Supplemental Materials

Molecular Biology of the Cell

Al-Zain et al.

Table S1 Yeast strains used in this study

Strain	Genotype	Source
RUY004	*MATa bar1 ORC6-wt::LEU2 lys2 ade2 ura3 leu2 his3 trp1 can1*	GW185-3-2 (S. Bell)
RUY005	*MATa bar1 ORC6-rxl::LEU2 lys2 ade2 ura3 leu2 his3 trp1 can1*	GW186-3-2 (S. Bell)
RUY006	*MATa bar1 ORC6-ps::LEU2 lys2 ade2 ura3 leu2 his3 trp1 can1*	GW187-3-2 (S Bell)
RUY007	*MATa bar1 ORC6-rxl,ps::LEU2 lys2 ade2 ura3 leu2 his3 trp1 can1*	GW188-3-2 (S. Bell)
BCY347	*MATalpha ORC6-wt::LEU2 URA3::GAL-CDC6ΔNT-HA(s) ADE2 ura3 leu2 his3 trp1 can1-100*	This study
BCY348	*MATa ORC6-rxl::LEU2 URA3::GAL-CDC6ΔNT-HA(s) ADE2 ura3 leu2 his3 trp1 can1-100*	This study
BCY349	*MATa ORC6-ps::LEU2 URA3::GAL-CDC6ΔNT-HA(s) ADE2 ura3 leu2 his3 trp1 can1-100*	This study
BCY350	*MATa ORC6-rxl,ps::LEU2 URA3::GAL-CDC6ΔNT-HA(s) ADE2 ura3 leu2 his3 trp1 can1-100*	This study
BCY351	*MATa ORC6-wt::LEU2 URA3::GAL-CDC6ΔNT-T368A-HA(s) ade2 ura3 leu2 his3 trp1 can1-100*	This study
BCY352	*MATalpha LEU2::ORC6-rxl URA3::GAL-CDC6ΔNT-T368A-HA(s) ade2 ura3 leu2 his3 trp1 can1-100*	This study
BCY353	*MATalpha LEU2::ORC6-ps URA3::GAL-CDC6ΔNT-T368A-HA(s) ade2 ura3 leu2 his3 trp1 can1-100*	This study
BCY354	*MATalpha LEU2::ORC6-rxl,ps URA3::GAL-CDC6ΔNT-T368A-HA(s) ade2 ura3 leu2 his3 trp1 can1-100*	This study
BCY234	*MATa bar1 URA3::GAL-CDC6-HA(s) ADE2 ura3 leu2 his3 trp1 can1-100*	This study
BCY235	*MATa bar1 URA3::GAL-CDC6-T368A-HA(s) ADE2 ura3 leu2 his3 trp1 can1-100*	This study
BCY360	*MATa bar1 URA3::GAL-CDC6-P369-HA(s) ADE2 ura3 leu2 his3 trp1 can1-100*	This study
BCY335	*MATa bar1 URA3::GAL-CDC6-P372-HA(s) ADE2 ura3 leu2 his3 trp1 can1-100*	This study
BCY333	*MATa bar1 URA3::GAL-CDC6-P373A-HA(s) ADE2 ura3 leu2 his3 trp1 can1-100*	This study
BCY412	*MATa bar1 URA3::GAL-CDC6-T39A-HA(s) ADE2 ura3 leu2 his3 trp1 can1-100*	This study
BCY416	*MATa bar1 URA3::GAL-CDC6-S43A-HA(s) ADE2 ura3 leu2 his3 trp1 can1-100*	This study
BCY420	*MATa bar1 URA3::GAL-CDC6-T39A,T368A-HA(s) ADE2 ura3 leu2 his3 trp1 can1-100*	This study
BCY372	*MATa bar1 URA3::GAL-CDC6-T368A,S372A(s)-HA ADE2 ura3 leu2 his3 trp1 can1-100*	This study
BCY254	*MATa bar1 mck1::KanMX URA3::GAL-CDC6-HA(s) ADE2 ura3 leu2 his3 trp1 can1-100*	This study
RUY508	*MATa bar1 ADE2 ura3 leu2 his3 trp1 can1-100*	F. Cross
BCY260	*MATa ORC6-rxl-LEU2 ade2 ura3 leu2 his3 trp1 can1-100*	F. Cross
BCY426	*MATa ORC6-rxl-LEU2 URA3::GAL-CDC6-HA(s) ade2 ura3 leu2 his3 trp1 can1-100*	This study
BCY508	*MATa ORC6-rxl::LEU2 URA3::GAL-CDC6-T368A-HA(s) ade2 ura3 leu2 his3 trp1 can1-100*	This study
BCY383	*MATa ORC6-rxl::LEU2 URA3::GAL-CDC6-P369A-HA(s) ade2*	This study

	ura3 leu2 his3 trp1 can1-100	
BCY516	*MATa ORC6-rxl::LEU2 URA3::GAL-CDC6-S372A-HA(s) ade2 ura3 leu2 his3 trp1 can1-100*	This study
BCY518	*MATa ORC6-rxl::LEU2 URA3::GAL-CDC6-P373A-HA(s) ade2 ura3 leu2 his3 trp1 can1-100*	This study
BCY380	*MATa ORC6-rxl::LEU2 URA3::GAL-CDC6-T368A,S372A-HA(s) ade2 ura3 leu2 his3 trp1 can1-100*	This study
BCY403	*MATa ORC6-rxl::LEU2 URA3::GAL-CDC6-T39A-HA(s) ade2 ura3 leu2 his3 trp1 can1-100*	This study
BCY434	*MATa ORC6-rxl::LEU2 URA3::GAL-CDC6-S43A-HA(s) ade2 ura3 leu2 his3 trp1 can1-100*	This study
BCY430	*MATa ORC6-rxl::LEU2 URA3::GAL-CDC6-T39A,T368A-HA(s) ade2 ura3 leu2 his3 trp1 can1-100*	This study
BCY514	*MATalpha URA3::GAL-CDC6-HA RAD52-YFP ADE2 ura3 leu2 his3 trp1*	This study (RAD52-YFP from R. Rothstein 2001)
BCY515	*MATalpha URA3::GAL-CDC6-T368A-HA RAD52-YFP ADE2 ura3 leu2 his3 trp1*	This study (RAD52-YFP from R. Rothstein 2001)
BCY545	*MATalpha URA3::GAL-CDC6-T39A,T368A-HA RAD52-YFP ADE2 ura3 leu2 his3 trp1*	This study (RAD52-YFP from R. Rothstein 2001)
BCY356	*MATa bar1::hisG GAL-CLB2-TAP::URA ade2 ura3 leu2 his3 trp1 can1-100*	DOM0073 (D. Morgan)
BCY397	*MATa bar1::hisG GAL-MCK1-TAP::URA ade2 ura3 leu2 his3 trp1 can1-100*	POM638 (D. Morgan) transformed into BCY355
BCY355	*MATa bar1::hisG ade2 ura3 leu2 his3 trp1 can1-100*	DOM0090 (D. Morgan)
BCY450	*MATa URA3::GAL-CDC6-HA(s) clb2::LEU2 ade2 ura3 leu2 his3 trp1 can1-100*	This study
BCY448	*MATa URA3::GAL-CDC6-HA(s) clb4::HIS3 ade2 ura3 leu2 his3 trp1 can1-100*	This study
BCY454	*MATa URA3::GAL-CDC6-HA(s) clb5::HIS3 ade2 ura3 leu2 his3 trp1 can1-100*	This study
BCY357	*MATa bar1 MCK1-9MYC::TRP ADE2 ura3 leu2 his3 trp1 can1-100*	A. Ikui 2012
BCY368	*MATa bar1 MCK1-GFP::HIS3 ADE2 ura3 leu2 his3 trp1 can1-100*	VR1328 (S. Yoshida) backcrossed with W303 three times
BCY539	*MATa GAL-CDC6-Δ370Δ371-HA ADE2 ura3 leu2 his3 trp1 can1-100*	This study
BCY476	*MATa GAL-CDC6-Δ370-HA ADE2 ura3 leu2 his3 trp1 can1-100*	This study
BCY488	*MATa GAL-CDC6-T370A-HA ADE2 ura3 leu2 his3 trp1 can1-100*	This study
BCY542	*MATa GAL-CDC6-T371A-HA ADE2 ura3 leu2 his3 trp1 can1-100*	This study
BCY077	*MATa bar1 CDC6-prA::HIS3 ade2 ura3 leu2 his3 trp1 can1-100*	F. Cross (2003)
BCY577	*MATa bar1 CDC6-T368A-prA::HIS3 ade2 ura3 leu2 his3 trp1 can1-100*	This study
BCY078	*MATa bar1 mck1::KanMX CDC6-prA::HIS3 ade2 ura3 leu2 his3 trp1 can1-100*	A. Ikui 2012
RUY269	*MATa CLB2-9MYC-TRP1 ORC6-PrA-HIS3 ade2 ura3 leu2 his3 trp1 can1-100*	F. Cross
BCY577	*MATa bar1 CDC6-T368A-prA::HIS3*	This study
BCY282	*MATa cdc4-1 CDC6-prA::HIS3 ura3 leu2 his3 trp1 can1-100*	This study
BCY474	*MATa bar1 dia2::KanMX CDC6-prA::HIS3 ADE2 ura3 leu2 his3 trp1 can1-100*	This study (*dia2::KanMX* from D. Koepp 2012)
BCY475	*MATa bar1 tom1::KanMX CDC6-prA::HIS3 ADE2 ura3 leu2*	This study (*tom1::KanMX*

	his3 trp1 can1-100	from D. Koepp 2012)
BCY512	*MATalpha SIC1-prA::HIS3 RAD52-YFP ADE2 ura3 leu2 his3 trp1*	This study (RAD52-YFP from R. Rothstein 2001)
BCY513	*MATalpha mck1::KanMX SIC1-prA::HIS3 RAD52-YFP ADE2 ura3 leu2 his3 trp1*	This study (RAD52-YFP from R. Rothstein 2001)
BCY514	*MATalpha mck1::KanMX RAD52-YFP URA3::GAL-CDC6-HA ADE2 ura3 leu2 his3 trp1*	This study (RAD52-YFP from R. Rothstein 2001)
BCY515	*MATalpha mck1::KanMX RAD52-YFP URA3::GAL-CDC6-T368A-HA ADE2 ura3 leu2 his3 trp1*	This study (RAD52-YFP from R. Rothstein 2001)
BCY600	*MATalpha CDC6-T368A-prA::HIS3 RAD52-YFP ADE2 ura3 leu2 his3 trp1*	This study (RAD52-YFP from R. Rothstein 2001)
BCY602	*MATalpha CDC6-T39A-T368A-prA::HIS3 RAD52-YFP ADE2 ura3 leu2 his3 trp1*	This study (RAD52-YFP from R. Rothstein 2001)
AAY029	*MATa his3 leu2 met1 ura3 trp1*	This study
AAY030	*MATa mre11::KanMX his3 leu2 met1 trp1*	This study
AAY031	*MATa mre11::KanMX mck1::KanMX his3 leu2 ura3 trp1*	This study
AAY032	*MATa URA3::GAL-CDC6-HA his3 met1*	This study
AAY033	*MATa URA3::GAL-CDC6-T368A-HA his3 leu2 trp1*	This study
AAY034	*MATa mre11::KanMX URA3::GAL-CDC6-HA his3 leu2 trp1*	This study
AAY035	*MATa mre11::KanMX URA3::GAL-CDC6-T368A-HA his3 leu2 trp1*	This study
BCY581	*MATa bar1 CDC6-T39A-T368A-prA::HIS3*	This study
BCY595	*MATa mre11::KanMX CDC6-T368A-prA::HIS3*	This study
BCY597	*MATa mre11::KanMX CDC6-T39A-T368A-prA::HIS3*	This study

Figure S1

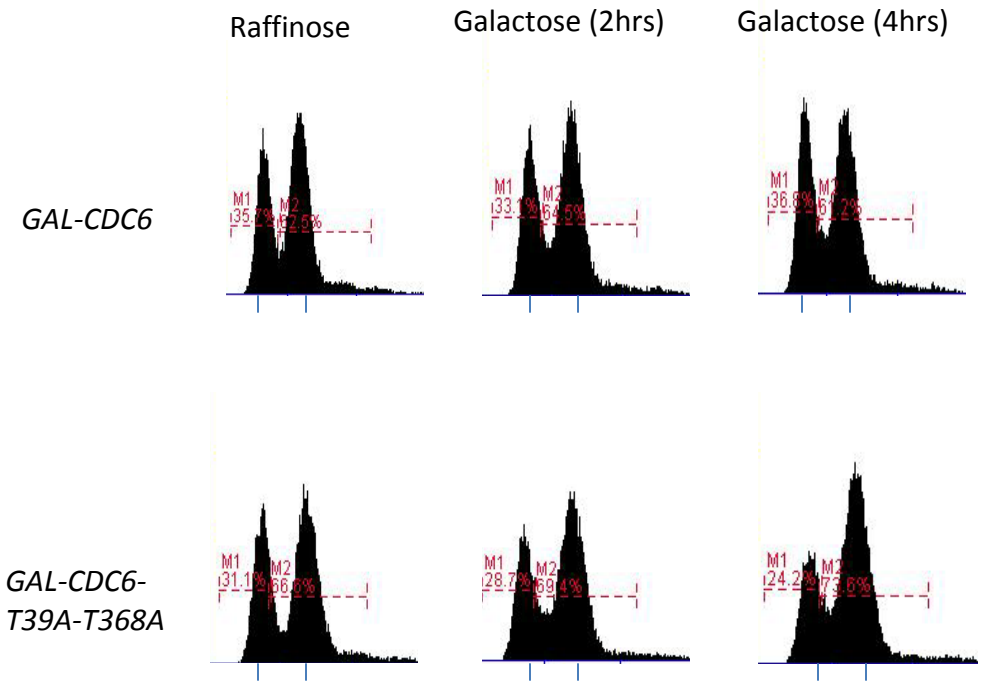

Figure S1. GAL-*CDC6-T39A-T368A* cells induce mitotic arrest. Cells were incubated in raffinose-comtaining media first, and used as a sample at time zero. Galactose was added to the media, and samples were collected after 2 hours or 4 hours. Samples were fixed and stained with propidium iodide. FACS analysis was performed to analyze cell cycle patterns.

Figure S2

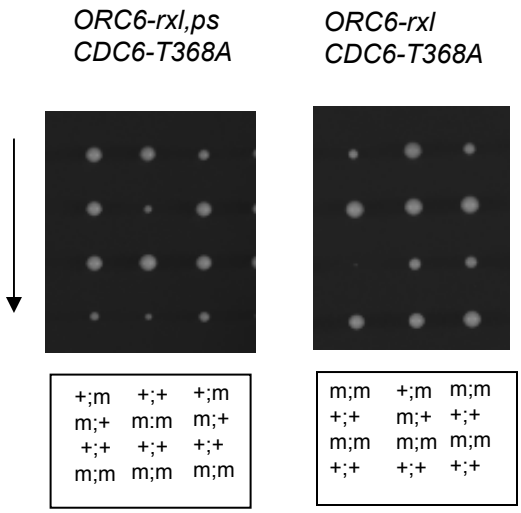

Figure S2. Synthetic lethality between *ORC6* mutants and *CDC6-T368A*. (A) *ORC-x::LEU2::HIS3/ORC6 CDC6-T368A/CDC6-wt* diploid strains were sporulated, tetrads were dissected on YEPD plates, and the plates were incubated for 3 days at 30 degrees. *ORC6* alleles and *CDC6-T368A* were identified based on the markers. Inviable spores were genotyped by assuming a 2:2 segregation. The *ORC6-rxl*, *ORC6-rxl,ps* alleles were indicated above each panel. The presence of *ORC6* mutant allele was marked as (m) and *ORC6* wild type as (+) on the left. The presence of *CDC6-T368A* (m) or *CDC6-wt* (+) was indicated on the right.

Figure S3

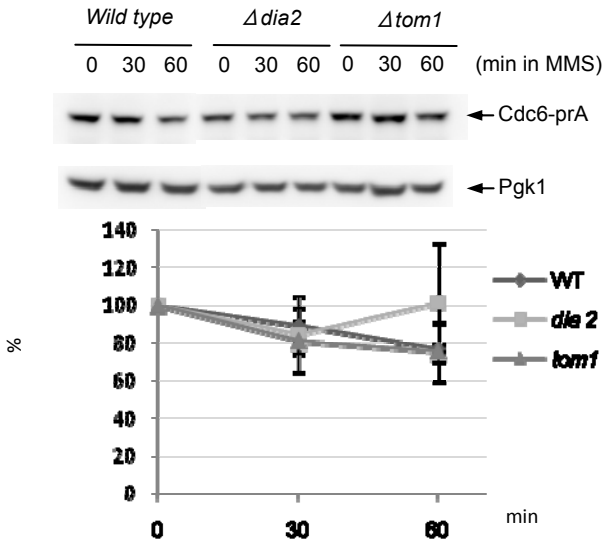

Figure S3 *CDC6-prA*, *Δdia2 CDC6-prA* or *Δtom1 CDC6-prA* cells were grown to log-phase, then MMS was added to the media (0.1% final). Samples were collected after 0, 30 or 60 minutes. The same experiment was performed three times and Cdc6 protein levels were quantified with the SD.

Figure S4

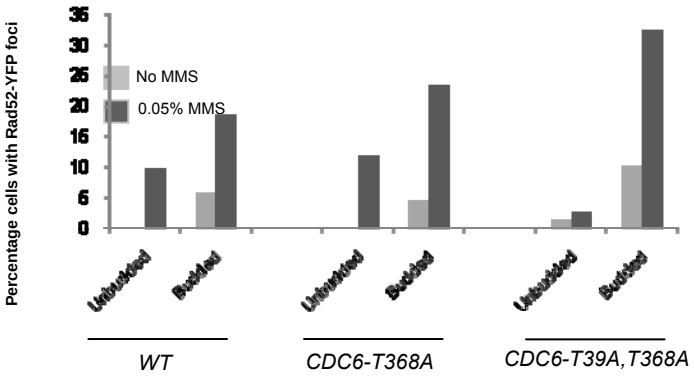

Figure S4. Wild type, *CDC6-T368A* or *CDC6-T39A,T368A* cells were grown to log phase. Cells were incubated for 1 hour in 0.05% MMS and Rad52-YFP foci formation was determined with or without MMS treatment. 100 cells were counted for each experiment.

Figure S5

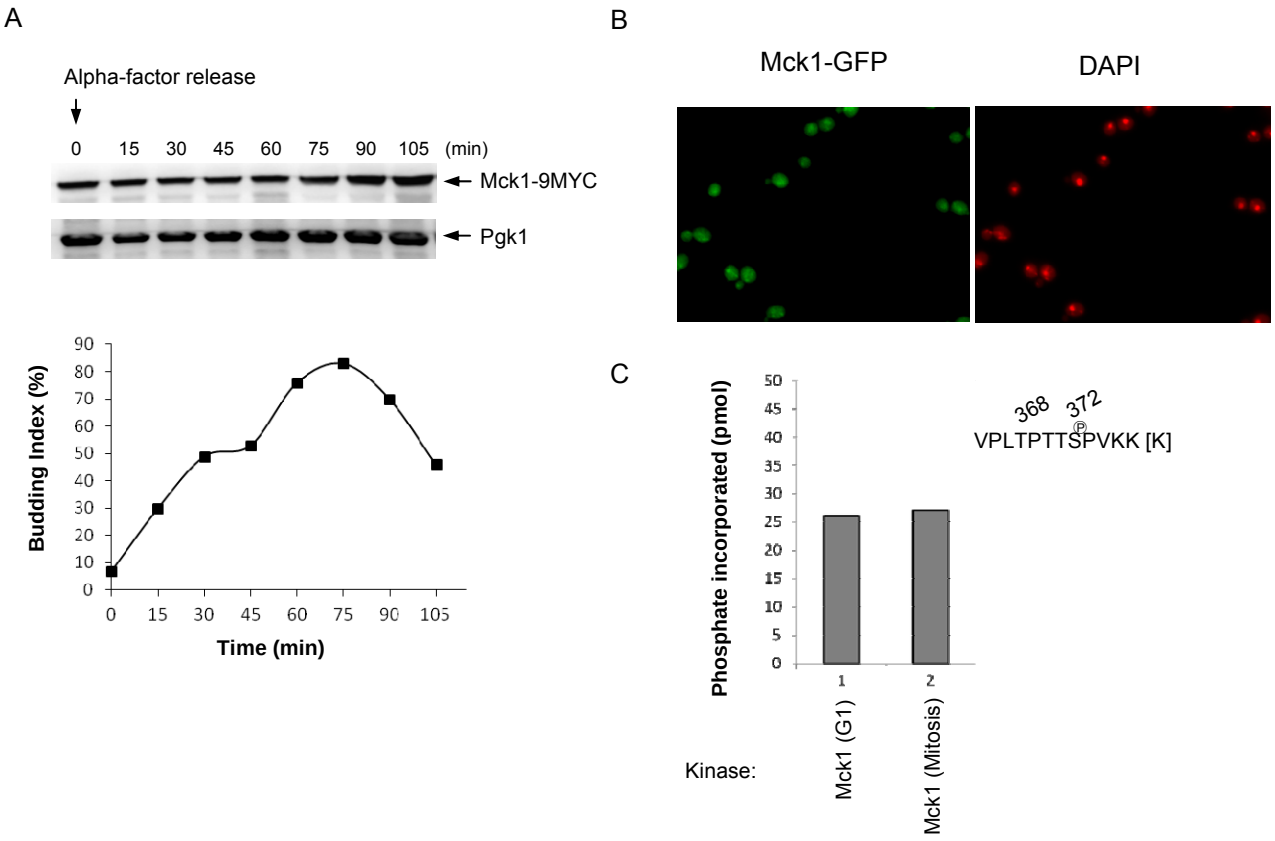

Figure S5. Mck1 phosphorylates the T368 site in Cdc6 after priming by Clb2-Cdk1. (A) To measure Clb2-Cdk1 and Mck1 kinase activities on Cdc6 phosphopeptides, various synthetic peptides of Cdc6 (residues 36-47 or 365-376; shown above) were incubated with purified kinases from asynchronous yeast cultures and γ-32P-ATP. For each kinase, phosphate incorporation was normalized to a control reaction without peptides. Values for the C-terminal peptides represent the average from three independent experiments. (B) Indicated strains were grown in raffinose-containing media first and then galactose was added to induce Cdc6 expression for 2 hours. Nocodazole or alpha-factor was added to the media and incubated for 2 hours. Western blotting was performed using anti-phosphoT368 of Cdc6, anti-HA, anti-Clb2 and anti-Pgk1 antibodies, respectively. (C) Indicated strains were grown in raffinose-containing media first and then galactose was added to induce Cdc6 expression for 2 hours. Nocodazole was added to the media for 2 hours and then western blotting was performed using anti-phosphoT368 of Cdc6, anti-HA, and anti-Pgk1 antibodies, respectively (left). Band intensity of the western blotting for phospho-Cdc6-T368 was quantified and normalized to the total amount of Cdc6 (right).

Figure S6

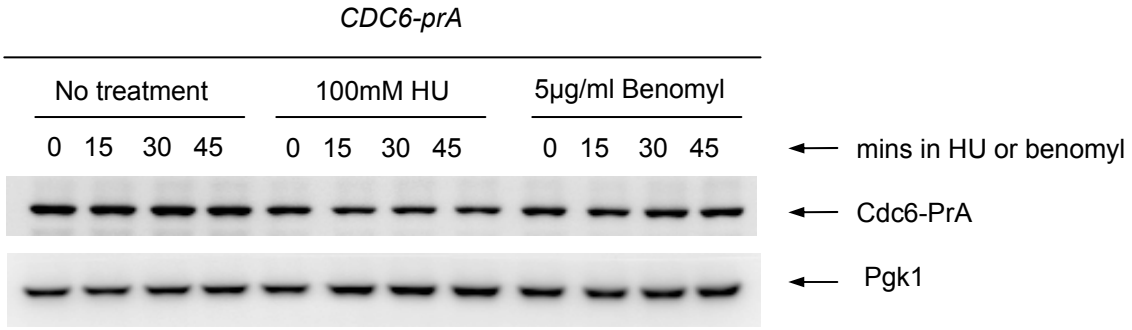

Figure S6. Hydroxyurea or Benomyl treatment does not trigger Cdc6 degradation. *CDC6-prA* cells were incubated in YEPD media to log phase, and hydroxyurea at the final concentration of 100mM or Benomyl at the final concentration of 5µg/ml was added to the media. Samples were taken after 0, 15, 30 or 45 mins, and protein extracts were subjected to western blotting to visualize endogenous Cdc6-prA. Pgk1 was used as a loading control.