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Supplemental Information

A WD-Repeat Protein Stabilizes

ORC Binding to Chromatin

Zhen Shen, Kizhakke M. Sathyan, Yijie Geng, Ruiping Zheng, Arindam Chakraborty, Brian Freeman, Fei Wang, Kannanganattu V. Prasanth, and Supriya G. Prasanth

Supplemental Figures **Page**

SUPPLEMENTAL FIGURES

Figure S1. ORCA is a highly conserved, novel, ORC-binding protein. (A) Percentage identity and percentage similarity of ORCA between humans and other organisms based on protein alignment. (B) ORCA and ORC interact with each other. T7-ORCA and HA-Orc2 were co-transfected in human cells for co-IP experiments. IP using T7 antibody could efficiently pull down Orc2 and similarly IP using HA antibody could bring down T7-ORCA.

A

Organism	Identity	Similarity	Gaps
Homo sapiens	100.0%	100.0%	0.0%
Pan troglodytes	99.2%	99.4%	0.3%
Mus musculus	77.8%	86.9%	0.8%
Canis familiaris	87.5%	91.8%	1.5%
Xenopus laevis	42.5%	59.4%	10.7%
Gallus gallus	45.3%	59.2%	13.7%
Danio rerio	36.5%	53.5%	15.0%
Drosophila melanogaster	37.3%	49.2%	0.0%
Nematostella vectensis	28.9%	48.1%	5.5%

B

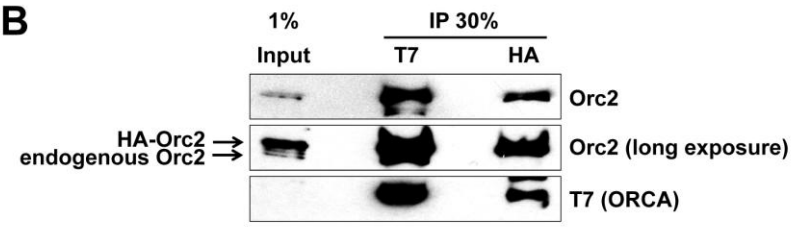


Figure S2. ORCA is highly dynamic. Time-lapse live cell imaging of YFP-ORCA in MCF7 cells. The images were obtained using a Delta Vision optical sectioning deconvolution instrument (Applied Precision) on an Olympus microscope with a 63X 1.42NA objective. Scale bar represents 10μm.

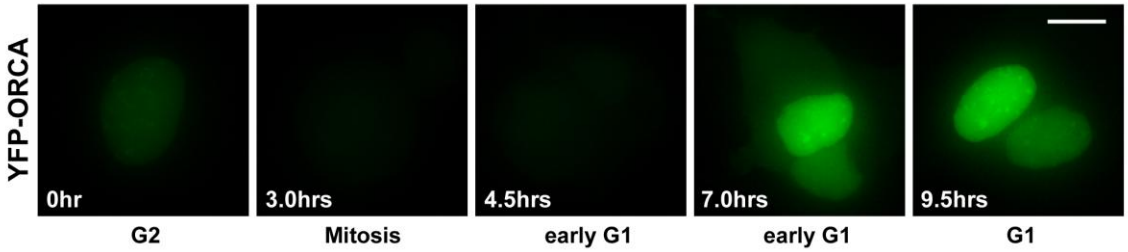


Figure S3. ORCA localizes to centromeres. (A) Immunolabeling using antibodies against centromere protein AnaC shows co-localization with YFP-ORCA at centromeric heterochromatin in MCF7 cells. Figure 4B is shown again (lower panel) with pearson co-efficient of correlation demonstrating significant co-localization. (B) Dynamics of YFP-ORCA and Orc2 during G1 and S phase in human U2OS cells. CAF1 (p150) is shown as an S-phase marker. (C) YFP-ORCA localizes to centromeres during mitosis. DNA was stained with DAPI (blue). Scale bar represents 10μm.

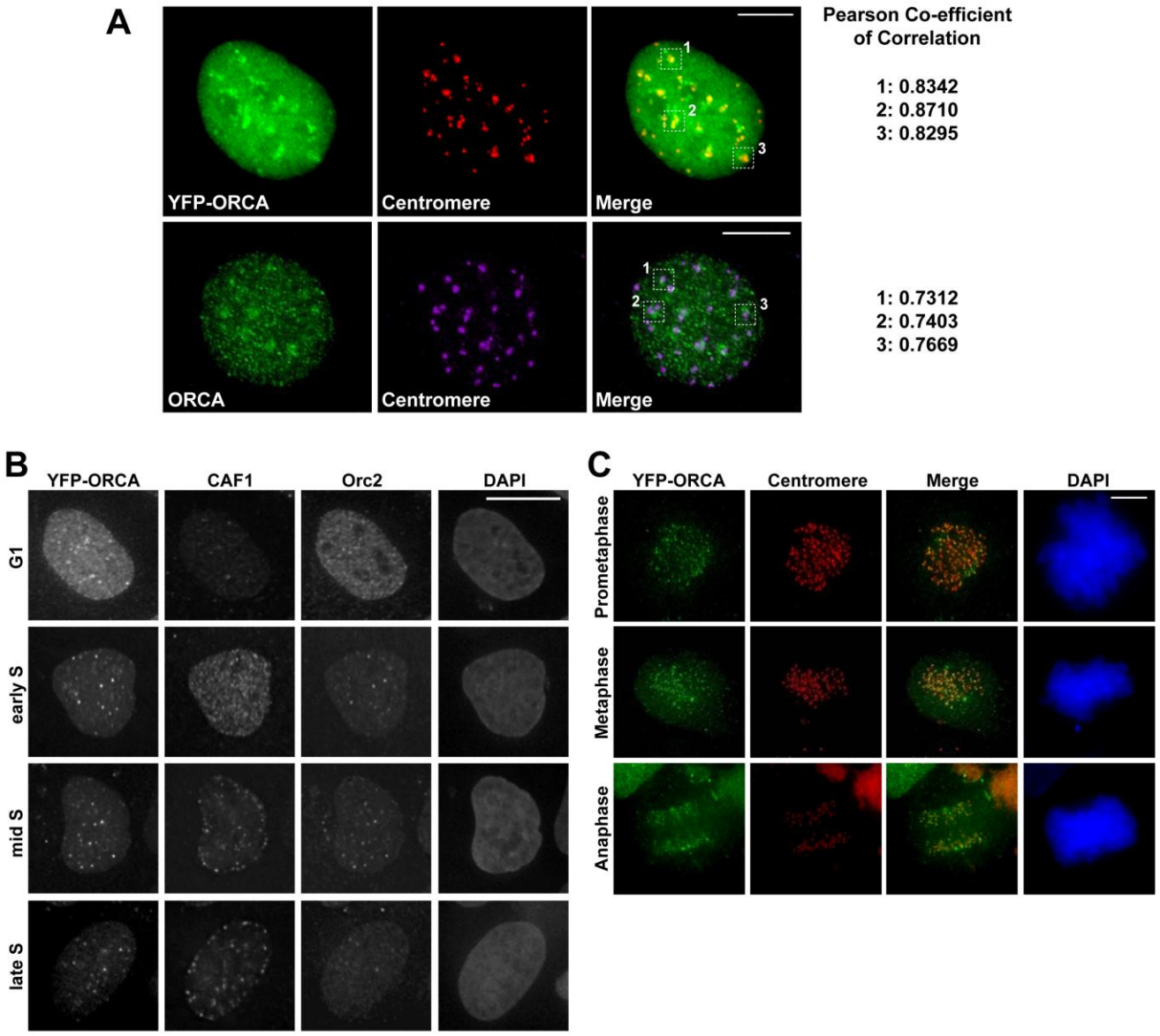


Figure S4. WD domain of ORCA is essential for chromatin binding. (A) Expression analysis of YFP-ORCA mutants in human cells following formaldehyde fixation shows the punctate localization of ORCA in cells containing an intact WD domain. (B) YFP-ORCA.128-647 localizes to heterochromatin as evident by immuno-localization with HP1α. DNA was stained with DAPI (blue). Scale bar represents 10μm. (C) IP using Orc2 mAb in extracts treated with (+) or without (-) DNaseI. Note the reduction of ORCA and Orc3 interaction with Orc2 in DNaseI-treated samples. Longer exposure of ORCA immunoblot is shown for input. '*' denotes the cross-reacting band.

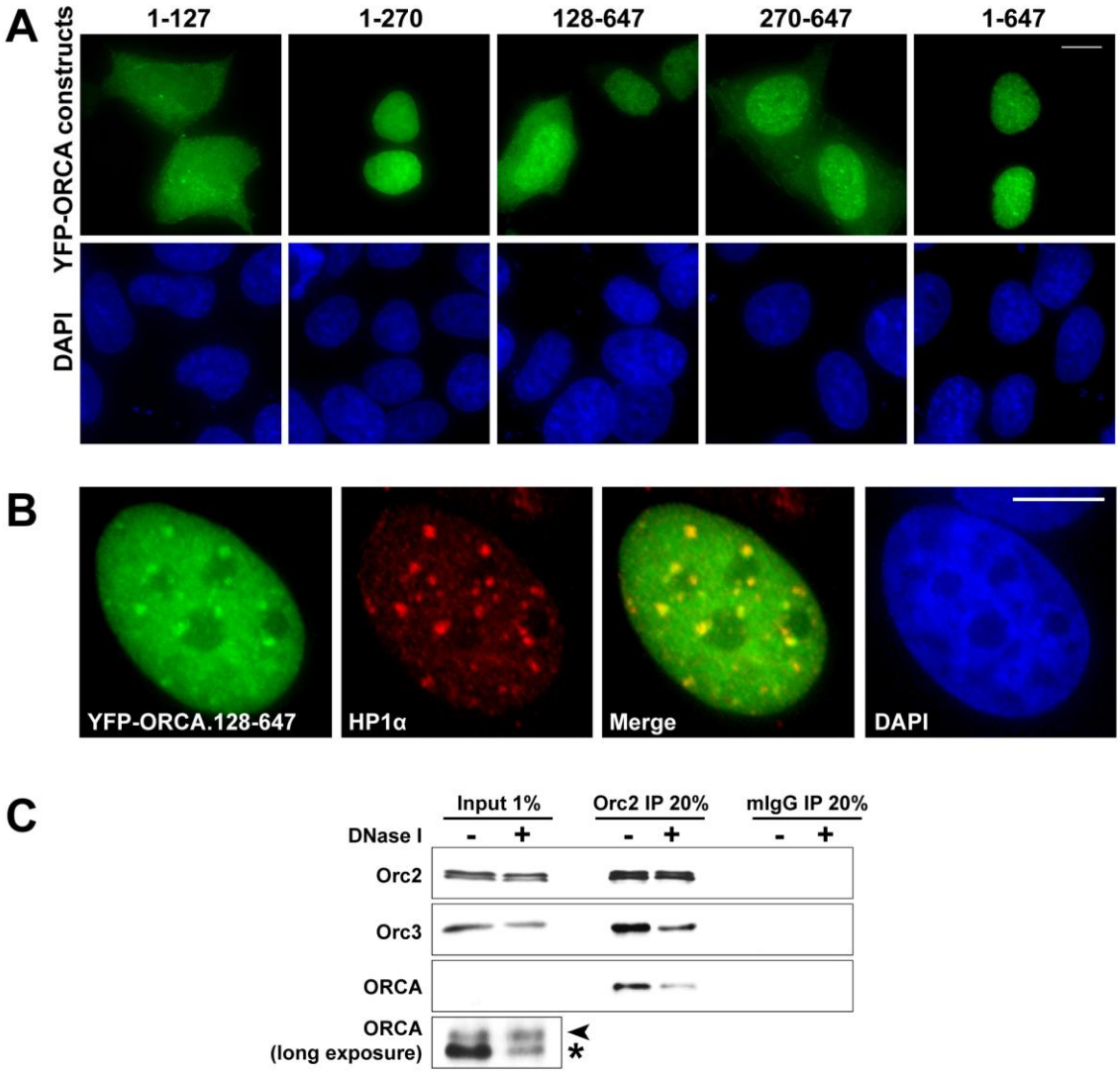
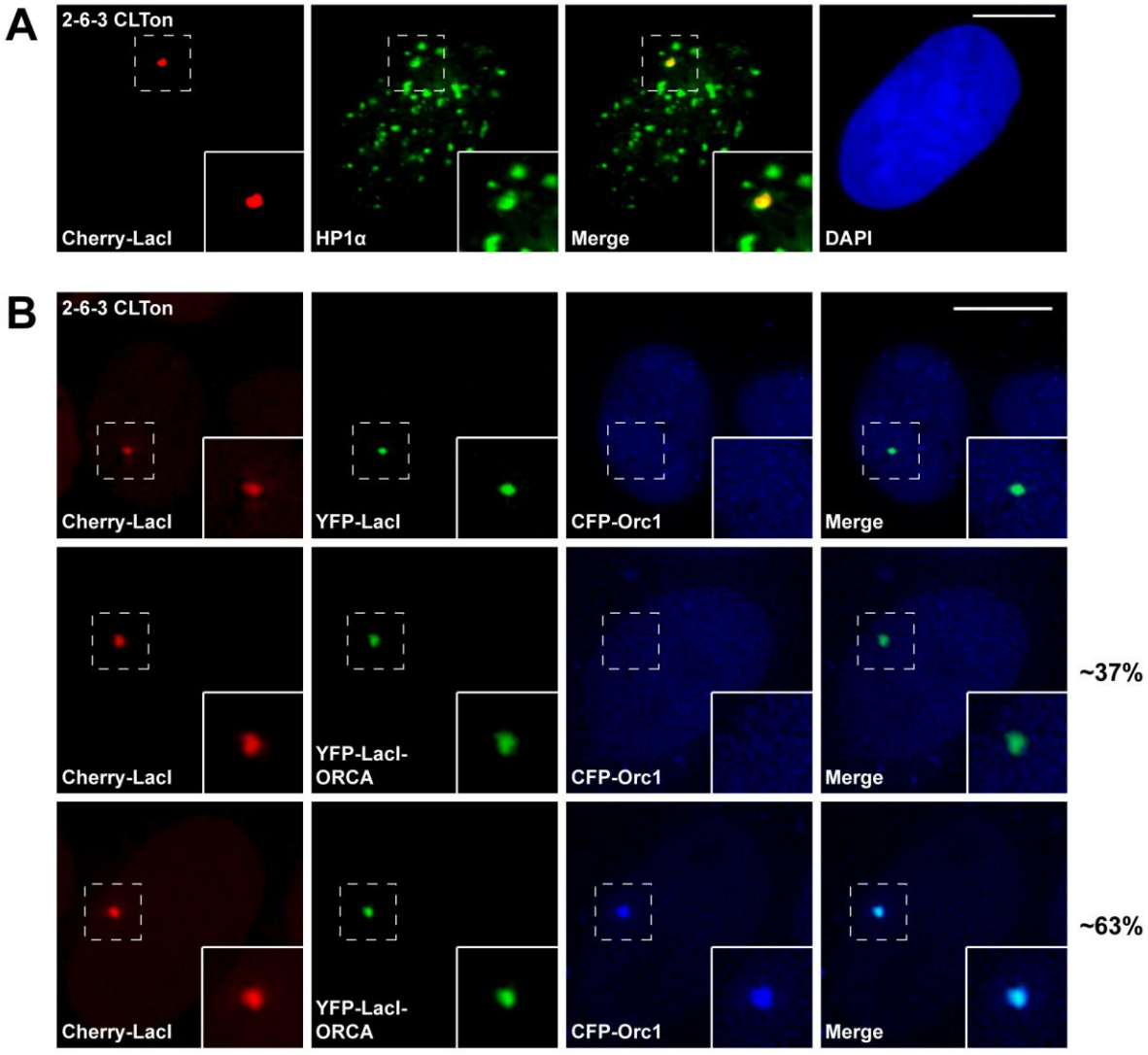
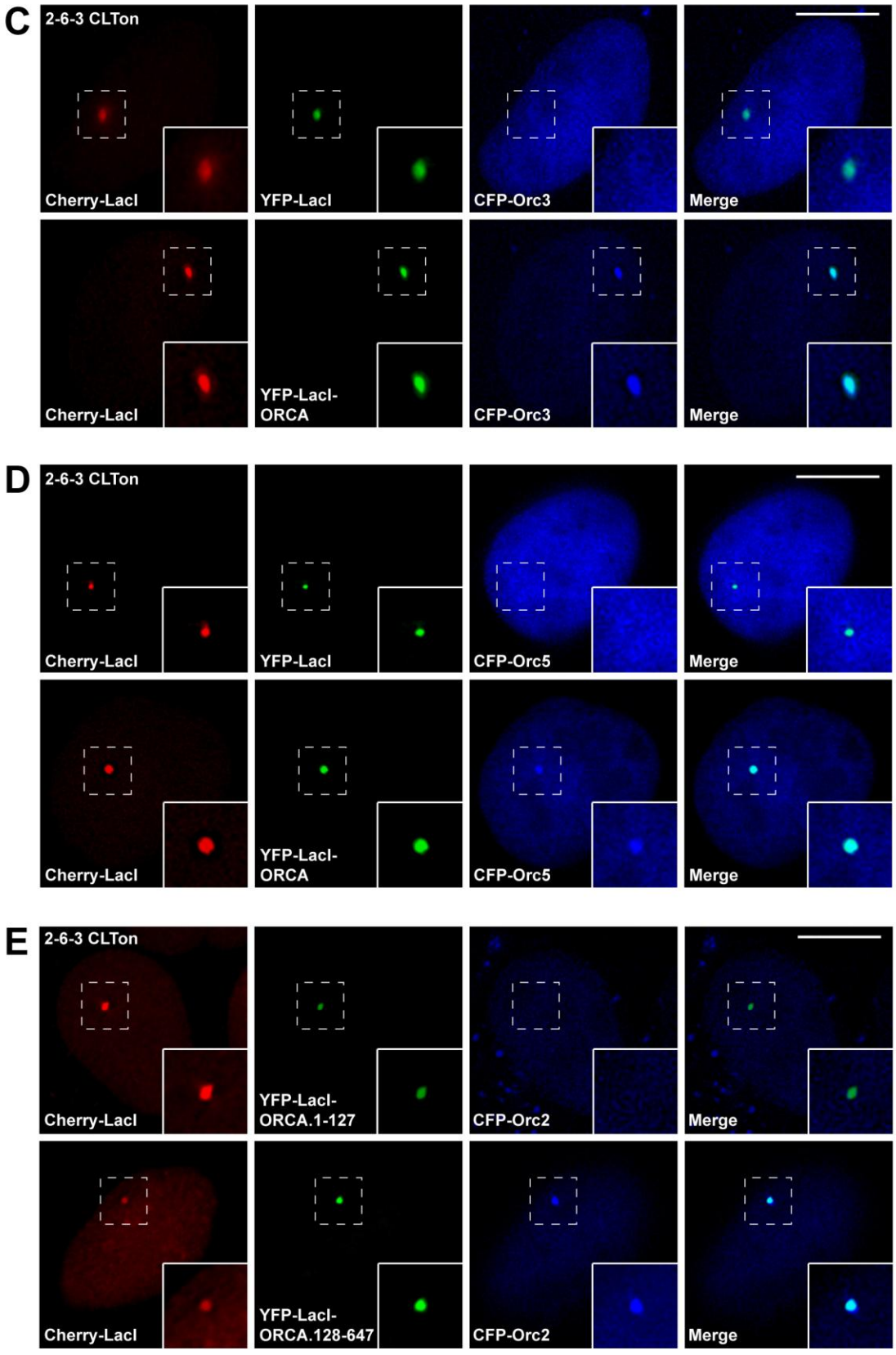


Figure S5. ORCA tethers ORC to chromatin. (A) The artificially generated chromatic locus in 2-6-3 CLTon cells is heterochromatic and shows accumulation of HP1α. ORCA recruits various ORC subunits to heterochromatic locus (B) Orc1, (C) Orc3 and (D) Orc5. (E) Co-transfection of YFP-LacI-ORCA.1-127 or YFP-LacI-ORCA.128-647 with CFP-Orc2 shows that YFP-LacI-ORCA.128-647 recruits Orc2 to the locus. (F) Tethering YFP-LacI-ORCA to the locus results in accumulation of MCM. Note the bottom cell (cell number 1) lacking YFP-LacI-ORCA does not show presence of MCM3 at the locus. DNA was stained with DAPI (blue). Scale bar represents 10μm.

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Cherry-LacI
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2-6-3 CLTon
Cherry-LacI
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CFP-Orc5
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YFP-LacI-ORCA
CFP-Orc5
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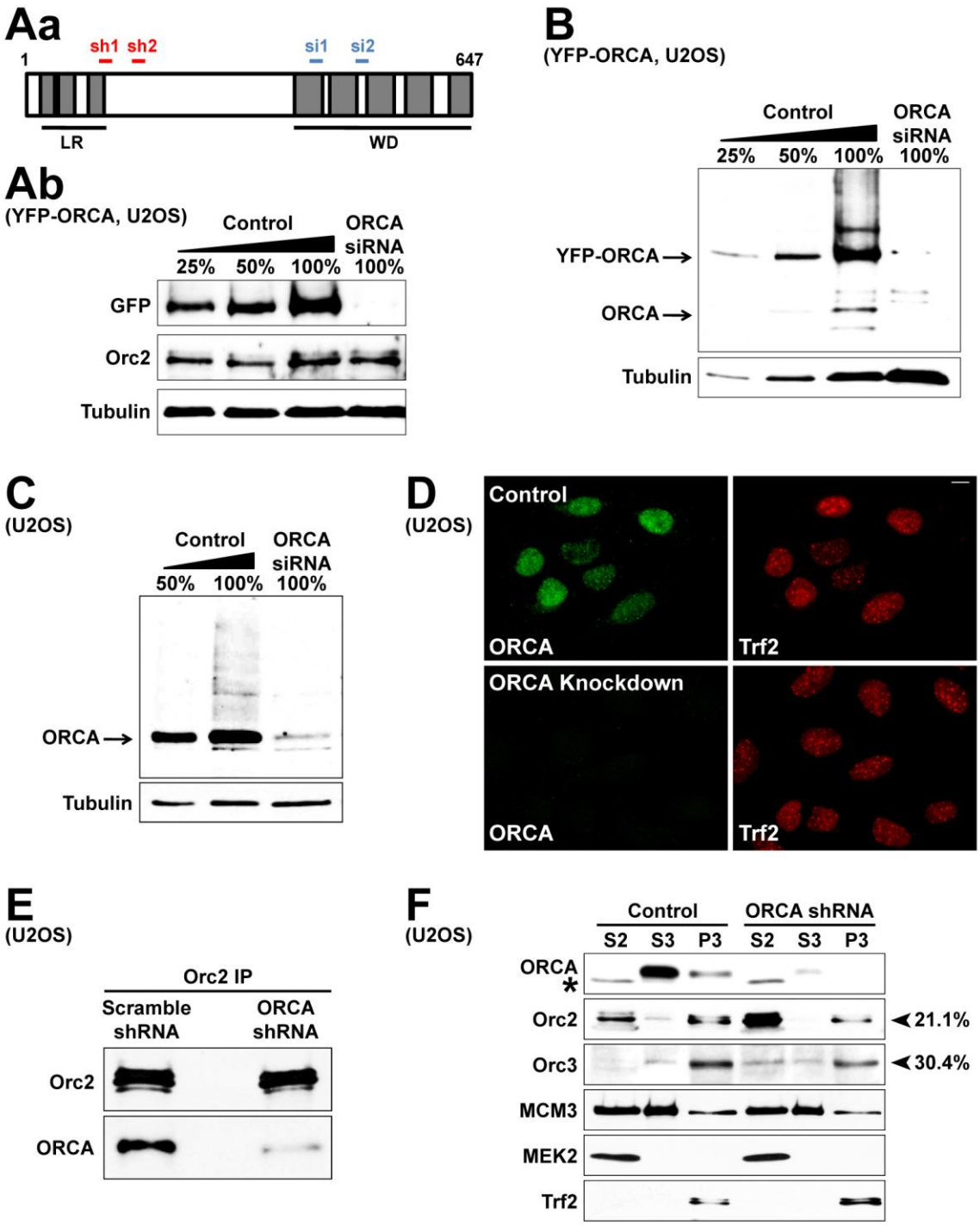
YFP-LacI-ORCA.1-127
CFP-Orc2
Merge
Cherry-LacI
YFP-LacI-ORCA.128-647
CFP-Orc2
Merge

Figure S6. ORCA is required for loading ORC to chromatin. (A) a. Schematic representation of the location of short hairpin 1 & 2 and siRNA oligo-1 and -2 against ORCA used in the present study. b. SiRNA oligonucleotide treatment of human cells using a pool of ORCA -1 and -2 oligos in YFP-ORCA cells shows the efficient depletion of ORCA. Note that Orc2 whole cell levels remain unchanged. (B and C) Immunoblot using ORCA antibody in YFP-ORCA cells or U2OS cells treated with ORCA-1 and -2 siRNAs show significant depletion of endogenous as well as YFP-ORCA from human cells. Note the disappearance of high molecular weight modifications of ORCA upon ORCA siRNA. (D) Knockdown of ORCA shows reduced ORCA in human cells as evident by immunofluorescence analysis. Scale bar represents 10μm. (E) Orc2 IP in ORCA depleted cells. Note the residual ORCA present in ORCA-depleted extracts forms complex with ORC. (F) Chromatin fractionation in control and ORCA shRNA-1 treated U2OS cells. Note that in ORCA depleted cells, Orc2 shows reduced binding to chromatin and a concomitant increase in S2 fraction. (G & I) Depletion of ORCA in WI38 as well as hESCs shows dramatically reduced proliferation rates. Error bar (Figure S6G) represents standard deviation from three independent experiments. (H) Orc2 and PCNA immunofluorescence in control and ORCA siRNA treated WI38 cells. (J) Immunofluorescence following detergent pre-extraction using Orc2 antibody in control and ORCA shRNA treated hESCs shows reduced levels of chromatin associated Orc2 in ORCA depleted hESCs. (K) Immunoblot analysis following depletion of ORCA using shRNA in hESCs. Note the increase in Cyclin E levels. (L) Chromatin fractionation in ORCA depleted hESCs and immunoblot analysis of ORC and MCM. Note the significant reduction of Orc1 and Orc2 and reduced MCM3 in P3 in ORCA depleted cells. SF2/ASF, a splicing factor is shown as loading control. Note that ORCA is found predominantly in the P3 fraction (chromatin/nuclear insoluble) in hESCs, whereas it is enriched in S3 (chromatin soluble) in human cancer cells, suggesting that ORCA is more strongly associated with the chromatin in hESCs. DNA was stained with DAPI (blue). Scale bar represents 10μm.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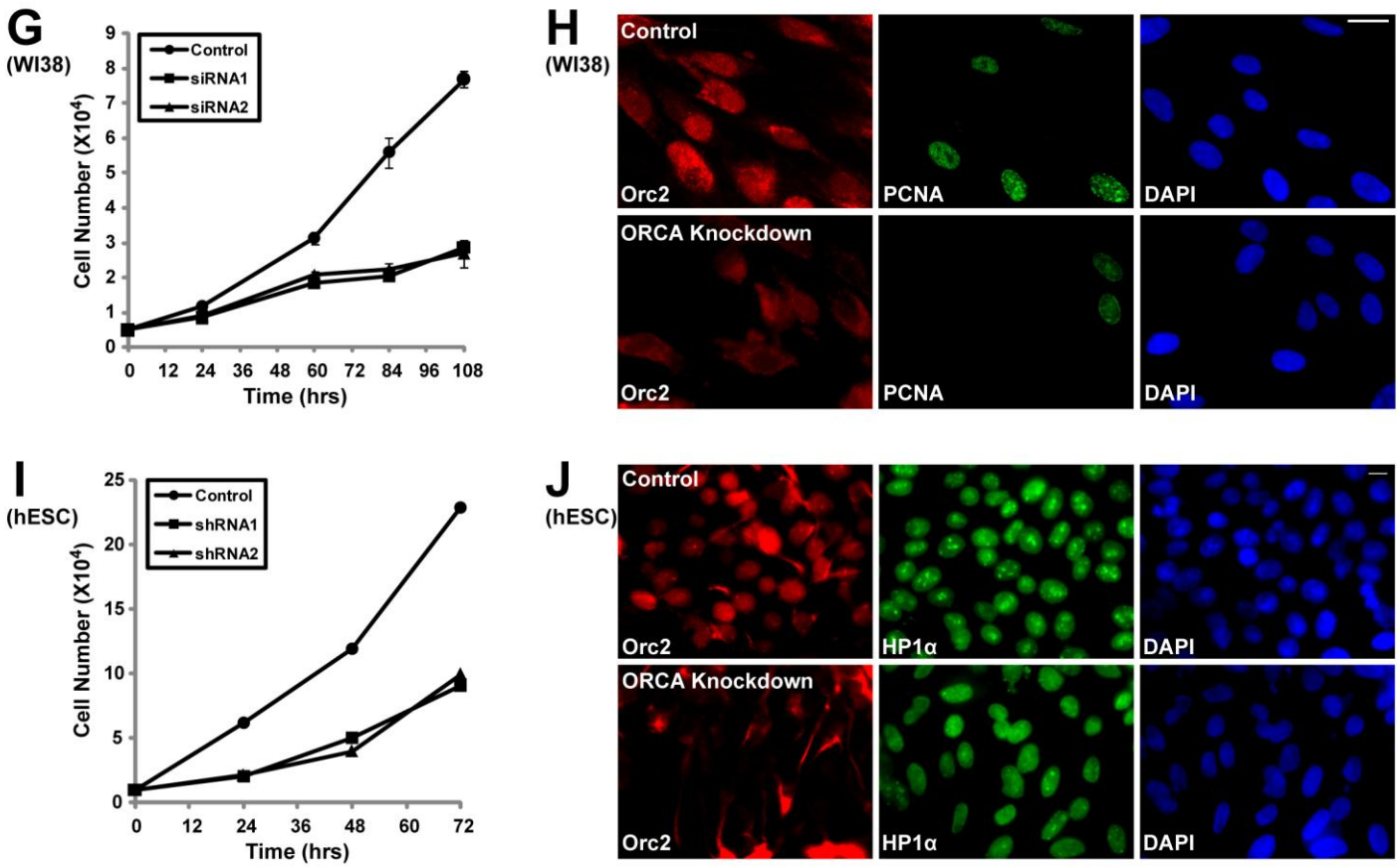
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MCM3
MEK2
SF2/ASF
5.2%
0.5%
24.2%
61.1%

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

ORCA sequences and plasmid constructs:

Human ORCA cDNA was cloned into pEYFP-C1 with CMV promoter (Clontech) and pCGT vector to generate YFP-ORCA and T7-ORCA. ORCA mutants were obtained using PCR from ORCA cDNA, (aa positions: 1-127, 1-270, 128-647 and 270-647) and also cloned into pEYFP or pCGT vectors. After confirmation by sequencing, constructs were used for transfection in human cells for immunofluorescence or immunoprecipitation experiments.

pEGFP-Lac repressor vector was a kind gift from Miroslav Dundr (Kaiser et al., 2008). pEYFP-Lac repressor was constructed by replacing GFP with YFP. pEYFP-LacI-ORCA was generated by ligating cDNA of ORCA into the pEYFP-LacI vector.

NCBI accession numbers for ORCA sequences: *Homo sapiens (*NP_690852.1), *Pan troglodytes* (XP_519556.2), *Mus musculus* (BAC39318.1), *Canis familiaris* (XP_536851.2), *Xenopus laevis* (AAH74207.1), *Gallus gallus* (XP_415759.2), *Danio rerio* (XP_001335047.1), *Drosophila melanogaster* (NP_723702.1), *Nematostella vectensis* (EDO47466.1). Sequence alignment was conducted using the website http://www.ebi.ac.uk/Tools/clustalw/

Live cell microscopy:

Human cells stably expressing YFP-ORCA or transiently transfected with 2µg pEYFP-ORCA were used for live-cell imaging. The cells were transferred to a FCS2 live-cell chamber (Bioptechs Inc.) mounted onto the stage of a Delta Vision optical sectioning deconvolution instrument (Applied Precision) on an Olympus microscope and kept at 37^{o}C in L-15 medium (minus phenol red) containing 30% FBS. Time-lapse images acquired with a 63X 1.42 N.A. objective lens were

captured with a Coolsnap CCD camera.

Immunofluorescence and immunoblots:

Cells were either fixed in 2% formaldehyde for 15 min at room temperature and then permeabilized by phosphate-buffered saline (PBS) + 0.5% Triton X-100 for 7 min on ice, or pre-extracted in CSK buffer (10mM PIPES pH 7.0, 100mM NaCl, 300mM sucrose, 3mM $MgCl_2$) + 0.5% Triton X-100 for 5 min on ice and then fixed with 2% formaldehyde. Chilled methanol was applied afterwards if needed, according to the specific requirement for the antibodies. Cells were then blocked in PBS + 1% normal goat serum (NGS), and incubated with primary antibodies in a humidified chamber for 1 hr and followed by secondary antibody for 45 min. All washes between each step were done in PBS + 1% NGS. DNA was stained with DAPI. Cells were mounted in Vectashieled (Vector Laboratories Inc.). Cells were examined using a Zeiss Axioimager z1 fluorescence microscope (Carl Zeiss Inc.) equipped with Chroma filters (Chroma Technology). Axiovision software (Zeiss) was used to collect digital images from a Hamamatsu ORCA cooled CCD camera. Cells were also examined on the Delta Vision optical sectioning deconvolution instrument (Applied Precision) on an Olympus microscope. The antibodies used for immunofluorescence were anti-Orc2 (1:400), anti-MCM3 (1:400), anti-PCNA mAbPC10 (1:150), anti-HP1α (1:75, Chemicon), anti-Centromere AnaC (1:10, Sigma-Aldrich), anti-Trf2 mAb4A794 (1:200, Upstate) and anti-CAF1 (p150, ss1, 1:400).

For immunoblots, anti-Orc1 mAbpKS1-40 (1:500), anti-Orc2 pAb205 (1:2000), anti-Orc3 goat pAb (1:500, Abcam), anti-Orc4 goat pAb (1:500, Abcam), anti-Orc5 pAb (1:500, BD Pharmingen), anti-Orc6 pAb982 (1:500), anti-Cdt1 guinea pig GP47 (1:1000), anti-Geminin (1:1000, Santa Cruz), anti-Cdc6 mAb37 (1:100), anti-MCM2 (1:1000), anti-MCM3 (1:1000), anti-PCNA mAbPC10

(1:1500), anti-Cyclin E (1:1000, Upstate), anti-Cyclin B (1:500, Oncogene Ab-3), anti-MEK2 (1:500, BD Pharmingen), anti-α-tubulin (1:10,000, Sigma-Aldrich) were used.

Nuclear extracts:

Cells were washed with cold PBS and extracted in hypotonic buffer (10mM HEPES-NaOH pH 7.9, 10mM KCl, 2mM $MgCl_2$, 0.34M sucrose, 10% glycerol, 0.1% Triton X-100) plus a supplement consisting of 1mM DTT, protein phosphatase inhibitors (10mM NaF, 1mM Na_3VO_4, 1mM $Na_2H_2P_2O_7$), protease inhibitor cocktail tablets (Roche) at 4^{o}C for 5 min. Nuclei were collected by centrifugation (1,500g, 5 min) and then extracted in nuclear extraction buffer (20mM HEPES-NaOH pH 7.9, 2mM $MgCl_2$, 1mM EGTA, 25% glycerol, 0.1% Triton X-100) plus a supplement consisting of 1mM DTT, protein phosphatase inhibitors, protease inhibitor cocktail tablets and 400mM NaCl at 4^{o}C for 30 min. The supernatant was collected after high-speed centrifugation (12,000 rpm, 5 min) and dialyzed in dialysis buffer containing 150mM NaCl overnight.

Immunoprecipitation:

Nuclear extract was incubated with GammaBind G Sepharose resin (Amersham) at 4^{o}C for 45 min for pre-clearing. Antibodies were added into the supernatant after the removal of the resin, and binding was carried out at 4^{o}C overnight. Resin was then added for 1 hr to capture the complex, washed three times in nuclear extraction buffer, and resuspended in Laemmli buffer for analysis.

If elution of the complex was needed, extract was incubated with antibody-conjugated resin overnight after pre-clearing. The resin was then washed thrice with TBS and incubated with the appropriate peptide at 30^{o}C for 15 min. The eluate was collected by low-speed centrifugation (5000 rpm, 1 min) and elution was repeated twice.

Gel filtration and glycerol gradient sedimentation:

Nuclear extract was subject to size exclusion chromatography using a Superdex-200 column (GE Inc.) at 0.25 ml/min and fractions (600µl each) were collected and precipitated by trichloroacetic acid. After centrifugation at 14,000 rpm for 5 min, the pellet was washed with acetone twice and resuspended in Tris pH 8.7 and Laemmli buffer for immunoblots.

For glycerol gradient sedimentation, IP eluate or marker (200µl) was layered on 10%-50% glycerol gradient (buffer: 27mM Tris pH 8.0, 100mM NaCl, 1mM EDTA, 0.1% NP-40, protease inhibitors). The mixture was centrifuged at 48,000 rpm for 16 hrs. Fractions (200µl each) were collected and prepared for immunoblots as described above.

Synchronization and flow cytometry:

To synchronize cells at G1/S boundary, 2mM thymidine was added. After 24 hrs, cells were washed thrice with fresh medium, grown for 12 hrs, and incubated with 2mM thymidine for an additional 24 hrs. Cells were then released, and aliquots were taken every 3 hrs for flow cytometry and chromatin fractionation. To arrest cells in mitosis, cells were treated with 50ng/ml nocodazole for 14-16 hrs.

For flow cytometry, cells were collected and washed in cold PBS, resuspended in PBS + 1% NGS, and fixed in chilled ethanol overnight. Cells were then washed and resuspended in PBS + 1% NGS with 120µg/ml propidium iodide (PI) and 10µg/ml RNase A for 30 min at 37^{o}C. DNA content was measured by flow cytometry.

Chromatin fractionation:

Detailed procedure was described previously (Mendez et al, 2000). To isolate chromatin, cells were resuspended in buffer A (10mM HEPES-NaOH pH 7.9, 10mM KCl, 1.5mM $MgCl_2$, 0.34M sucrose, 10% glycerol, 1mM DTT) plus protease inhibitor cocktail tablets and 0.5% Triton X-100 for 10 min on ice and centrifuged (1,300g, 5 min). Supernatant (S1) was then centrifuged at a high speed (20,000g, 5 min) to get S2. Nuclei (P1) were incubated with micrococcal nuclease (0.5U/ml micrococcal nuclease [Sigma-Aldrich] in buffer A plus 1mM $CaCl_2$) for 30 min at 37^{o}C and then 30 min on ice. S3 was collected by low-speed centrifugation. Pellet (P2) was then incubated with buffer B (3mM EDTA, 0.2mM EGTA, 1mM DTT) plus protease inhibitor cocktail tablets for 30 min on ice, and centrifuged again to get P3. WI38 cells were treated with 0.5% Triton-X in CSK for 5 min on ice, and the soluble fraction (S) was separated from the pellet (P) by centrifugation.

hES cell culture:

Federally registered hESC line H9 was purchased from WiCell Research Institute and routinely maintained under feeder conditions (Thomson et al., 1998). The culture medium consists of DMEM/F12 with 20% knockout serum replacement (KSR), 1 mM glutamine, 1% non-essential amino acid, 0.1 mM β-mercaptoethanol, and 4ng/ml bFGF (Invitrogen). For feeder-free cultures, cells are cultured on plates coated with Matrigel (BD Biosciences) in the presence of conditioned medium from MEFs, which replaces the MEF feeders (Xu et al., 2001). The experiments were conducted with H9 cells between passage 30 and 40. To infect hESCs, lentiviruses were mixed with MEF-CM, and the mixture was incubated with H9 cells (approximately 1000,000) for 16 hrs. To improve the efficiency of infection, Polybrene (Sigma-Aldrich) was added to the infection medium

to 6μg/ml. Virus-containing MEF-CM was replaced with complete feeder-free growth medium with 1μg/ml puromycin. Cells were continuously cultured for 7 days in puromycin containing medium. For proliferation assay the cells after selection were plated, and the cell number was counted 6 days after plating.

hES cells chromatin fractionation:

Chromatin fractionation of hESCs was conducted as described previously (Nimura et al, 2006) with minor modifications. ES cells were resuspended in nuclear isolation buffer (10mM Tris-HCl pH 7.5, 60mM KCl, 15mM NaCl, 1.5mM $MgCl_2$, 1mM $CaCl_2$, 0.25M sucrose, 10% glycerol, 1mM DTT) plus protease inhibitors and 0.15% NP-40 for 10 min on ice and centrifuged (1,300g, 5 min). Supernatant (S1) was then centrifuged at a high speed (20,000g, 5 min) to get S2. Nuclear pellets (P1) were incubated with 0.5U/ml micrococcal nuclease (Sigma-Aldrich) in nuclear isolation buffer with 100mM NaCl for 20 min at 37^{o}C and then 10 min on ice. 10mM EDTA was then added for 10 min incubation. S3 was separated from P3 by low-speed centrifugation.

SUPPLEMENTAL REFERENCES

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