Evolution of DNA Replication Origin Specification and Gene Silencing Mechanisms

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Supplementary Information

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Supplementary Fig. 1 | Orc2 loop DNA interaction exists in species with origin sequence specificity. a, Multiple sequence alignment of Orc2 among representing eukaryotic species as indicated. Orc2 DNA interacting loop region indicated with species that don’t have sequence specific origins shadowed in blue and species that sequence specific origins exist shadowed in pink. b, Orc2 AAA+ domain structure superposition among human Orc2 in teal color (from PDB code 5uj7), Drosophila Orc2 in wheat color (from PDB code 4xgc) and S. cerevisiae Orc2 in brown color (from PDB code 5udb). Orc2 loop that interact with DNA is colored in red. c-e, R390, Y395, W396, and H399 interact with DNA in base-specific (specificity) and base-nonspecific (affinity) manner in ORC-DNA structure at 3Å (PDB code 5zr1). Red asterisks denote the base interaction between amino acid and DNA base. Blue asterisks denote the base-nonspecific interaction between amino acid and DNA phosphate backbone. Prime symbols denote bases on
the opposite strand. Bases numbering denote as the positions in logo (see Fig. 2b). c shows Orc2 loop origin DNA minor groove insertion with base-specific interaction between W396 and G25, C25’ and T26, and base-nonspecific interaction between Y395 and phosphate backbone of T27. d-e, same as in c, but view in different angles. d shows the base-specific interaction between W396 and G25, C25’ and T26. e shows the base-nonspecific interaction between R390 and phosphate backbone of T23’ and base-nonspecific interaction between H399 and phosphate backbone of A24’.
**orc4Δ::TRP1 + pORC4/URA3 + plasmid indicated below:**

|                  | 15°C Day6 | 30°C Day2 | 37°C Day2 |
|------------------|-----------|-----------|-----------|
| **pORC4/LEU2**   | ![Image](YPD.png) | ![Image](FOA.png) | ![Image](YPD.png) | ![Image](FOA.png) |
| por4::LEU2       | ![Image](YPD.png) | ![Image](FOA.png) | ![Image](YPD.png) | ![Image](FOA.png) |
| por4<sup>cre</sup>LEU2<sub>c1</sub> | ![Image](YPD.png) | ![Image](FOA.png) | ![Image](YPD.png) | ![Image](FOA.png) |
| por4<sup>cre</sup>LEU2<sub>c2</sub> | ![Image](YPD.png) | ![Image](FOA.png) | ![Image](YPD.png) | ![Image](FOA.png) |
| por4<sup>cre</sup>LEU2<sub>c3</sub> | ![Image](YPD.png) | ![Image](FOA.png) | ![Image](YPD.png) | ![Image](FOA.png) |
| por4<sup>cre</sup>LEU2<sub>c4</sub> | ![Image](YPD.png) | ![Image](FOA.png) | ![Image](YPD.png) | ![Image](FOA.png) |

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Supplementary Fig. 2 | Orc4 mutants viability phenotypes in plasmid shuffle assay. Detailed procedure of plasmid shuffle assay is described in Method, Plasmid shuffle assay. Briefly, strains (orc4Δ::TRP1 + pORC4/URA3 + porc4 tested allele/LEU2) were grown overnight in YPD and spotted onto 5-FOA plates with 10-fold serial dilutions starting from 1.5x10^7 cells and spotted onto YPD plates as control. Mutations were indicated. Strain (orc4Δ::TRP1 + pORC4/URA3 + porc4 null allele/LEU2) and strain (orc4Δ::TRP1 + pORC4/URA3 + porc4 null allele/LEU2) were spotted as controls. Biological duplicates are denoted as c1 and c2. Plates were cultured under 25°C, 30°C, or 37°C for different days (as indicated) to test their cold or temperature sensitivity. Orc4 mutant phenotypes summarized in Supplementary Table 1.
**Supplementary Fig. 3** | **Orc2 mutants viability phenotypes in plasmid shuffle assay.** Detailed procedure of plasmid shuffle assay is described in Method, Plasmid shuffle assay. Briefly, strains \((orc2\Delta::TRP1 + pORC2/URA3 + porc2^\text{tested allele}/LEU2)\) were grown overnight in YPD and spotted onto 5-FOA plates with 10-fold serial dilutions starting from \(1.5 \times 10^7\) cells and spotted onto YPD plates as control. Mutations were indicated. Strain \((orc2\Delta::TRP1 + pORC2/URA3 + pORC2/LEU2)\) and strain \((orc2\Delta::TRP1 + pORC2/URA3 + porc2\text{null}/LEU2)\) were spotted as controls. Biological duplicates are denoted as c1 and c2. Plates were cultured under 25°C, 30°C, or 37°C for different days (as indicated) to test their cold or temperature sensitivity. Orc2 mutant phenotype summarized in Supplementary Table 2.
**Supplementary Fig. 4 | Orc4 protein expression detection and ORC complex formation detection.** Details of method is described in Method, Cell extract preparation, immunoprecipitation, immunoblot analysis and antibodies. NTAP-tagged Orc4 were immunoprecipitated via incubation with IgG beads. Wild type W303 strain, which contains non-tagged Orc4, is used parallelly as a control of pulldown assay. 2% of input and 16.7% of pulldown lysate were loaded and subsequently immunoblot with anti-Orc4 (SB12) and anti-Orc1 (SB13). Purified ORC complex (including Orc1 and non-tagged Orc4) was also loaded as control for immunoblotting of Orc1 and Orc4. NTAP-tagged Orc4 is around 83kDa (indicated with green arrows) and non-tagged Orc4 is around 56kDa (indicated with yellow arrows), while Orc1 is around 120kDa (indicated with red arrows). Both short and long exposure of blots are indicated. Source data are provided as a Source Data file.
Supplementary Fig. 5 | Cell cycle of NTAP-Orc4 integrated strains. Flow cytometry was done by growing cells into log phase, arresting at G1 phase with α-factor block for 3 hours (around 1~2
cell cycle time length) and then releasing into S phase for different time point (as indicated above on the left). Different time points were harvest and prepared for flow cytometry with method previously described\textsuperscript{50}. DNA strained with SYBR green. \textit{Orc4} mutants seemed to have hard time going through S phase and progression through mitosis.
Supplementary Fig. 6 | Analysis of plasmid borne and genome integrated Orc4 mutants. a, Schematic diagram of viability comparison assay between strains surviving dependent on single episomal origin (Orc4 on plasmid, denoted as [P]) or multiple chromosomal origins (Orc4 integrated into genome, denoted as [G]). The [P] strain relies on a CEN-based plasmid with a single replication origin to carry the tested Orc4 mutation and is therefore stringent. b, [P] strains (orc4::TRP1 + pORC4/URA3 + porc4/LEU2) and [G] strains (his3::NTAP-Orc4<sup>WT</sup>, orc4::TRP1, bar1Δ::TRP1, LEU2::BrdU-Inc + pOrc4/URA3) were grown overnight in YPD and spotted onto 5-FOA plates with 10-fold serial dilutions starting from 1.5x10<sup>7</sup> cells and spotted onto YPD plates as control. As controls for [P] strains, strain (orc4::TRP1 + pORC4/URA3 + pORC4/LEU2) and strain (orc4::TRP1 + pORC4/URA3 + porc4<sup>WT</sup>/LEU2) were spotted. As controls for [G] strains, strain (his3::NTAP-Orc4<sup>WT</sup>, orc4::TRP1, bar1Δ::TRP1, LEU2::BrdU-Inc + pOrc4/URA3) and strain (orc4::TRP1, bar1Δ::TRP1, LEU2::BrdU-Inc + pOrc4/URA3) were spotted. Plates were cultured under 25˚C, 30˚C, or 37˚C for different days (as indicated) to test their temperature sensitivity. The strain lacking a NTAP-tagged Orc4 did not grow on FOA. The viability deficient
phenotype of \textit{Orc4} mutants on single-origin plasmid seemed to be partially rescued when the mutants are integrated into the genome and survive on multiple origins.
Supplementary Fig. 7 | ARS motif logos generated from MPOS assay using ARS317 mutations. **a,** ARS motif logos for Orc4 integrated strains at A and B1 elements generated using mutation library with ARS317 sequence backbone. Same as Fig 3, top-half of logos representing the origin sequences that were selected-for in MPOS assay and bottom-half of logos representing the origin sequences that were selected-against in MPOS assay. **b,** Magnified view of A element region in a logo from Orc4<sup>WT</sup>, orc4<sup>F485A, Y486A</sup>, orc4<sup>F485A, Y486Q</sup> strains with bottom-half of logo faded. Dark purple circles indicate the major changes at A/T29, G/T30 logo positions in the Orc4 mutant strains.
Supplementary Fig. 8 | Principal component analysis and comparison of motif inference methods. a, PCA analyses of motifs (performed on the ARS416 library MPOS data, the ARS317 library MPOS data, or both libraries), and inferred using either information maximization (IM) or enrichment ratios (ER). The variance explained by the first two principal components, corresponding to the x- and y-axes of each plot, is indicated in the upper left corner. The dots within each plot represent biologically independent MPOS experiments, dot color indicates the Orc4 variant assayed, and dot shape indicates the library of mutated ARSs used as input. Note that motifs cluster according to the Orc4 variant assayed, and that this clustering is stronger for information maximization (IM)-inferred motifs compared to enrichment ratio (ER)-inferred motifs. b, Total variance across the motifs inferred for each Orc4 variant using either ER or IM inference. IM inference consistently yielded less intra-replicate variance than ER inference (48.3%
less on average for ARS416 motifs and 39.9% less for ARS317 motifs). This again reflects the robustness of IM inference in the face of experiment-to-experiment variation. c, Logos showing the ARS416 motifs for two Orc4 variants. For clarity, only 20 bp encompassing the essential A element are shown. ER motifs exhibited substantially more variability at key positions than did IM motifs (e.g. rose highlighted positions). Orc4 mutants resulted in consistently and clearly visible differences in the inferred IM motifs (e.g. cyan positions).
Supplementary Fig. 9 | Replicates of genome-wide replication origin profile. a, Schematic diagram for genome-wide replication origin profile analysis. Details of method is described in Methods, Genome-wide replication origin profile analysis. Briefly, Yeast cells were α-factor
blocked in G1 phase for 3 hours and then released into the growth medium (YPD with 200mM HU, 500uM EdU and 0.2mg/ml pronase E) for 90mins before harvest. Flow cytometry was done to check the stage of the cells. DNA is isolated from the harvested cells and sonicated using Bioruptor. EdU labeled newly synthesized DNA is pulldown by Click-iT chemistry with biotinylated azide and Streptavidin T1 magnetic beads. Then Illumina TruSeq Kit is used to establish and amplify the sequencing library. Sequencing data is then analyzed to show peaks on newly synthesized DNA with detailed computational method in Method, Computational analyses of replication origin profile and ChIP-seq data. **b-c**, Replicates of origin firing profiles in Fig. 3. Chromosome IV(ChrIV) is used as representation and replicates are from two independent experiments. **b** shows the direct comparison of two replicates with profiles from \textit{Orc4}^{WT} and \textit{mrc1Δ} strains shown as examples. **c** shows the genome-wide replication origin firing profiles from the all strains in replicate experiment.
Supplementary Fig. 10 | Chromatin immuno-precipitation (ChIP) of MCM in Orc4 strains. 

a-b, ChIP profile of MCM (anti-Mcm2) in G1 phase (Orc4<sup>WT</sup>, orc4<sup>F485I</sup>, Y486Q and mrc1Δ profiles in Fig. 3). Chromosome IV(ChrIV) is used as representation. Genome-wide replication origin firing profiles from Fig. 3 is attached for better reference of origin firing pattern and are shadowed in grey. a shows the direct comparison of two independent replicates with Mcm2-ChIP profiles from Orc4<sup>WT</sup> and mrc1Δ strains shown as examples. b shows the Mcm2-ChIP profiles of the all the strains from one of the two replicates.
specifically activated

specifically repressed

Orc4 WT height

$R^2$: 0.996

$R^2$: 0.699

$R^2$: 0.63

$R^2$: 0.394

$R^2$: 0.377

$R^2$: 0.159

$R^2$: 0.078

$R^2$: 0.112

$R^2$: 0.067
Supplementary Fig. 11 | Genomic origin firing peak heights scatter plot comparisons. Each dot represent a single replication origin that has its origin firing peak height in \( \text{Orc4}^{\text{WT}} \) (in b-j) or \( \text{mrc1}^{\Delta} \) (in k-t) as the x-value and its origin firing peak height in \( \text{orc4}^{\text{mut}} \) (in b-j, l-t) or \( \text{Orc4}^{\text{WT}} \) (in k) as y-value. Two origins exhibited aberrantly large height values, believed to have arisen from read mapping artefacts, and were removed from this analysis. Height values are normalized by...
computing the number of reads bounding 99.5% of positions within each profile and divided the entire profile by this number. Coefficient of determination values ($R^2$) are shown atop each panel. 

a, Illustration diagram for b-j showing the directions of activation (in green) and repression (in orange) for each replication origin (denote as black dot) in orc4 mutant strains. **b-j, orc4**$^{\text{mut}}$ strains direct comparison with *Orc4*WT strain. **k-t**, all ten *Orc4* strains direct comparison with *mrc1Δ* strain.
Supplementary Fig. 12 | Correlation of genomic origin peak height and MPOS motif scores. DNA sequences under origin firing peaks that were predicted to be ACS were obtained from OriDB\textsuperscript{13,37} and used for analysis. MPOS motif scores were assigned by how good the annotated ACSs matched to the MPOS motifs. Correlations between origin firing peak heights (in log\textsubscript{10}) and MPOS motif scores were evaluated for each annotated ACS. A section indicates the origin peak heights in wild-type strain or wild-type like orc4 mutant control strain. We took late vs early origin factors into consideration. However, a large value in origin height does not guarantee a high MPOS motif score. B section indicates the orc4 F485 and Y486 mutants that were shown to have their origin sequence recognition altered (Fig. 2c and Fig. 4). C section indicates the orc4 R478 and N489 mutation strains. P-values were also computed to assess the null hypothesis that log EdU heights and motif scores are not correlated; all P-values were Bonferoni corrected (by multiplying by the total number of tests). Significant correlations were indicated: *p<0.05, **p<0.01, ***p<0.001.
Supplementary Fig. 13 | Non-Y486 orc4 mutants efficiently use origins with the “AG” dinucleotide. Supplemental figure for Fig. 4. Box plots for the 6 orc4 mutant strains that do not have Y486 changed, except for the orc4^{F485Y, Y486F} strain that contains a conserved mutation whose strain grows similar to Orc4^{WT} and therefore is an exception of Y486 mutation strain. a. orc4^{F485Y, Y486F}, b. orc4^{R478K}, c. orc4^{N489A}, d. orc4^{F485I}, e. orc4^{R478A}, f. orc4^{N489W}. Y-axis is genomic origin firing peak heights in log_{10}. Each dot denotes an annotated ACS. Box plots elements: the minimum height, first (lower) quartile, median, third (upper) quartile, and maximum height. Diamond denotes outliers that exhibited aberrantly large values.
## Supplementary Table 1 | Summary of plasmid shuffle assay *Orc4* mutant phenotypes.

*Orc4* mutant viability phenotypes summarized from plasmid shuffle assay (Supplementary Fig. 1). Indicated symbols denotes different viability phenotypes.

| Orc4 Mutation | Phenotype |
|---------------|-----------|
| Kα-helix      | # @30°C (N=4) |
| Δα-helix      | # @all three temperature (N=2) |
| F485I, Y486Q  | *** @30°C; # @15°C (CS); # @37°C (TS) (N=3) |
| N489W         | *** @30°C; # @15°C (CS); # @37°C (TS) (N=3) |
| N489A         | *** @30°C; # @15°C (CS); # @37°C (TS) (N=3) |
| R478A         | ** @30°C; *** @15°C (CS); # @37°C (TS) (N=3) |
| F485I         | * @30°C; ** @15°C (CS); * @37°C (N=3) |
| F485A, Y486A  | * @30°C; ** @15°C (CS); * @37°C (N=3) |
| Y490L         | * @30°C; ** @15°C (CS); * @37°C (N=2) |
| F492S         | * @30°C; ** @15°C (CS); * @37°C (N=2) |
| R478K         | ~ @all three temperature (N=3) |
| Y486Q         | ~ @all three temperature (N=3) |
| F485A         | ~ @30°C; @15°C; ~ @37°C (N=3) |
| Y486A         | ~ @30°C; @15°C; ~ @37°C (N=3) |
| Y490A         | ~ @30°C; @15°C; ~ @37°C (N=3) |
| F492A         | ~ @30°C; @15°C; ~ @37°C (N=3) |
| Q493R         | ~ @all three temperature (N=2) |
| D479R         | ~ @all three temperature (N=2) |
| V226A         | ~ @all three temperature (N=2) |
| V226R         | ~ @all three temperature (N=2) |
| R227A         | ~ @all three temperature (N=2) |
| Q493A         | ~ @all three temperature (N=3) |
| K402A         | ~ @all three temperature (N=3) |
| K402E         | ~ @all three temperature (N=3) |
| T482D         | ~ @all three temperature (N=5) |
| T482R         | ~ @all three temperature (N=3) |
| T482A         | ~ @all three temperature (N=3) |
| T482I         | ~ @all three temperature (N=3) |
| F485Y, Y486F  | ~ @all three temperature (N=3) |
| Y490R         | ~ @all three temperature (N=3) |
| F492L         | ~ @all three temperature (N=3) |
| R227D         | ~ @all three temperature (N=3) |

Symbols denotes viability deficient phenotypes. ~ denotes minimal or not deficient; - denotes slightly deficient; * denotes moderate deficient; ** denotes strong deficient; *** denotes severe deficient; # denotes lethal.
Supplementary Table 2 | Summary of plasmid shuffle assay Orc2 mutant phenotypes.

Orc2 mutant viability phenotypes summarized from plasmid shuffle assay (Supplementary Fig. 2). Indicated symbols denotes different viability phenotypes.

| Orc2 Mutation | Phenotype                          |
|---------------|------------------------------------|
| Δ390-398      | # @all three temperature (N=2)     |
| Δ393-398      | # @all three temperature (N=2)     |
| W396A         | # @all three temperature (N=3)     |
| Y395A         | # @all three temperature (N=2)     |
| N398A         | ** @30°C; # @15°C (CS); # @37°C (TS) (N=2) |
| T393A         | ~ @all three temperature (N=3)     |
| K394A         | ~ @all three temperature (N=2)     |

Symbols denotes viability deficient phenotypes. ~ denotes minimal or not deficient; - denotes slightly deficient; * denotes moderate deficient; ** denotes strong deficient; *** denotes severe deficient; # denotes lethal
Supplementary Table 3 | Summary of doubling time of NTAP-Orc4 integrated strains.
Doubling time were calculated based on growth curves (see Fig. 1f) log phase cell concentration.

\[
k = \frac{\Delta \log(\text{Cell Conc.})}{\Delta \text{Time}} = \frac{\log(2)}{\text{Doubling Time}} \quad (4)
\]

\[
\text{Doubling Time} = \frac{\log (2)}{k} \quad (5)
\]

k dictates the slope of linear regression line of growth curves log phase.

| Orc4 Integrated Strains | Doubling Time |
|-------------------------|--------------|
| WT                      | 87mins       |
| F485Y, Y486F            | 89mins       |
| Y486Q                   | 91mins       |
| R478K                   | 91.5mins     |
| F485I                   | 93mins       |
| F485A, Y486A            | 97mins       |
| N489W                   | 112mins      |
| F485I, Y486Q            | 127mins      |
| R478A                   | 132mins      |
| N489A                   | 134.5mins    |
### Supplementary Methods

| Strain  | Genotype                                                                 | Source          |
|---------|---------------------------------------------------------------------------|-----------------|
| YB51    | MATα orc4::TRP1 ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS415/ORC4 | This lab        |
| YS856   | MATα ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100                   | This study      |
| YB410   | MATα orc2::TRP1 ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC2 | This lab        |
| YS3285  | MATα mrc1Δ::KanMX6 bar1Δ::TRP1 URA3::BrdU-Inc ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 | This study      |
| YB 1519 | MATα orc4::TRP1 ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 + pRS415/ORC4 | This study      |
| YB 1515 | MATα orc4::TRP1 ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 + pRS415 | This study      |
| YB1639  | MATα orc4::TRP1 ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 + pRS415/orc4Δα-helix | This study      |
| YB 1514 | MATα orc4::TRP1 ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 + pRS415/orc4Δκα-helix | This study      |
| YB 1520 | MATα orc4::TRP1 ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 + pRS415/orc4ΔY486Q  | This study      |
| YB 1521 | MATα orc4::TRP1 ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 + pRS415/orc4ΔY486A | This study      |
| YB 1522 | MATα orc4::TRP1 ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 + pRS415/orc4F485I  | This study      |
| YB 1523 | MATα orc4::TRP1 ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 + pRS415/orc4F485A  | This study      |
| YB 1524 | MATα orc4::TRP1 ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 + pRS415/orc4N489A | This study      |
| YB 1525 | MATα orc4::TRP1 ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 + pRS415/orc4N489W | This study      |
| YB 1526 | MATα orc4::TRP1 ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 + pRS415/orc4F492A  | This study      |
| YB 1527 | MATα orc4::TRP1 ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 + pRS415/orc4Y490A  | This study      |
| YB 1528 | MATα orc4::TRP1 ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 + pRS415/orc4R478A | This study      |
| YB 1555 | MATα orc4::TRP1 ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 + pRS415/orc4Q493A  | This study      |
| YB 1556 | MATα orc4::TRP1 ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 + pRS415/orc4K402A | This study      |
| YB 1557 | MATα orc4::TRP1 ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 + pRS415/orc4K402E | This study      |
| YB 1558 | MATα orc4::TRP1 ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 + pRS415/orc4T482A | This study      |
| YB 1559 | MATα orc4::TRP1 ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 + pRS415/orc4T482R | This study      |
| Yeast Strain | Genotype Details                                                                 | Source          |
|-------------|----------------------------------------------------------------------------------|-----------------|
| YB 1560     | MATα orc4::TRP1 ade2-1 ura3-1 his 3-11, 15 trp1-1 leu2-3,112 <br>can1-100 + pRS416/ORC4 + pRS415/orc4T482I | This study      |
| YB 1571     | MATα orc4::TRP1 ade2-1 ura3-1 his 3-11, 15 trp1-1 leu2-3,112 <br>can1-100 + pRS416/ORC4 + pRS415/orc4T482D | This study      |
| YB 1562     | MATα orc4::TRP1 ade2-1 ura3-1 his 3-11, 15 trp1-1 leu2-3,112 <br>can1-100 + pRS416/ORC4 + pRS415/orc4R478K | This study      |
| YB 1563     | MATα orc4::TRP1 ade2-1 ura3-1 his 3-11, 15 trp1-1 leu2-3,112 <br>can1-100 + pRS416/ORC4 + pRS415/orc4F485A, Y486A | This study      |
| YB 1564     | MATα orc4::TRP1 ade2-1 ura3-1 his 3-11, 15 trp1-1 leu2-3,112 <br>can1-100 + pRS416/ORC4 + pRS415/orc4F485Y, Y486F | This study      |
| YB1635      | MATα orc4::TRP1 ade2-1 ura3-1 his 3-11, 15 trp1-1 leu2-3,112 <br>can1-100 + pRS416/ORC4 + pRS415/orc4F485L, Y486Q | This study      |
| YKC01       | MATα orc4::TRP1 ade2-1 ura3-1 his 3-11, 15 trp1-1 leu2-3,112 <br>can1-100 + pRS416/ORC4 + pRS415/orc4F492L | This study      |
| YKC02       | MATα orc4::TRP1 ade2-1 ura3-1 his 3-11, 15 trp1-1 leu2-3,112 <br>can1-100 + pRS416/ORC4 + pRS415/orc4Y490L | This study      |
| YKC03       | MATα orc4::TRP1 ade2-1 ura3-1 his 3-11, 15 trp1-1 leu2-3,112 <br>can1-100 + pRS416/ORC4 + pRS415/orc4Y490R | This study      |
| YKC04       | MATα orc4::TRP1 ade2-1 ura3-1 his 3-11, 15 trp1-1 leu2-3,112 <br>can1-100 + pRS416/ORC4 + pRS415/orc4F492S | This study      |
| YB1637      | MATα orc4::TRP1 ade2-1 ura3-1 his 3-11, 15 trp1-1 leu2-3,112 <br>can1-100 + pRS416/ORC4 + pRS415/orc4R227D | This study      |
| YEH003      | MATα orc4::TRP1 ade2-1 ura3-1 his 3-11, 15 trp1-1 leu2-3,112 <br>can1-100 + pRS416/ORC4 + pRS415/orc4V226A | This study      |
| YEH004      | MATα orc4::TRP1 ade2-1 ura3-1 his 3-11, 15 trp1-1 leu2-3,112 <br>can1-100 + pRS416/ORC4 + pRS415/orc4V226R | This study      |
| YEH005      | MATα orc4::TRP1 ade2-1 ura3-1 his 3-11, 15 trp1-1 leu2-3,112 <br>can1-100 + pRS416/ORC4 + pRS415/orc4R227A | This study      |
| YEH002      | MATα orc4::TRP1 ade2-1 ura3-1 his 3-11, 15 trp1-1 leu2-3,112 <br>can1-100 + pRS416/ORC4 + pRS415/orc4Q493R | This study      |
| YEH001      | MATα orc4::TRP1 ade2-1 ura3-1 his 3-11, 15 trp1-1 leu2-3,112 <br>can1-100 + pRS416/ORC4 + pRS415/orc4D479R | This study      |
| YS2251      | MATα bar1Δ::TRP1 ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 | This study      |
| YB 1549     | MATα bar1Δ::TRP1 LEU2::BrdU-Inc ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 | This study      |
| YB1588      | MATα orc4::TRP1 bar1Δ::TRP1 LEU2::BrdU-Inc ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 | This study      |
| YB 1623     | MATα his3::TAP-ORC4 orc4::TRP1 bar1Δ::TRP1 LEU2::BrdU-Inc ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 | This study      |
| YB 1624     | MATα his3::TAP-orc4F485I orc4::TRP1 bar1Δ::TRP1 LEU2::BrdU-Inc ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 | This study      |
| YB 1625 | MATa his3::TAP-orc4N489A orc4::TRP1 bar1Δ::TRP1 LEU2::BrdU-Inc ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 | This study |
| YB 1630 | MATa his3::TAP-orc4KLaH orc4::TRP1 bar1Δ::TRP1 LEU2::BrdU-Inc ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 | This study |
| YB 1631 | MATa his3::TAP-orc4F485Y+Y486F orc4::TRP1 bar1Δ::TRP1 LEU2::BrdU-Inc ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 | This study |
| YB 1632 | MATa his3::TAP-orc4R478K orc4::TRP1 LEU2::BrdU-Inc ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 | This study |
| YB 1633 | MATa his3::TAP-orc4ΔaH orc4::TRP1 LEU2::BrdU-Inc ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 | This study |
| YB1647 | MATa his3::TAP-orc4N489W orc4::TRP1 LEU2::BrdU-Inc ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 | This study |
| YB1648 | MATa his3::TAP-orc4R478A orc4::TRP1 LEU2::BrdU-Inc ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 | This study |
| YB1652 | MATa his3::TAP-orc4Y486Q orc4::TRP1 LEU2::BrdU-Inc ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 | This study |
| YB1653 | MATa his3::TAP-orc4F485A+Y486A orc4::TRP1 LEU2::BrdU-Inc ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 | This study |
| YB1654 | MATa his3::TAP-orc4F485I+Y486Q orc4::TRP1 LEU2::BrdU-Inc ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 + pRS416/ORC4 | This study |
| YB1649 | MATa his3::TAP-ORC4 orc4::TRP1 LEU2::BrdU-Inc ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 | This study |
| YB1650 | MATa his3::TAP-orc4F485I orc4::TRP1 LEU2::BrdU-Inc ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 | This study |
| YB1655 | MATa his3::TAP-orc4N489A orc4::TRP1 LEU2::BrdU-Inc ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 | This study |
| YB1656 | MATa his3::TAP-orc4F485Y+Y486F orc4::TRP1 LEU2::BrdU-Inc ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 | This study |
| YB1657 | MATa his3::TAP-orc4R478K orc4::TRP1 LEU2::BrdU-Inc ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100 | This study |
Supplementary Methods Table 1 | Genotype and resources of yeast strains used in this study
| Primer Names | Primer Sequence | Note |
|--------------|----------------|------|
| YEH001       | GCGACGCAGCATTTCaaGCTAGCAATTATCAATTTTCAGGG | Used for Orc4 mutagenesis |
| YEH002       | CCCTGAAATTGATAATTGCTAGCttgAAATGCTGCGGGTCGC | Used for Orc4 mutagenesis |
| YEH003       | CGACCGCAGCATTTgccGCTAGCAATTATCAATTTCCAG | Used for Orc4 mutagenesis |
| YEH004       | CTGAAATTGATAATTGCTAGCggcAAATGCTGCGGGTCGC | Used for Orc4 mutagenesis |
| YEH005       | CGACCGCAGCAattTACGCTAGCAATTATCAATTTCCAGGG | Used for Orc4 mutagenesis |
| YEH006       | CCCTGAAATTGATAATTGCTAGCGTAaatTGCTGCGGGTCGC | Used for Orc4 mutagenesis |
| YEH007       | CGACCGCAGCAgtTACGCTAGCAATTATCAATTTCCAGGG | Used for Orc4 mutagenesis |
| YEH008       | CCCTGAAATTGATAATTGCTAGCGTAagcTGCTGCGGGTCG | Used for Orc4 mutagenesis |
| YEH009       | CGACCGCAGCATTTTACGCTAGCgtTATCAATTTCCAGGG | Used for Orc4 mutagenesis |
| YEH010       | CCTGAAATTGATAagcGCTAGCGTAAATGTCTGCGGGTCGC | Used for Orc4 mutagenesis |
| YEH011       | GCATTTTACGCTAGCttgTATCAATTTCCAGGCGACCATGATC CCG | Used for Orc4 mutagenesis |
| YEH012  | CGGGATCATGGTGCCCTGAAATTGATAccaGCTAGCGTAAAATGC | Used for Orc4 mutagenesis |
|---------|-----------------------------------------------|--------------------------|
| YEH013  | ACCGCAGCATTTTACGCTAGCAATTATCAAgtCAGGGCACC    | Used for Orc4 mutagenesis |
| YEH014  | GGTGCCCTGagcTTGATAATTGCTAGCGTAAAATGCTGCGGT  | Used for Orc4 mutagenesis |
| YEH015  | ACCGCAGCATTTTACGCTAGCAATgctCAATTTCAGGGGC     | Used for Orc4 mutagenesis |
| YEH016  | GCCCTGAAATTGagcATTGCTAGCGTAAAATGCTGCGGT      | Used for Orc4 mutagenesis |
| YEH017  | CCGAGAAATTCAGCCGTTGTTTtgcaGATAATGCGAC        | Used for Orc4 mutagenesis |
| YEH018  | GTCGCATTATCtgcCAAACCAACGGGCTGATTTCCTCGG      | Used for Orc4 mutagenesis |
| YEH024  | CCGTTGGTTTGAGAGATAATGAAAGACTCTGCTATACAATCA  | Used for Orc4 mutagenesis |
| YEH025  | GTAAACCGGGATCATGGTGCCGATGAATTGTAGTTTGTCTGAT | Used for Orc4 mutagenesis |
| YEH026  | GCCGTTGGTTTGAGAGATAATGGCACCATGATCCCGTTTGA   | Used for Orc4 mutagenesis |
| YEH027  | GTAAACCGGATCATGGTGCCATTATCTCTCTCAAACCAACGGC | Used for Orc4 mutagenesis |
| YEH032  | GCATTTTACGCTAGCAATTATCAAATTgccGGACCATGATCC | Used for Orc4            |
| YEHO33 | GGGATCATGGTGCTgccAAATTGATAATTGCTAGCGTAAAA TGC | Used for Orc4 mutagenesis |
|-------|---------------------------------------------|--------------------------|
| YEHO34 | GCATTGGTAGGCTAGCAATTATCAATTTagaGCCACACATGATC CCG | Used for Orc4 mutagenesis |
| YEHO35 | CCGGATCATGGTGCTctAAATTGATAATTGCTAGCGTAAAA TGC | Used for Orc4 mutagenesis |
| YEHO36 | GGTTGCGCTAAGGGCGgccGCACCGGATCTTTTTAATTTTAATT AGC | Used for Orc4 mutagenesis |
| YEHO37 | GCTAAATTAATAAAGATCCGTCgccCGCCCTTAAGGC AACC | Used for Orc4 mutagenesis |
| YEHO38 | GGTTGCGCTAAGGGCggagGCACCGGATCTTTTTAATTTTAATT AGC | Used for Orc4 mutagenesis |
| YEHO39 | GCTAAATTAATAAAGATCCGTCtcCGCCCTTAAGGC ACC | Used for Orc4 mutagenesis |
| YEHO40 | CGTTGGTTTAGAGATAATGCgccGCAGCATTCTCCTCAAACACCGC | Used for Orc4 mutagenesis |
| YEHO41 | GCTAGCGCTAAAAATGTGCgccGCAGATTCTCCTCAAACACCGG | Used for Orc4 mutagenesis |
| YEHO42 | CGTTGGTTTAGAGATAATGCggcGCAGCATTCTCCTCAAACACCGC | Used for Orc4 mutagenesis |
| YEHO43 | GCTAGCGCTAAAAATGTGCgccGCAGCATTCTCCTCAAACACCGG | Used for Orc4 mutagenesis |
| YEH180 | CAGCAgctgccGCTAGCAATTATCAATTTCAG | Used for Orc4 mutagenesis |
| YEH181 | CTGAAATTGATAATTGCTAGGgcagcTGCTG | Used for Orc4 mutagenesis |
| YEH182 | CGACCGCAGCAattcaaGCTAGCAATTATCAATTTCAG | Used for Orc4 mutagenesis |
| YEH183 | CTGAAATTGATAATTGCTAGTtgaatTGCTGCGGTCG | Used for Orc4 mutagenesis |
| YEH184 | CGACCGCAGCAactttGCTAGCAATTATCAATTTCAG | Used for Orc4 mutagenesis |
| YEH185 | CTGAAATTGATAATTGCTAGCaaagtaTGCTGCGGTCG | Used for Orc4 mutagenesis |
| YEH186 | CCGAGAAATCAGCCGTTGGTTTGaaaGATAATGCGAC | Used for Orc4 mutagenesis |
| YEH187 | GTCGCATTATCtttCAAACCAACGGCTGATTTCTCGG | Used for Orc4 mutagenesis |
| YEH188 | CGTTGGTTTGAGAGATAATGCGgatGCAGCATTTTACGCTAGC | Used for Orc4 mutagenesis |
| YEH189 | GCTAGCGTAAAATGCTGCaatCGCATTATCTCTCAAACCAACG | Used for Orc4 mutagenesis |
| YEH190 | CGTTGGTTTGAGAGATAATGCGgatGCAGCATTTTACGCTAGC | Used for Orc4 mutagenesis |
| YEH191 | GCTAGCGTAAAATGCTGCaatCGCATTATCTCTCAAACCAACG | Used for Orc4 |
| YEH235   | ACATTTGCTGGGCCTgcAGGCAAACTTTATTATACAATC | Used for Orc4 mutagenesis |
| YEH236   | GATTGTATAATAAAAGTTTGCCCTgcgAGGCCCCAGCAATGT | Used for Orc4 mutagenesis |
| YEH237   | ACATTTGCTGGGCCTgcgAGGCAAACTTTATTATACAATC | Used for Orc4 mutagenesis |
| YEH238   | GATTGTATAATAAAAGTTTGCCCTgcgAGGCCCAGCAATGT | Used for Orc4 mutagenesis |
| YEH239   | ACATTTGCTGGGCCTGgcCAAAATTTATTATACAATC   | Used for Orc4 mutagenesis |
| YEH240   | GATTGTATAATAAAAGTTTGggCACCAGCCGCAATGT   | Used for Orc4 mutagenesis |
| YEH241   | ACATTTGCTGGGCCTGTgcgAGGCCCAGCAATGT   | Used for Orc4 mutagenesis |
| YEH242   | GATTGTATAATAAAAGTTTGtcACAGGCCGCAATGT | Used for Orc4 mutagenesis |
| YEH304   | CCGAGAAATCGCCGTTGGTTTGAGAagaAATGCGAC | Used for Orc4 mutagenesis |
| YEH305   | GTGCATTtcTCTCAAACCAACGGCTATTCTCGG | Used for Orc4 mutagenesis |
| YEH352   | CCGCAGCATTTCAGCTAGCAATtgCAATTCAGGAC | Used for Orc4 mutagenesis |
| Yeast ORC1 Clone | Sequence                  | Used For                  |
|-----------------|--------------------------|---------------------------|
| YEH353          | GGTGCCCTGAAATTGcaaATTGCTAGCGTAAAATGCTGCGG | Orc4 mutagenesis          |
| YEH354          | CCGCAGCATTTTACGCTAGCAATgaCAATTTTCAGGGCACC | Orc4 mutagenesis          |
| YEH355          | GGTGCCCTGAAATTGctATTGCTAGCGTAAAATGCTGCGG | Orc4 mutagenesis          |
| YEH356          | CGCTAGCAATTATCAActaCAGGGCACCATGATCCCG   | Orc4 mutagenesis          |
| YEH357          | CGGGATCATGTTGCCCTGtagTTGATAATTGCTAGCG   | Orc4 mutagenesis          |
| YEH358          | CGCTAGCAATTATCAAtcaCAGGGCACCATGATCCCG   | Orc4 mutagenesis          |
| YEH359          | CGGGATCATGTTGCCCTGtaTTGATAATTGCTAGCG    | Orc4 mutagenesis          |
| YEH192          | GACAAGAAGCGAAgcAAGTACTGGGGCAATCATGTG    | Orc2 mutagenesis          |
| YEH193          | CACATGATTGCCCCAGTACTTgcTTGCCTTCTTGGTC   | Orc2 mutagenesis          |
| YEH194          | GACAAGAAGCGAAAACTgtTACTGGGGCAATCATGTG   | Orc2 mutagenesis          |
| YEH195          | CACATGATTGCCCCAGTAgcAGTTTCGCTTCTTGGTC   | Orc2 mutagenesis          |
| YEH196          | GACAAGAAGCGAAAACTAAGgcTTGGGGCAATCATGTG  | Orc2 mutagenesis          |
| Yeast  | Sequence                                                                 | Used for                  |
|--------|--------------------------------------------------------------------------|---------------------------|
| YEH197 | CACATGATTGCCCCAagcCTTAGTTTCGCTTCTTGTC                                  | mutagenesis               |
| YEH198 | GACAAGAAGCGAAAACCTAAGTACgetGGCAATCATGTG                                  | mutagenesis               |
| YEH199 | CACATGATTGCCagcGTACTTAGTTTCGCTTCTTGTC                                  | mutagenesis               |
| YEH200 | ACTAAGTACTGGGGCgetCATGTGATTTTCGAGA                                    | mutagenesis               |
| YEH201 | TCTGCAAAATCACATGagcGCCCCAGTACTTAGT                                     | mutagenesis               |
| YEH326 | CTGAGTTGACAAAGAAGCGAACATGTGATTTTCGAGATCCA                              | mutagenesis               |
| YEH327 | TGGATCTGCAAAATCACATGTGTTCTTTGTCAACTCAG                                 | mutagenesis               |
| YEH328 | TGGTCCCCGCTGAGTTGACACATGTGATTTTCAGATCCA                                | mutagenesis               |
| YEH329 | TGGATCTGCAAAATCACATGTGTAACACTCAGCGGGGACCA                               | mutagenesis               |
| YEH019 | ATGACTATAAGCGAAGCTCG                                                   | sequencing                |
| Code   | Sequence                        | Used for                                      |
|--------|---------------------------------|-----------------------------------------------|
| YEH020 | CTCAATTGAAACAGCAGTTG            | Orc4 sequencing /integrated NTAP-Orc4 sequencing |
| YEH021 | AAGAGTGATTTATATGCCGC            | Orc4 sequencing /integrated NTAP-Orc4 sequencing |
| YEH022 | TCAACTCCAGAATTCACCAC           | Orc4 sequencing /integrated NTAP-Orc4 sequencing |
| YEH023 | TCACAGTTGTGTCAGGAG            | Orc4 sequencing                               |
| YEH221 | GGAAGACTTTGTAGAGCAT          | Orc2 sequencing                               |
| YEH222 | CCTACACGTATGCAGGAA          | Orc2 sequencing                               |
| YEH223 | ATGCTCTACAAAGTCTTCC         | Orc2 sequencing                               |
| YEH224 | TTCCGATACGTGTAGG          | Orc2 sequencing                               |
| gYEH00 3-F | ATTTGTAATACGCTTTTACTAGTTTT | gRNA003 insertion to bRA89 plasmid            |
|   |   |   |
|---|---|---|
| gYEH00 3-R | TAGTAAAGCGTATTACAAATGATCA | Used for gRNA003 insertion to bRA89 plasmid |
| gYEH00 5-F | GAATATACTAAAAAAAAATGAGCGTTTT | Used for gRNA005 insertion to bRA89 plasmid |
| gYEH00 5-R | GCTCATTTTTTAGTATATCGATCA | Used for gRNA005 insertion to bRA89 plasmid |
| YEH263 | AGAAGAAGCATGGCAGGCCTTG | Used for CRISPR NTAP-Orc4 template construction/integrated NTAP-Orc4 sequencing |
| YEH264 | accagacaccTAGGCGAATTG | Used for CRISPR NTAP-Orc4 template construction |
| YEH265 | CTagtgtgtATGACCTATAAG | Used for CRISPR NTAP-Orc4 template construction |
| YEH266  | TTTTTCAACGCTAACTATAATTT | Used for CRISPR NTAP-Orc4 template construction/integrated NTAP-Orc4 sequencing |
|---------|--------------------------|----------------------------------------------------------------------------------|
| YEH267  | CTCGAGGCCACCAAGAAGAGAAAGAGAC | Used for CRISPR NTAP-Orc4 template construction |
| YEH268  | AGGCCTGCCATGCTTTTTTTTAATATTACCGATATT | Used for CRISPR NTAP-Orc4 template construction |
| YEH311  | TTCCTGCAGCCCGGGGGATCGAAGAGAAAGAGAGCCA | Used for CRISPR NTAP-Orc4 template construction |
| YEH312  | GCTCCACCGCGGGTGCGGCGCTTTTTCAACGCTAACTATAA | Used for CRISPR NTAP-Orc4 template construction |
| ssYEH0 01 | AATGAGCAGGCAAGATAAAACGAAAGGCAAAGATGACAGAGCAGAAAGCCGAAGAGAAAGAGAGCCAGATATTGAC TG | Used for CRIPSR NTAP-Orc4 integration yeast transformation (coupled with gRNA003) |
|-----------|--------------------------------------------------------------------------------|--------------------------------------------------------------------------------|
| ssYEH0 02 | ACAAGTGGCAATGAAAAAAATTATAGTTAGCGTTGAAAAA TgcgAAAGCGTATTACAAATGAAAACCAAGATTCAGATTGC | Used for CRIPSR NTAP-Orc4 integration yeast transformation (coupled with gRNA003) |
| ssYEH0 03 | TTATACATTATAAAGTAATGTGATTTTTCTCTAGGAAAGATAT ACTAAAAGAAGAGAAAAAGAGAGGAGCCAGATATTGACTG | Used for CRIPSR NTAP-Orc4 integration yeast transformation (coupled with gRNA005) |
| ssYEH0 04 | ACAAGTGGCAATGAAAAAAATTATAGTTAGCGTTGAAAAA ATateCAGGCAAGATAAAACGAAAGGCAAAGATGACAGAGCA | Used for CRIPSR NTAP-Orc4 integration yeast transformation (coupled with gRNA005) |
|   | Sequence | Use                  |
|---|----------|----------------------|
| **YEH298** | TTAAGAGCTTGGGTGAGCGCT | Used for CRISPR NTAP-Orc4 insertion checking |
| **YEH299** | ATGCTTGGCAGAGCATGTAT | Used for CRISPR NTAP-Orc4 insertion checking |
| **YEH173** | GTGCCCGGGAGGATGAGA | Used for integrated NTAP-Orc4 sequencing |
| **YEH279** | TCACCATCTTCAACAGTCAA | Used for integrated NTAP-Orc4 sequencing |
| **YEH281** | CGCGAATTGTGATATACCTA | Used for integrated NTAP-Orc4 sequencing |
| **YEH282** | ATATGAGTGATAATGGTTCC | Used for integrated NTAP-Orc4 sequencing |
| **YEH294** | GAAGAGAAAGAGAAGAGCCA | Used for integrated NTAP-Orc4 sequencing |
| **ARS416 L1 AR** | CTCGGCATTCTGCTGAACCGCTCTTCCGATCTCAATGATTTAGCATTATCTTTAC | Used for MPOS assay ARS416 sequencing library barcoding |
| Sample  | Primer Sequence | Use for                  |
|---------|-----------------|--------------------------|
| ARS317L1 AR | CTCGGCATTTCTGGCTGAACCGCTCTTCGATCTTTATGGAAAGATTAAAGCTCA | Used for MPOS assay ARS317 sequencing library barcoding |
| ARS416L1 AF01 | ACTCTTTCCCTACACGACGCTCTTCCGATCTACATAGACAAATGGTGTAAAGAC | Used for MPOS assay ARS416 sequencing library barcoding |
| ARS416L1 AF02 | ACTCTTTCCCTACACGACGCTCTTCCGATCTAGCCAGACAAATGGTGTAAAGAC | Used for MPOS assay ARS416 sequencing library barcoding |
| ARS416L1 AF03 | ACTCTTTCCCTACACGACGCTCTTCCGATCTATGTAGACAAATGGTGTAAAGAC | Used for MPOS assay ARS416 sequencing library barcoding |
| ARS416L1 AF04 | ACTCTTTCCCTACACGACGCTCTTCCGATCTCATAAGACAAATGGTGTAAAGAC | Used for MPOS assay ARS416 sequencing library barcoding |
| ARS416L1 AF05 | ACTCTTTCCCTACACGACGCTCTTCCGATCTCGATAGACAAATGGTGTAAAAGAC | Used for MPOS assay ARS416 sequencing library barcoding |
| ARS416 | L1  | AF  | Sequence                  | Used for                  |
|--------|-----|-----|---------------------------|---------------------------|
|        |     |     | ACTCTTTCCCTACACGACGCTCTTCGATCT CTCT AGACAAATGGTGTAAGAC | MPOS assay ARS416 sequencing library barcoding |
|        |     |     | ACTCTTTCCCTACACGACGCTCTTCGATCT GAGA AGACAAATGGTGTAAGAC |                           |
|        |     |     | ACTCTTTCCCTACACGACGCTCTTCGATCT GCTA AGACAAATGGTGTAAGAC |                           |
|        |     |     | ACTCTTTCCCTACACGACGCTCTTCGATCT GTAT AGACAAATGGTGTAAGAC |                           |
|        |     |     | ACTCTTTCCCTACACGACGCTCTTCGATCT TACA AGACAAATGGTGTAAGAC |                           |
|        |     |     | ACTCTTTCCCTACACGACGCTCTTCGATCT TCGG AGACAAATGGTGTAAGAC |                           |

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| ARS416 | L1   | AF12   | Used for MPOS assay | ARS416 sequencing library barcoding |
|--------|------|--------|---------------------|-----------------------------------|
|        |      |        | ACTCTTTCCCTACACGACGCTCTTCGGATCT TGTA AGACAAATGGTGTA AAAAGAC |                      |
| ARS416 | L1   | AF13   | Used for MPOS assay | ARS416 sequencing library barcoding |
|        |      |        | ACTCTTTCCCTACACGACGCTCTTCGGATCT AAAA AGACAAATGGTGTA AAAAGAC |                      |
| ARS416 | L1   | AF14   | Used for MPOS assay | ARS416 sequencing library barcoding |
|        |      |        | ACTCTTTCCCTACACGACGCTCTTCGGATCT ACCA AGACAAATGGTGTA AAAAGAC |                      |
| ARS416 | L1   | AF15   | Used for MPOS assay | ARS416 sequencing library barcoding |
|        |      |        | ACTCTTTCCCTACACGACGCTCTTCGGATCT AGGA AGACAAATGGTGTA AAAAGAC |                      |
| ARS416 | L1   | AF16   | Used for MPOS assay | ARS416 sequencing library barcoding |
|        |      |        | ACTCTTTCCCTACACGACGCTCTTCGGATCT ATTA AGACAAATGGTGTA AAAAGAC |                      |
| ARS416 | L1   | AF17   | Used for MPOS assay | ARS416 sequencing library barcoding |
|        |      |        | ACTCTTTCCCTACACGACGCTCTTCGGATCT CCAG AGACAAATGGTGTA AAAAGAC |                      |
| ARS416 L1 AF18 | ACTCTTTCCCTACACGACGCTCTTCCGATCT CGCA AGACAAATGGTGTAAGAC | Used for MPOS assay ARS416 sequencing library barcoding |
|----------------|----------------------------------------------------------|----------------------------------------------------------|
| ARS416 L1 AF19 | ACTCTTTCCCTACACGACGCTCTTCCGATCT CTGG AGACAAATGGTGTAAGAC | Used for MPOS assay ARS416 sequencing library barcoding |
| ARS416 L1 AF20 | ACTCTTTCCCTACACGACGCTCTTCCGATCT GATT AGACAAATGGTGTAAGAC | Used for MPOS assay ARS416 sequencing library barcoding |
| ARS416 L1 AF21 | ACTCTTTCCCTACACGACGCTCTTCCGATCT GGAA AGACAAATGGTGTAAGAC | Used for MPOS assay ARS416 sequencing library barcoding |
| ARS416 L1 AF22 | ACTCTTTCCCTACACGACGCTCTTCCGATCT GTCA AGACAAATGGTGTAAGAC | Used for MPOS assay ARS416 sequencing library barcoding |
| ARS416 L1 AF23 | ACTCTTTCCCTACACGACGCTCTTCCGATCT TAGC AGACAAATGGTGTAAGAC | Used for MPOS assay ARS416 sequencing library barcoding |
| ARS        | Primer Sequence                          | Used for                                      |
|------------|------------------------------------------|-----------------------------------------------|
| ARS416     | ACTCTTTCCCTACACGACGCTCTTCCGATCT TCTC AGACAAATGGTGTTAAAAGAC | MPOS assay                                   |
|            |                                          | ARS416 sequencing library barcoding          |
| ARS317     | ACTCTTTCCCTACACGACGCTCTTCCGATCT ACAT CAGTGTAAAAATTTTTTATTAAC | MPOS assay                                   |
|            |                                          | ARS317 sequencing library barcoding          |
| ARS317     | ACTCTTTCCCTACACGACGCTCTTCCGATCT AGCC CAGTGTAAAAATTTTTTATTAAC | MPOS assay                                   |
|            |                                          | ARS317 sequencing library barcoding          |
| ARS317     | ACTCTTTCCCTACACGACGCTCTTCCGATCT ATGT CAGTGTAAAAATTTTTTATTAAC | MPOS assay                                   |
|            |                                          | ARS317 sequencing library barcoding          |
| ARS317     | ACTCTTTCCCTACACGACGCTCTTCCGATCT CATA CAGTGTAAAAATTTTTTATTAAC | MPOS assay                                   |
|            |                                          | ARS317 sequencing library barcoding          |
| ARS317     | ACTCTTTCCCTACACGACGCTCTTCCGATCT CGAT CAGTGTAAAAATTTTTTATTAAC | MPOS assay                                   |
|            |                                          | ARS317 sequencing library barcoding          |
| Location  | Sequence                                                                 | Usage                                      |
|-----------|--------------------------------------------------------------------------|--------------------------------------------|
| ARS317    | ACTCTTTCCCTACACGACGCTTCCGATCT CTCT CAGTGGTTTCAATTATTATTAAAAAC             | Used for MPOS assay                        |
| L1        |                                                                         | ARS317 sequencing library barcoding        |
| AF06      |                                                                         |                                            |
| ARS317    | ACTCTTTCCCTACACGACGCTTCCGATCT GAGA CAGTGGTTTCAATTATTATTAAAAAC             | Used for MPOS assay                        |
| L1        |                                                                         | ARS317 sequencing library barcoding        |
| AF07      |                                                                         |                                            |
| ARS317    | ACTCTTTCCCTACACGACGCTTCCGATCT GCTA CAGTGGTTTCAATTATTATTAAAAAC             | Used for MPOS assay                        |
| L1        |                                                                         | ARS317 sequencing library barcoding        |
| AF08      |                                                                         |                                            |
| ARS317    | ACTCTTTCCCTACACGACGCTTCCGATCT GTAT CAGTGGTTTCAATTATTATTAAAAAC             | Used for MPOS assay                        |
| L1        |                                                                         | ARS317 sequencing library barcoding        |
| AF09      |                                                                         |                                            |
| ARS317    | ACTCTTTCCCTACACGACGCTTCCGATCT TACA CAGTGGTTTCAATTATTATTAAAAAC             | Used for MPOS assay                        |
| L1        |                                                                         | ARS317 sequencing library barcoding        |
| AF10      |                                                                         |                                            |
| ARS317    | ACTCTTTCCCTACACGACGCTTCCGATCT TCGG CAGTGGTTTCAATTATTATTAAAAAC             | Used for MPOS assay                        |
| L1        |                                                                         | ARS317 sequencing library barcoding        |
| AF11      |                                                                         |                                            |
| ARS317 | L1 | AF12 | Used for MPOS assay | ARS317 sequencing library barcoding |
|--------|----|------|----------------------|------------------------------------|
| ACTCTTTCCCTACACGACGCTCTTCGATCT | TGTA | CAGTGGTTTTCAATTTTTTATTAAC |

| ARS317 | L1 | AF13 | Used for MPOS assay | ARS317 sequencing library barcoding |
|--------|----|------|----------------------|------------------------------------|
| ACTCTTTCCCTACACGACGCTCTTCGATCT | AAAA | CAGTGGTTTTCAATTTTTTATTAAC |

| ARS317 | L1 | AF14 | Used for MPOS assay | ARS317 sequencing library barcoding |
|--------|----|------|----------------------|------------------------------------|
| ACTCTTTCCCTACACGACGCTCTTCGATCT | ACCA | CAGTGGTTTTCAATTTTTTATTAAC |

| ARS317 | L1 | AF15 | Used for MPOS assay | ARS317 sequencing library barcoding |
|--------|----|------|----------------------|------------------------------------|
| ACTCTTTCCCTACACGACGCTCTTCGATCT | AGGA | CAGTGGTTTTCAATTTTTTATTAAC |

| ARS317 | L1 | AF16 | Used for MPOS assay | ARS317 sequencing library barcoding |
|--------|----|------|----------------------|------------------------------------|
| ACTCTTTCCCTACACGACGCTCTTCGATCT | ATTA | CAGTGGTTTTCAATTTTTTATTAAC |

| ARS317 | L1 | AF17 | Used for MPOS assay | ARS317 sequencing library barcoding |
|--------|----|------|----------------------|------------------------------------|
| ACTCTTTCCCTACACGACGCTCTTCGATCT | CCAG | CAGTGGTTTTCAATTTTTTATTAAC |
| ARS317 | L1   | AF18   | Used for MPOS assay | ARS317 sequencing library barcoding |
|--------|------|--------|---------------------|-----------------------------------|
|        |      |        | ACTCTTTCCCTACACGACGCTCTTCCGATCT CGCA |                                  |
|        |      |        | CAGTGGTTTCAATTTTTTATTAAC |                                  |
| ARS317 | L1   | AF19   | Used for MPOS assay | ARS317 sequencing library barcoding |
|        |      |        | ACTCTTTCCCTACACGACGCTCTTCCGATCT CTGG |                                  |
|        |      |        | CAGTGGTTTCAATTTTTTATTAAC |                                  |
| ARS317 | L1   | AF20   | Used for MPOS assay | ARS317 sequencing library barcoding |
|        |      |        | ACTCTTTCCCTACACGACGCTCTTCCGATCT GATT |                                  |
|        |      |        | CAGTGGTTTCAATTTTTTATTAAC |                                  |
| ARS317 | L1   | AF21   | Used for MPOS assay | ARS317 sequencing library barcoding |
|        |      |        | ACTCTTTCCCTACACGACGCTCTTCCGATCT GGAA |                                  |
|        |      |        | CAGTGGTTTCAATTTTTTATTAAC |                                  |
| ARS317 | L1   | AF22   | Used for MPOS assay | ARS317 sequencing library barcoding |
|        |      |        | ACTCTTTCCCTACACGACGCTCTTCCGATCT GTCA |                                  |
|        |      |        | CAGTGGTTTCAATTTTTTATTAAC |                                  |
| ARS317 | L1   | AF23   | Used for MPOS assay | ARS317 sequencing library barcoding |
|        |      |        | ACTCTTTCCCTACACGACGCTCTTCCGATCT TAGC |                                  |
|        |      |        | CAGTGGTTTCAATTTTTTATTAAC |                                  |
| Primers and oligos sequences used in this study | Used for |
|------------------------------------------------|---------|
| ARS317 L1 AF24 ACTCTTTCCCTACACGACGCTCTTCCGATCT TCTC CAGTGGTTTTCAATTTTTTATTAAAC | MPOS assay ARS317 sequencing library barcoding |
| YEH229 GCCGCAAAAAAAGGGAATAAG | MPOS assay sequencing library construction |
| YEH230 CGTGTTCAAACGATACCTGG | MPOS assay sequencing library construction |
| YEH231 AATGATACGGCGACCACCAGATCTACACTCTTTCCAAGACG | MPOS assay sequencing library construction |
| YEH232 AAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCT | MPOS assay sequencing library construction |

**Supplementary Methods Table 2** | Primers and oligos sequences used in this study