Supplementary Information for

Orc6 is a component of the replication fork and enables efficient mismatch repair

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This PDF file includes:

  Supplementary text
  Figures S1 to S4
  Tables S1 to S2
  Legend for Dataset S1
  SI References

Other supplementary materials for this manuscript include the following:

  Dataset S1
MATERIALS AND METHODS

Cell lines and transfection

Human cell lines U2OS, A549, and HEK293T were grown in DMEM containing high glucose and supplemented with 10% fetal bovine serum (FBS). Plasmids and siRNAs were delivered using Lipofectamine 2000 or 3000 and Lipofectamine RNAiMax (Invitrogen), respectively. U2OS cells stably expressing HA-Orc6 or DD-Orc6 and Orc6 knockout cells were maintained in media containing selective antibiotics. Cell synchronization was done by nocodazole arrest for M and G1 phase samples, and by thymidine block for G1/S, S and G2 phase samples.

ORC6 CRISPR KO U2OS clones were made by transiently transfecting pT3.5 Caggs-FLAG-hCas9, two of the ORC6 gRNA (pCR4-TOPO-U6-gRNA backbone), pcDNA-PB7, and pPBSB-CG-Luc-GFP-Puro. KO clones developed by single cells were then selected by puromycin. Depletion of Orc6 was confirmed by western blotting.

For all siRNA experiments, siGl3, siRNA targeting firefly luciferase gene, was used as control and labeled as control-si. For control group in shRNA experiments, shGFP targeting green fluorescence protein was used.

Isolation of Proteins On Nascent DNA (iPOND)

A modified version of iPOND (1), described as aniPOND (2), was used. In brief, 1.5 x 10^8 HEK293T cells were pulsed with 10μM of EdU for 10 min. For mature DNA samples, cells were chased with 10μM Thymidine for 1h. Cells were lysed in NEB (20mM HEPES pH7.9, 50mM NaCl, 3mM MgCl2, 300mM sucrose, and 0.5% NP-40) on ice for 15 min and nuclei were harvested by centrifugation. After washing with PBS, nuclei were incubated in freshly prepared click reaction cocktail (2 mM copper sulfate, 10 µM biotin-azide, and 100 mM sodium ascorbate in PBS) at 4 °C for 1h. Nuclei were then washed in PBS and resuspended in B1 buffer (25mM NaCl, 2mM EDTA, 1% NP-40 in 50mM Tris-HCl pH8). Next, sonication was performed at 4 °C using a bioruptor (Diagenode). Samples were then centrifuged at max speed for 10 min at 4 °C and supernatants were collected. An equal volume of B2 buffer (150mM NaCl, 2mM EDTA, 1% NP-40 in 50mM Tris-HCl pH8) was added to bring up the NaCl concentration, and input was taken at this point. EdU labeled DNA was then pulled down with 50µl of streptavidin beads (Dynabeads MyOne Streptavidin C1) at 4 °C overnight. Beads were washed with B2 buffer three times before boiled in laemmlili sample buffer to elute captured proteins.
in Situ protein Interactions at Replication Forks (SIRF)

For SIRF experiments (3), U2OS cells were first pulsed with 125 µM EdU for 10 min. For Thymidine chases, cells were washed with PBS then 100 µM of Thymidine was added for 3h. Cells were then fixed in 2% paraformaldehyde (PFA) for 15 min at room temperature and permeabilized on ice with PBS containing 0.5% triton X-100. After washing with PBS, click reaction was performed with biotin-azide for 1h at room temperature. The coverslips were then blocked with blocking solution and preceded to standard PLA procedure using anti-biotin and antibodies indicated in the figures.

Proximity Ligation Assay (PLA)

PLA was performed using Sigma Duolink PLA as per the manufacture’s protocol. In brief, U2OS cells on coverslips were fixed in 2% PFA for 15 min at room temperature and permeabilized on ice with PBS containing 0.5% triton X-100. Coverslips were then blocked at 37 °C for 1h and incubated with primary antibodies overnight at 4 °C. After washing in buffer A, coverslips were incubated with Duolink PLA probes anti–mouse MINUS and anti–rabbit PLUS for 1h at 37 °C. Coverslips were then washed in buffer A and ligation was performed using ligation reaction mixture at 37 °C for 30 min. Next, coverslips were washed in buffer A and incubated in amplification reaction mixture for 100 min at 37 °C in the dark. Coverslips were subsequently washed twice in buffer B and once in 0.01x buffer B before DAPI staining. For PLA experiments together with EdU labeling, 10 µM EdU was added 30 min before fixation. Click reaction using AF488-azide (Invitrogen) was performed after the permeabilization step and before the blocking step mentioned above, and all steps were performed in the dark.

Immunostaining

Cells were pre-extracted in 0.5% triton X-100 in CSK buffer (10mM PIPES pH 6.8, 100mM NaCl, 300mM sucrose, 3mM MgCl2) for 5 min on ice before fixation if needed. Cells were then fixed in 2% PFA for 15 min. If the pre-extraction was not performed, then cells were permeabilized on ice with PBS containing 0.5% triton X-100 for 5 min. Coverslips were then blocked in 1% normal goat serum (NGS) in PBS for 30 min and incubated with primary antibodies. Next, cells were washed in NGS/PBS and incubate in fluorophore-conjugated
secondary antibodies for 45 min at room temperature. Cells were then washed in PBS and stained with DAPI.

For ssDNA visualization, U2OS cells were cultured in 10 µM BrdU for 36 h before any treatment to ensure same amount of BrdU incorporation. Cells were pre-extracted in CSK buffer containing 0.5% Triton X-100 for 5 min on ice, followed by fixing in 2% PFA for 20 min. Cells were washed with PBS and treated with chilled methanol for 15 min. Next, cells were treated with chilled acetone for 30 sec, washed with PBS again and blocked in PBST containing 2% BSA for 1 h. Cells were then incubated with the FITC-conjugated BrdU antibody at 4°C overnight. After washes with PBS, cells were stained with DAPI.

**Immunoprecipitation**

Cells were washed with PBS and lysed in IP lysis buffer (50mM Tris pH7.4, 150mM NaCl, 1mM MgCl2, 10% glycerol, 0.2% NP-40) containing protease inhibitors. Lysates were then sonicated and treated with benzonase nuclease (Sigma) for 30 min at room temperature, then EDTA was added to 2mM. Centrifugation was done at max speed for 10 min to remove insoluble debris. Next, lysates were pre-cleared with Gammabind G sepharose (GE healthcare Life Science) for 30 min at 4 °C. Antibodies were then added into lysates and incubated at 4 °C overnight. Proteins bound by antibodies were pulled down by Gammabind G Sepharose for 3h at 4°C. After incubation, beads were washed in lysis buffer and captured proteins were eluted and analyzed with western blot or mass spectrometry. Mass spectrometry and data analysis were performed by the Taplin Biological Mass Spectrometry Facility.

**Flow cytometry**

For PI cell cycle profile, cells were collected and washed once in ice cold PBS, resuspended in PBS + 1% NGS, and fixed in 90% chilled ethanol overnight. Cells were then washed and resuspended in PBS + 1% NGS with 120 µg/ml propidium iodide (PI) and 10 µg/ml RNase A for 45 min at 37 °C. DNA content was measured by flow cytometry. For BrdU-PI flow, cells were pulsed with BrdU for 30 min and stained with FITC-conjugated BrdU antibody before PI staining.

MCM-PI flow was done following methods previously described (4) with modifications. Briefly, U2OS cells were collected and pre-extracted with 0.5% triton X-100 in CSK buffer for 5 min on
ice to remove soluble MCMs. Cells were then fixed in 1% PFA for 15 min RT, and stained with MCM3 antibody for 1h at 37 °C in 1% BSA/PBS with 0.1% NP-40. Next, Cells were incubated with fluorophore-conjugated secondary antibody for 1h at 37 °C. Finally, after washes, cells were stained with PI as indicated above.

**Chromatin fractionation**

U2OS cells were resuspended with solution A (10mM HEPES pH7.9, 10mM KCl, 1.5mM MgCl2, 0.34M sucrose, 1mM DTT, 10% glycerol and 0.1% Triton X-100) and incubate on ice for 5min. The cytoplasmic fraction (S2) was then separated from the nuclei by centrifuging at 4°C at 1400g for 4min. Isolated nuclei were then washed with solution A without Triton X-100. The nuclei pellet was resuspended with solution B (3mM EDTA, 0.2mM EGTA, and 1mM DTT) and incubated on ice for 30min. The nuclear soluble fraction (S3) was then separated by centrifuging at 4°C at 1700g for 4min. The S2 and S3 fractions can be combined as total soluble fraction (S). The isolated chromatin pellets were then washed with buffer B. Finally, the chromatin pellets were resuspended in solution A and sonicated for 1min to get P3 fraction.

**Comet assay**

Comet assay was performed using CometAssay Kit (Trevigen) following the manufacturer’s instructions. Control and Orc6 siRNA knockdown were performed in U2OS cells for 48h, and cells were then treated with genotoxic drugs as indicated in the figure legends. Cells were collected by trypsinization, embedded in low-melting agarose and placed on CometSlides. After agarose solidifying, the slides were immersed in lysis solution for 30 min then subjected to electrophoresis for 30 min. For alkaline comet assay, the slides were incubated in alkaline unwinding solution before electrophoresis. After washing in water and 70% ethanol for 5 min each, the slides were allowed to dry and DNA was visualized using SYBR safe staining.

**DNA fiber assay**

Control and siRNA knockdown were performed in U2OS cells for 48h before starting labeling with thymidine analogs. Cells were labeled with 50µM CldU and 200µM IdU, with different treatments according to schemes in figures. DNA fibers were prepared on vinyl-silane coated coverslips using the FiberComb molecular combing system (Genomic Vision) as per the
manufacture’s protocol. To visualize the CldU and IdU tracks, DNA fibers on coverslips were denatured in denaturation solution (0.5M NaOH, 1M NaCl) for 8 min at room temperature. Coverslips were then washed with PBS and dehydrated in 70%, 90%, and 100% ethanol for 5 min each. Coverslips were blocked with 1% BSA in PBST, followed by incubating in antibodies against CldU and IdU. After washing in BSA/PBST, the coverslips were incubated in FITC-conjugated goat anti-rat IgG and Texas Red-conjugated goat anti-mouse IgG. The images were captured using Zeiss Axiovision system.

**Clonogenic survival assay**

U2OS cells were first treated with siRNA as indicated in each figure for 48 h, or Orc6 KO cells were used. Cells were seeded in 6-well plates at 500 cells/well. After treating with various concentration of H$_2$O$_2$ for 1 h or cisplatin for 4 h as labeled in the figures, cells were allowed to grow for two weeks or until colonies could be seen. Colonies were then stained using crystal violet and colony numbers were counted.

**Protein purification**

Human RPA and MutSα were purified as described previously (5, 6). Human Flag-MutLα was expressed in insect Sf9 cells and purified by chromatographies on α-Flag beads and MonoQ and MonoS columns. p11d-tRPA was a kind gift from Dr. Marc Wold (University of Iowa, Iowa City, IA). Human GST-Flag-Orc6 was induced for overexpression in E. coli BL21 (DE3) codon (+). The cells were collected and resuspended in GST buffer (25mM HEPES pH 7.5, 0.1mM EDTA, 150mM NaCl, 1mM DTT and 5% glycerol) containing lysozyme 0.5mg/ml and 0.1% Triton X-100 followed by sonication. The lysate was collected by centrifugation and the supernatant was subjected to GSTTrap column (GE healthcare). After washing with GST buffer containing 500mM NaCl, GST-Flag-Orc6 was either directly eluted in GST buffer containing 20mM reduced glutathione, or subjected to thrombin cleavage and gel filtration to obtain Flag-Orc6.

**GST pull down assay**

GST control or GST-Flag-Orc6 was induced for overexpression in E. coli BL21 (DE3) codon (+), and lysate was prepared as described in protein purification part. The lysate was incubated
with Glutathione-Agarose beads (Sigma) for 1h at 4°C. After protein binding, the beads were washed twice with GST buffer containing 500mM NaCl and once with GST buffer. For each GST pull down reaction, 2.5 µl of the packed beads containing 25 µg GST fusion protein was equilibrated in buffer A (20 mM HEPES pH 7.4, 100 mM NaCl, 0.1 mM EDTA, 0.01% Nonidet P-40, 5% glycerol, 0.1 mM DTT, and 0.2 mM PMSF). MutSα or MutLα were added as indicated in the figures to a final reaction volume of 30 µl containing buffer A. After incubated for 1h at 4°C, the beads were washed extensively with buffer A and bound proteins were eluted by boiling in laemmli sample buffer.

**In vitro MutLα recruitment by co-immunoprecipitation**

For each co-IP reaction, 5 µl packed GammaBind G sepharose beads were mixed with 5 µg of MSH2 antibody (Santa Cruz) overnight at 4°C. The beads with MSH2 antibody immobilized on were then equilibrated in MMR buffer (20 mM HEPES pH 7.4, 100 mM KCl, 5 mM MgCl2, 2 mM ATP, 4 mM DTT, 0.4 mg/ml BSA, 3–5% (v/v) glycerol), and MutSα was added in the final volume of 30 µl containing MMR buffer. The mixture was incubated for 2h at 4°C with gentle rotation. Afterward, the beads were washed, and 5′-nicked G-T or A-T DNA (6) and proteins (GST control/GST-Flag-Orc6 and MutLα) were subsequently added to the reaction mixture as indicated in the figures; the final reaction volume was 30 µl containing MMR buffer. After incubating for 1.5h at 4°C, the beads were extensively washed three times in MMR buffer containing 5% skim milk and twice in MMR buffer. The proteins bound were finally eluted by boiling in laemmli sample buffer.

**Mismatch repair (MMR) in vivo assay**

In vivo mismatch repair efficiency is measured using MMR assay previously described (7). pCS2-EGFP-WT (wild type) was a kind gift from Dr. Marcel Méchali (Laboratory of DNA Replication and Genome Dynamics, Institute of Human Genetics, CNRS, Montpellier, France). pCS2-EGFP-t456g was generated from WT by Quikchange Site-Directed mutagenesis procedure. The pCS2-EGFP-WT plasmid was amplified into linear form with two primers, WT_Fwd and WT_Rev (see supplemental material), of which only the reverse one was 5′-phosphorylated. The mutated pCS2-EGFP-t456g was amplified with another set of primers, MIS_Fwd and MIS_Rev, of which only the forward one was 5′-phosphorylated. The PCR
products were purified and digested using Lambda exonuclease (NEB) that degrades only the 5’-phosphorylated strands. The remaining single strands were then mixed (WT+t456g), denatured at 97°C for 5 min and annealed by slowly cooling to room temperature, allowing them to form nicked circular plasmids with T/C mismatch. The heteroduplex products were transfected into cells using lipofectamin 2000 or 3000. Cells were collected 16-18 h after transfection by trypsinization, washed in PBS and immediately analyzed by flow cytometry. The EGFP signal was measured to obtain mismatch repair efficiency.

**HPRT Assay**

The hypoxanthine phosphoribosyltransferase (HPRT) assay was performed using A549 cells as described in (8) with modifications. A549 cells were first cultured in 1x HAT (hypoxanthine, aminopterin, and thymidine) media for 10 days to eliminate background HPRT mutated cells. After this mutation cleansing step, cells were treated with control, Orc6 or MSH2 shRNA and selected in puromycin containing media. After selection, cells were either mock treated or treated with H2O2 to induce DNA damage and mutations. The cells were allowed to recover and seeded in 10cm dishes at $10^6$ in media containing 24 mM 6-thioguanine (6-TG) to screen for HPRT mutated cells. Cells were grown in 6-TG media for 20 days or until colonies could be seen. The number of colonies was counted and normalized with colony-formation efficiency calculated on cells grown in media without 6-TG.

**Single Molecule Pull down (SiMPull)**

For Orc6 interaction with RPA, SiMPull experiments were carried out in flow chambers prepared on quartz microscope slides, which were passivated with methoxy-polyethylene glycol (mPEG) doped with 1% biotin-PEG. Biotinylated HA antibody was immobilized on PEG passivated surfaces at approximately 20 nM concentration for 20 min after coating the flow chambers with 0.2 mg/ml NeutrAvidin for 5 min. Cells were collected 24 h after transient transfection, and lysed in high salt buffer (50 mM Tris-HCl pH 7.4, 500 mM NaCl, 10% glycerol, 0.25% Triton X-100 with protease inhibitors) at 4°C for 20 min. Equal amount of zero salt buffer were added and incubated for another 10 min. After centrifugation, the supernatants were used for SiMPull analysis. Samples were appropriately diluted with T50 buffer (10 mM
Tris-HCl pH 8.0, 50 mM NaCl, 0.1 mg/ml BSA) to obtain optimal single molecule density on the surface. Diluted Samples were incubated in the chamber for 20 min and washed with the buffer.

**Single-strand DNA in vitro pull down**

To generate ssDNA for the in vitro binding assay, synthetic 70-mer DNA oligomers with 3’ biotinylation were attached to streptavidin-coated magnetic beads (Dynabeads™ MyOne™ Streptavidin C1) in binding buffer (10mM Tris pH7.5, 100mM NaCl, 10% glycerol, 0.01% NP-40, 10mg/ml BSA). Generally, 100 pmol of biotinylated DNA oligomers were incubated with 100 µl of dynabeads for 30 min at room temperature. The ssDNA-bound beads were washed with binding buffer to remove unbound DNA oligomers. For each reaction, 5 µl ssDNA-bound beads were used. For RPA coated ssDNA-beads, 25 pmol of purified RPA was incubated with every 5 µl of ssDNA-beads. The beads were used for in vitro pull down as indicated in the figures.

**QUANTIFICATION AND STATISTICAL ANALYSES**

Microscopy image analyses and quantifications were done using CellProfiler (9). Comet assay quantifications were done using OpenComet (10). Normality test was performed using Shapiro-Wilk test and statistical analyses were performed by two-tailed Student’s t test or Mann-Whitney test as indicated in the figure legends. Quantifications represented as mean ± standard deviation. P value asterisk convention: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. Further statistical details of experiments can be found in the figure legends.
Figure S1. Human Orc6 is dispensable for G1 licensing

(A) Schematic illustration of SiMPull to examine interaction between Orc6 and RPA32. (B) Quantification of Orc6 and RPA32 interaction. One signal spot represents one RPA molecule. Mean ± SD. ****p < 0.0001 by unpaired two-tailed Student’s t test. (C) Immunostaining analysis of Orc6 knockdown U2OS cells. PCNA staining was used to mark S phase cells. Scale bar, 15μm. (D) Western blot showing the protein level of endogenous Orc6 and DD-Orc6 in the absence and presence of Shield1. (E) Cell cycle profile of samples from (D) by PI flow cytometry. (F) Schematic of the protocol for specifically depleting Orc6 in G1 phase. (G) Western blot analysis of the G1 phase chromatin fractionation. S2, cytosolic; S3, nuclear soluble; P3, chromatin fraction. α-tubulin and H4 serve as loading control for cytosolic and chromatin fraction, respectively. PI flow profiles show the synchrony of the cells. (H) Flow cytometric analysis of asynchronized U2OS cells to measure chromatin bound MCM in conjugation with PI staining cell cycle profile. Left panel: MCM3 antibody omitted as negative gating for MCM staining. Right panel: illustration of cell cycle progression; loading of MCM on chromatin increases through G1 (green); S phase cells (red) with increasing DNA content and decreasing chromatin MCM, until reaching G2/M (maroon). (I) MCM-PI flow of Orc6 and Orc1 knockdown cells. (J) G1 populations from (I) were highlighted for comparing the MCM loading.
Figure S2. Reduced ATR activation in Orc6-depleted cells is not due to changes in ATR signaling proteins’ recruitment to RPA-ssDNA

(A) Western blot of ATM/ATR signaling pathways for different DNA damage drug treatment in control and Orc6 knockdown U2OS cells. Asterisk indicates hyperphosphorylated RPA32. (B) In vitro ssDNA pull-down assay to determine the recruitment of ATR signaling proteins. ssDNA along or ssDNA pre-coated with purified RPA were incubated with control or Orc6-depleted nuclear extracts. Samples obtained after biotin-ssDNA pull-down were analyzed by western blotting. NE, nuclear extract. (C) In vitro ssDNA pull-down for effects of Orc6 to RPA’s ssDNA binding ability. Orc6 along, RPA along, Orc6 first then RPA or RPA first then Orc6 were added to ssDNA. Proteins bound to ssDNA were analyzed using western blot. (D) DNA fiber assay to determine nascent DNA resection/degradation after fork stalling at 15 or 40 min after the damage treatment. A representative experiment is shown. Mean ± SD. *p < 0.05 by unpaired two-tailed Student’s t test.
Figure S3. Orc6 interactome analysis and MMR phenotypic assays

(A) GO analysis of Orc6 interacting proteins. (B) Top events from KEGG pathway analysis of Orc6 interacting proteins. (C) Western blot showing the single and double depletion of MSH6 and Orc6 in U2OS cells. (D) Mutation frequency determined by HPRT assay. Mean ± SD. n=2 for left panel and n=3 for right panel. (E) Western blot showing the MSH2 and MSH2/Orc6 depletion in A549 cells.
Figure S4. Quantification of MutLα recruitment and MMR in vivo reporter assay

(A) Quantification of figure 5D in the main text. Intensity of bands was measured using imageJ from multiple repeats for MLH1 (upper panel) and PMS2 (lower panel). Mean ± SD. *p < 0.05 by unpaired two-tailed Student’s t test. (B) Quantification of figure 5E in the main text. Intensity of bands was measured for MLH1 (upper panel) and PMS2 (lower panel). Mean ± SD. *p < 0.05 by unpaired two-tailed Student’s t test. (C) Schematic illustration of the generation of the heteroduplex used for in vivo MMR reporter assay. (D) Left panel: quantification of MMR activity of Orc2 depleted U2OS cells. Mean GFP intensity was measured using flow cytometry. a.u.: arbitrary unit. Mean ± SD, n = 5. Right panel: western blot showing Orc2 knockdown efficiency.
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Mouse anti-α-Tubulin| Sigma-Aldrich | Cat# T5168; RRID:AB_477579 |
| Rabbit anti-Orc6    | Dr. Bruce Stillman lab | pAb982 |
| Rabbit anti-Orc2    | Dr. Bruce Stillman lab | pAb205 |
| Mouse anti-Biotin   | Sigma-Aldrich | Cat# B7653; RRID:AB_258625 |
| Rabbit anti-DNA polymerase delta | Abcam | Cat# ab186407 |
| Mouse anti-PCNA     | Santa Cruz Biotechnology | Cat# sc-56; RRID:AB_628110 |
| Mouse anti-RPA32    | Santa Cruz Biotechnology | Cat# sc-56770; RRID:AB_785534 |
| Rabbit anti-Histone H4 pan | Millipore | Cat# 05-858R; RRID:AB_10561768 |
| Mouse anti-RFC1     | Dr. Bruce Stillman lab | N/A |
| Rabbit anti-Cdt1    | Dr. Bruce Stillman lab | BWH131 |
| Rabbit anti-MCM3    | Dr. Bruce Stillman lab | pAb738 |
| Mouse anti-BrdU, FITC conjugated | BD Biosciences | Cat# 556028; RRID:AB_396304 |
| Rat anti-BrdU       | Bio-Rad | Cat# OBT0030G; RRID:AB_609567 |
| Mouse anti-BrdU     | BD Biosciences | Cat# 347580; RRID:AB_400326 |
| Rabbit anti-ATRIP   | Cell Signaling | Cat# 2737; RRID:AB_823659 |
| Rabbit anti-Chk1    | Cell Signaling | Cat# 2345; RRID:AB_10693648 |
| Rabbit anti-Phospho-Chk1 (Ser345) | Cell Signaling | Cat# 2348; RRID:AB_331212 |
| Rabbit anti-Phospho-Chk2 (Thr68) | Cell Signaling | Cat# 2661; RRID:AB_331479 |
| Rabbit anti-Phospho-RPA32 (Ser33) | Bethyl | Cat# A300-246A; RRID:AB_2180847 |
| Rabbit anti-Phospho-H2A.X (Ser139) | Cell Signaling | Cat# 9718; RRID:AB_2118009 |
| Mouse anti-Cyclin B1 | Cell Signaling | Cat# 4135; RRID:AB_2233956 |
| Mouse anti-MSH2     | Santa Cruz Biotechnology | Cat# sc-376384; RRID:AB_10988231 |
| Rabbit anti-MSH6    | Bethyl | Cat# A300-023A; RRID:AB_185543 |
| Mouse anti-MLH1     | BD Biosciences | Cat# 554073; RRID:AB_395227 |
| Mouse anti-PMS2     | Santa Cruz Biotechnology | Cat# sc-25315; RRID:AB_628163 |
| Mouse anti-HA       | Cold Spring Harbor Lab | 12CA5 |
| Mouse anti-T7       | Millipore | Cat# 69522; RRID:AB_11211744 |
| Mouse anti-Flag M2  | Sigma-Aldrich | Cat# F3165; RRID:AB_259529 |
### Chemicals, Peptides, and Recombinant Proteins

| Chemical/Protein                  | Manufacturer   | Catalog Number |
|-----------------------------------|----------------|----------------|
| Rabbit anti-GST, biotin conjugated| Abcam          | Cat# ab87834; RRID:AB_2041437 |
| EdU                               | Invitrogen     | Cat# A10044    |
| Alexa Fluor™ 488 Azide            | Invitrogen     | Cat# A10266    |
| Biotin Azide                      | Sigma-Aldrich  | Cat# B10184    |
| BrdU                              | Sigma-Aldrich  | Cat# B9285     |
| CldU                              | Sigma-Aldrich  | Cat# C6891     |
| IdU                               | MP Biomedicals | Cat# SKU02100357.2 |
| Benzonase                         | Sigma-Aldrich  | Cat# E1014     |
| Thymidine                         | Sigma-Aldrich  | Cat# T9250     |
| Nocodazole                        | Sigma-Aldrich  | Cat# M1404     |
| Hydroxyurea                       | Sigma-Aldrich  | Cat# H8627     |
| Hydrogen peroxide                 | Sigma-Aldrich  | Cat# H1009     |
| Neocarzinostatin                  | Sigma-Aldrich  | Cat# N9162     |
| Camptothecin                      | Sigma-Aldrich  | Cat# C9911     |
| Cisplatin                         | Sigma-Aldrich  | Cat# P4394     |
| Shield1                           | Takara         | Cat# 632189    |
| Calf intestinal phosphatase       | NEB            | Cat# M0290S    |
| Lambda exonuclease                | NEB            | Cat# M0262L    |

### Critical Commercial Assays

| Assay                           | Manufacturer   | Catalog Number |
|---------------------------------|----------------|----------------|
| Duolink™ In Situ Red Starter Kit Mouse/Rabbit | Sigma-Aldrich | Cat# DUO92101 |
| CometAssay® Kit                 | Trevigen       | Cat# 4250-050-K |
| FiberPrep® (DNA Extraction Kit) | Genomic vision | Cat# EXTR-001 |

### Deposited Data

| Description                       | Source          | Table |
|-----------------------------------|-----------------|-------|
| T7-Orc6 immunoprecipitation-mass spectrometry list | This paper | S1    |

### Experimental Models: Cell Lines

| Species        | Source          | Catalog Number |
|----------------|-----------------|----------------|
| Human: U2OS    | ATCC            | N/A            |
| Human: HEK293T | University of Illinois | N/A |
| Human: U2OS HA-Orc6 | This study | N/A |
| Human: U2OS DD-Orc6 | This study | N/A |
| Human: U2OS Orc6 KO (clone 3) | This study | N/A |
| Human: A549    | ATCC            | N/A            |

### Oligonucleotides

| siRNA            | Sequence Details                                      | Manufacturer | Catalog Number |
|------------------|-------------------------------------------------------|--------------|----------------|
| Orc6 siRNA1      | Sense 5’-AGAUAGAUAUACCGGAUUGGAGCCCA and Antisense 5’-UGGCUCCAAUUCCGCUUUAUCUAUCUAU | IDT          | N/A            |
| Orc6 siRNA2      | Sense 5’-UGUACAGCCUAGUUAUUAAUGTT and Antisense 5’-AACAUUUAAUACUUAGCUGUACAAU | IDT          | N/A            |
| Biotinylated ssDNA for in vitro pull down: 5'-TGCAGCTGGCAGCAGGTAAAAATGAAATCGGCCAACGCAGCCGGGAGAGGCGGTGATTGGGCGCT[BtnTg] | This paper | N/A |
| Primers used in this paper are listed in Table S2 | This paper | Table S2 |

**Recombinant DNA**

| Name | Authors | Year | Source |
|------|---------|------|--------|
| pCS2-EGFP | Traver et al., 2015 | N/A | De Novo software [https://denovosoftware.com/](https://denovosoftware.com/) |
| pPTuner-IRES2-DD-Orc6 | This study | N/A | N/A |
| pCD5-HA-Orc6 | This study | N/A | N/A |
| pCGT-T7-Orc6 | This study | N/A | N/A |
| pFlag-Orc6 | This study | N/A | N/A |
| pGEX-GST-Flag-Orc6 | This study | N/A | N/A |
| p11d-tRPA | Binz et al., 2006 | N/A | N/A |

**Software and Algorithms**

| Name | Source |
|------|--------|
| FCS express 5 | De Novo software [https://denovosoftware.com/](https://denovosoftware.com/) |
| Flowing software | N/A [http://flowingsoftware.btk.fi/](http://flowingsoftware.btk.fi/) |
| OpenComet | Gyori et al., 2014 [http://www.cometbio.org/](http://www.cometbio.org/) |
| CellProfiler | McQuin et al., 2018 [https://cellprofiler.org/](https://cellprofiler.org/) |
| Prism | GraphPad [https://www.graphpad.com/](https://www.graphpad.com/) |
| Zen | Zeiss [https://www.zeiss.com/microscopy/us/home.html](https://www.zeiss.com/microscopy/us/home.html) |

**Table S2. Primers used in this study**

| Name | Sequence | Reference |
|------|----------|-----------|
| WT_Fwd | 5'-GTTGCTCTTGCCCGGCG | Figure 5 and S4 |
| WT_Rev | 5'-[Phos]CGGTCGCCGATACACCTA | Figure 5 and S4 |
| MIS_Fwd | 5'-[Phos]GCTACAGAGTTCTGAAGTG | Figure 5 and S4 |
| MIS_Rev | 5'-CCGCCTACATACCCGT | Figure 5 and S4 |

**Dataset S1. Number of peptides, sum intensities and relative values of proteins identified by mass spectrometry in control, T7-Orc6, and T7-Orc6 with H2O2 treatment**
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