Supplementary Data

A RECONSTITUTED SYSTEM REVEALS HOW ACTIVATING AND INHIBITORY INTERACTIONS CONTROL DDK DEPENDENT ASSEMBLY OF THE EUKARYOTIC REPLICATIVE HELICASE

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Figure S1 (related to main Figure 2). Proteins used in this study

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Supplementary Material & Methods

Table S1. Oligonucleotides

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Supplementary References
Figure S1 (related to main Figure 2). Proteins used in this study. (A) Purified proteins. Please note that DDK is a dimer consisting of Cdc7 and Dbf4. (B) Immobilized proteins used for pull down reactions. An arrow marks the specific proteins, the asterisk degradation products and the hash indicates the heavy chain of the antibody. (C) Analysis of DDK activity in the context of the loaded MCM2-7 double-hexamer. (D) Analysis of DDK activity in the context of Sld3.
Figure S2 (related to main Figure 2 and 6). Complex formation is specific. (A) Cdc6 and DNA are essential to promote Sld3 recruitment to the pre-RC. “+ all” indicates the presence of both, Sld3 and the pre-RC proteins. The load indicates the presence of the factors before loading onto the gel-filtration column. (B) ORC does not support Cdc45 recruitment. 40 nM of each protein was used.
Figure S3 (related to main Figure 8). MCM2-7 bob-1 analysis. (A) MCM2-7 bob-1 promotes efficient MCM2-7 association in the presence of low salt (LS) and high salt (HS) resistant MCM2-7 loading. This assay was performed with the magnetic bead based method (2). (B) Cdc45 interacts weakly with the pre-RC MCM2-7 bob-1. We observed only very weak Cdc45 interactions with pre-RC. This interaction was analyzed using the gel-filtration based pre-RC assay. (C) Prolonged interactions of Cdc45 with the pre-RC MCM2-7 bob-1 indicate weak nonspecific Cdc45 interactions with DNA. (B and C) Lanes 3-6 show a dilution series representing % of total protein (40 nM), which were added into the reactions.
Oligonucleotides, plasmids and antibodies

Oligonucleotides, plasmids and antibodies used in this study are listed in Table S1, S2 and S3, respectively.

Cloning

The coding sequence of Sld2 and Dpb11 were amplified by PCR with primers (CS1144, CS1145 and CS1260, CS1261, respectively) that added NotI and EcoRI restriction sites. The PCR products were cloned into pGEX 6P1 vector.

The coding sequence of Sld7 was amplified by PCR with primers CS1653 and CS1654, adding NotI and BamHI restriction sites and cloned into pGEX-6P1 (GE Healthcare).

The coding sequence of Sld3 was amplified by PCR with primers CS785 and CS818, adding BamHI and SalI restriction sites and cloned into pESC-HIS (Agilent Technologies). This construct was then modified by insertion of an N-terminal MBP tag, amplified from pMALC2x with primes CS921 and CS922. The primers also coded for a PreScission protease site and a myc tag and BamHI restriction sites.

For yeast expression, the coding sequence of Cdc45 (missing the stop codon) was amplified by PCR with primers CS561 and CS572, adding NotI and SpeI restriction sites and cloned in pESC-HIS, pESC-LEU, pESC-TRP and pESC-URA vectors (Agilent Technologies). The stop codon was deleted in order to have a C-terminal FLAG tag.

The coding sequence of Cdc45 was amplified by PCR with primers CS591 and CS592 adding BamHI and NotI restriction sites and cloned in pGEX 6P1 vector (GE Healthcare) for bacterial expression.

MBP-PreScission-Dbf4 and Cdc7 were cloned into pESC-TRP (Stratagene). The dbf4 gene was amplified from genomic DNA of the *S. cerevisiae* strain S288C with the primers CS1052 and CS1053 via SmaI/NheI. The MBP-PreScission gene was amplified from pCS245 with primers CS921 and CS922 and cloned in before the dbf4 gene using a BamHI site. The cdc7 gene was amplified from genomic DNA of the *S. cerevisiae* strain S288C with the primers CS1058 and CS1059 and cloned via NotI/SpeI the vector. The resulting plasmid is pCS313.

Bacterial protein expression

The respective plasmids were transformed in BL21 Codon Plus RIL *E. coli* competent cells (Agilent). The cells were grown in Terrific Broth supplemented with appropriate antibiotics at 37°C to an OD600 of 1. Protein expression was then induced by addition of 0.5 mM IPTG for 5h at 16°C.

Yeast protein expression

Yeast strain AS499 (*MATa. bar1Δ, leu2-3,-112, ura3-52, his3-Δ200, trp1-Δ-63, ade2-1 lys2-801, pep4*) was transformed. The yeasts were grown overnight in selective medium at 30°C. The proteins were expressed as described previously (3).
**Protein purification**

**Sld3 purification**

Cells were lysed in a freezer mill. The cells were resuspended in buffer AS [50 mM HEPES/NaOH pH 8.1, 0.5 M NaCl 10% glycerol, 0.1% NP40, 1mM DTT] plus complete protease inhibitor cocktail (PIC) (Roche), extracted on a rotating wheel for 2h at 4 °C and centrifuged at 27,600 g for 1 h at 4 °C. The extract was incubated for 2h at 4 °C with Amylose Resin (NEB). The beads were afterwards rinsed with 10 column volumes buffer AS + PIC and washed twice with 20 cv Buffer AS + PIC for 30 min and once with 20 cv Buffer AS without PIC for 30 min. MBP-myc-Sld3 was eluted at that stage with buffer AS + 10 mM Maltose. To elute myc-Sld3, instead, beads were incubated with 1 cv buffer AS (without protease inhibitors and PreScission protease for 2 h at 4 °C and rinsed afterwards additionally with 1 cv buffer AS. Elutions were diluted to 0.1 M NaCl, bound 30 min to SP-Sepharose (GE Healthcare). The resin was rinsed once with 10 cv buffer BS [50 mM HEPES/NaOH pH8.1, 0.1 M NaCl 10% glycerol, 0.1% NP40, 1mM DTT] and once with 10 cv buffer CS [50 mM HEPES/NaOH pH 8.1, 0.2 M NaCl 10% glycerol, 0.1% NP40, 1mM DTT]. The protein was eluted in buffer AS.

**Sld2 purification**

The cells were lysed by sonication in buffer A [50 mM PIPES (pH 6.5), 500 mM AS, 10 mM MgCl₂, 5 mM DTT, and 1% Triton, 10% Glycerol, 2 mM ATP]. The fusion protein was bound to glutathione agarose (Sigma) in buffer A at 4 °C for 2 h, and then the protein was eluted by addition of PreScission Protease (GE Healthcare) for 2h at 4 °C. The eluate was diluted with 1 volume buffer A with no salt and bound to SP Sepharose (GE Healthcare). Sld2 was eluted with buffer B [50 mM PIPES (pH 6.5), 500 mM AS, 5 mM DTT, and 0.1% Triton, 10% Glycerol].

**Cdc45 purification**

The protein was purified as previously described (4). To remove the FLAG peptide the sample was incubated for 30 min with DEAE beads (GE Healthcare), the resin was extensively washed and the protein was eluted with purification buffer containing 500 mM KCl.

**Sld7 purification**

The cells were lysed by sonication in buffer C [50 mM HEPES-KOH (pH 7.5), 300 mM NaCl, 10 mM MgCl₂, 1 mM DTT, and 1% Triton, 10% Glycerol, 2 mM ATP]. Ammonium sulphate was added to a final concentration of 250 mM to the extract. Nucleic acids were precipitated from the cell extract by the addition of 30 µl 10 % polymin P/HCl (pH 6.5) per ml of extract. GST-Sld7 was precipitated by adding 0.4 g fine powdered ammonium sulphate per ml of extract. The fusion protein was bound to glutathione agarose (Sigma) in buffer C at 4 °C for 2 h, and then Sld7 was eluted by addition of PreScission Protease (GE Healthcare) for 2h at 4 °C. The eluate was concentrated using a centricon concentrator (Millipore).
DDK purification

Cells were lysed in a freezer mill. The resulting extract was incubated with amylose resin (NEB) pre-equilibrated with buffer DK [50 mM Hepes-KOH pH 7.5, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.02% NP40, 1 mM DTT] and supplemented with proteases and phosphatases inhibitors for 2 h at 4°C. The protein was eluted by cleaving the MBP tag via addition of PreScission protease for 2 h at 4°C. The eluate was incubated with Glutathione agarose for 2 h to remove the PreScission protease. The GST flow-through was collected and concentrated by centricon (Millipore).

Dpb11 purification

The cells were lysed by sonication in buffer D [50 mM PIPES (pH 6.5), 100 mM NaCl, 3 mM DTT, and 1% Triton, 10% Glycerol]. Ammonium sulphate was added to a final concentration of 250 mM to the extract. Nucleic acids were precipitated from the cell extract by the addition of 45 µl 10 % polymin P/HCl (pH 6.5) per ml cell extract. Protein from the supernatant was precipitated by adding 0.35 g fine powdered ammonium sulphate per ml of extract. The fusion protein was bound to glutathione agarose (Sigma) in buffer D at 4 °C for 2 h, and then Dpb11 was eluted by addition of PreScission Protease (GE Healthcare). The eluate was diluted with 1 volume buffer D with no salt and bound to SP Sepharose (GE Healthcare). Dpb11 was eluted with buffer E [50 mM PIPES (pH 6.5), 500 mM NaCl, 5 mM DTT, 10 mM MgCl₂, 0.1% Triton, 10% Glycerol].

Competitor DNA

Two complementary oligonucleotides (CS1126 and CS 1127, table S1) were incubated in buffer M [100 mM Tris-HCl (pH 7.9), 100 mM NaCl, 1 mM DTT, 10 mM MgCl₂] for 5 min at 95 °C and then slowly cooled down to RT over 30 min. The hybridized oligos were concentrated using Microcon (Millipore) to a concentration of 5 µM.

In vitro transcription and translation

To generate 35S-Met-labelled proteins, the coding sequences of the genes were amplified using a sense primer and anti-sense primers, as suggested by the supplier's instruction (Promega). To compensate for different methionine content additional methionines were incorporated into the anti-sense primer. 400 ng of the resulting PCR product was used as template in the TNT-T7 Quick for PCR DNA system (Promega), combined with 20 µl of TNT Quick Master Mix and 20 µCi of 35S-Met (Perkin-Elmer) in a total volume of 25 µl and incubated for 90 min at 30°C with mixing.

Sld3 pull-down reactions

Immobilisation (MBP-Sld3): MBP-Sld3 (400 ng) and an equimolar amount of MBP were immobilized on anti-MBP antibody (NEB) beads (protein G - Sigma) for 15 min at 24°C with mixing in 50 µl of Sld3 buffer CS [pH 7.5] followed by three washes with 100 µl of buffer I (225 mM K-Glu, 1% BSA).

Immobilisation (Sld3): Sld3 (150 ng) was immobilized on anti-Sld3 318 beads (protein G) for 15 min at 24°C with mixing in 50 µl of Sld3 binding buffer followed by three washes and used for pull downs, together with IgG control beads.
Pull-down: *In vitro* translated proteins or purified proteins (0.5 µg) were incubated with MBP or MBP-Sld3 beads for 2h (*in vitro* translated proteins) or 1 h (purified proteins) with mixing at 4°C in 500 µl (*in vitro* translated proteins) or 200 µl (purified proteins) of buffer I (225 mM K-Glu, 1% BSA); afterwards the beads were washed three times with 200 µl of buffer I (225 mM K-Glu) and separated by SDS/PAGE followed by phosphor-imager analysis (*in vitro* translated proteins) or Western blot (purified proteins).

**Sld2 pull-down reactions**

**Extract preparation:** The pellets of 50 ml of GST and GST-Sld2 cultures were resuspended in 2.5 ml of Sld2 buffer A. 0.1 mg/ml of lysozyme and 25 U/ml Benzonase (Merck) were added and the mixture was incubated on ice for 30 min. The extract was sonicated, the supernatant separated by centrifugation and aliquoted.

**Immobilisation (GST-Sld2):** GST-Sld2 (400 ng) and an equimolar amount of GST were immobilized by incubating 50 µl of GST and GST-Sld2 containing extracts with anti-GST antibody (Sigma) beads (protein G – GE Healthcare) for 1h at 4°C with mixing in 100 µl of Sld2 buffer A followed by three washes with 100 µl of buffer I (225 mM K-Glu, 1% BSA).

**Pull-down: *In vitro* translated proteins or 0.5 µg of purified proteins were incubated for 2 h (*in vitro* translated proteins) or 1 h (purified proteins) with mixing at 4°C in 500 µl (*in vitro* translated proteins) or 200 µl (purified proteins) of buffer I (225 mM K-Glu, 1% BSA); afterwards the beads were washed three times with 200 µl of buffer I (225 mM K-Glu) and analysed by SDS/PAGE followed by phosphorimaging analysis (*in vitro* translated proteins) or Western blot (purified proteins).

**Cdc45 pull-down reactions**

**Extract preparation:** The pellets of 50 ml of GST and GST-Cdc45 culture were resuspended in 2.5 ml of Cdc45 binding buffer (C45), then 0.1 mg/ml of lysozyme were added and the mixture was incubated on ice for 30 min. The extract was sonicated and the supernatant separated by centrifugation and aliquoted.

**Immobilisation and pull-down:** GST-Cdc45 (400 ng) and an equimolar amount of GST were immobilized by incubating 50 µl of GST and GST-Cdc45 containing extracts with anti-GST antibody (Sigma) beads for 1h at 4°C with mixing in 100 µl of buffer C45 and used for pull downs as described for Sld2.
| Primers name | Usage | Gene | Primers sequence |
|--------------|-------|------|------------------|
| CS1144       | Cloning | Sld2 | ATGACGCGGGCGCTACCTTTCTCCCACATCGT |
| CS1145       | Cloning | Sld2 | AGTCTGGGATTCATGTACTTCATTGGAACCTGGACAATTTG |
| CS1260       | Cloning | Dpb11 | CCGCTCGAGTCAAGAATCATCTAATCTCTTTGCTGATTTTC |
| CS1261       | Cloning | Dpb11 | ATAAAGATGCGCGCCCATGAAGCCTTTCTAAGGACAA |
| CS1653       | Cloning | Sld7 | TATTCACGTGGATCCATGTAGTGAATACAC |
| CS1654       | Cloning | Sld7 | CATTATATATGCAGCGCTCATGATTTGGTGAAAGAGC |
| CS785        | Cloning | Sld3 | CGCGGAGTCCATGGAAACATGGGAAAGTCATAGC |
| CS818        | Cloning | Sld3 | ATGGCCGACGTCGACCTATGTGGATTCTGGAGCAATATAA |
| CS561        | Cloning | Cdc45 | GCTAGCGCGCCCATGTATTATGGAATCAGCCAGTTTAG |
| CS572        | Cloning | Cdc45 | GCTAACTAGTCCTAAACAATCCACTCAAGGTCAGC |
| CS591        | Cloning | Cdc45 | GACTGCGGATCCATGTATTATGGAATCAGCCAGTTTAG |
| CS592        | Cloning | Cdc45 | GACTGCGCGGGCGCTTTAAACAATCCACTCAAGGTCAGC |
| CS1052       | Cloning | Dbf4 | TCCTCCCCCAGGATGGTTTCTCAACCGAAATGAA |
| CS1053       | Cloning | Dbf4 | CTACTACTAGCTAGCTATATTGGAATCTGGATTTTC |
| CS1058       | Cloning | Cdc7 | AAGGAAAGAAACGCGCGCATGACAAGCAAACGAAAGATA |
| CS1059       | Cloning | Cdc7 | CTAAGCTAGACTAGTCTATTCAGATATAAGGAGAA CAT |
| CS1126 | Competitor DNA | - | TCCTGCTGGAAATATACAGATGAACCCGGGACGGGTCGGTTC |
| CS1127 | Competitor DNA | - | AGAACGACCCGTCCCCGGGTTTCTCCTGTATATTC CAGCAGGA |
| CS1670 | In vitro translation | Mcm2 | ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG TCTGATAATAGAACGCTAGAC |
| CS1671 | In vitro translation | Mcm2 | AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA GTG ACC CAA GGT ATA AAT TGC |
| CS1667 | In vitro translation | Mcm3 | ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG GAAGGCTCAACGCGATTGG |
| CS1674 | In vitro translation | Mcm3 | AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA TCA GAC TCT CCA AAC TTT ATC G |
| CS1677 | In vitro translation | Mcm4 | ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG TCTCAACAGTCTAGCTCTCCAA |
| CS1678 | In vitro translation | Mcm4 | AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA TCA GAC AC G TT ATT CAG GCG |
| CS1681 | In vitro translation | Mcm5 | ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG TCATTTGATAGACCAGAACATAC |
| CS1682 | In vitro translation | Mcm5 | AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA TCA TAC ACC ACT TCT GTA AAT ATT C |
| CS1685 | In vitro translation | Mcm6 | ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG TCATCCCCTTTTCAGC |
| CS1686 | In vitro translation | Mcm6 | AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA GCT GGA ATC CTG TGG TTC |
| CS1689 | In vitro translation | Mcm7 | ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG AGTGCGGCACCTCCATC |
| CS1690 | In vitro translation | Mcm7 | AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA TCA AGC GTC TTG TAG ATC GAT AC AG |
| CS1766 | In vitro translation | Sld3 | ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG GAA ACA TGG GAA GTC ATA G |
| CS1767 | In vitro translation | Sld3 | AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA CTA TGT GGA TTC TGG AGC AAA TA |
| CS2044 | In vitro translation | Sld2 | ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG TAC TCA TTT GAA CTG GAC AAA TTG |
| CS2050 | In vitro translation | Sld2 | AAACACAAAAACAAAAACAAAAAATA TCA TCT TCC CCA TCG TC |
| CS2040 | In vitro translation | Sld7 | ACTGATC TAA TACGACTCACTATAGGG CAT CCACCAG TCA CGG AAA TTA TGC ACA CTA AAT TTT |
| CS2041 | In vitro translation | Sld7 | AAACACAAAAACAAAAACAAAAAATA TCA TTA TTT GGT AAA GAG TTT CAG |
| CS1764 | In vitro translation | Cdc45 | ACTGATC TAA TACGACTCACTATAGGG CAT CCACCAG TAT TAT GGA ATC AGC CAG TTT AG |
| CS1765 | In vitro translation | Cdc45 | AAACACAAAAACAAAAACAAAAAATA TTA CAA TCC ACT CAA GGT C |
| CS2042 | In vitro translation | Dpb11 | ACTGATC TAA TACGACTCACTATAGGG CAT CCACCAG TAT TAT GGA ATC AGC CAG TTT AG |
| CS2043 | In vitro translation | Dpb11 | AAACACAAAAACAAAAACAAAAAATA TCA AGA ATC TAA TTC CTT TGT CTG |
| CS921  | MBP-Protease-BamHIb | MBP | CGCGCGGGATCCCCGGGCCCCGCGGGGACAGACT TCCAGGTATCAGTATCCGAGGT |
| CS922  | MBP-Protease-myc-BamHIb | MBP | CGCGCGGGATCCGGGAGTCTTTCTGTGCCCGAATCAA CTCCGCTGGGCCCCGCGGGGACAGACTTCCAG GGTCGTATCGATCCCCGAGGT |
### Table S2. Plasmids

| Plasmid name | Gene/DNA Sequence | Vector   | Source                      |
|--------------|-------------------|----------|-----------------------------|
| pSC372       | ARS1              | pUC19    | Evrin C et al, PNAS. 2009   |
| pCS245       | sld3              | pESC-HIS | This study                  |
| pCS8         | cdc45             | pESC-HIS | This study                  |
| pCS241       | cdc45             | pESC-LEU | This study                  |
| pCS242       | cdc45             | pESC-TRP | This study                  |
| pCS243       | cdc45             | pESC-URA | This study                  |
| pCS229       | cdc45             | pGEX-6P1 | This study                  |
| pSC290       | sld2              | pGEX-6P1 | This study                  |
| pSC486       | sld7              | pGEX-6P1 | This study                  |
| pSC300       | dpb11             | pGEX-6P1 | This study                  |
| pCS313       | cdc7/dbf4         | pESC-Trp | This study                  |
### Table S3. Antibodies

| Antibody name | Antigen | Peptide Sequence          | Source                        |
|---------------|---------|---------------------------|-------------------------------|
| Cdc6          | Cdc6    | -                         | mouse Santa Cruz (SC53218)   |
| ORC1          | ORC1    | -                         | mouse Stillman (SB13)         |
| MCM2          | MCM2    | -                         | mouse Zou and Stillman, Mol Cell Biol. 2000 |
| Sld3 (318)    | Sld3    | CTKKGLVRRRSKKTSE          | rabbit This study            |
| Sld3 (418)    | Sld3    | CKLKGPSMRPKRALKKVND       | rabbit This study            |
| Sld2          | Sld2    | CRKRPKRKVRIRRLRDNPETE     | rabbit This study            |
| Sld7          | Sld7    | THRDELKRISMGSEEVS         | rabbit This study            |
| Dpb11         | Dpb11   | CGSIQDKKRTASLEKPMRRQTRNQTE| rabbit This study            |
| FLAG          | FLAG    | -                         | mouse Sigma (A8592)          |
| MBP           | MBP     | -                         | mouse NEB (E8032L)           |
| GST           | GST     | -                         | mouse Pierce (P08263)        |
Supplementary References

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3. Burgers, P.M. (1999) Overexpression of multisubunit replication factors in yeast. *Methods*, **18**, 349-355.

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