Supplemental material

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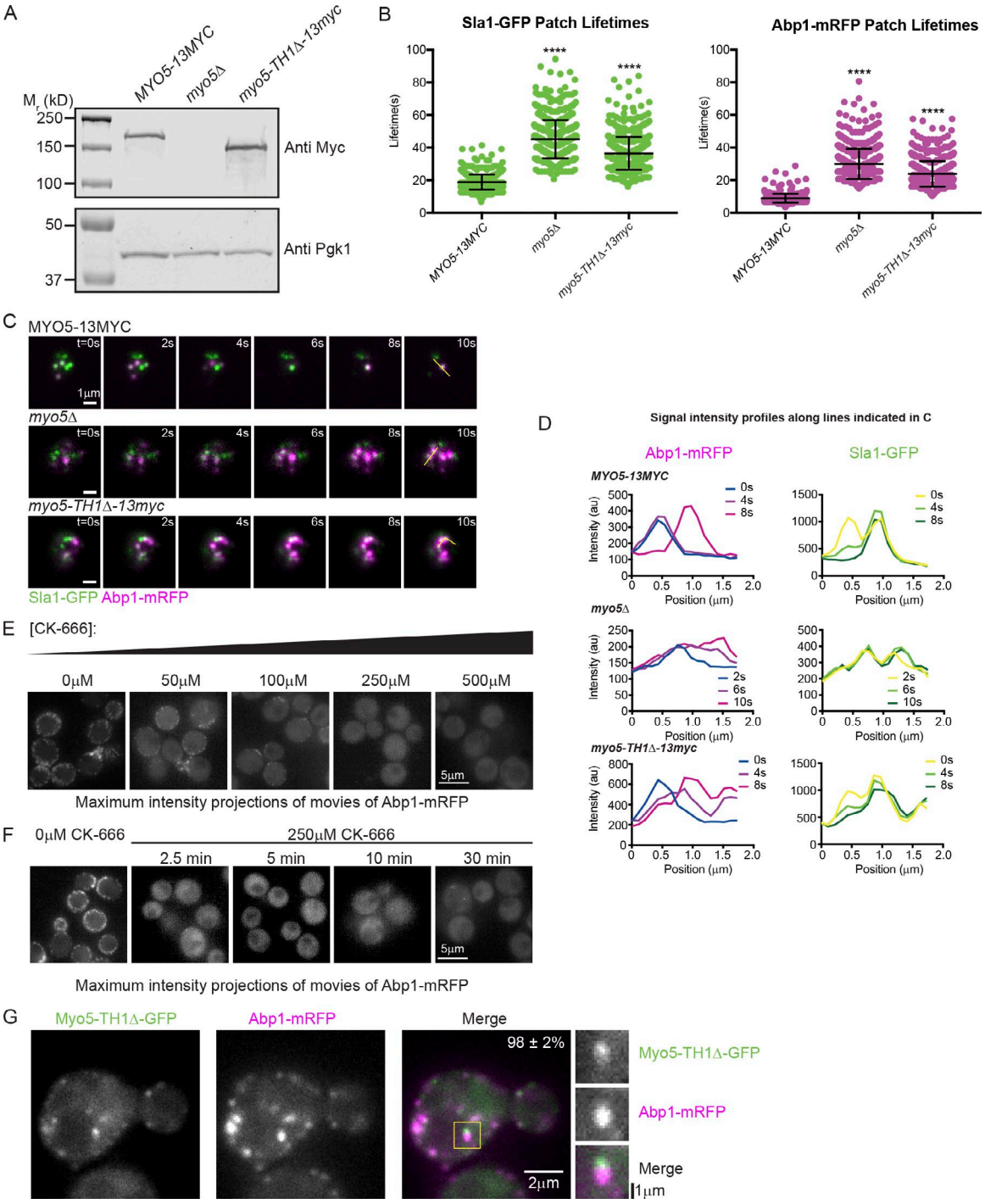


Figure S1. **Membrane-bound Myo5 restricts actin assembly and actin assembly factors to endocytic sites.** Related to Figs. 1 and 2. **(A)** Immunoblot of *MYO5-13Myc*, *myo5Δ*, and *myo5-TH1Δ-13Myc* strains used in this study. Myo5 variants were recognized via a 13Myc epitope tag with the 9E10 anti myc antibody. Pgk1 was probed as a loading control. **(B)** Sla1-GFP and Abp1-mRFP patch lifetimes for each indicated genotype. *n* = 587 (*MYO5*), 600 (*myo5Δ*), and 596 (*myo5-TH1Δ*) patches measured. Asterisks denote statistical significance compared with *MYO5* as in Fig. 1 C (F_{Sla1} = 1,212 and F_{Abp1} = 1,342). Error bars are SD. **(C)** Additional montages from TIRFM videos of cells of the indicated genotypes endogenously expressing Sla1-GFP (green) and Abp1-mRFP (magenta). Yellow lines indicate selections used for intensity profiles in D. See Video 2. **(D)** Abp1-mRFP fluorescence intensity profiles from the indicated time points along the line indicated in C. **(E)** Effects of increasing concentrations of CK-666 on assembly of endocytic actin networks, labeled by Abp1-mRFP in wild-type *MYO5* cells. Cells were treated with the indicated concentration of CK-666 for 45 min. **(F)** Effects of increasing CK-666 treatment times on assembly of endocytic actin networks, labeled by Abp1-mRFP in wild-type *MYO5* cells. **(G)** Still from an epifluorescence video of a *myo5-TH1Δ-GFP* (green) cell endogenously expressing Abp1-mRFP (magenta). The yellow box indicates an Abp1-mRFP comet with Myo5-TH1Δ-GFP at the tip. This selection is enlarged twofold and shown in grayscale at right. The percentage shown is the proportion of Abp1-mRFP comets with associated GFP signal ± SD; *n* = 153 total comets observed. See Video 9. ****, P < 0.0001. All cells are *myo3Δ*.

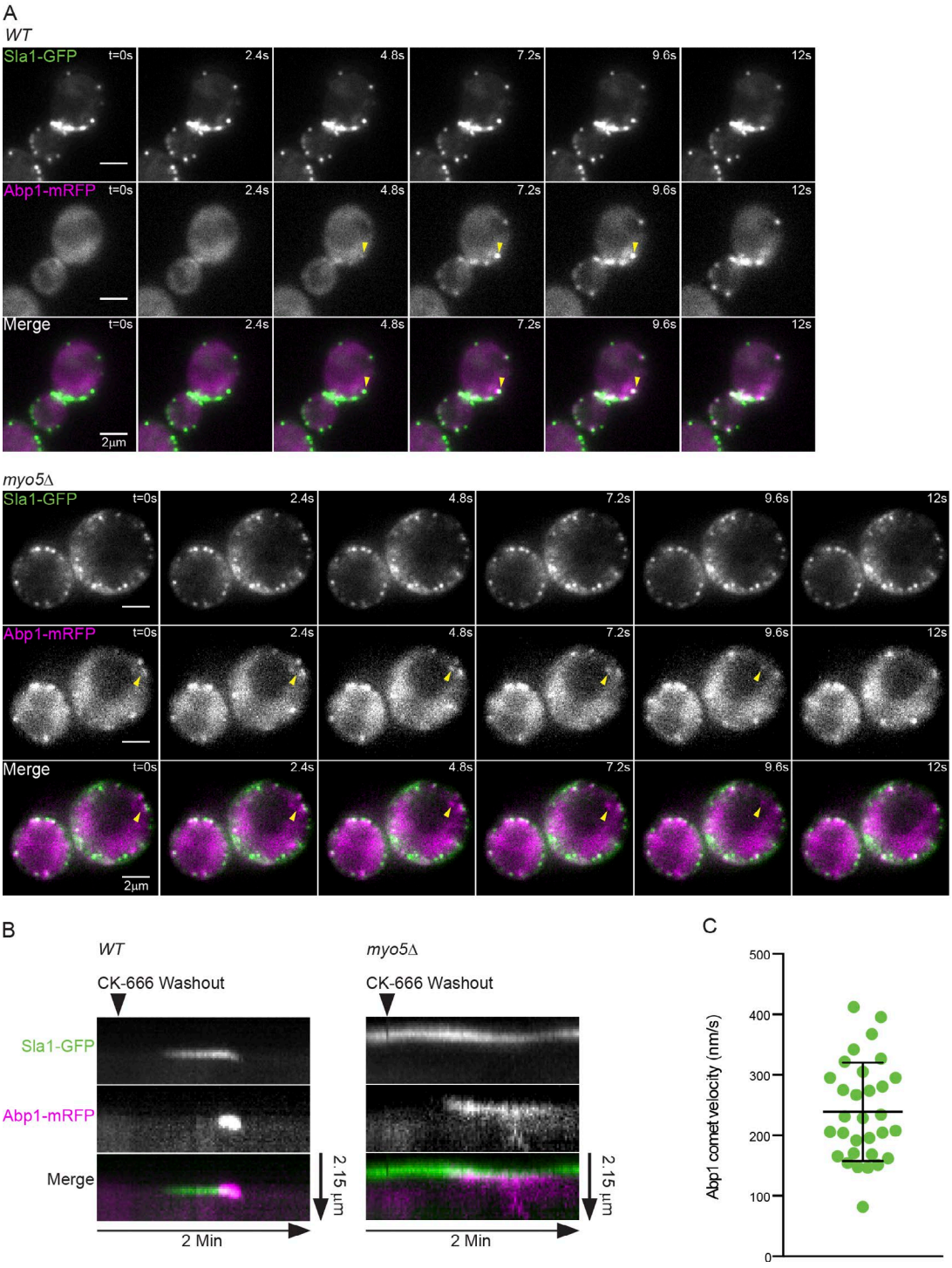


Figure S2. **Myo5 couples the assembling actin network to the PM during CME.** Related to Fig. 3. **(A)** Montages from epifluorescence videos of cells of the indicated genotypes endogenously expressing Sla1-GFP (green) and Abp1-mRFP (magenta) after washout of CK-666. Yellow arrowheads mark endocytic actin assembly events. **(B)** Kymographs of additional individual endocytic events after washout of CK-666 from the videos shown in A. **(C)** Quantification of the velocity of Abp1-mRFP actin comets as they leave the PM after washout of CK-666 in *myo5Δ* cells. $n = 31$ comets tracked from 28 cells. Error bars are SD. See Video 10. All cells are *myo3Δ*.

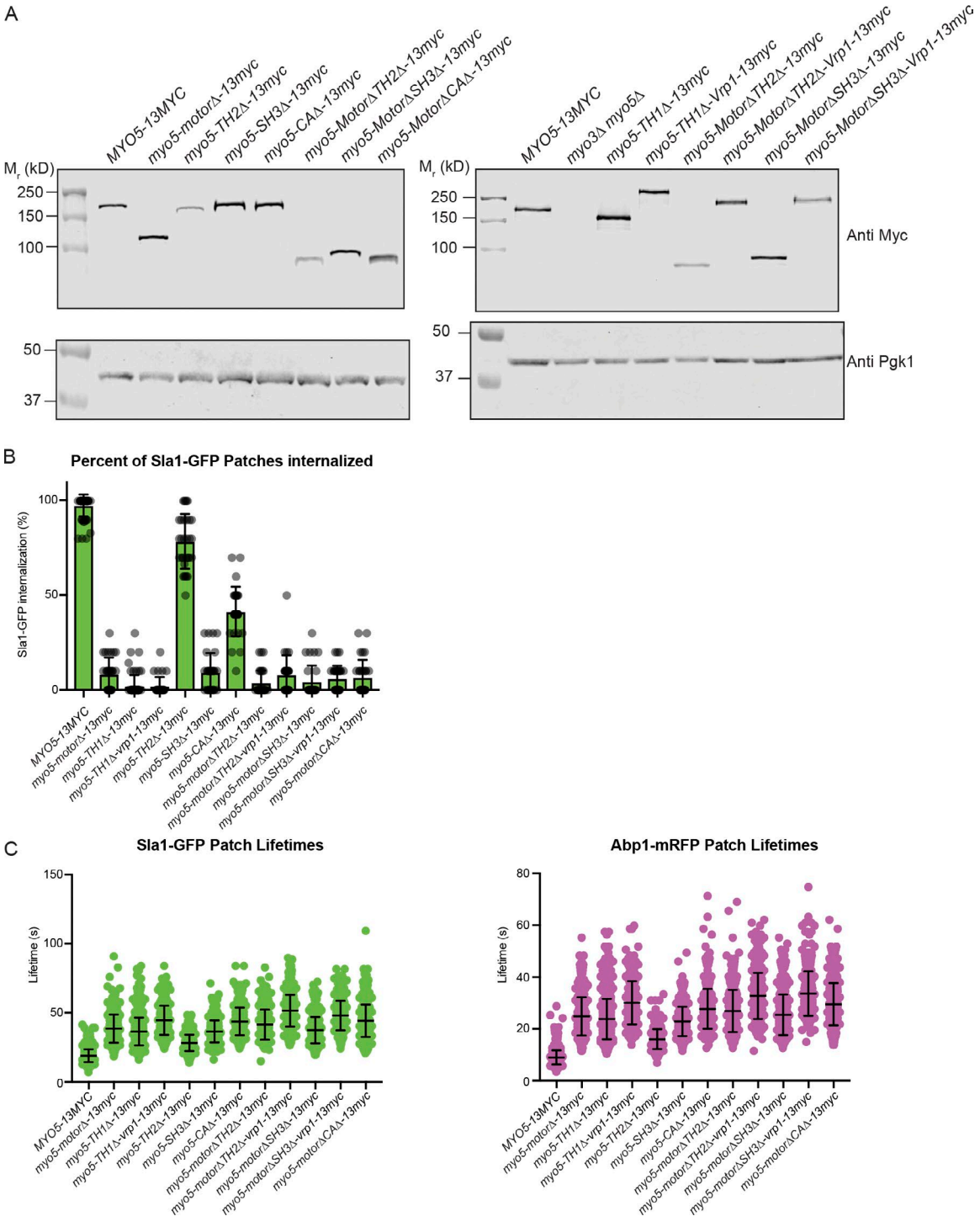
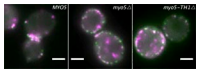
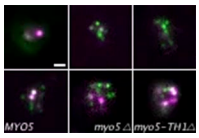


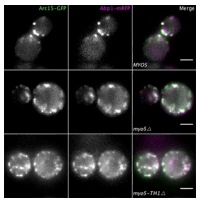
Figure S3. **Molecular determinants of myosin-mediated coupling of actin assembly to endocytic sites.** Related to Fig. 4. **(A)** Immunoblot of *MYO5* truncation mutant strains used in this study. Myo5 variants were recognized via a 13Myc epitope tag with the 9E10 anti myc antibody. Pgk1 was probed as a loading control. **(B)** Quantification of the proportion of Sla1-GFP vesicles internalized in individual cells of each indicated genotype during a 2-min video. Data for *MYO5* and *myo5-TH1Δ* are repeated from Fig. 1 D. *n* = 61 (*MYO5*), 65 (*myo5-TH1Δ*), 31 (*myo5-TH2Δ*), and 30 cells for all other variants. All variants are statistically significantly different (P < 0.0001) compared with *MYO5* by one-way ANOVA (F = 641.2) followed by Dunnett's test. **(C)** Sla1-GFP and Abp1-mRFP patch lifetimes for each indicated genotype. Data for *MYO5* and *myo5-TH1Δ* are repeated from Fig. S1 B. *n* = 587 (*MYO5*), 596 (*myo5-TH1Δ*), 306 (*myo5-TH2Δ*), and 300 patches measured for all other variants. All variants are statistically significantly different (P < 0.0001) compared with *MYO5* by one-way ANOVA (F_{Sla1} = 373.8 and F_{Abp1} = 409.6) followed by Dunnett's test. All cells analyzed were *myo3Δ*.



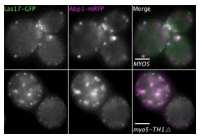
Video 1. **Epifluorescence videos of *MYO5-13Myc*, *myo5Δ*, and *myo5-TH1Δ-13Myc* cells endogenously expressing Sla1-GFP (green) and Abp1-mRFP (magenta).** Related to Fig. 1 (A–D). Motile cytoplasmic actin comets form in the absence of membrane-bound Myo5. Frames are separated by 1.15 s and played back at 15 frames per second (fps). Scale bars are 2 µm. All cells are *myo3Δ*.



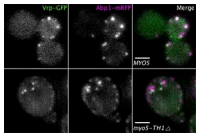
Video 2. **TIRFM videos of *MYO5-13Myc*, *myo5Δ*, and *myo5-TH1Δ-13Myc* cells endogenously expressing Sla1-GFP (green) and Abp1-mRFP (magenta).** Related to Fig. 1 (E and F) and Fig. S1 (C and D). Actin waves spread from CME sites across the PM in the absence of membrane-bound Myo5. Frames are separated by 1 s and played back at 15 fps. Scale bar is 1 µm. All cells are *myo3Δ*.



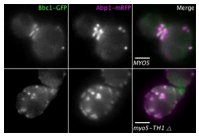
Video 3. **Epifluorescence videos of *MYO5-13Myc*, *myo5Δ*, and *myo5-TH1Δ-13Myc* cells endogenously expressing Arc15-GFP (green) and Abp1-mRFP (magenta).** Related to Figs. 2 A and 3A. Yellow arrowheads mark cytoplasmic actin comets emerging from the PM in mutants. The Arp2/3 complex colocalizes with cytoplasmic actin comets in *myo5* membrane-binding mutants. Frames are separated by 2 s and played back at 15 fps. Scale bars are 2 µm. All cells are *myo3Δ*.



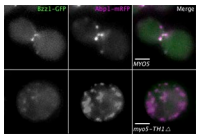
Video 4. **Epifluorescence videos of *MYO5-13Myc* and *myo5-TH1Δ-13Myc* cells endogenously expressing Abp1-mRFP (magenta) and Las17-GFP (green).** Related to Fig. 2 D. Individual channels are shown in grayscale at left. Las17 localizes to the tips of cytoplasmic actin comets in *myo5* membrane-binding mutants. Frames are separated by 1.15 s and played back at 15 fps. Scale bars are 2 µm. All cells are *myo3Δ*.



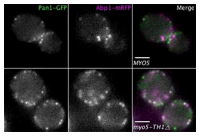
Video 5. **Epifluorescence videos of *MYO5-13Myc* and *myo5-TH1Δ-13Myc* cells endogenously expressing Abp1-mRFP (magenta) and Vrp1-GFP (green).** Related to Fig. 2 D. Individual channels are shown in grayscale at left. Vrp1 localizes to the tips of cytoplasmic actin comets in *myo5* membrane-binding mutants. Frames are separated by 2 s and played back at 15 fps. Scale bars are 2 µm. All cells are *myo3Δ*.



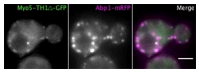
Video 6. **Epifluorescence videos of *MYO5-13Myc* and *myo5-TH1Δ-13Myc* cells endogenously expressing Abp1-mRFP (magenta) and Bbc1-GFP (green).** Related to Fig. 2 D. Individual channels are shown in grayscale at left. Bbc1 localizes to the tips of cytoplasmic actin comets in *myo5* membrane-binding mutants. Frames are separated by 1.15 s and played back at 15 fps. Scale bars are 2 µm. All cells are *myo3Δ*.



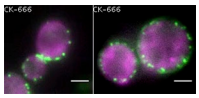
Video 7. **Epifluorescence videos of *MYO5-13Myc* and *myo5-TH1Δ-13Myc* cells endogenously expressing Abp1-mRFP (magenta) and Bzz1-GFP (green).** Related to Fig. 2 D. Individual channels are shown in grayscale at left. Bzz1 localizes to the tips of cytoplasmic actin comets in *myo5* membrane-binding mutants. Frames are separated by 1.15 s and played back at 15 fps. Scale bars are 2 µm. All cells are *myo3Δ*.



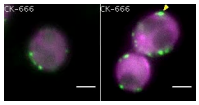
Video 8. **Epifluorescence videos of *MYO5-13Myc* and *myo5-TH1Δ-13Myc* cells endogenously expressing Abp1-mRFP (magenta) and Pan1-GFP (green).** Related to Fig. 2 D. Individual channels are shown in grayscale at left. Pan1 does not localize to the tips of cytoplasmic actin comets in *myo5* membrane-binding mutants. Frames are separated by 2 s and played back at 15 fps. Scale bars are 2 µm. All cells are *myo3Δ*.



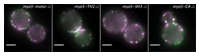
Video 9. **Epifluorescence video of a cell endogenously expressing Abp1-mRFP (magenta) and Myo5-TH1Δ-GFP (green).** Related to Fig. S1 G. Individual channels are shown in grayscale at left. Myo5-TH1Δ localizes to the tips of cytoplasmic actin comets. Frames are separated by 1.15 s and played back at 15 fps. Scale bars are 2 µm. The cell is *myo3Δ*.



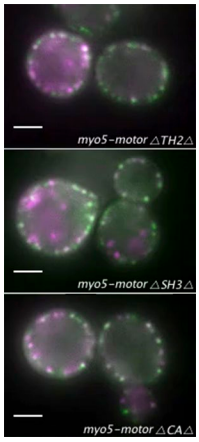
Video 10. **Epifluorescence videos of *MYO5 and myo5Δ* cells endogenously expressing Sla1-GFP (green) and Abp1-mRFP (magenta) after washout of CK-666 (time of washout indicated by text in the upper left).** Related to Fig. 3 D and Fig. S2. Yellow arrowheads mark endocytic actin assembly events. Actin flares internalize from the PM in *myo5* membrane-binding mutants without coincident internalization of the endocytic coat. Frames are separated by 1.15 s and played back at 15 fps. Scale bars are 2 µm. All cells are *myo3Δ*.



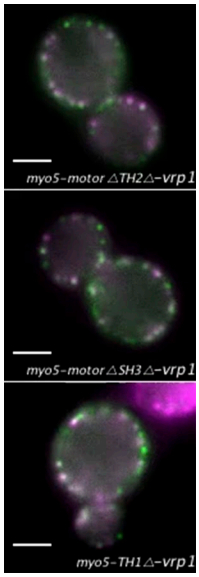
Video 11. **Epifluorescence videos of *MYO5 and myo5Δ* cells endogenously expressing Vrp1-GFP (green) and Abp1-mRFP (magenta) after washout of CK-666 (time of washout indicated by text in the upper left).** Related to Fig. 3 E. Yellow arrowhead marks an endocytic actin assembly event with fragmenting of the Vrp1-GFP patch. Actin flares internalize from the PM in *myo5* membrane-binding mutants along with the WASP/myosin complex. Frames are separated by 1.15 s and played back at 15 fps. Scale bars are 2 µm. All cells are *myo3Δ*.



Video 12. **Epifluorescence videos of cells of the indicated genotypes endogenously expressing Sla1-GFP (green) and Abp1-mRFP (magenta).** Related to Fig. 4 C. Single domain deletion mutants of Myo5 still anchor the actin cytoskeleton to CME sites. Frames are separated by 1.15 s and played back at 15 fps. Scale bars are 2 µm. All cells are *myo3Δ*.



Video 13. **Epifluorescence videos of cells of the indicated genotypes endogenously expressing Sla1-GFP (green) and Abp1-mRFP (magenta).** Related to Fig. 4 D. Deletion of the Myo5 motor domain in tandem with a tail domain recapitulates cytoplasmic actin comet formation. Frames are separated by 1.15 s and played back at 15 fps. Scale bars are 2 µm. All cells are *myo3Δ*.



Video 14. **Epifluorescence videos of cells of the indicated genotypes endogenously expressing Sla1-GFP (green) and Abp1-mRFP (magenta).** Related to Fig. 4 E. Linkage of *myo5* actin-binding mutants but not *myo5* membrane-binding mutants to Vrp1 rescues cytoplasmic comet formation. Frames are separated by 1.15 s and played back at 15 fps. Scale bars are 2 µm. All cells are *myo3Δ*.

Table S1. **Strains used in this study**

Name	Genotype	Source
DDY1102	*MATa/MATα his3-Δ200/his3-Δ200, leu2-3, 112/leu2-3, 112, ura3-52/ura3-52, ade2-1/ADE2, lys2-801/LYS2*	Drubin laboratory collection
DDY5684	*MATa/MATα his3-Δ200/his3-Δ200, leu2-3, 112/leu2-3, 112, ura3-52/ura3-52, MYO5/myo5Δ::HygMX, myo3Δ::cgLEU2/myo3Δ::cgLEU2, ABP1-mRFP::HIS3/ABP1-mRFP::HIS3, SLA1-GFP::KanMX6/SLA1-GFP::KanMX6*	This study
DDY5685	*MATa his3-Δ200, leu2-3, 112, ura3-52, MYO5-13MYC::URA3, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, SLA1-GFP::KanMX*	This study
DDY5686	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo5Δ::URA3, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, SLA1-GFP::KanMX6*	This study
DDY5687	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo5-TH1Δ-13myc::URA3, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, SLA1-GFP::KanMX6*	This study
DDY5688	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, ARC15-GFP::KanMX*	This study
DDY5689	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo5Δ::URA3, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, ARC15-GFP::KanMX*	This study
DDY5690	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo5-TH1Δ-13myc::URA3, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, ARC15-GFP::KanMX*	This study
DDY5691	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, LAS17-GFP::HIS3*	This study
DDY5692	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo5-TH1Δ-13myc::URA3, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, LAS17-GFP::HIS3*	This study
DDY5693	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, VRP1-GFP::KanMX*	This study
DDY5694	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo5-TH1Δ-13myc::URA3, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, VRP1-GFP::KanMX*	This study
DDY5695	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, BBC1-GFP::HIS3*	This study
DDY5696	*MATα his3-Δ200, leu2-3, 112, ura3-52, myo5-TH1Δ-13myc::URA3, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, BBC1-GFP::HIS3*	This study
DDY5697	*MATα his3-Δ200, leu2-3, 112, ura3-52, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, BZZ1-GFP::HIS3*	This study
DDY5698	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo5-TH1Δ-13myc::URA3, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, BZZ1-GFP::HIS3*	This study
DDY5699	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo3Δ::cgLEU, ABP1-mRPF::HIS3, PAN1-GFP::HIS3*	This study
DDY5700	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo5-TH1Δ-13myc::URA3, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, PAN1-GFP::HIS3*	This study
DDY4773	*MATa his3-Δ200, leu2-3, 112, ura3-52, MYO5-13MYC::URA3, myo3Δ::cgLEU2*	Lewellyn et al., 2015
DDY4828	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo5Δ::URA3, myo3Δ::cgLEU2*	Lewellyn et al., 2015
DDY4775	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo5-TH1Δ-13myc::URA3, myo3Δ::cgLEU2*	Lewellyn et al., 2015
DDY5701	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo5-motorΔ-13myc::URA3, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, SLA1-GFP::KanMX*	This study
DDY5702	*MATα his3-Δ200, leu2-3, 112, ura3-52, myo5-TH2Δ-13myc::URA3, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, SLA1-GFP::KanMX6*	This study
DDY5703	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo5-SH3Δ-13myc::URA3, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, SLA1-GFP::KanMX6*	This study
DDY5704	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo5-CAΔ-13myc::URA3, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, SLA1-GFP::KanMX*	This study
DDY5705	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo5-motorΔTH2Δ-13myc::URA3, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, SLA1-GFP::KanMX*	This study
DDY5706	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo5-motorΔSH3Δ-13myc::URA3, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, SLA1-GFP::KanMX*	This study
DDY5707	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo5-motorΔCAΔ-13myc::URA3, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, SLA1-GFP::KanMX*	This study
DDY4786	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo5-TH1Δ-vrp1-13myc:URA3, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, SLA1-GFP::KanMX6*	Lewellyn et al., 2015

Table S1. **Strains used in this study *(Continued)***

Name	Genotype	Source
DDY5708	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo5-motorΔTH2Δ-vrp1-13myc::URA3, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, SLA1-GFP::KanMX*	This study
DDY5709	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo5-motorΔSH3Δ-vrp1-13myc::URA3, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, SLA1-GFP::KanMX*	This study
DDY5710	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, SLA1-GFP::KanMX*	This study
DDY5711	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo5Δ::URA3, myo3Δ::cgLEU2, ABP1-mRFP::HIS3, VRP1-GFP::KanMX6*	This study
DDY5712	*MATa his3-Δ200, leu2-3, 112, ura3-52, myo5-TH1Δ-GFP::URA3, myo3Δ::cgLEU2, ABP1-mRFP::HIS3*	This study

Table S2. **Plasmids used in this study**

Plasmid	Description
pDD2679	pBluescript containing *myo5-motorΔTH2Δ-13myc* marked with *URA3*
pDD2680	pBluescript containing *myo5-motorΔSH3Δ-13myc* marked with *URA3*
pDD2681	pBluescript containing *myo5-motorΔCAΔ-13myc* marked with *URA3*
pDD2682	pBluescript containing *myo5-motorΔTH2Δ-vrp1-13myc* marked with *URA3*
pDD2683	pBluescript containing *myo5-motorΔSH3Δ-vrp1-13myc* marked with *URA3*
pDD2684	pBluescript containing *myo5-motorΔCAΔ-vrp1-13myc* marked with *URA3*

Reference

Lewellyn, E.B., R.T.A. Pedersen, J. Hong, R. Lu, H.M. Morrison, and D.G. Drubin. 2015. An Engineered Minimal WASP-Myosin Fusion Protein Reveals Essential Functions for Endocytosis. *Dev. Cell.* 35:281–294. https://doi.org/10.1016/j.devcel.2015.10.007