

Fig. S1 Point-mutations introduced into Myo3

The deduced amino acid sequence of *S. pombe* Myo3 (Sp Myo3; GenBank accession no. AAC04615) was aligned with *S. pombe* Myo2 (Sp Myo2; AAC49908), the *D. discoideum* myosin-II heavy chain (Dd myosin-II; EAL64202), *H. sapiens* non-muscle myosin-IIA (MYH9) (Hs NMHCIIA; EAW60098), *H. sapiens* non-muscle myosin-IIB (MYH10) (Hs NMHCIIIB; AAI17692), *S. pombe* Myo1 (type-I myosin) (Sp Myo1; CAB46766), and *S. pombe* Myo51 (type-V myosin) (Sp Myo51; CAA21172) by CLC Sequence Viewer 7. A part of the alignment (around residues 200-800) was shown. Boxed regions indicate conserved residues into which the mutations were introduced.

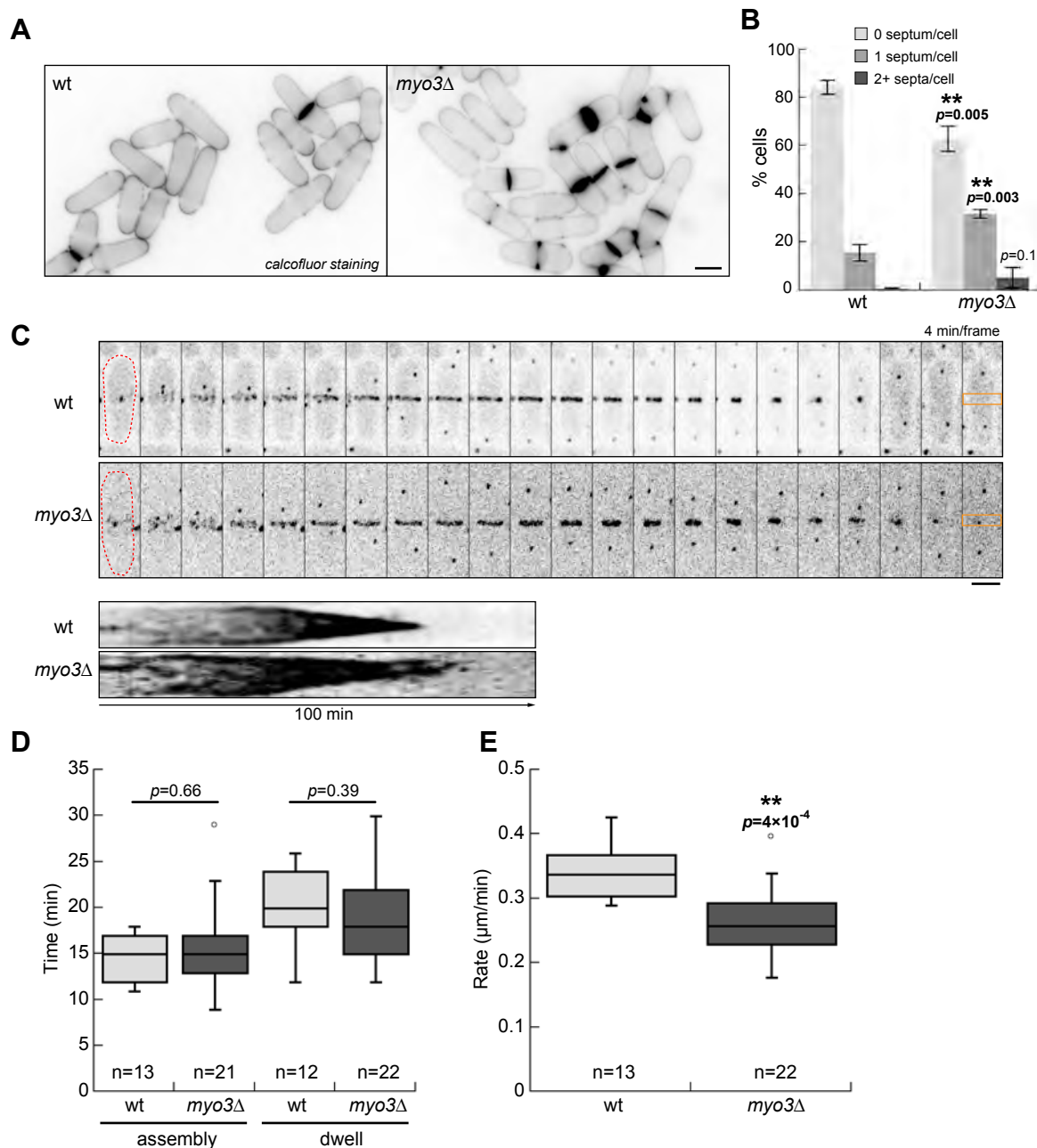


Fig. S2 Deletion of *myo3+* reduced the constriction rate of the CR and septation efficiency (A) Septa of wt and *myo3Δ* cells grown at 30°C were stained and imaged. (B) Quantification of the number of septa per cell. Data are the mean \pm SD (error bars) from three independent experiments (300-700 cells were scored at each measurement). (C) Time-lapse imaging of the assembly and constriction of Rlc1 rings in wt and *myo3Δ* cells. Bottom, kymographs of the boxed regions. (D) Box plots of the time required for the assembly of Rlc1 rings from the SPB separation and time of the dwell phase. (E) Box plot of the rate of Rlc1 ring constriction. Data in (D-E) were pooled from two independent experiments. Double asterisks indicate a significant difference ($p < 0.01$). Bars, 5 μm .

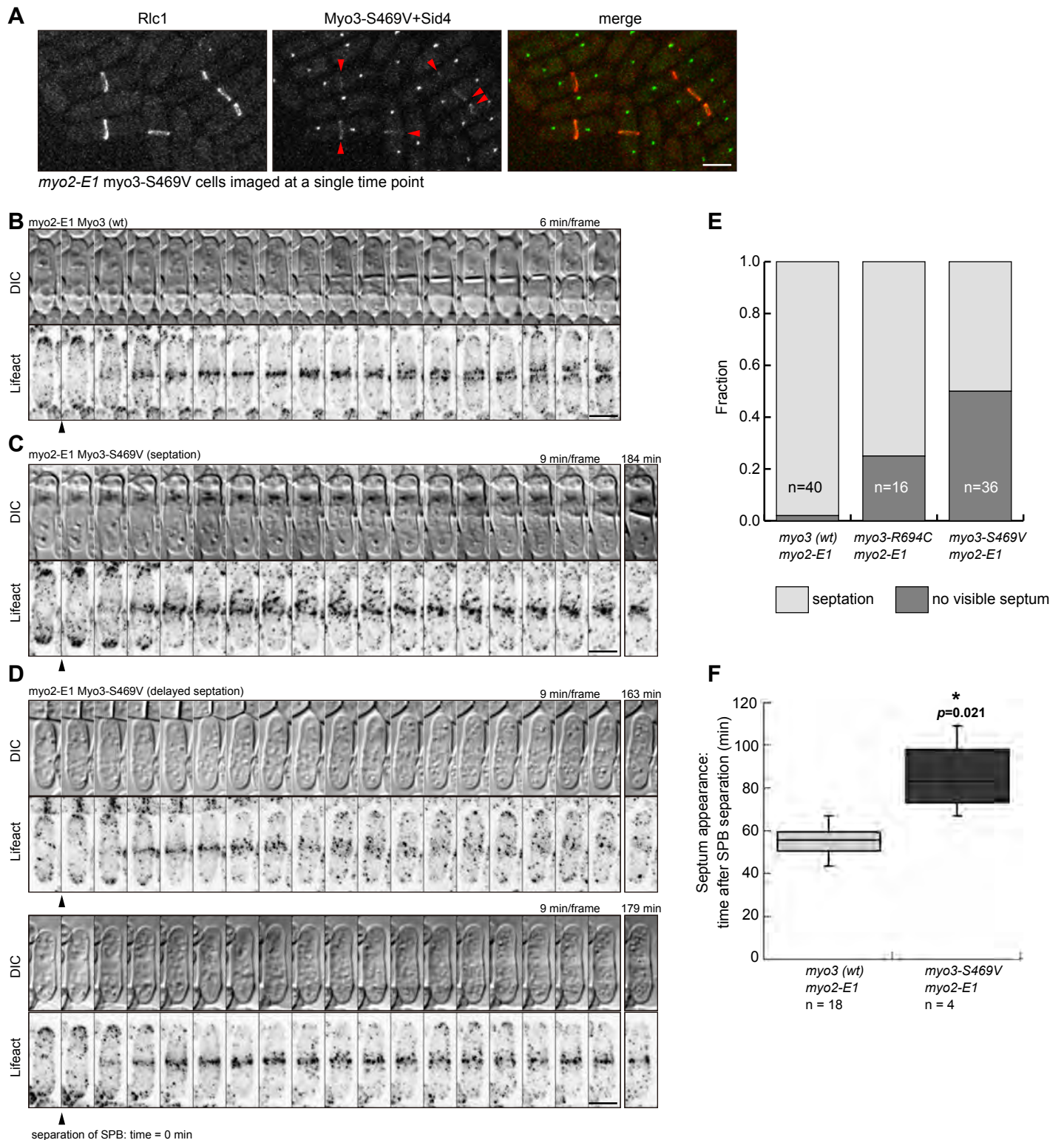


Fig. S3 Behavior of the medial actin ring in *myo2-E1 myo3-S469V* cells

(A) A stack of z-sections of *myo2-E1 rlc1-3mCherry myo3-S469V-3mYFP sid4-3mYFP* cells was acquired at a single time point using a confocal microscope (LSM 700; Carl Zeiss, Inc.). The experimental conditions were the same as those used in Figure 5, except that cells were not repeatedly scanned by lasers. Arrowheads indicate Myo3-S469V-3mYFP localizing to the CR in the *myo2-E1* background. (B-D) Observation of the medial actin ring during cytokinesis in cells expressing Myo3-3mYFP (B) or Myo3-S469V (C, D) in the *myo2-E1* background. Arrowheads indicate the timing of the SPB separation. (E) The cells of the indicated genotypes were scored according to septum formation in bright field images. These cells were observed for at least 70 (wt) or 95 (R694C and S469V) min after the SPB separation. (F) Box plot of the time from SPB separation to the appearance of a visible septum. An asterisk indicates a significant difference (vs. wt, $p < 0.05$). Data in (E-F) were pooled from two independent experiments. Bars, 5 μ m.

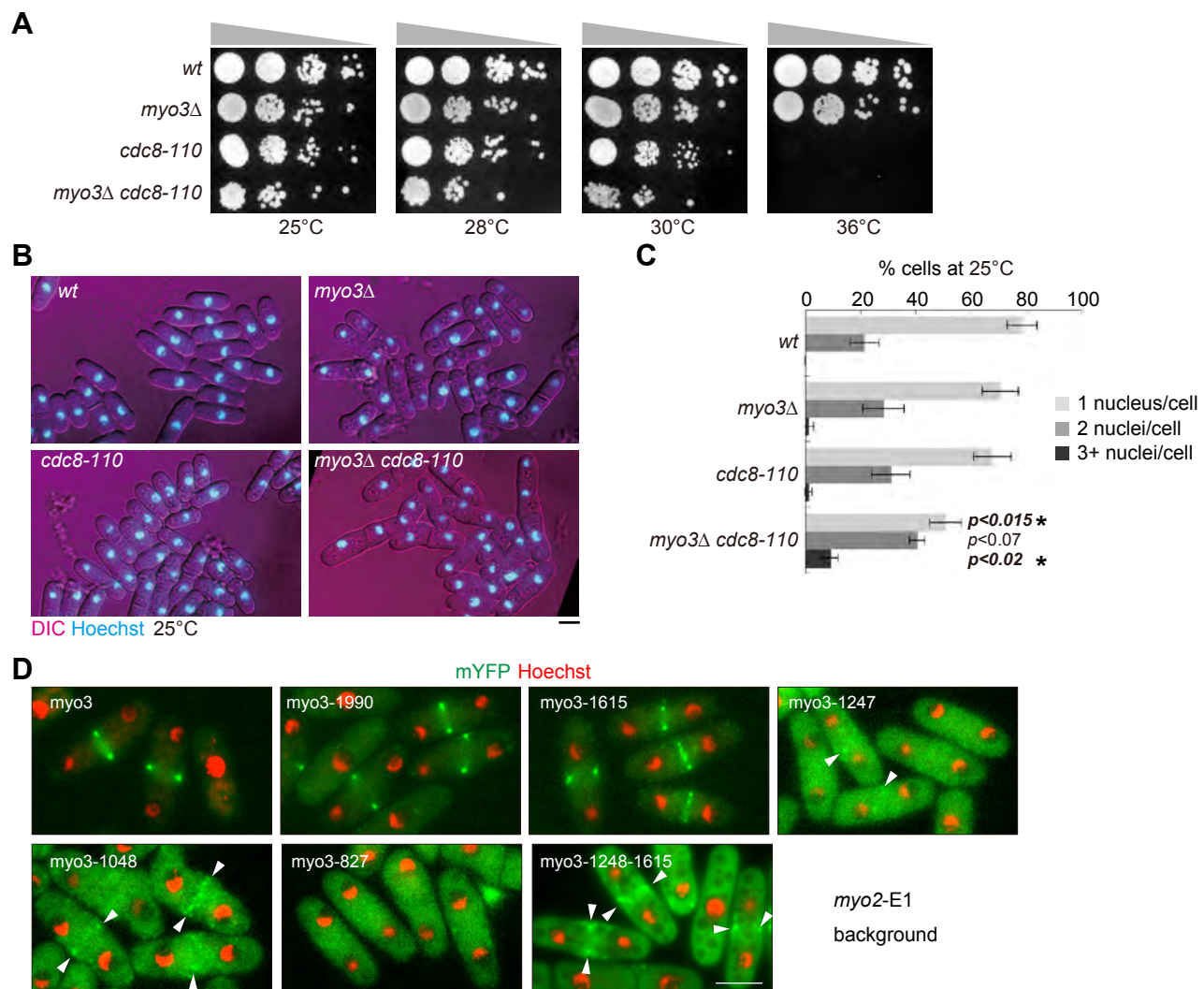


Fig. S4 Genetic interaction between *myo3Δ* and *cdc8-110*, and localization of the *myo3* deletion forms in the *myo2-E1* background

(A) Cells of the indicated genotypes were serially diluted, spotted on plates, and grown for 3 days at the indicated temperature. (B) Cells were grown at 25°C, stained with Hoechst, and then imaged in a single focal plane. (C) Quantification of the number of nuclei per cell. Data are the mean \pm SD (error bars) from three independent experiments (250–500 cells were scored at each measurement). P-values were calculated versus the three other strains, and the most stringent conditions were shown. Asterisks indicate a significant difference ($p < 0.05$). (D) Localization of the *myo3* deletion forms in the *myo2-E1* background. Cells expressing the indicated forms of Myo3-3mYFP were imaged in a single focal plane. Myo3-1245–1615-3mYFP was expressed under the control of the *nmt41* promoter in the absence of thiamine for 36 h. Arrowheads indicate the medial localization of mutant *myo3*s. Bars, 5 μ m.

Table S1. Strains used in this study

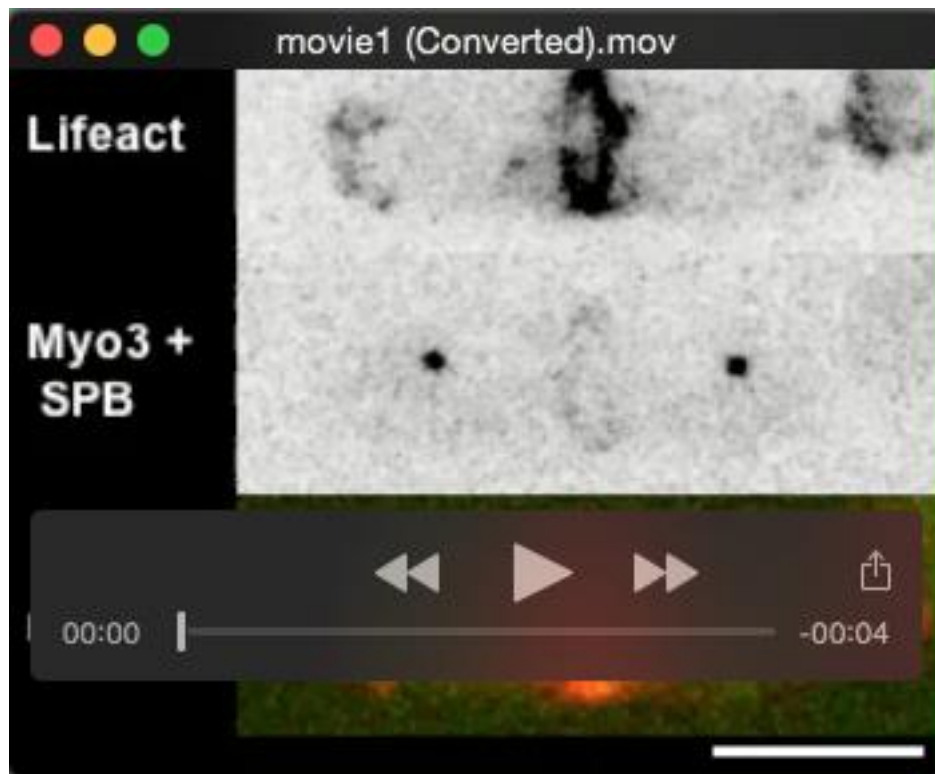
Name	Genotype	Source	Figure
MTY672	myo3 Δ head::ura4 (delete 0.4-2.4kbp)-3mYFP-3HA:hphMX6	This study	
MTY616	mYFP-Myo2 Pcdc4-lifeact-4mCherry sid4-3mYFP-3HA:hph	Lab stock	1
MTY597	myo3-3mYFP-3HA:hph sid4-3mYFP-3HA:kan Pcdc4-lifeact-4mCherry	This study	1, 2
MTY904	mid1 Δ ::ura4 myo3-3mYFP-3HA:hph sid4-3mYFP-3HA:kan Pcdc4-lifeact-4mCherry	This study	2
MTY1020	myo3-G688V-3mYFP-3HA-hphMX6 sid4-4mCherry:kanMX6	This study	3
MTY465	myo3-3mYFP-3HA:hphMX6	This study	3
MTY473	myo3-S469V-3mYFP-3HA:hphMX6	This study	3
MTY474	myo3-R694C-3mYFP-3HA:hphMX6	This study	3
MTY497	myo3-E480K-3mYFP-3HA:hphMX6	This study	3
MTY498	myo3-R240A-3mYFP-3HA:hphMX6	This study	3
MTY642	kan:Pnmt41-myo3-3mYFP-3HA:hph	This study	3
MTY685	myo3-G688V-3mYFP-3HA:hphMX6	This study	3
MTY693	myo3-G688V-3mYFP-3HA rlc1-3mCherry sid4-3mYFP-3HA	This study	3
MTY716	myo3-E480K-3mYFP-3HA sid4-3mYFP-3HA	This study	3
MTY718	myo3-R240A-3mYFP-3HA sid4-3mYFP-3HA:kan	This study	3
MTY719	myo3-G688V-3mYFP-3HA sid4-3mYFP-3HA	This study	3
MTY724	myo3-S469V-3mYFP-3HA sid4-3mYFP-3HA:kan	This study	3
MTY528	myo3-3mYFP-3HA:hphMX6 sid4-3mYFP-3HA:kan	This study	3, 7
MTY569	kan:Pnmt41-myo3tail-3mYFP-3HA:hphMX6	This study	3, 7
MTY717	myo3-R694C-3mYFP-3HA sid4-3mYFP-3HA:kan	This study	3, 7
MTY551	myo3-3mYFP-3HA:hphMX6 rlc1-3mCherry sid4-3mYFP-3HA	This study	4
MTY704	myo3-S469V-3mYFP-3HA rlc1-3mCherry sid4-3mYFP-3HA	This study	4
MTY913	myo3-R694C-3mYFP-3HA rlc1-3mCherry sid4-3mYFP-3HA	This study	4
MTY636	rlc1-3mYFP-3HA sid4-3mYFP-3HA:kan	Lab stock	6, S2
MTY684	myo3 Δ :kan rlc1-3mYFP-3HA sid4-3mYFP-3HA:kan	This study	6, S2
MTY777	myo2-E1 myo3-3mYFP-3HA rlc1-3mCherry sid4-3mYFP-3HA	This study	4, 5
MTY778	myo2-E1 myo3-R694C-3mYFP-3HA rlc1-3mCherry sid4-3mYFP-3HA	This study	4, 5
MTY780	myo2-E1 myo3-S469V-3mYFP-3HA rlc1-3mCherry sid4-3mYFP-3HA	This study	4, 5, S3
MTY982	cde8-110 rlc1-3mYFP-hphMX6 sid4-3mYFP-3HA	This study	6
MTY983	cde8-110 myo3 Δ ::kan rlc1-3mYFP-hphMX6 sid4-3mYFP-3HA	This study	6

MTY1001	myo3-1990-3mYFP-3HA:hphMX6	This study	7
MTY1002	myo3-1615-3mYFP-3HA:hphMX6	This study	7
MTY1003	myo3-1247-3mYFP-3HA:hphMX6	This study	7
MTY1004	myo3-827-3mYFP-3HA:hphMX6	This study	7
MTY1008	myo3-1048-3mYFP-3HA:hphMX6	This study	7
MTY1010	kanMX6:Pnmt41-myo3-1248-1615-3mYFP:hphMX6	This study	7
MTY838	myo2-E1 myo3-3mYFP-3HA:hph sid4-3mYFP-3HA:kan Pcdc4-lifeact-4mCherry	This study	S3
MTY839	myo2-E1 myo3-S469V-3mYFP-3HA sid4-3mYFP-3HA Pcdc4-lifeact-4mCherry:ura4	This study	S3
MTY840	myo2-E1 myo3-R694C-3mYFP-3HA sid4-3mYFP-3HA Pcdc4-lifeact-4mCherry:ura4	This study	S3
MTY10	ura4D-18 leu1-32	Lab stock	S4
MTY16	cdc8-110	Lab stock	S4
MTY78	myo3Δ::kanMX6	This study	S4
MTY88	myo3Δ::kanMX6 cdc8-110	This study	S4
MTY1078	myo2-E1 myo3-3mYFP-3HA:hphMX6	This study	S4
MTY1079	myo2-E1 myo3-1990-3mYFP-3HA:hphMX6	This study	S4
MTY1080	myo2-E1 myo3-1615-3mYFP-3HA:hphMX6	This study	S4
MTY1081	myo2-E1 myo3-1247-3mYFP-3HA:hphMX6	This study	S4
MTY1082	myo2-E1 myo3-1048-3mYFP-3HA:hphMX6	This study	S4
MTY1083	myo2-E1 myo3-827-3mYFP-3HA:hphMX6	This study	S4
MTY1084	myo2-E1 kanMX6:Pnmt41-myo3-1248-1615-3mYFP:hphMX6	This study	S4

Table S2. Plasmids used in this study

Name	Structure	Source	Purpose
MTP467	pREP81-GFP-myo3	Lab stock	
MTP461	pREP81-GFP-myo3-R240A	This study	MTY498
MTP465	pREP81-GFP-myo3-G688V	This study	MTY685
MTP466	pREP81-GFP-myo3-E480K	This study	MTY497
MTP486	pREP81-GFP-myo3-S469V	This study	MTY473
MTP487	pREP81-GFP-myo3-R694C	This study	MTY474

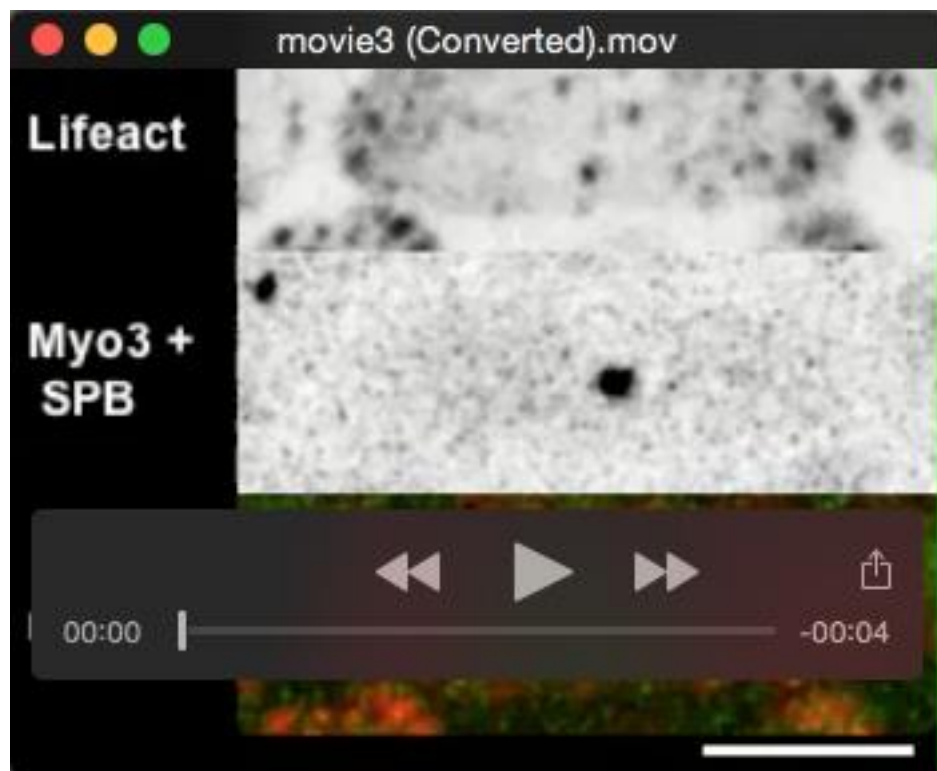
Movies



Movie 1. Myo3 and actin rings in DMSO-treated cells. Related to Figure 1C. Strain: MTY597 (*myo3-3mYFP sid4-3mYFP Pcdc4-lifeact-4mCherry*). Time-lapse imaging of Myo3-3mYFP and actin rings using an inverted fluorescence microscope (BX71; Olympus) equipped with a UPlanSApo 100 \times /1.40 NA objective lens (Olympus), spinning-disk confocal scanner (CSU22; Yokogawa), piezo objective positioner (E-665; Physik Instrumente), electron multiplying CCD camera (iXon 3 885; Andor), and emission beam splitter (Dual View; Roper Scientific) with excitation by 488-nm and 568-nm lasers using GFP and mCherry filters and MetaMorph software (version 7.7.5.0; Molecular Devices). Myo3, Lifeact, and SPBs in late anaphase cells were simultaneously imaged at 1-min intervals. A total of 0.36% DMSO was supplemented at $t = 00:00$. Stacks of 12 confocal z -sections spaced by 0.45 μm were collected every minute for 30 min, and projected to an xy image using a maximum intensity projection. Green, Myo3-3mYFP and Sid4-3mYFP; red, Lifeact-4mCherry. Bar, 5 μm .



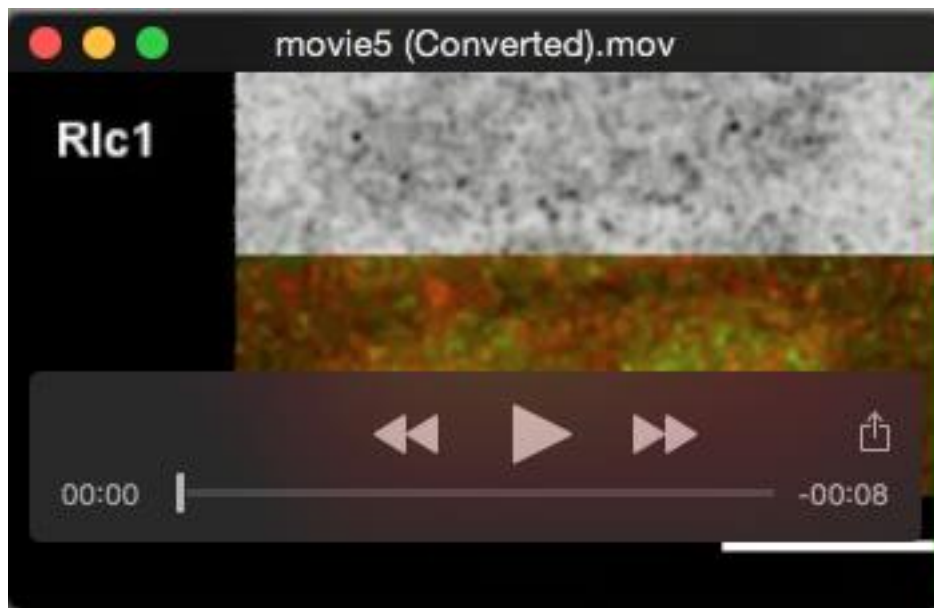
Movie 2. Myo3 and actin rings in Lat-A-treated cells. Related to Figure 1D. Strain: MTY597 (*myo3-3mYFP sid4-3mYFP Pcdc4-lifeact-4mCherry*). Time-lapse imaging of Myo3-3mYFP and actin rings using an inverted fluorescence microscope (BX71; Olympus) equipped with a UPlanSApo 100 \times /1.40 NA objective lens (Olympus), spinning-disk confocal scanner (CSU22; Yokogawa), piezo objective positioner (E-665; Physik Instrumente), electron multiplying CCD camera (iXon 3 885; Andor), and emission beam splitter (Dual View; Roper Scientific) with excitation by 488-nm and 568-nm lasers using GFP and mCherry filters and MetaMorph software (version 7.7.5.0; Molecular Devices). Myo3, Lifeact, and SPBs in late anaphase cells were simultaneously imaged at 1-min intervals. A total of 0.36% DMSO plus 7 μ M Lat-A was supplemented at $t = 00:00$. Stacks of 12 confocal z -sections spaced by 0.45 μ m were collected every minute for 30 min, and projected to an xy image using a maximum intensity projection. Green, Myo3-3mYFP and Sid4-3mYFP; red, Lifeact-4mCherry. Bar, 5 μ m.



Movie 3. Assembly of Myo3 and actin rings. Related to Figure 2A. Strain: MTY597 (*myo3-3mYFP sid4-3mYFP Pcdc4-lifeact-4mCherry*). Time-lapse imaging of Myo3-3mYFP and actin rings using a confocal microscope (LSM 700; Carl Zeiss, Inc.) equipped with an alpha Plan-Apochromat 100×/1.46 NA objective lens (Carl Zeiss, Inc.). The duplicated SPBs separated at $t = 00:00$. Stacks of 12 confocal z -sections spaced by $0.62\ \mu\text{m}$ were collected every 2 minutes for 60 min, and projected to an xy image using a maximum intensity projection. Green, Myo3-3mYFP and Sid4-3mYFP; red, Lifeact-4mCherry. Bar, $5\ \mu\text{m}$.



Movie 4. Assembly of Myo3 and Rlc1 rings. Related to Figures 4A-B. Strains: MTY551 (*myo3-3mYFP rlc1-3mCherry sid4-3mYFP*) (left column) and MTY913 (*myo3-R694C-3mYFP rlc1-3mCherry sid4-3mYFP*) (right column). Time-lapse imaging of Myo3 and Rlc1 rings using a confocal microscope (LSM 700; Carl Zeiss, Inc.) equipped with an alpha Plan-Apochromat 100 \times /1.46 NA objective lens (Carl Zeiss, Inc.). The duplicated SPBs separated at $t = 00:00$. Stacks of 13 confocal z -sections spaced by 0.62 μm were collected every 2 minutes for 90 min, and projected to an xy image using a maximum intensity projection. Green, Myo3-3mYFP and Sid4-3mYFP; red, Rlc1-3mCherry. Bar, 5 μm .



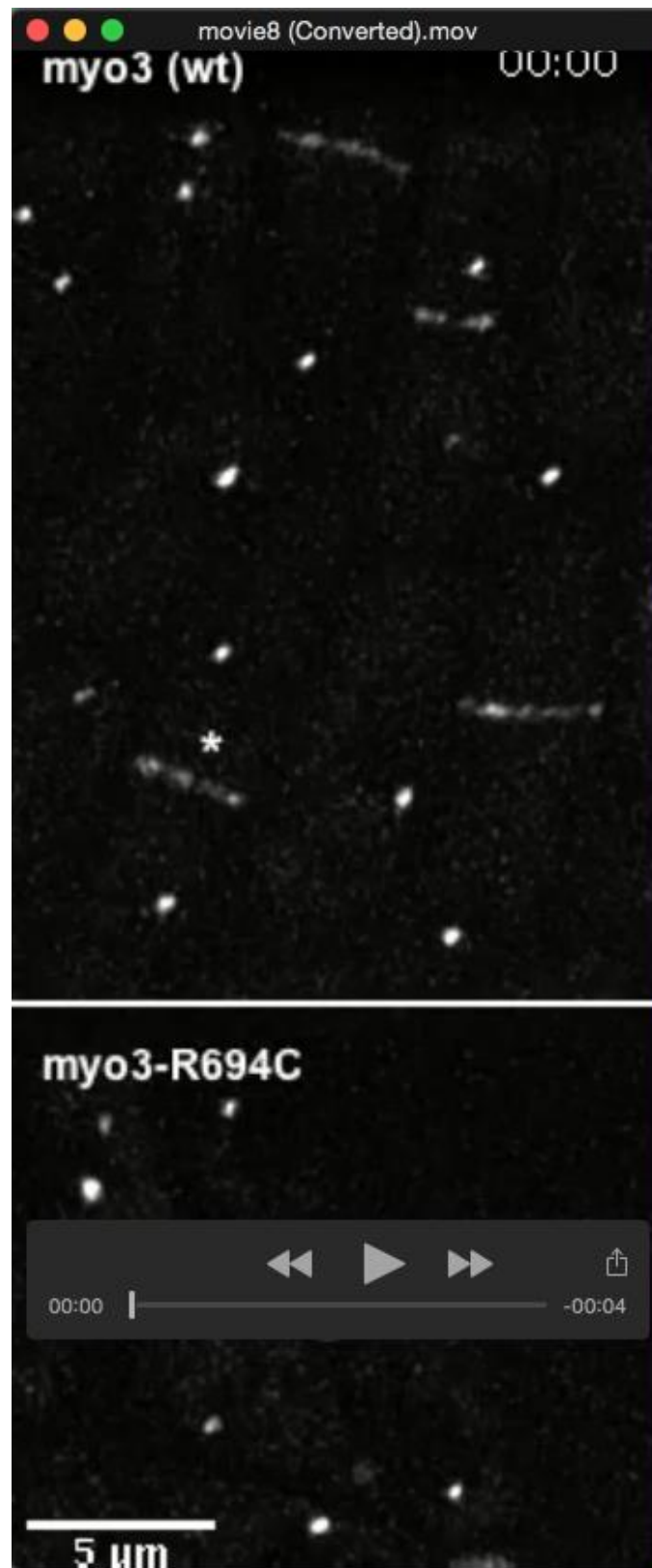
Movie 5. Constriction of the Rlc1 ring in the *myo2-E1 myo3-S469V* background. Related to the upper panel of Figure 5C. Strain: MTY780 (*myo2-E1 myo3-S469V-3mYFP rlc1-3mCherry sid4-3mYFP*). Time-lapse imaging of Myo3-S469V and Rlc1 rings using a confocal microscope (LSM 700; Carl Zeiss, Inc.) equipped with an alpha Plan-Apochromat 100×/1.46 NA objective lens (Carl Zeiss, Inc.). The duplicated SPBs separated at $t = 00:00$. Stacks of 13 confocal z-sections spaced by 0.62 μm were collected every 3 minutes for 180 min, and projected to an *xy* image using a maximum intensity projection. Green, Myo3-S469V-3mYFP and Sid4-3mYFP; red, Rlc1-3mCherry. Bar, 5 μm .



Movie 6. Collapse of the Rlc1 ring in the *myo2-E1 myo3-S469V* background. Related to the middle and bottom panels of Figure 5C. Strain: MTY780 (*myo2-E1 myo3-S469V-3mYFP rlc1-3mCherry sid4-3mYFP*). Time-lapse imaging of Myo3-S469V and Rlc1 rings using a confocal microscope (LSM 700; Carl Zeiss, Inc.) equipped with an alpha Plan-Apochromat 100×/1.46 NA objective lens (Carl Zeiss, Inc.). Left and right columns correspond to the middle and bottom panels shown in Figure 5C, respectively. Stacks of 13 confocal *z*-sections spaced by 0.62 μm were collected every 3 minutes for 180 min, and projected to an *xy* image using a maximum intensity projection. Green, Myo3-S469V-3mYFP and Sid4-3mYFP; red, Rlc1-3mCherry. Bar, 5 μm .



Movie 7. Behavior of Rlc1 rings in *myo3Δ cdc8-110* cells. Related to Figure 6B. Strain: MTY983 (*cdc8-110 myo3Δ rlc1-3mYFP sid4-3mYFP*). Time-lapse imaging of the Rlc1 ring and SPBs using an inverted fluorescence microscope (BX71; Olympus) equipped with a UPlanSApo 100×/1.40 NA objective lens (Olympus), a spinning-disk confocal scanner (CSU22; Yokogawa), piezo objective positioner (E-665; Physik Instrumente), and electron multiplying CCD camera (iXon 3 885; Andor) with excitation by a 488-nm laser using a GFP filter and MetaMorph software (version 7.7.5.0; Molecular Devices). An asterisk indicates the cell shown in the bottom panel of Figure 6B. Stacks of 13 confocal z-sections spaced by 0.60 μm were collected every 2 minutes for 240 min, and projected to an *xy* image using a maximum intensity projection. Bar, 5 μm .



Movie 8. Motile Myo3 clusters on the contractile ring. Related to Figure 7D. Strains: MTY528 (*myo3-3mYFP sid4-3mYFP*) and MTY717 (*myo3-R694C-3mYFP sid4-3mYFP*).

Time-lapse imaging of the Myo3 ring and SPBs using an inverted fluorescence microscope (BX71; Olympus) equipped with a UPlanSApo 100×/1.40 NA objective lens (Olympus), spinning-disk confocal scanner (CSU22; Yokogawa), piezo objective positioner (E-665; Physik Instrumente), and electron multiplying CCD camera (iXon 3 885; Andor) with excitation by a 488-nm laser using a GFP filter and MetaMorph software (version 7.7.5.0; Molecular Devices). Asterisks indicate the cells shown in Figure 7D (the bottom panel for wt). Stacks of 9 confocal *z*-sections spaced by 0.31 μm (corresponding to the lower half of the contractile ring) were collected every 15 s for 8 min. The stacked images were deconvolved using Huygens Essential software (version 4.5; Scientific Volume Imaging) based on the classic maximum likelihood estimation method, and projected to an *xy* image using a maximum intensity projection. Bar, 5 μm .