Supplemental Information:

Supplemental figure legends

Figure S1. Yeast kinetochores form clusters during the cell cycle. *MTW1-GFP TUB1-mCherry* cells were arrested in G_1 phase and then transferred to an agarose pad on a microscope slide for live-cell imaging at 25°C. The localization of kinetochore (Mtw1-GFP) and the spindle morphology (Tub1-mCherry) in a representative cell are shown.

Figure S2. The kinetochore protein foci are functional kinetochores. G_1-arrested *CEN4-GFP NUF2-mCherry* cells were released into 25°C YPD medium containing 20 μg/ml nocodazole. Cells were harvested after 120 min and the images were acquired by confocal microscopy. The images show the co-localization of *CEN4*-GFP with either the larger or the smaller Nuf2-mCherry focus.

Figure S3. Yeast cohesion mutants do not show a dramatic kinetochore clustering defect. **A.** G_1-arrested WT or *GAL-SCC1 (MCD1)* cells with *MTW1-GFP TUB1-mCherry* were released from galactose medium into glucose medium containing 20μg/ml of nocodazole to repress Scc1 expression and disrupt the spindle structure. The cells were harvested after 120 min incubation at 25°C and the number of Mtw1-GFP foci was counted. The average of three independent experiments is shown. **B.** G_1-arrested WT, *ctf8*Δ, *ctf18*Δ, and *dcc1*Δ mutants with *MTW1-GFP* were released into YPD media containing 20 μg/ml nocodazole. Cells were harvested after 120 min incubation at 25°C and the number of Mtw1-GFP foci in each cell was counted using fluorescence microscopy. For each sample, at least 100 cells were counted and the percentage of cells with different number of Mtw1-GFP foci is shown on the top. Some representative images from confocal microscopy are shown at the bottom panel to indicate the distribution of Mtw1-GFP.

Figure S4. *slk19*Δ mutants do not show kinetochore clustering defect during an undisturbed cell cycle. G_1-arrested *slk19*Δ mutant cells were transferred onto an agarose pad on a microscope slide to perform live-cell imaging at 25°C. The spindle structure (Tub1-mCherry) and the kinetochore (Mtw1-GFP) signal in a representative *slk19*Δ cell are shown.

Figure S5. Kinetochore mutants *ydr532*Δ and *ctf3*Δ did not show obvious kinetochore clustering defect. WT, *ydr532*Δ, and *ctf3*Δ cells with *MTW1-GFP* were first arrested in G_1 phase and then released into YPD medium containing 20 μg/ml nocodazole. After incubation at 25°C for 120 min, the cells were fixed for fluorescence microscopy. The percentage of cells with different number of GFP foci is shown on the top panel after counting for more than 100 cells for each sample. The bottom panel shows the localization of Mtw1-GFP in some representative cells.

Figure S6. The kinetochore clustering defect in *slk19*Δ mutant cells is not a consequence of compromised mitotic exit pathway. G_1-arrested WT, *slk19*Δ, and *spo12*Δ mutant cells with *MTW1-GFP* were released into YPD media containing 20 μg/ml nocodazole. Cells were harvested after 120 min incubation at 25°C and the number of Mtw1-GFP foci per cell was counted using fluorescence microscopy. For each sample, more than 100 cells were counted. The percentage of cells with different GFP foci is shown on the top. Confocal microscopy was used to project representative maximum intensity images (bottom).

Figure S7. The expression of Slk19 proteins from bacteria. Left: Coomassie blue staining of SDS-PAGE gel of cleared lysates from bacteria expressing pET-20b(+) (vector only), pET-Slk19-His, pET-Slk19-HA-His, or pET-Slk19-Myc-His. Right: Coomassie blue staining of SDS-PAGE gel of purified pET-Slk19-His, pET-Slk19-HA-His, or pET-Slk19-Myc-His. The samples

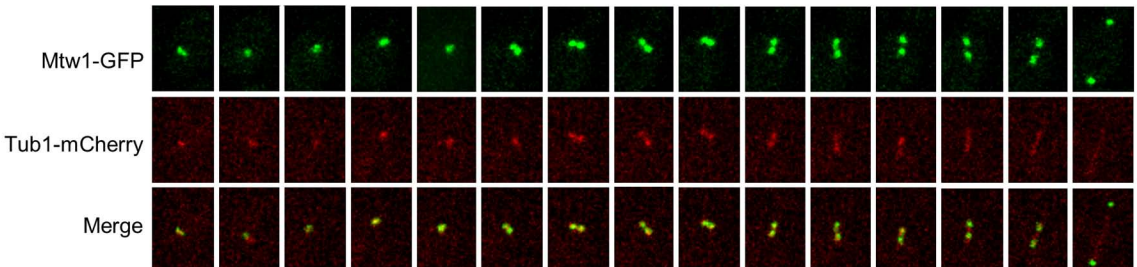
of the purified proteins were also tested by Western blot analysis using anti-HA and anti-Myc antibodies.

Figure S8. *slk19* mutants exhibit separated sister centromeres in the absence of spindle structure. **A.** *slk19*Δ mutants show separated sister centromeres. G_1-arrested WT and *slk19*Δ cells in *TUB1-mCherry* background with either *CEN4-GFP* or GFP-marked *URA3* locus (*URA3-GFP)* were released into YPD containing 20 μg/ml nocodazole for 120 min at 25°C. Confocal microscopy was used to project maximum intensity images (right panel). More than 100 cells were counted for the percentage of cells with separated GFP dots and the average of three independent experiments is shown. The scale bar represents 3μm. The arrow indicates a cell with separated *CEN4*-GFP dots. **B.** *slk19*Δ mutant cells fail to keep excised sister *CEN4*-GFP together. WT and *slk19*Δ strains with two recombination sites (RS) flanking the *CEN4-GFP* were constructed. The strains with a vector or the P_{GAL}*Recombinase (*P_{GAL}*Recom)* were grown up in raffinose at 25°C until log phase. Cells arrested in G_1 were released into medium containing galactose and 20 μg/ml nocodazole and incubated at 30°C to induce the expression of the recombinase. After 150 min, cells were harvested, fixed and the separation of *CEN4*-GFP dots was examined using fluorescence microscopy. More than 100 cells were counted for each experiment and shown is the average for three separate experiments. Confocal microscopy was used to project representative maximum intensity images (right). The arrow indicates a cell with fully separated *CEN4*-GFP dots. The scale bar = 3 μm.

Table S1: Strains used in this study

Stains	Relevant genotypes	Source
Y300	*MATa trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100*	Lab stock
Y308	*MATa slk19Δ::HIS3*	Uhlmann Lab
2588-7-4	*MATa MTW1-GFP::HIS3 TUB1-mCherry::URA3*	This study
2588-2-2	*MATa MTW1-GFP::HIS3 TUB1-mCherry::URA3 slk19Δ::HIS3*	This study
2694-2-4	*MATa SPC42-mApple::HIS3 MTW1-GFP::HIS3*	This study
2694-1-3	*MATa SPC42-mApple::HIS3 MTW1-GFP::HIS3 cdc13-1*	This study
2763-5-4	*MATa scc1-73 MTW1-GFP::HIS3 TUB1-mCherry::URA3*	This study
2003-1-4	*MATa kre28::KanMX MTW1-GFP::HIS3 bar1*	This study
2492-5-2	*MATa ctf3Δ::KanMX MTW1-GFP::HIS3 TUB1-mCherry::URA3*	This study
2669-1-1	*MATa promURA3::tetR::GFP::LEU2 CENIV::tetOX448::URA3 CENV::tetOX448::HIS3 TUB1-mCherry::URA3*	This study
2668-2-3	*MATa promURA3::tetR::GFP::LEU2 CENIV::tetOX448::URA3 CENV::tetOX448::HIS3 TUB1-mCherry::URA3 slk19Δ::HIS3*	This study
900-8-1	*MATa spo12Δ::KanMX*	Lab stock
YYW187	*MATa mad1Δ::HIS3*	Lab stock
2019-8-1	*MATa PDS1-18myc::LEU2 slk19Δ::HIS3*	Lab stock
JBY649	*MATa PDS1-18myc::LEU2*	Bachant Lab
YYW141	*MATa promURA3::tetR::GFP::LEU2 CENIV::tetOX448::URA3 TUB1-mCherry::URA3*	Lab stock
2152-13-1	*MATa promURA3::tetR::GFP::LEU2 CENIV::tetOX448::URA3 TUB1-mCherry::URA3 slk19Δ::HIS3*	This study
2257-5-2	*MATa promURA3::tetR::GFP::LEU2 ura3::tetOX448::URA3 slk19Δ::HIS3*	This study
172-1-2	*MATa promURA3::tetR::GFP::LEU2 ura3::tetOX448::URA3*	Lab stock
543-5-3	*MATa promURA3::tetR::GFP::LEU2 TELV::tetOX448::URA3*	Lab stock
YYW305	*MATa RS::HIS3* (5.6kb-*CENIV*) *RS::KAN* (6.8kb + CENIV) *promURA3::tetR::GFP::LEU2 CENIV::tetOX448::URA3* (p1217 vector)	This study
YYW308	*MATa RS::HIS3* (5.6kb-*CENIV*) *RS::KAN* (6.8kb + *CENIV*) *promURA3::tetR::GFP::LEU2 CENIV::tetOX448::URA3* (P_{GAL}*R-R-Recombinase::TRP1)*	This study
YYW306	*MATa RS::HIS3* (5.6kb -*CENIV*) *RS::KanMX* (6.8kb + *CENIV*) *promURA3::tetR::GFP::LEU2 CENIV::tetOX448::URA3 slk19Δ::HIS3* (p1217 vector)	This study
YYW307	*MATa RS::HIS3* (5.6kb-*CENIV*) *RS::KAN* (6.8kb + *CENIV*) *promURA3::tetR::GFP::LEU2 CENIV::tetOX448::URA3* (P_{GAL}*R-Recombinase::TRP1) slk19Δ::HIS3*	This study
1091-5-3	*MATa cdc13-1 promURA3::tetR::GFP::LEU2 CENIV::tetOX448::URA3 TUB1-mCherry::URA3*	Lab stock
2032-14-1	*MATa cdc13-1 promURA3::tetR::GFP::LEU2 CENIV::tetOX448::URA3 TUB1-mCherry::URA3 slk19Δ::HIS3*	This study
2083-9-3	*MATa cdc13-1 promURA3::tetR::GFP::LEU2 CENIV::tetOX448::URA3 TUB1-mCherry::URA3 sgo1Δ::KAN*	This study
2315-26-2	*MATa cdc13-1 promURA3::tetR::GFP::LEU2 CENIV::tetOX448::URA3 sgo1Δ::KanMX slk19Δ::HIS3*	This study
2650-1-3	*MATa scc1-73 promURA3::tetR::GFP::LEU2 CENV::tetOX448::HIS3 TUB1-mCherry::URA3*	This study
2281-2-4	*MATa scc1-73 promURA3::tetR::GFP::LEU2 CENV::tetOX448::HIS3 TUB1-mCherry::URA3 slk19Δ::HIS3*	This study
2847-4-1	*MATa scc1-73 promURA3::tetR::GFP::LEU2 CENV::tetOX448::HIS3 TUB1-mCherry::URA3 spo12::KanMX*	This study
YYW277-1	*MATa/α SLK19-13myc::Sphis5*$^{+}$*/SLK19*	This study
YYW314	*MATa/α SLK19-13myc::Sphis5*$^{+}$*/SLK19-6HA::URA3*	This study
934-1-4	*MATa CEN4-GFP::URA3, LEU2 NUF2-mCherry*	Lab stock
920-24-1	*MATa spo12::KAN MTW1-GFP::HIS3*	Lab stock
777-2-2	*MATa TUB1-GFP:URA3*	Lab stock
2582-4-1	*MATa TUB1-GFP:URA3 slk19Δ::HIS3*	This study
891-7-1	*MATa TUB1-GFP:URA3 spo12Δ::KanMX*	Lab stock
2660-4-3	*MATa GAL-MCD1::KanMX MTW1-GFP::HIS3 TUB1-mCherry::URA3*	This study

Richmond et al. Fig. S1



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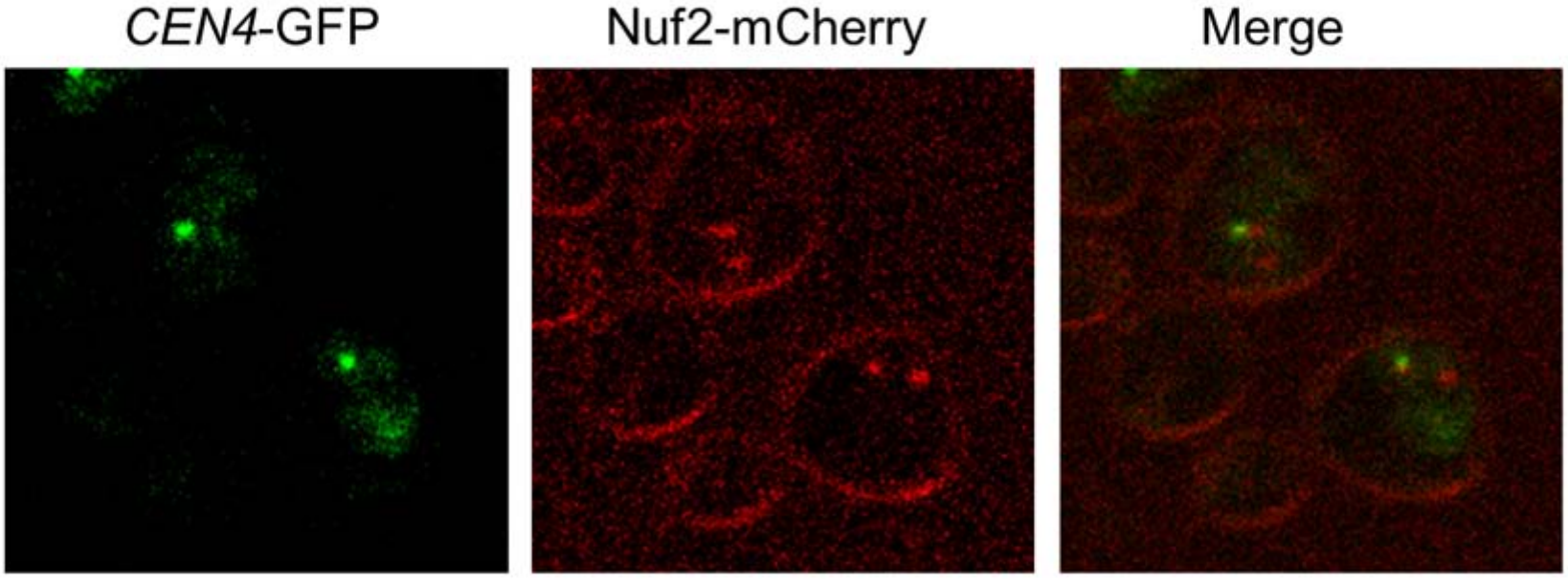

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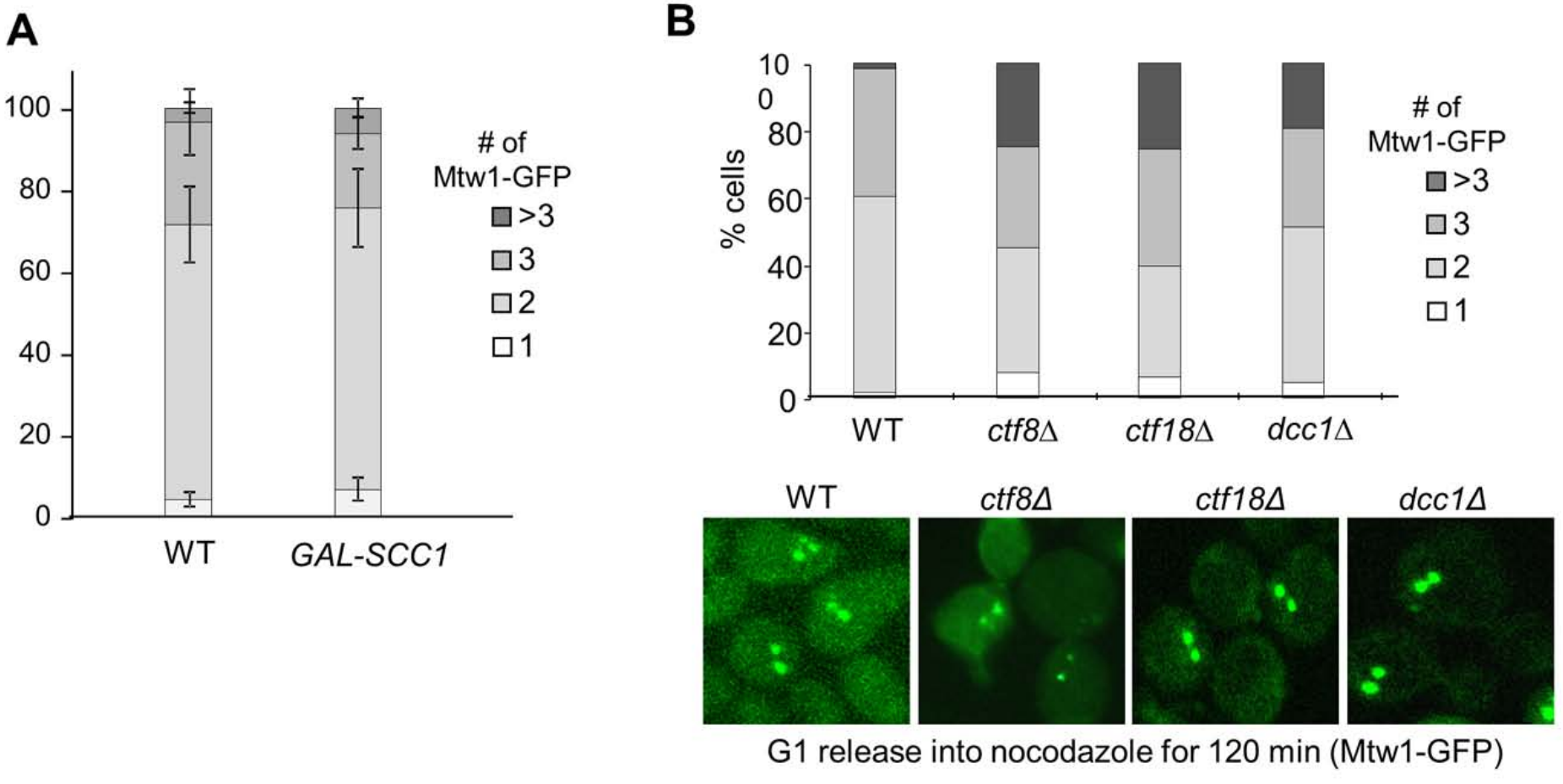

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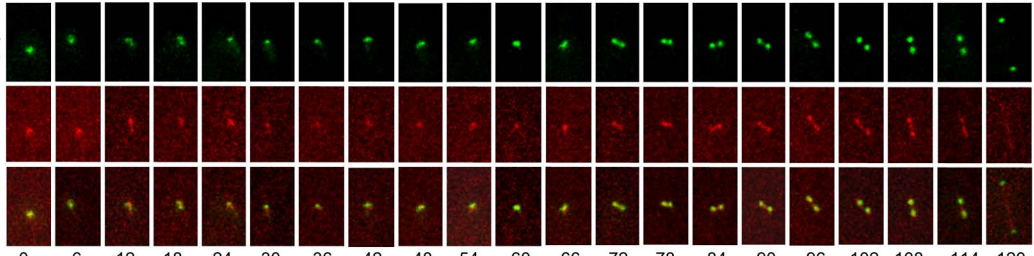

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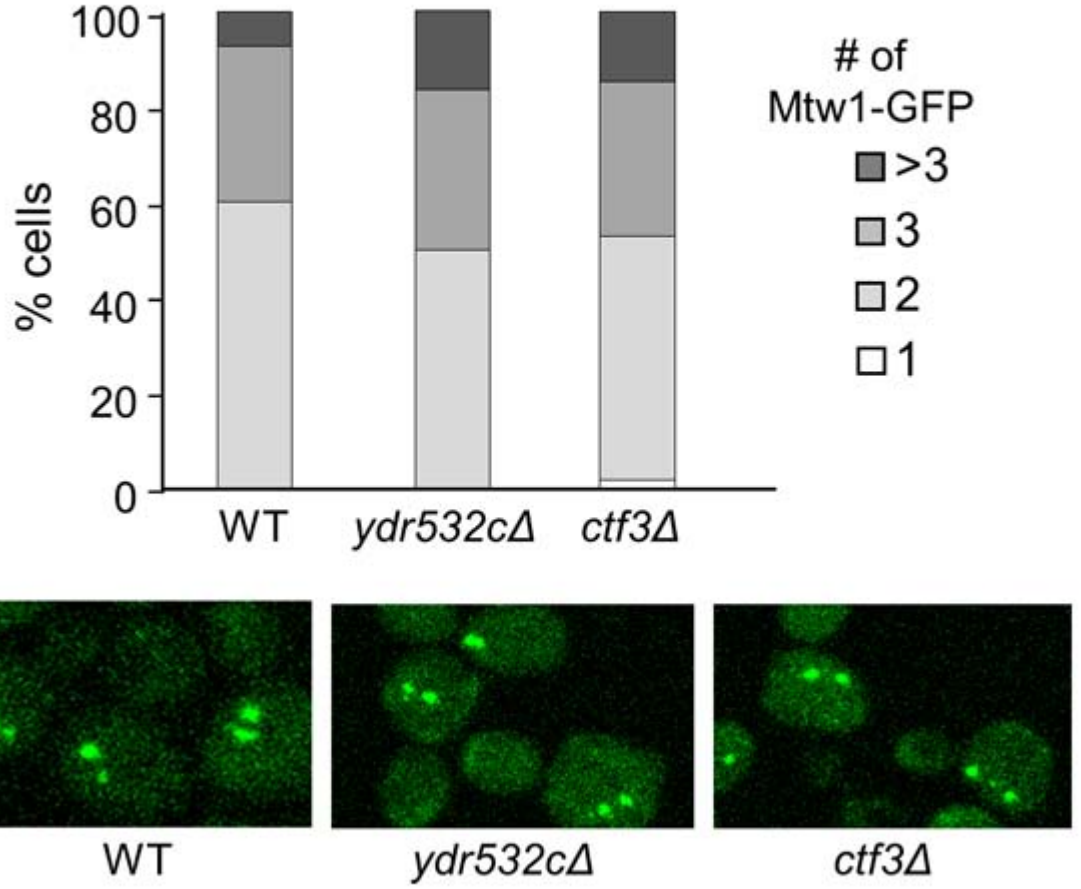
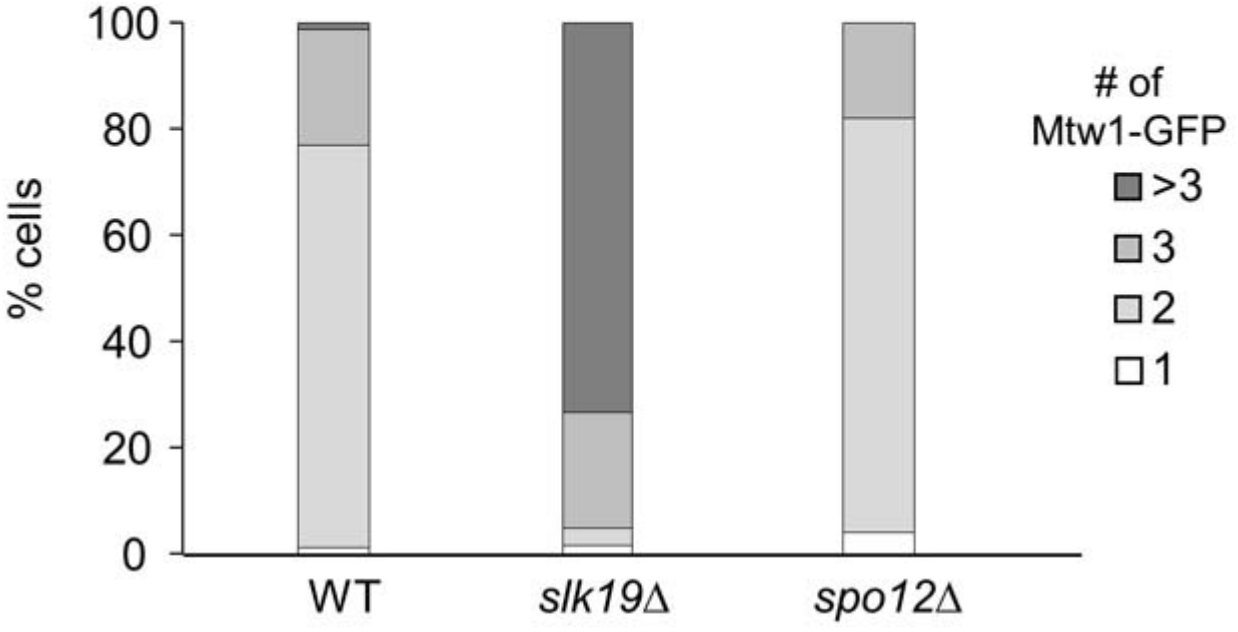
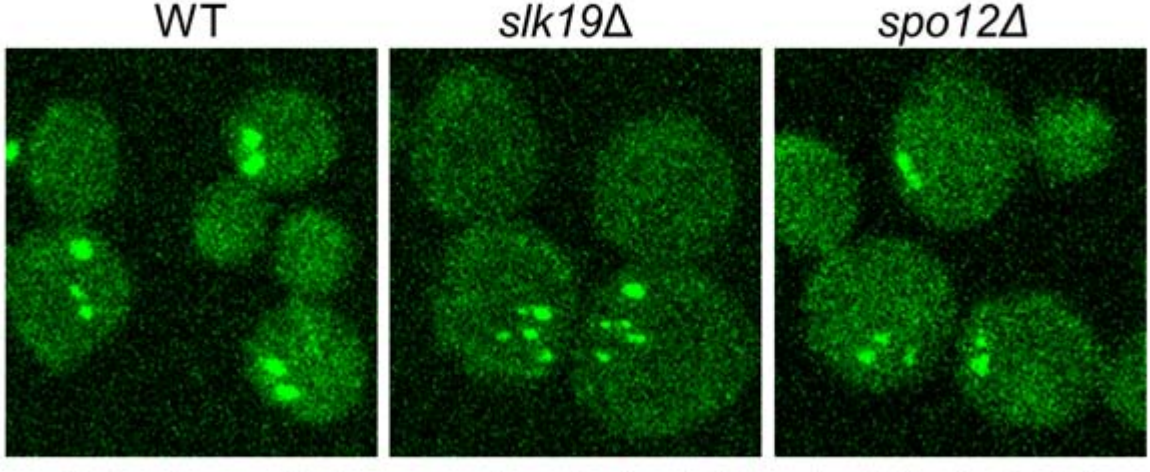


G1 release into nocodazole for 120 min (Mtw1-GFP)

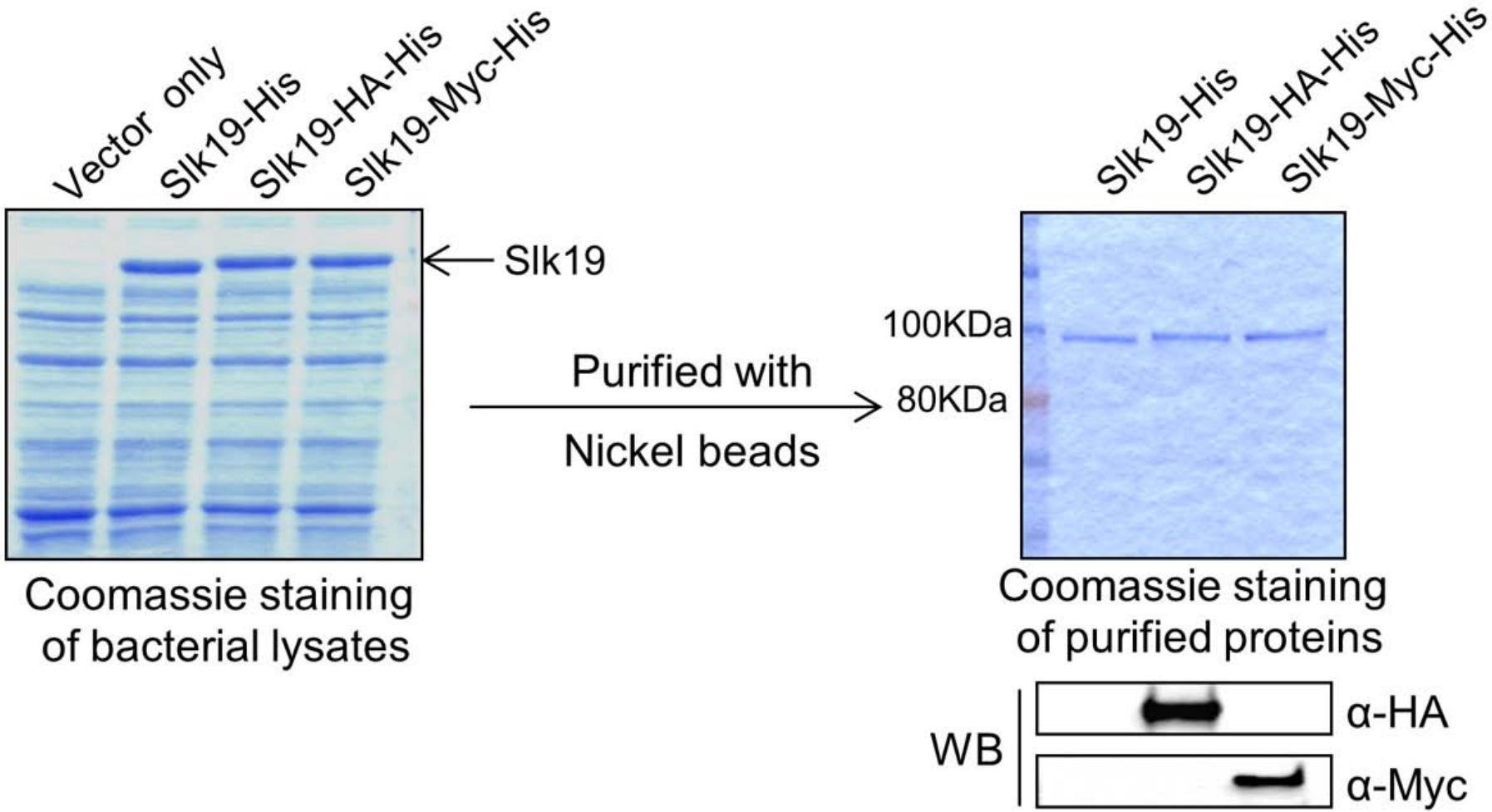
Richmond et al. Fig. S6





G1 release into nocodazole for 120 min (Mtw1-GFP)

Richmond et al. Fig. S7



Richmond et al. Fig. S8