Supplementary Information: The Dbf4-Cdc7 kinase promotes S phase by alleviating an inhibitory activity in Mcm4
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Supplementary Table

Yeast strains used in this study

| Strain   | genotype                                                                 | source                  |
|----------|---------------------------------------------------------------------------|-------------------------|
| y2007    | MATα sld3-600,609,622A-dpb11(253-764)::KanMX, his3::PGAL1-4HA-sld2 T84D::HIS3, bar1Δ::TRP1 | 1                       |
| YB291    | MATα cdc7-4 leu2-3, 112, trp1-1, ura3-1, his3-11, 15, ade2-1               | This lab                |
| YB558    | MATα dbf4-1 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100            | This lab                |
| YB528    | MATα mcm5-bob1 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100         | This lab                |
| YB529    | MATα cdc7Δ::HIS3 mcm5-bob1 ade2-1 his3-11,15 leu2-3,112 can1-100           | This lab                |
| YS1114   | MATα mcm4Δ2-174 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100        | This study              |
| YS1790   | MATα sld3-600,609,622A-dpb11(253-764)::KanMX, his3::PGAL1-4HA-sld2 T84D::HIS3, bar1Δ::TRP1 URA3::GAL-DBF4 | This study; derivative of y2007 |
| YS1797   | MATα mcm4Δ2-174 dbf4-1 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 | This study              |
| YS1798   | MATα mcm4Δ2-174 dbf4-1 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 | This study              |
| YS1802   | MATα MCM4 dbf4-1 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100        | This study              |
| YS1887   | MATα mcm4Δ2-174 cdc7-4 leu2-3, 112, trp1-1, ura3-1, his3-11, 15, ade2-1    | This study              |
| YS1888   | MATα mcm4Δ2-174 cdc7-4 leu2-3, 112, trp1-1, ura3-1, his3-11, 15, ade2-1    | This study              |
| YS1890   | MATα MCM4 cdc7-4 cdc7-4 leu2-3, 112, trp1-1, ura3-1, his3-11, 15, ade2-1    | This study              |
| YS1994   | MATα mcm4Δ2-174 cdc7Δ::KanMX6 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112   | This study              |
| YS2041   | MATα cdc7Δ::HIS3 mcm4Δ::TRP1 ade2 ade2 his3 leu2 trp1?+ pRS416/CDC7 + pRS416/MCM4 | This study              |
| YS2178   | MATα mcm4Δ2-174 sld3-600,609,622A-dpb11(253-764)::KanMX, his3::PGAL1-4HA-sld2 T84D::HIS3, bar1Δ::TRP1 | This study; derivative of y2007 |
| YS2251   | MATα bar1Δ::TRP1 ade2-1 can1-100 his3-11,-15 leu2-3,112 trp1-1 ura3-1     | This study              |
| YS2261   | MATα mcm4Δ2-174 bar1Δ::TRP1 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 | This study              |
YS2263  |  \( \text{MAT}a \ mcm2^{1-200}\cdot\text{mcm}4^{A2-174} \ bar1\Delta::\text{TRP1} \ ade2-1 \ ura3-1 \ his3-11,15 \ trp1-1 \ leu2-3,112 \ can1-100 \)  |  This study  
YS2321  |  \( \text{MAT}a \ mcm4^{A2-174} \ bar1\Delta::\text{TRP1} \ cdc7\Delta::\text{HIS3} \ ade2-1 \ ura3-1 \ his3-11,15 \ trp1-1 \ leu2-3,112 \ can1-100 \)  |  This study  
YS2325  |  \( \text{MAT}a \ mcm2^{1-200}\cdot\text{mcm}4^{A2-174} \ bar1\Delta::\text{TRP1} \ cdc7\Delta::\text{HIS3} \ ade2-1 \ ura3-1 \ his3-11,15 \ trp1-1 \ leu2-3,112 \ can1-100 \)  |  This study  
YS2380  |  \( \text{MAT}a \ bar1\Delta::\text{hisG} \ cdc7-4 \ \text{JET1} \ trp1-1::\text{GALp-sld2-11D-Myc-His}_{\gamma-}\cdot\text{TRP1} \ DBF4::\text{pST266} \ (\text{CMV}1_p\cdot\text{tTA- TetO}_{2}\cdot\text{DBF4-Myc- His}_{\gamma-}\cdot\text{kanMX}) \ \text{CDT1-GFPS65T::kanMX ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100} \)  |  This study; derivative of YST619  
YS2400  |  \( \text{MAT}a \ mcm4^{A2-174} \ bar1\Delta::\text{hisG} \ \text{JET1} \ trp1-1::\text{GALp-sld2-11D-Myc-His}_{\gamma-}\cdot\text{TRP1} \ DBF4::\text{pST266} \ (\text{CMV}1_p\cdot\text{tTA-TetO}_{2}\cdot\text{DBF4-Myc- His}_{\gamma-}\cdot\text{kanMX}) \ \text{CDT1-GFPS65T::kanMX ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100} \)  |  This study; derivative of YST630  
YS2411  |  \( \text{MAT}a \ mcm4^{A2-174} \ bar1\Delta::\text{hisG} \ cdc7-4 \ \text{JET1} \ trp1-1::\text{GALp-sld2-11D-Myc-His}_{\gamma-}\cdot\text{TRP1} \ DBF4::\text{pST266} \ (\text{CMV}1_p\cdot\text{tTA-TetO}_{2}\cdot\text{DBF4-Myc- His}_{\gamma-}\cdot\text{kanMX}) \ \text{CDT1-GFPS65T::kanMX ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100} \)  |  This study; derivative of YST619  
YST630  |  \( \text{MAT}a \ bar1\Delta::\text{hisG} \ \text{JET1} \ trp1-1::\text{GALp-sld2-11D-Myc-His}_{\gamma-}\cdot\text{TRP1} \ DBF4::\text{pST266} \ (\text{CMV}1_p\cdot\text{tTA-TetO}_{2}\cdot\text{DBF4-Myc- His}_{\gamma-}\cdot\text{kanMX}) \ \text{CDT1-GFPS65T::kanMX ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100} \)  |  \( ^2 \)  

**Supplementary Figures:**

![Figure S1](image.png)

**Figure S1. Deletion of the Mcm4 NSD allows cells to grow in the absence of \( \text{CDC7} \).**

Yeast strains of indicated genotypes at endogenous loci were grown on YPD plates for 4 days at indicated temperatures.
Figure S2. The Mcm4 NSD contains an inhibitory activity that renders DDK essential for viability.

a and b, The MCM4 plasmid failed to yield transformed colonies in cdc7-4 mcm4Δ2-174 or cdc7Δ mcm4Δ2-174 cells, while CDC7 efficiently rescued cdc7Δ mcm4Δ2-174 cells. c, The inhibitory activity of mcm4 vectors were examined by transformation of cdc7Δ mcm4Δ2-174 cells. The cdc7-4 mcm4Δ2-174 (a) or cdc7Δ mcm4Δ2-174 (b and c) cells were transformed with indicated plasmids and plated on selective media at 30°C.
Figure S3  Removal of the inhibitory domain within the Mcm4 NSD allows DDK-independent cell proliferation.
A modified plasmid shuffle assay (see main text) was used to identify MCM4 alleles that allow DDK-independent cell proliferation. The tester cells were transformed with the indicated plasmids and assayed for growth on YPD and 5-FOA media. All mcm4 alleles used in the assay complement mcm4Δ.

Figure S4 The essential function of DDK is to counteract the inhibitory domain within the Mcm4 NSD.
Alanine substitution of 11 potential DDK phosphorylation sites within 74-174 in the full length NSD is lethal. The 74-174DDK11A mutation includes 5A+2A and four other predicted DDK sites within proximal NSD (residues 74-174 of Mcm4). This mutant is lethal presumably because DDK can no longer exert its essential due to the ablation of the phosphorylation sites. It remains to be tested whether the 74-174DDK11D mutation is sufficient to bypass DDK.
Figure S5. Growth of mcm4<sup>Δ74-174</sup> derivatives with serine phosphorylation sites mutated within the distal NSD domain.

Plasmid constructs were introduced into yeast strain YS2007 (mcm4Δ + pMCM4/URA3) for plasmid shuffle. The transformed colonies were then isolated and grown in SC-LEU media overnight and plated on 5-FOA media to select for loss of pMCM4/URA3. All of these mcm4 alleles can rescue mcm4Δ. The yeast strains carrying the indicated mcm4<sup>Δ74-174</sup> derivatives as the sole copy of the MCM4 gene were grown on YPD plates at various temperatures.
Figure S6. NTDs of Mcm2 and Mcm6 do not function like the Mcm4NSD for CDC7 bypass.

a, Diagram of the 6 MCM subunits of *S. cerevisiae*. The extended N-terminal regions of Mcm2, Mcm4 and Mcm6 are hi-lighted in red, orange and yellow, respectively. These unstructured regions contain DDK target sites and biased amino-acid composition. The zinc-finger motif (Zn finger), walker A and B boxes and the Arginine finger (R finger) within the conserved region of the 6 MCM paralogs are indicated. b and c, Mutants of *mcm2* and *mcm6* with the indicated internal deletions within their NTDs were tested for DDK bypass ability by the plasmid shuffle assay described above using, where in indicated, the tester strains YS2508 (*cdc7*Δ *mcm2*Δ + p*CDC7*/URA3 + p*MCM2*/URA3) for *mcm2* (b) and YS2506 (*cdc7*Δ *mcm6*Δ + p*CDC7*/URA3 + p*MCM6*/URA3) for *mcm6* (c). All *mcm2* alleles presented here complement *mcm2Δ*. Asterisks indicate *mcm6* alleles that fail to rescue *mcm6Δ*. DDK bypass control: *cdc7*Δ *mcm4*Δ + p*CDC7*/URA3 + p*MCM2*/URA3+ *pmcm4*Δ/174 /LEU2 (left patches) or *pmcm2*Δ/200 - *pmcm4*Δ/174/LEU2 (right patches).
Figure S7. The positive and negative contributions of the Mcm4 NSD to cell proliferation.

a, Removal proximal NSD allows DDK-independent cell growth. b, The distal NSD of Mcm4 (residues 2-73) facilitates cell proliferation in the absence of DDK. Cell proliferation curves of the indicated yeast strains in YPD media at 30ºC.
Figure S8 Immunoblot analysis of samples from Fig. 3c for Orc6 and galactose-dependent over-expression of sld2-T84D (4HA-sld2-T84D) and Dbf4 (2HA-db4).

Figure S9. DDK-independent increase in DNA content in G1 arrested cells.  

**a.** Flow cytometry analysis of DNA content in CDK bypass cells containing CDC45/JET1 and GAL-sld2-11D with the indicated genotypes for the CDC7 and MCM4 loci. Cells were synchronized in α-F and held in G1 at 25ºC in the continued presence of α-F. G1 arrested cells from each strain were split in two and each half was incubated at 25ºC or 36ºC for one more hour before adding galactose to induce CDK bypass.  

**b.** Immunoblot analysis of protein samples from a. Sld2-11D-Myc was detected using an anti Myc antibody and Orc6 was detected by an anti Orc6 monoclonal antibody.
Figure S10. The contribution of DDK and Mcm4 to growth of \textit{CDC45/JET1 GAL-sld2-11D} cells upon induction of CDK bypass.

Cells were grown on media containing glucose (YPD) or galactose (YPGal) at 30ºC or 37ºC. The \textit{mcm4^{A74-174}} or \textit{mcm4^{A74-174,(3D+3D+2D+2D)P}}} allele was introduced to \textit{CDC45/JET1 GAL-sld2-11D} cells by two-step gene replacement. Shown here are parental strains (sectors 1 and 8) and the second-step homologous recombination products of the \textit{mcm4^{A74-174}} integrant (sectors 2 and 7) and of the \textit{mcm4^{A74-174,(3D+3D+2D+2D)P}}} integrant (sectors 3-6). The phosphorylation site mutations were further verified by sequencing the PCR products derived from relevant locus of the yeast genomic DNA. Note one of the second-step homologous recombination products of the \textit{mcm4^{A74-174,(3D+3D+2D+2D)P}}} integrant has only one SSP (68-70) to DDP mutation (sector 6).

\textbf{Comment on Figures S9 and S10:}

For studies presented in Figure S9 and S10, the chromosomal \textit{MCM4} was replaced with \textit{mcm4^{A74-174}} in S-CDK bypass strains with either \textit{CDC7} or \textit{cdc7-4}. In these strains, the dominant \textit{CDC45/JET1} that can bypass essential CDK phosphorylation of Sld3 was combined with \textit{GAL-sld2-11D} (encoding a galactose-inducible phospho-mimetic form of Sld2). In the continuous presence of \(\alpha\)-factor, DNA content of these S-CDK bypass strains increased upon induction of Sld2-11D at the permissive temperature (25ºC), while at high temperature (36ºC) DNA synthesis did not occur in \textit{cdc7-4} cells under the otherwise same conditions (Fig. S9a). In contrast, \textit{cdc7-4 mcm4^{A74-174}} cells initiated DNA synthesis at 36ºC, as evident by a distinct shift in the DNA content compared to the \textit{cdc7-4 MCM4} control strain (Fig. S9a). However, the increase of DNA content is modest, suggesting that DNA replication was limited. This suggests that this genetic combination of \textit{cdc7-4 CDC45/JET1 GAL-sld2-11D mcm4^{A74-174}}} cannot efficiently bypass CDK and DDK simultaneously. We suggest that DDK bypass is apparent in this strain background because at 36ºC \textit{cdc7-4 MCM4} (sector 8) was not viable while \textit{cdc7-4 mcm4^{A74-174}}} (sector 7) was. From the study presented in Figure 2b, we found that, in the absence of DDK, CDK control of \textit{mcm4^{A74-174}}} became critical. Thus, the inability of \textit{cdc7-4 CDC45/JET1 GAL-sld2-11D mcm4^{A74-174}}} cells to efficiently replicate DNA in G1
arrested cells at high temperature in the presence of galactose could be due to the additional requirement of mcm4Δ74-174 for CDK when DDK is inactivated. Consistent with this idea, as CDK bypass for Sld2 and Sld3 was constitutively induced on YPGal, JET1 GAL-sld2-11D cells with active DDK could not grow (sectors 1 and 2 at 36°C and sectors 1, 2, 7 and 8 at 30°C) while cdc7-4 mcm4Δ74-174 JET1 GAL-sld2-11D cells were viable when DDK was inactivated at 36°C (sector 7). It is possible that, by inactivating DDK, cell viability was restored in cdc7-4 mcm4Δ74-174 JET1 GAL-sld2-11D cells because DNA replication in this strain was again under CDK control through CDK sites in mcm4Δ74-174. While inefficient DDK-mediated function may attenuate uncontrolled initiation of DNA replication due to constitutive CDK bypass, it could not account for the viability of these cells because cdc7-4 MCM4 cells, which have attenuated DDK activity at 30°C (sector 8 on YPD) were dead under the constitutive CDK bypass conditions (sector 8 on YPGal at 30°C). This observation adds another aspect of the interplay between CDK and DDK in regulating origin activation through the NSD of Mcm4.

We have tried to introduce the phospho-mimetic version of mcm4Δ74-174 (mcm4Δ74-174, (3D+3D+2D+2D)P), which can bypass DDK efficiently (Fig. 2b and Fig. S10 sector 5 on YPD at 36°C), into the JET1 GAL-sld2-11D background. However, unlike MCM4 or mcm4Δ74-174, this mutant allowed cells to grow on YPGal (Fig. S10, sectors 3-5). Moreover, flow cytometry analysis also showed that, in contrast to the robust DNA synthesis, this mutant allowed only modest increase of DNA content in G1 in the presence of galactose (data not shown), suggesting that constitutive CDK bypass for Sld2 and Sld3 is not sufficient for deregulated DNA replication in this mutant, even in the presence of DDK. It is possible that this DDK bypass mutant with phosphomimetic residues at the distal NSD is compromised in other important step of initiation, such as pre-RC assembly. Consistent with this idea, it has been shown that phosphorylation of MCM4 by cdc2 protein kinase inhibits the activity of the MCM complex and that a certain level of Mcm4 dephosphorylation occurred after mitotic exit prior to pre-RC assembly. Moreover, in yeast, pre-RC assembly is also negatively regulated by CDKs through nuclear exclusion of the MCM complex.

Together, these observations highlight the importance of coordinated control of initiation of DNA replication by cell-cycle regulated kinases both temporally and spatially.
Figure S11  Cell cycle block by HU and resumption of DNA synthesis after removal of the blocking agent.

a, Flow cytometry analysis of DNA content of cells used in Fig. 4b. Note that a small population of cells with less than 1C DNA content is present in the asynchronous cdc7Δ mcm4Δ74-174 culture. This population further increases progressively after release into HU from G1 arrest (compare sample collected 180 min after release and samples from G1 arrest and earlier time points). b, Flow cytometry analysis of DNA content of cells synchronized in G1, released into 200 mM HU for 150 min (G1→HU sample) and then further released into fresh YPD media at 25ºC.

A recent report showed that DDK is needed for recovery from an S phase block to resume DNA synthesis. However, like MCM4 and mcm4Δ74-174 cells, there was no obvious problem for cdc7Δ mcm4Δ74-174 cells to resume S phase progression after the HU block was removed (Fig. S11b), suggesting that either mcm4Δ74-174 can also bypass the requirement of DDK for recovery or DDK is not required for recovery from a replication block. Interestingly, the amount of cells with less than 1C DNA content increased progressively after release from HU, consistent with the inability of these cells to activate the checkpoint in the presence of HU. Thus, the lethality of cdc7Δ mcm4Δ74-174 cells is likely due to failure to coordinate mitosis and DNA replication, rather than the inability to recover from checkpoint arrest.

It is worthy of noting that the mouse and fission yeast homologs of Mrc1, a replication fork protein required for Rad53 activation during replication stress, appear to be substrates of DDK. Whether DDK affects Rad53 activation through Mrc1 remains to be addressed. Alternatively, cells without DDK may adapt to chronic defects in S phase progression and constantly attenuate their intra-S phase checkpoint response.
Figure S12  Spontaneous Rad53 activation did not occur in $\text{cdc7}^{\Delta} \text{mcm4}^{474-174}$ cells despite its defects in S phase progression

Immunoblot analysis for Rad53 and Orc6 phosphorylation status of protein samples prepared from WT, $\text{mcm4}^{474-174}$ and $\text{cdc7}^{\Delta} \text{mcm4}^{474-174}$ cells synchronized in G1 and released into YPD for the indicated times.

Supplementary References

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