Supplemental Information

Dual Control of Yen1 Nuclease Activity and Cellular Localization by Cdk and Cdc14 Prevents Genome Instability

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Figure S1. Catalog of Yen1 Phosphomutants

Related to Figure 1. Schematic representation of all the Yen1 mutants employed in this study. The pairs Yen1^{2A}/Yen1^{7A}, Yen1^{4A}/Yen1^{5A}, and Yen1^{8A}/Yen1^{S679A} contain complementary sets of substitutions with respect to those in Yen1^{ON}. The color code of protein domains is shown in Figure 1A. Substitutions in Cdk sites are indicated in red. On the right, the level of nuclease activity in immunoprecipitates of the different Yen1 mutants from cells synchronized in G1 or S phase is indicated. (-), no activity detected; (+), equivalent level of activity to Yen1 in G1; (++), activity higher than Yen1, but lower than Yen1^{ON}; (+++), level of activity equivalent to Yen1^{ON} in G1; nd, not determined.
Table S1. Number of crossover (CO), noncrossover (NCO) and break-induced replication (BIR) events scored in red/white sectored colonies. The Chi-Square test P-values (Pearson) for each type of event between the two strains are indicated (related to Figure 2). The total loss of heterozygosity (LOH), calculated as the sum of CO and BIR events, is shown in the right column.

|       | CO  | NCO | BIR | Total | Total LOH (CO+BIR) |
|-------|-----|-----|-----|-------|-------------------|
| YEN1  | 202 | 1140| 76  | 1418  | 278               |
| YEN1^ON | 310 | 1234| 100 | 1644  | 410               |
| P-value| 0.000652 | 0.000422 | 0.392883 |       | 0.000422          |
Table S2. *S. cerevisiae* strains used in this study.

| Strain    | Genotype                     | Used in Figure |
|-----------|------------------------------|----------------|
| W165      | BY4741                       | 1,2            |
| W455      | YEN1-myc9::KANMX4            | 1,3            |
| W1122     | YEN1<sup>EeA</sup>-myc9::KANMX4 | 1             |
| W1739     | YEN1<sup>IN</sup>-myc9::KANMX4 | 1,3             |
| W166      | yen1Δ::KANMX4                | 2             |
| W1398     | YEN1<sup>EeA</sup>          | 2             |
| W1458     | YEN1<sup>IN</sup>           | 2             |
| W1676     | mus81Δ::HPHX4               | 2             |
| W1769     | mus81Δ::HPHX4 yen1Δ::KANMX4  | 2             |
| W1680     | mus81Δ::HPHX4 YEN1<sup>EeA</sup> | 2             |
| W1736     | mus81Δ::HPHX4 YEN1<sup>IN</sup> | 2,3         |
| W167      | sgs1Δ::HIS3                 | 2             |
| W168      | sgs1Δ::HIS3 yen1Δ::KANMX4    | 2             |
| W1709     | sgs1Δ::HIS3 YEN1<sup>EeA</sup> | 2             |
| W1770     | sgs1Δ::HIS3 YEN1<sup>ON</sup> | 2             |
| W1772<sup>2</sup> | MATα/MATα MUS81/mus81Δ::HPHX4 sgs1Δ::HIS3/SGS1 | 2       |
| W1774<sup>3</sup> | MATα/MATα MUS81/mus81Δ::HPHX4 sgs1Δ::HIS3/SGS1 YEN1<sup>EeA</sup>/YEN1<sup>EeA</sup> | 2       |
| W1776<sup>2</sup> | MATα/MATα MUS81/mus81Δ::HPHX4 sgs1Δ::HIS3/SGS1 yen1Δ::KANMX4/yen1Δ::KANMX4 | 2       |
| W1783<sup>3</sup> | MATα/MATα MUS81/mus81Δ::HPHX4 sgs1Δ::HIS3/SGS1 YEN1<sup>ON</sup>/YEN1<sup>ON</sup> | 2       |
| W2530<sup>4</sup> | MATα/MATα ade2-I lys2::GAL-ISCEI his3::HPHX4 YEN1-myc9::KANMX4 | 2       |
| W2536<sup>5</sup> | MATα ade2-n his3::NATMX4 met22::klURA3 YEN1-myc9::KANMX4 | 2       |
| W2534<sup>4</sup> | MATα/MATα ade2-I lys2::GAL-ISCEI his3::HPHX4 YEN1<sup>ON</sup>-myc9::KANMX4 | 2       |
| W2537<sup>5</sup> | MATα ade2-n his3::NATMX4 met22::klURA3 YEN1<sup>ON</sup>-myc9::KANMX4 | 2       |
| W2543<sup>6</sup> | YEN1-myc18::URA3 | 3,4     |
| W2544<sup>6</sup> | YEN1<sup>ON</sup>-myc18::URA3 | 3     |
| W2545<sup>4</sup> | YEN1<sup>EeA</sup>-myc18::URA3 | 3     |
| W2546<sup>6</sup> | YEN1<sup>58/70A</sup>-myc18::URA3 | 3     |
| W2547<sup>6</sup> | YEN1<sup>58/70A</sup>-myc18::URA3 | 3     |
| W169<sup>7</sup> | mus81Δ::HIS3 | 3     |
| W2428<sup>8</sup> | mus81Δ::HIS3 YEN1<sup>EeA</sup> | 3     |
| W2434<sup>8</sup> | mus81Δ::HIS3 YEN1<sup>58/70A</sup> | 3     |
| W2438<sup>8</sup> | mus81Δ::HIS3 YEN1<sup>58/70D</sup> | 3     |
| W1848<sup>8</sup> | YEN1<sup>EeA</sup>-myc9::KANMX4 | 3     |
| W1850<sup>8</sup> | YEN1<sup>58/70A</sup>-myc9::KANMX4 | 3     |
| W1852<sup>8</sup> | YEN1<sup>58/70D</sup>-myc9::KANMX4 | 3     |
| W2310<sup>9</sup> | cdc28-as1 NATMX4:: P<sub>GAL1</sub>-YEN1-myc9::KANMX4 | 4     |
| W2701<sup>9</sup> | cdc28-as1 NATMX4:: P<sub>GAL1</sub>-YEN1<sup>ON</sup>-myc9::KANMX4 | 4     |
| W2444<sup>4</sup> | MATα YEN1-myc9::KANMX4 | 4     |
| W2448<sup>4</sup> | MATα YEN1-myc9::KANMX4 cdc14-1 | 4     |
| W2452<sup>4</sup> | MATα YEN1-myc9::KANMX4 cdc15-2 | 4     |
| W2454<sup>4</sup> | MATα YEN1-myc9::KANMX4 cdc14-1 cdc15-2 | 4     |
|   | Genotype                                                                 | Reference |
|---|--------------------------------------------------------------------------|-----------|
| W2682 | MATa YEN1<sup>14X</sup>-myc::KANMX4 cdc14-1                             | 4         |
| W2553 | MATa YEN1-myc18::URA3                                                   | 4         |
| W2559 | MATa YEN1-myc18::URA3 cdc14-1                                           | 4         |
| W2686 | MATa YEN1<sup>14X</sup>-myc18::URA3 cdc14-1                             | 4         |
| W2044 | MATa YEN1-myc9::KANMX4                                                  | 5         |
| W2548 | MATa YEN1-myc9::KANMX4 CDC14<sup>1406-1</sup>                           | 5         |
| W2050 | MATa                                                                     | 5         |
| W2088 | MATa leu2-3,112::P<sub>GAL1</sub>-CDC14-FLAG::LEU2                     | 5         |
| W2084 | MATa YEN1-myc9::KANMX4 leu2-3,112::P<sub>GAL1</sub>-CDC14-FLAG::LEU2   | 5         |
| W2323 | MATa leu2-3,112::P<sub>GAL1</sub>-CDC14<sup>4253A</sup>-FLAG::LEU2     | 5         |
| W2327 | MATa leu2-3,112::P<sub>GAL1</sub>-CDC14<sup>4253A</sup>-FLAG::LEU2 YEN1-myc9::KANMX4 | 5         |
| W2325 | MATa leu2-3,112::P<sub>GAL1</sub>-CDC14<sup>4253A</sup>-FLAG::LEU2     | 5         |
| W2329 | MATa leu2-3,112::P<sub>GAL1</sub>-CDC14<sup>4253A</sup>-FLAG::LEU2 YEN1-myc9::KANMX4 | 5         |
| W2305 | MATa ura3-52::P<sub>GAL1</sub>-YEN1-FTH::URA3 yen1Δ::HIS3 pep4Δ::KANMX4 bar1Δ::HPHNT1 | 6         |
| W2309 | MATa ura3-52::P<sub>GAL1</sub>-YEN1<sup>14X</sup>-FTH::URA3 yen1Δ::HIS3 pep4Δ::KANMX4 bar1Δ::HPHNT1 | 6         |
| W2017 | MATa ura3-52::P<sub>GAL1</sub>-YEN1<sup>14X</sup>-FTH::URA3 yen1Δ::HIS3 pep4Δ::KANMX4 bar1Δ::HPHNT1 | 6         |
| W2507 | MATa ura3-52::P<sub>GAL1</sub>-YEN1-FTH::URA3 yen1Δ::HIS3 pep4Δ::KANMX4 bar1Δ::HPHNT1 cdc14-1 | 6         |

1 Unless indicated, all the strains are BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) derivatives.
2 Diploids obtained by mating of BY4741 and BY4742 (MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) derivatives.
3 W303 (ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1) derivative.
EXTENDED EXPERIMENTAL PROCEDURES

Yeast strain and plasmid constructions

All strains were derivatives of *S. cerevisiae* BY4741, BY4742 and W303. Their genotypes are detailed in Table S2. The following alleles have been described previously: *cdc28-as1* (F88G) (Bishop et al., 2000), *YEN1-myc9* (Matos et al., 2011), *TAB6-1* (*CDC14*<sup>116L</sup>) (Shou et al., 2001), *cdc14-1* (*CDC14*<sup>G123D</sup>), *cdc15-2* (*CDC15*<sup>4206D</sup>) (Hartwell et al., 1974), *CDC14*<sup>D253A</sup> and *CDC14*<sup>C283S</sup> (Bloom et al., 2011). Deletion, promoter replacement and epitope-tagging of endogenous genes were carried out by standard one-step PCR replacement. The *cdc28-as1* allele, originally in a W303 background, was backcrossed to BY4741/BY4742.

The *YEN1* coding sequence was cloned into pDONR221 by Gateway recombination (Life Technologies) for site-directed mutagenesis with the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent). *YEN1* variants with the following amino acid substitutions were generated and are listed in Figure S1: *YEN1<sup>EEAA</sup>* (E193A E195A), *YEN1<sup>ON</sup>* (S67A, S71A, S245A, S500A, S507A, S513A, S583A, S655A, S679A), *YEN1<sup>S679A</sup>*, *YEN1<sup>S679D</sup>* and *YEN1<sup>1A</sup>* (S67A, S71A, S245A, S500A, S507A, S513A, S583A), *YEN1<sup>1A</sup>* (S67A, S71A, S245A, S500A, S507A, S513A, S583A, S655A, S679A), *YEN1<sup>4A</sup>* (S67A, S71A, S245A, S500A, S507A, S513A, S583A, S655A, S679A), *YEN1<sup>7A</sup>* (S67A, S71A, S245A, S500A, S507A, S513A, S583A, S655A, S679A), *YEN1<sup>8A</sup>* (S67A, S71A, S245A, S500A, S507A, S513A, S583A, S655A, S679A), *YEN1<sup>11A</sup>* (S67A, S71A, S245A, S500A, S507A, S513A, S583A, S655A, T610A, T614A, S615A, T664A, S679A, T703A), *YEN1<sup>17A</sup>* (S67A, S71A, S245A, S497A, S498A, S500A, T610A, T614A, S615A, T664A, S679A, T703A), *YEN1<sup>10A</sup>* (S243A, S245A, S497A, S498A, S500A, S507A, S513A, S583A, T610A, T614A, S615A, S655A, T664A, S679A, T703A).

All versions of *YEN1* were shuttled into pAG416GPD-ccdB-HA (Alberti et al., 2007) for constitutive expression in yeast. Alternatively, for physiological expression levels, the same mutations were introduced into the endogenous *YEN1* locus by delitto perfetto mutagenesis (Storici and Resnick, 2006).

For purification purposes, codon optimized versions of *YEN1*, *YEN1<sup>ON</sup>* and *YEN1<sup>EEAA</sup>* followed by a C-terminal 3xFLAG-2xTEV-10xHIS (FTH) tag were cloned into a pRS306 derivative under the control of the *GAL1* promoter. The resulting vectors were linearized with StuI and integrated at the *ura3-1* locus of W303 strains.
The coding sequence of *CDC14* followed by a C-terminal FLAG tag was cloned into pDONR221 and site-directed mutagenesis was carried out as described for *YEN1*. The wild-type, D253A and C283S alleles were shuttled to pAG305GAL-cedB and the resulting vectors were linearized with BstEII and integrated at the *leu2-3,112* locus of W303 for galactose-dependent inducible expression. The *CDC14* dominant allele *TAB6-1* was generated in a similar manner and used to replace endogenous *CDC14* by one-step PCR.

**Yeast cultures**

Mitotic time courses were carried out as described (Matos et al., 2011). Briefly, BY4741 *MATa* derivatives were grown exponentially in YPD (OD$_{600}$ ~0.3) at 30°C and synchronized by addition of α-factor (final concentration 5 µg/ml). After 2 h, the cells (>95% unbudded) were harvested, washed once in YPD and released into one half volume of YPD at 30°C. Samples (100 ml cells) were collected at time 0 (G1), 25 min (S/G2-phase) and 60 min (G2/M) for DNA content analysis and for preparation of protein extracts.

For S-phase arrest, solid hydroxyurea was added to cultures growing exponentially (OD$_{600}$ ~0.4-0.5) in YPD or YP$_{Raff}$ (1% yeast extract, 2% peptone, 2% raffinose) to a final concentration of 150 mM and incubated for a further 2 h. Inducible expression of Cdc14-FLAG was achieved by addition of 0.5-2% galactose to cultures either growing exponentially or HU-arrested in YP$_{Raff}$.

For Cdc28-as1 kinase inhibition during S-phase, 5 µM 1NM-PP1 (Santa Cruz) was added to cultures previously arrested in HU. For the induction of Yen1-myc9 before Cdk inhibition, 0.02% galactose was added. Since Cdk inhibition affects transcription, we experimentally titrated the amount of galactose required to achieve similar levels of Yen1 expression after Cdk inhibition, and therefore used 0.1% galactose.

To inhibit the Cdc14-1 and Cdc15-2 temperature sensitive proteins, cultures growing exponentially at 25°C were switched to 37°C for 2 h before harvesting.
Phosphorylation Site Analysis

The phosphorylation sites in Yen1 were determined as previously described for Mms4 (Matos et al., 2011) using affinity-purified myc9- or TAP-tagged Yen1 from both cycling cultures or cultures synchronized in S-phase.

Protein analyses

Protein analysis was performed as described (Matos et al., 2008). Briefly, cell pellets were disrupted using glass beads in 10% TCA. Precipitates were collected by centrifugation, resuspended in 2x NuPAGE sample buffer, and neutralized with 1 M Tris-base. Samples were boiled at 95°C for 5 min, cleared by centrifugation, and separated in either NuPAGE 3-8% Tris-Acetate or 4-12% MOPS polyacrylamide gradient gels (Life Technologies).

Immunoprecipitates were prepared from 50-100 ml of either cycling or synchronized cultures. Cells were lysed with glass beads in buffer A150 (40 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% NP40, 1 mM DTT) containing protease and phosphatase inhibitors. Protein extracts were cleared and normalized (typically ~750 µl, 5-10 mg/ml total) and epitope-tagged proteins were captured using monoclonal antibodies to myc (9E10) or FLAG (Sigma) immobilized on agarose beads (AminoLink Plus, Thermo Scientific).

For Western blotting, proteins were transferred onto Immobilon-P membranes (Millipore) and detected with the following antibodies: myc HRP-conjugated (rabbit, 1:3000, Abcam), myc (mouse 9E10, 1:3000, Cancer Research UK), FLAG-HRP (1:1000, Sigma), Cdc14 (Goat, 1:1000, Santa Cruz), Clb2 (Goat, 1:500, Santa Cruz), and Pgk1 (mouse, 1:5000, Life Technologies).

DNA damage sensitivity assays

Cells grown to mid-log phase were normalized to 0.5x10⁷ cells/ml, and 10-fold serial dilutions were spotted onto YPD plates, or SC-URA plates containing different concentrations of MMS or HU. Plates were incubated in the dark for 3 days at 30°C.
Fluorescence microscopy

Yeast cells were processed for immunostaining as described (Matos et al., 2013). Analyses of Yen1-myc18 localization were performed by examination of >200 cells for each condition. For the quantifications displayed in Figures 3C and 4F, the standard error of the mean was below 3.5% for all samples.

Genetic analysis of DSB-induced recombination

Analysis of recombination outcome during mitotic DSB repair was performed as described (Ho et al., 2010; Matos et al., 2013; Mazón et al., 2012) using diploids that were freshly generated for each experiment by mating the appropriate MATα and MATa haploids: YEN1, W2530 x W2536; YEN1ON, W2534 x W2537 (Table S2).

Yen1 purification

FTH-tagged Yen1, Yen1ON and Yen1EEAA were expressed from the GAL1 promoter by addition of 2% galactose to cultures in YPraffinose. Preparations were made either from cells growing exponentially at 30°C, or from cdc14-1 mutants arrested at the restrictive temperature (37°C) in anaphase (to obtain fully phosphorylated Yen1). Cells (4 liters at ~2-4x10⁷ cells/ml), were harvested, washed and resuspended in a small volume of A500 buffer (40 mM Tris-HCl, pH 7.5, 500 mM NaCl, 20% glycerol, 0.1% NP-40, 1 mM DTT) containing phosphatase and protease inhibitors and disrupted in a freezer mill. The powder was resuspended in ~ 2x vol of A500, cleared by ultracentrifugation and incubated with anti-FLAG M2 agarose beads (Sigma) for 1 h at 4°C. The beads were extensively washed in A500 and proteins eluted with three volumes of A500 supplemented with 0.5 mg/ml 3xFLAG peptide. If it was wanted to dephosphorylate Yen1-FTH, the appropriate preparation was supplemented with 1 mM MnCl₂, split into two, and one half either mock-treated or incubated with 6,000 units of lambda phosphatase (New England Biolabs) for 30 min at room temperature. Next, to all preparations, imidazole was added to 5 mM and the proteins were loaded onto a Ni-NTA column (Qiagen), the column was washed with
A500 buffer containing increasing concentrations of imidazole up to 50 mM, and finally Yen1 was eluted with A500 containing 300 mM imidazole. Yen1 was dialyzed extensively against A500, and stored in aliquots at -80°C. Protein concentrations were determined using the Bradford assay (BioRad) and on Coomassie-stained PAGE gels using BSA as the standard. Typical yields were in the range of 0.5-1.1 mg at a concentration of ~ 5-10 µM. Control experiments confirmed the absence of non-specific endo- or exonuclease activities.

DNA binding and nuclease assays

5'-32P-end-labeled synthetic DNA substrates were prepared as described (Rass and West, 2006). Unlabeled substrates were purified by native 12% PAGE and visualized by UV-shadowing. Binding assays were carried out for 15 min on ice by mixing Yen1 with unlabeled DNA (10 nM) supplemented with a small amount of 5'-32P-end-labeled DNA (~0.2 nM) in 10 µl of binding buffer (50 mM Tris-HCl pH 7.5, 125 mM NaCl, 5 mM EDTA, 1 mM DTT, 100 µg/ml BSA, 11% glycerol, and 2 ng/µl duplex poly dIdC). Protein-DNA complexes were separated by 5% PAGE using 0.5x TBE and radiolabeled DNAs were visualized by autoradiography.

Nuclease assays were carried out with either immobilized Yen1-myc9 (immuno-affinity purification was carried out using α-myc beads) or with purified Yen1-FTH proteins. The beads (10 µl) were washed extensively, and then mixed with 10 µl reaction buffer (50 mM Tris-HCl pH 7.5, 1 mM MgCl2) containing ~0.5 nM 5'-32P-end-labeled synthetic Holliday junction X0 or X26 DNA (Ip et al., 2008). Reactions were incubated for 15-20 min with gentle rotation at 30°C and stopped by addition of 2.5 µl 10 mg/ml proteinase K and 2% SDS, and further incubation at 37°C for 1 h. Loading buffer was added and radiolabeled products were separated by 10% PAGE, and analyzed by autoradiography or by phosphorimaging using a Typhoon scanner and ImageQuant software. The nuclease activity of purified Yen1-FTH was analyzed using 10 nM unlabeled DNA (10 nM) supplemented with 5'-32P-end-labeled DNA (~0.2 nM) in 10 µl reaction buffer (50 mM Tris-HCl pH 7.5, 1 mM MgCl2, 50 mM NaCl and 2% glycerol). The products were analyzed by autoradiography and phosphorimaging.
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