Supplementary Materials and methods

Construction of off-aid strains

Construction of \( P_{nmt81}^{\text{pol1}} \)-aid, \( P_{nmt81}^{\text{spp2}} \)-aid, \( cdc20 \)-aid and \( dpb2 \)-aid strains has been described previously (Kanke et al., 2011; Kanke et al., 2012). A strain expressing Cdc20-aid from \( P_{nmt81} \) was constructed as described below. The 5'-UTR of \( cdc20^+ \) was amplified by PCR using the primers SPNCRNA.418-F2 (primers used in this study are listed in Supplementary Table S3) and SPNCRNA.418DW-BamHI-R from 972 (\( h^+ \), wild-type) genomic DNA, and a 0.6-kb \( \text{SpeI-BamHI} \)-digested product and a 1.7-kb \( \text{BamHI-EcoRV} \)-digested fragment containing the \( \text{hphMX6}^+ \) marker gene from the pFA6a-hphMX6 plasmid (Hentges et al., 2005) were introduced into the \( \text{SpeI-EcoRV} \) sites of pBluescript SK+ (pBS), yielding the plasmid pBS-SPNCRNA.418tail-hphMX6. The N-terminal region of \( cdc20^+ \), linking with \( P_{nmt81} \), was amplified using the primers Pnmt-EcoRV-F and cdc20NTD-SpeI-R from the plasmid pREP81-cdc20, in which the \( \text{NdeI-XmaI} \) fragment containing the full-length \( cdc20^+ \) was introduced into pREP81. The 1.5-kb \( \text{EcoRV-SpeI} \)-digested product was cloned into the \( \text{EcoRV-SpeI} \) sites of pBS, yielding the plasmid pBS-Pnmt81-cdc20NTDhead. Two \( \text{SpeI-EcoRV} \)-digested fragments from pBS-SPNCRNA.418tail-hphMX6 and pBS-Pnmt81-cdc20NTDhead were cloned into the \( \text{SpeI} \) site of pBS, yielding the plasmid pBS-SPNCRNA.418tail-hphMX6-Pnmt81-cdc20NTDhead. This plasmid was digested with \( \text{SpeI} \) and used for transformation of the \( cdc20 \)-aid strain to create the \( P_{nmt81}^{\text{pol1}} \)-aid strain. The insertion was confirmed by genomic PCR. A strain expressing Dpb2-aid from \( P_{nmt81} \) was constructed using the two-step PCR method. The first PCR amplified 0.3-kb and 0.2-kb fragments containing, respectively, the 5'-UTR of \( dpb2^+ \) linking with part of a selective marker (\( \text{hphMX6} \)), and the N-terminal region of \( dpb2^+ \) linking with part of a \( P_{nmt81} \), using respective pairs of primers – ‘dpb2UP-F’ and ‘dpb2UP-hphMX6-R’, and ‘Pnmt81-dpb2-F’ and ‘dpb2-HeadR’ – from 972 genomic DNA. The second PCR amplified the integration cassette from the pBS-SPNCRNA.418tail-hphMX6-Pnmt81cdc20NTDhead plasmid with the primers: ‘dpb2UP-F’ and ‘dpb2-HeadR’ and the two short fragments obtained in the first PCR. The integration cassette obtained above was then used for transformation into the \( dpb2 \)-aid strain to create the \( P_{nmt81}^{\text{pol1}} \)-dpb2-aid strain. Correct integration of the cassette at the \( dpb2^+ \) locus was confirmed by genomic PCR, and the sequence was confirmed to
have no mutation in dpb2⁺.

**Epitope-tagging of Cdc20, Pol1, and Cdc6**

All epitope-tagged strains were made by integration of a 3xFLAG- or 3xHA- gene fragment into the original chromosome loci at the C-terminus. The cdc20⁺ gene was tagged with a DNA sequence encoding six consecutive histidine residues and 3xFLAG or 3xHA epitopes using the two-step PCR method, essentially as described previously (De Antoni and Gallwitz, 2000). The first PCR amplified 0.3-kb and 0.2-kb fragments containing, respectively, the C-terminal region of cdc20⁺ linking with part of an epitope, and the downstream region of cdc20⁺ linking with part of a selective marker (kanMX6⁺) gene, using respective pairs of primers – ‘cdc20CterF’ and ‘Tag5-His+cdc20CterR’, and ‘Tag3-ter+cdc20DWF’ and ‘cdc20DW2R’ – from 972 genomic DNA. The second PCR amplified the integration cassette from the pU6H3FLAG or pU6H3HA plasmid with the primers ‘cdc20CterF’ and ‘cdc20DW2R’ and two short fragments obtained in the first PCR. The integration cassette obtained above was then used for transformation of the 972 strain. Correct integration of the cassette at the cdc20⁺ locus was confirmed by genomic PCR, and the sequence was confirmed to have no mutation in cdc20⁺. The presence of Cdc20-FLAG or Cdc20-HA protein in the cell extracts was then confirmed by Western blotting analysis with anti-FLAG or anti-HA antibody. The resulting strains showed wild-type growth, suggesting that the Cdc20-FLAG and Cdc20-HA were functional. pol1-flag and cdc6-flag were constructed similarly. The integration cassette for the pol1⁺ gene was amplified from plasmid pU6H3FLAG using the primers ‘pol1CterF’ and ‘pol1DWR’ and the first PCR products obtained below. Two 0.3-kb fragments were amplified using respective pairs of primers – ‘pol1CterF’ and ‘Tag5-His+pol1CterR’, and ‘Tag3-ter+pol1DWF’ and ‘pol1DWR’ – from 972 genomic DNA. The integration cassette for the cdc6⁺ gene was amplified from plasmid pU6H3FLAG using the primers ‘cdc6CterF’ and ‘cdc6DWR’ and the first PCR products obtained below. Two 0.2-kb fragments were amplified using respective pairs of primers – ‘cdc6CterF’ and ‘Tag5-His+cdc6CterR’, and ‘Tag3-ter+cdc6DWF’ and ‘cdc6DWR’ – 972 genomic DNA. The correct integration and sequence were confirmed. The resulting pol1-flag and cdc6-flag strains showed wild-type growth.
**Isolation of cdc20 carboxyl-terminal domain mutants**

In order to introduce a random mutation in the C-terminal domain of *cdc20*+, a mutating plasmid containing the *cdc20*+ CTD region (from 3653 to 6600 bp of *cdc20*+ ORF), was constructed as described below. Genomic fragments of 1.5-kb and 1.2-kb containing the upward and downward regions of the *cdc20*+ CTD region, respectively, were amplified from 972 genomic DNA using respective pairs of primers – ‘*cdc20Cter1F*’ and ‘*cdc20Cter1R*’, and and ‘*cdc20Cter2F*’ and ‘*cdc20Cter2R*’. The PCR products, which had the EcoRI sites at both 5’ and 3’ ends, were cloned into the EcoRI site of PBS resulting in the plasmids pBS-cdc20Cterm1 and pBS-cdc20Cterm2. Genomic fragments of 0.7-kb and 0.2-kb containing the C-terminal region and the downstream region of *cdc20*+, respectively, were amplified using respective pairs of primers – ‘*cdc20DW1F*’ and ‘*cdc20DW1R*’, and ‘*cdc20DW2F*’ and ‘*cdc20DW2R*’ – to create the HindIII site 0.2-kb downstream of the *cdc20*+ gene. The 0.7-kb fragment was cloned into the XhoI-HindIII sites of PBS, yielding the plasmid pBS-cdc20DW1. The 0.2-kb fragment was cloned into the HindIII site of PBS, yielding the plasmid pBS-cdc20DW2. The HindIII-BamHI fragment of pBS-cdc20DW2 was cloned into the HindIII-BamHI site of pBS-cdc20DW1, yielding the plasmid pBS-cdc20DW. The HindIII-digested 1.8-kb fragment containing the *ura4*+ gene was inserted at the HindIII site of pBS-cdc20DW, yielding the plasmid pBS-cdc20DW-ura4. Finally, the XhoI-PstI-digested 1.4-kb fragment from pBS-cdc20Cterm1 and the PstI-SalI-digested 1.2-kb fragment from pBS-cdc20Cterm2 were cloned into the XhoI-SalI site of pBS-cdc20DW-ura4, yielding the plasmid pBS-cdc20Cterm-ura4, which was then used for mutagenesis of *cdc20*+ CTD. Mutating PCR was carried out with ampliTaq Gold (Perkin Elmer) containing 1.5 mM MgCl₂ and 0.125 mM MnCl₂ using ‘*cdc20mutF*’ and ‘*cdc20mutR*’ as primers. The PCR products were used for transformation of HM83 (*h*+ *ura4-D18). Among *ura4*+ transformants grown on EMM plates at 25°C, temperature-sensitive mutants at 36°C on EMM and YE plates were selected, and the correct integration at the *cdc20*+ locus was confirmed by genomic PCR.

**Construction of the cdc20ΔN strain**

A strain expressing Cdc20 CTD from *P*ₙ₅₁ or *P*ₙ₅₄₁ was constructed as described below. The C-terminal region of *cdc20*+, linking with *P*ₙ₅₁ or *P*ₙ₅₄₁, was amplified using the
primers Pnmt-EcoRV-F and cdc20Cter1R from plasmid pREP1-cdc20 CTD or pREP41-cdc20 CTD, in which the NdeI-XmaI fragment containing the CTD (amino acids 1218-2199) of cdc20+ was introduced into pREP1 or pREP41, and the product was digested with EcoRV and BamHI. The 1.8-kb digested product was then cloned into the EcoRV-BamHI sites of pBS, yielding the plasmid pBS-Pnmt1-cdc20CTDhead or pBS-Pnmt41-cdc20CTDhead. Two SpeI-EcoRV-digested fragments from pBS-SPNCRNA.418tail-hphMX6, and pBS-Pnmt1-cdc20CTDhead or pBS-Pnmt41-cdc20CTDhead, 2.3 kb and 1.8 kb long, respectively, were cloned into the SpeI site of pBS, yielding the plasmid pBS-SPNCRNA.418tail-hphMX6-Pnmt1-cdc20CTDhead or pBS-SPNCRNA.418tail-hphMX6-Pnmt41-cdc20CTDhead, which was then digested with SpeI and used for transformation to create PnPnmt1-cdc20-CTD or PnPnmt41-cdc20-CTD strain. The insertion was confirmed by genomic PCR.

Construction of the cdc20-dpb2 dpb2Δ strain
A strain expressing Cdc20-Dpb2 from the cdc20+ original chromosome loci was constructed as described below. The C-terminal region of cdc20+ was amplified using the primers cdc20tail-speI-F and cdc20tail-5GA-NdeI-R from 972 genomic DNA. A SpeI-NdeI-digested 0.3-kb PCR product and an NdeISacI-digested fragment from pREP1-dpb2, in which the NdeI-BamHI fragment containing the full-length dpb2+ was introduced into pREP1, were cloned into the SpeI-SacI sites of pBS, yielding the plasmid pBS-cdc20tail-5GA-dpb2-nmtT. The downstream region of cdc20+ was amplified using the primers cdc20DW-BglII-F and cdc20DW-SpeI-R from 972 genomic DNA. A BglII-SpeI-digested 0.2-kb PCR product and a BglII-SacI-digested fragment from pFA6a-kanMX6 were cloned into the SpeI-SacI sites of pBS, yielding the plasmid pBS-kanMX6R-cdc20DW. The two SpeI-SacI-digested products from pBS-cdc20tail-5GA-dpb2-nmtT and pBS-kanMX6R-cdc20DW were cloned into the SpeI site of pBS, yielding the plasmid pBS-cdc20tail-5GA-dpb2-nmtT-kanMX6R-cdc20DW, which was then digested with SpeI and used for transformation to create the cdc20-dpb2 strains. The insertion was confirmed by genomic PCR. In the cdc20-dpb2 strains, the dpb2+ gene was replaced with hphMX6, essentially as described previously (Hentges et al., 2005).
**Yeast two-hybrid assay**

The BD MATCHMAKER GAL4 2-Hybrid System 3 (BD-Clontech) was used for yeast two-hybrid analysis. Derivatives of pGBK7, Gal4-DNA binding domain (BD) vector, and pGADT7, an activation-domain (AD) vector, were used. A pair of pGBK7 and pGADT7 derivatives were introduced into *S. cerevisiae* AH109 (MAT1, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, URA3::MEL1UAS-MEL1TATA-lacZ MEL1) cells. Trp⁺ Leu⁺ transformants harboring both plasmids were selected on synthetic glucose medium (SD) lacking tryptophan and leucine (-WL) and the interaction was assayed for activation of the HIS3 and ADE2 reporter genes by growth on selective media lacking histidine (-WLH) or histidine and adenine (-WLHA) at 30°C for 3-4 days. When indicated, 2 mM 3-aminotriazole (3AT), an inhibitor of histidine synthesis, was added to -WLH (-WLH+3AT) and -WLHA (-WLHA+3AT).
References in Supplementary Materials

De Antoni, A., and Gallwitz, D. (2000). A novel multi-purpose cassette for repeated integrative epitope tagging of genes in *Saccharomyces cerevisiae*. Gene 246, 179-185.

Hentges, P., Van Driessche, B., Tafforeau, L., Vandenhaute, J., and Carr, A.M. (2005). Three novel antibiotic marker cassettes for gene disruption and marker switching in *Schizosaccharomyces pombe*. Yeast 22, 1013-1019.

Kanke, M., Kodama, Y., Takahashi, T.S., Nakagawa, T., and Masukata, H. (2012). Mcm10 plays an essential role in origin DNA unwinding after loading of the CMG components. EMBO J 31, 2182-2194.

Kanke, M., Nishimura, K., Kanemaki, M., Kakimoto, T., Takahashi, T.S., Nakagawa, T., and Masukata, H. (2011). Auxin-inducible protein depletion system in fission yeast. BMC Cell Biol 12, 8.
Legends to Supplementary Figures

Supplementary Figure S1. Recruitment of GINS, Pol α, Pol δ and PCNA onto the origins followed by limited DNA synthesis at the origins in cdc20Δct1.

(A) As described in Fig. 2A, aliquots of nda3-KM311 (wild-type) and nda3-KM311 cdc20-ct1 (cdc20-ct1) derivatives carrying pol1-flag or cdc6-flag were analyzed by ChIP assay at 60 min after M release. ChIP recoveries of two early origins, ars2004 and ars3002, and the non-origin fragments located 30 kb distant from ars2004, were measured by qPCR. Mean ± s.d. obtained from multiple measurements in qPCR is presented.

(B) As described in Fig. 2B, HM1427 P\textsubscript{nmt1}TK P\textsubscript{adhl}hENT nda3-KM311 (wild-type) and HM1431 cdc20-ct1 P\textsubscript{nmt1}TK P\textsubscript{adhl}hENT nda3-KM311 (cdc20-ct1) cells were synchronously released from M-phase at 36°C in the presence of 5-bromo-2'-deoxyuridine (BrdU, 200 mM), with addition of hydroxyurea (HU, 15 mM) in the case of wild-type. Samples taken at 60 min after M release were analyzed using the BrdU-IP assay. BrdU-IP DNAs were analyzed by qPCR using primers for ars2004, the regions located 1 kb, 2 kb and 4 kb distant from ars2004, denoted as ‘+1kb’, ‘+2kb’ or ‘+4kb’, and the non-origin region. Mean ± s.d. obtained from multiple measurements in qPCR is presented.

Supplementary Figure S2. Cdc45 migrates from the origin in cdc20ΔN.

(A) HM4544 cdc20-flag cdc45-myc cdc25-22 (wild-type) or HM4553 P\textsubscript{nmt41}-cdc20-CTD-flag cdc45-myc cdc25-22 (cdc20ΔN) strain grown at 25°C were arrested at G2/M-phase by incubation at 36°C for 3.5 h, and then released at 25°C in the presence of HU (10 mM). DNA contents of the wild-type (left) and cdc20ΔN (right) cells taken at the indicated time points were analyzed by flow cytometry. Positions of 1C and 2C DNA contents are shown. (B) Samples taken at 70 min after G2/M release were analyzed by ChIP assays. Chromatin-immunoprecipitated DNAs with anti-myc, anti-Rpa2 or anti-FLAG were analyzed by qPCR using primers for ars2004, the regions located 2 kb and 4 kb distant from ars2004 and the non-origin region. Mean ± s.d. obtained from multiple measurements in qPCR is presented.

(C) Wild-type (HM3017 P\textsubscript{nmt1}-cdc20), cdc20ΔN (HM3101 P\textsubscript{nmt1}-cdc20-CTD), cdc20-ct1 (HM4338 P\textsubscript{nmt1}-cdc20-ct1) and cdc20-S1904PΔN (HM4353 P\textsubscript{nmt1}-cdc20-S1904P-CTD) cells in log phase were serially diluted ten-fold and spotted onto EMM plates, followed by
incubation at 25°C, 33°C or 36°C. (D) Wild-type (HM3017), cdc20ΔN (HM3101) cdc20-ct1 (HM4338) and cdc20-S1904PΔN (HM4353) cells grown at 25°C in EMM medium were incubated at 36°C for the indicated times and the DNA content was then analyzed by flow cytometry. Positions of 1C and 2C DNA contents are indicated.

**Supplementary Figure S3.** Cdc45 and Pol ε migrate from the origin without significant DNA synthesis under Pol α depletion.

(A) As described in Figs. 3A-D and 6A-C, wild-type, Pol α-depletion and Cdc20-depletion derivatives carrying P\textsubscript{adh1}-TK P\textsubscript{adh1}-hENT, were analyzed by BrdU-labeling assay at 90 min after G2/M release. BrdU-IP DNAs were analyzed by qPCR for the non-origin, ars2004, and the regions located 1 kb, 2 kb and 4 kb distant from ars2004. Mean ± s.d. obtained from multiple measurements in qPCR is presented.

(B) As described in Fig. 3E, wild-type and Pol α-depletion derivatives carrying flag-cdc45, cdc20-flag or cdc6-flag were analyzed by ChIP assay at 80 min after G2/M release. Mean ± s.d. obtained from multiple measurements in qPCR is presented.

**Supplementary Figure S4.** Origin association of Cdc20 and Dpb2 is impaired by the cdc20-ct1 mutation.

As described in Fig. 5B, wild-type and cdc20-ct1 derivatives carrying cdc20-flag or flag-dpb2 were analyzed by ChIP assay at 60 min after M release. Mean ± s.d. obtained from multiple measurements in qPCR is presented.

**Supplementary Figure S5.** Pol ε is required for replisome assembly.

(A) As described in Fig. 6D, aliquots of HM3452 psf2-flag cdc45-myc 2xTIR1 cdc25-22 (wild-type) and HM3450 P\textsubscript{nmT81}-cd20-aid psf2-flag cdc45-myc 2xTIR cdc25-22 (Cdc20 depletion) taken at the indicated time points were analyzed by ChIP assays with anti-Mcm6, anti-FLAG or anti-myc, except for HU-free wild-type cells as a control. Mean ± s.d. obtained from multiple measurements in qPCR is presented. (B) As described in Figs. 6E, aliquots of HM3240 slf3-flag cut5-myc 2xTIR1 cdc25-22 (wild-type) and HM3400 P\textsubscript{nmT81}-cd20-aid slf3-flag cut5-myc 2xTIR cdc25-22 (Cdc20 depletion) taken at the indicated time points were analyzed as (A).
**Supplementary Figure S6.** Dpb2 is required for loading of CMG components onto replication origins. Dpb2-aid protein was depleted, similarly to the methods used for Pol α depletion described in Fig. 3A, and thiamine was added at 6 h before G2/M arrest (-9.5 h). (A) Dpb2-aid protein in whole-cell extracts prepared at the indicated time points was analyzed by immunoblotting with anti-IAA17 antibody. Tag- (lane 1) and Pnative (lane 2) indicate the samples of the untagged strain (HM3021) and the strain for Dpb2-aid, expressed from native promoter (HM3063, dpb2-aid 2xTIR1 cdc25-22) grown at 25°C, respectively. Non-specific protein bands are indicated by asterisks (*). CBB represents total proteins bound on the membrane stained with Coomassie brilliant blue. (B) DNA contents of the wild-type (left) and Dpb2-depleted (right) cells were analyzed by flow cytometry. Positions of 1C, 2C and 4C DNA contents are shown. (C) Dpb2-depletion derivatives carrying sld3-flag cut5-myc, drc1-flag or psf2-flag cdc45-myc were analyzed using ChIP assays with anti-Mcm6, anti-FLAG or anti-myc, similarly to the procedures described in Figs. 6D and 6E.

**Supplementary Figure S7.** Mutually dependent association of Cdc20 and Dpb2 with the replication origins. (A) Cdc20-aid and FLAG-Dpb2 proteins in whole-cell extracts prepared at the indicated time points (see Fig. 6) were analyzed by immunoblotting using anti-IAA17 or anti-FLAG antibodies. Lanes 1 and 2 indicate the samples of the Dpb2-untagged strain (HM3021) and the Cdc20-untagged strain (HM3310) grown at 25°C, respectively. CBB represents total proteins bound on the membrane stained with Coomassie brilliant blue. (B) Aliquots of HM3310 flag-dpb2 2xTIR1 cdc25-22 (wild-type) and HM3375 Pnmt81-cdc20-aid flag-dpb2 2xTIR cdc25-22 (Cdc20 depletion) taken at the indicated time points were analyzed by flow cytometry. (C) Localizations of Mcm6 and Dpb2 were analyzed by ChIP assays as described for Figs. 6D and 6E. In the case of wild-type cells, HU (10 mM) was added at Time 0. Mean ± s.d. obtained from multiple measurements in qPCR is presented. (D) Dpb2-aid and Cdc20-FLAG proteins in whole-cell extracts prepared at the indicated time points (see Supplementary Fig. S6) were analyzed by immunoblotting using anti-IAA17 or anti-FLAG antibodies. Lanes 1 and 2 indicate the samples of the Cdc20-untagged strain (HM3021) and the
Dpb2-untagged strain (HM3575) grown at 25°C, respectively. CBB represents total proteins bound on the membrane stained with Coomassie brilliant blue. (E) Aliquots of HM3575 \( cdc20\)-flag 2xTIR1 \( cdc25\)-22 (wild-type) and HM3361 \( P_{\text{nmt81}}\)-dpb2-aid \( cdc20\)-flag 2xTIR \( cdc25\)-22 (Dpb2 depletion) taken at the indicated time points were analyzed by flow cytometry. (F) Samples taken in (E) were analyzed by ChIP assays with anti-Mcm6 or anti-FLAG, described in (C).

**Supplementary Figure S8.** Yeast two-hybrid analysis of Cdc20 or Dpb2 with replication factors.

(A) Yeast two-hybrid analyses performed using pGADT7 and pGBK7 derivatives are shown. \( S.\ cerevisiae \) AH109 co-transformed with pGADT7-cdc20 or pGADT7-cdc20-ct1 and pGBK7 derivatives was assayed for activation of the \( HIS3 \) and \( ADE2 \) reporter genes on non-selective (-WL) plates, and also on selective medium lacking histidine and/or adenine (-WLH, -WLHA) and with addition of 2 mM 3-aminotriazole (3AT) to a -WLH or -WLHA (-WLH+3AT, -WLHA+3AT) plate. (B) \( S.\ cerevisiae \) AH109 co-transformed with pGADT7-cdc20 or pGADT7-cdc20-ct1 and pGBK7 derivatives was assayed for activation of the \( HIS3 \) and \( ADE2 \) reporter genes. ‘++’ and ‘+’ indicate growth of co-transformed cells on -WLHA and -WLH selective medium plates, respectively, and ‘–’ indicates no growth on selective medium. ‘NT’ indicates not tested. (C) \( S.\ cerevisiae \) AH109 co-transformed with pGBK7-dpb2 and pGADT7 derivatives was assayed for activation of the \( HIS3 \) and \( ADE2 \) reporter genes. Mcm5* encodes amino acids 1-213 of Mcm5. ‘++’ and ‘+’ indicate growth of co-transformed cells on -WLHA and -WLH+3AT selective medium plates, respectively, and ‘–’ indicates no growth on selective medium.

**Supplementary Figure S9.** Pol ε is required for DNA replication after the initiation process.

(A) For synchronous release from HU-arrest, HM3623 flag-dpb2 \( cdc45\)-myc 2xTIR1 \( cdc25\)-22 (wild-type) and HM3621 \( P_{\text{nmt81}}\)-dpb2-aid flag-dpb2 \( cdc45\)-myc 2xTIR1 \( cdc25\)-22 (Cdc20 depletion) cells were arrested at G2/M-phase by incubation at 36°C for 3.5 h and released at 25°C in the presence of HU (13 mM, -3 h). To shut off the transcription of \( cdc20\)-aid, thiamine (10 µg/ml) was added 2 h before G2/M release (-5
h). After cultured with HU for 2 h (-1 h), the cells were incubated with auxin (0.5 mM) for 1 h to degrade Cdc20-aid proteins and then released into fresh media containing thiamine (10 µg/ml) and auxin (0.5 mM), but without HU (Time 0). (B) Aliquots taken at indicated time points were analyzed for flow cytometry. Positions of 1C and 2C DNA contents are indicated. (C) Samples taken at -1 h were analyzed by ChIP assays with anti-Mcm6, anti-FLAG, anti-myc or anti-Rpa2 and quantified using primers for ars2004 and the non-origin region. Mean ± s.d. obtained from multiple measurements in qPCR is presented. The results indicate that replication factors were loaded onto origins before depletion of Cdc20.
## Supplementary Table S1: Yeast strains used in this study

| Strain     | Genotype                                                                 | Source     |
|------------|---------------------------------------------------------------------------|------------|
| HM3017     | h cdc20::hphMX6-P<sub>myc</sub>-cdc20                                      | This work  |
| HM3021     | h cdc25-22 ade6::ade6<sup>-P<sub>myc</sub>-skp1-1-OsTIR1-natMX6-P<sub>myc</sub>-skp1-1-A<sub>TIR</sub>1-2NLS</sup> | Our stock |
| HM3032     | h cdc43::flag-cdc43-ura4<sup>-P<sub>myc</sub>-skp1-1-OsTIR1-natMX6-P<sub>myc</sub>-skp1-1-A<sub>TIR</sub>1-2NLS</sup> | This work  |
| HM3063     | h dpb2::dpb2-aid-ura4<sup>-P<sub>myc</sub>-skp1-1-OsTIR1-natMX6-P<sub>myc</sub>-skp1-1-A<sub>TIR</sub>1-2NLS</sup> | This work  |
| HM3101     | h cdc20::hphMX6-P<sub>myc</sub>-cdc20-CTD                                  | This work  |
| HM3118     | h dpb2::hphMX6-P<sub>myc</sub>-dpb2-aid-ura4<sup>-P<sub>myc</sub>-skp1-1-OsTIR1-natMX6-P<sub>myc</sub>-skp1-1-A<sub>TIR</sub>1-2NLS</sup> | This work  |
| HM3240     | h sld3-30 ura4::ura4<sup>-P<sub>myc</sub>-skp1-1-OsTIR1-natMX6-P<sub>myc</sub>-skp1-1-A<sub>TIR</sub>1-2NLS</sup> | This work  |
| HM3284     | h drc1::drc1-3flag-kanMX6<sup>-P<sub>myc</sub>-skp1-1-OsTIR1-natMX6-P<sub>myc</sub>-skp1-1-A<sub>TIR</sub>1-2NLS</sup> | This work  |
| HM3310     | h dpb2::4flag-dpb2-sup3-5<sup>-P<sub>myc</sub>-skp1-1-OsTIR1-natMX6-P<sub>myc</sub>-skp1-1-A<sub>TIR</sub>1-2NLS</sup> | This work  |
| HM3327     | h cdc20::hphMX6-P<sub>myc</sub>-cdc20-aid-ura4<sup>-P<sub>myc</sub>-skp1-1-OsTIR1-natMX6-P<sub>myc</sub>-skp1-1-A<sub>TIR</sub>1-2NLS</sup> | This work  |
| HM3361     | h dpb2::hphMX6-P<sub>myc</sub>-dpb2-aid-ura4<sup>-P<sub>myc</sub>-skp1-1-OsTIR1-natMX6-P<sub>myc</sub>-skp1-1-A<sub>TIR</sub>1-2NLS</sup> | This work  |
| HM3375     | h cdc20::hphMX6-P<sub>myc</sub>-cdc20-aid-ura4<sup>-P<sub>myc</sub>-skp1-1-OsTIR1-natMX6-P<sub>myc</sub>-skp1-1-A<sub>TIR</sub>1-2NLS</sup> | This work  |
| HM3400     | h cdc20::hphMX6-P<sub>myc</sub>-cdc20-aid-ura4<sup>-P<sub>myc</sub>-skp1-1-OsTIR1-natMX6-P<sub>myc</sub>-skp1-1-A<sub>TIR</sub>1-2NLS</sup> | This work  |
| HM3445     | h dpb2::4flag-dpb2-sup3-5<sup>-P<sub>myc</sub>-skp1-1-OsTIR1-natMX6-P<sub>myc</sub>-skp1-1-A<sub>TIR</sub>1-2NLS</sup> | This work  |
| HM3450     | h cdc20::hphMX6-P<sub>myc</sub>-cdc20-aid-ura4<sup>-P<sub>myc</sub>-skp1-1-OsTIR1-natMX6-P<sub>myc</sub>-skp1-1-A<sub>TIR</sub>1-2NLS</sup> | This work  |
| HM3452     | h dpb2::hphMX6-P<sub>myc</sub>-dpb2-aid-ura4<sup>-P<sub>myc</sub>-skp1-1-OsTIR1-natMX6-P<sub>myc</sub>-skp1-1-A<sub>TIR</sub>1-2NLS</sup> | This work  |
| HM3454     | h cdc20::hphMX6-P<sub>myc</sub>-cdc20-aid-ura4<sup>-P<sub>myc</sub>-skp1-1-OsTIR1-natMX6-P<sub>myc</sub>-skp1-1-A<sub>TIR</sub>1-2NLS</sup> | This work  |
| HM3462     | h cdc20::hphMX6-P<sub>myc</sub>-cdc20-aid-ura4<sup>-P<sub>myc</sub>-skp1-1-OsTIR1-natMX6-P<sub>myc</sub>-skp1-1-A<sub>TIR</sub>1-2NLS</sup> | This work  |
| Strain  | Description                                                                 | Comment       |
|---------|------------------------------------------------------------------------------|---------------|
| HM4544  | \texttt{h cdc20::cdc20-3flag-kanMX6 cdc45::cdc45-9myc-ura4^+ cdc25-22}     | This work     |
| HM4553  | \texttt{h cdc20::hphMX6-P_nmt41-cdc20-CTD-3flag-kanMX6 cdc45::cdc45-9myc-ura4^+ cdc25-22} | This work     |
| HM4661  | \texttt{h cdc20::hphMX6-P_nmt41-cdc20-aid-ura4^+ drc1::drc1-3HA-kanMX6 cdc25-22} <br> \texttt{ade6::ade6-^P_{adh15-}skp1-OsTIR1-natMX6-^P_{adh15-}skp1-AtTIR1-2NLS} | This work     |
| HM4664  | \texttt{h cdc20::hphMX6-P_nmt41-cdc20-aid-ura4^+ dpb2::4flag-dpb2-sup3-5} <br> \texttt{drc1::drc1-3HA-kanMX6 cdc25-22} <br> \texttt{ade6::ade6-^P_{adh15-}skp1-OsTIR1-natMX6-^P_{adh15-}skp1-AtTIR1-2NLS} | This work     |
Supplementary Table S2: qPCR primers used in this study

| Locus         | Primer name   | Sequence                        |
|---------------|---------------|---------------------------------|
| non-origin    | non-ars1 region-514F | 5’-TACGCGACGAACCTTGCATAT-3’       |
|               | non-ars1 region-583R | 5’-TTATCAGACCATGGAGGCCCAT-3’      |
| ars2004       | ars2004 region-273F | 5’-CGGATCCGTAATCCCCAAACA-3’       |
|               | ars2004 region-338R | 5’-TTTGCTTACATTTTCGGGAATTTTA-3’   |
| ars3002       | ars3002-F       | 5’-TCATTAGCAAAACAAAGGAATTTTA-3’   |
|               | ars3002-R       | 5’-AATTTCGGGCAATTTAACCAACG-3’     |
| +0.5kb        | ars2004+R0.5kb-F | 5’-GGCACTATACCAAACCTCATTGTTTA-3’  |
|               | ars2004+R0.5kb-R | 5’-TCAATGGATCGTGCTTTTTA-3’        |
| +1kb          | ars2004+R1.0kb-F | 5’-GGCACTATACCAAACCTCATTGTTTA-3’  |
|               | ars2004+R1.0kb-R | 5’-ATGATGAAAGACTCGGTCAGCTA-3’     |
| +2kb          | ars2004+R2kb-F  | 5’-GGGTTATCTCTCTCTCCTC-3’         |
|               | ars2004+R2kb-R  | 5’-GTAATAACGCTTGATAAAGCAGAA-3’    |
| +4kb          | ars2004+R4kb-F  | 5’-GGTGATCTTGCTAAAGAATCAT-3’      |
|               | ars2004+R4kb-R  | 5’-CAGACTGAGACCTTTCAGATAA-3’      |
Supplementary Table S3: Primers used in this study

| Primer name         | Sequence                                                                 |
|---------------------|--------------------------------------------------------------------------|
| SPNCRNA.418-F2      | 5'-GCATTCCCTTGACATTACAC-3'                                               |
| SPNCRNA.418-DW-BamHI-R | 5'-AAAGGATCCACCAACAAAGTCCGCCGCTGC-3'                                    |
| Pmnt-EcoRV-F        | 5'-AAAGATATCTCTCAGGTCGATCGACT-3'                                        |
| cdc20NTD-Spel-R     | 5'-AAAAACTAGTGTAAGGAGATGGAATGCT-3'                                      |
| dpb2UP-F            | 5'-CTGCCTAGCTGACACAGTGTATTG-3'                                          |
| dpb2UP-hphMX6-R     | 5'-CTTAATTTACGCCGAGATCCTGTTATGAGCTCCACTC-3'                             |
| Pmnt81-dpb2-F       | 5'-CTTAATGCTGCGTTATTAAATCATATGAAACATTTCCATTACGGATCTCGG-3'               |
| dpb2-HeadR          | 5'-AACGCCATTGTGACAGCGACACT-3'                                           |
| cdc20CterF          | 5'-GAATGATGCGGTTTCTGAGA-3'                                              |
| Tag5-ter+cdc20CterR | 5'-CCGTCGATGATAGGTGTCGATCACAGCAGGAAGATCT-3'                             |
| Tag3-ter+cdc20DWF   | 5'-CGCCCTGACATCATCTGCCCAGGTAAGCTTGGTCCAGATTACAAATG-3'                   |
| cdc20DWR2R          | 5'-TACAGGTACCTATTGAGGAGATCT-3'                                          |
| pol1CterF           | 5'-GTGGAATTCGCCACAGCAGAAATG-3'                                          |
| pol1DWR             | 5'-GAATGATTTCACATATAGCTATATCATGTCT-3'                                   |
| Tag5-ter+pol1CterR  | 5'-CCGTCGATGATAGGTGTCGATCACAGCAGGAAGATCT-3'                             |
| Tag3-ter+pol1DWF    | 5'-CGCCCTGACATCATCTGCCCAGGTAAGCTTGGTCCAGATTACAAATG-3'                   |
| cdc6CterF           | 5'-GTCTAAATAGATCGCGCAACT-3'                                             |
| cdc6DWR             | 5'-CCCTTCTGATTATCTGGAGATCT-3'                                           |
| Tag5-ter+cdc6CterR  | 5'-CCGTCGATGATAGGTGTCGATCACAGCAGGAAGATCT-3'                             |
| Tag3-ter+cdc6DWF    | 5'-CGCCCTGACATCATCTGCCAGAGGATGTTTATTTAATTACAAATGTG-3'                   |
| cdc20Cter1F         | 5'-AAGAAATTTCTAGGAGAAGTGTGACTCAA-3'                                    |
| cdc20Cter1R         | 5'-AAGAAATTTCTAGGAGAAGTGTGACTCAA-3'                                    |
| cdc20Cter2F         | 5'-AAGAAATTTCTAGGAGAAGTGTGACTCAA-3'                                    |
| cdc20Cter2R         | 5'-AAGAAATTTCTAGGAGAAGTGTGACTCAA-3'                                    |
| cdc20DWF1F          | 5'-AAACTCGAGGAGCATTTGACATCTTGG-3'                                       |
| cdc20DWF1R          | 5'-AAACTCGAGGAGCATTTGACATCTTGG-3'                                       |
| cdc20DWR1            | 5'-AAAAAGCTTGTAGGAAGAAATGGA-3'                                         |
| cdc20DWF2R          | 5'-AAAAAGCTTGTAGGAAGAAATGGA-3'                                         |
| cdc20DWF2F          | 5'-AAAAAGCTTGTAGGAAGAAATGGA-3'                                         |
| cdc20DWF2R          | 5'-AAAAAGCTTGTAGGAAGAAATGGA-3'                                         |
| cdc20mutF           | 5'-CTCGAGTAAACACGCTA-3'                                                 |
| cdc20mutR           | 5'-GGATTTCAAACACTTCTATGTAAC-3'                                         |
| cdc20tail-Spel-F    | 5'-TTTACTATAATGCCGTCGATGACTTGAGT-3'                                     |
| cdc20tail-5GA-NdeI-R | 5'-AAGAATTTCTAGGAGAAGTGTGACTCAA-3'                                   |
| cdc20DW-BglII-R     | 5'-AAAAAGCTTGTAGGAAGAAATGGA-3'                                         |
| cdc20DW-Spel-R      | 5'-AAAAAGCTTGTAGGAAGAAATGGA-3'                                         |
Supplementary Fig. S4

Cdc20 IP

IP recovery (%)

wild-type +HU  cdc20-ct1

non-ori  ars2004  ars3002

Dpb2 IP

IP recovery (%)

wild-type +HU  cdc20-ct1

non-ori  ars2004  ars3002
**A**

Supplementary Fig. S9

- \( P_{nmt}^{c} \text{cdc20-aid} 2xTIR1 \)
- \( cdc25-22 \)

\[ 25°C \begin{align*}
\text{G2/M-arrest} & \quad 36°C \quad 3.5 \text{ h} \\
\text{+thiamine} & \quad \text{(-6.5 h)} \\
\text{+HU} & \quad \text{(-3 h)} \\
\text{+auxin (Time 0)} & \\
\text{HU-release} & \\
\end{align*} \]

**B**

- Wild-type
- Cdc20 depletion

**C**

IP recovery (%)

### Mcm6 IP

- Wild-type
- Cdc20 depletion

### Cdc45 IP

- Wild-type
- Cdc20 depletion

### Dpb2 IP

- Wild-type
- Cdc20 depletion

### Rpa2 IP

- Wild-type
- Cdc20 depletion