure binding ability. Representative images of different mutants of purified GST-Flag-Orc6 (upper) and quantified results (lower). Mean ± SD. (C) Immunoprecipitation of different mutants of Flag-Orc6 from U2OS cells. Samples were analyzed by western blotting.

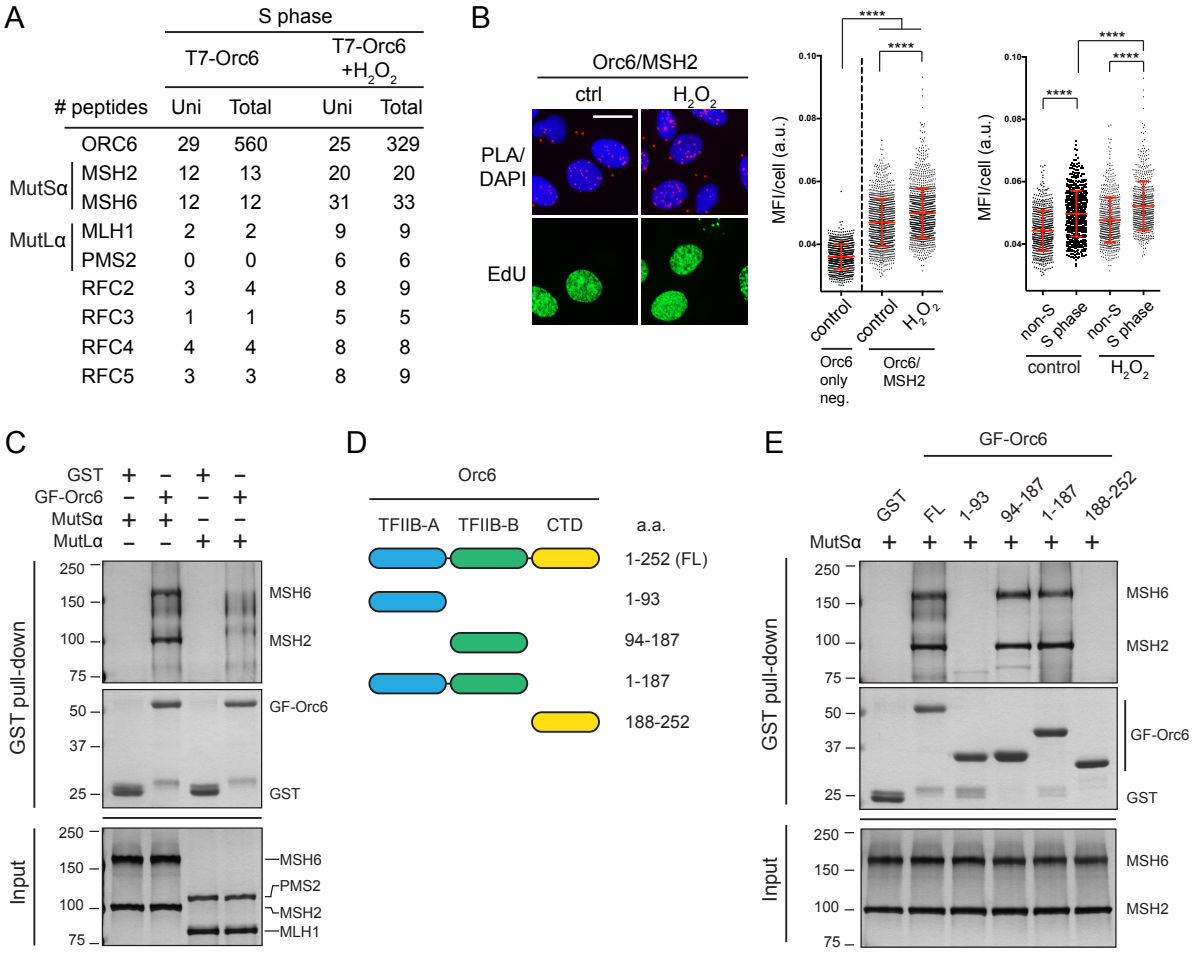

Figure 2.12 Orc6 interacts with MutSα using its middle TFIIB domain

(A) MMR proteins identified by IP-MS analysis from S phase synchronized U2OS cells expressing T7-Orc6, with or without H_2O_2 treatment. For each protein, numbers of unique peptides (Uni) and total peptides (Total) are presented. (B) Left panel: Orc6 and MSH2 association by PLA. EdU incorporation for determining S phase cells. Scale bar, 25μm. Middle panel: quantification of PLA. First column represents a negative control where MSH2 antibody was omitted. Right panel: further analysis of the quantification where S phase (EdU positive) and non-S phase (EdU negative) cells were separated in both control and H_2O_2 groups. Mean ± SD. ****$p < 0.0001$ by unpaired two-tailed Student's t test. MFI: mean fluorescence intensity. a.u.: arbitrary unit. (C) Direct interaction of Orc6 with MutSα or MutLα examined by GST pull-down assay. Proteins on SDS-PAGE gels were visualized by silver stain (upper image and input) or Coomassie stain (middle image). GF-Orc6 stands for GST-Flag-Orc6. GST as a negative control.

Figure 2.12 (cont.) (D) Schematic illustration of Orc6 domains and different truncations. (E) Interaction of different truncations of GST-Flag-Orc6 with MutSα by GST pull-down assay. Proteins were visualized by silver stain (upper image and input) or Coomassie stain (middle image).

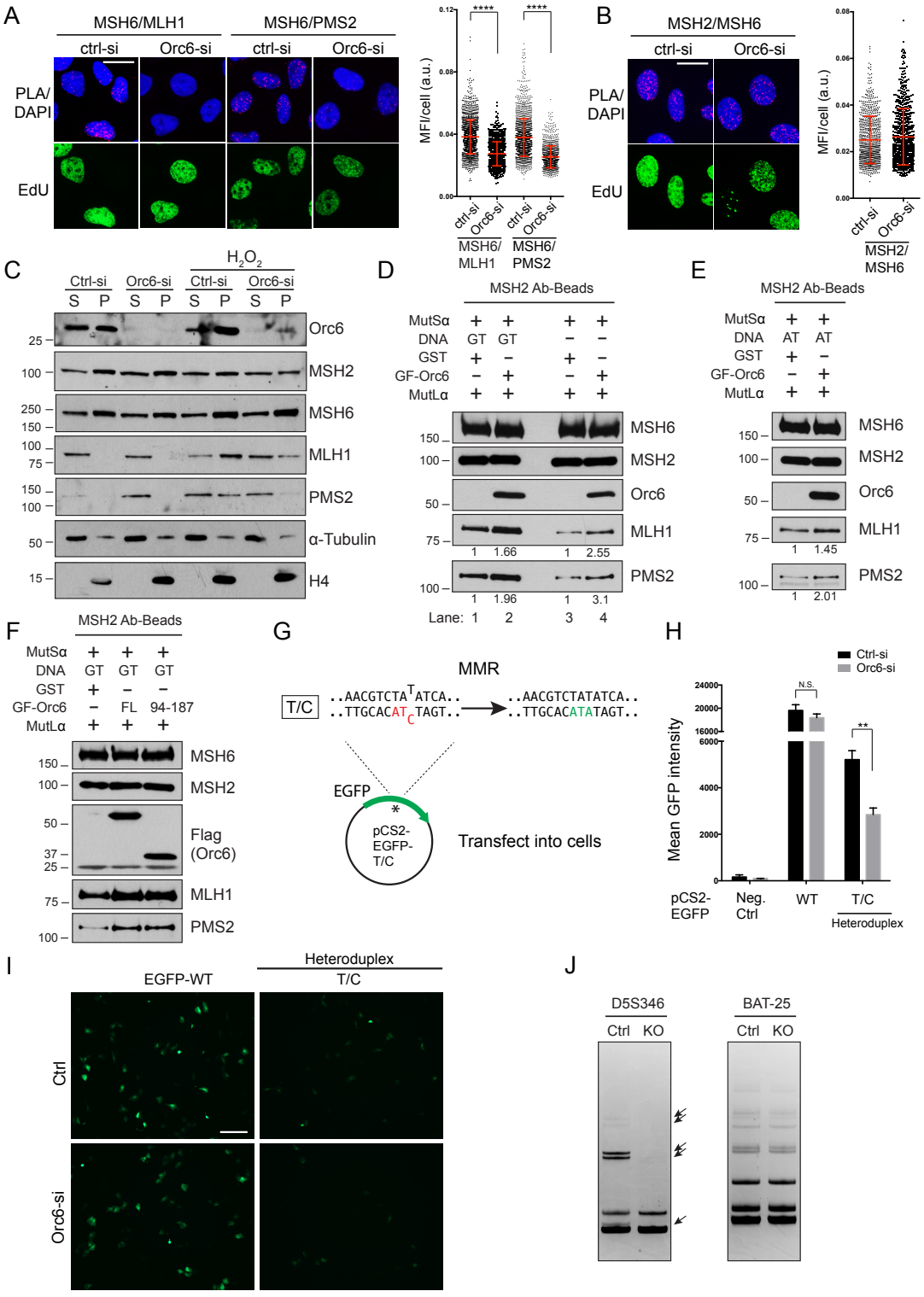

Figure 2.13 Orc6 facilitates MutLα binding to MutSα and promotes MMR efficiency

(A) Interaction between MSH6/MLH1 and MSH6/PMS2 quantified by PLA in control and Orc6-depleted cells. Representative images (left) and quantification (right) are shown. Scale bar,

Figure 2.13 (cont.) 25μm. Mean ± SD. ****p < 0.0001 by unpaired two-tailed Student's t test. (B) Formation of MSH2/MSH6 complex (MutSα heterodimer) examined by PLA; representative images (left) and quantification (right) are shown. Scale bar, 25μm. Mean ± SD. (C) Western blot analysis of chromatin fractionation samples. The samples were prepared following the same protocol as in figure 5D. S, soluble; P, chromatin fraction. (D) Effect of Orc6 on the *in vitro* recruitment of MutLα to MutSα in the presence of G/T mismatch DNA (lane 1 and 2) or no DNA (lane 3 and 4). GF-Orc6 stands for GST-Flag-Orc6. GST as a control. (E) Effect of Orc6 on the *in vitro* recruitment of MutLα to MutSα in the presence of A/T homoduplex DNA. (F) *In vitro* recruitment of MutLα to MutSα using Orc6 full-length (FL) or TFIIB-B domain (94-187). (G) Illustration of the heteroduplex used in MMR assay. (H) Quantification of MMR activity. Mean GFP intensity was measured using flow cytometry. Mean ± SD, n = 3. **p < 0.01 by unpaired two-tailed Student's t test. (I) Representative images of MMR assay are shown. Scale bar, 200μm. (J) Microsatellite loci D5S346 and BAT-25 were PCR-amplified from genomic DNA of wild-type U2OS control or Orc6 knockout (hypomorph) cells. The products were resolved on 15% polyacrylamide gels and stained with EtBr. Arrows indicate the differences observed.

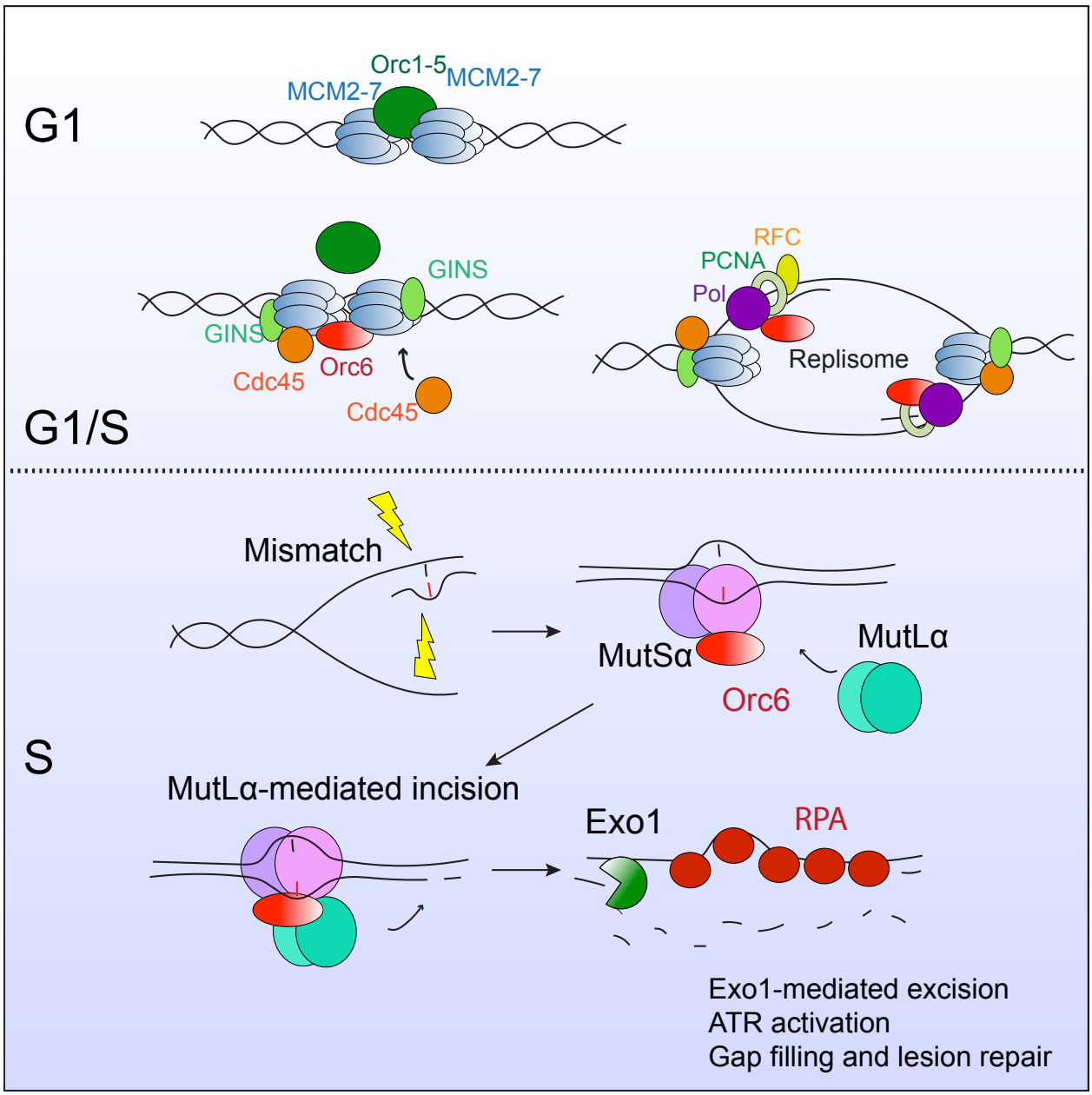

Figure 2.14 Model for Orc6 functioning in S phase entry and facilitating the assembly of MMR complex for efficient repair and ATR activation.

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CHAPTER 3. PCNA-MEDIATED STABILIZATION OF E3 LIGASE RFWD3 AT THE REPLICATION FORK IS ESSENTIAL FOR DNA REPLICATION

3.1 Introduction

Maintenance of genome integrity is key to cell survival. Defects in DNA replication and errors in the DNA damage response contribute to genome instability and are key contributing factors in many diseases, including cancer (Techer et al., 2017). Replication stress is a leading cause of genome instability and occurs when replication forks progress slowly or stall. A huge repertoire of cellular factors can mediate replication stress-induced DNA damage, including deprivation of dNTP pools, defects in DNA replication proteins, and decreased firing of origins due to defects in replication initiation (Toledo et al., 2017). Intricate checkpoint pathways operate to ensure that the entry into or progression through S-phase is blocked when the cells encounter DNA damage.

Cells encounter many assaults to their genome that are repaired accurately and efficiently to maintain genome integrity. Ubiquitination is emerging as an important player in DNA replication, repair, and damage signaling pathways. Non-degradative ubiquitin signaling involving either monoubiquitination or polyubiquitination have been implicated in the maintenance of genome integrity, including in processing of DNA double strand breaks, repair of interstrand crosslink lesions (ICL), and bypass of lesion during DNA replication [for review see (Ulrich and Walden, 2010)]. The signaling events are triggered by ubiquitin protein ligases that can initiate monoubiquitination or polyubiqtuination through non-standard linkage.

DNA interstrand crosslink lesions (ICLs) are links between the two strands of DNA with a covalent bond, and many pathways, including nucleotide excision repair, structure specific endonucleases, translesion synthesis, and homologous recombination (HR), have been implicated in resolving such errors (Hashimoto et al., 2016). ICLs inhibit DNA replication and transcription, and the dominant mode of ICL repair is believed to happen during S-phase and requires

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converging replication forks (Akkari et al., 2000; Taniguchi et al., 2002). Mutations in ICL repair are associated with Fanconi anemia (FA), a rare heritable disorder, and ~21 FA genes reported thus far have been implicated in ICL repair (Ceccaldi et al., 2016; Federico et al., 2017). The role of novel factors in ICL repair and the importance of ubiquitination in the damage response is becoming an intense area of research.

RFWD3 (RING finger and WD repeat domain-containing protein 3) is an E3 ubiquitin ligase that was initially identified in a proteomic study as a substrate of ATM/ATR (Matsuoka et al., 2007). Biallelic mutations in RFWD3 were reported in patients with FA (Knies et al., 2017). RFWD3 is emerging as an important component in the FA/BRCA pathway and has also been assigned the alias FANCW. Previous studies have shown that RFWD3 functions synergistically with MDM2 to regulate the ubiquitination of tumor suppressor protein p53 in response to DNA damage (Fu et al., 2010). RFWD3 associates with the single stranded binding protein RPA, facilitates the RPA-mediated DNA damage response, and affects HR at stalled replication forks (Elia et al., 2015; Gong and Chen, 2011; Liu et al., 2011). Recent studies have shown that RPA-mediated recruitment of RFWD3 is essential for ICL repair (Feeney et al., 2017) and that RFWD3-mediated ubiquitination promotes the removal of RPA and RAD51 from damage sites to allow HR (Inano et al., 2017). All of these data support the role of RFWD3 in DNA-damage repair.

While RFWD3 is emerging as a new component of the FA pathway, up until now, this role has been thought to be mediated primarily by its interactions with the single-stranded DNA binding protein RPA that are important for repair of ICL DNA damage. In the present study, we report that RFWD3's role in the FA pathway may be related to its role in normal DNA replication via its direct interaction with PCNA at the replication fork. We propose that the association of RFWD3 at the replication fork mediates the ubiquitination of key replication fork components that is essential for efficient progression of DNA replication. This seems to be a fundamentally different way of considering how RFWD3 functions in DNA metabolism, as it raises the possibility that a major role of RFWD3 in genome stability occurs via its role in normal, unstressed DNA replication at the replication fork as opposed to solely functioning in DNA repair processes.

3.2 Results

3.2.1 RFWD3 localizes to the replication fork and interacts with PCNA

RFWD3 is an E3 ligase that is known to play important roles in DNA damage response. A recent nascent chromatin capture proteomics study showed the enrichment of RFWD3 at the replication fork (Alabert et al., 2014), supporting the notion that RFWD3 might play important roles in unperturbed DNA replication. To test this model, we examined the presence of RFWD3 at the fork and monitored the spatiotemporal dynamics of RFWD3 in a quantitative manner by performing iPOND (isolate proteins on nascent DNA) (Sirbu et al., 2012). RFWD3 showed enrichment at the unperturbed replication fork, but not following thymidine chase (matured DNA) (Figure 3.1A). PCNA, the DNA clamp, was also found to be enriched on the nascent DNA but not on the mature DNA, confirming the specificity of the iPOND assay. Histone H4 was found on nascent as well as on the mature DNA. Further, our lab has previously identified that, by immunoprecipitation (IP) and in vitro co-immunoprecipitation, RFWD3 associated with the fork protein PCNA in unperturbed human cells as well as in DNA damaged cells (Wang, 2017). Finally, examination of the localization of RFWD3 in asynchronously grown human cells revealed colocalization of RFWD3 with the replication protein PCNA in a subset of cells (Figure 3.1B). All these data strongly support the existence of RFWD3 at the replication fork.

PCNA is known to be the master regulator of events at the replication fork (Mailand et al., 2013). PCNA provides a molecular platform that enables protein-protein and protein-DNA interactions at the fork. A large repertoire of proteins associate with PCNA via a general motif, PIP box sequence (Q-XX-[L/I/M]-XX-[HF/DF/Y]) (Warbrick, 1998). Our previous data have also revealed a PIP box consensus on WD40 domain of RFWD3 (Wang, 2017). Further, disruption of the PIP box by mutation abolished RFWD3 interaction with PCNA (Wang, 2017). To further evaluate and examine this interaction together with previously known RPA interaction, we conducted immunoprecipitation experiments using the HA antibody in cell lines stably expressing HA-RFWD3-WT, HA-RFWD3-PIPm, or HA-RFWD3-I639K (mutant known to abolish RFWD3 interaction with RPA) showed that WT as well as I639K mutant (albeit weaker than WT) interacted with PCNA (Figure 3.1C). On the other hand, PIP box mutant failed to interact with PCNA. RFWD3 has previously been reported to associate with the single stranded binding protein RPA. We find that the RPA interaction to RFWD3 was abrogated in the I639K

mutant, as expected (Figure 3.1D). RPA interacted with WT-RFWD3 and with the PIP mutant, but to a lesser extent compared to the WT (Figure 3.1D).

To further investigate the complex assembly of RFWD3, PCNA, and RPA, purified proteins or nuclear extract were fractionated on a Superdex-200 gel filtration column. A significant portion of RFWD3 was found in fraction 7 (~669kDa) co-fractionating with RPA and PCNA, indicating that RFWD3 is in a high molecular weight complex containing RPA and PCNA (Figure 3.1E and 3.1F), in addition to subcomplexes of RFWD3-PCNA and RFWD3-RPA. Further, GST-pull down assays demonstrated that RFWD3, PCNA and RPA could exist in one single complex, as RPA can bind to RFWD3 without displacing PCNA (Figure 3.1G). Our results suggest that RFWD3 associates to the replication fork at S-phase and interacts with the fork components PCNA and RPA.

3.2.2 RFWD3 is required for S-phase progression

Because RFWD3 localized to the replication fork in unperturbed cells, we addressed the role of RFWD3 during the normal cell cycle. Despite numerous efforts, our attempts to knockout RFWD3 in human U2OS cells utilizing the CRISPR/Cas9-mediated genome editing method were unsuccessful, as we failed to get clones with complete loss of the RFWD3 protein. This is consistent with results from other groups, suggesting that RFWD3 is an essential gene (Feeney et al., 2017; Inano et al., 2017). We used two independent siRNAs (RFWD3 si-1 and RFWD3 si-2) to deplete RFWD3. Depletion of RFWD3 in U2OS showed an increase in the 4C DNA content and accumulation of cells in S-phase (Figure 3.2A-3.2C), similar to our previous results from multiple cell lines (Wang, 2017).

We have also previously examined fork progression in RFWD3-depleted cells using a DNA fiber assay (Wang, 2017). We observed a consistent reduction in replication fork speed in cells depleted of RFWD3. These suggest that the reduced fork speed leads to prolonged S phase; hence more cells are accumulated in S phase. Further, live cell imaging of YFP-PCNA in control as well as RFWD3-depleted cells demonstrated that cells lacking RFWD3 displayed an average S-phase length of 16 hours, while that of the control was less than 14 hours (Figure 3.2D and 3.2E). These results revealed that the depletion of RFWD3 caused a prolonged S-phase.

We next addressed if the association of RFWD3 and PCNA and/or RPA were required for proper DNA replication. We performed RFWD3 depletion in HA-RFWD3-WT, HA-RFWD3-PIPm, HA-RFWD3-C315A (catalytically inactive), and HA-RFWD3-I639K mutants and tested for cell cycle distribution using PI and BrdU-PI flow analyses. Depletion of RFWD3 showed increased population in S-phase (Figure 3.2F and 3.2H) as well as reduced incorporation of BrdU with a population of cells accumulating between 2C and 4C DNA content (Figure 3.2G). SiRNA resistant version of WT-RFWD3 rescued the S-phase accumulation defects in endogenous RFWD3-depleted cells, however the other mutants did not (Figure 3.2F-3.2H). Similarly, WT-RFWD3 was able to rescue the defects in DNA fiber length, but the PIP mutant failed to do so (Figure 3.2I). These results suggest that the association of RFWD3 and PCNA are important for efficient DNA replication.

3.2.3 Loss of RFWD3 causes fork stalling

We performed chromatin fractionation to determine the loading of the replisome components in control and RFWD3-depleted cells that had been synchronized in S-phase. We previously observed an increase in the total as well as chromatin-associated levels of the single stranded binding protein RPA, DNA polymerases, and preIC component Cdc45 (Wang, 2017). Further analysis also revealed an increase in TLS polymerases on chromatin (Figure 3.3A). All these results point to stalling of the replication forks in the absence of RFWD3. Replication fork stalling upon RFWD3 depletion were corroborated by cell biological studies using DNA fiber assay (Figure 3.3B). Also, an increased number of RPA and RAD51 foci in RFWD3-depleted cells supports our model that the loss of RFWD3 causes replication fork stalling (Figure 3.3C and 3.3D).

3.2.4 RFWD3 mediates ubiquitination of fork components to enable DNA replication

We have observed that RFWD3 is required for efficient DNA replication. We demonstrated that RFWD3 interacts with PCNA and localizes to the replication fork during S-phase. To address the functional significance of PCNA interaction with RFWD3, we depleted PCNA using previously validated siRNA oligonucleotide (Prasanth et al., 2004) and monitored

the levels and chromatin binding of RFWD3. Cells lacking PCNA showed reduction of both total levels and chromatin-bound levels of RFWD3, suggesting that the binding of PCNA to RFWD3 stabilizes RFWD3 (Figure 3.4A). Moreover, chromatin-associated RPA levels remained unaltered, suggesting that RPA is not sufficient to recruit and stabilize RFWD3 on chromatin in unperturbed cells (Figure 3.4A). Previous work has suggested that RFWD3 is recruited to stalled forks during interstrand crosslink repair via RPA. We found that the depletion of RPA in undamaged cells did not affect the total or the chromatin associated pool of RFWD3 (Figure 3.4B and 3.4C). Furthermore, the association of RFWD3 to PCNA remained unaltered in cells lacking RPA (Figure 3.4D), suggesting that RPA-independent mechanisms could act in recruiting and/or stabilizing RFWD3 on chromatin in unperturbed cells.

Next, we depleted RFWD3 in U2OS cells stably expressing either HA-RFWD3-WT or the PIPm. The increased levels of chromatin-associated RPA observed in RFWD3-depleted cells were rescued in the WT-RFWD3 expressing cells, but not in the PIP mutant lines (Figure 3.4E). These results support our conclusion that the binding of RFWD3 to PCNA is essential for DNA replication and upon abrogating this interaction, replication is stalled.

In order to address the mechanism that causes fork stalling in the absence of RFWD3, we evaluated the ubiquitination of select replisome components, considering that RFWD3 is an E3 ligase. We performed an *in vivo* ubiquitination assay in control as well as RFWD3-depleted cells treated with the proteasome inhibitor MG132 or the p97 inhibitor DBeQ (to inhibit degradation of ubiquitinated proteins). We observed a significant reduction in RPA ubiquitination upon RFWD3 depletion (Figure 3.4F). There was a marginal reduction in a specific ubiquitinated form of PCNA (labeled*), but not in other forms. Our results support previous observations that RFWD3 ubiquitinates RPA, although previous reports observed RPA ubiquitination in cells treated with DNA damaging agent such as MMC. Our results support a model in which RFWD3 associates with PCNA, and this interaction is critical for the stabilization of RFWD3 to the fork. At the fork, RFWD3 ubiquitinates substrates essential for DNA replication progression. RPA is one of RFWD3's substrates, and its ubiquitination triggers its removal and faithful completion of DNA replication (Figure 3.4G).

3.3 Discussion

RFWD3, originally identified as a substrate for ATM/ATR in a large-scale proteomic screen, is an E3 ligase that plays a crucial role in the DNA damage response (Matsuoka et al., 2007). RFWD3 is known to ubiquitinate p53 and stabilize p53 in response to DNA damage (Fu et al., 2010). Several recent studies have pinpointed the role of RFWD3 in replication checkpoint control (Elia et al., 2015; Feeney et al., 2017; Inano et al., 2017). RFWD3 interacts with replication protein A (RPA), at stalled forks and facilitates RPA-mediated DNA damage signaling (Gong and Chen, 2011; Liu et al., 2011). RPA-ubiquitination by RFWD3 was found to be critical for HR at stalled forks, and was found to be required for fork restart (Elia et al., 2015). Other recent studies have highlighted the importance of RFWD3 in ICL repair (Feeney et al., 2017; Inano et al., 2017). HR was found to be disrupted in RFWD3-mutant cells and biallelic mutations in RFWD3 have been linked to Fanconi anemia (FA) (Knies et al., 2017). Specifically, RFWD3 has been implicated in ubiquitinating RPA and RAD51 and in their subsequent removal for allowing HR progression (Inano et al., 2017). However, the mechanism of how RPA and RAD51 are recognized by RFWD3 remains to be determined.

RFWD3 and several FA genes function at different stages of HR. Evidence indicates that FANCD2 and RFWD3 as well as RAD51 and BRCA2/FANCD1 accumulate at the same damage sites and show functional convergence (Knies et al., 2017). The FA pathway is known to suppress genome instability upon encountering replication fork stalling (Ceccaldi et al., 2016). Other than their bonafide roles in ICL repair, FA genes are known to play roles in replication by promoting fork stability, and during mitosis in controlling chromosome segregation (Naim and Rosselli, 2009). The role of the FA pathway in stabilizing stalled forks is beginning to emerge as an important mechanism for the maintenance of genome stability and this function is clearly independent of its role in ICL repair.

In this study, we demonstrate that RFWD3 plays an important role during unperturbed cell cycle as cells lacking RFWD3 show defects in cell survival, S-phase progression, and sister chromatid cohesion. Cells without RFWD3 show slower fork progression and a prolonged S-phase, suggesting its role in DNA replication. Consistent with its role in replication, RFWD3 is enriched at the replication fork as observed by nascent strand capture assay (Alabert et al., 2014). We support the model that RFWD3 is a component of the FA pathway that has an essential

function in ICL repair, and that PCNA-mediated recruitment of RFWD3 to the fork and its stabilization are essential for DNA replication. PCNA is the sliding clamp at the replication fork that is required for the processivity of DNA replication (Prelich et al., 1987). In addition to PCNA's primary function to tether different replication factors to the DNA template, PCNA also acts as the interacting scaffold at the center of the replication fork to coordinate various processes such as nucleosome assembly and epigenetic inheritance, and plays a role during DNA damage repair. Interestingly, PCNA is also known to be monoubiquitinated by the RAD18 E3 ligase in response to ICL lesions and by CRL4(Cdt2) to promote translesion synthesis, a lesion associated with endogenous replication stress (Choe and Moldovan, 2017; Terai et al., 2010). We observed that in the absence of RFWD3, many components of the fork showed increased binding to the chromatin, including RPA, DNA polymerases, Cdc45, and TLS polymerases, all of which point to stalling of the replication fork. The uncoupling of the helicase from the stalled polymerase is known to cause an increased accumulation of ssDNA (as evidenced by increased RPA), resulting in ubiquitination of PCNA (Chang et al., 2006; Zhuang et al., 2008). A recent study has reported the presence of p53 bound to PCNA at stalled forks for suppressing the extension from these forks (Hampp et al., 2016). It is interesting to note that RFWD3 is known to positively regulate p53 stability (Fu et al., 2010). It is a possibility that in the absence of RFWD3, persistence of stalled forks is because of defects in ubiquitination of substrates like p53 and RPA. The identification of the entire repertoire of substrates of RFWD3 during unperturbed DNA replication and during DNA damage would tremendously improve our understanding.

Depletion of RFWD3 has been shown to induce Chk1 and Chk2 phosphorylation in the absence of DNA damage, corroborating our results that RFWD3 has a fundamental role in promoting the stability of unperturbed replication forks (Elia et al., 2015). We find that PCNA is required for stabilizing RFWD3 to the chromatin. PCNA is known not only to influence the association of a large number of factors to chromatin, but also to affect their activity in many instances (Choe and Moldovan, 2017). The PIP domain within RFWD3 is close to I639 moiety, a site that was found to be mutated in FA patient and has previously been shown to be crucial for RPA binding. Furthermore, it has previously been reported that RPA-mediated recruitment of RFWD3 to stalled forks is critical for ICL repair (Feeney et al., 2017). We find that RFWD3 can be stabilized on chromatin in the absence of RPA, and our results support a model where RFWD3 associates with PCNA and localizes to the replication fork. The fork-associated

RFWD3 is also important for the ubiquitination of replication fork components including RPA, to ensure proper fork progression. One model would posit that the ubiquitination of RPA enables removal of RPA, thus allowing fork progression. Cells lacking RFWD3, show increased accumulation of RPA, increased PCNA monoubiquitination, and therefore fork stalling. We propose that RFWD3 plays a key role in resolving the DNA breaks and stalled forks that are natural occurrences during regular DNA replication.

3.4 Material and methods

3.4.1 Cell culture and synchronization

U2OS and HEK293T cells were grown in DMEM containing high glucose and supplemented with 10% fetal bovine serum. Vectors and siRNA were delivered using Lipofectamine 2000 and Lipofectamine RNAiMax (Invitrogen) respectively. U2OS cells stably expressing HA-RFWD3 were generated by lentivirus infection.

3.4.2 Plasmids, siRNA and antibodies

HA-RFWD3 vector was generated by cloning RFWD3 into the pCGN vector. The GST-RFWD3 construct was a kind gift from Dr. Yi Wang (Baylor College of Medicine). Mutant RFWD3 constructs were generated using the QuikChange Site-Directed Mutagenesis Kit (Agilent) as per the manufacturer's protocol.

The siRNA oligos used in this study were synthesized by Sigma.

RFWD3 si-1 (5'-GGACCUACUUGCAAACUAU-3')

RFWD3 si-2 (5'- GCAGUCAUGUGCAGGAGUU-3')

PCNA si (5'- CGGUGACACUCAGUAUGUC-3')

RPA1 si (5'- GGUGCUACAUAGUUGGUAA-3')

RPA2 si (5'- CCUAGUUUCACAAUCUGUU-3')

The following antibodies were used for immunoblotting: anti-RFWD3 (1:250, Bethyl, A301-397A; 1:500, Abcam, ab138030), anti-HA (1:500, 12CA5), anti-α-tubulin (1:10000,

Sigma-Aldrich, T5168), anti-Histone H4 (1:500, Millipore, 05-858R), anti-PCNA (1:1000, Santa Cruz Biotechnology, sc-56), anti-RPA1 (1:300, Bethyl, A300-241A), anti-RPA2 (1:250, Santa Cruz, sc-56770), anti-REV (1:200, Santa Cruz, sc-393022), anit-Pol-ι (1:200, Santa Cruz, sc-101026).

The following antibodies were used for immuno-staining and DNA fiber staining: anti-PCNA (1:200, PC10), anti-RPA2 (1:500, Cell Signaling, 2208), anti-Rad51 (1:300, Santa Cruz, sc-8349), anti-BrdU (for CldU, 1:200, Bio Rad, OBT0030G), anti-BrdU (for IdU, 1:200, BD, 347580).

3.4.3 iPOND

A modified version of iPOND (Sirbu et al., 2012), described as aniPOND in (Leung et al., 2013), was used to isolate proteins at the replication fork. In brief, 1.5 x 10^8 293T cells per condition were labeled with 10μM of EdU for 15 min. For Thymidine chase, cells were washed with PBS to remove EdU and incubated in medium containing 10μM Thymidine for 1h. After all pulse-chase treatments, cells were immediately lysed in NEB (20mM HEPES pH7.9, 50mM NaCl, 3mM MgCl2, 300mM sucrose, and 0.5% NP-40) on ice for 15 min and nuclei were harvested by centrifugation. After washing with PBS, nuclei were incubated in click reaction with biotin-azide for 1h at 4 °C. Afterwards, nuclei were washed in PBS and resuspended in B1 buffer (25mM NaCl, 2mM EDTA, 1% NP-40 in 50mM Tris-HCl pH8) for sonication. Sonication was done at 4 °C using a bioruptor (Diagenode) at high intensity in 30-second on/off cycles for 30 min. Samples were then centrifuged at max speed for 10 min at 4 °C and supernatants were collected. An equal volume of B2 buffer (150mM NaCl, 2mM EDTA, 1% NP-40 in 50mM Tris-HCl pH8) was added to bring up the NaCl concentration, and input was taken at this point. EdU labeled DNA was then captured with 50μl of streptavidin beads (Dynabeads® MyOne™ Streptavidin C1) at 4 °C overnight. Beads were washed with B2 buffer three times and boiled in laemmli sample buffer to elute captured proteins.

3.4.4 Co-Immunoprecipitation

U2OS cells were lysed in IP buffer containing 50mM Tris pH7.4, 500mM NaCl, 0.5% NP-40, 1mM DTT, 10% glycerol, and protease and phosphatase inhibitors for 30min at 4°C. The lysate was then diluted with an equal volume of IP buffer containing no NaCl to bring down the NaCl concentration to 250mM. The diluted lysate was pre-cleared with Gammabind Sepharose beads for 1h at 4°C and incubated with the appropriate antibody overnight at 4°C. Protein complexes bound by antibody were pulled down by Gammabind Sepharose beads for 1.5h at 4°C. The beads were then washed with IP buffer supplemented with 250mM NaCl three times and denatured in Laemmli buffer.

For direct interaction assay, 500ng of each protein were incubated in PBS supplemented with 0.2% Triton for 30min at 4°C. The mixture was then incubated with antibodies at 4°C for another 1h. Protein complexes bound by antibody were pulled down by Gammabind Sepharose beads for 1h at 4°C. The beads were then washed with PBS supplemented with 0.2% Triton three times and denatured in Laemmli buffer.

3.4.5 Flow cytometry

Cells were collected and washed once in ice cold PBS, resuspended in PBS + 1% normal goat serum (NGS), and fixed in 90% chilled ethanol overnight. Cells were then washed and resuspended in PBS + 1% NGS with 120 μg/ml propidium iodide (PI) and 10 μg/ml RNase A for 45 min at 37 °C. DNA content was measured by flow cytometry.

3.4.6 Chromatin fractionation

U2OS cells were resuspended with solution A (10mM HEPES pH7.9, 10mM KCl, 1.5mM MgCl2, 0.34M sucrose, 1mM DTT, 10% glycerol and 0.1% Triton X-100) and incubate on ice for 5min. The cytoplasmic fraction (S2) was then separated from the nuclei by centrifuging at 4°C at 1400g for 4min. Isolated nuclei were then washed with solution A without Triton X-100. The nuclei pellet was resuspended with solution B (3mM EDTA, 0.2mM EGTA, and 1mM DTT) and incubated on ice for 30min. The nuclear soluble fraction (S3) was then separated by centrifuging at 4°C at 1700g for 4min. The isolated chromatin fraction was then

washed with buffer B. Finally, the chromatin pellet (P3) was resuspended in solution A and sonicated for 1min to get the lysate.

3.4.7 *In vivo* ubiquitination assay

293T cells transfected with flag-ubiquitin were lysed using denature buffer (20mM Tris pH7.5, 250mM NaCl, 1mM EDTA, 0.5% NP40, 0.5% SDS, 0.5% sodium deoxycholate) supplemented with protease and phosphatase inhibitors at 4°C for 20 min, followed by sonication for 15 min and passage through a 27G needle to shear the DNA. Ubiquitinated proteins were then pulled down by anti-flag M2 agarose beads (Sigma) at 4°C overnight. The beads were washed in denature buffer three times and captured ubiquitinated proteins were eluted by boiling in Laemmli buffer.

3.4.8 DNA fiber assay

U2OS cells were labeled with 25mM CldU for 30min followed by 30 min of 250mM IdU. DNA fibers were prepared using the FiberComb molecular combing system (Genomic Vision) as per the manufacture's protocol.

To visualize the CldU and IdU track, coverslips with DNA fibers were stained with antibodies specific to CldU or IdU. DNA fibers on coverslips were denatured in denaturation solution (0.5M NaOH, 1M NaCl) for 8min at room temperature. Coverslips were then washed with PBS and dehydrated in 70%, 90%, and 100% ethanol for 5min each. Re-hydrated coverslips were then immunostained with CldU and IdU specific antibodies.

3.4.9 Protein purification

Human RPA and PCNA were purified as described previously (Binz et al., 2006; Fien and Stillman, 1992). Human GST-RFWD3 was induced for overexpression in *E. coli* BL21 (DE3) codon (+). The cells expressing GST-RFWD3 were collected and lysed in GST buffer (50mM Tris pH7.5, 0.1mM EDTA, 150mM NaCl, 1mM DTT and 5% glycerol) containing lysozyme 0.5 mg/ml and 0.1% Triton X-100 followed by sonication. The lysate was collected by

centrifugation and the supernatant was subjected to GSTrap column (GE healthcare) using an AKTA pure system. After washing with GST buffer containing 500mM NaCl, GST-RFWD3 was eluted in GST buffer containing 20mM reduced glutathione.

3.4.10 GST pull-down

After obtaining the bacterial lysate containing GST-RFWD3, the lysate was incubated with glutathione-agarose (Sigma) for 1h at 4°C. The beads with GST-RFWD3 immobilized on were then washed with GST buffer three times to remove unbound proteins. For PCNA-RPA titration, 5 µl of beads containing 400ng of GST-RFWD3 were used per reaction. The GST-RFWD3 beads were first saturated with exceeding amount of PCNA for 1h at 4°C, followed by washing with GST buffer to remove unbound PCNA. The GST-RFWD3-PCNA beads were then incubated with different amount of RPA for another 1h at 4°C. After washing with GST buffer three times, the beads were boiled in laemmli sample buffer and preceded to western blot.

3.4.11 Live cell microscopy

Human U2OS cells stably expressing YFP-PCNA were used for live-cell imaging. The cells were grown in glass bottom culture dishes (MakTek). The dish was mounted onto the stage of a Delta Vision optical sectioning deconvolution instrument (Applied Precision) on an Olympus microscope and kept at 37°C in L-15 medium (minus phenol red) containing 30% FBS. Time-lapse images acquired with a 63X 1.42 N.A. objective lens was captured with a Coolsnap CCD camera.

3.5 Figures

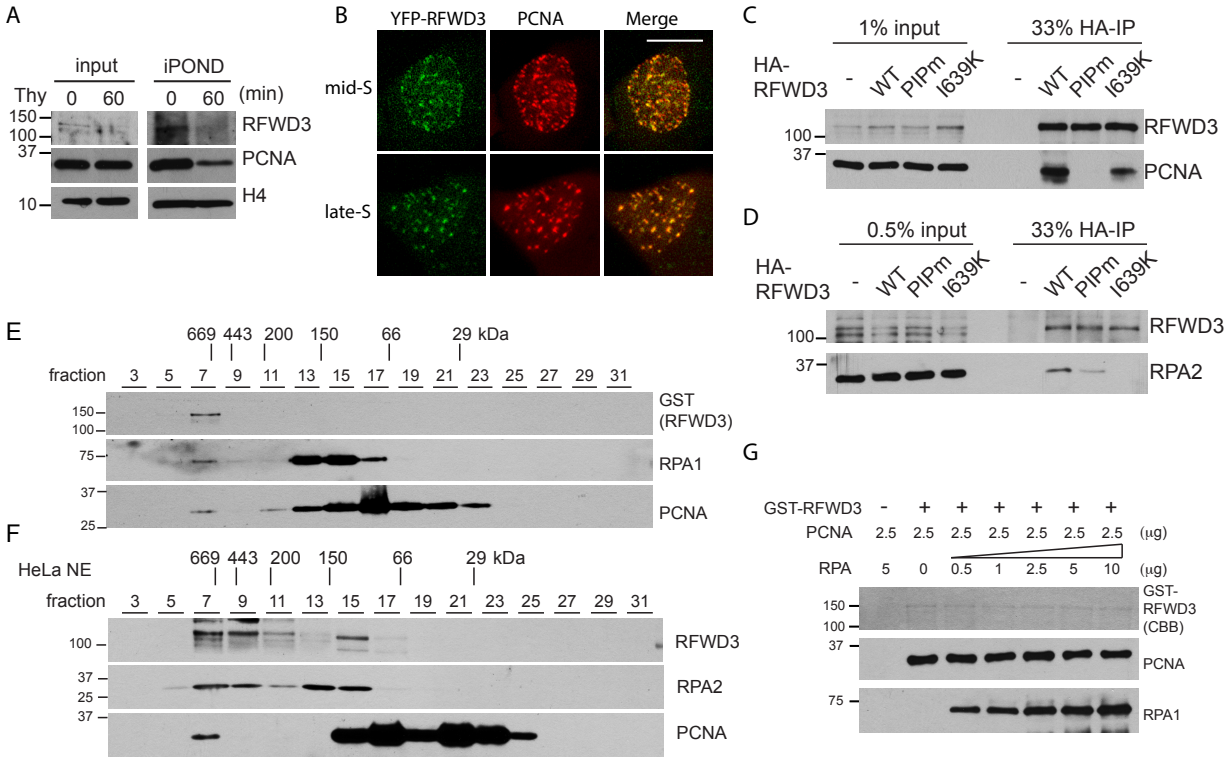

Figure 3.1 RFWD3 is at the replication fork and associates with PCNA

(A) Cells labeled with EdU, processed by iPOND (0 and 60 min Thymidine chase) and immunoblotted using RFWD3, PCNA, and H4 antibodies. (B) Localization of YFP-RFWD3 with PCNA. Scale bar: 15μm. (C-D) IP in U2OS cells expressing various HA-RFWD3-WT, HA-RFWD3-PIPm, and HA-RFWD3-I639K mutant using HA Ab. (E-F) Purified proteins or HeLa nuclear extract fractionated over a Superdex 200 gel filtration column and fractions analyzed for RFWD3, PCNA and RPA by immunoblot. Molecular weight markers are labeled on top of the panel. (G) GST pull down assay to determine the complex assembly of GST-RFWD3, PCNA and RPA.

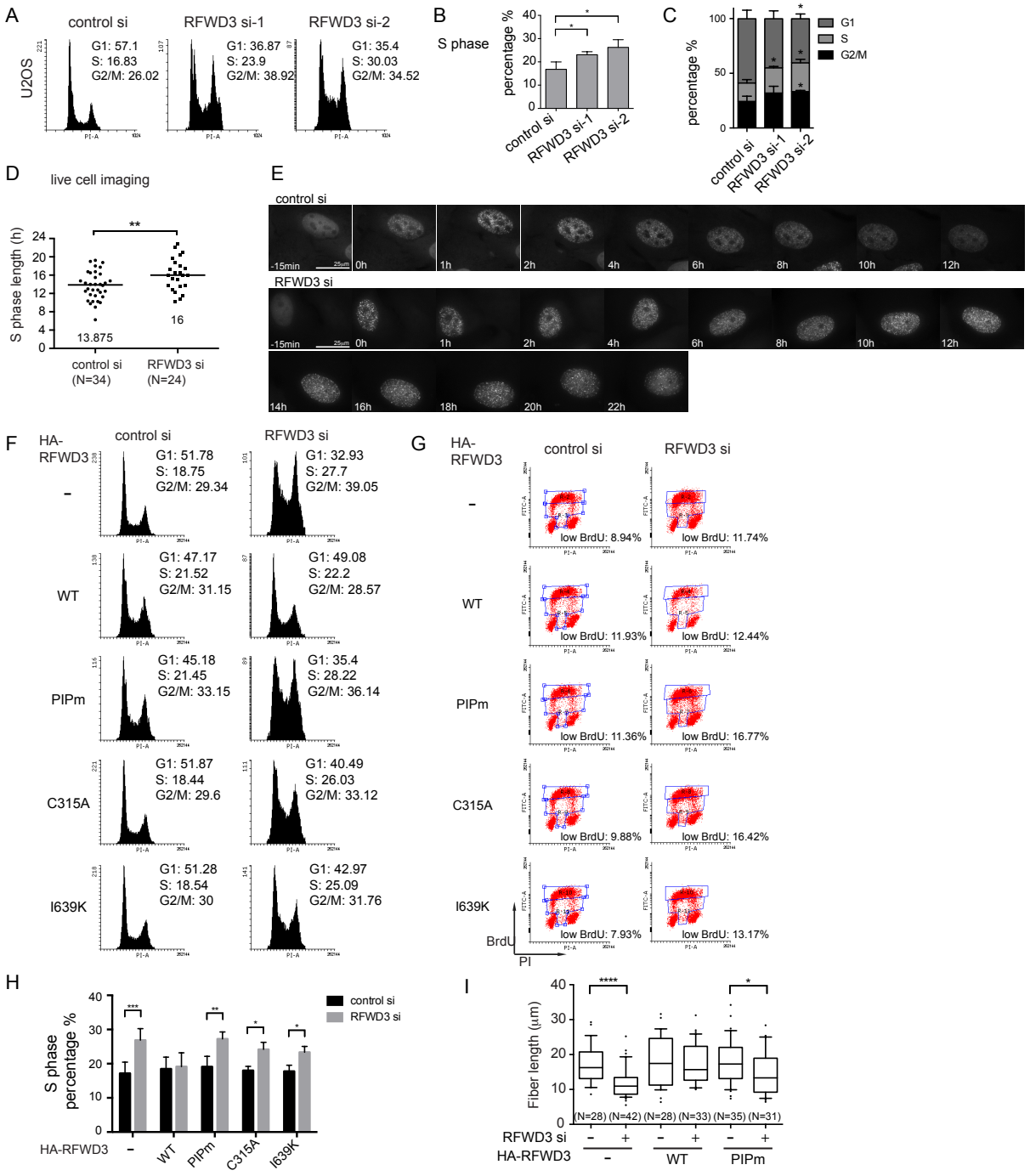

Figure 3.2 RFWD3 is required for S-phase progression

(A) Cell cycle profile (PI flow cytometry) of U2OS cells depleted of RFWD3. (B) Quantification of S-phase population from three independent experiments. (C) Distribution of cells in different stages of the cell cycle in control and RFWD3-siRNA treated. (D) Length of S-phase quantitated by live cell imaging of YFP-PCNA in control and RFWD3-siRNA-treated cells.

Figure 3.2 (cont.) (E) Still images of YFP-PCNA in control and RFWD3-siRNA-treated cells. 0h time point denotes the starting of replication, as observed by PCNA pattern on chromatin. (F) Cell cycle profile by flow cytometry of various U2OS cell-lines (HA-RFWD3-WT, HA-RFWD3 -PIPm, HA-RFWD3-C315A, and HA-RFWD3-I639K mutants) depleted of endogenous RFWD3. Note that only the RFWD3-WT can rescue the cell cycle defect. (G) BrdU-PI profile of various U2OS cell-lines (HA-RFWD3-WT, HA-RFWD3-PIPm, HA-RFWD3-C315A and HA-RFWD3-I639K mutants) depleted of endogenous RFWD3. (H) Quantification of S-phase population (from three independent experiments) in HA-RFWD3-WT, HA-RFWD3-PIPm, HA-RFWD3-C315A, and HA-RFWD3-I639K mutants depleted of endogenous RFWD3. (I) DNA fiber assay in HA-RFWD3-WT, HA-RFWD3-PIPm cell lines that are depleted of endogenous RFWD3. *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$ by unpaired two-tailed student's t-test.

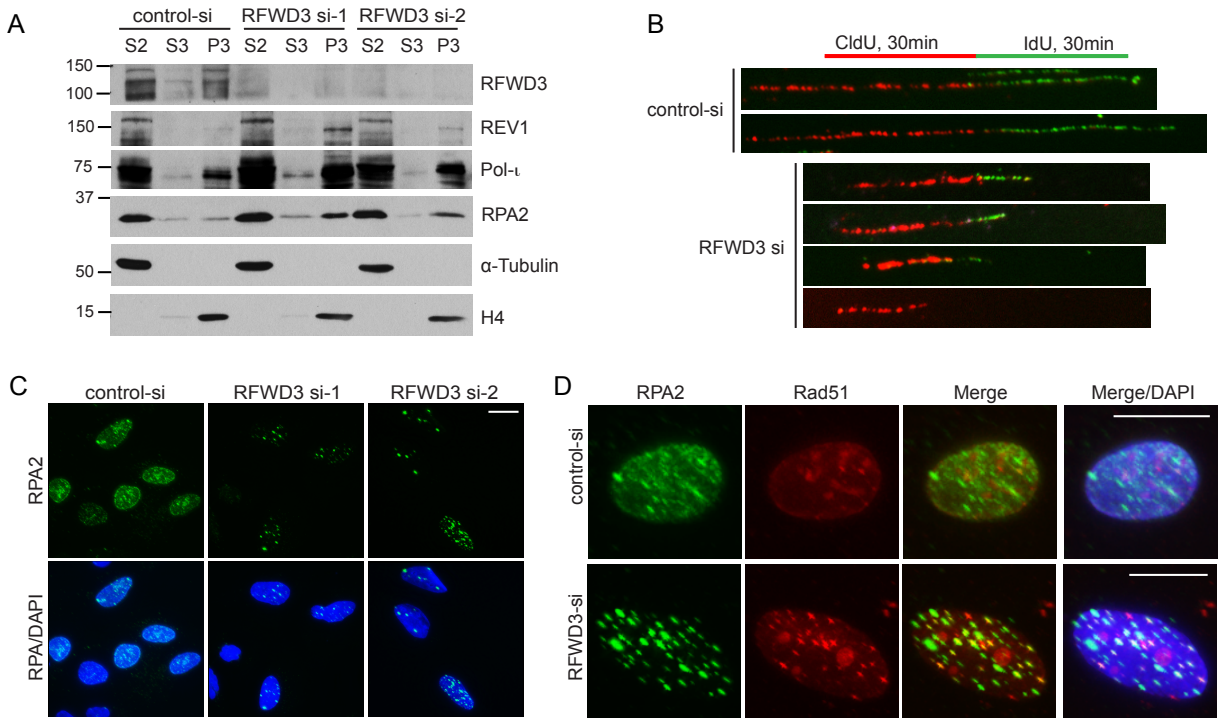

Figure 3.3 Loss of RFWD3 causes fork stalling

(A) Chromatin fractionation in RFWD3-depleted U2OS cells (siRNA 1 and siRNA 2) followed by IB analysis. H4 and α-Tubulin are loading controls. S2- cytosolic; S3- nuclear soluble; P3- chromatin bound fraction. (B) Representative fluorescence image showing fibers from control (control siRNA) and RFWD3-depleted (RFWD3 si-1) U2OS cells that were labeled with CldU and IdU. Note increased fork stalling in the absence of RFWD3 (control siRNA-progressing fork 76.3%, stalled fork 11.3%, newly fired or terminated fork 12.4%; RFWD3 siRNA-progressing fork 65.5%, stalled fork 19.5%, newly fired or terminated fork 15%). (C) RPA and (D) RPA with RAD51 immunofluorescence in control and RFWD3-depleted cells. (RPA staining in Control- 47.4% replication and 10.3% damage-like foci; RFWD3-siRNA 1- 50.9% replication and 23.4% damage-like foci; RFWD3-siRNA 2- 36.4% replication and 31.3% damage-like foci). Scale bar: 15μm.

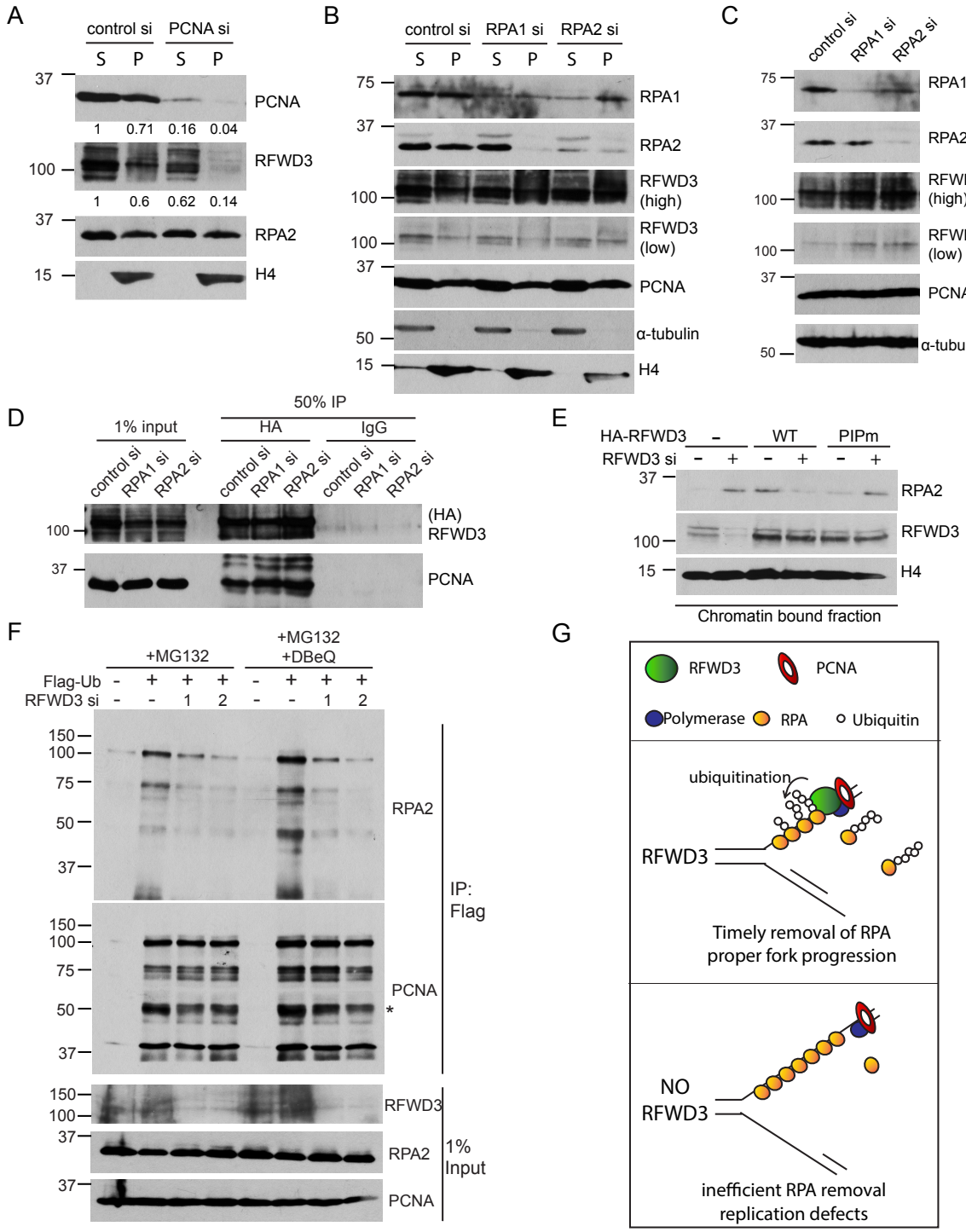

Figure 3.4 PCNA-mediated stabilization of E3 ligase RFWD3 to the replication fork is essential for ubiquitination of RPA

(A) Chromatin fractionation in PCNA-depleted U2OS cells and immunoblot analysis. Note that in the PCNA-siRNA treated cells, there is significant reduction of total as well as chromatin

Figure 3.4 (cont.) associated-RFWD3. S-soluble fraction, P-chromatin bound fraction. (B) Chromatin fractionation in RPA1- and RPA2-depleted U2OS cells and immunoblot analyses using RPA, PCNA, and RFWD3 antibodies. (C) Depletion of RPA1 and RPA2 using siRNA. Immunoblotting using RFWD3 and PCNA. (D) IP using HA Ab from U2OS cells stably expressing HA-RFWD3 that are treated with control, RPA1 or RPA2 siRNAs. RFWD3 and PCNA were analyzed by immunoblotting (IB). (E) Depletion of endogenous RFWD3 in U2OS cells stably expressing HA-RFWD3-WT or HA-RFWD3-PIPm, and immunoblot analyses using RPA and RFWD3 antibodies. Note the increase in RPA in the absence of RFWD3 is rescued by the RFWD3-WT but not the PIP-mutant. (F) *In vivo* Ub assay in control and RFWD3 siRNA treated cells (+MG132; +MG132 and DBeQ) transfected with Flag-Ub. Immunoprecipitation was performed with FLAG and immunoblotting with RPA2 and PCNA. Note the reduction in RPA ubiquitination and a specific form of PCNA (*) (G). Cartoon demonstrating the role of RFWD3 in ubiquitination of RPA and DNA replication progression.

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CHAPTER 4. CONCLUSIONS AND PERSPECTIVES

Given the critical role of DNA replication in cell physiology, from G1 origin licensing to S phase replication elongation, the regulatory mechanism governing this process is an active field of study. Meanwhile, how the DNA replication coordinates with DNA repair and DNA damage response is also critical to understand. In this thesis, I studied two human proteins that are involved in these processes, Orc6 and RFWD3. Orc6, as one of the ORC subunits, has been suggested for a long time to be functioning in origin licensing during G1, but not in S phase. On the other hand, studies of RFWD3 have focused almost exclusively on its role in DNA damage and HR, but not in unperturbed cell cycle progression. Here I report that both of them have previously un-identified roles in regulating genome stability during DNA replication.

As I described in Chapter 2, I characterized the function of human Orc6, the smallest ORC subunit. As part of the ORC, Orc6 has been suggested to function in origin licensing. This is true in yeast and *Drosophila* (Bleichert et al., 2013; Miller et al., 2019). However, the function of human Orc6 has not been fully examined. One most prominent phenotype of human Orc6 depletion is reduction in the S phase population, which has been observed for nearly two decades (Prasanth et al., 2002). The S phase reduction can be due to defects in G1 licensing process as suggested in other model systems, or defects in S phase entry. I showed that human Orc6 is dispensable for origin licensing. In contrast, Orc6 functions after pre-RC loading to promote Cdc45 association to MCMs and thus is crucial for CMG helicase function and S phase entry. On the other hand, during S phase, Orc6 travels along at replication fork and works as an accessory factor for MMR protein complex to promote MMR efficiency. These findings suggest a completely unexpected function of Orc6 as a S-phase factor.

Although the detailed molecular mechanism is not fully understood, it has been suggested that there is a p53-dependent origin-licensing checkpoint, where cells are able to sense the status of origin licensing in G1 (McIntosh and Blow, 2012). Only when cells have licensed a sufficient amount of origins are they able to initiate S phase. Therefore, it is reasonable to hypothesize that the accumulation of G1 population in Orc6-depleted cells is due to defects in licensing. However, we did not observe defects in MCM licensing in Orc6-depleted cells using MCM-PI flow as well as chromatin fractionation in cells depleted of Orc6 specifically in G1. Given the

fact that even the knockdown of MCM proteins does not cause reduction but instead an increase in the S phase population in asynchronized U2OS cells (Cortez et al., 2004), our results suggest that human Orc6 functions in regulating different aspects of G1/S transition.

Recently, Orc1, Orc2, Orc5 or Orc2/5 have been successfully depleted from human cells using CRISPR knockout (Shibata and Dutta, 2020; Shibata et al., 2016). The fact that subunits in the core ORC are dispensable is striking. Nevertheless, it is interesting to note that, in these cells, Orc6 was not affected at all in terms of its chromatin association. Although it is unclear how MCMs can still be loaded in the absence of core ORC, these results strongly imply that Orc6 might function independently of ORC. Another evidence in our study to support this idea is the fact that C-terminal truncated Orc6 could rescue the S phase defects similar to WT Orc6. Meanwhile, the fact that Orc6 complete knockout cannot be generated indicates the essentiality of Orc6 for cell survival. Although it is a negative result and we cannot rule out the possibility of getting Orc6 knockout cells in the future.

We have concluded that human Orc6 is dispensable for MCM licensing. These results are based on the observation that despite the absence of Orc6, cells were able to load an equal amount of MCMs onto chromatin in the presence of an intact Orc1-5. However, it is impossible to completely rule out that Orc6 is involved in G1 licensing origin selection. The fact that human Orc6 is sub-stoichiometric in ORC complex, it is possible that miniscule amount of Orc6 enable licensing. It is worth noting that in my Orc6 IP-MS screening, several chromatin remodeler proteins are identified as Orc6 interacting partners. Although the significance of these interactions remains to be studied, one hypothesis would be that Orc6 in ORC facilitates the selection of ORC binding to certain sites by modulating chromatin environment. Thus, even though we observed that the depletion of Orc6 does not cause reduction in ORC and MCM chromatin loading level, there might be alterations in the distribution of their binding regions and/or efficiency. It would therefore be interesting to see the genome-wide ORC and MCM binding in Orc6-depleted G1 phase cells using ChIP-seq to understand if Orc6 has a role in the origin selection program.

In addition to our characterization of human Orc6's dispensability in licensing, the role of Orc6 in mismatch repair was a surprising finding. Specifically, my work shows that binding of Orc6 with MutSα facilitates the recruitment of MutLα. Despite being extensively studied, it is

well known that the interaction between MutSα and MutLα is transient and difficult to capture. Thus, the involvement of Orc6 in the protein complex can potentially be valuable for future structural studies. The dominant model currently for MutSα-MutLα assembly is that, the MutS and MutLα form a 1:1 MutSα-MutLα complex after MutSα encounters a mismatch. Interestingly, recent studies on MMR suggest a different model, showing that multiple MutSα and multiple MutLα complexes accumulate around the mismatch DNA. (Bradford et al., 2020; Hao et al., 2020; Putnam, 2020). It would be interesting to see if Orc6 is involved in this higher-order complex formation. In addition, while this thesis was being written, the full-length structure of human Orc6 was published (Xu et al., 2020). Consistent with previous studies, the structure of Orc6 contains three independent domains, TFIIB-A, TFIIB-B and CTD. The structural information could facilitate the experimental design for future studies. TFIIB-B is the domain that interacts with MutSα based on our findings. It is worth noting that a study using bioinformatic analysis reported that Orc6 contains a putative SHIP (MSH2-Interacting-Peptide) box at a.a. 134-140 within TFIIB-B (Goellner et al., 2018). However, the Orc6-MutSα interaction was not disrupted when we used SHIP box mutated Orc6. Nevertheless, it is important to narrow the interacting region within Orc6 TFIIB-B domain. The identification of the minimal peptide required for MutSα binding would have a broad application in many experiments for MMR study. One of our hypotheses is that, Orc6 binding to MutSα stabilizes MutSα at a conformation that allows MutLα to bind. Different biochemical and biophysical tools could therefore be used, such as fluorescence anisotropy or circular dichroism, to determine the MutSα conformational changes upon Orc6 binding. One long-term goal would be solving the structure of Orc6 bound to MutSα.

We have also identified that Orc6 is phosphorylated at T229 upon oxidative stress and Orc6 protein level is significantly upregulated. Despite several attempts with different experiments, we have not yet established the functional significance of this phosphorylation. The T229 is close to Y232, the residue critical for Orc3 binding. However, we observed that T229 phospho-mimetic mutant did not disrupt the binding to core ORC. One potential issue is that the phospho-mimetic residue might not behave as an actual phosphorylation. Synthetic peptides can be used for the *in vitro* examination of the actual phospho-group in terms of protein interaction. However, it might be harder to prove that T229 phospho-mimetic mutant is functional *in vivo*. On the other hand, we did observe that T229 phospho-dead mutant was not able to rescue ATR

defects, while phospho-mimetic partially rescued. We also observed that Orc6 T229 mutants bind stronger to DNA, even when T229 is not in the reported DNA binding region of Orc6 (Xu et al., 2020). Together with the fact that Orc6 is at replication fork, our hypothesis is that Orc6 phosphorylated at T229 may halt the fork progression. This can be tested by performing single molecular DNA fiber assay and observing the fork rate in the presence of H_2O_2 in Orc6 WT or T229 phospho-dead background. Meanwhile, the fact that Orc6 protein level is rapidly upregulated upon oxidative stress within 20 min suggests that it is more likely the protein stability being altered instead of it being transcriptionally regulated. We have preliminary data showing that Orc6 is ubiquitinated. How this ubiquitination is regulated in normal and DNA damage conditions, and the functional relevance of it remain to be studied. The phosphorylation might also crosstalk with ubiquitination and affect the stability of Orc6 protein level.

It is also worth noting that Orc6 has been shown to correlate with cancer progression, including pediatric brain tumors, breast cancer, colorectal cancer, prostate cancer, hepatocellular carcinoma (Gavin et al., 2008; Hu et al., 2019; Idilli et al., 2020; Koleck and Conley, 2016; Wang et al., 2020; Wei et al., 2020; Xi et al., 2008). Although the molecular explanation has yet to be established, the newly identified function of Orc6 in MMR would be most relevant to colorectal cancer, where MMR is often defective. Thus, it would be interesting to study the Orc6 function in colorectal cancer development. For example, cancer phenotypic analysis can be done in MMR-proficient and MMR-deficient colorectal cancer cell lines with or without Orc6. Chemotherapeutic drug sensitivity can also be analyzed in cell lines expressing different level of Orc6. However, the results should be carefully interpreted given that Orc6 plays multiple essential functions in S phase entry, MMR, and cytokinesis.

In Chapter 3, I focused on RFWD3, a protein thought to be functioning only during DNA damage conditions. Together with our lab's previous graduate student's work (Wang, 2017), we identified that the direct binding of RFWD3 to PCNA stabilizes it at the replication fork, and hence ubiquitinates RPA and promotes proper fork progression. Without RFWD3, cells showed reduced fork rate and increased replication stress phenotypes. The architecture of human replisome and the minimal constitution of replisome are not fully understood. Our findings that RFWD3 localizes at replication fork could suggest that RFWD3 is a constituent component in replisome across the entire genome. It is well known that PCNA acts as a binding platform for a great number of proteins required in DNA metabolism at the fork. On the contrary, RFWD3 may

be required for replication fork movement at intrinsic hard-to-replicate regions, such as common fragile sites, which are prone to stall. Thus, the function of RFWD3 on RPA would be more critical at certain genomic regions. Therefore, for future direction, it would be interesting to determine if RFWD3 preferentially associates with certain replication domains or common fragile sites in the genome during S phase.

Current known RFWD3 targets are p53, RPA and Rad51. Although identifying and validating E3 ligase substrates are not easy tasks, it would be informative to identify more RFWD3 substrates, either by IP-MS interactome analysis or SILAC-based ubiquitination profile analysis. Since we observed the association of RFWD3 at the replication fork, candidate-approach can also be conducted for searching replisome components as potential substrates. In the present study of this thesis, we have concluded that the RFWD3-depleted phenotype is contributed partially, if not fully, by defects in RPA ubiquitination. However, regarding the consequence of RFWD3 mediated ubiquitination of RPA, there are still unanswered questions. While the role of RFWD3 on ubiquitinating RPA and facilitating its removal were seen in both DNA damage and normal replication condition as we reported (Inano et al., 2017; Lin et al., 2018), a recent study made an unexpected observation that RFWD3-mediated RPA ubiquitination in BRCA2-deficient cells was removal- and repair-resistant (Duan et al., 2020). Thus, the function of RFWD3 is context-specific and should be considered carefully when performing further experiments.

In summary, my studies in this thesis have elucidated the molecular details of two important replication proteins, Orc6 and RFWD3, and their roles in preventing genome instability. Orc6 facilitates MMR function in S phase to ensure the fidelity of DNA replication; RFWD3 promotes proper fork movement to prevent intrinsic replication stress. Together, these findings provide more insights and novel regulatory mechanisms in how genome integrity is maintained during cell cycle progression.

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