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Supplemental Information

Origin Licensing Requires ATP Binding and Hydrolysis by the MCM Replicative Helicase

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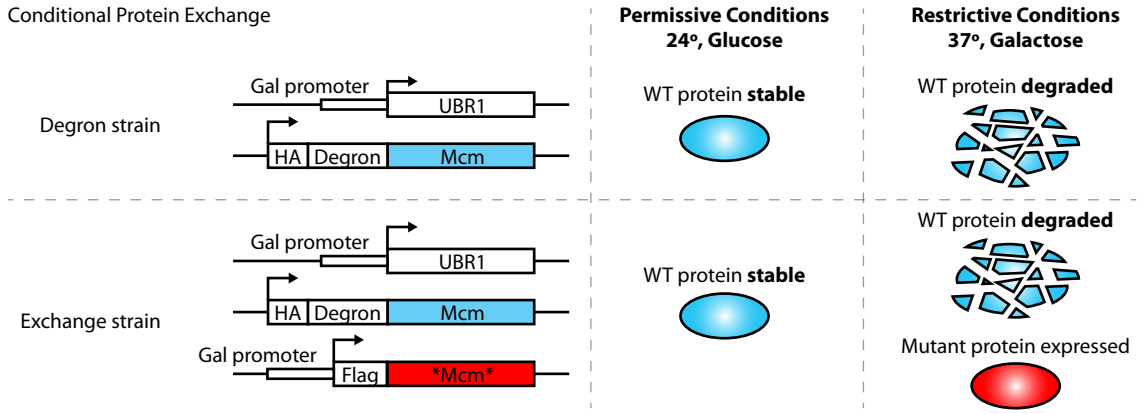
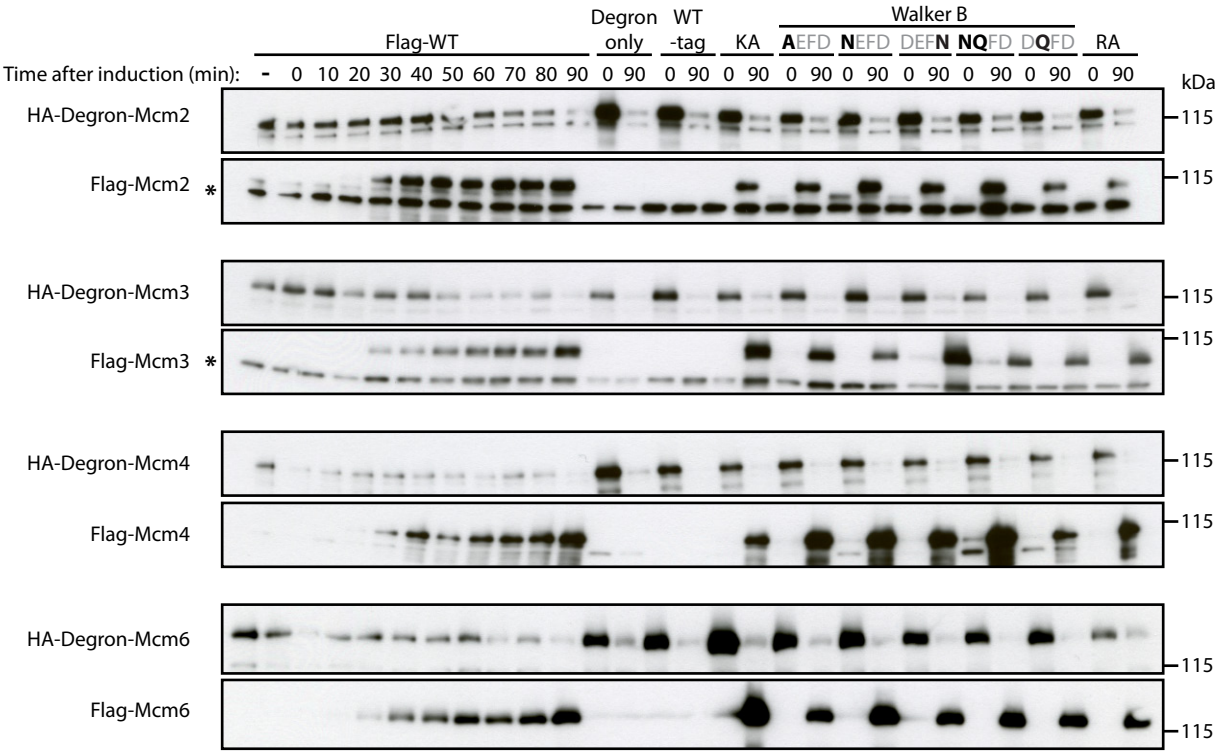
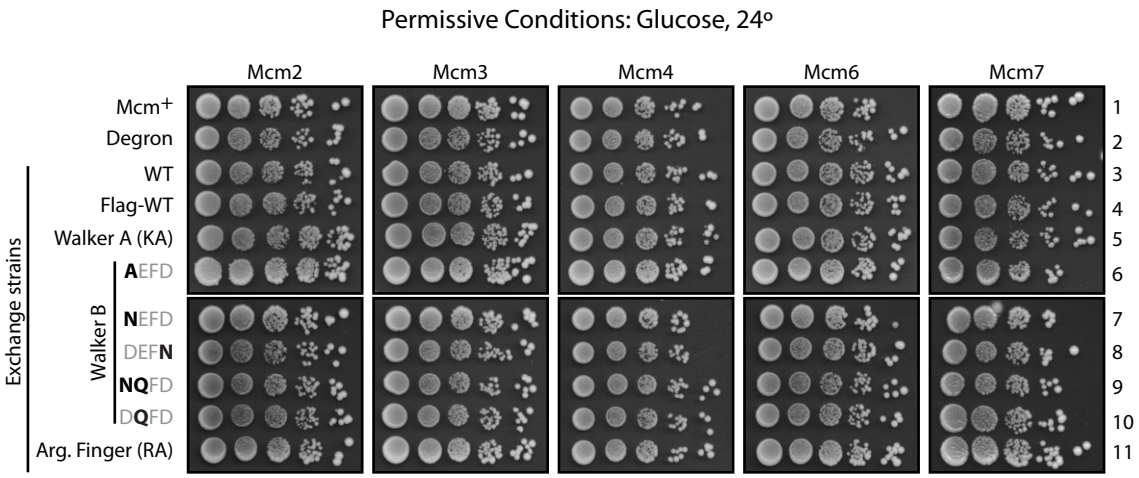
A


Conditional Protein Exchange
Permissive Conditions
24°, Glucose
Restrictive Conditions
37°, Galactose
Degron strain
Gal promoter
UBR1
HA
Degron
Mcm
WT protein stable
WT protein degraded
Exchange strain
Gal promoter
UBR1
HA
Degron
Mcm
Gal promoter
Flag
Mcm
WT protein stable
WT protein degraded
Mutant protein expressed

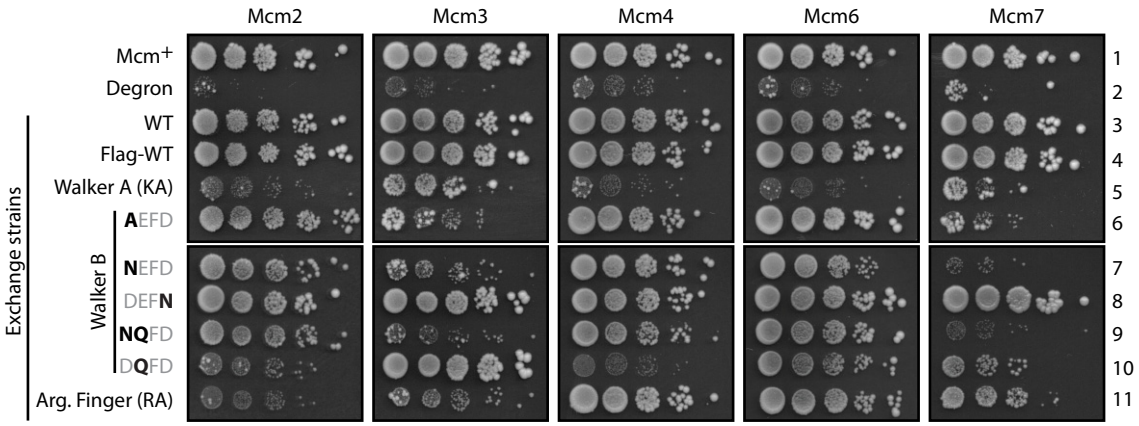
B


Flag-WT
Degron only
WT -tag
KA
Walker B
AEFD
NEFD
DEFN
NQFD
DQFD
RA
Time after induction (min):
- 0 10 20 30 40 50 60 70 80 90 0 90 0 90 0 90 0 90 0 90 0 90 0 90 0 90 0 90
kDa
HA-Degron-Mcm2
Flag-Mcm2 *
HA-Degron-Mcm3
Flag-Mcm3 *
HA-Degron-Mcm4
Flag-Mcm4
HA-Degron-Mcm6
Flag-Mcm6
115

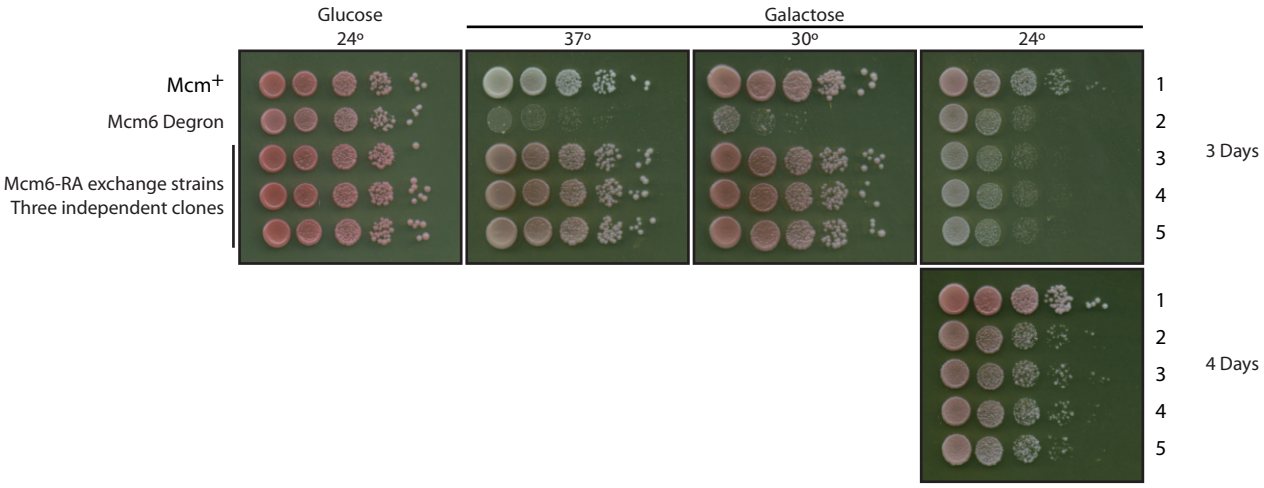
A


Permissive Conditions: Glucose, 24°
Mcm2
Mcm3
Mcm4
Mcm6
Mcm7
Mcm+
Degron
WT
Flag-WT
Walker A (KA)
AEFD
NEFD
DEFN
NQFD
DQFD
Arg. Finger (RA)
Exchange strains
Walker B
1
2
3
4
5
6
7
8
9
10
11

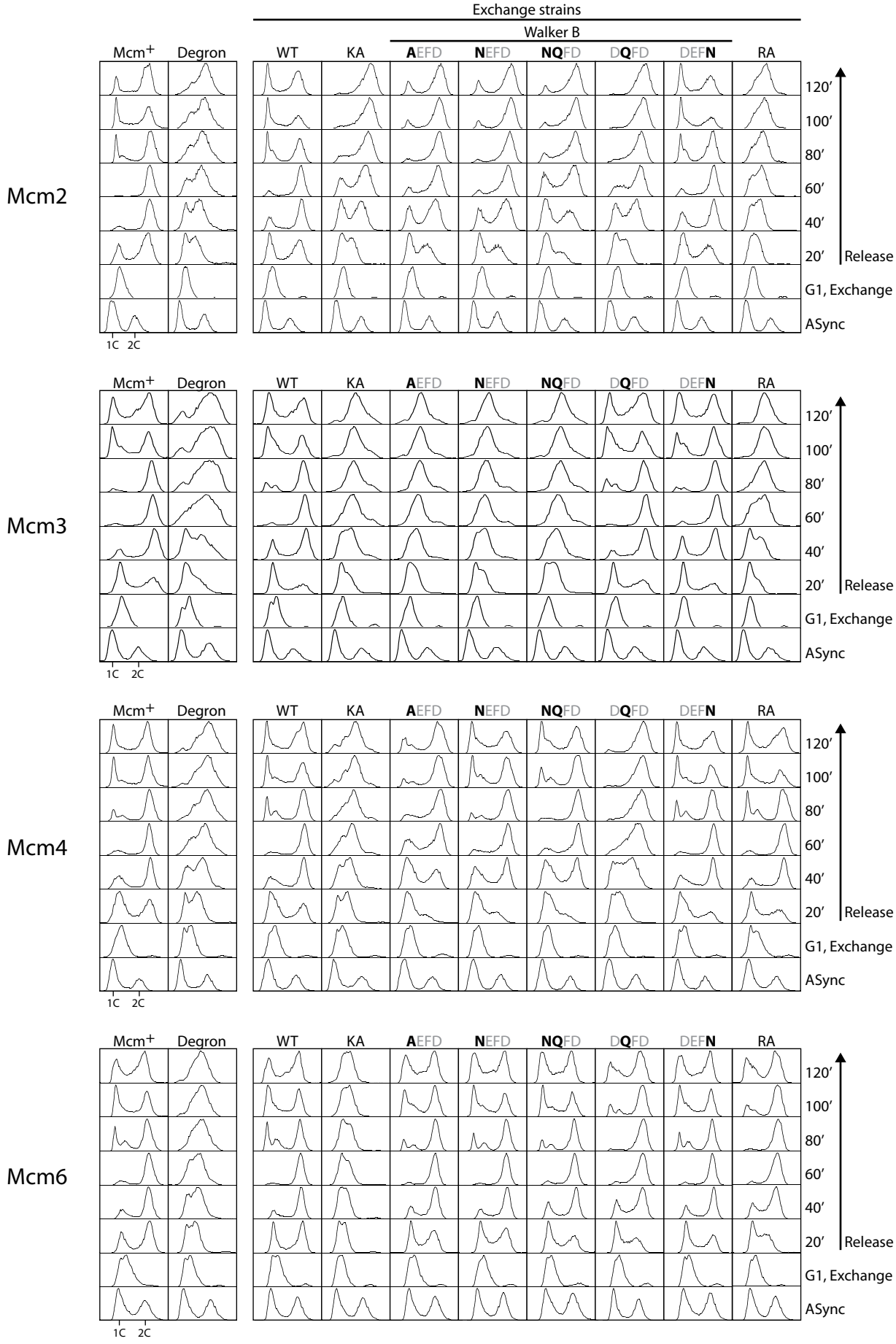
B


Restrictive Conditions: Galactose, 37°
Mcm2
Mcm3
Mcm4
Mcm6
Mcm7
Mcm+
Degron
WT
Flag-WT
Walker A (KA)
AEFD
NEFD
DEFN
NQFD
DQFD
Arg. Finger (RA)
Exchange strains
Walker B
1
2
3
4
5
6
7
8
9
10
11

C


Glucose
24°
Galactose
37°
30°
24°
Mcm+
Mcm6 Degron
Mcm6-RA exchange strains
Three independent clones
1
2
3
4
5
3 Days
1
2
3
4
5
4 Days

Coster et al. Figure S3



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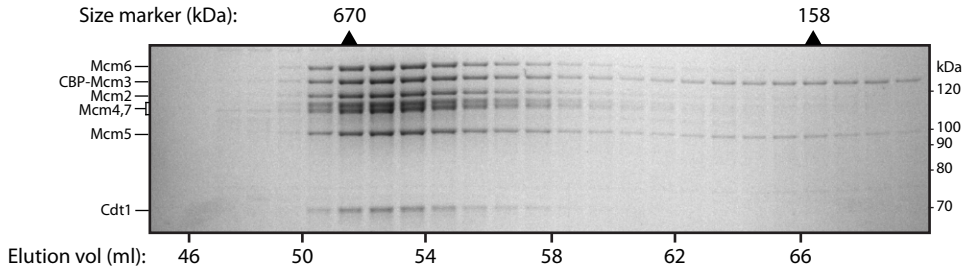
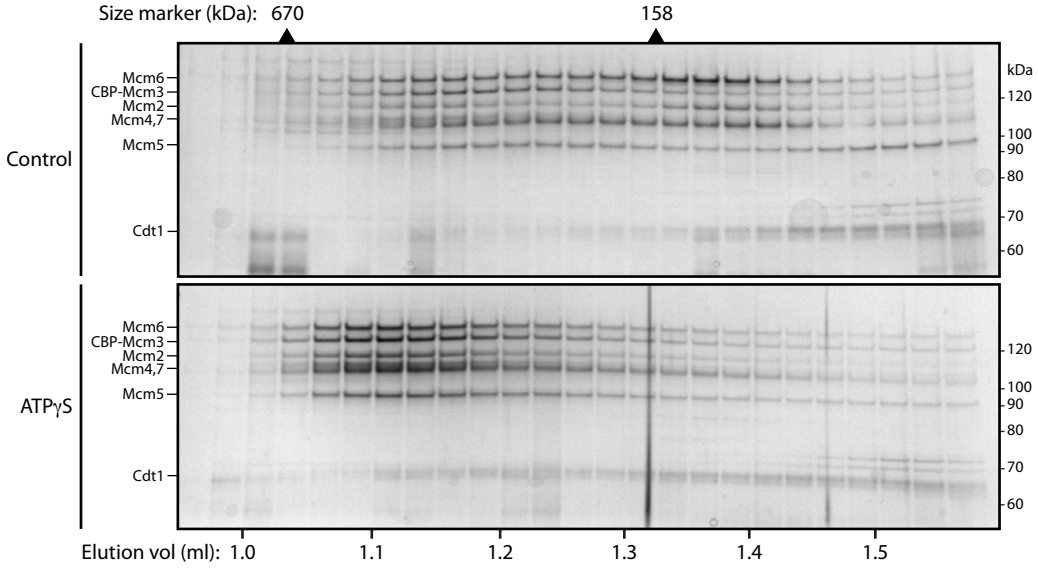


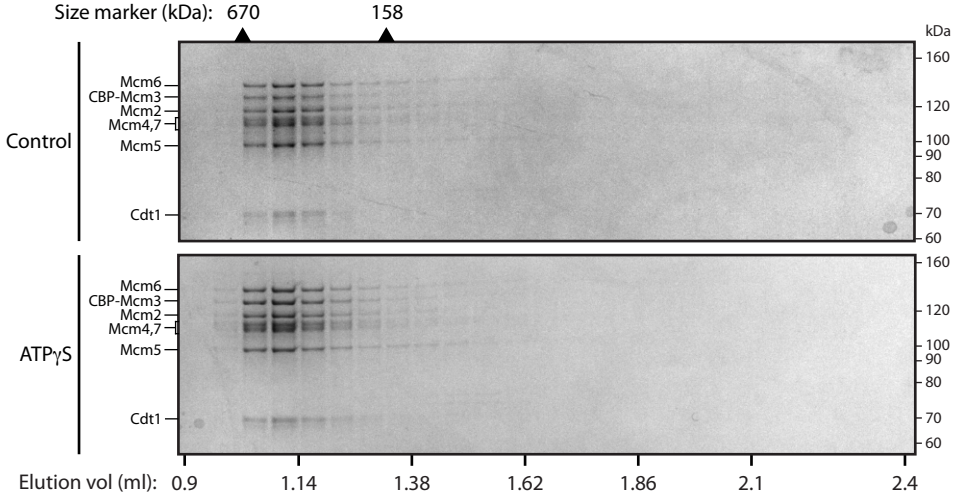
Preparative gel filtration of WT MCM•Cdt1 on a 120 ml Superdex 200 column

B



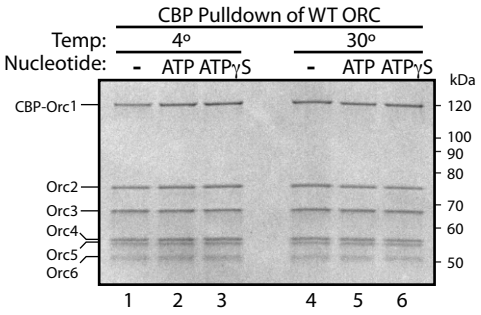
Analytical gel filtration of 320 nM WT MCM•Cdt1 on a 2.4 ml Superdex 200 column

C

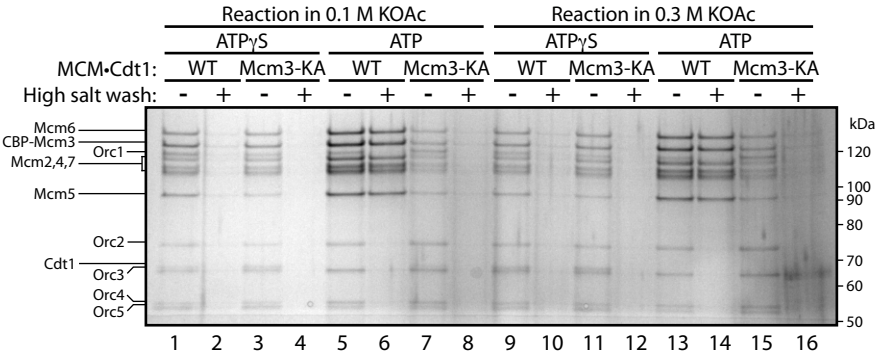


Analytical gel filtration of 1.92 µM WT MCM•Cdt1 on a 2.4 ml Superdex 200 column

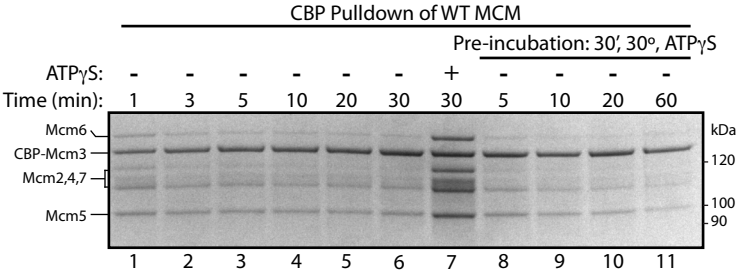
D


CBP Pulldown of WT ORC
Temp:
4°
30°
Nucleotide:
- ATP ATPγS
- ATP ATPγS
kDa
CBP-Orc1
Orc2
Orc3
Orc4
Orc5
Orc6
120
100
90
80
70
60
50
1 2 3 4 5 6

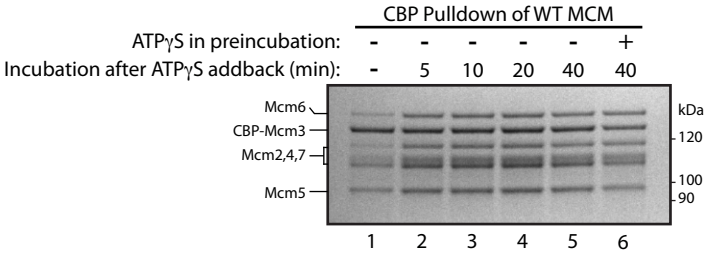
E


Reaction in 0.1 M KOAc
Reaction in 0.3 M KOAc
ATPγS
ATP
ATPγS
ATP
MCM•Cdt1:
WT
Mcm3-KA
WT
Mcm3-KA
WT
Mcm3-KA
WT
Mcm3-KA
High salt wash:
- + - + - + - + - + - + - + - +
kDa
Mcm6
CBP-Mcm3
Orc1
Mcm2,4,7
Mcm5
Orc2
Cdt1
Orc3
Orc4
Orc5
120
100
90
80
70
60
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

F

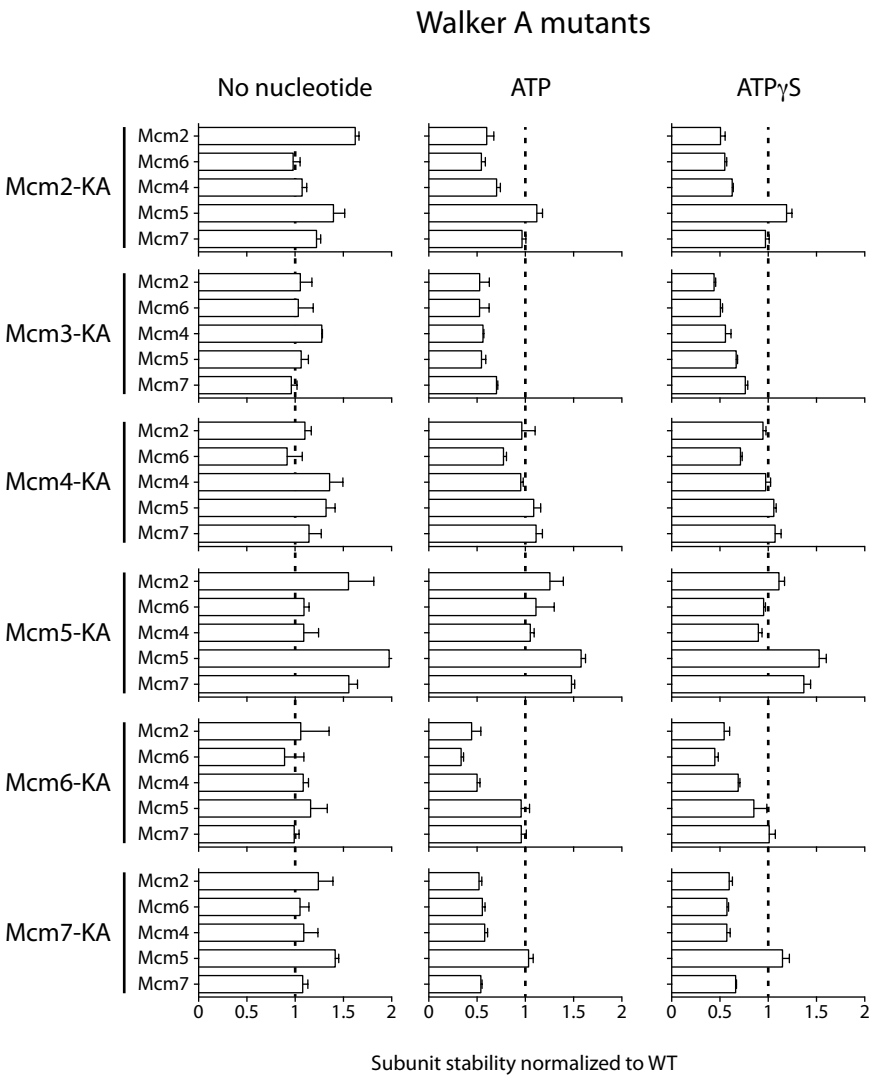

CBP Pulldown of WT MCM
Pre-incubation: 30', 30°, ATPγS
ATPγS:
- - - - - - + - - - -
Time (min):
1 3 5 10 20 30 30 5 10 20 60
kDa
Mcm6
CBP-Mcm3
Mcm2,4,7
Mcm5
120
100
90
1 2 3 4 5 6 7 8 9 10 11

G

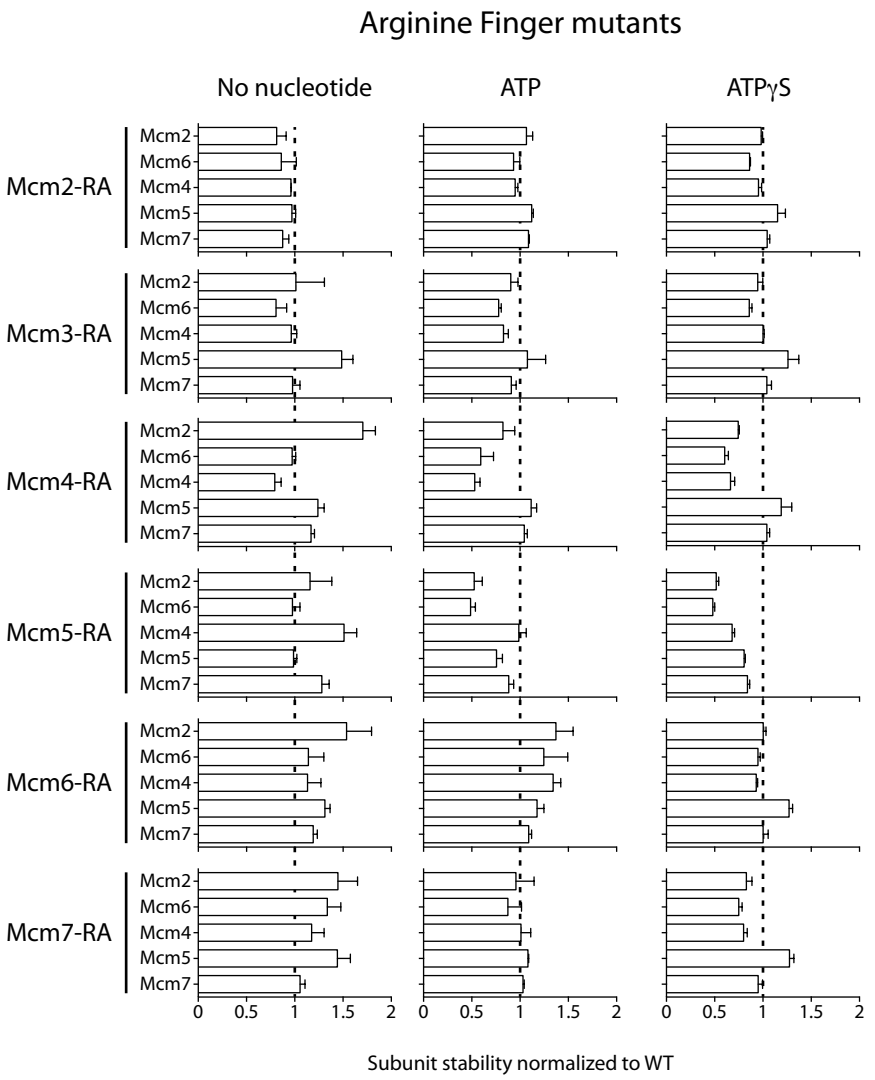

CBP Pulldown of WT MCM
ATPγS in preincubation:
- - - - - +
Incubation after ATPγS addback (min):
- 5 10 20 40 40
kDa
Mcm6
CBP-Mcm3
Mcm2,4,7
Mcm5
120
100
90
1 2 3 4 5 6

Coster et al. Figure S5

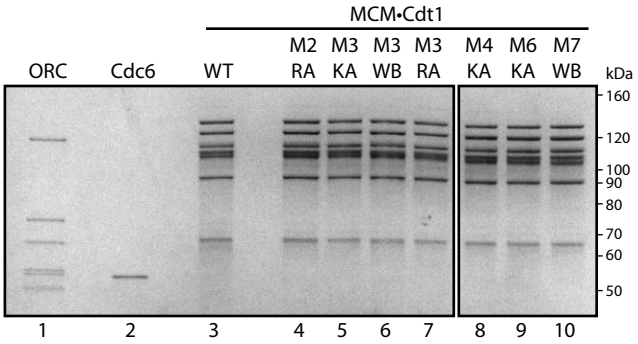
A



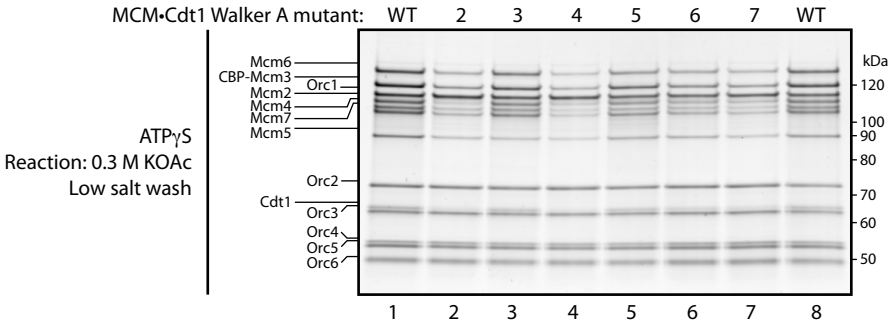
B



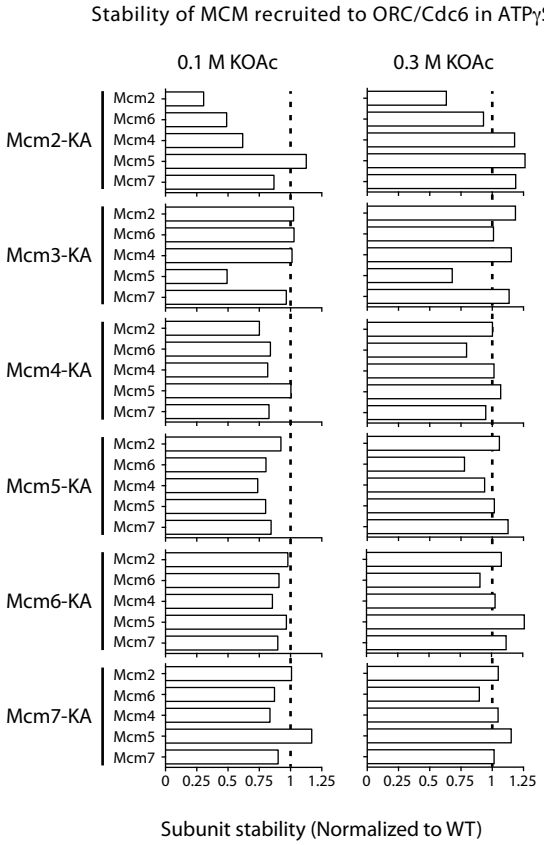
A


MCM•Cdt1
ORC
Cdc6
WT
M2 RA
M3 KA
M3 WB
M3 RA
M4 KA
M6 KA
M7 WB
kDa
160
120
100
90
80
70
60
50
1 2 3 4 5 6 7 8 9 10

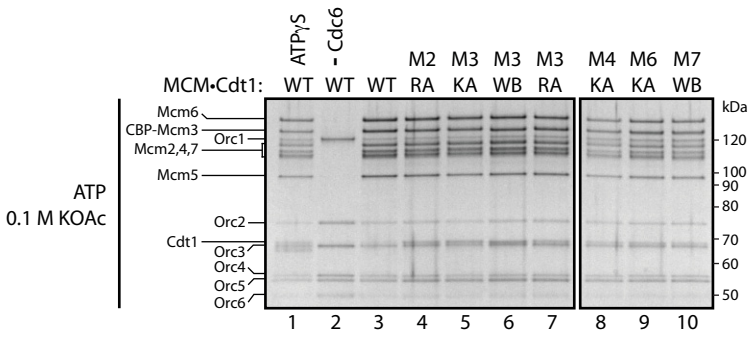
B


MCM•Cdt1 Walker A mutant: WT 2 3 4 5 6 7 WT
ATPγS
Reaction: 0.3 M KOAc
Low salt wash
Mcm6
CBP-Mcm3
Orc1
Mcm2
Mcm4
Mcm7
Mcm5
Orc2
Cdt1
Orc3
Orc4
Orc5
Orc6
kDa
120
100
90
80
70
60
50
1 2 3 4 5 6 7 8

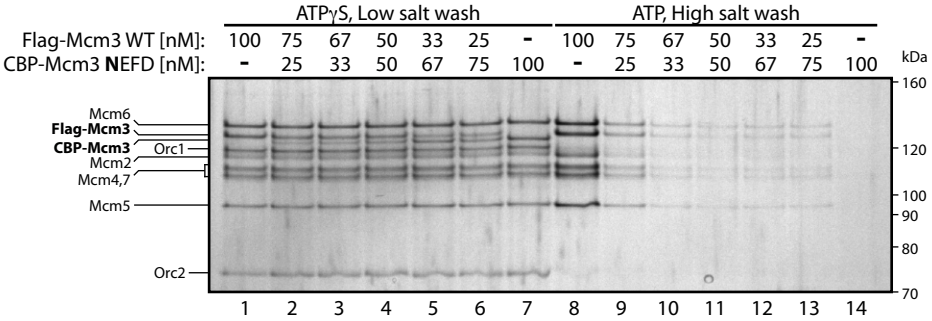
C


Stability of MCM recruited to ORC/Cdc6 in ATPγS
0.1 M KOAc
0.3 M KOAc
Mcm2-KA
Mcm3-KA
Mcm4-KA
Mcm5-KA
Mcm6-KA
Mcm7-KA
Mcm2
Mcm6
Mcm4
Mcm5
Mcm7
0 0.25 0.5 0.75 1 1.25
Subunit stability (Normalized to WT)

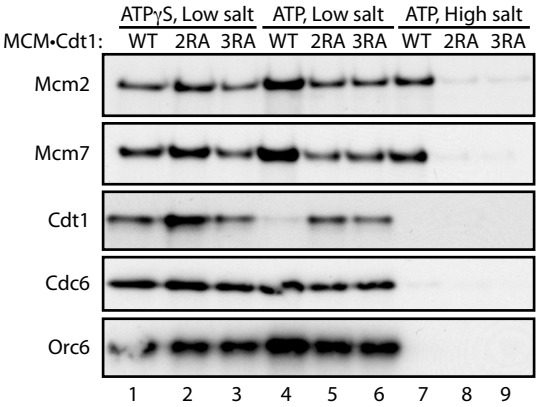
D


ATPγS
- Cdc6
MCM•Cdt1:
WT WT WT M2 RA M3 KA M3 WB M3 RA M4 KA M6 KA M7 WB
kDa
Mcm6
CBP-Mcm3
Orc1
Mcm2,4,7
Mcm5
ATP
0.1 M KOAc
Orc2
Cdt1
Orc3
Orc4
Orc5
Orc6
120
100
90
80
70
60
50
1 2 3 4 5 6 7 8 9 10

E


ATPγS, Low salt wash
ATP, High salt wash
Flag-Mcm3 WT [nM]: 100 75 67 50 33 25 - 100 75 67 50 33 25 -
CBP-Mcm3 NEFD [nM]: - 25 33 50 67 75 100 - 25 33 50 67 75 100
kDa
Mcm6
Flag-Mcm3
CBP-Mcm3
Orc1
Mcm2
Mcm4,7
Mcm5
Orc2
160
120
100
90
80
70
1 2 3 4 5 6 7 8 9 10 11 12 13 14

F


ATPγS, Low salt
ATP, Low salt
ATP, High salt
MCM•Cdt1:
WT 2RA 3RA WT 2RA 3RA WT 2RA 3RA
Mcm2
Mcm7
Cdt1
Cdc6
Orc6
1 2 3 4 5 6 7 8 9

Supplemental Figure Legends

Figure S1, related to Figure 3

A. Diagram describing the conditional protein exchange approach. In degron strains, the only copy of each Mcm gene is tagged by a degron sequence. Transfer to restrictive conditions induces the expression of the E3 ubiquitin ligase Ubr1, which then targets the degron protein for degradation. Complementation of a degron strain with a second, Galactose-inducible, copy of the corresponding Mcm gene results in what we refer to as an Exchange strain. The same restrictive conditions that result in degradation of the WT degron protein lead to induction of the second allele, effectively leading to an exchange between the two proteins.

B. Western blot analysis of protein exchange. See Figure 3B.

Figure S2, related to Figure 3

A-B. Viability of yeast strains was analysed by plating 10-fold dilutions onto YP plates containing Glucose (A) or Galactose (B). Growth was assessed after four days incubation at 24º (A) or 37º (B).

C. Viablity of Mcm6-RA mutant strains. Three independently derived clones of the Mcm6-RA exchange strain were analysed together with the parental strain and the Mcm6 degron strains as in A and B. Growth was also assessed at 30º.

Figure S3, related to Figure 3

Cell cycle phenotypes of MCM ATPase mutants. See Figure 3C.

Figure S4, related to Figure 4

A. The last step of the purification of WT MCM•Cdt1. Peak fractions for the CBP pulldown were concentrated and loaded onto a 120 ml Superdex 200 column. 5 µl from each 1 ml fraction were analysed by SDS-PAGE and coomassie staining.

B-C. Analytical gel filtration of WT MCM•Cdt1 pre-incubated in low salt wash buffer at 30º for 30 min in the absence or presence of 5 mM ATPγS at a final concentration of 320 nM (B) or 1.92 µM (C). Samples were loaded onto a 2.4 ml Superdex 200 column. Fractions were analysed by SDS-PAGE and silver staining.

D. Stability assay with WT ORC. The same experiment as shown in Fig. 4C was carried out but with 50 nM ORC instead of 100 nM MCM.

E. The loading reaction works in either 0.1 M or 0.3 M KOAc. Loading reaction were carried out with WT or Mcm3-KA MCM•Cdt1 with the only difference being the salt concentration in the reaction itself as shown.

F. Disassembly of MCM is rapid and cannot be delayed by pre-incubation with ATPγS. A time course of the stability assay. A stability assay was carried out in the absence or presence of 5 mM ATPγS and samples were taken at the indicated time and analysed for bound proteins. For lanes 8-11, reactions were first pre-incubated with ATPγS (essentially as in lane 7), beads were washed once, resuspended in buffer lacking nucleotide for the indicated time and then washed and analysed for remaining bound proteins.

G. MCM can reassemble upon addition of nucleotide. A stability assay was carried out in two steps. A pre-incubation was carried out in the absence or presence of ATPγS for 20 min at 30º. ATPγS was then directly added to all

reactions that did not already contain a nucleotide. Reactions were then incubated further for the indicated times and bound proteins were assessed.

Figure S5, related to Figure 4

Stability of Walker A and Arginine finger mutants. See Fig.4F.

Figure S6, related to Figure 6

A. Comparison of purified proteins used in loading reactions. Purified samples were analysed by SDS-PAGE and coomassie staining.

B. Walker A mutants can be recruited to ORC•Cdc6 even at 0.3 M KOAc. Loading reactions were carried out as in Fig. 5C but with a final concentration of 0.3 M KOAc.

C. Quantification of MCM subunit stoichiometry for experiments shown in Fig. 5C (left) and S6B (right). The intensity of each MCM subunit was quantified and divided by that of Mcm3 (similar to the stability assay quantification). Note that this analysis does not reflect overall recruitment efficiency to ORC•Cdc6 but rather reflects the stoichiometry of the recruited MCM hexamer.

D. Samples for analysis by EM. See Fig. 6E.

E. Loading requires ATP hydrolysis by both MCM hexamers. Loading reactions were carried out with ATPγS or ATP by mixing WT and mutant MCM complexes at different ratios, but keeping the total amounts of MCM constant. The WT complex contains a Flag-tagged Mcm3. The mutant complex contains a CBP-tagged Mcm3 Walker B NEFD mutant.

F. Arginine finger mutants are defective in Cdt1 release. See Fig. 6F.

Supplemental Table 3, related to Experimental Procedures

PCR reactions for detection of correct plasmid integration

	Primer	Primer	Product	Product
Mcm2	GC83	GC77	Trp1 locus	1266
	GC83	GC11	Integration of Mcm2 at Trp1 5'	2758
	GC72	GC77	Integration of a pRS at Trp1 3'	2292
Mcm3	GC82	GC79	Ura3 locus	1437
	GC82	GC23	Integration of Mcm3 at Ura3 5'	2361
	GC72	GC79	Integration of a pRS at Ura3 3'	2345
Mcm4	GC83	GC77	Trp1 locus	1266
	GC83	GC38	Integration of Mcm4 at Trp1 5'	1853
	GC72	GC77	Integration of a pRS at Trp1 3'	2292
Mcm6	GC84	GC85	Leu2 Locus	2275
	GC84	GC72	Integration of a pRS at Leu2	3374
	GC85	GC52	Integration of Mcm6 at Leu2 3'	3343
Mcm7	GC82	GC79	Ura3 locus	1437
	GC82	GC66	Integration of Mcm7 at Ura3 5'	2953
	GC72	GC79	Integration of a pRS at Ura3 3'	2345

Supplemental Table 4, related to Experimental Procedures

Primers used for detection of correct plasmid integration

Primer	Sequence	Target
GC11 Mcm2 R 207	TCGTCCATCAAATCTACTTCG	Mcm2, 207 bp from start, 3'>5'
GC23 Mcm3 F 2630	GATTACCTAACGTATCTTCTGC	Mcm3, 2630 bp from start, 5'>3'
GC38 Mcm4 F 2794	TCAGTTCGCCTGAATAACC	Mcm4, 2794 bp from start, 5'>3'
GC52 Mcm6 F 2719	AAACAACGGTGACGTATG	Mcm6, 2719 bp from start, 5'>3'
GC66 Mcm7 R 210	ATTCAGTTCCCTATTAGCC	Mcm7, 210 bp from start, 3'>5'
GC72 Amp F	GTGCTGCAATGATACCGCGAG	Internal part of the Amp resistance cassette
GC77 Trp1 R	ACAAGTTTGATTCCATTGCGGTG	Genomic region 190 bp downstream Trp1
GC79 Ura3 R	GTTACTTGGTTCTGGCGAGG	Genomic region 172 bp downstream Ura3
GC82 Ura3 b F	GAGGCTACTGCGCCAATT	Genomic region 423 bp upstream Ura3
GC83 Trp1 b F	GAGCTGACAGGGAAATGGT	Genomic region 359 bp upstream Trp1
GC84 Leu2 F	GCAGATTCCCTTTTATGGATTCC	Genomic region 655 bp upstream Leu2
GC85 Leu2 R	GGTAGATTTAGTACTGAAGAGGAGGTCG	Genomic region 474 bp downstream Leu2

Supplemental Experimental Procedures

Plasmids

All yeast plasmids are based on pRS vectors, each with one or two MCM subunits under the dual Gal1,10 promoter. Plasmids carrying each of the single Mcm2-7 subunits were fully sequenced to ensure their WT sequence prior to cloning of mutants. Fragments containing the desired mutations were obtained from the GeneArt Gene Synthesis service (Life Technologies). A 3xFlag sequence was added to each synthetic gene fragment, including a WT fragment. Synthetic fragments were then used to replace the corresponding sequence within the full-length MCM subunit, using restriction sites present in the original sequence. Plasmids were then verified by sequencing and linearized for transformation into yeast.

Yeast strains

All yeast strains are haploids based on the W303 background. Strains were established either by transformation with linearized integrating plasmids or by mating, sporulation, tetrad dissection and selection of haploids with the correct marker combination, as detailed in Supplemental Table 2. Several of the degron strains (Labib et al., 2000) were rederived so that all resulting exchange strains carry the same selection markers. Transformation, preparation of genomic DNA and PCR were performed as described in (Sanchez-Diaz et al., 2004). Transformants were analysed by PCR of genomic DNA and correct integration events were identified by the absence of a genomic product and the presence of 5' and 3' integration products. Supplemental Table 3 shows the primer combinations used for testing

integrations and Supplemental Table 4 shows the primers sequences. Protein levels for both the degron and complementing alleles were analysed by western blotting of whole cell extracts prepared according to (Kushnirov, 2000) and probed with an anti-HA antibody (monoclonal 12CA5) or an anti-Flag antibody (Sigma monoclonal M2).

Purification of Mcm2-7/Cdt1 via CBP-Mcm3

Yeast strains yAM33 (WT) or yGC212-yGC219 (mutants) were grown in 4 L [YP + 2% Raffinose] at 30º. When cells reached ~5x10^7 cells/ml, alpha factor was added at a final concentration of 100 ng/ml. After three hours, expression was induced with 2% Galactose for three hours at 30º. Cells were harvested, washed twice in [25 mM HEPES-KOH pH=7.6, 1 M Sorbitol], once in Lysis buffer [45 mM HEPES-KOH pH=7.6, 0.1 M KOAc, 5 mM MgOAc, 0.02% NP-40, 10% Glycerol], resuspended in [Lysis buffer, Protease inhibitors (Roche), 2 mM b-mercaptoethanol] and frozen drop-wise in liquid nitrogen. Frozen cells were crushed in a freezer mill (SPEX CertiPrep 6850), thawed and resuspended in 1 vol of [Lysis buffer, Protease inhibitors (Roche), 2 mM β-mercaptoethanol]. The final KOAc concentration was adjusted to 0.5 M and the lysate was clarified by ultracentrifugation at 45,000 RPM for one hour in a Type 45 rotor (Beckman). The clear phase was recovered and dialyzed twice for one hour against Lysis buffer to reduce KOAc concentration to 0.1 M. The cleared lysate was supplemented with 2 mM $CaCl_2$ and incubated for three hours at 4º with 1 ml Calmodulin beads (Agilent Technologies). Beads were washed with at least 20 ml [Lysis buffer, 2 mM $CaCl_2$] and bound proteins were eluted with [Lysis buffer, 1 mM EDTA, 2 mM EGTA]. The eluate was then concentrated on a 100K Centricon (Millipore) and loaded onto a 120 ml

HiLoad Superdex 200 16/60 Gel filtration column (GE Healthcare) in Lysis buffer. Peak fractions were pooled, concentrated and aliquoted.

Loading Assay

DNA templates, 1 kb in size, were generated by PCR using a 5' primer containing a photocleavable biotin (IDT) and a plasmid containing ARS305 as a template. PCR products were purified and for each reaction 300 ng (0.45 pmol) were coupled to 5 µl slurry Dynabeads M-280 streptavidin magnetic beads (Life Technologies). Loading reactions were assembled on ice in a final volume of 40 µl of [25 mM HEPES-KOH pH=7.6, 10 mM MgOAc, 1 mM DTT, 0.02% NP-40, 5% Glycerol and 100 mM KOAc]. Reactions contained either 5 mM ATP (Affymetrix) or ATPγS (Roche) as noted. DNA-beads were added, following by 50 nM ORC, 50 nM Cdc6 and 250 nM Mcm2-7/Cdt1. Reactions were immediately transferred to 30º with mixing at 1250 RPM for 20 min. Reactions were then washed either twice with a low salt wash [45 mM HEPES-KOH pH=7.6, 5 mM MgOAc, 1 mM EDTA, 1 mM EGTA, 0.02% NP-40, 10% Glycerol and 300 mM KOAc] or once with a high salt wash (same as low salt wash, but with 500 mM NaCl instead of 300 mM KOAc) and once with low salt. Samples were then resuspended in low salt wash and photocleaved by exposure to UV (330 nm) for 10 min on ice. Released DNA-bound proteins were mixed with sample buffer and analyzed by SDS-PAGE.

Cell cycle time course experiments

An overnight culture grown in [YP + 2% Raffinose] was diluted to $5x10^6$ cells/ml in [YP + 2% Raffinose + 5 µg/ml alpha factor] in a final volume of 25 ml. After three hours at 25º, cells were collected and resuspended in [YP +

2% Galactose + 5 µg/ml alpha factor]. After 30' at 25º, cells were collected and resuspended in [YP + 2% Galactose + 5 µg/ml alpha factor] prewarmed to 37º. After 60' at 37º, cells were collected and resuspended in [YP + 2% Galactose] prewarmed to 37º. Cell were then incubated at 37º and 1 ml samples were collected every 20' for analysis by flow cytometry.

Flow cytometry

Approximately $1x10^7$ cells were resuspended in 1 ml ice-cold 70% ethanol and stored overnight at 4º. Cells were washed once in [50 mM Tris pH=8] and then incubated at 37º for four hours in [50 mM Tris pH=8, 0.1 mg/ml RnaseA]. Cells were then incubated for 30' at 37º in [5 mg/ml Pepsin]. Cells were washed twice in [50 mM Tris pH=8] then diluted 10-fold in [50 mM Tris pH=8, 1 µM SYTOX Green]. Samples were briefly sonicated and analysed on a FACSCalibur flow cytometer (BD Biosciences). Data analysis was carried out using FlowJo (Tree Star).

Supplemental References

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