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Phosphorylation of Orc6 During Mitosis Regulates DNA Replication and Ribosome Biogenesis

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ABSTRACT

The human Origin Recognition Complex (ORC) is required not only for the initiation of DNA replication, but is also implicated in diverse cellular functions, including chromatin organization, centrosome biology, and cytokinesis. The smallest subunit of ORC, Orc6, is poorly conserved amongst eukaryotes. Recent studies from our laboratory have suggested that human Orc6 is not required for replication licensing, but is needed for S-phase progression. Further, ATR-dependent phosphorylation of Orc6 at T229 is implicated in DNA damage response during S-phase. In this study, we demonstrate that the CDK-dependent phosphorylation of Orc6 at T195 occurs during mitosis. While the phosphorylation at T195 does not seem to be required to exit mitosis, cells expressing the phosphomimetic T195E mutant of Orc6 impede S-phase progression. Moreover, the phosphorylated form of Orc6 associates with ORC more robustly, and Orc6 shows enhanced association with the ORC outside of G1, supporting the view that Orc6 may prevent the role of Orc1-5 in licensing outside of G1. Finally, Orc6 and the phosphorylated Orc6 localize to the nucleolar organizing centers and regulate ribosome biogenesis. Our results suggest that phosphorylated Orc6 at T195 prevents replication.

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Mitosis; nucleolus; Orc6; phosphorylation; replication

Introduction

The origin recognition complex (ORC) is a six-subunit complex that is required for the initiation of DNA replication in eukaryotes.[1] The individual subunits of ORC as well as multiple sub-complexes of ORC, play diverse roles in cell cycle progression and chromatin organization.[2] The largest subunit of ORC, Orc1, and the core ORC, consisting of Orc2-5, are well conserved across species.[3] However, the smallest subunit, Orc6, is the least conserved and has been implicated to play diverse roles in a species-dependent manner.[4–7] Most recently, our lab demonstrated that the human Orc6 is not required for replication licensing but plays an important role in S-phase progression.[8] Further, we showed that Orc6 is associated with the mismatch repair complex during S-phase and facilitates mismatch repair to maintain genome stability. Finally, we showed that Orc6 is phosphorylated at T229 in response to DNA damage in S-phase, which is critical for fork pausing and activating the repair process.[9]

The phosphorylation of proteins is an important mode of modulating their cellular function. Cyclin-dependent kinases (CDKs) are well known for their roles in phosphorylating substrates that govern cell cycle progression.[10,11] Many studies have pointed to the functional relevance of ORC phosphorylation in regulating ATP-binding and, hence, replication initiation.[12,13] Human Orc2 is phosphorylated during S-phase by CDKs, which results in the dissociation of the core ORC from the chromatin.[14] Further, the phosphomimetic mutant of Orc2 fails to bind to origins, supporting the argument that phosphorylation is an important mode of replication licensing control. Orc2 is also phosphorylated in the budding and fission yeast.[15,16] and mutations within CDK consensus sites result in cell cycle defects and aberrant replication control.[17,18] Orc1 also contains CDK consensus sites in human cells and is phosphorylated during mitosis. Orc1 must be dephosphorylated for the core ORC to associate with Orc1 to promote pre-RC assembly.[19] Similarly, hamster Orc1 is also phosphorylated during G2/M, which inhibits chromatin reloading of ORC during mitosis.[20] In budding yeast, Orc1, Orc2, and Orc6, all contain CDK consensus sites,[21] and Orc6 is phosphorylated in a cell cycle-dependent manner.[22]

Human Orc6 also contains a CDK consensus site (S/T)PX(K/R) at threonine 195. Work from the DePamphilis group showed that this phosphorylation is required for the nuclear localization of Orc6, and this localization is independent of the other ORC subunits.[23] In this study, we report that Orc6 phosphorylation at T195 occurs predominantly at the start of mitosis, just prior to the breakdown of the nuclear envelope. The phosphorylated Orc6 localizes to nucleolar organizing

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regions and rapidly gets dephosphorylated as the cells complete mitosis. We provide evidence that the expression of Orc6 protein pushes cells into S-phase and that the phosphorylation of Orc6 delays replication progression, supporting the model that phosphorylation of Orc6 prevents DNA replication. Orc6 localizes within the nucleolus and ORC has previously been implicated in ribosome biogenesis, which occurs within the nucleolus.[24,25] We provide evidence that human Orc6 is required for ribosome biogenesis. Our results suggest that Orc6 is a multifunctional protein that regulates DNA replication and ribosome biogenesis, key cellular processes that must be accurately coordinated to ensure genome integrity.

Results

Human Orc6 is phosphorylated at T195 during mitosis

In yeast, cell-cycle-dependent phosphorylation of Orc6 plays an important role in preventing rereplication.[16] We performed Phos-Tag gel electrophoresis,[9,26] and identified two key phosphorylation sites on human Orc6. A subset of hOrc6 was found to be phosphorylated at T229 in response to treatment with okadaic acid (OA) (and in response to other oxidative DNA damaging methods),[9] while phosphorylation of T195 was found to be present with and without damage (Figure 1A and B). The pT195 antibody was generated in this study while pT229 antibody was used in the previous paper[9]. Please see Materials and Methods and Supplementary Table 1 for details. We transfected U2OS cells with various Orc6 constructs, including Flag-Orc6-WT (wild-type), T195A (phospho-dead), T195E (phospho-mimic), T229A, T229E or M2 (both T195 and T229 were mutated to Ala) and treated the cells with either DMSO or with okadaic acid. We recently reported that cells expressing Flag-Orc6-WT display four major bands (using phos-tag gel electrophoresis-unphosphorylated, * corresponding to Orc6 phosphorylated on both T195 and T229; *1 indicating T229 phosphorylation, *2 indicating T195 phosphorylation).[9] Note that T229 phosphorylation was only observed in cells treated with okadaic acid[9] (Figure 1A and B). Phosphorylation at T195 and T229 was verified by treating the cells with calf intestinal phosphatase (CIP). Further, treating the cells with increasing concentration of H_2O_2 to induce oxidative damage (like treatment with OA) showed robust phosphorylation of T229, and increased intensity of T195-Orc6 (Figure 1C). Using the T195 phospho-specific antibody, we confirmed that the *2 band represents phosphorylation at T195 (Figure 1D, please note that parts of this figure were reproduced from Figure 1A, Lin et al., to show phosphorylation at T229). Finally, the T195 phosphoantibody did not detect the phospho-dead (T195A) or phosphomimic (T195E)-expressing protein (Figure 1E). Moreover, CIP-treated samples confirmed the specificity of the antibody. We have previously shown that the endogenous Orc6 is phosphorylated at T229 in response to okadaic acid and H_2O_2 treatment.[9]

To evaluate the cell cycle dynamics of the T195 phosphorylation in an endogenous context, we performed cell cycle synchronization and collected cells at the G1, G1/S, S, G2, and M phases of the cell cycle. Flow cytometry was used to confirm the synchrony (Figure 2A). Using Phos-tag gel electrophoresis, we found that a phosphorylated form of endogenous Orc6 was predominant at G2 and mitosis, with almost all Orc6 being phosphorylated during mitosis (Figure 2A). We evaluated the T195 phosphorylation using a T195-phospho-specific antibody and found the onset of T195 phosphorylation during G2 and robust T195 phosphorylation during mitosis (Figure 2B). We further collected cells after nocodazole arrest and release and, based on visualizing the cells under a microscope, categorized them for enrichment at prometaphase, metaphase, anaphase, telophase and G1. Using Phos-tag gel electrophoresis, we found that Orc6-phosphorylation was maximal at prometaphase and metaphase and was rapidly dephosphorylated by the end of mitosis (Figure 2C). We confirmed that T195-phosphorylation of Orc6 was primarily found in prometaphase and then rapidly dephosphorylated by the end of mitosis (Figure 2D). The specificity of the phospho-antibody was confirmed by siRNA experiments (Figure 2E). We also confirmed the phosphorylation of Orc6 at T195 at mitosis by synchronizing cells with double thymidine, followed by releasing them into mitosis (Figure 2F). Finally, treatment of mitotic cells with a CDK1 inhibitor, RO-3306, showed loss of T195-phosphorylation of Orc6 (Figure 2G), confirming that this is a CDK-dependent phosphorylation.

T195-phosphorylated Orc6 localizes to the nucleolar-organizing regions

Using T195-specific antibody, we conducted immunofluorescence (IF) imaging studies in U2OS cells. We found a robust signal of the phospho-Orc6 in a small population of cells, containing chromosomes showing a "worm-like appearance" reminiscent of cells beginning to condense their chromatin prior to the nuclear envelope breakdown (Figure 3A). Detailed examination of T195p-Orc6 localization showed decoration within DAPI-less regions of the G2/M transitioning cells. Further, Orc6-antibody signals were reduced in cells treated with Orc6 siRNA, confirming the specificity of the localization of Orc6 (Figure 3B). The phospho-Orc6 was found to be colocalizing with a nucleolar marker, fibrillarin (Figure 3C).

The nucleolus is sub-compartmentalized into fibrillar center (FC), which is enriched for the unengaged rDNA transcriptional machinery, including RNA polymerase I.[27,28] The dense fibrillar component (DFC, marked by fibrillarin) surrounds the FC and is enriched for pre-RNA processing factors. The RNA Poll transcription occurs at the boundary of FC and DFC. The granular component (GC) is located at the peripheral region of the nucleolus where maturation and assembly into ribosomes occur. High-resolution imaging to address the distribution of T195-Orc6 in the nucleolus, along with immunolocalization of fibrillarin and RNA Pol I, showed that the phosphorylated Orc6 was enriched at the DFC ring (Figure 3D). The fibrillarin ring structures have been proposed to be sites for the interaction of rRNA, noncoding RNAs, and phospholipids.[29]

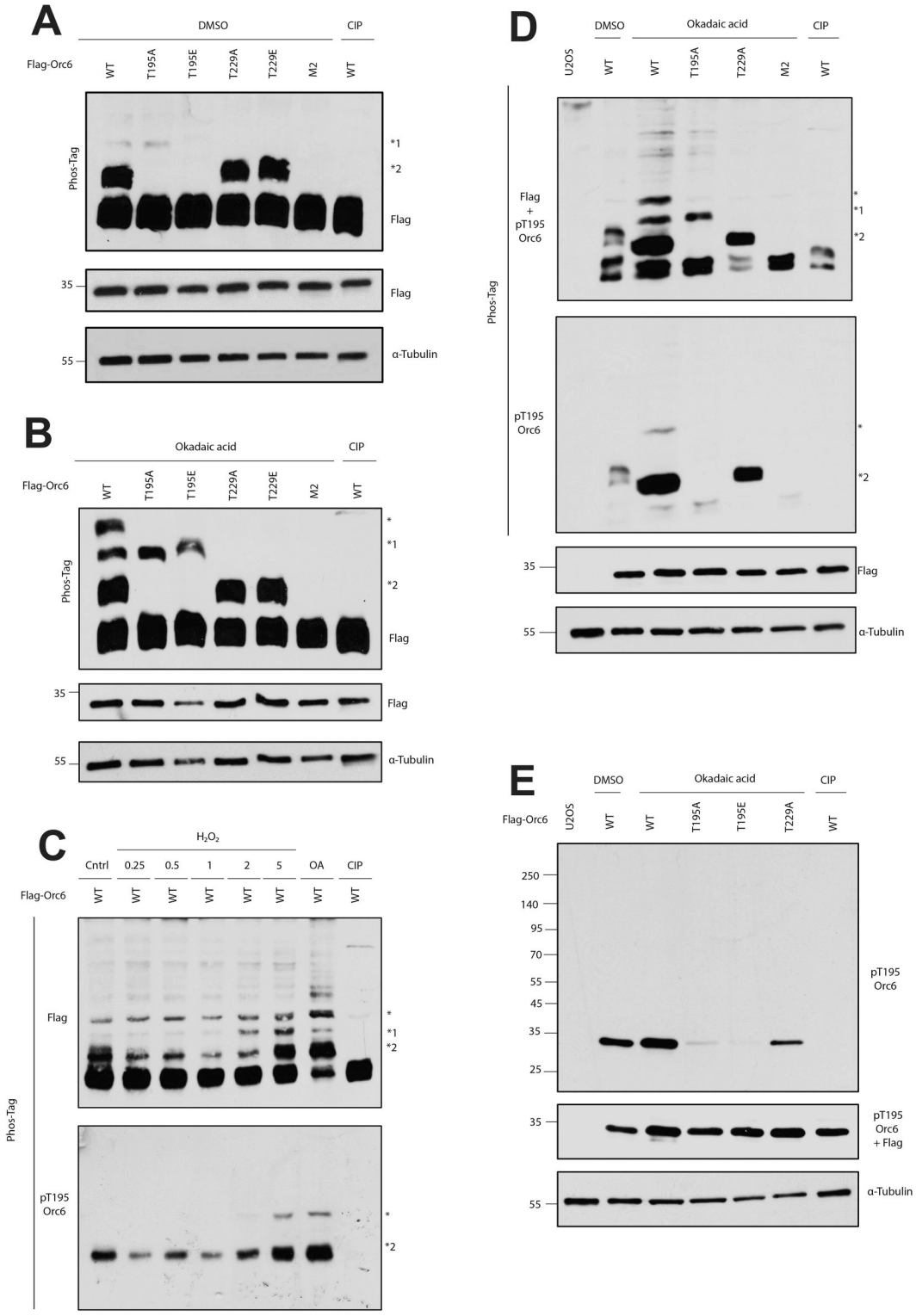

Figure 1. Phosphorylation of Orc6 at threonine 195 (T195) is enhanced upon DNA damage. (A) Phos-tag gel of Orc6 phosphorylation in DMSO-treated cells. (B) Phos-tag gel of Orc6 phosphorylation in okadaic acid-treated (OA) cells. (C) Phos-tag gel of Orc6 phosphorylation in H_2O_2-treated cells. (D) Validation of Orc6 T195 antibody specificity in Phos-tag gel. Please note that parts of this figure (FLAG antibody and alpha-tubulin) is used in Figure 1A of Lin et al.).[9] (E) Validation of Orc6 T195 antibody specificity in Western blot. *indicates Orc6 phosphorylated on both T195 and T229; *1 indicates T229 phosphorylation; *2 indicates T195 phosphorylation, M2 indicates both T195A and T229A mutation.

Finally, to confirm the localization of phospho-Orc6, we exogenously expressed YFP-Orc6, YFP-Orc6-T195A, and YFP-Orc6-T195E in U2OS cells. We also performed immunostaining with the T195-phospho-Orc6 antibody in these cells and found that the antibody did not recognize T195A or the T195E forms of Orc6 (Figure 4A), consistent with the Phos-tag experiments (Figure 1E). The YFP-Orc6 and the phospho-Orc6 antibody showed co-occupancy at the nucleolar regions in G2/M transitioning cells (Figure 4B). Furthermore, Orc6 was found to be present ubiquitously within the nucleolus as a diffused signal in interphase as well as in mitosis (Figure 4A and B). Orc6 has also been reported to be a component of

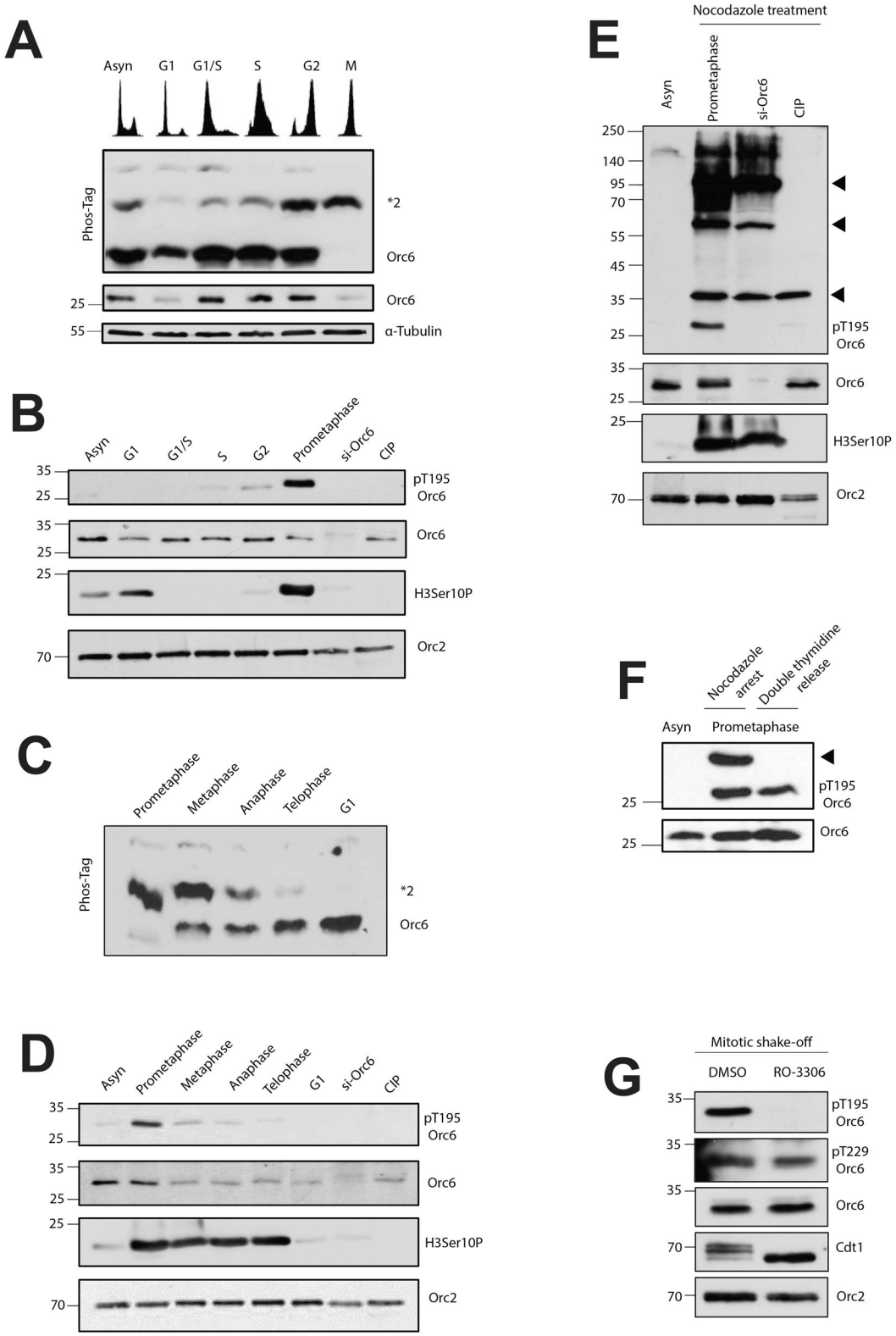

Figure 2. Orc6 is phosphorylated at T195 during mitosis. (A) Phos-tag gel of Orc6 phosphorylation in different cell cycle stages (B) Western blot showing Orc6 phosphorylation in different cell cycle stages (C) Phos-tag gel of Orc6 phosphorylation in different mitotic cell cycle stages. (D) Western blot analysis showing Orc6 phosphorylation at T195 in different mitotic cell cycle stages. (E) Western blot analysis showing Orc6 phosphorylation at T195 in nocodazole treated cells synchronized in prometaphase. (F) Western blot analysis of cells synchronized in prometaphase with both nocodazole and thymidine treatments show Orc6 phosphorylation at T195. (G) Loss of Orc6 T195 phosphorylation signal upon treatment with RO-3306, a CDK inhibitor in cells synchronized in mitosis. H3Ser10p serves as the marker for mitosis. Arrowhead indicates nonspecific bands. Cells were treated with calf intestinal phosphatase (CIP) and loss of phosphorylation-specific signal after CIP treatment confirms loss of phosphorylation.

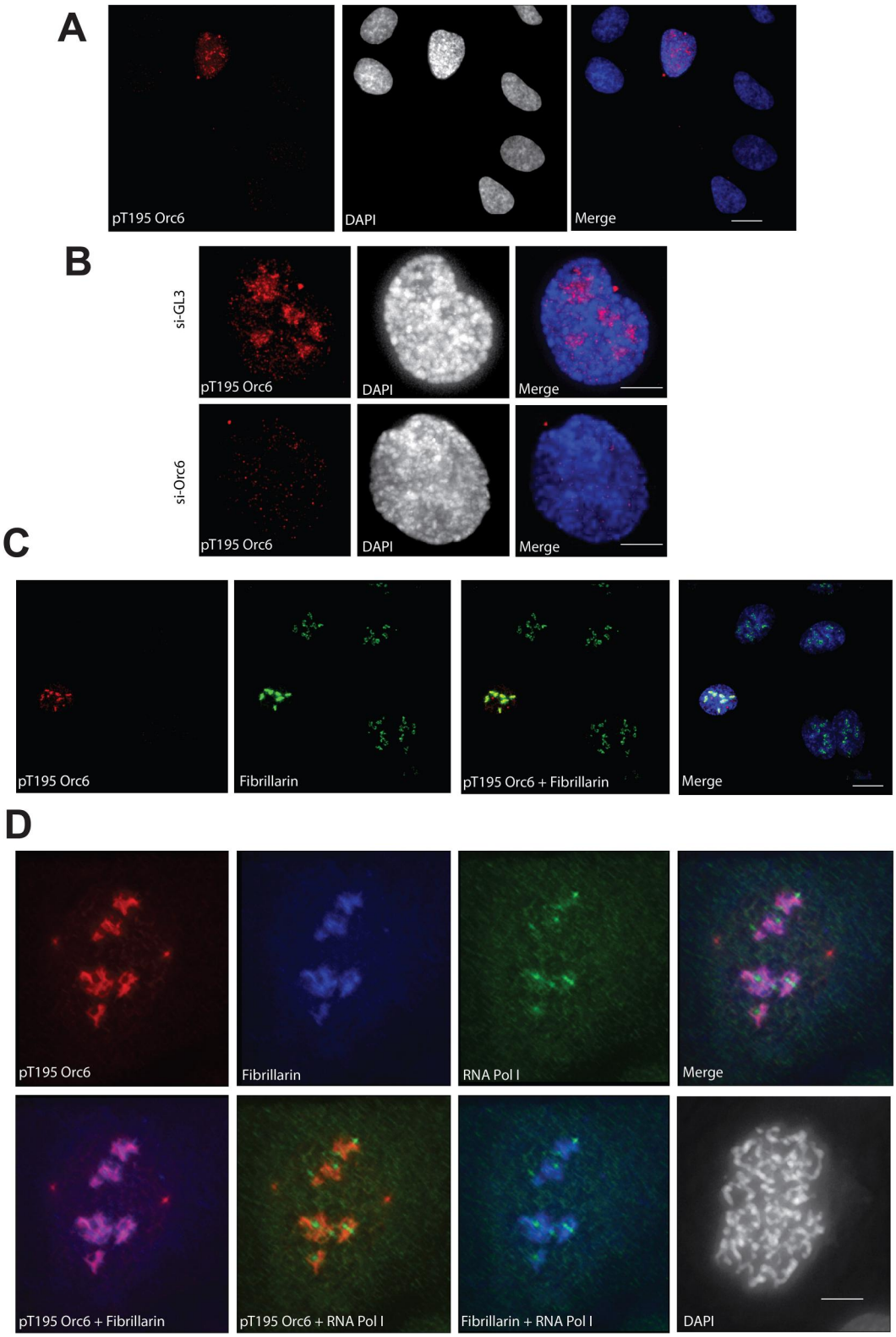

Figure 3. Orc6 is localized at the nucleolus. (A) Immunostaining showing Orc6 T195 phosphorylation localizes to the nucleus (scale bar = 10 μm). (B) Validation of Orc6 pT195 antibody specificity in siGL3 and Orc6-depleted U2OS cell line (scale bar = 5 μm). (C) Orc6 pT195 colocalizes with fibrillarin, a nucleolar marker (scale bar = 10 μm). (D) Immunostaining of Orc6 pT195, fibrillarin, and RNA Pol I in the U2OS cell line (scale bar = 5 μm).

the nucleolus and was identified as one of the ~1425 proteins enriched within the nucleolus by the Human Protein Atlas (https://www.proteinatlas.org/humanproteome/subcellular/nucleoli). It was also among the 470 proteins in the nucleoli, which were confirmed by experimental evidence.

Orc6 is needed for the maintenance of nucleolar structure and for ribosome biogenesis

The number of nucleoli remains relatively constant in each cell line. For example, in the hTERT-immortalized near-diploid retinal pigment epithelial RPE1 cells (hTERT-RPE1), their

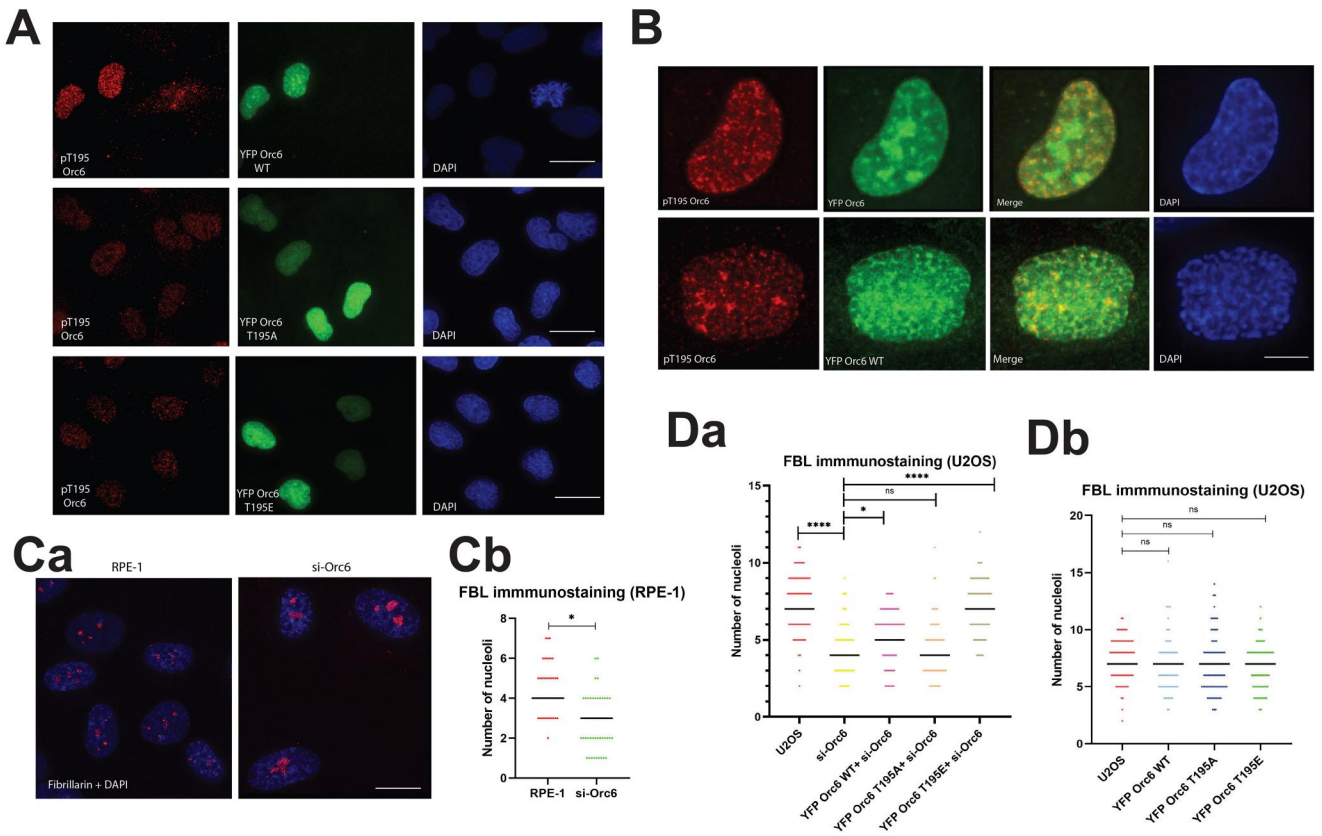

Figure 4. Depletion of Orc6 results in changes in nucleolar number. (A) Validation of Orc6 pT195 antibody in cells expressing YFP Orc6 WT/T195A/T195E (scale bar = 10 μm). (B) Immunostaining shows Orc6 pT195 in cells expressing YFP Orc6 WT in interphase (top) and mitosis (bottom) (scale bar = 5 μm). (Ca) Representative images of immunostaining of fibrillarin in wild-type and Orc6-depleted RPE-1 cell line (scale bar = 5 μm). (Cb) Quantification of nucleolar number in wild-type and Orc6-depleted RPE-1 cell line ($n = 50$). (Da) Quantification of nucleolar number in Orc6-depleted U2OS cells rescued with YFP Orc6 WT/T195A/T195E ($n = 100$). (Db) Quantification of nucleolar number in U2OS control and U2OS cells expressing YFP Orc6 WT/T195A/T195E ($n = 100$). Fibrillarin (FBL) is used as the nucleolar marker. ns $P > 0.05$,$^{*}P < .05$, $^{****}P < .0001$, by unpaired Student's two-tailed *t*-test.

number ranged from 3 to 5 per nucleus (Figure 4Ca and b). Similarly, in the transformed mammary epithelial MCF10a cells, the number ranged from 2 to 3,[30] and in the transformed osteosarcoma cells, U2OS, the number ranged from 6 to 8 per nucleus (Figure 4Da and b). Orc6-depleted cells (U2OS and hTERT-RPE-1) showed a significant decrease in the overall number of nucleoli (Figure 4C and D). Further, expression of the wild-type Orc6, as well as the phosphomimic (T195E), rescued the nucleolar number, nearly reaching the control levels (Figure 4Da). In contrast, overexpression of the WT, T195A, or T195E Orc6 in cells with intact endogenous Orc6 did not impact the nucleolar number (Figure 4Db). In this context, it is interesting to note that a recent work by the Baserga lab revealed that changes in nucleolar number are observed in the absence of regulators of ribosome biogenesis.[30]

We performed a series of experiments to address the role of Orc6 within the nucleolus and, specifically, assessed its involvement in ribosome biogenesis. We addressed whether Orc6 is associated with the rDNA promoter by performing ChIP in cells expressing HA-Orc6. Orc6 was enriched at the rDNA promoter, as observed by the primer set 41.9. However, no Orc6 enrichment was observed at the transcribed and intergenic spacer regions (primer sets H 0.9, H4, H8, and H27) (Figure 5A). To address the functional relevance of Orc6 at the rDNA locus, we tested whether Orc6 regulates rDNA transcription and/or if it has a role in rRNA processing. RNA Pol I transcription was monitored using the dual-luciferase reporter assay, as a way to quantitatively measure Pol I transcription.[31,32] We found a significant decrease in the rDNA transcription from the reporter locus in the absence of Orc6 (Figure 5B). Next, we performed rRNA FISH (using a 5′ETS probe, 980-1641) to assess the levels of nascent rRNA in the nucleolus in hTERT-RPE1 cells. As noted earlier, we found a reduction in the number of nucleoli but surprisingly the signal intensity of the 5′ETS/pixel seemed to increase (Figure 5C). It is possible that there is either a potential stabilization of the pre-rRNA despite reduced transcription or likely a defect in rRNA processing. Consistent with reduced transcription, we found a decrease in global cellular protein translation, as observed by the puromycin incorporation assay[33] (Figure 5D). A low dose of puromycin was fed to the cells, and Western blotting with an antipuromycin antibody was used to detect the level of protein synthesis in control and Orc6-depleted cells. Using two different siRNA oligonucleotides, it was evident that in the absence of Orc6, global cellular translation was impacted, further confirming its role in ribosome biogenesis (Figure 5D). Finally, we addressed whether Orc6 affects the processing of pre-rRNAs by performing a Northern blot analysis. We used three different probes, spanning the 5′ETS (5′ external transcribed spacer 2-414), ITS1 (internal transcribed spacer 1) and ITS2 (Figure 5E).

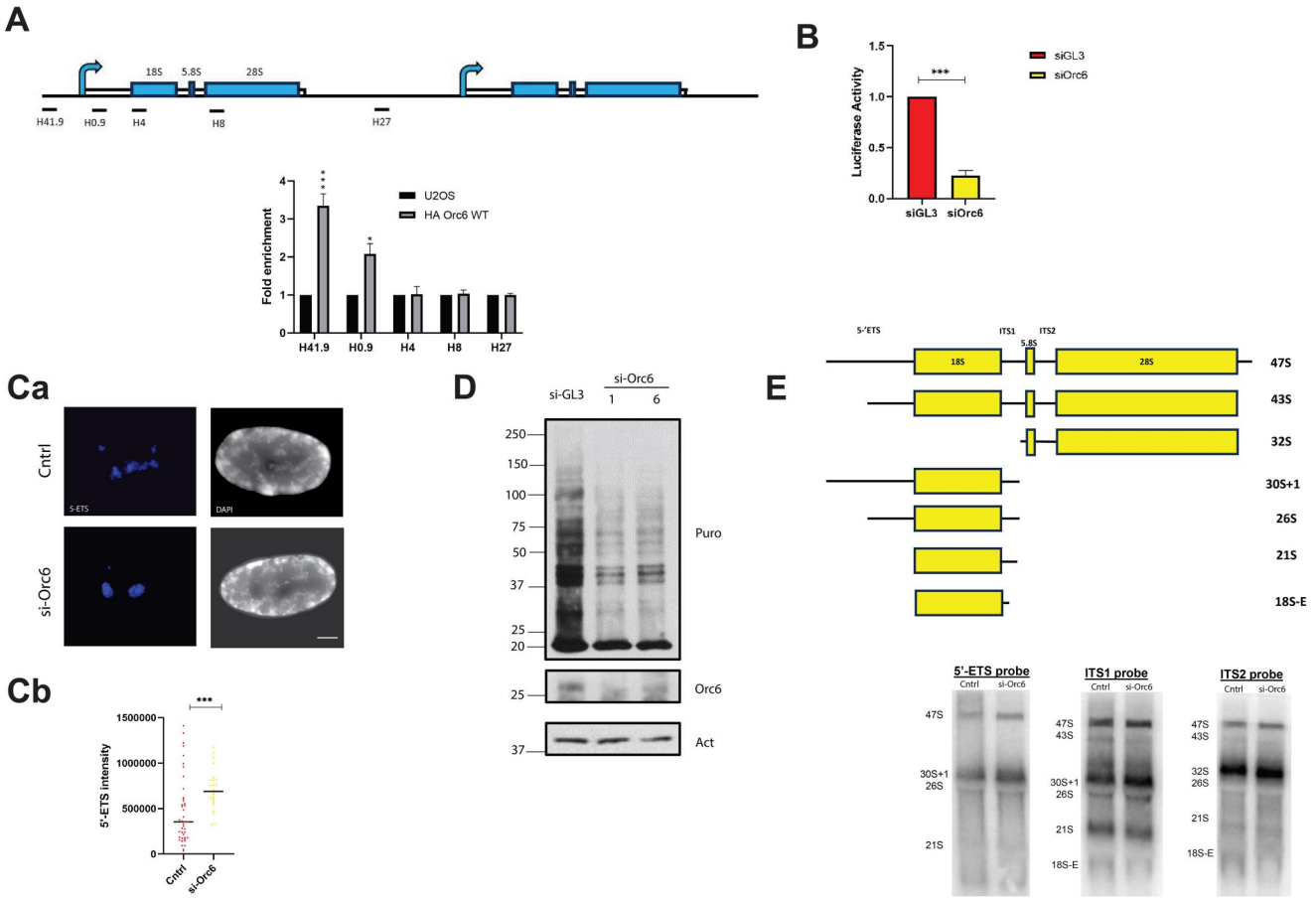

Figure 5. Orc6 is involved in ribosome biogenesis. (A) HA ChIP-qPCR analysis at rDNA promoter regions in U2OS cells expressing HA Orc6 WT. (B) Luciferase activity of siGL3 and Orc6-depleted U2OS cells. $*P < .05$, $***P < .001$, by unpaired Student's two-tailed *t* test. (Ca) Representative images of RNA FISH between control and Orc6-depleted U2OS cells with 5′ETS probe. (Cb) Quantification of 5′ETS signal intensity in control and Orc6-depleted U2OS cells. (D) Western blot analysis of puromycin incorporation assay in siGL3 and Orc6-depleted U2OS cells. (E) Northern blot analysis of control and Orc6-depleted U2OS cells with probes targeting multiple rDNA promoter regions.

The loss of Orc6 did not demonstrate any apparent defects in pre-rRNA processing (Figure 5E), although this cannot be completely ruled out based on the use of the three probes.

These results highlight an important role for Orc6 in ribosome biogenesis, likely at the level of rDNA transcription. It is also likely that Orc6 plays a role in rDNA copy number maintenance by regulating the replication of the rDNA locus. Future experiments would address such a hypothesis.

Phosphorylation of Orc6 at T195 inhibits DNA replication

Orc6 phosphorylation at T195 mostly occurs at the onset of mitosis, and therefore, we tested whether Orc6-T195 phosphorylation regulates mitotic entry or exit. Control and Orc6-KO U2OS cells (these are not complete knockouts, but are hypomorphic lines with significantly reduced total Orc6[8]) were synchronized in prometaphase using nocodazole and then released from this arrest and collected at various times points: prometaphase (0 h), entrance into G1 phase (released from nocodazole for 4 h), entrance into S phase (12 h), and exit out of S phase (16 h). Control and Orc6 KO (clone 3) cells all showed an expected PI flow profile for asynchronous cells, validating cell viability. Comparing both control (Figure 6Aa) and Orc6 KO (Figure 6Ab) cells demonstrated that all cells (Control, cells expressing WT, T195A and T195E Orc6) were arrested in prometaphase efficiently upon nocodazole treatment (as observed at 0 h). It also appeared that cells transfected with wild-type Orc6, Orc6-T195A, and Orc6-T195E mutants in both U2OS and Orc6 KO cells were all able to successfully exit mitosis and enter G1 phase (see 4 h). Strikingly, even Orc6 KO cells that were not transfected with Orc6 (column labeled "control") were able to exit mitosis and enter G1 phase, suggesting that low levels of Orc6 in the U2OS cells are sufficient for the mitotic roles of Orc6 (Figure 6Ab).

Looking at 12 h across in cells not transfected with any plasmid, the Orc6 KO could not enter the S phase as efficiently compared to control U2OS cells, as seen by the significant decrease in the S phase cell population. In contrast, the U2OS cells could enter the S phase efficiently (column labeled "control" in Figures 6Aa and 6Ab). This finding suggests that the Orc6 is essential for cells to progress into the S phase. In Orc6 KO cells (Figure 6Ab), the addition of wild-type Orc6 into the Orc6 KO cells greatly restored the ability of the cells to enter the S-phase. The expression of WT-Orc6 in control, as well as the Orc6 KO, seemed to push cells into S-phase. Orc6 KO cell, transfected with T195A also rescued the ability to enter the S-phase, showing an increase of cells in the S phase. A similar result was observed in Figure 6Aa, in which U2OS control cells transfected with wild-type Orc6

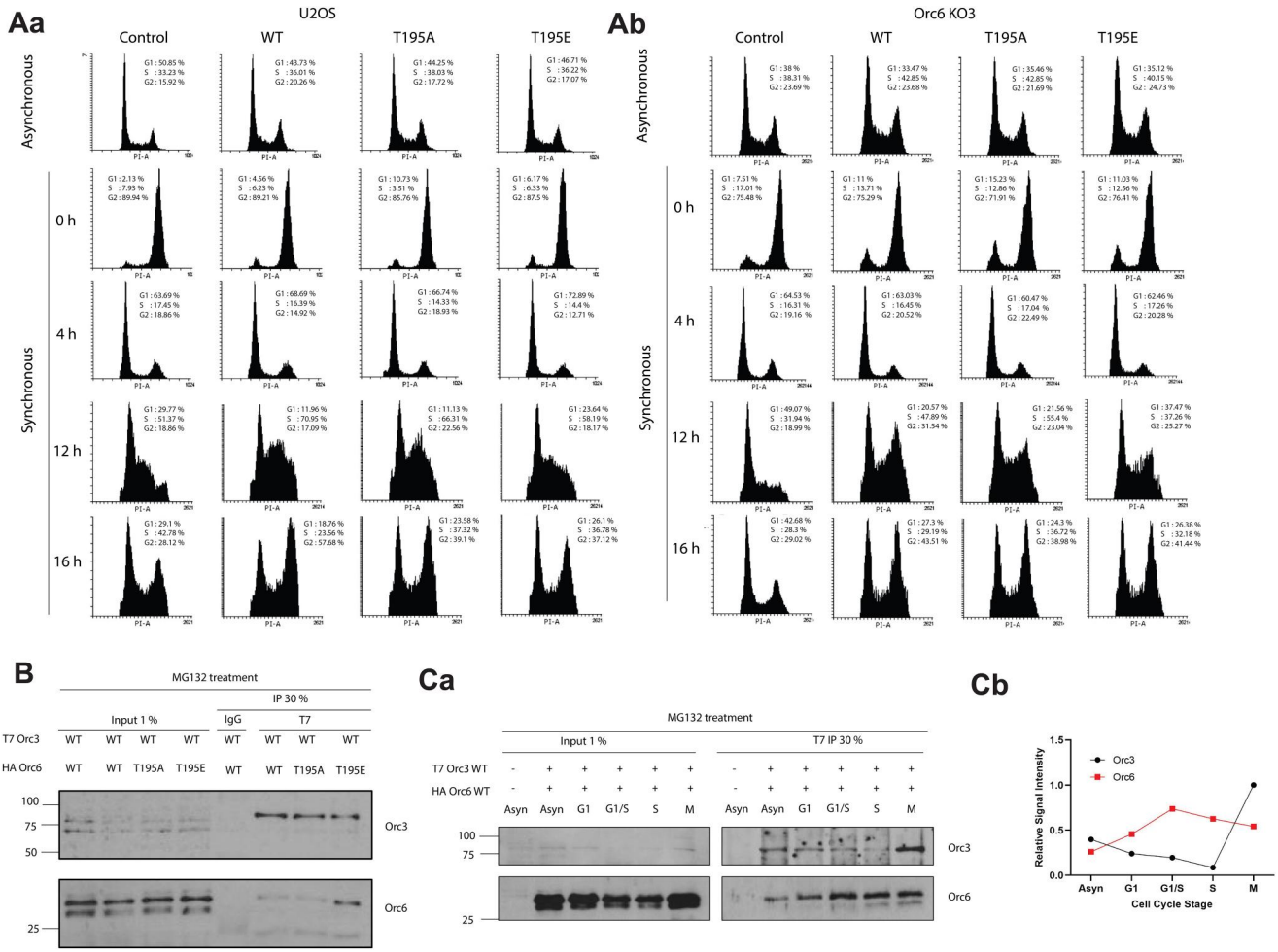

Figure 6. Phosphorylation of Orc6 T195 impacts its association with ORC and cell cycle progression. (A) Representative image of cell cycle profile between control (Aa) and Orc6 knockout (KO3, Ab) U2OS cells expressing wild-type (WT-Orc6), phospho-dead (Orc6-T195) or phosphomimetic (Orc6-T195E) mutants of Orc6. (B) Immunoprecipitation of U2OS cells expressing T7 Orc3 WT and HA Orc6 WT/T195A/T195E. (Ca) Immunoprecipitation of U2OS cells expressing T7 Orc3 WT and HA Orc6 WT in different stages of the cell cycle. (Cb) Relative signal intensity between Orc3 and Orc6 in different cell cycle stages.

or Orc6 T195A promoted cell entry into the S phase, as observed by the increased number of S phase cells. Interestingly, Orc6 KO cells transfected with T195E was not able to rescue the phenotype of the Orc6 KO's inability to enter S phase as efficiently as did WT-Orc6 or Orc6-T195A. This is also observed in U2OS cells transfected with Orc6-T195E, which shows a lower number of cells in the S phase than wild-type or T195A (Figure 6Aa). It is also important to note that cells expressing WT-Orc6 show an increased ability to enter DNA replication, consistent with our results that Orc6 is required for S-phase.[9] The results for entry into S phase suggest that phosphorylation of Orc6 may prevent entry and efficient progression into S phase. At the same time, unphosphorylated Orc6 facilitates entry into the S phase. Notably, it appears that once cells entered the S phase, different Orc6 mutants (both the T195A and T195E) did not affect the ability to proceed with DNA replication. Our results suggest that phosphorylation of Orc6 might be a way to ensure that replication is inhibited outside of the S-phase. However, for the re-establishment of pre-RC, Orc6 dephosphorylation must occur during mitosis.

Finally, the 16-h Orc6 KO histogram showed that most cells still stayed in G1 and did not enter S and/or did not reach G2 without Orc6 (as observed by the significantly smaller G2/M peak). In addition, the wild-type, T195E, and T195A transfected Orc6-KO cells appeared to be able to finish the S phase successfully, as seen in the presence of a large G2/M cell population in the 16-h row (Figure 6Ab). In U2OS, cells transfected with wild-type, T195E- or T195A- Orc6 showed overall similar profiles as in KO cells but with higher S and G2, reflecting more cells entering S phase at the earlier point.

Due to the above observations showing that T195E-Orc6, the phosphomimetic Orc6, slowed cell entry into the S-phase, we further explored the mechanism behind this observation. To test the mechanism by which T195E-Orc6 prevents entry into S-phase, we tested the ability of Orc6 to associate with ORC, specifically Orc3, since it is known to bind to Orc3 directly. Cotransfection of Orc3 and Orc6 (WT, 195 A or 195E), followed by immunoprecipitation of Orc3, demonstrated the most robust association of Orc3 with the phosphorylated Orc6 (Figure 6B). This suggested to us that the binding of Orc6 to Orc3 might be a mechanism to prevent replication licensing. Further, cell synchronization experiments showed that Orc6 associates with ORC most robustly outside of G1, supporting the model that the binding of Orc6 to ORC may

prevent licensing (Figure 6C). Future work will determine how the overexpression of Orc6 rapidly increases S-phase progression. Does Orc6 enable increased origin firing, or does the increased rRNA expression lead to rapid S-phase progression? These questions remain.

Discussion

The smallest subunit of ORC, Orc6 in human cells, is emerging to be a multifunctional protein, governing DNA replication, cytokinesis, and DNA damage response. The human Orc6 is an abundant protein with a vast pool of Orc6 that is not in a complex with the rest of the ORC. There is growing evidence indicating that it has a limited role in replication licensing yet plays a crucial role in replication progression and mismatch repair during S-phase.[8] We have previously reported that the ATR-mediated phosphorylation of Orc6 at T229 is critical for fork pausing at damaged sites.[9] Prior work has also pointed to the CDK-mediated phosphorylation of Orc6 at T195 as being implicated in the nuclear localization of Orc6, which is independent of the rest of the ORC.[23]

In the current study, we demonstrate the intricate role of human Orc6 in the regulation of DNA replication, and how posttranslational modification of Orc6 during the cell cycle regulates the process of replication control. Orc6 undergoes dynamic changes in phosphorylation and interaction with ORC throughout the cell cycle, playing a crucial role in coordinating DNA replication with cell cycle progression. Orc6 appears to serve as a complementary and balancing factor to the core ORC components. While ORC is essential for the initiation of DNA replication, its dynamic interactions and posttranslational modifications modulate ORC activity to ensure precise control and coordination of replication timing. Specifically, Orc6's phosphorylation at threonine 195 (T195) enhances its interaction with ORC during mitosis, thereby regulating ORC loading onto chromatin and preventing premature licensing. Conversely, dephosphorylation of Orc6 in the subsequent G1 phase reduces its interaction with ORC, allowing efficient licensing of replication origins. In vitro reconstitution experiments suggest that hOrc6 is not needed for Mcm2-7 loading, but it likely has a role in late stages of licensing, perhaps in loading a second Mcm2-7 hexamer.[34,35] The exact role of hOrc6 remains an intense area of study. Nevertheless, the complementary regulation ensures that ORC activity is finely tuned and temporally coordinated with the cell cycle progression, highlighting the critical role of Orc6 in balancing and modulating ORC function.

During mitosis, Orc6 undergoes phosphorylation at threonine 195 (T195), with levels of phosphorylation reaching maximum during prometaphase, and then gradually decreasing thereafter. We observe that the phosphomimic mutant of Orc6, T195E, associates more strongly with the core ORC component, Orc3, suggesting that phosphorylation of Orc6 at T195 enhances its interaction with ORC. As cells transition from mitosis onto the subsequent G1 phase, Orc6 phosphorylation diminishes, and its interaction with ORC decreases. As commonly understood, the initiation of replication is inhibited outside of G1, which is facilitated collectively by the inhibition of pre-RC formation and through preventing ORC binding to chromatin. In addition, posttranslational modifications such as phosphorylation or ubiquitination of key components can lead to their inactivation or degradation, thus preventing the formation of functional pre-RCs. At the end of mitosis or beginning of G1, components of pre-RC, like Orc1, are dephosphorylated, enabling pre-RCs to be reassembled at origins.

Recent reports have suggested that ubiquitination-mediated degradation of Orc6 from chromatin is important for preventing MCM reloading.[36] We propose that the phosphorylation of Orc6 and its enhanced interaction with ORC during mitosis constitute an inherent mechanism for cells to inhibit premature ORC loading onto chromatin, thereby preventing premature licensing. Conversely, following prometaphase and in the subsequent G1 phase, dephosphorylation of Orc6 and its decreased binding to ORC allows efficient licensing of replication origins. This observation is consistent with a previous report from our laboratory, showing that Orc6 is dispensable for licensing origins as its depletion does not affect the loading of MCMs during G1.[8] As the cell cycle progresses, and following licensing of origins as observed in post-G1 cells, we see an increased association of Orc6 with ORC. This observation supports previous findings of Orc6 having a critical role in S-phase progression as Orc6-depleted cells were unable to efficiently progress through S-phase even with proper licensing.[8] We speculate that Orc6 phosphorylation and dephosphorylation may function in regulating the licensing of replication origins, highlighting the importance of Orc6 in maintaining proper timing and fidelity of genome duplication (Figure 7A).

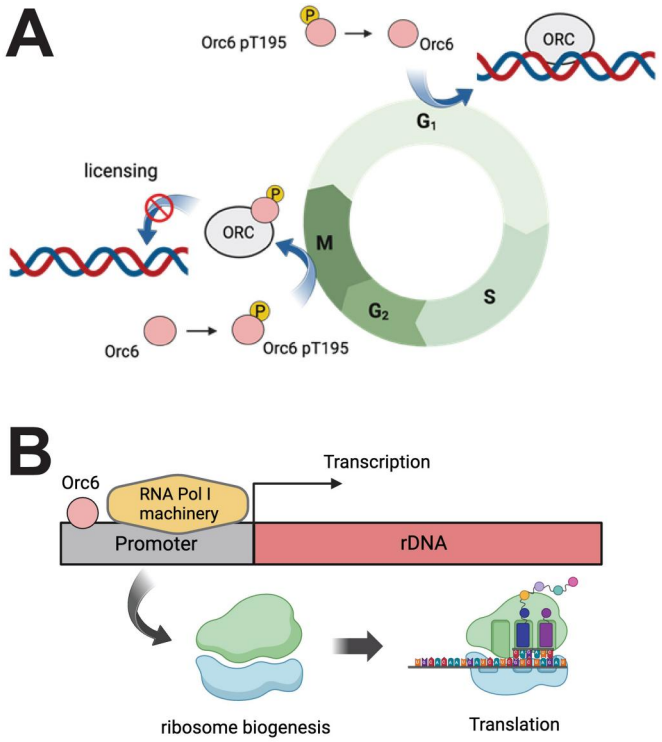

Figure 7. Schematic representation of (A) the role of Orc6 phosphorylation at T195 during mitosis and our model on how it impacts ORC function, (B) the role of Orc6 in ribosome biogenesis. (Created with BioRender).

The findings presented in this study show that Orc6 is localized at the nucleolus, an observation which aligns with that in *Drosophila*.[37] The Chesnokov lab has previously found that the *Drosophila* Orc6 is localized in the nucleolus of the salivary gland cells. The yeast Orc6 has also been found to associate with nucleolar proteins, such as RRB1 and YPH1, suggesting a link between DNA replication and ribosome biogenesis.[24,38] Most interestingly, the human Orc6 (and some other subunits of ORC) were also reported to localize to the nucleolus along with its interacting partner HMGa1,[39] indicating a potential role of ORC with factors that control ribosome biogenesis. We report that Orc6 binds to the rDNA promoter and influences rDNA transcription (Figure 7B), as evidenced by ChIP and loss of luciferase activity in the absence of Orc6, respectively. Furthermore, the reduction in translation efficiency, as observed on the puromycin assay post Orc6 depletion, implicates Orc6 in modulating ribosome function or protein synthesis. It is possible that the function of Orc6 in the nucleolus is to act as a mediator between proteins involved in ribosome biogenesis and rDNA transcription regulation. In an alternative model, Orc6 may occupy the rDNA promoter region, thereby impeding the binding of transcription repressors and potentially facilitating transcriptional activation. Notably, BEND3,[40] a transcription repressor, involved in rDNA silencing was initially identified by our laboratory as an ORC-interactor.

Our observations in the study also indicate that phosphorylated Orc6 at T195 localizes in the nucleolus, preferably in early mitotic cells. Considering that transcription by RNA Pol I exhibits oscillatory patterns throughout the cell cycle and is typically repressed during mitosis,[41] it is plausible that Orc6 phosphorylation at T195 plays a role in orchestrating transcription repression during this phase. This suggests a potential mechanism where Orc6 phosphorylation modulates transcription activity: repressing transcription while phosphorylated but enhancing transcription upon dephosphorylation.

The yeast Orc1 has been shown to protect rDNA repeats from double-strand breaks during meiosis.[42] Also, tetrahymena ORC is reported to localize to rDNA and non-rDNA replication origins for replication initiation.[43] Our prior work has shown that Orc6 is required for DNA damage response. The nucleolus harbors rDNA repeats that are known to be subject to replication stress. Therefore, it is possible that Orc6 is required to maintain genome stability at the rDNA loci. Further experiments aimed at elucidating the molecular mechanisms underlying the observed phenotypes will enhance our understanding of the functional significance of Orc6 in these pathways.

Material and methods

Cell culture

U2OS cell lines are grown in Dulbecco's modified Eagle's medium (DMEM) containing high glucose, 5% fetal bovine serum (FBS), and 1% penicillin and streptomycin. U2OS cell lines stably expressing HA-Orc6 WT/HA-Orc6 T195A/HA-Orc6 T195E are maintained in DMEM and selected by puromycin. For cell synchronization, nocodazole arrest (50 ng/mL) was performed for M and G1 phase samples, and double thymidine block (2 mM) was done for G1/S, S, and G2 phase samples.[9] HTERT-RPE1 15/3 cell line is a gift from Brian McStay laboratory.[44] HTERT-RPE1 wild-type and HTERT-RPE1 15/3 cells were grown in DMEM/F12 medium supplemented with 10% FBS.

Antibody generation

Polyclonal pT195 and pT229 Orc6 antibodies were produced by Abgent, a Wuxi AppTec company. One phosphopeptide was used to immunize two New Zealand rabbits for polyclonal antibody production and purification. The produced antisera was affinity purified using the immunizing peptide.

Orc6 pT195 peptide: VDREPGDVA pThr PPRKRKKIVVEAC

Orc6 pT229 peptide: HKPQKDEDL pThr QDYEEWKRKC

Whole-cell lysis

Cells were collected, washed with PBS once and lysed with buffer containing 80 mM Tris pH6.9, 2% SDS, 15% glycerol, 0.1 M DTT on ice for 15 min. Loading dye was added to the protein samples prior to protein denaturation at 95 °C for 5 min. Samples were then analyzed by Western blotting. Phos-tag analysis was performed as described in.[26]

Immunoprecipitation

U2OS cells were seeded at $3*10^6$ cells in 10 cm plate. Following 20 h, 3 μg of T7-Orc3 and 1 μg of HA Orc6 plasmid were transfected to U2OS cells. Lipofectamine 2000 or Lipofectamine 3000 (Thermo Fisher Scientific) were used for plasmid DNA delivery. After 48 h incubation, cells were collected, washed with PBS and lysed with lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, 0.2% NP-40) containing protease inhibitors. Lysed cells were sonicated and treated with benzonase nuclease (Sigma) for 30 min at room temperature. EDTA was added at a concentration of 2 mM, followed by sample centrifugation at 15000 rpm for 10 min to remove insoluble debris. Protein lysates were pre-cleared with Gammabind G Sepharose (GE Healthcare Life Science) for 1 h at 4 °C. Antibodies were added to the lysates and incubated at 4 °C overnight. Proteins bound by the antibodies were pulled down by Gammabind G Sepharose at 4 °C for 3 h. Beads were washed with lysis buffer and captured proteins were eluted for Western blot analysis. Three biological replicates were performed for the experiment.

Flow cytometry

Cells were collected, washed once with ice cold PBS, and resuspended in PBS + 1% normal goat serum (NGS) before sample fixation with 90% chilled ethanol at 4 °C overnight. The next day, cells were washed and resuspended in PBS + 1% NGS containing 24 μg/mL propidium iodide (PI) and 10 μg/mL RNase A for 45 min at 37 °C. DNA content was

measured by flow cytometry and the data was analyzed with FCS Express 5 software. Three biological replicates were performed for the experiment.

Immunostaining

U2OS cells were seeded at 1×10^6 cells per well in 6-well plate. Twenty-four and 48 h later, first and second rounds of Orc6 siRNA (final concentration 40 nM) were delivered to U2OS cells using Lipofectamine RNAimax (Thermo Fisher Scientific). Five hundred nanograms of HA-Orc6 WT/T195A/T195E plasmid were transfected to cell 5 h prior to the first round of Orc6 knockdown. Five hundred nanograms of HA-Orc6 WT/T195A/T195E was transfected to cell 5 h prior to first round of Orc6 knockdown. After 48-h incubation following the second knockdown, cells were fixed with 2% PFA for 15 min at room temperature and 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 at room temperature for 1 h. Next, cells were washed with PBS containing 1% NGS and incubated with fluorophore-conjugated secondary antibodies at room temperature for 1 h. Cells were then washed with PBS prior to staining with DAPI. Three biological replicates were performed for the experiment. Significance was calculated by unpaired Student's *t* test using Graphpad prism, https://www.graphpad.com/w.

Northern blot

Northern blot analysis was done as previously described.[45] RNA was extracted with TRIzol reagent (Invitrogen) and DNA was removed using DNAse I (Qiagen, Cat# 74104). Then, 2 mg of total RNA extracted from U2OS control and siORC6 cells was separated on 1% denature agarose gel prepared with NorthernMax Denaturing Gel Buffer (Ambion) and run in NorthernMax Running Buffer (Ambion) at 80 V for 2 h. RNA was then transferred to Amersham Hybond-N + blot (GE Healthcare) by capillary transfer in 10 × SSC for 48 h and crosslinked to the blot by UV (254 nm, 120mJ/cm^2). The DNA probes were labeled with [a-32P] dCTP by Prime-It II Random Primer Labeling Kit (Stratagene) as per manufacturer's instructions. Hybridization was carried out using ULTRAhyb Hybridization Buffer (Ambion) containing 1×10^6 cpm/mL of denatured radiolabeled probes targeting 5′ETS, ITS-1, or ITS-2 region, respectively at 42 °C overnight. Blots were then washed and developed using phosphor-imager.

Three biological replicates were performed for the experiment.

RNA-Fluorescence in situ hybridization (FISH)

The smFISH was done as previously described.[45] 5′ETS tag probe sequence was previously described.[44] Oligonucleotide probes were hybridized with cells for overnight at 37 °C. Posthybridization washes were done with 2 × SSC at 42 °C three times, 5 min each. Cells were then mounted with VectaShield. Three biological replicates were performed for the experiment.

Imaging acquisition and analyses

smFISH images were taken using Axioimager.Z1 microscope (Zeiss) equipped with 63X/1.4 NA oil immersion objective and Zeiss Axiocam 506 mono camera with a z-interval of 0.24 mm. For the measurement of pre-rRNA signal in U2OS control and si-Orc6 cells, z-stacks were imported into Fiji/ImageJ and maximum intensity projection was performed. 5′ETS signal was segmented separately by optimized threshold and inverted into binary masks. The integrated density of 5′ETS signal in each cell was measured. Significance was calculated by unpaired Student's *t* test using Graphpad prism, https://www.graphpad.com/w

Puromycin incorporation assay

siGl3-treated and Orc6-depleted U2OS cells were generated as previously mentioned in "immunostaining" protocol. Final concentration of 10 μg/mL of puromycin was added to U2OS siGl3-treated and Orc6-depleted cells. After incubation for 10 min, cells were collected and processed as mentioned in "Whole-cell lysate preparation". Samples were analyzed by SDS pages with each respective primary and secondary antibodies. Three biological replicates were performed for the experiment.

Dual luciferase assay

siGl3-treated and Orc6-depleted U2OS cells were generated as previously mentioned in "immunostaining" protocol. Twenty hours prior to cell collection, 500 ng of firefly luciferase plasmid, containing human RNAP1 promoter (Addgene, plasmid #194250), and 50 ng of Renilla luciferase plasmids were transfected in U2OS siGl3-treated and Orc6-depleted cells. The luciferase activity of both firefly and Renilla luciferase were measured with dual-luciferase Reporter Assay System (Promega, E1910). The ratio between firefly and Renilla luciferase activity were calculated to measure the rDNA transcription activity. Three biological replicates were performed for the experiment. Significance were calculated by unpaired Student's *t* test using GraphPad prism, https://www.graphpad.com/w.

Details of the antibodies and their dilution, siRNA oligonucleotides, and plasmids are included in Supplementary Table S1.

Contacts for reagent and resource sharing

Requests for reagents and published resources should be directed to Supriya G. Prasanth (supriyap@illinois.edu).

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Author contributions

A.C., F.K., H.Z.O., M.L., M.K.A., R.P., D.C., Y-C.L., designed, performed, and analyzed most experiments. G.O., helped with statistics of the microscopy data; S.G.P. and K.V.P. supervised the project. F.K., H.Z.O., and S.G.P. wrote the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability statement

Data sharing not applicable – no new data generated.

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