SI Text

Table S1. Yeast strains used in this study. All strains are in the W303 background and have the basic genotype *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 lys2::hisG can1-100*, except for yTJT73, which is in the S288C background. yTJT73 was constructed by non-integrative transformation of pRS316-*pCdt1-CDT1* into the Mat**a**/α diploid BY4743 (*CDT1*/*cdt1*::KanMX) followed by sporulation and selection for **a** mating type, pRS316-*pCdt1-CDT1*, and G418 resistance.

Strain	**Genotype**	**Source**
yTJT1	*bar1::hisG pep4::kanMX HIS3::pRS403-GAL1,10-MCM2,MCM3 LYS2::pRS307-GAL1,10-MCM4,MCM5 TRP1::pRS404-GAL1,10-MCM6,MCM7 URA3::pRS306-GAL1,10-CDT1-1xFLAG MATa*	This study
yTJT9	*bar1::hisG pep4::kanMX HIS3::pRS403-GAL1,10-MCM2,MCM3 LYS2::pRS307-GAL1,10-MCM4,MCM5 TRP1::pRS404-GAL1,10-MCM6,MCM7 URA3::pRS306-GAL1,10-ΔN271CDT1-1xFLAG MATa*	This study
yTJT67	*bar1::hisG pep4::kanMX HIS3::pRS403-GAL1,10-MCM2,MCM3 LYS2::pRS307-GAL1,10-MCM4,MCM5 TRP1::pRS404-GAL1,10-MCM6,MCM7 URA3::pRS306-GAL1,10-ΔN292CDT1-1xFLAG MATa*	This study
yTJT38	*bar1::hisG pep4::kanMX HIS3::pRS403-GAL1,10-MCM2,MCM3 LYS2::pRS307-GAL1,10-MCM4,MCM5 TRP1::pRS404-GAL1,10-MCM6,MCM7 URA3::pRS306-GAL1,10-ΔN301CDT1-1xFLAG MATa*	This study
yTJT46	*bar1::hisG pep4::kanMX HIS3::pRS403-GAL1,10-MCM2,MCM3 LYS2::pRS307-GAL1,10-MCM4,MCM5 TRP1::pRS404-GAL1,10-MCM6,MCM7 URA3::pRS306-GAL1,10-ΔN433CDT1-1xFLAG MATa*	This study
yTJT11	*bar1::hisG pep4::kanMX HIS3::pRS403-GAL1,10-MCM2,MCM3 LYS2::pRS307-GAL1,10-MCM4,MCM5 TRP1::pRS404-GAL1,10-MCM6,MCM7 URA3::pRS306-GAL1,10-ΔN471CDT1-1xFLAG MATa*	This study
yTJT32	*bar1::hisG pep4::kanMX HIS3::pRS403-GAL1,10-*	This study

	MCM2,MCM3 LYS2::pRS307-GAL1,10-MCM4,MCM5 TRP1::pRS404-GAL1,10-MCM6,MCM7 URA3::pRS306-GAL1,10-ΔC17CDT1-1xFLAG MATa	
yTJT33	*bar1::hisG pep4::kanMX HIS3::pRS403-GAL1,10-MCM2,MCM3 LYS2::pRS307-GAL1,10-MCM4,MCM5 TRP1::pRS404-GAL1,10-MCM6,MCM7 URA3::pRS306-GAL1,10-ΔC38CDT1-1xFLAG MATa*	This study
yTJT14	*bar1::hisG pep4::kanMX HIS3::pRS403-GAL1,10-MCM2,MCM3 LYS2::pRS307-GAL1,10-MCM4,MCM5 TRP1::pRS404-GAL1,10-MCM6,MCM7 URA3::pRS306-GAL1,10-ΔC133CDT1-1xFLAG MATa*	This study
yTJT16	*bar1::hisG pep4::kanMX HIS3::pRS403-GAL1,10-MCM2,MCM3 LYS2::pRS307-GAL1,10-MCM4,MCM5 TRP1::pRS404-GAL1,10-MCM6,MCM7 URA3::pRS306-GAL1,10-ΔC333CDT1-1xFLAG MATa*	This study
yTJT19	*bar1::hisG pep4::kanMX URA3::pRS306-GAL1,10-CDT1-1xFLAG MATa*	This study
yTJT22	*bar1::hisG pep4::kanMX URA3::pRS306-GAL1,10-ΔN271CDT1-1xFLAG MATa*	This study
yTJT71	*bar1::hisG pep4::kanMX URA3::pRS306-GAL1,10-ΔN292CDT1-1xFLAG MATa*	This study
yTJT48	*bar1::hisG pep4::kanMX URA3::pRS306-GAL1,10-ΔN301CDT1-1xFLAG MATa*	This study
yTJT56	*bar1::hisG pep4::kanMX URA3::pRS306-GAL1,10-ΔN433CDT1-1xFLAG MATa*	This study
yTJT24	*bar1::hisG pep4::kanMX URA3::pRS306-GAL1,10-ΔN471CDT1-1xFLAG MATa*	This study
yTJT34	*bar1::hisG pep4::kanMX URA3::pRS306-GAL1,10-ΔC17CDT1-1xFLAG MATa*	This study
yTJT35	*bar1::hisG pep4::kanMX URA3::pRS306-GAL1,10-ΔC38CDT1-1xFLAG MATa*	This study
yTJT27	*bar1::hisG pep4::kanMX URA3::pRS306-GAL1,10-ΔC133CDT1-1xFLAG MATa*	This study
yTJT29	*bar1::hisG pep4::kanMX URA3::pRS306-GAL1,10-ΔC333CDT1-1xFLAG MATa*	This study
yTJT73	*his3Δ1 leu2Δ0 ura3Δ0 cdt1::kanMX pRS316-pCDT1-CDT1 MATa*	This study
yTJT83	*bar1::hisG pep4::kanMX HIS3::pRS403-GAL1,10-MCM2,MCM3 LYS2::pRS307-GAL1,10-MCM4,MCM5 TRP1::pRS404-GAL1,10-MCM6,MCM7 URA3::pRS306-GAL1,10-CDT1-*	This study

	4xMyc-1xFLAG MATa	
yTJT84	*bar1::hisG pep4::kanMX HIS3::pRS403-GAL1,10-MCM2,MCM3 LYS2::pRS307-GAL1,10-MCM4,MCM5 TRP1::pRS404-GAL1,10-MCM6,MCM7 URA3::pRS306-GAL1,10-ΔN292CDT1-4xMyc-1xFLAG MATa*	This study
ySC17	*bar1::hisG pep4::kanMX TRP1::pRS404-GAL1,10-ORC3,ORC4 LYS2::pLys2-GAL1,10-ORC2,ORC5 HIS3::pRS403-GAL1,10-ORC1,6 ura::pSF322-CDC6-HA MATa*	Bowers, et al. (2004)
yTJT230	*bar1::hisG pep4::kanMX HIS3::pRS403-GAL1,10-MCM2,MCM3 LYS2::pRS307-GAL1,10-MCM4,MCM5 TRP1::pRS404-GAL1,10-MCM6,MCM7 URA3::pRS306-GAL1,10-CDT1-6xGly-PP-1xFLAG MATa*	This study
yRH182	*bar1::hisG pep4::Hph KanMX::Psf2-13myc NAT::Mcm10-13myc HIS3::Cdc45-3HA LYS2::HisG leu2::Gal1,10-Cdc45/Dpb11-FLAG LYS2::Gal1,10-Sld2-FLAG/Sld3-FLAG*	Heller et al., 2011
ySK100 (aka F1ORC1)	*bar1::HisG orc1::HisG leu2::ORC1 TRP1::p404-GAL1-10-ORC3,4 LYS2::pLYS2-GAL1-10-ORC2,5 HIS3::p403-GAL1-10-1xFlag-ORC1,6 pep4::KanMX MATa*	Tsakraklides and Bell (2010)

Supplementary Figure Legends

Fig. S1. *In vivo* complementation analysis of ScCdt1 deletion mutants. (A) Each Cdt1 deletion mutant was placed under its native promoter and transformed into a Cdt1 swapper strain (yTJT73). Complementation was scored as the ability of the Cdt1 mutant to support yTJT73 cell proliferation on 5-FOA, which selects against cells containing a plasmid with wild-type Cdt1. (B) Full-length and N-terminal domain Cdt1 deletion constructs were N-terminally tagged with the SV-40 NLS, placed under control of the native promoter, and transformed into a Cdt1 swapper strain (yTJT73) and complementation was scored as in *A*.

Fig. S2. C-terminal deletions of Cdt1 do not support Cdt1 origin recruitment or Mcm2-7 loading. (A) Cdt1-specific immunodepletion from whole cell extracts (WCE). Cdt1 was immunodepleted from G1-arrested ySC17 WCE competent for helicase loading by three successive rounds of incubation with beads coupled to anti-Cdt1 antibody (HM5352). The resulting extracts were separated by SDS-PAGE and immunoblotted with antibodies against Mcm2-7 (UM-174) and Cdt1 (HM5352). All detectable Cdt1 was removed after the first round of Cdt1 depletion and Mcm2-7 was not co-depleted. (B) Cdt1-depleted WCE does not support wild-type Cdt1 origin recruitment. Helicase-loading assays were performed with untreated and Cdt1-depleted and G1-arrested WCEs in the presence of ATP or ATPγS using 1 pmol *ARS1* DNA. Following three low-salt washes, DNA was released from beads and DNA-associated proteins were analyzed by 8% SDS-PAGE followed by immunoblotting with anti-Cdt1 antibodies (HM5352). (C) C-terminally deleted Cdt1 does not support Cdt1 origin recruitment. Helicase-loading

assays were performed with Cdt1-depleted ySC17 WCE in the presence of ATPγS and the indicated purified Cdt1 using 1 pmol *ARS1* DNA and analyzed as in *B.* All four forms of Cdt1 tested here are detected by the HM5352 anti-Cdt1 antibody (data not shown). * indicates the location of a cross-reacting protein. (D) Wild-type but not C-terminally deleted Cdt1 supports Mcm2-7 loading. Helicase-loading assays were performed with Cdt1-depleted ySC17 WCE in the presence of ATP and the indicated purified Cdt1 using 1 pmol of *ARS1* DNA coupled to beads. After incubation in extract, the DNA beads were either washed with three low-salt washes or two low salt and one high salt wash as indicated. DNA was then released from beads and DNA-associated proteins were analyzed by 8% SDS-PAGE followed by immunoblotting with anti-Mcm2-7 (UM174) and anti-Orc4 (SB6, Chen et al., 2007) antibodies.

Fig. S3. Myc-tagged Cdt1/Mcm2-7 complexes support Mcm2-7 helicase loading. (A) Purified Myc-tagged Cdt1/Mcm2-7 complexes. G1-arrested whole cell extract was generated from a strain over-expressing all six Mcm proteins and Cdt1 tagged with 4xMyc and 1xFLAG at its C-terminus (yTJT83). Tagged Cdt1/Mcm2-7 complexes were purified by FLAG purification, separated by SDS-PAGE and stained with Coomassie. (B) Reconstituted helicase-loading assays were performed as described in Fig. 6A, except that Cdc6 was omitted from the reaction mixture in lanes 3 and 4. DNA-protein complexes were analyzed by 8% SDS-PAGE followed by immunoblotting with anti-Mcm2-7 (UM174) and anti-Orc4 (SB6) antibodies. (C) Reconstituted Cdt1 recruitment assays were performed as described in *B*, except that the reactions were supplied with ATPγS instead of ATP. Anti-Cdt1 (HM5352) antibody was used to detect Cdt1. (D)

Tagged and untagged Cdt1 exhibit equivalent levels of incorporation into the pre-RC. Fully reconstituted Cdt1 recruitment assays were performed on 2 pmol *ARS1* DNA using 6 pmol purified ORC, Cdc6, and indicated molar ratio of tagged and untagged Cdt1/Mcm2-7 (6 pmol total) in the presence of ATPγS. Assembled complexes were washed, DNA-protein complexes were released from beads, and samples were separated by 7% SDS-PAGE and analyzed by immunoblot with anti-Mcm2-7 (UM174) and anti-Cdt1 (HM5352) antibodies.

Fig. S4. Only one ORC molecule is present in the ATPγS-arrested helicase loading intermediate. (A) Epitope tagged ORC does not co-precipitate untagged ORC when both are present in the Cdt1-recruitment assay. ORC including either full-length ORC tagged with a single FLAG-epitope, untagged Orc1 lacking amino acids 1-235, or an equimolar combination of both were used in Cdt1-recruitment assays. DNA-associated complexes were washed and the protein-DNA complexes were released from the attached magnetic beads (input). The released protein-DNA complexes were subjected anti-FLAG immunoprecipitation and the resulting immunoprecipitated material (IP) and supernatant (Sup) were separated. Each sample was separated by SDS-PAGE and immunoblotted with antibodies directed against ORC (1108) and Mcm2-7 (UM-174). (B) The B2-element is not required for the formation of the multi-Cdt1 helicase-loading intermediate. Experiment was performed as described in Fig. 5A, except a 1-kb linear *ARS1 B2*$^{-}$ template was used instead of the wild-type *ARS1* DNA.

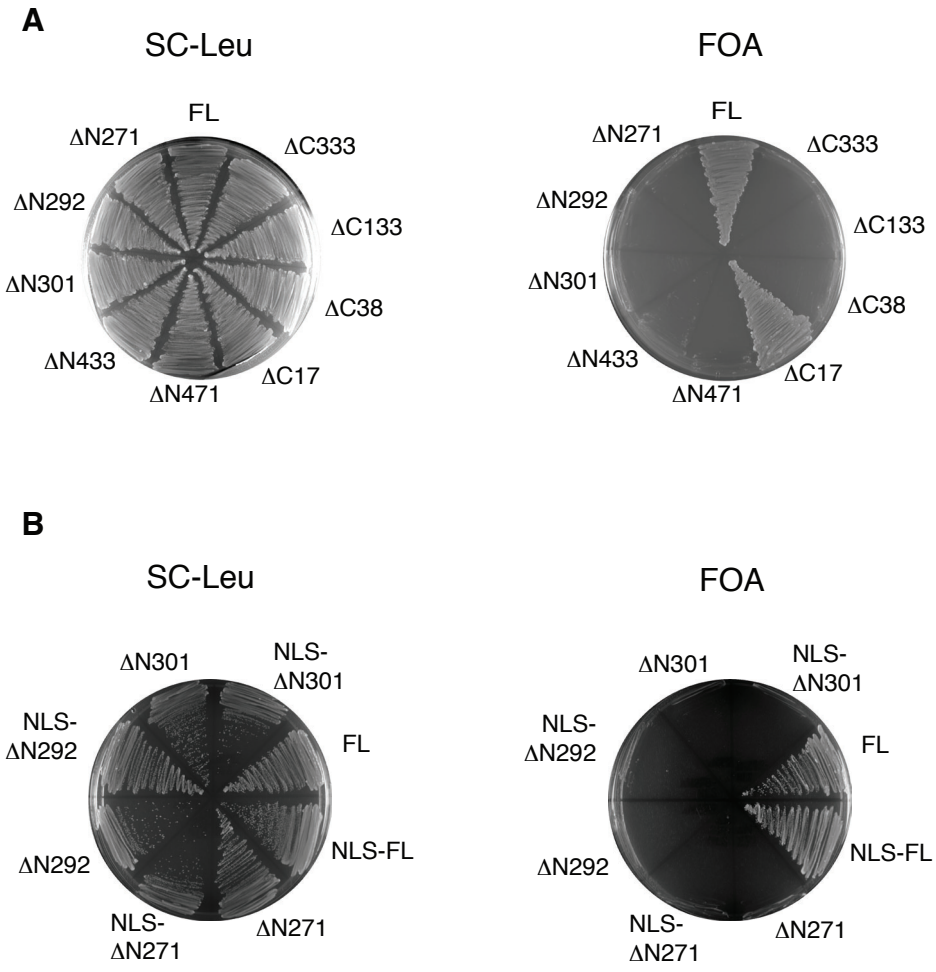
Fig. S5. S-CDK phosphorylation of ORC interferes with recruitment ΔN292 Cdt1. (A) S-CDK phosphorylation of ORC partially inhibits Cdt1 recruitment. Reconstituted Cdt1-recruitment assays were performed with unmodified or S-CDK-modified ORC (Chen and Bell, 2011). To ensure that S-CDK did not modify other aspects of the assays, ORC was phosphorylated by purified Clb5-Cdc28 and then purified away from the kinase. Origin-DNA associated proteins were separated by SDS-PAGE and immunoblotted with antibodies directed against Mcm2-7 (UM-174, *top panel*), Cdt1 (HM-5352, *top panel*) and ORC (1108, *bottom panel*). Quantification of the data determined the CDK-phosphorylated ORC:unphosphorylated ORC ratios of origin-associated proteins were: Mcm2-7, 0.55; Cdt1 0.48; ORC, 0.98. (B) S-CDK phosphorylation of ORC prevents ΔN292-Cdt1 origin recruitment. Reconstituted Cdt1-recruitment assays were performed as described in (A) except ΔN292 Cdt1 was substituted for full-length Cdt1. The relative levels of origin-associated proteins were: Mcm2-7, 0.04; Cdt1, 0.06; ORC, 1.04.

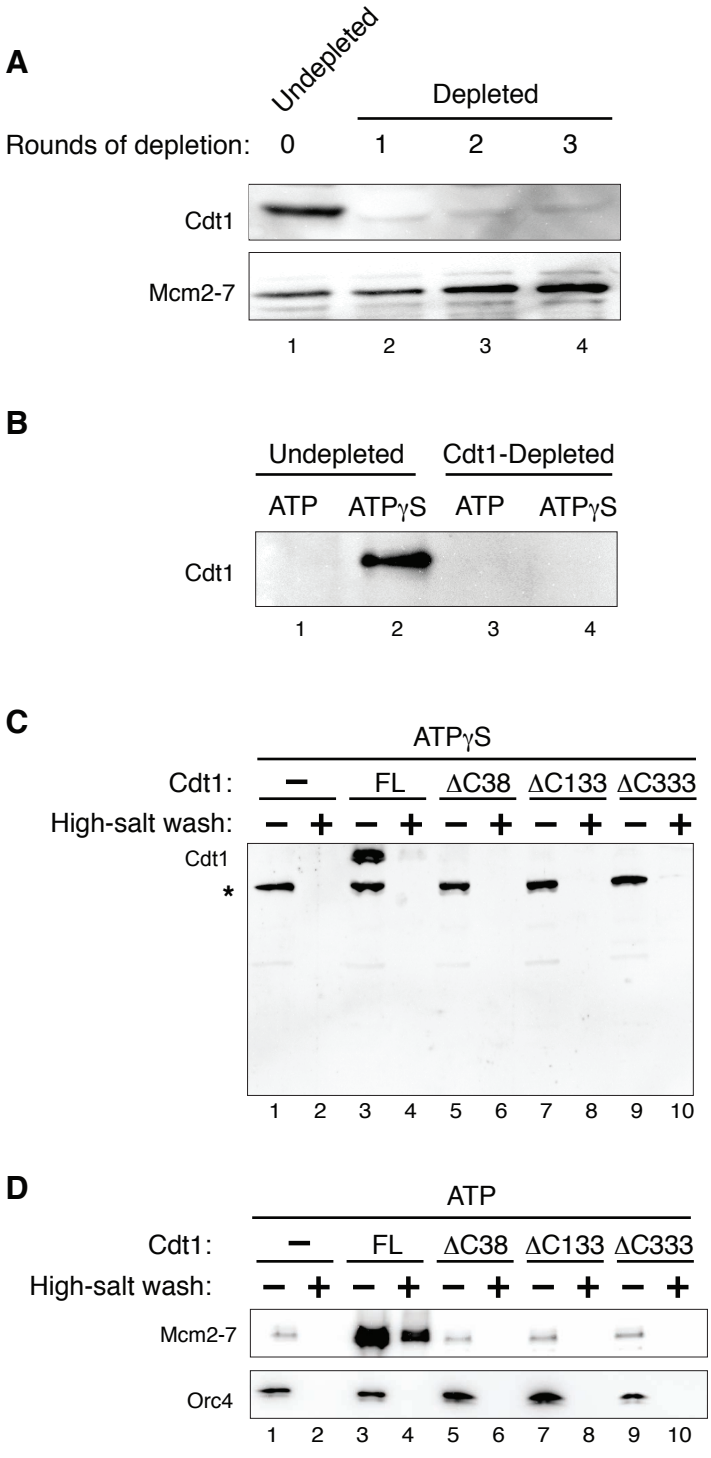
Fig. S6. CDK and DDK phosphorylation of wild-type and ΔN271 Cdt1 replication reactions. Enlarged version of Mcm2-7, Mcm4-phospho-S82-D83, and ORC immunoblots of Fig. 7C. Bands corresponding to unmodified and modified substrates of DDK (Mcm6) and CDK (Orc2 and Orc6) are indicated.

Fig. S7. ΔN271 Cdt1 is not associated with origin DNA after Mcm2-7 loading. Mcm2-7 loading reactions were performed with Cdt1-depeleted, G1-arrested cell extracts in the presence of full-length or ΔN271 Cdt1 and 175 fmoles of pUC19-coupled magnetic

beads. G1-extract was removed from the beads and the origin-DNA-associated proteins were analyzed by SDS-PAGE and immunoblotting using antibodies against Cdt1 (HM-5352).

Takara and Bell, Fig. S1

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Cdt1
1 2 3 4
C
ATPγS
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High-salt wash: – + – + – + – + – +
Cdt1
*
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Cdt1: – FL ΔC38 ΔC133 ΔC333
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Orc4
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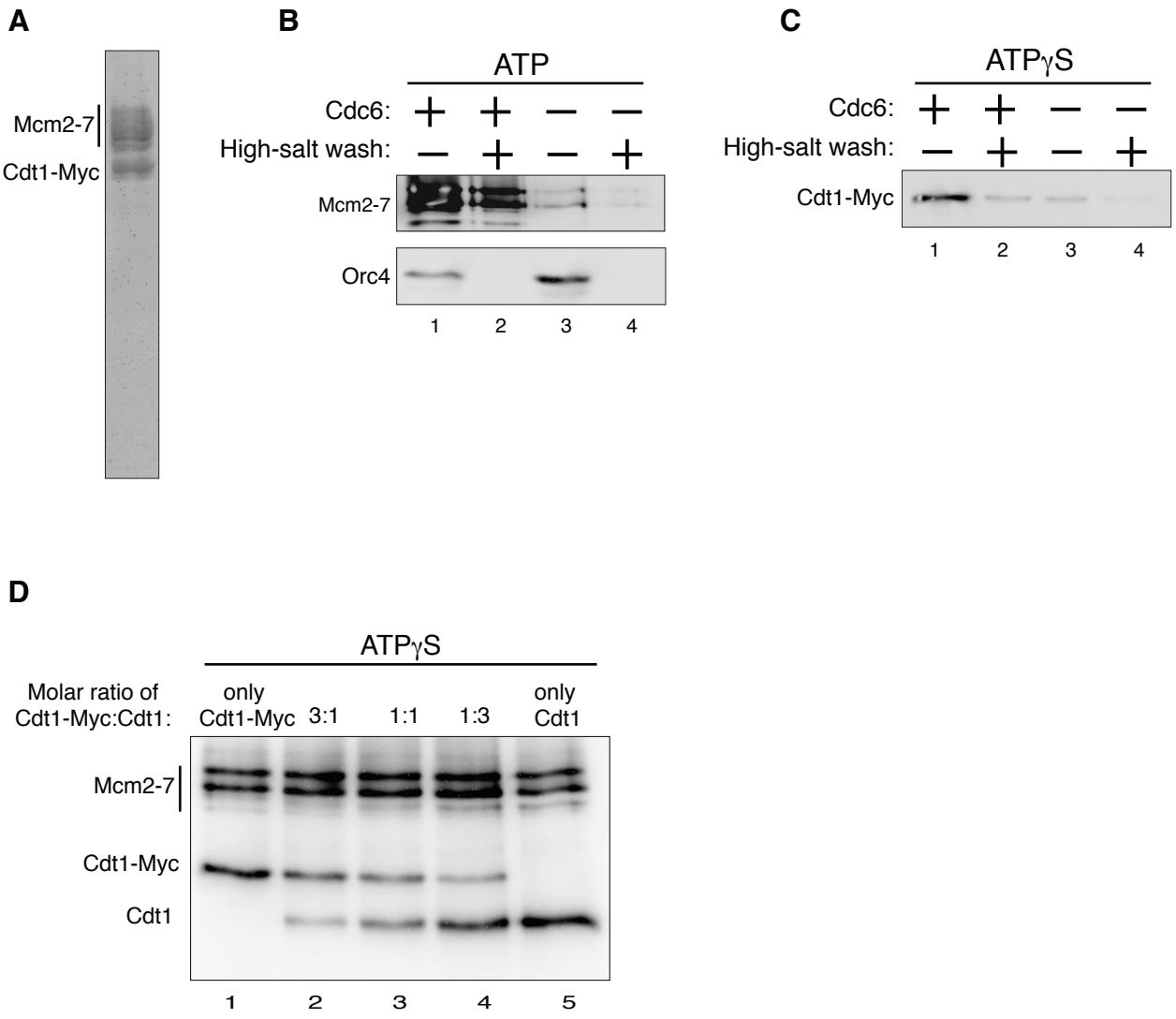
Takara and Bell, Fig. S3



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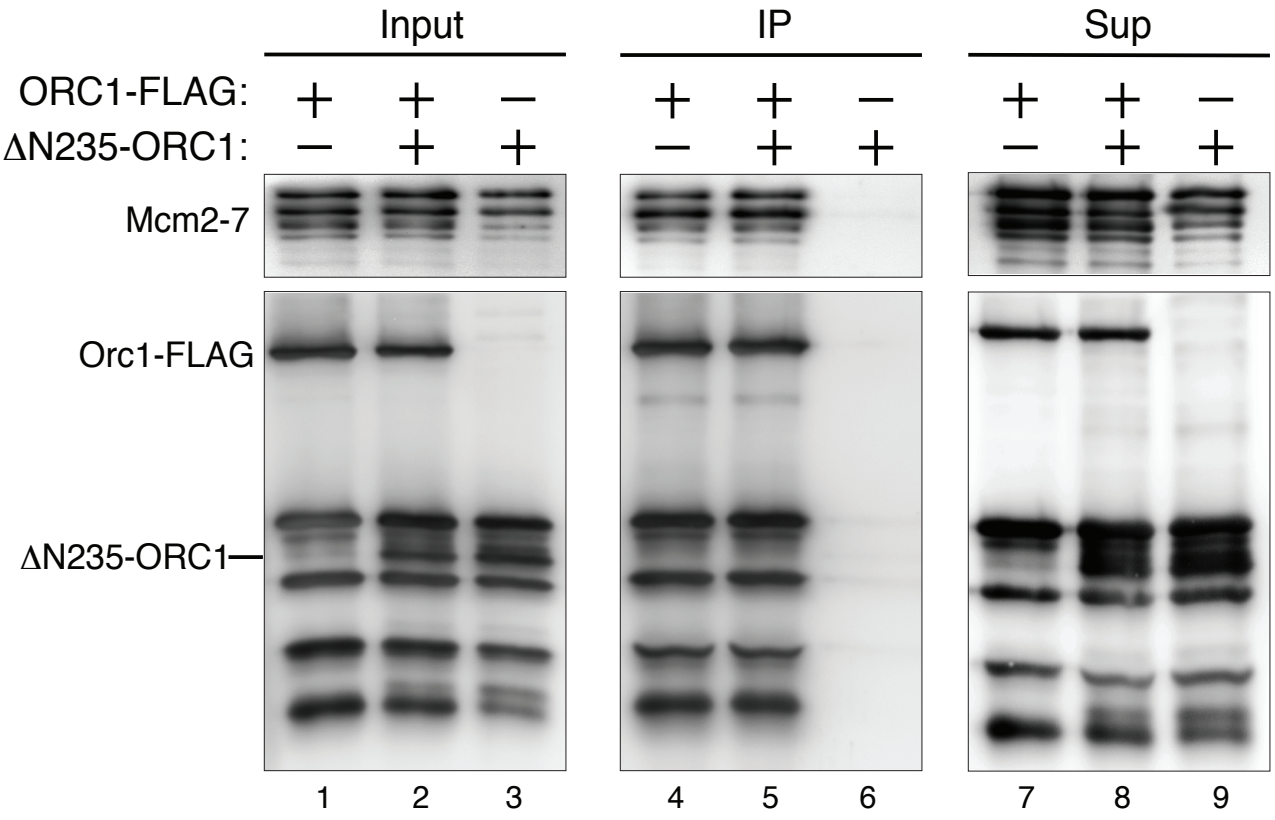
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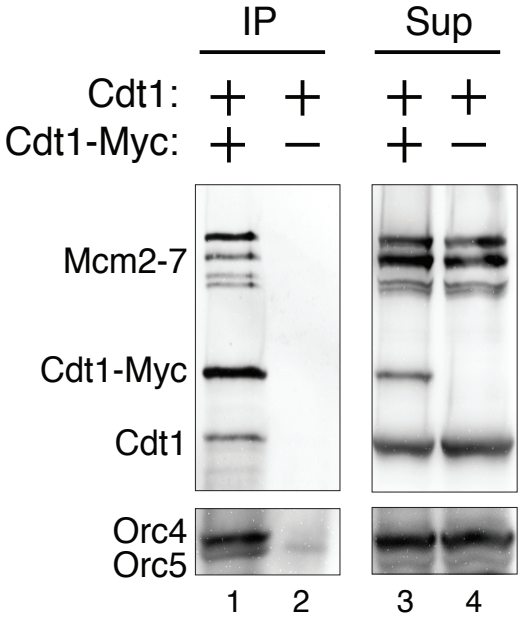
B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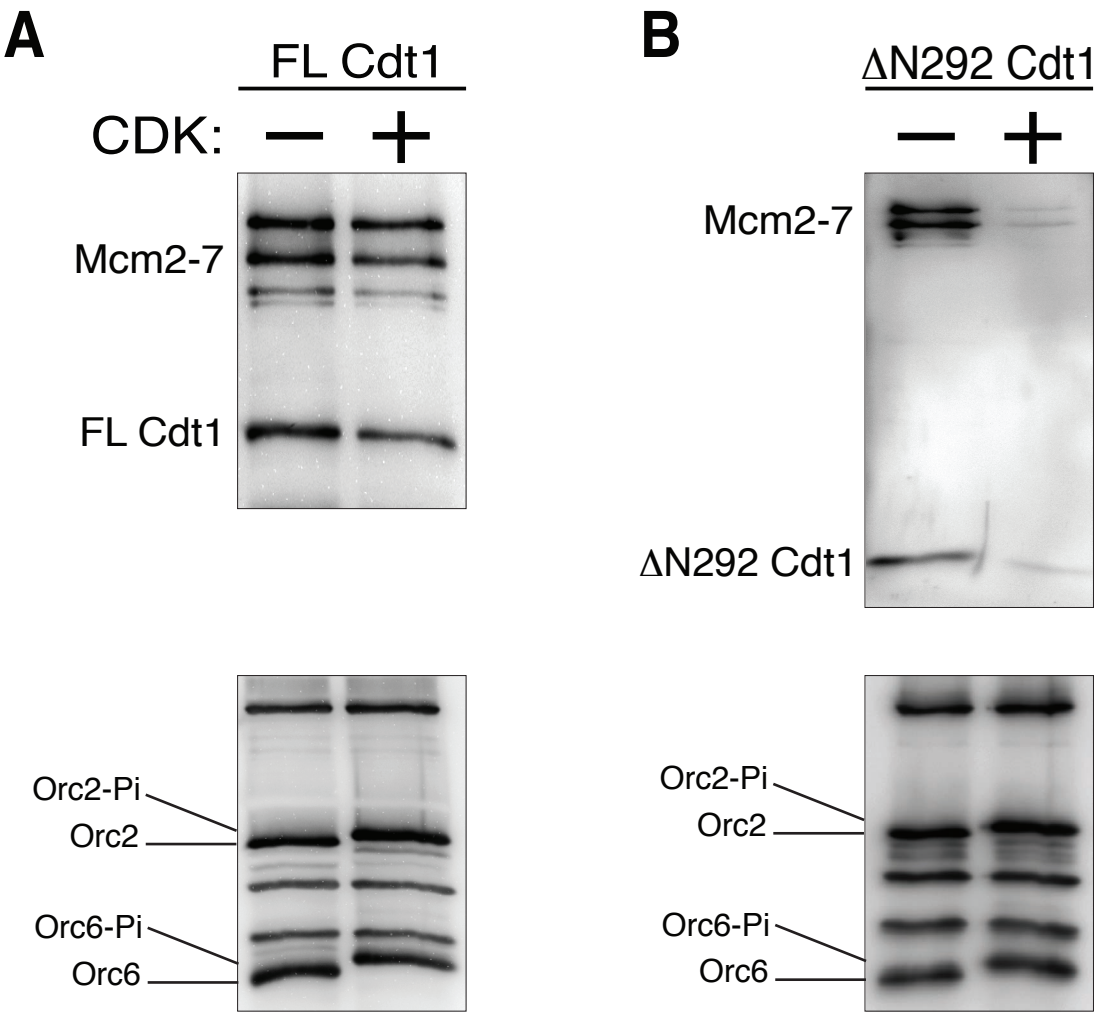
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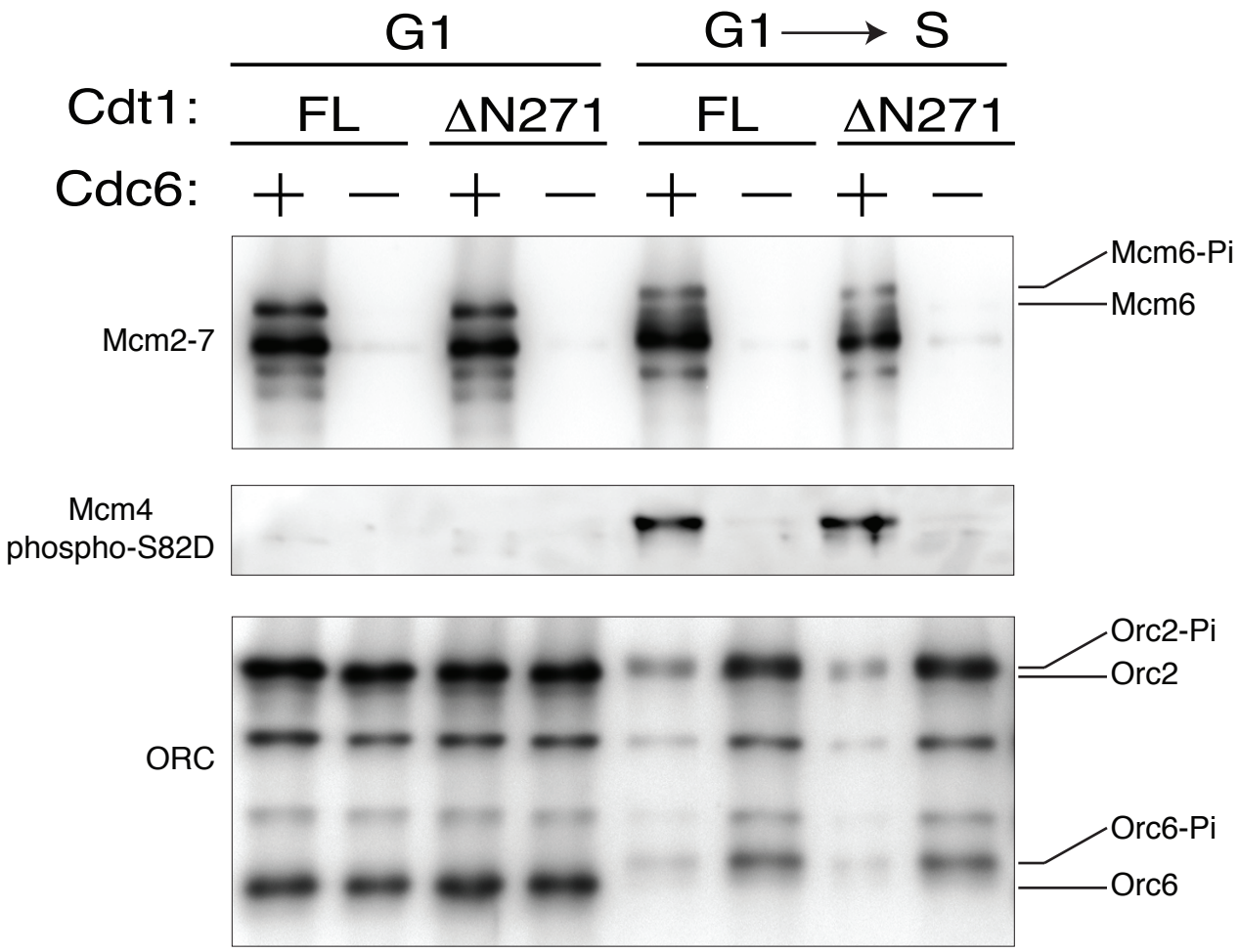
Takara and Bell, Fig. S6



Takara and Bell, Fig. S7