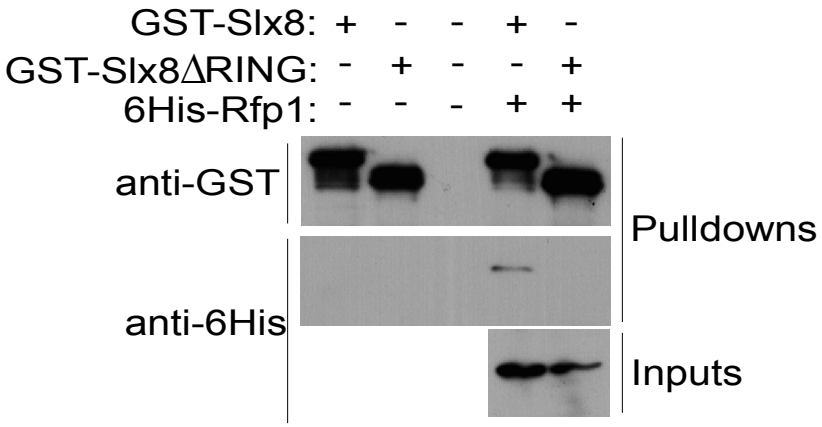
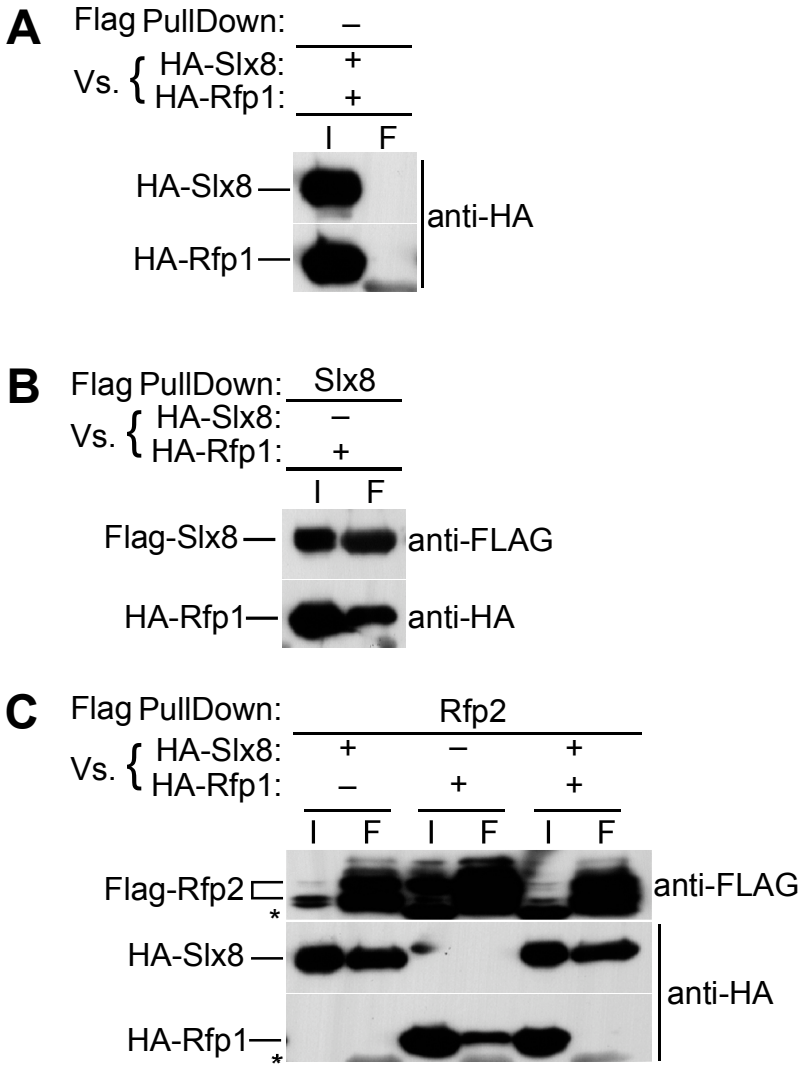
Supplementary Figure 1 Prudden *et al*., 2007



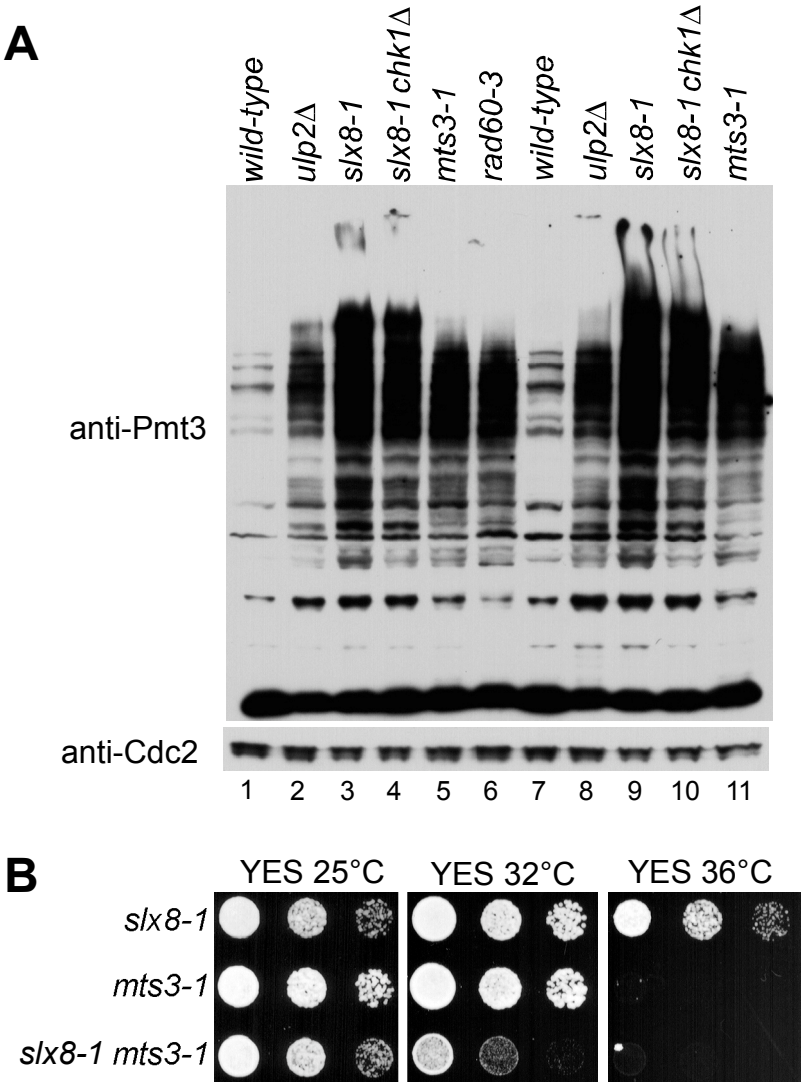
Supplementary Figure 1. The interaction of Slx8 with Rfp1 is dependent on the Slx8 RING-domain. The indicated proteins were either expressed as single plasmids, or co-expressed in bacteria. Cell lysates were purified on GST beads (Pulldowns), washed samples were resolved by SDS-PAGE and immuno-blotted with antisera to the GST or 6His epitopes. The soluble 6His-Rfp1 inputs are indicated. (+), present; (-), absent.

Supplementary Figure 2 Prudden *et al*., 2007



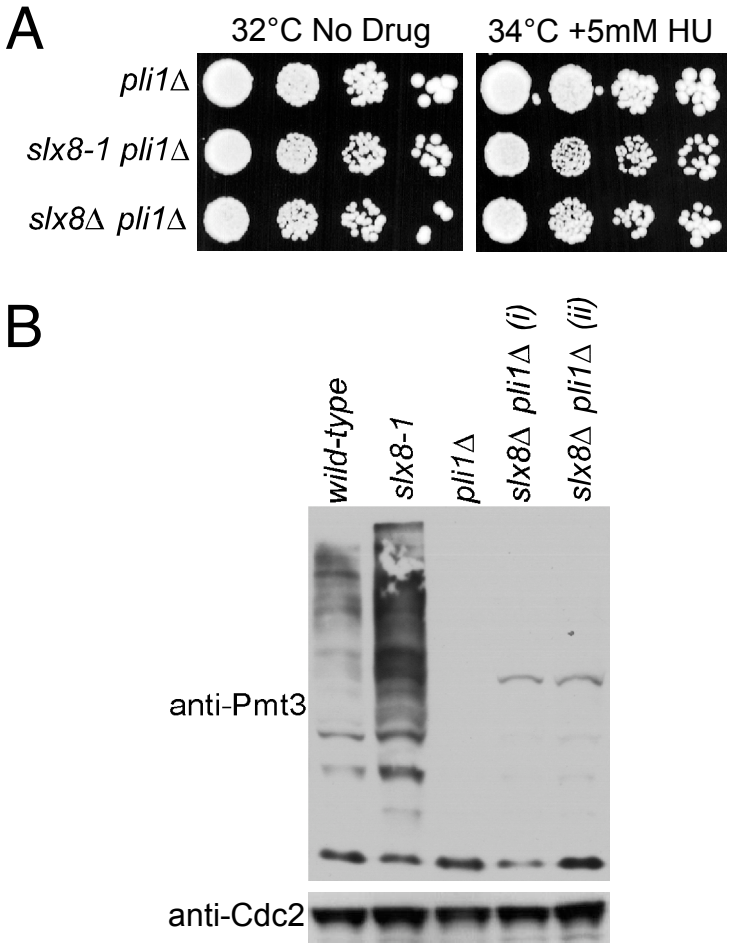
Supplementary Figure 2. Slx8 forms mutually exclusive heterodimers with either Rfp1 or Rfp2 in an insect cell expression system. Insect cells were simultaneously infected with baculoviruses to co-express the indicated epitope-tagged recombinant proteins. After lysis, an input sample was taken (I: Input), and the rest was immunoprecipitated with anti-FLAG beads (F; Flag pull-down). Samples were resolved by SDS-PAGE and immunoblotted with antisera to the HA or FLAG epitopes. (A) Control for background binding of HA-tagged proteins to anti-FLAG affinity gel. (B) FLAG-Slx8 co-precipitates with HA-Rfp1. (C) FLAG-Rfp2 co-precipitates with HA-Slx8 or weakly with HA-Rfp1, but competes with HA-Rfp1 for binding to HA-Slx8. +, present; -, absent; *, background band.

Supplementary Figure 3 Prudden *et al*., 2007



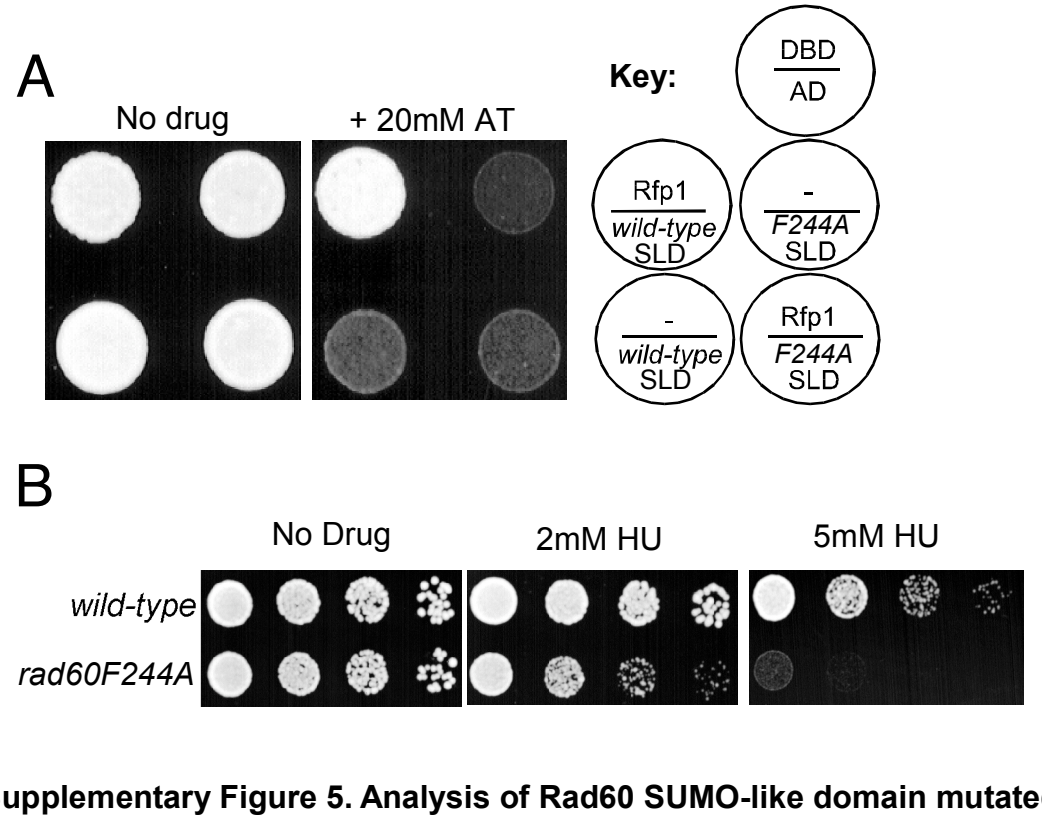
Supplementary Figure 3. Accumulation of SUMO conjugates in *rad60-3* and *mts3-1* mutant backgrounds. (A) Total lysates were prepared under denaturing conditions from the indicated strains, following 7 hrs incubation at 36˚ C, resolved by SDS-PAGE and immunoblotted with antisera for SUMO (Pmt3), or Cdc2 (loading control). Lanes 7 to 10 are shown in Figure 4C in the manuscript. Lanes 7 to 11 are the same protein samples as in Lanes 1 to 5, however, twice the relative amount of protein was resolved in Lanes 7 to 11. (B) Serial dilutions of the indicated strains, grown at the indicated temperatures.

Supplementary Figure 4 Prudden *et al*., 2007



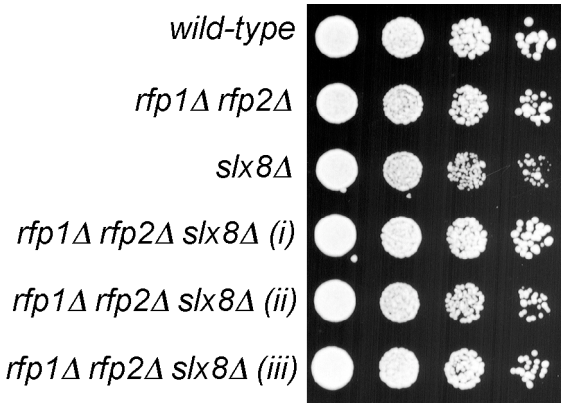
Supplementary Figure 4. Deletion of Pli1 also rescues deletion of Slx8. (A) Serial dilutions of the indicated strains were grown in the presence or absence of 5mM HU. (B) Total lysates were prepared under denaturing conditions from the indicated strains, resolved by SDS-PAGE and immunoblotted with antisera for SUMO (Pmt3), or Cdc2 (loading control). All strains were incubated for 7 hrs at 36˚ C.

Supplementary Figure 5 Prudden *et al*., 2007



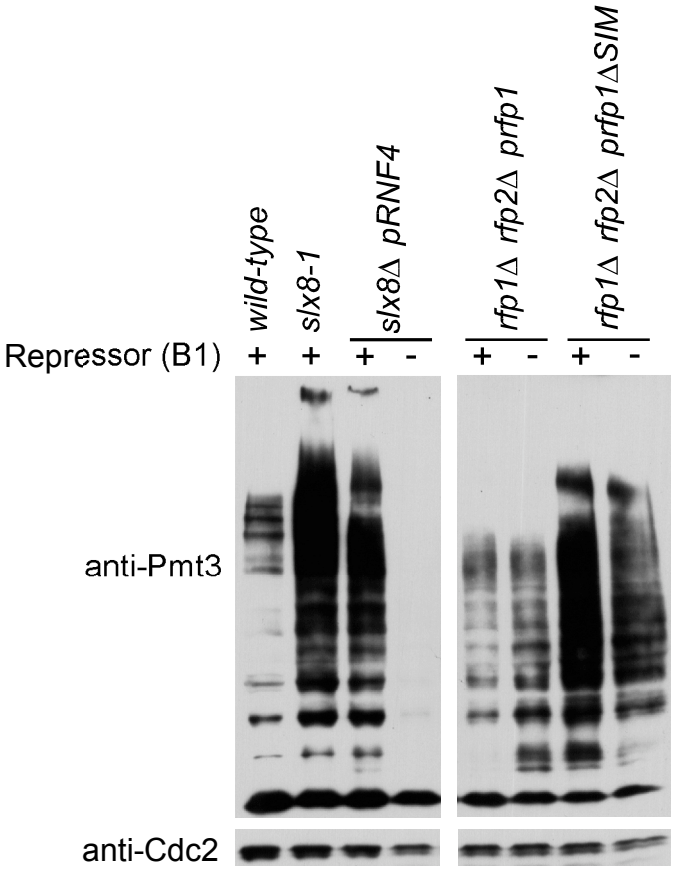
Supplementary Figure 5. Analysis of Rad60 SUMO-like domain mutated within its SIM binding pocket. (A) Two-hybrid analysis shows that *rad60F244A* SLD mutant no longer interacts with Rfp1. The indicated yeast two hybrid strains were spotted onto selective plates, which were untreated (No drug), or drug treated (20mM 3-AT), to identify interacting proteins. Key indicates genes placed into the Gal4 DNA binding (DBD) or the Gal4 activating (AD) domains. Empty vector controls (-) are shown. **(B) The *rad60F244A* mutation results in Hydroxyurea sensitivity in fission yeast .** Serial dilutions of the indicated strains were grown at 32°C, and were either non-treated (No Drug), or treated with the indicated concentrations of Hydroxyurea (HU).

Supplementary Figure 6 Prudden *et al*., 2007



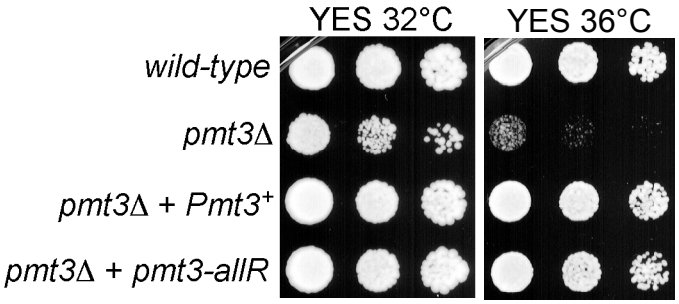
Supplementary Figure 6. Expression of *H. Sapiens* RNF4 rescues the viability of an *rfp1Δ*, *rfp2Δ*, *slx8Δ* triple mutant. Serial dilutions of the indicated strains were grown under conditions which induced expression of *RNF4*.

Supplementary Figure 7 Prudden *et al*., 2007



Supplementary Figure 7. Analyzing sumoylation pathway homeostasis in *rfp1*Δ *rfp2*Δ double mutants. Total lysates were prepared under denaturing conditions from the indicated strains, resolved by SDS-PAGE and immunoblotted with antisera for SUMO (Pmt3), or Cdc2 (loading control). The *wild-type* and *slx8-1* controls were incubated for 7 hrs at 36˚ C. the remaining strains were grown in inducing conditions (-B1) or repressing conditions (+B1).

Supplementary Figure 8 Prudden *et al*., 2007



Supplementary Figure 8. Expression of *pmt3-allR* suppresses the lethality of *pmt3*Δ. Serial dilutions of the indicated strains were grown at the semi-permissive temperature (32°C), or the restrictive temperature (36°C) for the *pmt3*Δ strain. The *pmt3*Δ + *Pmt3*$^{+}$ and *pmt3*Δ + *pmt3-allR* integrants were expressed from endogenous Pmt3 promoters.

Supplemental Materials and Methods:

Mammalian transfections and pulldown experiments: pCDNA3-Myc-Rnf4 is a derivative of pCDNA3 with a single 5' Myc-tag on RNF4 cDNA. pCDNA3-NIP45-Flag has a single 3' FLAG-tag on NIP45 cDNA. pCDNA3-Flag-NIP45 and derivative mutants have a single 5' FLAG-tag on NIP45 cDNA. The Flag-NIP45-Nterm mutant contains amino acids 1-265 of NIP45. The pHH10B-Ha SUMO-1DC4 was kindly provided by Dr. Frauke Melchior (University Goettingen, Goettingen, Germany). The Flag-NIP45-Nterm-SUMO-1DC4 is a fusion protein between Flag-NIP45-Nterm and SUMO-1DC4. HEK293T cells were transfected with the indicated plasmids using Effectene (Invitrogen) following the manufacturers instructions. Where indicated, 2mM Hydroxurea was added 36 hours following transfection, and cells were harvested after an additional 12 hours incubation. Cells were washed with 1xPBS, and lysed in 20mM Tris-HCl pH 7.4, 150mM NaCl, 1% Nonidet P-40, 5mM EDTA, protease inhibitors and 20mM N-Ethylmaleimide. Cell lysates were subjected to immunoprecipitation with anti-Flag M2 agarose beads (Sigma-Aldrich), and analyzed by Western blot using anti-myc (9E10, Covance) and anti-Flag (Sigma-Aldrich) antibodies.

Expression of recombinant *S. pombe* proteins and interaction assays in insect cells: DNA sequences encoding the open reading frames of Slx8, Rfp1 and Rfp2 were amplified by PCR from an *S. pombe* cDNA library using Pfu Turbo (Stratagene, La Jolla, CA) and the following primer pairs. For Slx8, 5'-ATCGGGATCCGATGCCACCCGCACATAAG-3' and 5'-ATGCAAGCTCGAGTTAAGATTTCTTT TTTT-GAGATCCGAGC-3' ; for Rfp1, 5'-ATCGGGATCC GATGCAATTTAATGGAAGCAATGG-3' and 5'-ATGCAAGCTCGAGTCAATAAAATAATTCCCACATG GC-3'; and for Rfp2, 5'-ATGCAAGCTCGAGTTAT AAATATAAAGGAAATATAAATTTTTTGGTTATAC-3' and 5'- ATCGGGATCCGATGAATCTGCATGGACT AGAATTAC -3'. These PCR fragments were cloned between the *BamHI* and *XhoI* restriction sites in frame into the Bac-to-Bac baculovirus expression vector pFastBac HT (Invitrogen, Carlsbad, CA) where the 6xHis-TEV tag was replaced by a single Hemagglutinin (HA) or FLAG tag in the N-terminus. Transposition of these vectors into bacmids, as well as baculovirus production methods were described elsewhere (Pebernard *et al*., 2006). Direct interaction between Slx8, Rfp1 and Rfp2 was tested by FLAG immunoprecipitation in Sf9 cells co-infected with the indicated baculoviral stocks, as described previously (Pebernard *et al*., 2006). Co-precipitated proteins were resolved on 12% SDS/PAGE and immunoblotted for the HA and FLAG epitopes using the mouse monoclonal 12CA5 (BABCO) and anti-FLAG M2 (Sigma-Aldrich, Saint Louis, MI) antibodies, respectively.

Determining the Rad60 SUMO-like domain F244A SIM binding mutation: Searching with Rad60 sequence (Uniprot ID: Q9USX3) for homologues, using PSI-BLAST (Altschul *et al*., 1997) and SAM-T06 (Karplus *et al*., 1998) revealed similarity to homologues with experimentally determined three-dimensional structures. Notably, the Rad60 N-terminal SLD is most similar to SUMO-1, while the C-terminal SLD has greater similarity to human SUMO-3. For comparative modeling, the sequence alignments of the Rad60 N and C-terminal SLDs to their structural homologues was optimized by iterative multiple sequence alignment methods (Burke *et al*., 1999), meta-server fold recognition (Kurowski & Bujnicki, 2003; Wallner B *et al*., 2007) and manual adjustment. Rad60 comparative models were generated using Modeler 8v1 (Sali & Blundell, 1993). Analysis of the structural models and conservation pattern in the multiple sequence alignment indicated that a *F244A* mutation likely perturbs SIM binding to the N-terminal SLD, without affecting the domain's structural integrity.

Supplementary References:

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997 Sep 1;25(17):3389-402.

Burke DF, Deane CM, Nagarajaram HA, Campillo N, Martin-Martinez M, Mendes J, Molina F, Perry J, Reddy BV, Soares CM, Steward RE, Williams M, Carrondo MA, Blundell TL, Mizuguchi K. An iterative structure-assisted approach to sequence alignment and comparative modeling. Proteins. 1999;Suppl 3:55-60.

Karplus K, Barrett C, Hughey R. Hidden Markov models for detecting remote protein homologies. Bioinformatics. 1998;14:846– 56.

Kurowski MA, Bujnicki JM. GeneSilico protein structure prediction meta-server. Nucleic Acids Res. 2003 Jul 1;31(13):3305-7.

A. Sali & T.L. Blundell. Comparative protein modelling by satisfaction of spatial restraints. J. Mol. Biol. 234, 779-815, 1993

Wallner B, Larsson P, Elofsson A. Pcons.net: protein structure prediction meta server. Nucleic Acids Res. 2007 Jun 21; [Epub ahead of print]

Supplemental Strains:

Fission yeast Strains. All strains are *ura4-D18 leu1-32* unless otherwise stated.

NBY700, *mts3-1*.
NBY1457, *pmt3::ura4*$^{+}$.
NBY1510, *rad60::hphMx6* pJK148-*rad60F244A:myc:kanMx6*.
NBY1515, *rad60::hphMx6* pJK148-*Rad60:myc:kanMx6*.
NBY1525, *pmt3::ura4*+ pREP41 (-nmt), *Pmt3*$^{+}$.
NBY1526, *pmt3::ura4*+ pREP41 (-nmt), *pmt3-K9R*.
NBY1580, *mts3-1 slx8-1:myc:kanMx6*.
NBY1596, *rfp1::kanMx6 rfp2::hphMx6 slx8::hphMx6* pREP41-EGFP-*RNF4*.

Budding yeast Strains*. All strains are Y190 derived.

NBY1503, pAS404 pGADT7-*Rad60-SLDs* (aa 188-406) *F244A*.
NBY1504, pAS404-Rfp1 pGADT7-*Rad60*-SLDs (aa 188-406) *F244A*.

*Two-hybrid interactions were analyzed on -LEU -HIS -TRP plates that contained no drug, or 20mM 3-aminotriazole.