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Orc1 Controls Centriole and Centrosome Copy Number in Human Cells

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Abstract

Centrosomes, each containing a pair of centrioles, organize microtubules in animal cells, particularly during mitosis. DNA and centrosomes are normally duplicated once prior to cell division to maintain optimal genome integrity. We report a new role for the Orc1 protein, a subunit of the Origin Recognition Complex (ORC) that is a key component of the DNA replication licensing machinery in controlling centriole and centrosome copy number in human cells, independent of its role in DNA replication. Cyclin A promotes Orc1 localization to centrosomes where Orc1 prevents Cyclin E-dependent re-duplication of both centrioles and centrosomes in a single cell division cycle. The data suggest that Orc1 is a regulator of centriole and centrosome re-duplication as well as the initiation of DNA replication.

The assembly of a bipolar, microtubule spindle during mitosis is essential for accurate chromosome segregation. In animal cells, spindle formation is organized by centrosomes, organelles that contain of a pair of centrioles surrounded by pericentriolar material (PCM) that need to be duplicated exactly once every cell division cycle, in coordination with DNA replication to maintain genome stability (1). Licensing DNA for replication is a critical regulatory step involving the Origin Recognition Complex (ORC), the first component for assembly of a pre-Replicative-Complex (pre-RC) at each origin (2). Accumulated evidence supports roles for ORC subunits in addition to licensing DNA replication (3). In particular, human Orc2 subunit localizes to centrosomes and depletion of Orc2 and Orc3 causes centrosome amplification in mitosis (4).

Several regulators of the DNA licensing machinery have been reported to be involved in the control of both DNA and centriole duplication (5). Both cyclin E and cyclin A, as well as Cdk2 activity are well known positive regulators of DNA replication and also promote centrosome duplication (or re-duplication) (6–11). Depletion of the DNA replication licensing inhibitor Geminin causes re-duplication of both DNA and centrosomes in human cells (12).

In a screen using short interfering RNA (siRNA) for human ORC proteins with roles in centrosome biology, we found that depletion of the largest human ORC subunit, HsOrc1, leads to excess centrosomes (fig. S1, Fig. 1A). Orc1 siRNA-treated U2OS cells were analyzed for centrosome defects by dual-color indirect immunofluorescence (IF) using anti-centrin 2 (stains centrioles) and anti--tubulin (stains centrosomes) antibodies. 72 hr post siRNA, 39.77 ± 3.5% of cells transfected with Orc1-1 siRNA and 25.53 ± 0.3% of cells transfected with Orc1-2

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siRNA showed multiple centrosomes and centrioles, in comparison to 2.4 ± 1.2% of control cells (fig. S1C). In Orc1-1 treated cells 33.29 ± 2.6% of the multiple centrosomes were separated rather than linked and the re-duplication occurred in different human cell lines (fig. S1 and S2).

To study the kinetics of centriole and centrosome re-duplication after Orc1 depletion, U2OS cells were synchronized in mitosis by nocodazole treatment and released into the next cycle. Centriole and centrosome numbers were assessed 12 hr, 36 hr and 60 hr after nocodazole release (Fig. 1B, fig. S3). Disorganized disengagement of duplicated centrioles (2Pairs - Dis.) was observed 12 hr after release when most of the cells were in G1 or early S phase (Fig. 1B, fig S3; centrin 2). This was followed by an increase in the percentage of cells showing centriole re-duplication (>2P). The complete centrosome structure, as evidenced by -tubulin staining, was observed later, mostly 60 hr after release (-tubulin). The very early effect of Orc1 depletion on centriole disengagement and re-duplication suggests that Orc1 might have a more direct effect on these processes, whereas complete centrosome formation follows centriole re-duplication. Similar dynamics of centriole re-duplication after Orc1 depletion were observed in HeLa cells (fig. S4). It has been proposed that centriole engagement blocks re-duplication (13). In Orc1 depleted cells, however, the duplicated centrioles were found disengaged and underwent re-duplication, suggesting that Orc1 may block re-duplication by participating in processes of centriole engagement of the newly duplicated centrioles.

Different mechanisms for centrosome amplification have already been reported. Apparent multiple centrosomes can arise by centrosome fragmentation, centriole splitting that occurs during early mitosis after DNA damage (14) or authentic re-duplication. Analysis of multiple parameters including DNA content (fig. S1B), cell cycle markers (fig. S5), centriolin staining of mature centrosomes (fig. S6 and S7) and the numbers of centrioles per PCM (Fig. 1A) all indicate that Orc1 depletion causes *bona fide* centriole and centrosome re-duplication in U2OS cells (see supplemental text).

To address whether over-expression of Orc1 would affect centrosome re-duplication caused by prolonged S-phase arrest in the presence of hydroxyurea (HU) (9,15), U2OS cells were transiently transfected with YFP-Orc1^{WT}, YFP-Orc2 or a vector carrying only YFP in the presence of HU (Fig. 2A and D; fig. S8). Centrosome re-duplication after HU treatment, but not normal duplication, was substantially inhibited in YFP-Orc1^{WT} cells, whereas YFP or YFP-Orc2 did not have any effect, suggesting a role for Orc1 as an inhibitor of centrosome re-duplication.

YFP-Orc1 associated with centrosomes in addition to its nuclear localization in approximately 35% of transfected cells (fig. S9A and Video 1). Furthermore, endogenous Orc1 and other ORC subunits co-fractionated with purified centrosomes (fig. S9B). Transiently expressed Orc1 fused to the PACT domain, a conserved motif that targets proteins to centrosomes (16), resulted in inhibition of centrosome re-duplication in HU-treated U2OS cells (Fig. 2B and D), suggesting that Orc1 controls centrosome copy number when located at centrosomes.

Centrosome re-duplication was significantly inhibited by the full length and N terminal region of Orc1 (aa 1 to 500; Orc1^{WT}. Nterm. Flag; Fig. 2C), the latter of which is deficient ORC assembly (17). Both Orc1 versions also inhibited centrosome re-duplication when expressed after the application of the HU block, indicating that the inhibition was not caused by a G1 arrest in these cells (fig. S10). Thus Orc1 control of centrosome copy-number is separate from its DNA replication role, does not require assembly of into ORC, but such assembly may facilitate a more efficient block.

Orc1 depletion in synchronized U2OS cells did not trigger early responses of DNA damage checkpoint pathways that are known to affect centriole and centrosome copy number (18,19).

Activation of Chk1 by phosphorylation on serine 317 was not detected and p27 and the p53 pathways were activated well after centriole amplification was initiated (Fig. 1B, fig. S11).

Several reports have shown that Cyclin E over-expression in mammalian cells is linked to genome and centrosome abnormalities (20,21) and that centrosome re-duplication requires Cdk2 activity (7–9,11). Elevated levels of Cyclin E were identified as early as 12hr after release of synchronized Orc1-depleted U2OS cells in Cyclin A negative cells (Fig. 3A and 3B, also see fig. S5A and S5B) and continued to increase. We thus hypothesized that Orc1 control of centriole and centrosome re-duplication involved Cyclin E and Cdk2. The Cdk2 inhibitor roscovitine suppressed centriole and centrosome re-duplication in Orc1 depleted cells (fig. S12). In a second experiment, simultaneous depletion of Orc1 and Cyclin E blocked centriole and centrosome re-duplication, whereas normal duplicated centriole and centrosome numbers were observed (Fig. 3C, fig. S13A). Cyclin E siRNA did not change the number of cells with a 4C DNA content after Orc1-1 treatment (fig. S13B), implying that the block of centrosome over-duplication in Orc1-1 depleted cells by Cyclin E siRNA was not due to a compete G1 arrest. In contrast, Cyclin A siRNA did not abolish centrosome re-duplication in Orc1 depleted cells (Fig. 3C).

We tested the ability of elevated levels of cyclins to cause centrosome re-duplication in HU treated cells expressing YFP. $Orc1^{WT}$ (Fig. 4A). Co-expression of Cyclin E with YFP. $Orc1^{WT}$ blocked YFP. $Orc1^{WT}$ inhibition of HU-induced centrosome re-duplication, whereas neither Cyclin A nor Cyclin B had any effect (Fig. 4A and B). HU treated cells co-expressing YFP. $Orc1^{WT}$ with Cyclin A showed a cytoplasmic pool of over-expressed YFP. $Orc1^{WT}$ in addition to the nuclear localization and an enhancement of YFP-Orc1 located at centrosomes (Fig. 4B, b–b; the number of YFP-Orc1 positive centrosomes in the presence of Cyclin A increased six-fold compared to YFP-Orc1 alone). Co-expression of Cyclin E with YFP. $Orc1^{WT}$. PACT also eliminated the ability of YFP. $Orc1^{WT}$. PACT to block centrosome re-duplication, whereas Cyclin A or Cyclin B had no effect (Fig. 4A and B). Altogether, the results suggest that Orc1 might directly regulate centriole and centrosome copy number in cells, and the mechanism involves Cyclin E.

Orc1 co-precipitated with Cyclin E and Cyclin A, as previously shown (22), whereas no association was observed with Cyclin B (Fig. 4C). Association in a complex with Cyclin E was stronger than with Cyclin A; however, so far we have not been able to find evidence that Orc1 binds Cyclin E directly. Nevertheless, purified wild type Orc1 inhibited Cyclin E-Cdk2 and Cyclin A-Cdk2 kinase activity using histone H1 as substrate (fig. S14B).

The Orc1 ‘RXL’ mutant (K235A-L237A, $Orc1^{A\text{-}A}$) impairs the direct interaction with Cyclin A, but does not affect Cyclin E binding (Fig. 4C; fig. S14A) and cannot block Cyclin A associated kinase activity (fig. S14B). Although some inhibition was observed, $Orc1^{A\text{-}A}$ and $Orc1^{A\text{-}A.}$ Nterm did not block centrosome re-duplication as efficiently as wild type Orc1, suggesting that Orc1-Cyclin A interaction is required for optimal centrosome copy number control (Fig. 4D). $Orc1^{A\text{-}A}$. PACT blocked centrosome re-duplication to the same extent as $Orc1^{WT}$. PACT (Fig. 4E). Since Cyclin A function has been previously linked to the cytoplasmic localization of Orc1 (23), possibly $Orc1^{A\text{-}A}$ and $Orc1^{A\text{-}A.}$ Nterm mutants were not exported efficiently to centrosomes to block HU-induced re-duplication, whereas $Orc1^{A\text{-}A}$. PACT was directly targeted to centrosomes. Accordingly, Cyclin A stimulation of Orc1 association with centrosomes was significantly reduced when co-expressed with the ‘RXL’ $Orc1^{A\text{-}A}$ mutant . Altogether, the data indicates that both Cyclin E and Cyclin A play a role in Orc1 mediated control of centrosome numbers.

ORC in human cells is dynamically assembled and disassembled during the cell cycle and participates in many aspects of chromosome duplication and segregation, including functions

at kinetochores, centromeres and heterochromatin (3). Orc2 is localized to centrosomes, but its depletion affects centrosome copy number in mitosis in addition to controlling DNA replication, centromere activity and chromosome structure (4). In contrast, Orc1 controls Cyclin E-dependent centrosome copy number by directly preventing re-duplication of centrioles immediately after centrioles duplicate upon commitment to cell division. Without Orc1, duplicated centrioles are disengaged and extensive Cyclin E-dependent centriole re-duplication occurs. Note that in the absence of Orc1 and Cyclin E, centrioles duplicate, but they do not re-duplicate, suggesting that such centriole duplication is Cyclin A dependent, consistent with previous studies (24). But in the absence of Cyclin E and Orc1, centrosome re-duplication does not occur. Similarly, Cyclin E, but not Cyclin A over-rides the Orc1 inhibition of centrosome-re-duplication.

Cyclin E is required for centrosome duplication and re-duplication and co-precipitates with Orc1 (7,11,25–27). We have not been able to detect direct binding between Cyclin E and Orc1, suggesting that a mediator protein may facilitate the binding. The mediator may be another DNA replication protein implicated in centrosome biology (12,28). Furthermore, depletion of Orc1 increases Cyclin E protein levels in cells that may contribute to centriole re-duplication. Both proteins are degraded by SCF, which localizes to centrosomes and has also been implicated in control of centrosome duplication (29,30).

There is a short time during the cell division cycle after centrioles have duplicated when Cyclin E is still present in cells (late G1 and early S phase). During this time, if Cyclin E activity is not checked, centriole re-duplication might occur. We suggest that a mechanism involving Cyclin A-dependent localization of Orc1 to centrosomes that prevents Cyclin E-dependent centriole re-duplication during this time window (fig. S15). In this manner, Orc1 plays a dual role in coordinating DNA replication and centrosome copy number.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References and Notes

1. Nigg EA. Trends in Cell Biology 2007;17:215. [PubMed: 17383880]
2. Blow JJ, Dutta A. Nature Reviews Molecular Cell Biology 2005;6:476.
3. Sasaki T, Gilbert DM. Current Opinion in Cell Biology 2007;19:337. [PubMed: 17466500]
4. Prasanth SG, Prasanth KV, Siddiqui K, Spector DL, Stillman B. Embo Journal 2004;23:2651. [PubMed: 15215892]
5. Bettencourt-Dias M, Glover DM. Nature Reviews Molecular Cell Biology 2007;8:451.
6. Coverley D, Laman H, Laskey RA. Nature Cell Biology 2002;4:523.
7. Lacey KR, Jackson PK, Stearns T. Proceedings of the National Academy of Sciences of the United States of America 96 1999:2817.
8. Matsumoto Y, Hayashi K, Nishida E. Current Biology 1999;9:429. [PubMed: 10226033]
9. Meraldi P, Lukas J, Fry AM, Bartek J, Nigg EA. Nature Cell Biology 1999;1:88.
10. De Boer L, et al. Oncogene 2008;27:4261. [PubMed: 18372919]
11. Hinchcliffe EH, Li C, Thompson EA, Sluder MJLG. Science 1999;283:851. [PubMed: 9933170]
12. Tachibana KEK, Gonzalez MA, Guarguaglini G, Nigg EA, Laskey RA. Embo Reports 2005;6:1052. [PubMed: 16179947]
13. Tsou MFB, Stearns T. Current Opinion in Cell Biology 2006;18:74. [PubMed: 16361091]
14. Hut HMJ, et al. Molecular Biology of the Cell 2003;14:1993. [PubMed: 12802070]
15. Wong C, Stearns T. Nature Cell Biology 2003;5:539.
16. Gillingham AK, Munro S. Embo Reports 2000;1:524. [PubMed: 11263498]

17. Siddiqui K, Stillman B. Journal of Biological Chemistry 2007;282:32370. [PubMed: 17716973]
18. Bourke E, et al. Embo Reports 2007;8:603. [PubMed: 17468739]
19. Sugihara E, et al. Cancer Res 2006;66:4020. [PubMed: 16618721]
20. Spruck CH, Won KA, Reed SI. Nature 1999;401:297. [PubMed: 10499591]
21. Saavedra HI, et al. Cancer Cell 2003;3:333. [PubMed: 12726860]
22. Mendez J, et al. Molecular Cell 2002;9:481. [PubMed: 11931757]
23. Laman H, Peters G, Jones N. Experimental Cell Research 2001;271:230. [PubMed: 11716535]
24. Hanashiro K, Kanai M, Geng Y, Sicinski P, Fukasawa K. Oncogene 2008;27:5288. [PubMed: 18490919]
25. Tsou MFB, Stearns T. Nature 2006;442:947. [PubMed: 16862117]
26. Matsumoto Y, Maller JL. Science 2004;306:885. [PubMed: 15514162]
27. Duensing A, et al. Oncogene 2006;25:2943. [PubMed: 16331279]
28. Ferguson RL, Maller JL. J Cell Sci 2008;121:3224. [PubMed: 18799789]
29. Freed E, et al. Genes & Development 1999;13:2242. [PubMed: 10485847]
30. Nakayama K, et al. Embo Journal 2000;19:2069. [PubMed: 10790373]
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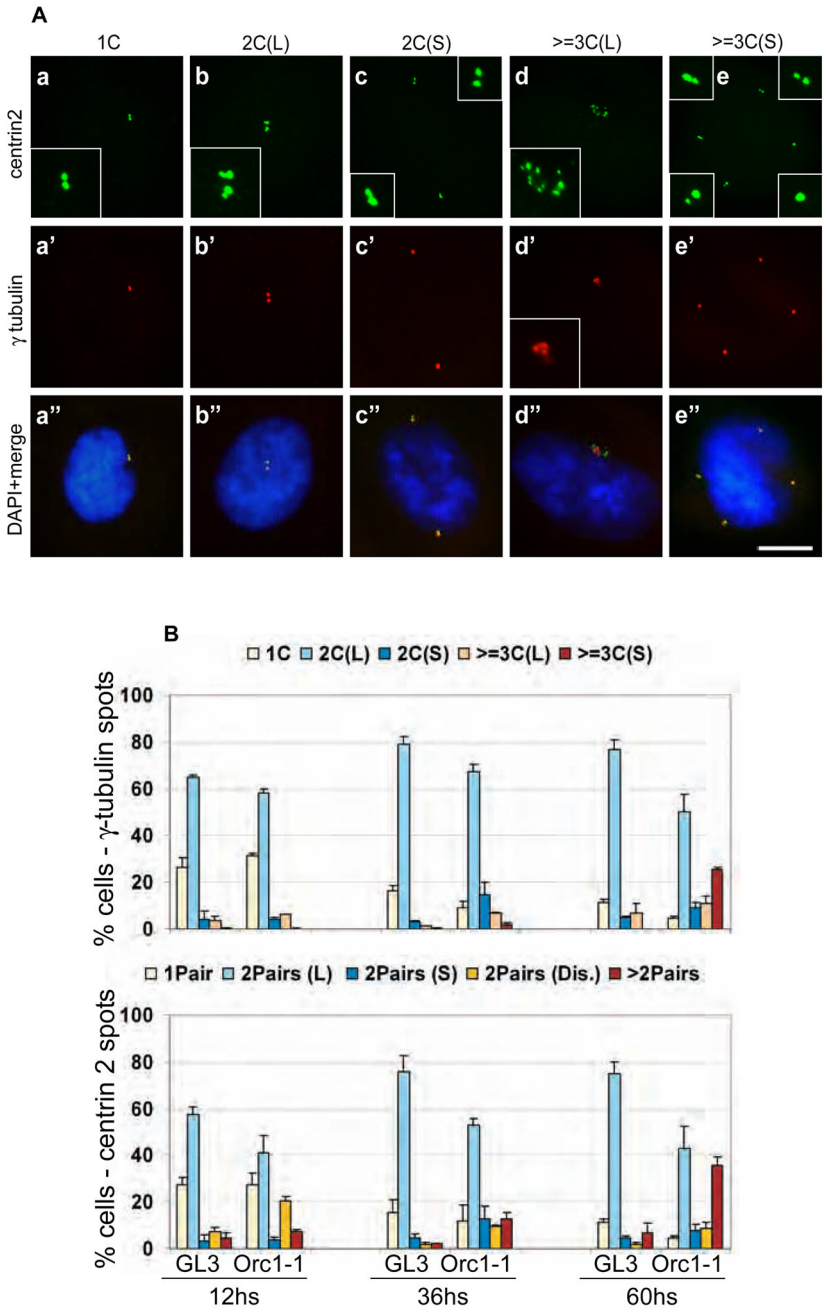

Fig. 1. Orc1 depletion causes centriole and centrosome re-duplication

(A) Human U2OS cells treated for 72 hours with Orc1-1 siRNA duplex were co-immunostained for centrioles with anti-centrin 2 (green, a to e) and centrosomes with anti--tubulin (red, a to e) antibodies. Insets are higher magnification. DNA was stained with DAPI (blue) and merged images are shown in a to e. Scale bar, 10 m. **(B)** Quantification of centrosome and centriole numbers by -tubulin (upper panel) and centrin 2 (Bottom panel) immunostaining, respectively. Cells were harvested at 12, 36 or 60 hours after nocodazole release. Error bars represent one standard error.

1C, one centrosome; 2C(L), two centrosomes linked; 2C(S), two centrosomes separated; >=3C (L), three or more centrosomes linked; >=3C(S), three or more centrosomes separated. 1Pair, one centriole pair; 2 Pairs (L), two centriole pairs linked; 2 Pairs (S), two centriole pairs

separated; 2 Pairs (Dis.), two centriole pairs with disorganized disengaged; >=2 Pairs, more than two centriole pairs.

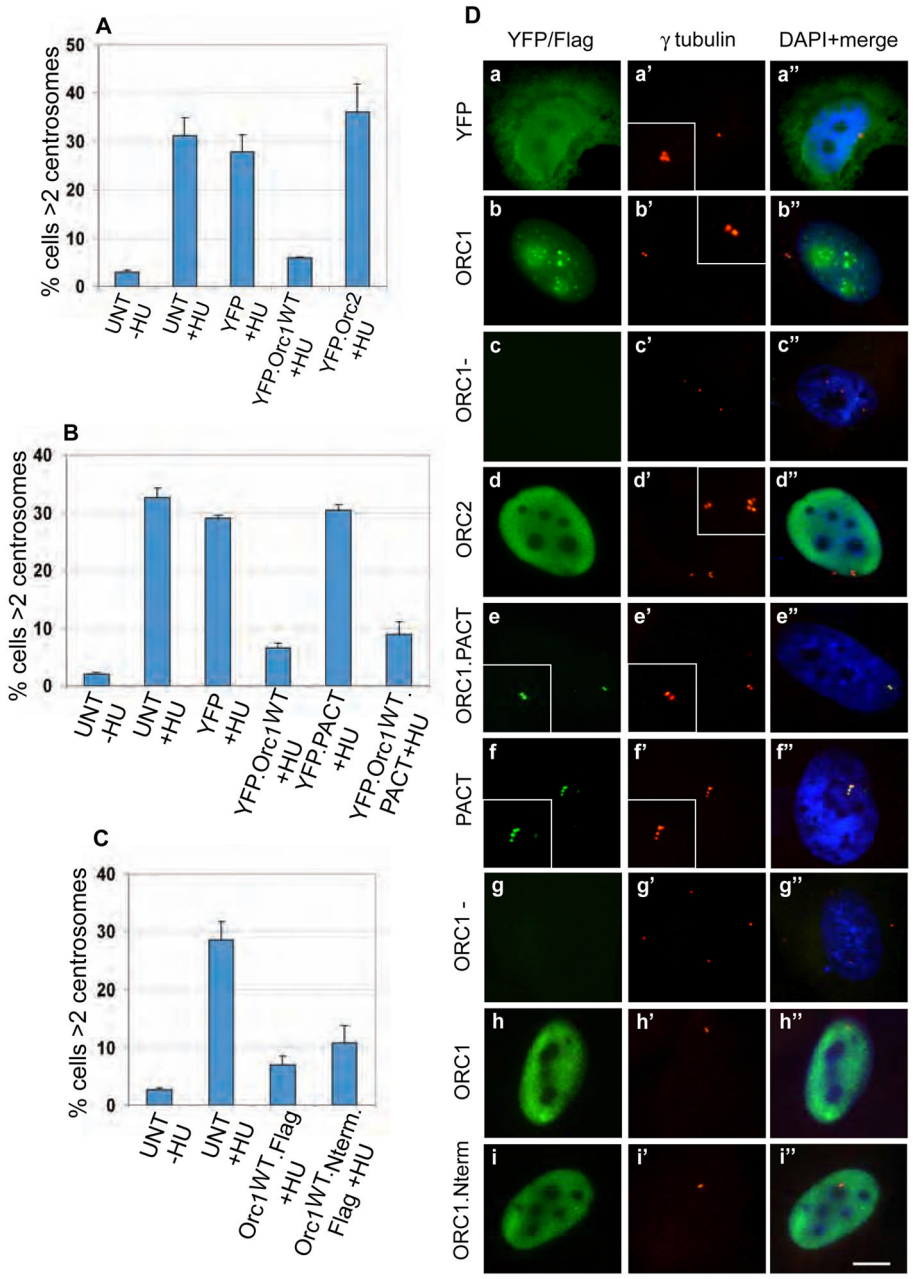

Fig. 2. Over-expression of Orc1 blocks centrosome re-duplication

U2OS asynchronous cells were transfected (or not, UNT) with the indicated constructs (see text) and treated with 16mM hydroxyurea (+ or HU). Cells were harvested at 68 hours after HU-treatment. Centrosome numbers were scored by -tubulin immunostaining in YFP or Flag positive cells. **(A–C)** Quantification of multiple centrosomes in HU-arrested cells. **(D)** Cells were immunostained for centrosomes with anti -tubulin (red, a to i); YFP expression was immunostained with anti-GFP in (A) (green, a to d) or visualized directly in (B) (e, f); and Flag expression was immunostained with anti-Flag in (C) (g to i). DNA was stained with DAPI (blue) and merged images are shown in a to i. Scale bar, 10 m. Insets are higher magnification.

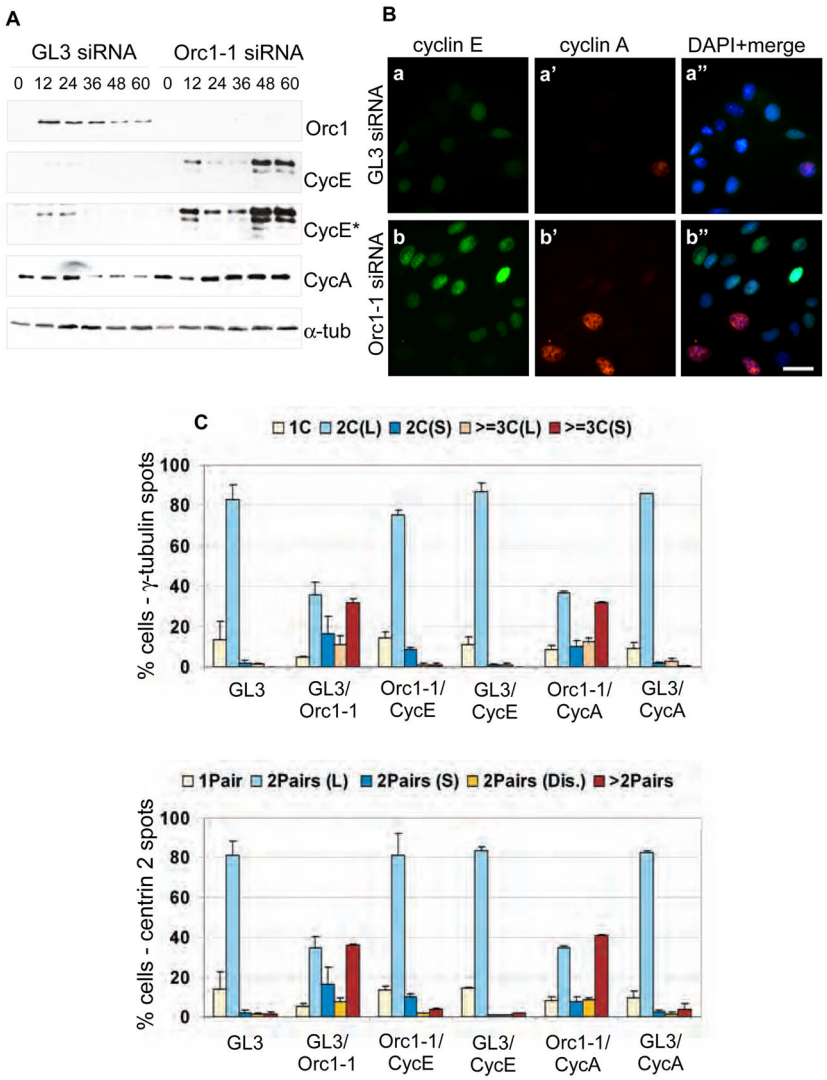

Fig. 3. Orc1 depletion causes Cdk2 and Cyclin E-dependent centriole and centrosome re-duplication

(A) and (B) Nocodazole arrested U2OS cells were transfected with control (GL3) or Orc1-1 siRNA, then released into the next cycle and re-transfected with the same siRNA duplexes. **(A)** Immunoblot of whole-cell extract of cells harvested at the indicated time points. Orc1, Cyclin E, Cyclin A and -tubulin levels were assessed by immunoblotting with specific antibodies. Cyclin E*, a longer exposure. **(B)** Cells were harvested at 12 hours after nocodazole release and co-immunostained with anti-Cyclin E (green) and anti-Cyclin A (red). DNA was stained with DAPI (blue) and merged images are shown in a and b. Scale bar, 30 m. **(C)** Quantification of centrosome and centriole numbers by -tubulin and centrin 2 immunostaining, respectively, of asynchronous U2OS cells transfected with the indicated siRNAs. Cells were analyzed 72 hours after the first transfection. Error bars represent one standard error.

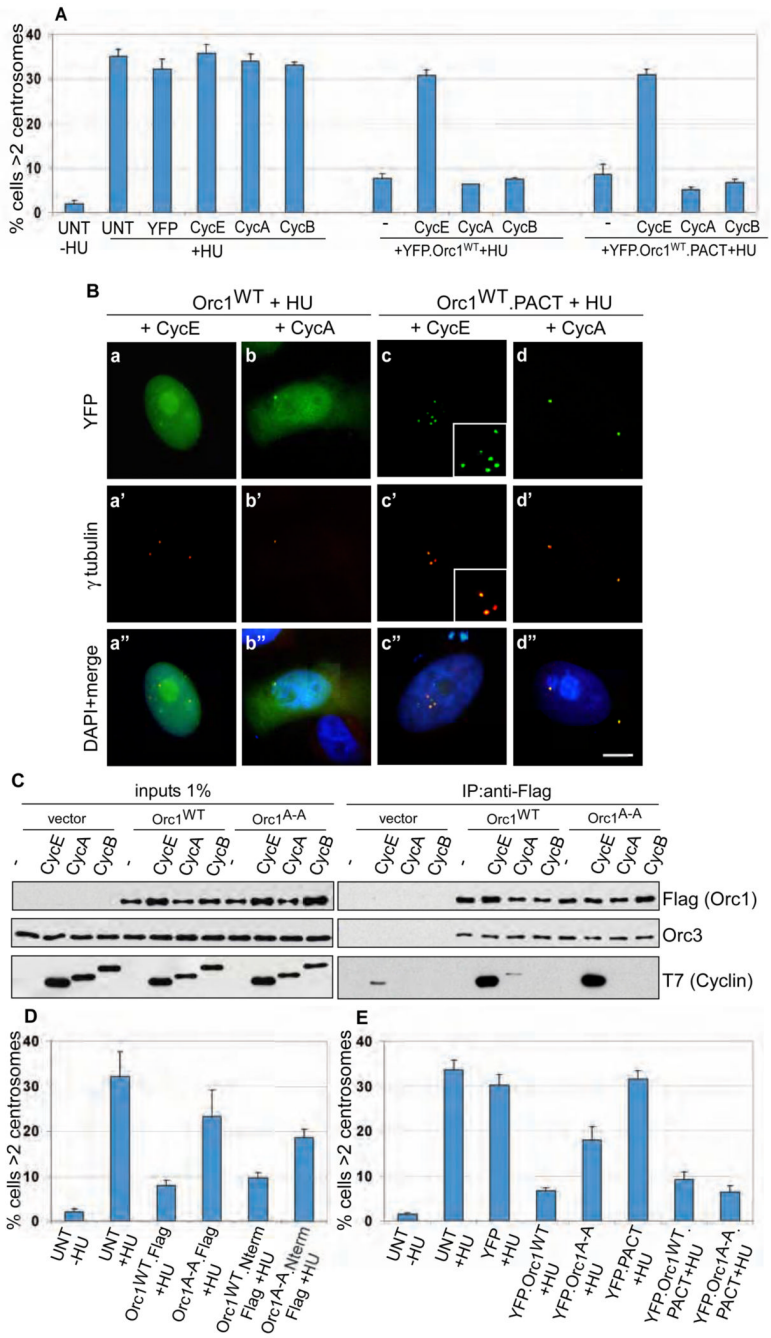

Fig. 4. Cyclin E suppresses Orc1 inhibition of centrosome re-duplication in S-phase-arrested cells

In A, B, C and D: U2OS asynchronous cells were transfected with the indicated constructs (see text) and treated with 16mM hydroxyurea (+HU). Untransfected cells were treated or not with HU (UNT +HU and UNT HU, respectively). Cells were harvested at 68 hours after transfection and HU-treatment. **(A)** Quantification of multiple centrosomes in transfected HU-arrested cells. Centrosome numbers were scored by -tubulin immunostaining in YFP positive cells. Error bars represent one standard error. **(B)** Transfected, HU-treated cells were immunostained for centrosomes with anti -tubulin (red, a to d) and YFP expression was visualized directly (green, a to d). DNA was stained with DAPI (blue) and merged images are shown in a to d. Scale bar, 10 m. **(C)** Immunoprecipitation with anti-Flag antibody from whole-cell extract from

HEK293 cells transiently co-expressing the indicated constructs and immunoblotting with the indicated antibodies. Vector, full-length Orc1^{WT}. Flag (wild type) or full-length Orc1^{A-A}. Flag (mutant) were transiently transfected alone (); or with either T7-Cyclin E, T7-Cyclin A or T7-Cyclin Right panel: 10% of the immunoprecipitates. **(D)** and **(E)** Quantification of multiple centrosomes in transfected HU-arrested cells. Centrosome numbers were scored by -tubulin immunostaining in Flag positive cells in (D), and by -tubulin immunostaining in YFP positive cells directly visualized in (E). Error bars represent one standard error.