Online Supplemental Materials

Video 1: A mitotic arrested cell following Orc2 depletion (72 hr.) showing multipolar asters. Note as many as 8 asters can be observed. The movie was obtained using a Delta Vision optical sectioning deconvolution instrument (Applied Precision) using an Olympus IX70 microscope base with a 63x 1.4NA objective.

Video 2: A control cell (luciferase siRNA treated) showing the labeling of Condensin I on the metaphase chromosomes. The antibody used was against the non-SMC subunit CAP-G. Note the sister chromatid lining up the arms like beads on a string. The movie was obtained using a Delta Vision deconvolution microscope (Applied Precision) as in Video 1.

Video 3: Cells treated with Orc2 siRNA showing the labeling of Condensin I on the short, condensed metaphase chromosomes. The chromosomes are much thicker and wider as compared to the control chromosomes. The condensin seems to be accommodated in these abnormally condensed chromosomes in a spiral fashion. The movie was obtained using a Delta Vision deconvolution instrument (Applied Precision) as described in Video 1.

Supplementary figures

Figure S1 **Anti-Orc2 antibody specificity.** Two antibodies directed against human Orc2 were used in these studies, a polyclonal and monoclonal antibody. To confirm the specificity of Orc2 antibody, a synthetic peptide (see materials and methods) that blocked

the monoclonal antibody recognizing the epitope on Orc2 protein was used for epitope blocking experiments. Pre-incubating the monoclonal antibody with the peptide abolished Orc2 specific signals in immunoblots and immunoprecipitation (Siddiqui and Stillman, unpublished observations). Anti-Orc2 mAb decorated the centrosomes and soluble Orc2 in HeLa cells (a) whereas following incubation of the mAb with the blocking peptide the soluble and centrosome specific signals were lost. Using the same peptide to block polyclonal antibody did not abolish the polyclonal signal of both soluble and centrosome associated signals suggesting that polyclonal antibody recognized another epitope (c and d). DNA was stained with DAPI. Bar represents 5 M.

Figure S2 **Orc2 localization at heterochromatin. A.** Immunofluorescence following paraformaldehyde fixation of Orc2 (pAb) in MCF7 cells. **B.** Immunofluorescence following detergent pre-extraction was carried out to study colocalization of Orc2 (pAb labeling in a) and HP1 (a') in HeLa cells. Merge (a'') represent the areas of colocalization. Compared to human MCF7 cells, HeLa cells have diffuse heterochromatin localization, but Orc2 still localized with these structures. Bar represents 5 M.

Figure S3 **Localization of YFP-Orc2 and T7-Orc2 in MCF7 cells. A.** Transient transfection (24 hr.) of YFP-Orc2 (a) in MCF7 cells revealed heterochromatin association and diffuse nuclear staining of Orc2. DNA was stained with DAPI (a'). **B.** Immunoblot of YFP-Orc2 transfected cells and untransfected (UT) cells with GFP antibody revealed ~ 97kDa YFP-Orc2 fusion protein. **C.** Transient transfection (24 hr.) into MCF7 cells of T7-Orc2 revealed co-localization of endogenous Orc2 (pAb in a) and exogenous T7-Orc2

(anti-T7 a') and shown as a merge (a''). **D.** Immunoblot of T7-Orc2 (12 and 24 hrs post transfection) and UT cells using T7 and pAb Orc2 antibodies. Bar represents 5□M.

Figure S4 **Silencing of Orc2 expression by siRNA.** Immunoblot of whole cell extract from cells transfected with Orc2 siRNA duplexes (2-1 and 2-2), control luciferase (Con) and untreated asynchronous cells (Asy) and harvested at 48 hrs (lanes 1-3) and 72 hrs (lanes 4-6). Cells were transfected at 0 hr, 24 hrs and 48 hrs at about 20% starting confluence. Efficacy of RNAi was assessed by immunoblotting with Orc2. □-tubulin levels were used as loading controls.

Figure S5 **hOrc3 regulated the stability of hOrc2 and vice-versa. A.** Immunoblot of whole cell extracts from Orc3 siRNA treated HeLa cells at 24, 48 and 72 hrs as compared to luciferase control siRNA treated cells. Immunoblots were probed with antibody against Orc2, Orc3 and □-tubulin. In cell with reduced Orc3, Orc2 levels decreased. □-tubulin levels were used as loading controls. **B.** DNA content assessed by propidium iodide staining of DNA and flow cytometry showed an increase in the S phase and G2/M population in Orc3 siRNA treated cells (b) as compared to the normal profile of control cells (a). Cells were harvested at 72hr.

Figure S6 **Detection of chromatin bound MCM3 following Orc2 knockdown. A.** Immunostaining with anti-MCM3 polyclonal antibodies in Orc2 depleted cells. MCM staining patterns change throughout the cell cycle and characteristic patterns can distinguish G1, S and G2 cells (Prasanth et al., 2004). The staining pattern of MCM

localization in Orc2 depleted cells is consistent with cells arrested in S-phase or G2 phase of the cell cycle, suggesting that the Orc2-depleted cells were not all arrested in G1 phase. Note G1 phase cells were also observed (data not shown). **B.** Dual color IF of chromatin bound MCM3 and centrosome-associated -tubulin in Orc2 depleted cells showed an S-phase pattern of MCM localization and duplicated centrosomes. Also note the MCM3 negative cell with duplicated centrosomes spread wide apart suggests that the cell is in very late S phase or G2 phase. Bar represents 5 M.

Figure S7 **Localization of heterochromatin associated proteins following Orc2 depletion A.** Immuno-staining with anti-HP1 antibodies show HP1 localization in heterochromatic foci (a) in both control siRNA treated cells and in Orc2 siRNA treated cells (72 hr. post transfection) (b). Immuno-localization of trimethylated K9-histone H3 was detected by immunofluorescence. Staining was comparable in control (a') and Orc2 siRNA treated cells (b'). Chromatin was stained with DAPI (blue, a'' & b''). Bar in A represents 5 M. **B,** Immunoblot analysis of whole cell extracts from cells transfected with Orc2 siRNA duplex or control luciferase siRNA at 72 hours post transfection. Efficacy of RNAi was assessed by immunoblotting to detect Orc2. HP1, HP1 and trimethyl K9 modification on histone H3 levels were comparable in control versus Orc2 siRNA treated cells suggesting that the HP1 and HP1 proteins were not degraded after Orc2 depletion. Luciferase siRNA treated cells were loaded in dilutions representing 25%, 50% and 100%, and Orc2 siRNA was at 100%. Note that more than 80% knockdown of Orc2 was achieved.

Figure S8. **Aberrant mitotic structures in Orc2 depleted cells. A.** Anti-□-tubulin staining in Orc2 siRNA treated cells revealed the increased number of apparent mitotic cells. Chromatin was stained with DAPI (red) and showed segregation and congression defects. Note the mitotic population is in a different focal length compared to the interphase population, which appeared as unfocussed cells in the background. Bar represents 10 □M. **B.** Phospho-H3 serine-10 antibody staining in control (a) and Orc2 depleted (b) cells both show characteristic decoration of the mitotic chromosomes. Chromatin was stained with DAPI (blue). Bar represents 5□M.

Figure S9. **Orc2 depletion results in spindle checkpoint mediated prometaphase arrest A.** Mitotic index analysis during a time course of control (luciferase siRNA treated) versus Orc2 siRNA treatement. Cells were stained with anti-□-tubulin and those cells with mitotic asters were counted and plotted as a percentage of total cells. **B.** Fraction of the mitotic population in different stages of mitosis in control versus Orc2 siRNA treated cells at 72 hrs post siRNA (N=1000 each). Note the dramatic increase in prophase+prometaphase cells (87% in Orc2 siRNA as compared to 32% in control). The 11% metaphase population in Orc2 siRNA includes the metaphases with several lagging chromosomes. Also note the very significant reduction in the number of anaphases and telophases in Orc2 siRNA treated cells (2% in Orc2 siRNA versus 28% in control) suggesting that the cells arrested in prometaphase and did not complete mitosis.

Figure S10 **Multiple centrosome-like structures in mitotic cells lacking Orc2. A,**HeLa cells were treated with Orc2-1 siRNA for 72 hr, and then round mitotic cells were fixed

and stained with auto-antibody against human centrosome (yellow) and □-tubulin (green). Dual labeling revealed that each aster had a centrosome structure (a). IF using □-tubulin (red) and DNA staining with DAPI (blue) showed multiple centrosomes in a cell with unaligned chromosomes (b). Scale bar denotes 5 □M. **B,** Treatment with Orc2 siRNA abolishes centrosome labeling with anti-Orc2 antibodies. Round, mitotic cells were isolated following RNAi treatment for 72 hr. with control (a; luciferase) and Orc2-1 (b) and Orc2-2 (c) siRNAs. The cells were stained with antibodies against □-tubulin (a, b, c) and Orc2 (a', b' c') and with the DNA dye DAPI (a", b", c"). The cells treated with Orc2 siRNA lacked Orc2 antibody labeling at the centrosomes. Scale bar denotes 5 □M.

In control RNAi treated mitotic cells, 87% contained bipolar spindles and stained positive for Orc2 at centrosomes; 8% had bipolar spindles and were negative; 1% were multipolar spindles and stained positive for Orc2 at centrosomes; 3% were negative; and 1% showed Orc2 labeling at some, but not all centrosomes. In the Orc2-1 RNAi treated, mitotic arrested cells, 20% had a bipolar spindle with aligned chromosomes and were Orc2 positive at centrosomes; 15% of the bipolar spindles were Orc2 negative; 1% had bipolar spindles with unaligned chromosomes and were Orc2 positive; 23% had bipolar spindles with unaligned chromosomes and were Orc2 negative; 31% showed multipolar asters and were Orc2 negative at centrosomes; 4% had multipolar spindles and partial Orc2 labeling at some centrosomes and the remaining 6% were negative. In Orc2-2 RNAi treated cells. 10% of cells had bipolar spindles with aligned chromosomes and were Orc2 positive at centrosomes; 6% were negative; 7% had bipolar spindles with unaligned chromosomes and were positive; 13% were negative; 40% had multipolar spindles and were negative; 13% had multipolar spindles with partial labeling for Orc2 at some

centrosomes; the remaining 11% were Orc2 negative at centrosomes and had other unusual spindles such as mono-polar.

Figure S11 **Orc2 depletion disrupted HP1 binding at centromeres in aberrant mitotic cells. A.** Prominent HP1 labeling at centromeres (a) in control (luciferase) siRNA treated cells compared to loss of these foci in Orc2 siRNA treated cells (72 hr. post transfection; b and c). DNA was stained with DAPI (blue). Scale bar 5 M.

Figure S12 **Spiral arrangement of condensin in aberrant mitotic chromosomes present in Orc2 depleted cells. A.** Round cells from either control or Orc2 siRNA treated cells were lysed with hypotonic buffer and the chromosomes were stained with DAPI and the associated condensin protein was detected by immunofluorescence using an antibody against non-SMC subunit of condensin I (h-CAP-G, red). Metaphase chromosomes of control (a) and Orc2 depleted cells (b). **B.** One arm of the sister chromatid from control (a) Orc2 (b and c) siRNA treated cells were stained with CAP-G antibody. Note the railroad track-like appearance of condensin in the control, normal sister chromatid compared to the spiral arrangement of condensin in the sister chromatid from Orc2 depleted cells. DNA was stained with DAPI (blue).

Supplementary Note 1: PCNA knockdown results in cells undergoing apoptosis.

To address whether the mitotic arrest following Orc2 knockdown was a consequence of a defect in DNA replication, depletion of a crucial DNA replication protein PCNA was studied (Supplementary Figure S13). Compared to control cells, the depletion of PCNA

resulted in an accumulation of S-phase cells and an increase in G2/M and sub 2C population, as observed flow cytometry of DNA content (Supplementary Figure S13B). Following siRNA transfection for 72 hr., more than 90% of the cells lacked PCNA as determined by IF and these cells did not incorporate BrdU in a 10 min. pulse label (Supplementary Figure S13) or a 24 hr. long-term label (data not shown). Importantly, we did not observe any increase in the number of mitotic cells (by □-tubulin staining) in the population of PCNA siRNA treated cells. Thus defective DNA replication does not ineluctably cause an aberrant mitotic arrest. We did, however, observe prominent DAPI dense, fragmented nuclei typical of apoptotic cells following PCNA depletion. Immunoblots using antibodies against caspases-7 and –9 clearly showed the cleaved and activated forms of these caspases following PCNA depletion, similar to cells treated with the drug etoposide (Supplementary Figure S13A). In case of Orc2 depletion we observed significant caspase activation only after 96 hours which could well be a consequence of DNA replication defect (Supplementary Figure S14).

Figure S13 **Loss of PCNA triggers apoptosis. A,** Immunoblot demonstrating depletion of PCNA following siRNA treatment of HeLa cells for 72 hrs. "Con" represents control luciferase siRNA treated cells. Etoposide marked lanes represent cells treated with this drug at 50 □M for 28 and 56 hours to induce apoptosis, as positive control for caspase induction. Caspase-7 and caspase-9 immunoblots reveal apoptosis in cells lacking PCNA. **B,** Assessment of DNA content by propidium iodide staining and flow cytometry in control and PCNA siRNA treated cells. Cells lacking PCNA arrested in S-phase and had and altered G1 to G2/M ratio, as well as sub-2C DNA. **C,** Immuno-labeling of cells

transfected with PCNA siRNA duplexes showed considerable reduction of signal with PCNA antibody, corroborating the immunoblot (b). The cells lacking the PCNA labeling did not show BrdU incorporation during a 10 minute (shown) and 24 hr (not shown) pulse label (b'), whereas luciferase control showed uniform PCNA labeling (a) and complete colocalization with BrdU based on a 10 min BrdU pulse (a'). Nomarski images are a'' and b''. Scale bar 10 μM.

Figure S14 **Apoptosis in Orc2 depleted cells.** Immunoblot of whole cell extracts from cells transfected with Orc2 siRNA duplex or control (luciferase) siRNA and harvested at different times post-transfection. Cells were transfected at 0 hr, 24 hrs and 48 hrs. The cells were initially about 20% confluent. "Con" represents control siRNA treated cells. Etoposide marked lanes represent cells treated with this drug at 50 μM for 28 and 56 hours to induce apoptosis, and represented a positive control for caspase induction. Caspase-9 immunoblot revealed the smaller molecular mass forms of caspase-9, indicating apoptosis in cells lacking Orc2 after 96 hours and 120 hours.

Supplementary Note 2. Immunofluorescence and antibodies.

The following section contains detailed information about antibodies and immunofluorescence methods that were used in the paper. Cells were rinsed briefly in phosphate buffered saline (PBS) pH 7.4 then fixed for 15 min in 1.0% formaldehyde in PBS (pH 7.4) (optimal for cell-cycle localization studies of Orc2) or for 7 min in cold methanol (-20ºC) for cytoskeletal (α-tubulin) and centrosomal (γ-tubulin and Orc2) antibodies. Cells were permeabilized in PBS +0.5% Triton-X-100 + 1% goat serum for 7-

10 min on ice, and primary antibodies were added for 1 hr at room temperature: Anti-Orc2 pAb205 (1:400), Anti-Orc2 mAb920 (1:200) {anti human Orc2 mAb was raised against a recombinant GST-fusion protein consisting of the N-terminal 303 amino acids of human Orc2. The epitope was determined to be between aa 124 and 143 (Siddiqui and Stillman, unpublished observations) and this peptide was used for blocking experiments}, α-tubulin mAb (1:800, Sigma), β-tubulin mAb (1:800, Sigma), Anti-BrdU (1:750, Sigma), Anti-PCNA (PC10 mAb) (1:150), Anti-MCM3 pAb738 (1:400), Anti-HP1α (1:75, Chemicon), Anti-HP1β (1:700; Chemicon), Anti-CAP-G pAb (1:2500, Ono et al., 2003), Anti-Centromere, AnaC (Sigma), and anti-CenpF (1:2000; Calbiochem). Cells were rinsed thrice in PBS + 1% goat serum, then secondary anti-species-specific antibodies (Jackson Laboratories, West Grove, PA) were added for 30 min at room temperature: goat anti-mouse (GAM) IgG_1-Texas Red (1:1000), GAM IgG H+L Texas Red (1:500), GAM IgM-Cy5 (1:1000), goat anti-rat IgG-fluorescein (1:1200). DNA was stained with 4'. 6-diaminido-2-phenylindole (DAPI). Cells were mounted in medium containing 90% glycerol, 10% PBS pH 8.0 plus 0.1% paraphenylenediamine. Cells were examined using a Zeiss Axioplan 2i fluorescence microscope (Carl Zeiss Inc., Thornwood, NY) equipped with Chroma filters (Chroma Technology, Brattleboro, VT). OpenLab software (Improvision, Boston, MA) was used to collect digital images from a Hamamatsu ORCA cooled CCD camera.

For visualizing PCNA (Martini et al., 1998), MCM, HP1 and Orc2 labeling on the chromatin, cells were pre-extracted in 0.5% Triton-X in Cytoskeletal buffer (10mM PIPES, pH 7; 100mM NaCl, 300mM Sucrose, 3mM $MgCl_2$) for 5 min on ice, followed by 1.7% PF fixation. For PCNA and MCM an additional step of methanol extraction was

used. Rest of the procedure was followed as described above. For BrdU labeling, cells were incubated in BrdU (10μM for 10 min or 100nM for 24 hrs), fixed in 2%PF followed by permeabilization in 0.5% Triton X-100. DNA was denatured with 4N HCl for 30 min at room temperature (RT). Rest of the IF was as above.

For immunoblots Anti-Orc2 rabbit pAb (1:4000), Anti-Orc3 goat pAb (1:700, Novus biologicals), α-tubulin (1:10,000, Sigma), Anti-Orc1 rabbit pAb769 (1:150), Anti-Orc6 rabbit pAb982 (1:1000), Anti-Cdc6 mouse mAb (1:500, Neomarkers), Anti-MCM-2 pAb732, MCM-3 pAb738, MCM-4 pAb739 (all 1:2000), Anti-MCM-7 mouse mAb (1:1000, Neomarkers), Anti-PCNA mAbPC10 (1:1500), Anti-MEK (1:500, BD Pharmingen), Anti-HP1α (1:1500, Chemicon), Anti-HP1β (1:2500; Chemicon), Anti-HP1γ(1:2500; Chemicon), Anti-trimethyl K-9 H3 (1:2000; Upstate biotechnology), Anti-caspase-7 mouse mAb and Anti-caspase-9 mouse mAb (both at 1:1000, gift from Y. Lazebnik, Cold Spring Harbor Laboratory) were used.