Supporting Online Material

Orc1 Controls Centriole and Centrosome Copy Number in Human Cells

Adriana S. Hemerly,[1,2] Supriya G. Prasanth,[1,3] Khalid Siddiqui,[1,4] Bruce Stillman [1,5]

[1] Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor 11724, NY, USA
[2] Instituto de Bioquímica Médica, UFRJ, 21941-590, Rio de Janeiro, Brazil

Footnotes
[3] (present address) Department of Cell and Developmental Biology, 601 S. Goodwin Avenue, University of Illinois at Urbana-Champaign, IL 61801 USA
[4] (present address) Clare Hall Laboratories, Cancer Research UK, South Mimms, Herts., EN6 3LD, UK

[5] To whom correspondence should be addressed. E-mail: stillman@cshl.edu

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Materials and Methods

Cell culture and drugs
U2OS osteosarcoma, HeLa adenocarcinoma and HCT116 $p53^{+/+}$ colorectal human cancer cells were grown as monolayers in complete Dulbecco's modified Eagle's medium (DMEM – Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). To synchronize U2OS cells at mitosis, 100ng/ml Nocodazole (Sigma, St Louis, CA) was added to the medium for 16 hr. Subsequently, cells were released from the block by washing twice in PBS and re-plating in fresh medium. Cdk2 activity was inhibited in U2OS cells by treatment with 10mM roscovitine (Sigma, St Louis, CA) for 24hr. In siRNA treated cells, roscovitine was added at 48 hr after the first transfection. DMSO was used as the vehicle control.

Plasmids
The human Orc1 and Orc2 cDNAs were cloned into pEYFP-C1 and pEYFP-C3 with CMV promoter (CLONTECH Laboratories Inc.), respectively, generating the plasmids $YFP.Orc1^{WT}$ (Prasanth et al., to be submitted) and YFP.Orc2 (*1*). To generate PACT fusions, the C terminus of AKAP450 (PACT domain), amino acids 3643-3808, was amplified as described in (*2*) and cloned into pEYFP-C1 using the restriction sites SalI(5') and BamHI(3') generating the plasmid YFP.PACT. The plasmid $YFP.Orc1^{WT}.PACT$ was constructed by cloning the Orc1 cDNA into the plasmid YFP.PACT, at the C terminus of YFP and N terminus of PACT, using the restriction sites HindIII(5') and SalI(3'). The plasmids $Orc1^{WT}.Flag$ and $Orc1^{WT}.Nterm.Flag$ (also called $Orc1^{1-500}$) were described in (*3*). Site direct mutagenesis was performed on $Orc1^{WT}.Flag$, $Orc1^{WT}.Nterm.Flag$ and $YFP.Orc1^{WT}.PACT$, changing the amino acids K235A and L237A, according to (*3*). Cyclins A, B and E were all cloned by PCR to be expressed with an N terminal T7 tag into BamHI/ XhoI sites of pLPC (gift from Masashi Narita) and verified by sequencing. Oligonucleotides sequences used to generate the plasmids are available under request.

Antibodies
The following primary antibodies were used for: (i) *immunostaining:* rabbit pAb anti- g-tubulin (#T5192, Sigma), mouse mAb anti-centrin 2 (gift from J.L Salisbury), rabbit pAb anti-centriolin (gift from S. Doxsey), mouse mAb anti-Cyclin B (clone 18, BD Biosciences), rabbit mAb anti-phospho-Histone H3 (Ser 10) (clone MC463, Upstate), rabbit pAb738 anti-MCM3; rabbit pAb anti-Cyclin E (C-19, Santa Cruz Biotech.) was used only for co-staining with anti-GFP; (ii) *immunostaining and immunoblotting*: mouse mAb anti- g-tubulin (GTU-88, Sigma), mouse mAb anti- GFP (7.1 and 13.1, Roche), mouse mAb anti-Cyclin E (HE12, Santa Cruz Biotech.), rabbit pAb anti-Cyclin A (H-432, Santa Cruz Biotech.), mouse mAb anti-Flag (M2, Sigma); (iii) *immunoblotting:* mouse mAb anti- a-tubulin (B512, Sigma), mouse mAb anti-T7 (#69522-4,Novagen), mouse mAb anti-Lamin A/C (clone 14, BD Biosciences), mouse mAb anti-MEK2 (clone 96, BD Biosciences), mouse mAb anti-p53 (Ab-6, Calbiochem), mouse mAb anti-p27 (clone 57, BD Biosciences), rabbit pAb anti-p21 (C-19, Santa Cruz Biotech.), rabbit pAb anti-Chk1 (#2345, Cell Signaling), rabbit pAb anti-phospho-Chk1 (Ser317) (#2344, Cell Signaling), mouse mAb PKS1-40 anti-Orc1, rabbit pAb205 anti-Orc2, rabbit pAb CS1890 anti-Orc3 (*3*), goat pAb anti-Orc4 (ab9641, Abcam).

To develop monoclonal antibodies against human Orc1, a peptide antigen designed from the sequence ELFEDDSDPPPKKRAR derived from human Orc1 was coupled to Keyhole Limpet Hemocyanin (KLH) and injected into mice. After fusion and screening, clone no. PKS1-40 was tested on HEK293 cell extracts and recognized a

band of ~ 100 kD by western blotting. This was confirmed to be Orc1 protein by western blot analysis on Orc1-1 siRNA treated extracts that showed the signal was eliminated.

To develop monoclonal antibodies against human Orc3, HsOrc3 was purified from *E. coli* as a GST tagged protein and injected into mice. Monoclonal antibodies were raised according to standard protocols.

Immunofluorescence, and Immunoblot

Immunostaining was performed by dual-color indirect immunofluorescence (IF) essentially as described in (*1*). For visualizing centrosomes with anti-g̃tubulin and anti-centrin 2, or anti-centriolin and anti-centrin 2, cells were fixed seven minutes with cold methanol. For visualizing MCM3, cells were pre-extracted in 0.5% Triton-X in Cytoskeletal buffer, followed by 2% paraformaldehyde fixation and subsequent cold methanol extraction, according to (*1*). All other IF was carried out with 2% paraformaldehyde fixation and subsequent cold methanol extraction. For g-tubulin immunostaining and direct visualization of YFP and CFP in transfected cells, the cold methanol extraction was performed for 4 min. The following secondary antibodies were used for indirect immunofluorescence: Fluorescein (FITC)-conjugated AffiniPure Goat Anti-mouse (Jackson ImmunoResearch Lab.), Texas Red dye-conjugated AffiniPure Donkey Anti-Rabbit (Jackson ImmunoResearch Lab.)

Unfortunately, the endogenous Orc1 protein could not be localized to centrosomes since all antibodies developed against human Orc1 were not suitable for *in situ* immuno-localization.

To prepare total cell extracts for immunoblots, cells pellets were resuspended in Laemmli Buffer without dye and with protease and phosphatase inhibitors. Samples were boiled for 5 minutes and protein concentrations were determined by Bradford (BioRad). Centrosomes of HeLa cells were purified as described in (*4*). Immunoblots were performed according to standard procedures.

Live cell imaging

For live cell imaging, electroporation was performed on trypsinized cells resuspended in 250 µl of growth medium and transferred to cuvettes containing 2 µg of pEYFP-Orc1 plasmid plus 20 µg salmon sperm DNA. Cells were seeded onto acid-washed coverslips and processed for immunofluorescence localization or live cell imaging.

Flow cytometry

Flow cytometry using Propidium Iodide (PI) (Sigma, St Louis, CA) as label was performed to measure DNA content. Cells were collected by trypsinization, resuspended in PBS with 10% calf serum (EB) and fixed in 90% cold ethanol overnight at -20^{0}C. Fixed cells were stained for 30 minutes at 37^{0}C with 25mg/mL PI in EB containing 10mg/mL RNase A. DNA-associated red fluorescence for PI (488 nM Argon Laser for excitation) was measured on a LSR-II cell analyzer (Becton Dickinson) using FACS Calibur software.

RNA interference

RNAi was performed as described previously (*5*). siRNA oligonucleotides (Dharmacon Inc., Lafayette, CO) were made to the following sequences (sense-strand): Orc1-1 CUGCACUACCAAACCUAUAdTdT (*6*); Orc1-2 GGUUGUUCCACCGAGAUUCdTdT; luciferase GL3 CUUACGCUGAGUACUUCGAdTdT (*5*); Cyclin E1/E2 AACCAAACUUGAGGAAAUCUA (*7*) Cyclin A AACTACATTGATAGGTTCCTG (*8*). Two transfections were carried out with 100mM siRNA duplexes with Oligofectamine (Invitrogen, CA), in an interval of 24 hours, according to the manufacturer's instructions.

Co-transfections were carried with 100mM of each siRNA duplex. In the experiment with U2OS cells synchronized at mitosis, the first transfection was carried out concomitantly with the addition of nocodazole, and the second transfection was performed 24 hours after (at 8 hours after nocodazole release).

Centrosome duplication assay
U2OS cells were grown on glass coverslips in 6-well culture vessels till 80 – 90 % confluency before transfection. They were transfected with either Fugene6 (Roche) or Lipofectamine 2000 (Invitrogen, CA), according to the manufacturer's instructions, with 2mg plasmid DNA, except for 0.5mg pEYFP-C1 and 0,5mg pECFP-C1 (CLONTECH Laboratories Inc). pECFP-C1 was added to transfected DNA in the assays with PACT protein fusions to help the identification of cells expressing PACT protein fusions located to centrosomes. Transfected and untransfected U2OS cells were directly treated with 16mM hydroxyurea (HU; Sigma, St Louis, CA) for 68 hours, to arrest cells at S-phase and promote centrosome amplification. Cells were stained with rabbit pAb anti- g-tubulin (#T5192, Sigma) for centrosome visualization. Over-expressed proteins with the Flag-tag were visualized by co-immunostaining with mouse mAb anti-Flag (M2, Sigma); and with the T7-tag by co-immunostaining with mouse mAb anti-T7 (#69522-4, Novagen). YFP, CFP and YFP-tagged proteins were visualized directly or by immunostaining with anti-GFP, as indicated. Quantitative analyses of centrosome numbers were performed on 100-300 Flag-, CFP- or YFP-positive cells, depending on the expressed proteins, and three to five independent experiments were performed for each transfection.

Immunoprecipitation
HEK293 cells were grown in 10 cm plates till 80 – 90 % confluency and then transfected with plasmid DNA using Lipofectamine 2000. Wild type Orc1 co-expressed with cyclin A shows reduced expression, since cyclin A overexpression promotes Orc1 degradation (data not shown). To stabilize Orc1 and normalize Orc1 expression levels across the experiment, 10 mM MG132 was added 24 hours after transfection. Cells were incubated for an additional 12 hours then harvested. Whole cell extracts were prepared and Orc1-flag immunoprecipitations were done using Flag polyclonal antibody (Sigma) as described previously (*3*). 1% of the inputs and 10% of the immunoprecipitates were analyzed by SDS PAGE followed by western blotting using the following antibodies: Orc1 (anti-flag M2 Sigma 1:2500) Cyclin (anti-T7 mAb Novagen 1:10,000) Orc3 (anti-Orc3 mAb 1:1000). Plasmids are described in Supplementary Methods.

Cyclin-dependent protein kinase assay
1.5 pmole of substrate Histone H1 (Millipore) alone or in combination with purified GST-ORC1[101-861] (containing human Orc1 with amino acids 101-861) or the mutant, purified GST-ORC1[101-861, A-A], (containing human Orc1 amino acids 101-861 with the changes K235A-L237A, named Orc1[A-A] in the text). Increasing amounts of GST-Orc1 were titrated in between 0.1 pmol – 4.0 pmol in the presence of 10 ng of either Cdk2/Cyclin A (Millipore, Upstate) or Cdk2/Cyclin E (Cell Signaling) per 10 ul reaction volume, containing 0.1 mg/ml BSA, 0.2 mM cold ATP, 0.02 mCi g-^{32}P ATP (6,000 Ci/mMol PerkinElmer) in reaction buffer; 50 mM Tris-HCl pH 7.5, 10 mM MgCl$_2$, 1 mM DTT, for 20 minutes at 37˙C. Reactions were quenched with an equal volume SDS sample buffer, boiled and run on 12% SDS-polyacrylamide gels. The intensity of the histone H1 bands were determined and plotted as arbitrary units relative to the kinase activity in the absence of GST-Orc1.

Supporting Movie and Figures

Supplementary Movie 1: Time lapse imaging of YFP-Orc1 showing Orc1 localization at the centrosomes in MCF7 cells. The images were obtained using a Delta Vision optical sectioning deconvolution instrument (Applied Precision) on an Olympus microscope with a 63x 1.4NA objective.
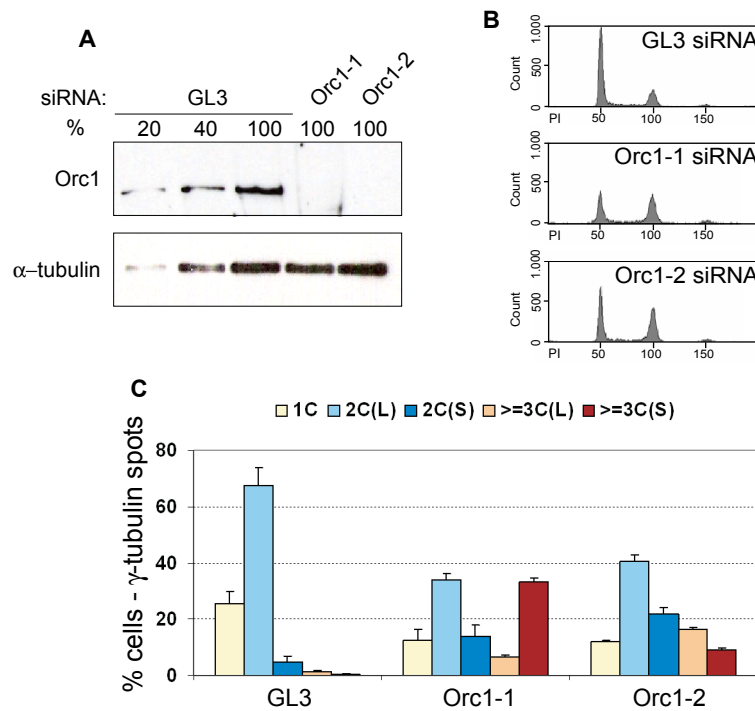
Supporting Figures

Fig. S1. Depletion of Orc1 using siRNA causes centrosome over duplication.

(A) Immunoblot with anti-Orc1 antibody of whole-cell extract from U2OS cells transfected with two Orc1 siRNA duplexes (Orc1-1 and Orc1-2) or control luciferase (GL3) and harvested at 72 hours. γ-tubulin levels were used as loading control. Three dilutions of control siRNA treated cells were loaded and Orc1 siRNA treated cells were at 100%. **(B)** DNA content of control (GL3), Orc1-1 and Orc-2 siRNA treated U2OS cells determined by flow cytometry. **(C)** Quantification of centrosome numbers by γ-tubulin staining in U2OS cells treated for 72 hours with control (GL3), Orc1-1 and Orc1-2 siRNA duplexes. At least 200 cells in three independent experiments were counted. Error bars represent one standard error.

Note that transfection of U2OS cells using two independent siRNAs caused a loss of more than 80% of Orc1 protein (fig. S1A). Flow cytometry showed an increase of cells with a 4C DNA content 72 hr after the Orc1 siRNA treatment (46.1% with Orc1-1 and 43% with Orc1-2 siRNAs), in comparison to 18.8% of 4C cells transfected with control siRNA. After more than 72 hr of Orc1 depletion, cells eventually formed apoptotic nuclei.
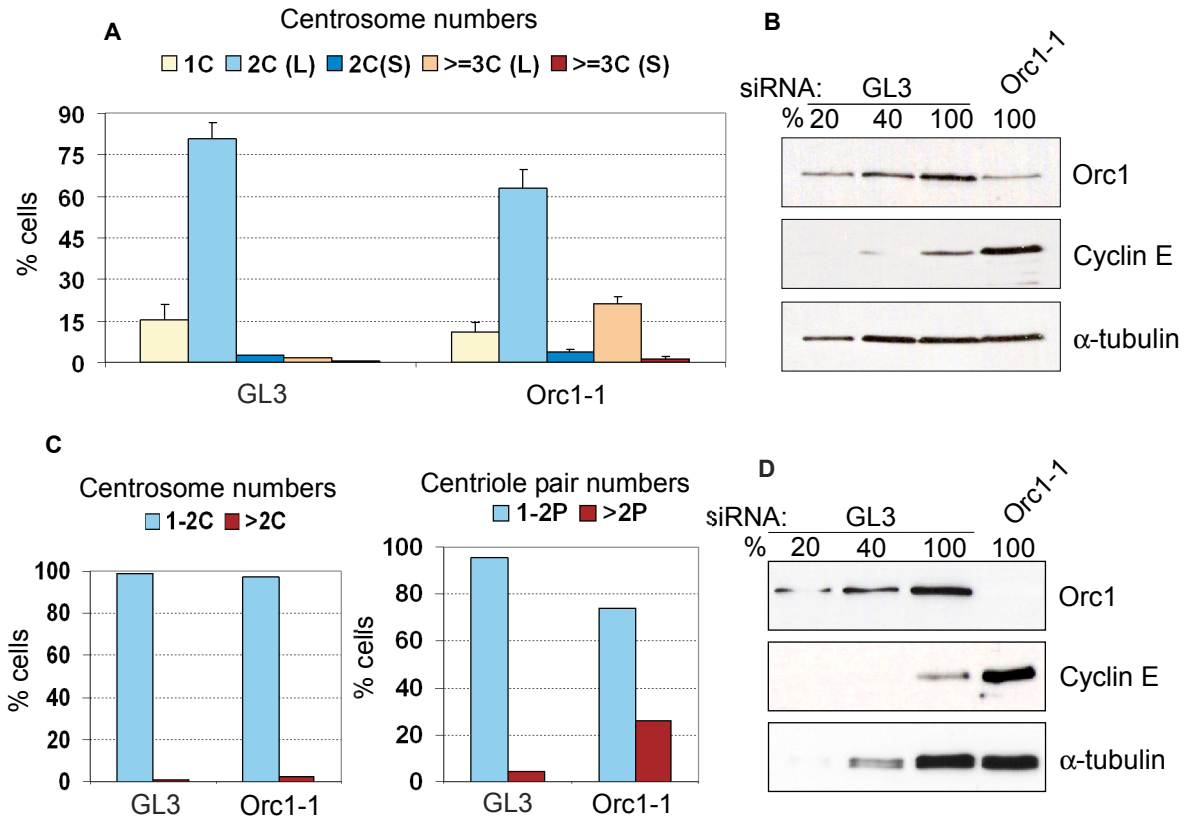
Fig. S2: Orc1 silencing by siRNA treatment causes centriole and centrosome amplification in different human cell lines.

(A) Quantification of centrosome numbers by γ-tubulin staining in HCT116 cells (p53+/+) treated for 72 hours with control (GL3) and Orc1-1 siRNA duplexes. 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. **(B)** Immunoblot of whole-cell extract from HCT116 (p53+/+) cells transfected with GL3 and Orc1-1 siRNA duplexes and harvested at 72 hours. Efficacy of siRNA was assessed by immunoblotting with anti-Orc1 antibody and Cyclin E levels were assessed with anti-Cyclin E antibody. γ-tubulin levels were used as loading control. GL3 siRNA treated cells were loaded in dilutions representing 20%, 40% and 100%, and Orc1 siRNA treated cells were at 100%. **(C)** Quantification of centrosome and centriole numbers in HeLa cells by γ-tubulin and centrin 2 immunostaining, respectively. Cells were harvested at 72 hours. Left panel: 1-2C, one or two centrosomes; >2C, more than two centrosomes. Right panel: 1-2P, one or two centriole pairs; >2P, more than two centriole pairs. **(D)** Immunoblot of whole-cell extract from HeLa cells transfected with GL3 and Orc1-1 siRNA duplexes and harvested at 72 hours. Efficacy of siRNA was assessed by immunoblotting with anti-Orc1 antibody and Cyclin E levels were assessed with anti-Cyclin E antibody. γ-tubulin levels were used as loading control. GL3 siRNA treated cells were loaded in dilutions representing 20%, 40% and 100%, and Orc1 siRNA treated cells were at 100%.

Comment: Similar effects of Orc1 depletion on centrosome numbers identified in U2OS cells were observed in HCT116 cells (fig. S2, A and B). Depletion of Orc1 in HeLa cells did not affect centrosome numbers, but caused centriole overduplication (fig. S2, C and D).
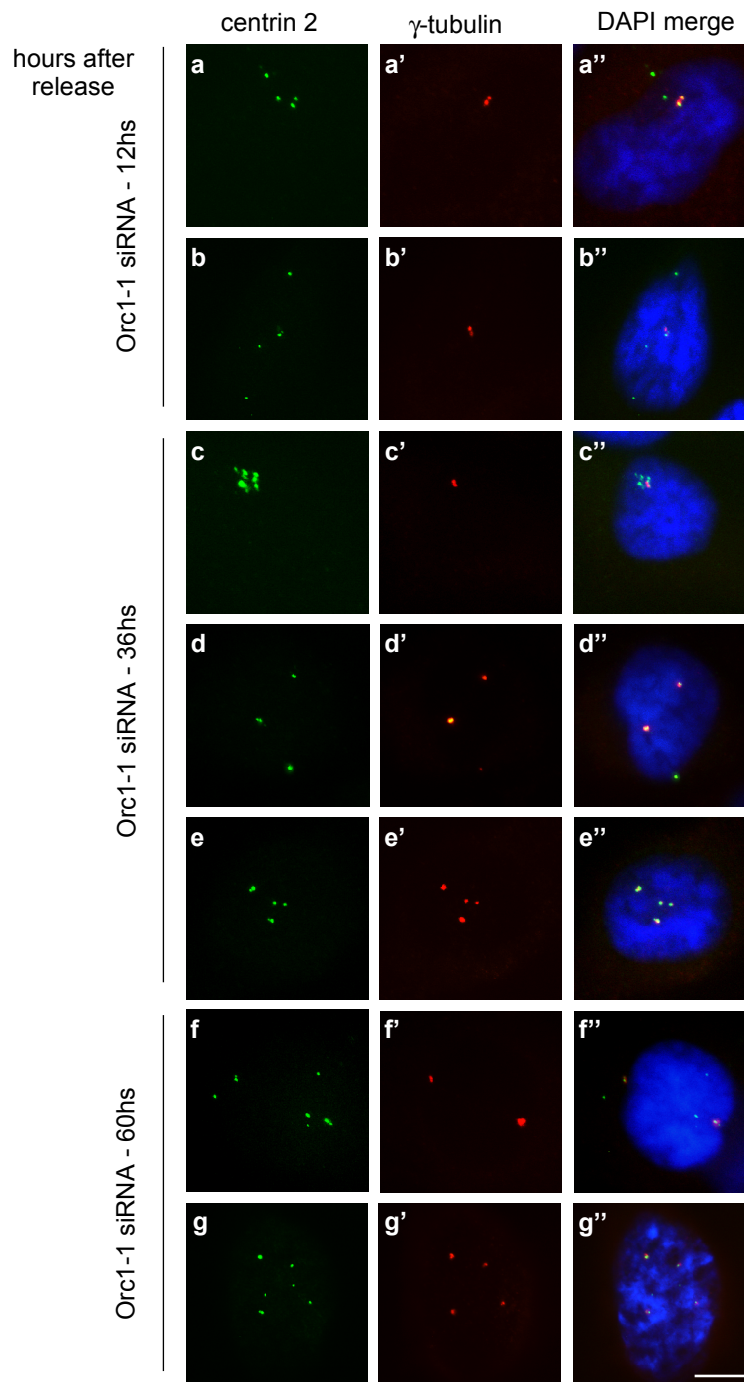
Fig. S3: Orc1 depletion has a very early effect on centriole disengagement and re-duplication in U2OS cells.

U2OS cells were transfected with control (GL3) or Orc1-1 siRNAs, synchronized in mitosis by nocodazole treatment, released into the next cycle and re-transfected with the same siRNAs. Cells were harvested at 12 hours, 36 hours and 60 hours after nocodazole release and were immunostained for centrosomes with γ-tubulin (red, a' to g') and centrin 2 (green, a to g) antibodies. DNA was stained with DAPI (blue) and merged images are shown in a" to g". Scale bar, 10 μm. Quantification of data in this experiment is shown in Fig. 1B.
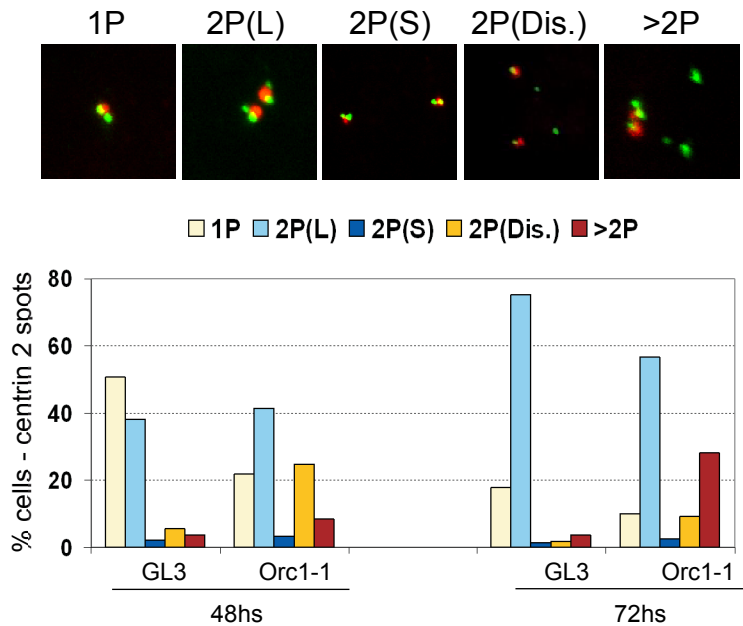
Fig. S4: Orc1 depletion in HeLa cells causes centriole re-duplication.

Asynchronous HeLa cells were treated for 48 hours and 72 hours with GL3 (control) or Orc1-1 siRNA duplexes and subjected to dual-color IF. Treated cells were immunostained for centrosomes with γ-tubulin (red) and centrin 2 (green). The upper panel of shows examples of classes of HeLa cells treated Orc1 siRNA and the various patterns of centrin 2 and γ-tubulin staining. Centriole numbers were quantified by centrin 2 immunostaining. 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. Merged images representative of the centriole pairs counting are shown. The graph below shows the percentage of cells the different centrin 2 patterns. Note in the absence of Orc1, we first observe the accumulation of cells with disorganized centriole disengagement (2P(Dis) at 48 hours), followed by an increase in the number of cells with multiple centrioles (>2P at 72 hours). HeLa cells, unlike U2OS, did not progress to multiple (>=3C) γ-tubulin positive centrosomes.
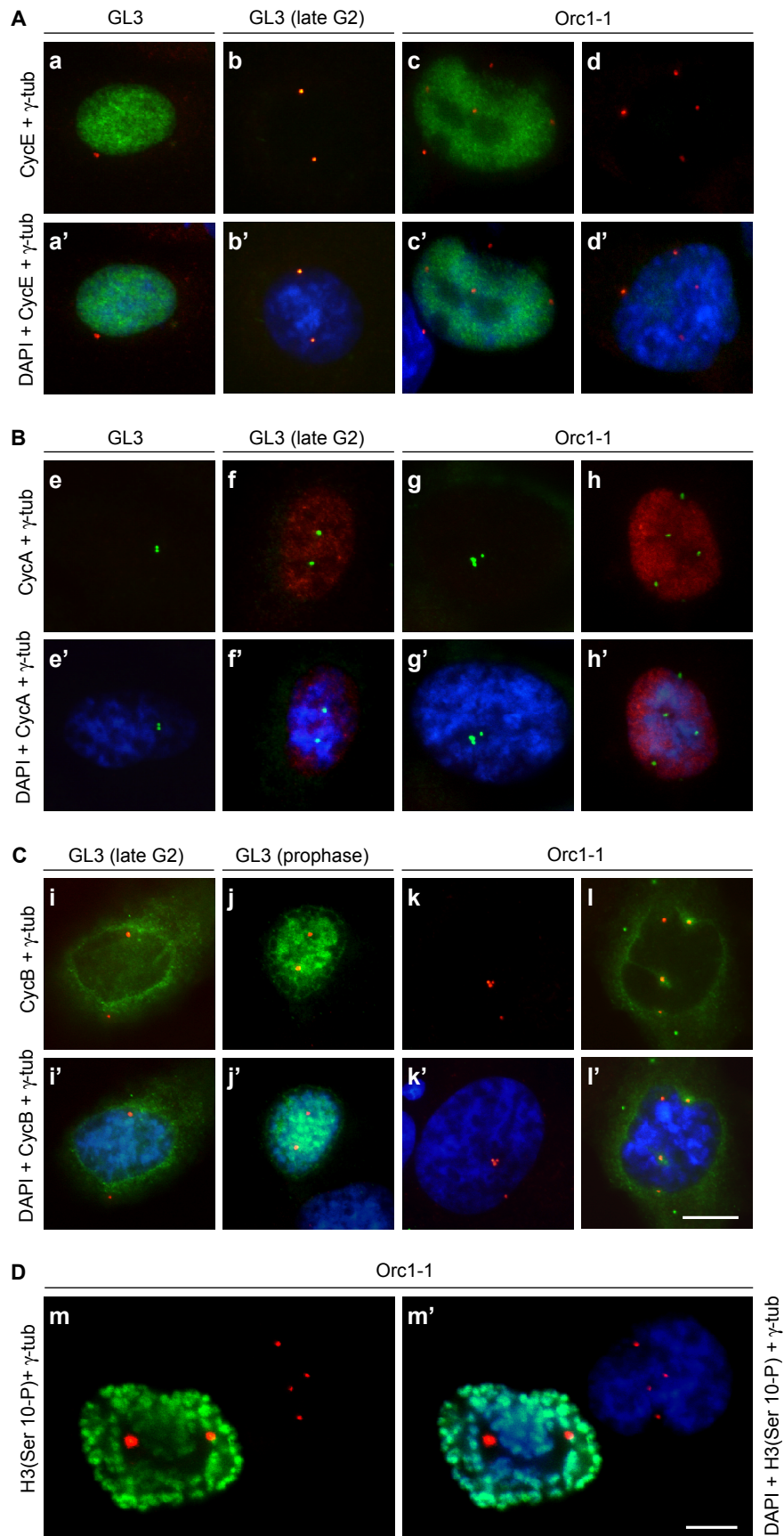
Fig S5: Orc1 depleted U2OS cells with multiple separated centrosomes are mostly at late G2.

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The cell cycle phase of Orc1 depleted cells with multiple centrosomes was inferred based on the accumulation and localization of cell cycle markers. Asynchronous U2OS cells were treated for 72 hours with GL3 (control) or Orc1-1 siRNA duplexes and co-immunostained with anti-γ-tubulin (to visualize centrosomes) and with antibodies against cell cycle markers. DNA was stained with DAPI (blue) and merged images are shown in a' to m'. Scale bar in (a) to (l'), 10 μm. Scale bar in (D), 10 μm. The majority of cells with separated multiple centrosomes showed a late G2 profile of accumulation of nuclear Cyclin A and cytoplasmic Cyclin B1 and they were negative for histone H3 phosphorylation on serine 10, a mitotic marker that first appears at prophase. The data indicated that these cells were in late G2-phase.

(A) Cells were immunostained with anti-Cyclin E (green) and anti-γ-tubulin (red) and merged images are shown in (a) to (d). In control, Cyclin E was present in cells with one or two centrosomes linked (a-a'), and never observed at late G2 (two separated centrosomes) (b-b'). A similar percentage of Orc1 depleted cells with multiple centrosomes were found expressing Cyclin E (c-c') or not (d-d'). A similar observation was made for cells with multiple linked centrosomes (data not shown).

(B) Cells were immunostained with anti-Cyclin A (red) and anti-γ-tubulin (green) and merged images are shown in (e) to (h). In control, Cyclin A was observed only in cells at late G2 (two separated centrosomes) (f-f'). All Orc1 depleted cells with multiple separated centrosomes expressed Cyclin A (h-h'), and cells with multiple linked centrosomes did not exhibit Cyclin A (g-g').

(C) Cells were immunostained with anti-Cyclin B1 (green) and anti-γ-tubulin (red) and merged images are shown in (i) to (l). Cyclin B1 localization is a good marker to differentiate cells at late G2 and early prophase, since it is normally found in the cytoplasm until late G2 (i-i'), and during early mitosis it moves into the nuclei (j-j'). All Orc1 depleted cells with multiple centrosomes showed a cytoplasmic localization of Cyclin B (l-l'). Cells with multiple linked centrosomes did not express Cyclin B.

(D) Cells were immunostained with anti-H3(Ser10-P) (green) and anti-γ-tubulin (red) and a merged image is shown in (m). Phosphorylation of histone H3 in Ser10 marks the beginning of mitosis, as shown by the cell at left side (m-m'). None of the Orc1 depleted cells with multiple centrosomes (right side) showed staining for H3(Ser10-P).
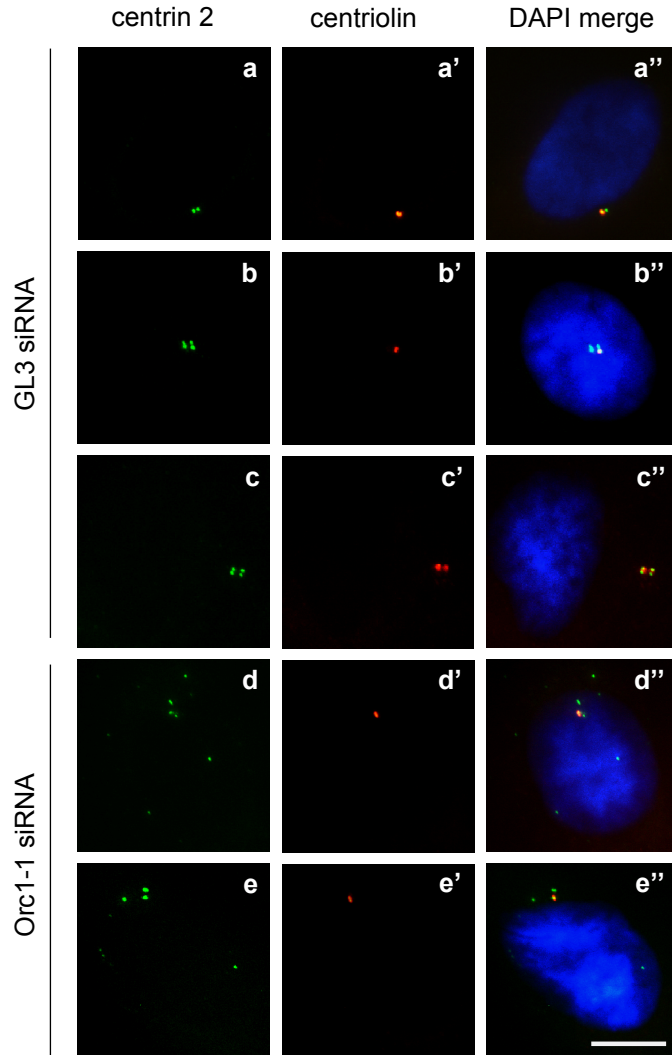
Fig. S6: Centrosome amplification in Orc1 depleted cells did not arise from cell division failure.

Asynchronous U2OS cells were treated for 72 hours with GL3 (control) or Orc1-1 siRNA duplexes and co-immunostained with anti- centrin 2 to visualize centrioles (green, a to e) and with anti centriolin (red, a' to e'). DNA was stained with DAPI (blue) and merged images are shown in a" to e". Scale bar, 10 μm.

Comment: Centriolin is a marker for maternal centrioles and centrosome maturation *(8)*. It associates with only one centriole during the duplication process in G1-, S- and G2-phase cells (a"- b"); and centriolin binds to the two mature centrioles during late G2 (c") and mitosis. Normally, more than two mature centriolin positive centrioles arise from cell division failure. Note that Orc1 depleted cells with multiple centrosomes contained mostly one centriolin positive centriole (d"- e"), arguing against the cytokinesis defect model for centrosome amplification.

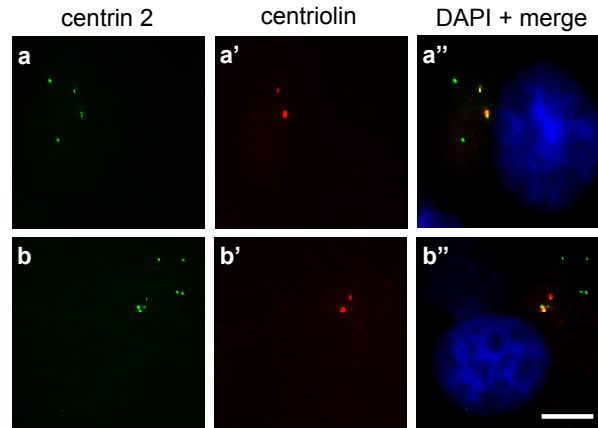centrin 2   centriolin   DAPI + merge

Fig. S7. Centriolin staining of HeLa cells depleted of Orc1.

Orc1-1 treated HeLa cells harvested at 72 hours were immunostained with anti- centrin 2 to visualize centrioles (green, a and b) and with anti-centriolin (red, a' and b') to visualize mature centrosomes (see fig. S6). DNA was stained with DAPI (blue) and merged images are shown in a" and b". Scale bar, 10 μm. Note that Orc1 depleted HeLa cells with multiple centriole pairs contained mostly one centriolin positive centriole, arguing against the cytokinesis defect model for centriole amplification.

Comment: Depletion of Orc1 causes bona fide re-duplication of centrioles and centrosomes. As discussed in the text of the paper, various mechanisms for centrosome amplification have already been reported. Apparent multiple centrosomes can arise by centrosome fragmentation or splitting that occurs during early mitosis after DNA damage, in addition to authentic re-duplication *(9)*. The flow cytometry data showed an increase in the 4C DNA population after Orc1 depletion and immunostaining of cell cycle markers indicated that cells with multiple centrosomes were in interphase, well before entering mitosis (fig. S1B; fig. S5). In addition, the great majority of the separated multiple centrosomes show γ-tubulin stained PCM associated with two centrin 2 spots, suggesting they arise mainly by centriole re-duplication, rather than by centriole splitting (Fig. 1A). Centrosome amplification can also occur through accumulation by cell division failure *(10, 11)*, however flow cytometry did not show more than 4C DNA content in Orc1 depleted cells, arguing against increased centrosome numbers caused by a cytokinesis failure (fig. S1B). This conclusion was further corroborated by the absence of multiple centriolin positive centrioles in U2OS cells depleted of Orc1 (fig. S6). Centriolin is a marker of mature mother centrioles in cells and the fact that we did not see more then one or two centriolin spots indicates that multiple cell cycles did not occur. Moreover, Orc1 depleted HeLa cells with multiple centrioles also exhibited only one or two centriolin positive centrioles, suggesting a bona fide centriole overduplication (fig. S7). Note that the effect of depletion of Orc1 differs from siRNA mediated depletion of Orc2, which causes centrosome amplification in cells arrested in mitosis *(1).*
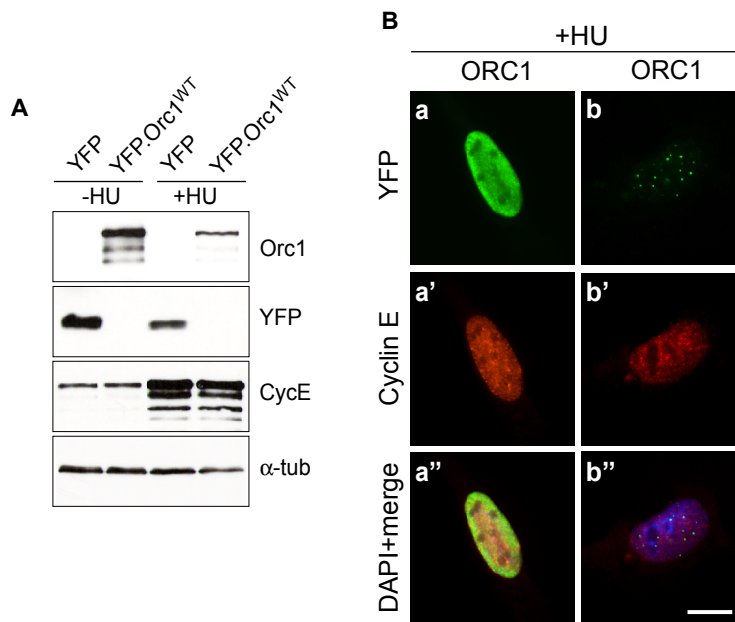
Fig. S8: Expression of Orc1 and Cyclin E in transfected S-phase arrested cells.

U2OS asynchronous cells were transfected with the vector alone (YFP) or YFP-tagged-Orc1 (YFP.Orc1[WT]) and treated (+HU) or not with 16mM hydroxyurea (-HU). **(A)** Immunoblot of whole-cell extract of transfected cells. YFP.Orc1[WT], YFP and Cyclin E levels were assessed by immunoblotting with anti-Orc1, anti-GFP and anti-Cyclin E antibodies. The endogenous Orc1 protein is not detected at this exposure. γ-tubulin levels were used as loading control. **(B)** HU-arrested cells transfected with YFP.Orc1[WT] were harvested at 48 hours and co-immunostained with anti-Cyclin E (red) and anti-GFP (green). DNA was stained with DAPI (blue) and merged images are shown in a" to b". Scale bar, 10 μm.

Comment: Although endogenous Orc1 levels are cell cycle regulated in human cells, dropping in S-phase *(2, 12)*, over-expressed YFP-Orc1[WT] was present in moderate levels in HU-arrested cells. Note that Cyclin E abundance is not reduced in YFP.Orc1[WT] transfected cells. Since Orc1 depletion increased Cyclin E levels, YFP.Orc1[WT] overexpression might trigger Cyclin E instability and inhibition of centrosome overduplication, which then would be rescued by co-expressing Cyclin E with Orc1. Nevertheless, immunoblotting and immunostaining assays showed that overall Cyclin E accumulation was not decreased in YFP-Orc1[WT] expressing cells compared to cells expressing YFP alone, suggesting that the Orc1 mechanism of action does not exclusively involve the control of Cyclin E levels.
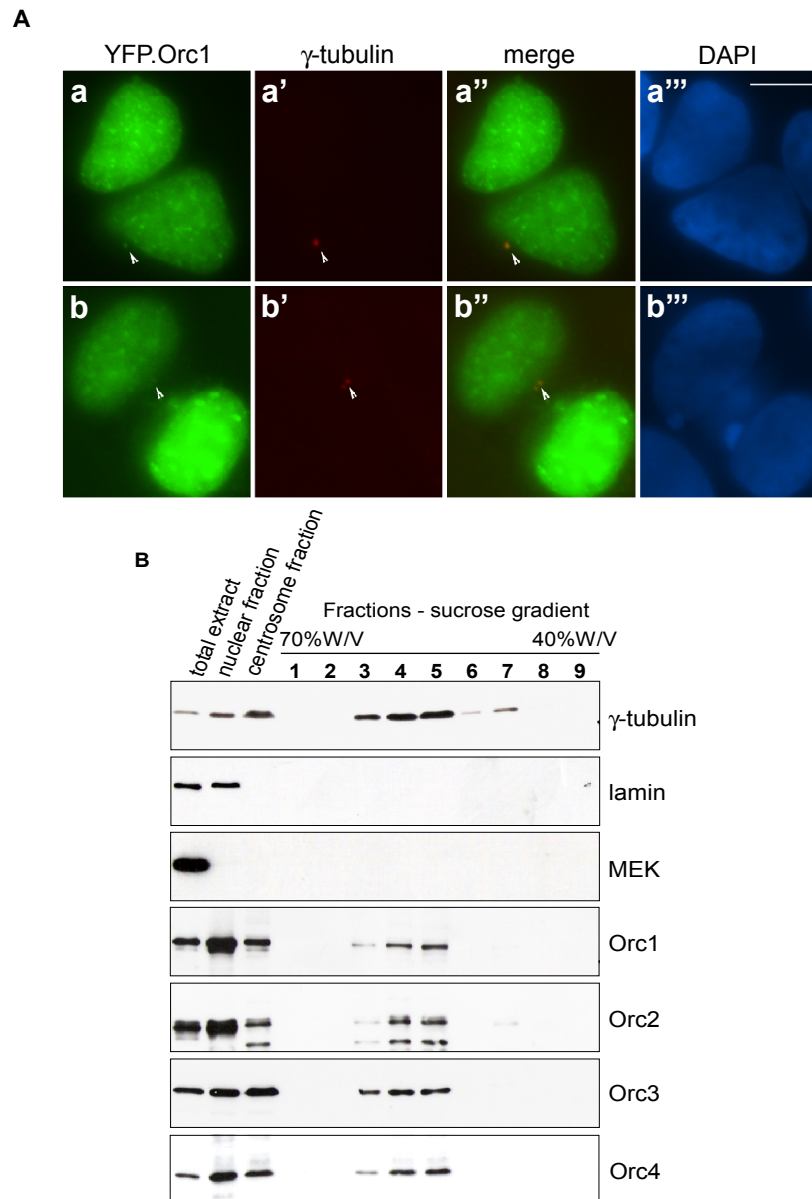
Fig. S9: Orc1 localization at centrosomes

(A) Immuno-fluorescence analysis of MCF7 cells transiently transfected with YFP-Orc1[WT] (green, a-b) and labeled with anti-γ-tubulin antibody (red, a'-b'). Note the localization of Orc1 at the centrosomes (arrowhead). Merges highlight the colocalization of Orc1 and γ-tubulin at the centrosomes (yellow, a''-b''). Chromatin was stained with DAPI (blue, a'''- b'''). Bar represents 5 μm. The centrosome localization was observed in approximately 35% of U2OS cells expressing YFP-Orc1, suggesting that this localization might be transient. It was associated with unduplicated (one γ-tubulin) and duplicated (two γ-tubulin) interphase centrosomes. Similar centrosomal localization patterns were seen in U2OS cells and MCF7 cells transiently expressing YFP-Orc1. **(B)** Centrosomes from HeLa exponentially growing cells were purified on a discontinuous sucrose gradient (70% to 40%) according to *(3)*. The proteins in each fraction from the final discontinuous sucrose gradient were separated on SDS-PAGE gels and were subjected to immunoblotting. The first three lanes show protein extracts from different steps of the purification: total HeLa cell lysate (lane 1), " nuclear" fraction that was pelleted at the first centrifugation after cell lyses (lane 2) and crude centrosome fraction that was sedimented into the first sucrose cushion by centrifugation (lane 3). Lanes 4 to 12 show the nine centrosomal fractions collected after discontinuous sucrose gradient from the bottom of the tube. The centrosomal marker γ–tubulin copurified largely with the fractions 3, 4 and 5. The nuclear envelope protein lamin A/C and the cytosolic kinase MEK2 were not detected in any of the centrosomal fractions, confirming they were free of nuclear and cytoplasmic contamination. In agreement with our previous observations, Orc2 copurified with the centrosomal fractions, confirming the localization to the centrosomes. Orc1, Orc3 and Orc4 also co-fractionated with the centrosomal fractions, consistent with their location to centrosomes. Orc1, Orc2, Orc3 and Orc4 were also localized to centrosomes in murine cells *(13)*.
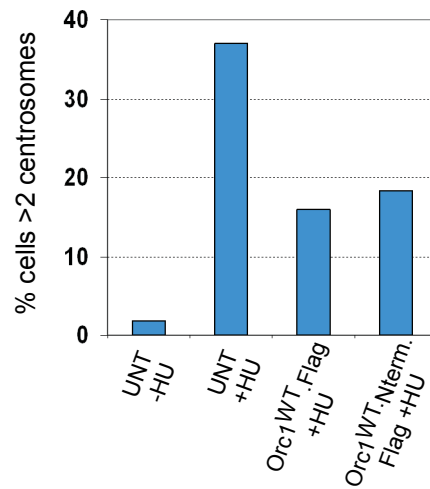
Fig. S10: Orc1 inhibition of centrosome re-duplication is not an indirect effect of G1 arrest.

U2OS asynchronous cells were pretreated with 16mM hydroxyurea for 24 hours followed by transfection with the Flag tagged full-length Orc1 (Orc1^{WT}.Flag) and Flag tagged N terminus region (aa 1 to 500) of Orc1 (Orc1^{Nterm}.Flag), and re-incubated with 16mM hydroxyurea (+HU) for 44 hours. As control, untransfected cells were treated or not with HU (UNT +HU and UNT -HU, respectively). Transfected cells were identified by anti-Flag immunostaining and centrosome numbers were scored by γ-tubulin immunostaining.
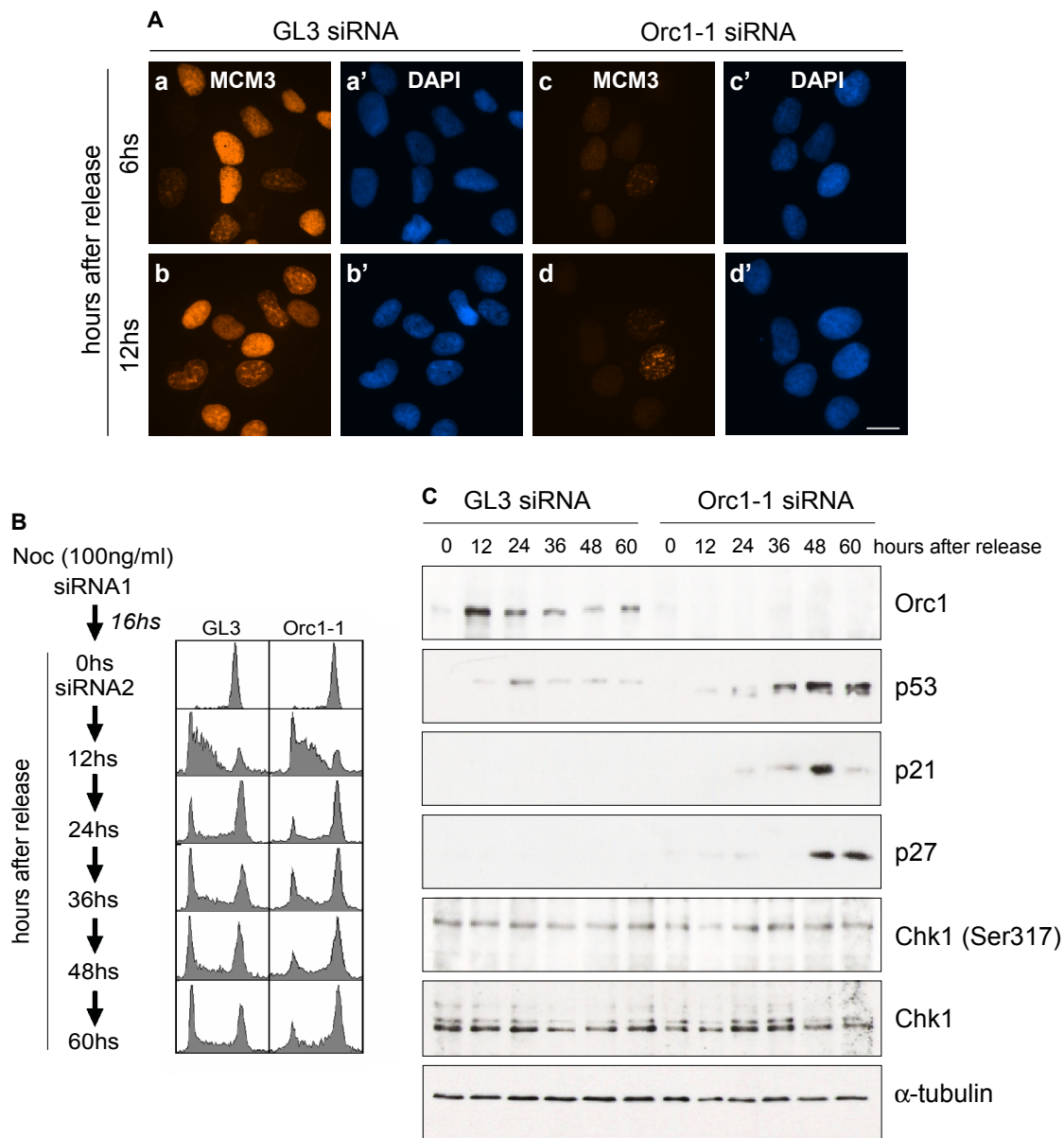
Fig. S11: Orc1 depleted U2OS cells showed reduced MCM3 loading on chromatin and activation of DNA damage checkpoint pathways as a late response.

U2OS cells were transfected with control (GL3) or Orc1-1 siRNA duplexes, synchronized in mitosis by nocodazole treatment, released into the next cycle and re-transfected with the same siRNA duplexes (experiment shown in Fig. 1B). **(A)** MCM3 loading onto chromatin was assessed in cells harvested at 6 hours and 12 hours after nocodazole release. Cells were immunostained with anti-MCM3 (red) following pre-extraction procedure (Methods). Scale bar, 20 μm. DNA was stained with DAPI (blue). MCM3 loading was dramatically reduced (but not eliminated) in Orc1 depleted cells at 6 hours after release, when cells are mostly at G1- phase (c, c'), and in S-phase cells at 12 hours after release (d,d') Orc1 siRNA treatment greatly reduced but did not eliminate Orc1 proteins levels and as a consequence, some MCM protein loaded onto chromatin and allowed one S phase (see Fig. 2A). **(B)** Schematic outline of the experimental procedure showing DNA content of GL3 and Orc1-1 siRNA treated cells harvested at the indicated time points and analyzed by flow cytometry. **(C)** Immunoblots of a whole-cell extract of cells harvested at the indicated time points. Orc1, p53, p21, p27, Chk1 phosphorylated in Ser317 and Chk1 levels were assessed by immunobloting with specific antibodies. γ-tubulin levels were used as loading control.

Comment on fig. S11: DNA damage response does not induce centriole re-duplication. Bona fide centrosome re-duplication was reported to occur as a result of DNA damage responses mediated by p27 loss or Chk1 activation in cells arrested in G2 phase for an extended period *(14-16)*. Also, centrosome defects are commonly associated with the loss of p53 activity *(17)*. In the Orc1 siRNA-treated U2OS cells released from a nocodazole block, cells progressed into S phase and accumulated with a 4C DNA content (fig. S11B). Some MCM3 was loaded onto chromatin in these cells, although as expected with markedly reduced levels (fig. S11A). It is therefore likely that depletion of Orc1 was not complete, allowing some pre-RC assembly and activation of a reduced number of origins. Note that in Orc1 depleted cells, the Chk1 pathway is not activated by Chk1 phosphorylation in Ser317. p53, p27 and p21 levels increased after centriole amplification was initiated (Fig. 1B, fig. S3, fig S11B and C), indicating that they did not participate in the mechanisms mediating centriole re-duplication in Orc1 depleted cells. Moreover, the cells are clearly wild type for a p53 response, demonstrating that the increased centriole and centrosome numbers in the absence of Orc1 are not due to a lack of p53. These results argue that the effect of Orc1 depletion on centriole copy number is not directly due to the induction of DNA damage responses.
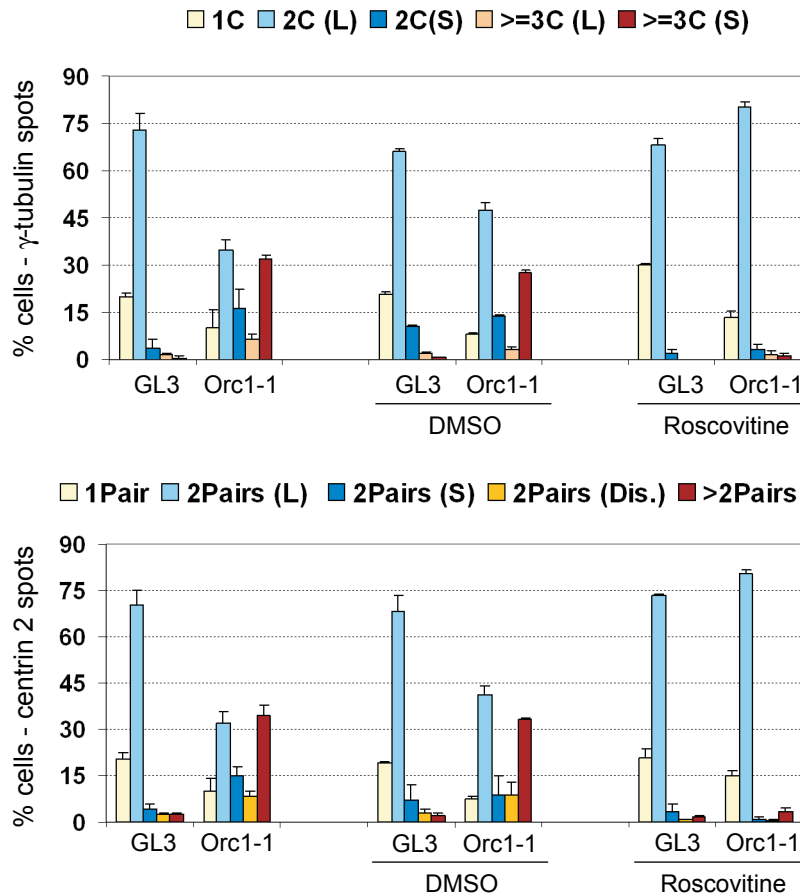
Fig. S12: Roscovitine abolishes centriole and centrosome overduplication in Orc1 depleted cells

Quantification of centrosome and centriole numbers by γ-tubulin and centrin 2 immunostaining, respectively, of asynchronous U2OS cells transfected with GL3 or Orc1-1 siRNA duplexes and not treated, or treated 24 hours with 10 M roscovitine. The drug was added 48 hours after the first transfection. As control, transfected cells were incubated with DMSO. Cells were analyzed 72 hours after the first transfection. Inhibition of Cdk2 activity with roscovitine suppressed centriole and centrosome overduplication in Orc1 depleted cells. The percentage of cells with multiple centrioles dropped from 33.52 ± 0.25% in Orc1-1 transfected cells to 3.28 ± 1.9% in Orc1 depleted cells that were treated with roscovitine. Error bars represent one standard error. Upper panel: 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. Bottom panel: 1Pair, one centriole pair; 2 Pairs (L), two centriole pairs linked; 2 Pairs (S), two centriole pairs separated; 2 Pairs (Dis.), two centriole pairs disoriented and disengaged; >2 Pairs, more than two centriole pairs.
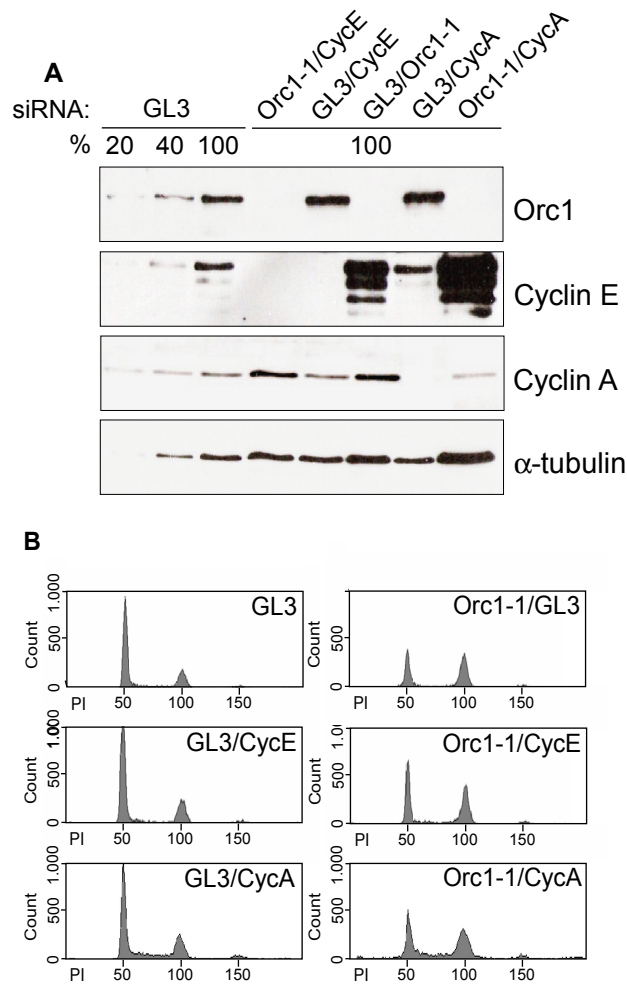
Fig. S13. Simultaneous depletion of Orc1 and Cyclin E, but not Cyclin A, blocks centriole and centrosome re-duplication

U2OS cells were transfected with GL3, Cyclin E, or Cyclin A siRNA alone; and cells co-transfected with Orc1-1 and GL3; Orc1-1 and Cyclin E; Orc1-1 and Cyclin A. Cells were analyzed 72 hours after the first transfection. Error bars represent one standard error. **(A)** Immunoblot of whole-cell extract from U2OS cells transfected with the indicated siRNAs and harvested at 72 hours. Efficacy of siRNA was assessed by immunoblotting with the indicated antibodies; and γ-tubulin levels were used as loading control. GL3 siRNA treated cells were loaded in dilutions representing 20%, 40% and 100%, and the co-transfected cells were at 100%. This data was collected in parallel with data shown in Fig. 3C and represents the controls for the levels of proteins present in cells in which centriole and centrosome numbers were determined. **(B)** DNA content of U2OS cells transfected as in **(C)** and harvested at 72 hours was determined by flow cytometry. As mentioned in the main text, note that Cyclin E siRNA did not change the number of cells with a 4C DNA content after Orc1-1 treatment, implying that the block of centrosome overduplication in Orc1-1 depleted cells by Cyclin E siRNA was not an indirect effect of a G1 arrest
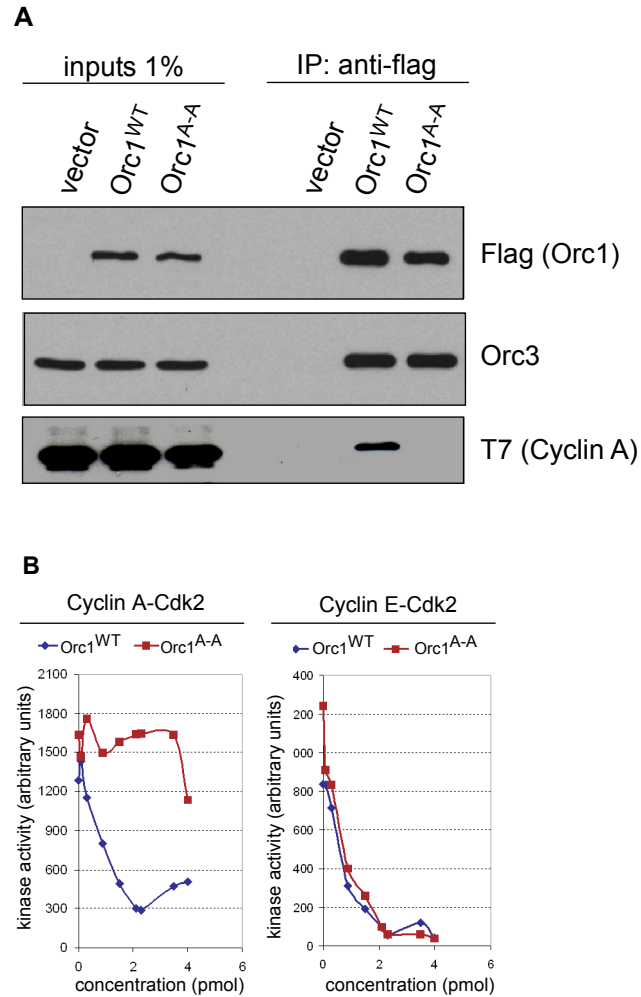
A

B

Figure S14. Orc1 binds to Cyclin A and inhibits both Cyclin E and Cyclin A activities.

(A) Immunoprecipitation with anti-Flag antibody from whole-cell extract from HEK293 cells transiently co-expressing full-length Orc1^{WT}.Flag (wild type) or full-length Orc1^{A-A}.Flag (mutant, K235A-L237A) with T7-Cyclin A and immunoblotting with the indicated antibodies. 1% input of total protein extracts and 10% of the immunoprecipitates are presented. Orc1 interaction with Cyclin A requires K235 and L237, part of the RXL motif. **(B)** Effect of increasing amounts of GST-Orc1^{WT} or GST-Orc1^{A-A} proteins on Cyclin-Cdk2 kinase assay using histone H1 as a substrate. Increasing amounts (pmol) of GST-Orc1$^{WT(101-861)}$ or GST-Orc1$^{A-A(101-861)}$ proteins were added to reactions that contained 1.5 pmol of histone H1 as substrate. Relative H1 phosphorylation is plotted in arbitrary units
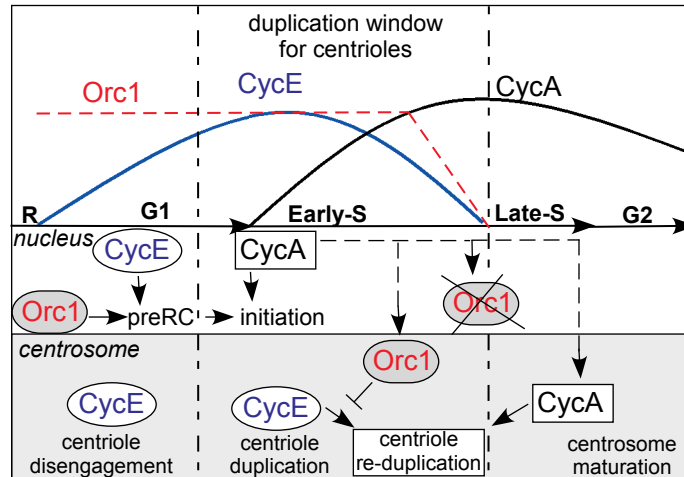
Fig. S15. Model for the role of Orc1 in control of DNA replication licensing and centriole and centrosome re-duplication.

As cells pass through the restriction point in G1 phase and commit to cell division, Orc1 in the nucleus has either already participated in the licensing of DNA replication origins or is acting in cooperation with Cyclin E to form pre-RCs at origins *(19)*. Cyclin E also promotes centriole duplication, possibly by allowing centriole disengagement *(20-27)*. If Cyclin E persists, then it might promote centriole re-duplication. Cyclin E is normally transient (see Fig. 3A) but there is a window of opportunity when Cyclin E is still present at centrosomes after normal centriole duplication has taken place. During this time, before Cyclin E destruction, Orc1 could block the Cyclin E function at centrosomes, thereby limiting centriole duplication to once per cell cycle. The Orc1 localization to centrosomes could be facilitated by Cyclin A promoting nuclear export of Orc1 *(28)* and localization to centrosomes (Fig. 4B). Cyclin A also promotes the destruction of the bulk of Orc1 as cells enter S phase, the same time when Cyclin E is degraded. Once Cyclin E is degraded, the possibility of re-duplication is lost since Cyclin A cannot induce centriole and centrosome re-duplication if the duplicated centrioles are engaged, consistent with data in Fig. 3C and Fig. 4A. It is known that Cyclin A is involved in centrosome maturation later in the cell cycle *(29)*.

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