Figure S1

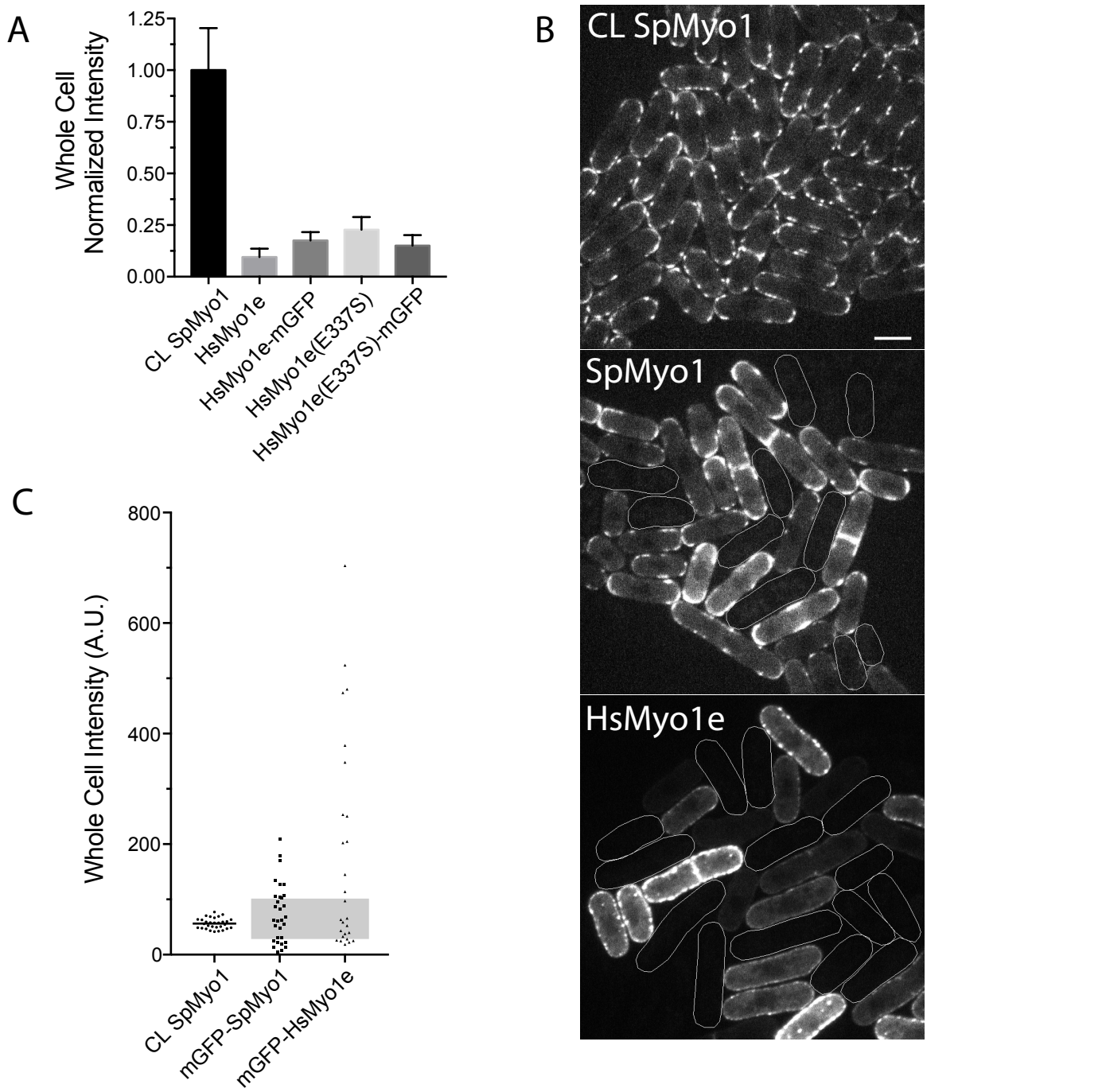

Figure S1: HsMyo1e fails to express from endogenous *S. pombe myo1* locus. (A) Bar graph (mean ± SD) of whole cell fluorescence intensities of *S. pombe* cells expressing from the *myo1* locus under control of endogenous *Pmyo1* promoter mGFP-SpMyo1 (CL SpMyo1), untagged HsMyo1e, mGFP-tagged HsMyo1e, untagged HsMyo1e(E337S), or mGFP-tagged HsMyo1e(E337S). Fluorescence intensities were measured in a single confocal section through the middle of the cell, subtracted for extracellular background, and normalized to the intensity of CL SpMyo1. Untagged strains were used as controls for autofluorescence. N= 18-20 cells. (B, C) Variation in the protein expression levels from the multi-copy plasmid under the control of thiamine-repressible *3xPnmt1* promoter. (B) Single confocal sections through the middle of CL SpMyo1 cells expressing mGFP-tagged SpMyo1 from the endogenous *myo1* locus (top panel), *myo1Δ* cells expressing mGFP-SpMyo1 for 12 hours in the absence of thiamine (middle panel), or expressing mGFP-HsMyo1e for 19 hrs in the absence of thiamine (bottom panel) from the plasmid. (C) Dot plot of background-subtracted whole cell intensities of individual cells. N=27-31 cells. The shaded box depicts myosin expression at 0.5-2X the level of the endogenous SpMyo1, the range used for analysis of actin patch dynamics.

Figure S2

Figure S2: Alignment of *S. pombe* Myo1 (SpMyo1) and *H. sapiens* Myo1e (HsMyo1e) protein sequences. Protein domains of SpMyo1 and HsMyo1e are color-coded and labeled: motor domain (black), IQ1 (red), IQ2 (orange), TH1 (green), TH2 (blue), SH3 (purple), CA (bright green). Identical and similar amino acid residues are indicated on the middle consensus line. Black arrowheads mark domain boundaries used to create chimeric and deletion constructs. TH, tail homology domain; SH3, Src Homology 3 domain; CA, central-acidic domain.

Figure S3

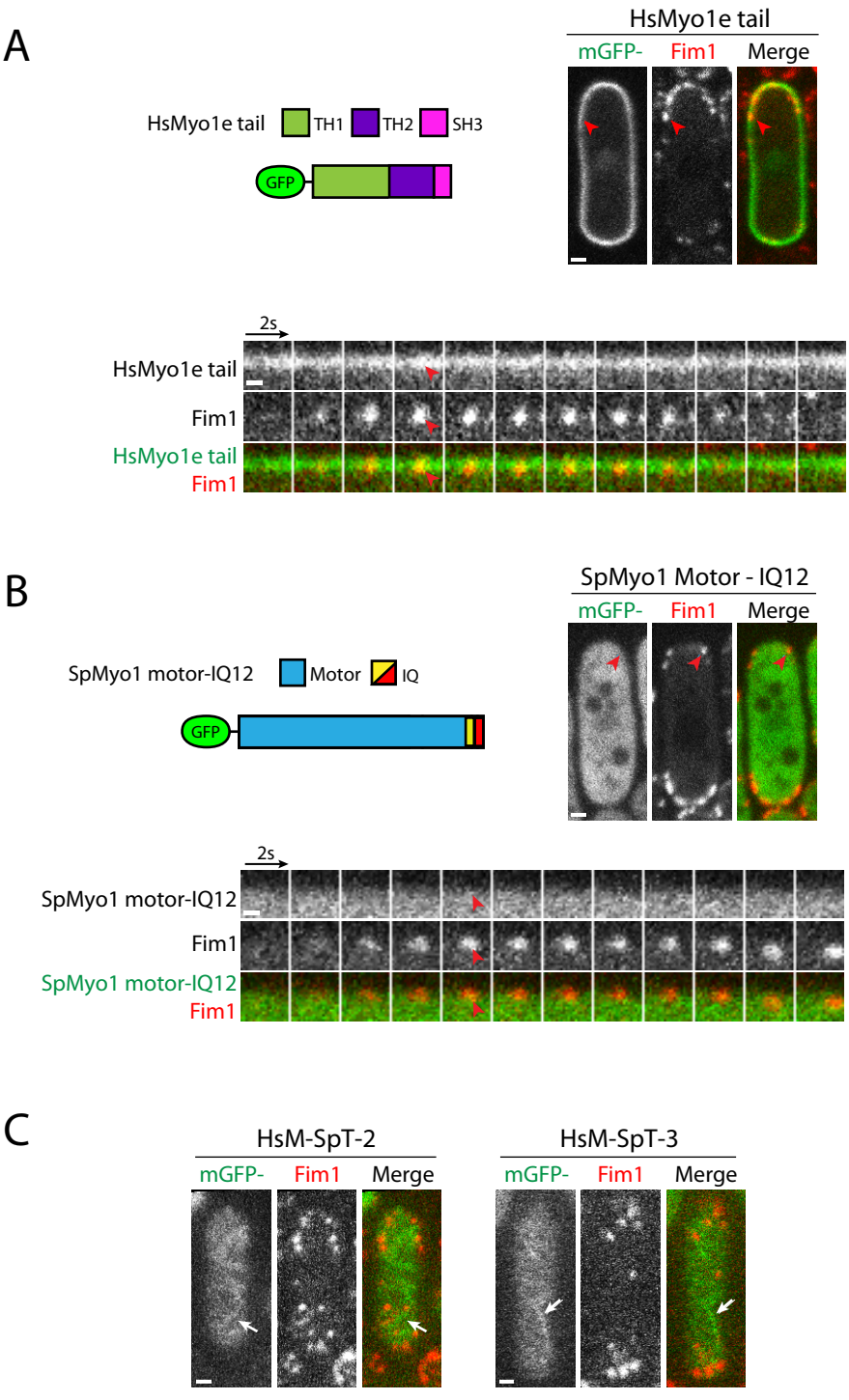

Figure S3: HsMyo1e tail, SpMyo1 motor-IQ12, and HsMyo1e motor – SpMyo1 tail chimeras do not localize to actin patches. (A-C) Analysis of colocalization of mGFP-tagged myosin constructs (green) with Fim1-mCherry (red) in actin patches in *myo1Δ* cells. mGFP-tagged constructs were expressed from plasmids under control of *3xPnmt1* promoter for 12-18 hours in the absence of thiamine in *myo1Δ* cells expressing Fim1-mCherry. (A, B) Top left, schematic diagram of mGFP-tagged (A) HsMyo1e tail and (B) SpMyo1 motor constructs. Top right, representative images in single confocal sections through the middle of the cells. Scale bars, 1 µm. Bottom panel, montages of individual patches at 2-second intervals. Scale bars, 0.5 µm. Red arrowheads indicate the absence of mGFP-tagged proteins in Fim1-mCherry-labeled actin patches. (C) Representative images in single confocal sections of the top surface of HsM-SpT-2 and HsM-SpT-3 expressing cells. Scale bars, 1 µm. The white arrows indicate HsM-SpT-2 and HsM-SpT-3 in the filamentous thread-like structures on the surface of the cell.

Figure S4

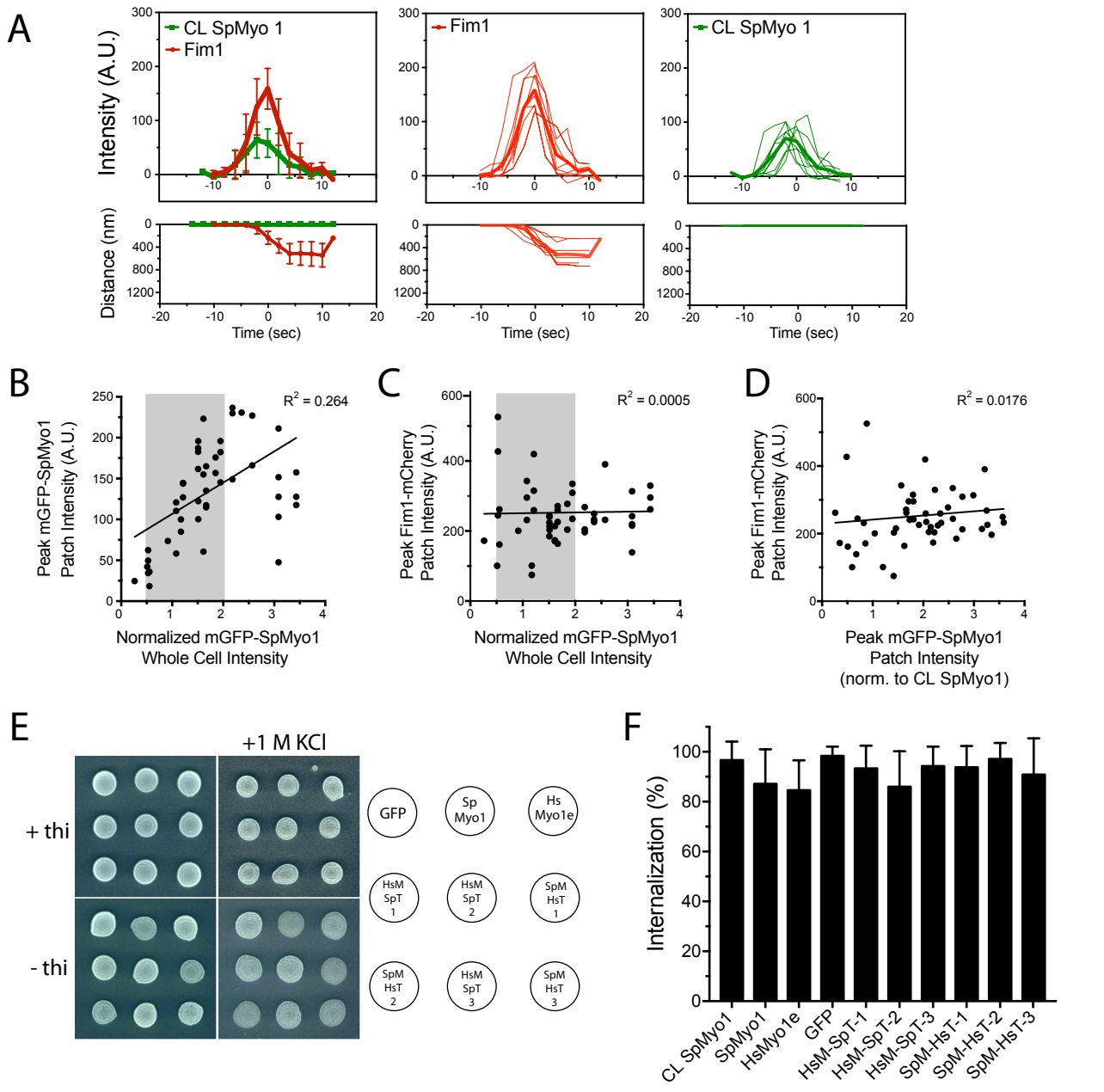

Figure S4: Tracking dynamics of mGFP-tagged SpMyo1 and human-yeast myosin-I chimeras and Fim1-mCherry in endocytic actin patches. (A) Raw (thin lines) and average (thick lines; error bars in the graphs on the left indicate SD) time courses of (upper panels) fluorescence intensity and (lower panels) distance traveled for mGFP-SpMyo1 (green) and Fim1-mCherry (red) in actin patches in control wild-type cells expressing mGFP-SpMyo1 (CL SpMyo1) from the endogenous *myo1* locus. Patch dynamics were tracked in time series of images acquired at 2-second intervals in a single confocal section through the middle of the cells. The time courses of cortical background-subtracted intensities and distances from the origin for individual patches were aligned to the peak of Fim1-mCherry patch intensity (time zero) and averaged at each time point. N= 9 patches in 3 cells. (B-D) Correlation plots of (B) peak intensities of mGFP-SpMyo1 in patches versus whole cell mGFP-SpMyo1 intensities, (C) peak intensities of Fim1-mCherry in patches versus whole cell mGFP-SpMyo1 intensities, and (D) Fim1-mCherry peak patch intensities versus mGFP-SpMyo1 peak patch intensities. mGFP-SpMyo1 was expressed from the plasmid under control of *3xPnmt1* promoter for 12 hours in the absence of thiamine in *myo1Δ* cells expressing Fim1-mCherry. Whole cell intensities and peak patch intensities were measured in time series of images acquired at 2-second intervals in a single confocal section through the middle of the cells. Whole cell intensities representing mGFP-SpMyo1 expression levels were normalized to the intensities of control wild-type cells expressing mGFP-Myo1 from the endogenous *myo1* locus. Lines represent the best linear fits with corresponding R^2 values from linear regression analysis. N=50 patches from 20 cells. Shaded areas indicate the 0.5-2-fold range of expression levels that was accepted for tracking dynamics of myosin and Fim1 in actin patches. (E) Analysis of salt sensitivity of *myo1*$^+$ cells expressing mGFP alone, mGFP-tagged SpMyo1, HsMyo1e, or SpMyo1-HsMyo1e chimeras off of plasmid under control of *3xPnmt1* promoter in the presence and the absence of thiamine at 25°C on EMM agar plates containing 1 M KCl. These cells also express actin patch marker Fim1-mCherry. (F) Bar graph of percent internalization (± SD) of Fim1-mCherry patches in wild-type cells expressing mGFP alone, mGFP-tagged SpMyo1, HsMyo1e, or human-yeast myosin-I chimeras from the plasmid under control of *3xPnmt1* promoter for 12-16 hours in the absence of thiamine. N= 28-45 patches in at least 5 cells. No significant differences from wild-type cells (CL SpMyo1), expressing mGFP-SpMyo1 from the endogenous *myo1* locus, were determined by a one-way ANOVA (p=0.3839).

Figure S5

A

```
Hs CaM        1  MQADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAEL
Sp Cam1       1  MTTRNLTDEQIAEFREAFSLFDRDQDGNITSNELGVVMRSLGQSPTAAEL
Sp Cam2       1  ---MPASKEQTDEMKEAFVLYDIDKDGLIPTSHVGSVLRSLGINVTDAEL

Hs CaM       51  QDMINEVDADGNGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNG
Sp Cam1      51  QDMINEVDADGNGTIDFTEFLTMMARKMKDTDNEEEVREAFKVFDKDGNG
Sp Cam2      48  AKLSNELG----DAIDEKKFMSFVSNKLRETESEEEYIKAFRVFDKDNSG

Hs CaM      101  YISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVNYEEFVQMMTAK
Sp Cam1     101  YITVEELTHVLTSLGERLSQEEVADMIREADTDGDGVINYEEFSRVISSK
Sp Cam2      94  YIETAKFADYMKTLGEKLSDNEVQLMVQEADPTNSGSFDYYDFVQRIMAK
```

B

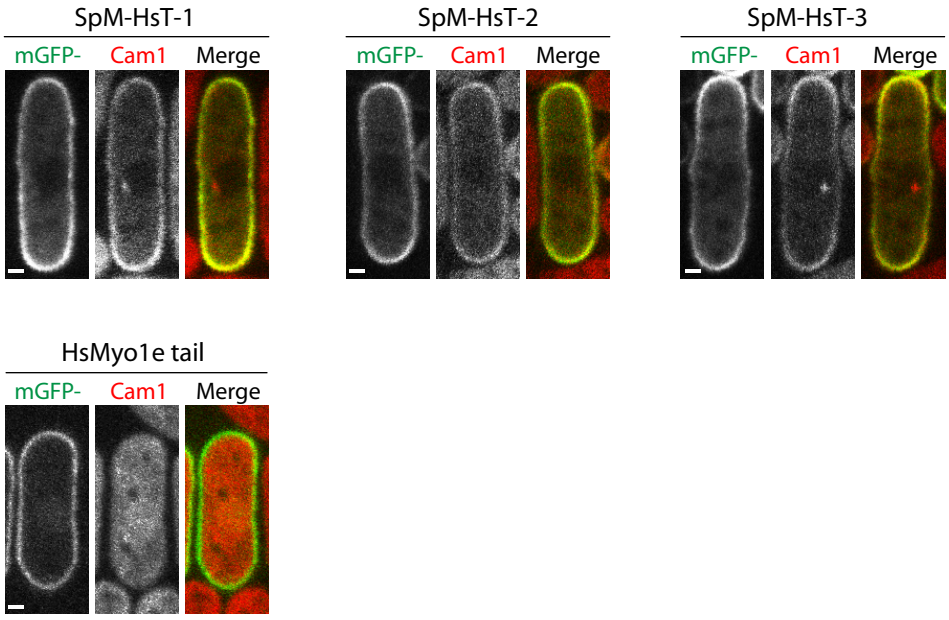

Figure S5: Human-yeast myosin-I chimeras recruit Cam1. (A) Alignment of *H. sapiens* calmodulin 1 (CaM) (NP_001350599.1), *S. pombe* Cam1 (NP_593340.1), and Cam2 (NP_594877.1) protein sequences. Red color denotes identical amino acids and blue color denotes similar amino acids. (B) Colocalization analysis of mGFP-tagged human-yeast myosin-I chimeras and HsMyo1e tail (green) with mCherry-tagged calmodulin Cam1 (red) in confocal sections through the middle of *myo1Δ* cells overexpressing myosin constructs. mGFP-tagged chimeras and HsMyo1e tail were expressed from plasmids under control of *3xPnmt1* promoter for 12-18 hours in the absence of thiamine. Scale bars, 1µm.

Figure S6

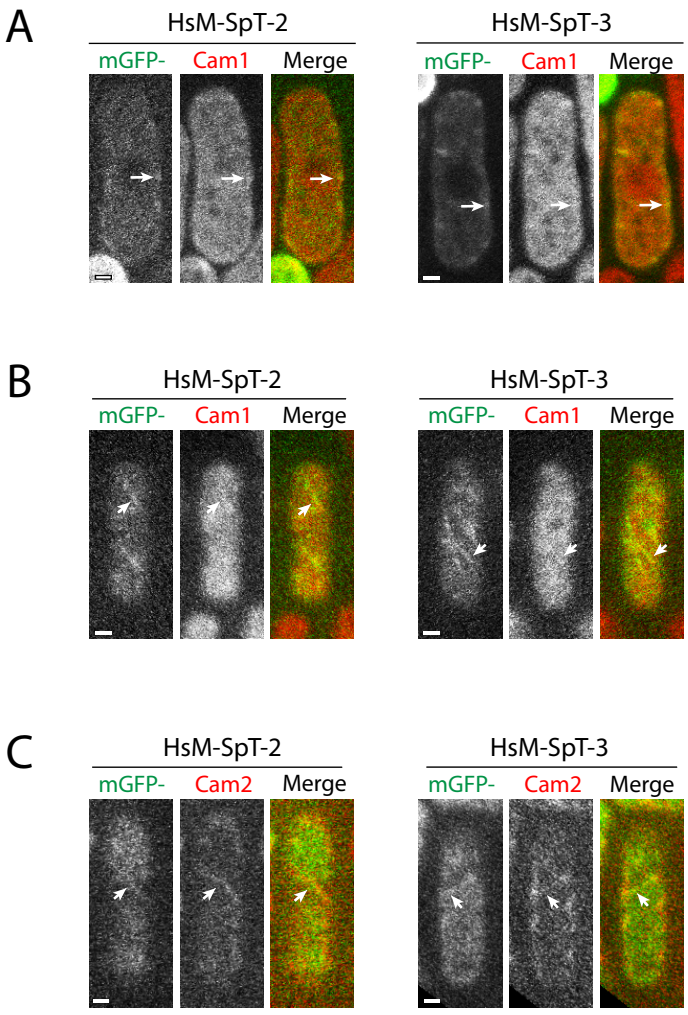

Figure S6: HsMyo1e motor - SpMyo1 tail chimeras HsM-SpT-2 and HsM-SpT-3 localize to cortical filamentous structures and recruit Cam1 and Cam2. (A, B) Images from colocalization analysis of mGFP-tagged HsMyo1e motor - SpMyo1 tail chimeras HsM-SpT-2 and HsM-SpT-3 (green) with mCherry-tagged calmodulin Cam1 (red) in *myo1Δ* cells in sections through the (A) middle and (B) top surface of the cells. (C) Images from colocalization analysis of mGFP-tagged HsMyo1e motor - SpMyo1 tail chimeras HsM-SpT-2 and HsM-SpT-3 (green) with mCherry-tagged Cam2 (red) in *myo1Δ* cells in sections through the top surface of the cells. HsM-SpT-2 and HsM-SpT-3 were expressed from plasmids under control of *3xPnmt1* promoter. White arrows indicate colocalization of Cam1 or Cam2 with HsM-SpT-2 and HsM-SpT-3 in filamentous thread-like cortical structures. Scale bars, 1µm.

Figure S7

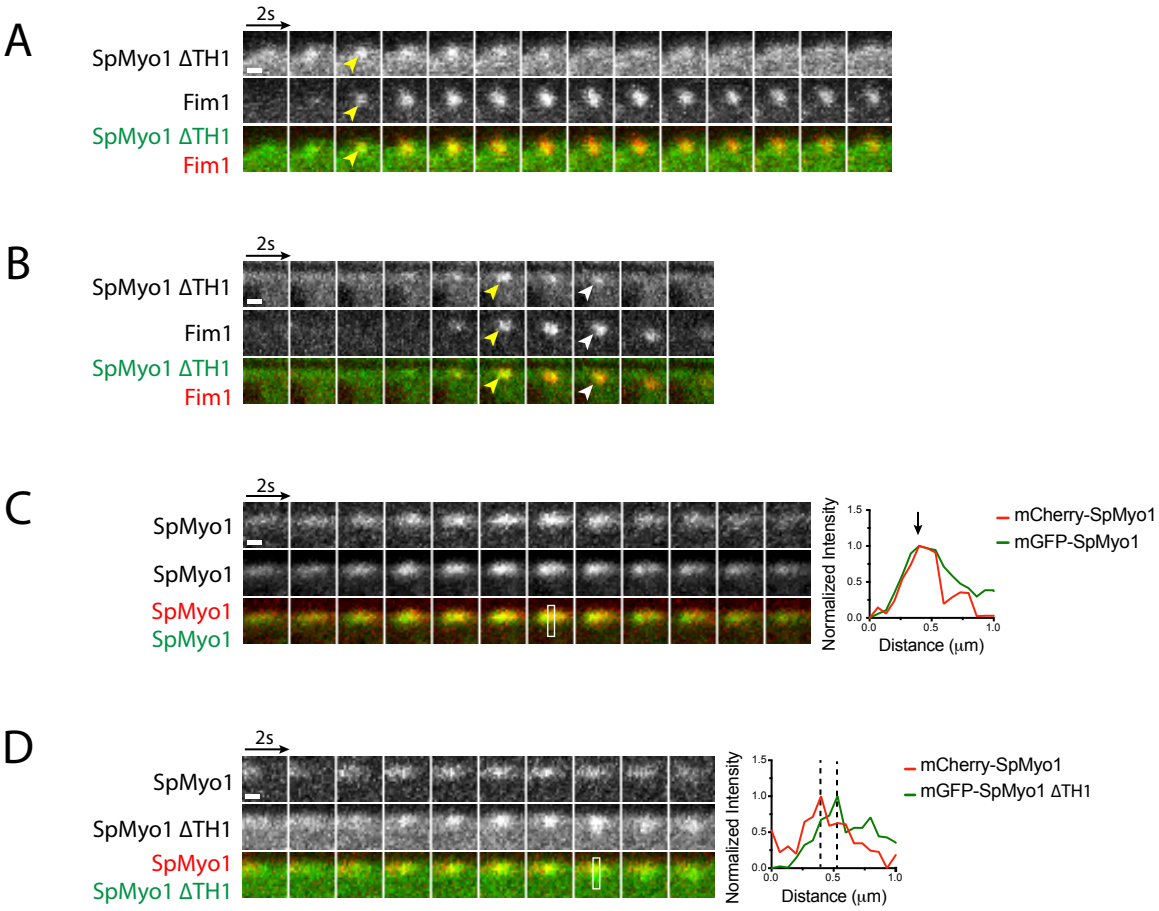

Figure S7: SpMyo1 does not require the TH1 domain for localization to endocytic actin patches. (A, B) Example montages of mGFP-tagged SpMyo1ΔTH1 (green) and Fim1-mCherry (red) in (A) a non-internalizing patch in a *myo1Δ* cell and (B) an internalizing patch in a wild-type cell. Yellow arrowheads indicate colocalization of myosin constructs and Fim1-mCherry in actin patches. White arrows depict internalization of mGFP-SpMyo1ΔTH1 with Fim1-mCherry. (C, D) Example montages of (C) mGFP-tagged SpMyo1 (green) or (D) mGFP-tagged SpMyo1ΔTH1 (green) with SpMyo1-mCherry (red) to monitor SpMyo1 localization. Normalized line scans on the side were made across areas indicated by white boxes. Black arrow on line scan indicates coincident SpMyo1 peaks, while dotted lines denote peak separation. All montages are presented at 2-second intervals in a single confocal section through the middle of the cells. Scale bars, 0.5 μm.

Table S1. *S. pombe* strains used in this study.

Strain	Genotype	Source
Figure 1		
VS1123A	*h$^+$ ade6-M210 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 kanMX6-Pmyo1-mGFP-myo1*	Lab stock
SB1	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP-573-3xPnmt1-mGFP-L*	This study
TP192	*h$^-$ ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::ura4$^+$*	Sirotkin et al. (2005)
VS1257	*h$^-$ ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::MYO1E*	This study
SB70	*h$^-$ ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::MYO1E(E337S)*	This study
VS1263-D7	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6*	Bi et al. (2017)
SB3	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP-573-3xPnmt1-mGFP-L-HsMyo1e*	This study
SB92	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP-573-3xPnmt1-mGFP-L-HsMyo1e(E337S)*	This study
Figure S1		
VS1123A	*h$^+$ ade6-M210 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 kanMX6-Pmyo1-mGFP-myo1*	Lab stock
VS1257	*h$^-$ ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::MYO1E*	This study
SB70	*h$^-$ ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::MYO1E(E337S)*	This study
SB71	*h$^+$ ade6-M210 leu1-32 ura4-D18 his3-D1 myo1Δ::MYO1E(E337S)-mGFP kanMX6*	This study
SB72	*h$^+$ ade6-M210 leu1-32 ura4-D18 his3-D1 myo1Δ::MYO1E-mGFP kanMX6*	This study
SB2	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP-573-3x Pnmt1-mGFP-L-SpMyo1*	This study
SB3	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP-573-3xPnmt1-mGFP-L-HsMyo1e*	This study
Figure 2, 3, 4		
VS1123A	*h$^+$ ade6-M210 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 kanMX6-Pmyo1-mGFP-myo1*	Lab stock
VS1263-D7	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6*	Bi et al. (2017)
SB1	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP-573-3xPnmt1-mGFP-L*	This study
SB2	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP-573-3x Pnmt1-mGFP-L-SpMyo1*	This study
SB3	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP-573-3xPnmt1-mGFP-L-HsMyo1e*	This study
SB4	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP-573-3xPnmt1-mGFP-L-HsM-SpT-1*	This study
SB5	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP-573-3xPnmt1-mGFP-L-HsM-SpT-2*	This study
SB6	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP-573-3xPnmt1-mGFP-L-SpM-HsT-1*	This study
SB7	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP-573-3xPnmt1-mGFP-L-SpM-HsT-2*	This study
SB9	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP-573-3xPnmt1-mGFP-L-HsM-SpT-3*	This study
SB10	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP-573-3xPnmt1-mGFP-L-SpM-HsT-3*	This study

Figure S3		
SB5	*h+ ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP-573-3xPnmt1-mGFP-L-HsM-SpT-2*	This study
SB9	*h+ ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP-573-3xPnmt1-mGFP-L-HsM-SpT-3*	This study
SB37	*h+ ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP-573-3xPnmt1-mGFP-L-HsMyo1etail*	This study
SB106	*h+ ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP-573-3xPnmt1-mGFP-L-SpMyo1-motor-IQ12*	This study
Figure S4		
VS1123A	*h+ ade6-M210 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 kanMX6-Pmyo1-mGFP-myo1*	Lab stock
SB2	*h+ ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP-573-3x Pnmt1-mGFP-L-SpMyo1*	This study
VS888-3	*h- ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6*	Lab stock
SB48	*h- ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 pSGP-573-3xPnmt1-mGFP-L*	This study
SB49	*h- ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 pSGP-573-3xPnmt1-mGFP-L-SpMyo1*	This study
SB50	*h- ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 pSGP-573-3xPnmt1-mGFP-L-HsMyo1e*	This study
SB51	*h- ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 pSGP-573-3xPnmt1-mGFP-L-HsM-SpT-1*	This study
SB52	*h- ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 pSGP-573-3xPnmt1-mGFP-L-HsM-SpT-2*	This study
SB53	*h- ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 pSGP-573-3xPnmt1-mGFP-L-SpM-HsT-1*	This study
SB54	*h- ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 pSGP-573-3xPnmt1-mGFP-L-SpM-HsT-2*	This study
SB55	*h- ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 pSGP-573-3xPnmt1-mGFP-L-HsM-SpT-3*	This study
SB56	*h- ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 pSGP-573-3xPnmt1-mGFP-L-SpM-HsT-3*	This study
Figure 5 and S5		
VS1449-3A	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::kanMX6 kanMX6-Pcam1-mCherry-cam1*	This study
SB59	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::kanMX6 kanMX6-Pcam1-mCherry-cam1 pSGP-573-3xPnmt1-mGFP-L*	This study
SB60	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::kanMX6 kanMX6-Pcam1-mCherry-cam1 pSGP-573-3xPnmt1-mGFP-L-SpMyo1*	This study
SB62	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::kanMX6 kanMX6-Pcam1-mCherry-cam1 pSGP-573-3xPnmt1-mGFP-L-HsMyo1e tail*	This study
SB63	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::kanMX6 kanMX6-Pcam1-mCherryVS-cam1 pSGP-573-3xPnmt1-mGFP-L-HsM-SpT-1*	This study
SB64	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::kanMX6 kanMX6-Pcam1-mCherry-cam1 pSGP-573-3xPnmt1-mGFP-L-HsM-SpT-2*	This study
SB65	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::kanMX6 kanMX6-Pcam1-mCherry-cam1 pSGP-573-3xPnmt1-mGFP-L-SpM-HsT-1*	This study
SB66	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::kanMX6 kanMX6-Pcam1-mCherry-cam1*	This study

	pSGP-573-3xPnmt1-mGFP-L-SpM-HsT-2	
SB67	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::kanMX6 kanMX6-Pcam1-mCherry-cam1 pSGP-573-3xPnmt1-mGFP-L-HsM-SpT-3*	This study
SB69	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::kanMX6 kanMX6-Pcam1-mCherry-cam1 pSGP-573-3xPnmt1-mGFP-L-SpM-HsT-3*	This study
Figure 6 and S6		
TP364-2	*h^- ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::kanMX6 pUR19-myo1$^+$*	Lab stock
VS1275	*h^+ ade6-M210 leu1-32 ura4-D18 his3-D1 cam2-mCherry-kanMX6*	Lab stock
VS2193-3A	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::kanMX6 cam2-mCherry-kanMX6*	This study
SB39	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 myo1DΔ::kanMX6 cam2-mCherry-kanMX6 pSGP-573-3xPnmt1-mGFP-L-SpMyo1*	This study
SB41	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::kanMX6 cam2-mCherry-kanMX6 pSGP-573-3xPnmt1-mGFP-L-HsM-SpT-1*	This study
SB42	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 myo1ΔD::kanMX6 cam2-mCherry-kanMX6 pSGP-573-3xPnmt1-mGFP-L-HsM-SpT-2*	This study
SB43	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 myo1DΔ::kanMX6 cam2-mCherry-kanMX6 pSGP-573-3xPnmt1-mGFP-L-SpM-HsT-1*	This study
SB44	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::kanMX6 cam2-mCherry-kanMX6 pSGP-573-3xPnmt1-mGFP-L-SpM-HsT-2*	This study
SB45	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::kanMX6 cam2-mCherry-kanMX6 pSGP-573-3xPnmt1-mGFP-L-HsM-SpT-3*	This study
SB46	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::kanMX6 cam2-mCherry-kanMX6 pSGP-573-3xPnmt1-mGFP-L-SpM-HsT-3*	This study
SB89	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::kanMX6 cam2-mCherry-kanMX6 pSGP-573-3xPnmt1-mGFP-L*	This study
Figure 7 and S7		
TP364-2	*h^- ade6-M216 leu1-32 ura4-D18 his3-D1 myo1Δ::kanMX6 pUR19-myo1$^+$*	Lab stock
VS1010-A	*h^+ ade6-M210 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6*	Lab stock
VS1459-6	*h^+ ade6-M210 leu1-32 ura4-D18 his3-D1 kanMX6-mCherry(VS)-myo1*	Lab stock
VS2345-1B	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6*	This study
SB93	*h^+ ade6-M216 leu1-32 ura4-D18 his3-D1fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP573-Pnmt1-3x-mGFP-L-SpM-HsT-1ΔTH2-SH3*	This study
SB99	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP573-Pnmt1-3x-mGFP-L-SpMyo1ΔTH1*	This study
SB98	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP573-Pnmt1-3x-mGFP-L-SpMyo1ΔTH2-SH3-CA*	This study
SB100	*h? ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 myo1Δ::kanMX6 pSGP573-Pnmt1-3x-mGFP-L-SpM-HsT-1ΔTH1*	This study
SB103	*h^- ade6-M216 leu1-32 ura4-D18 his3-D1 fim1-mCherry-natMX6 pSGP-573-3xPnmt1-mGFP-L-SpMyo1ΔTH1*	This study
SB107	*h^+ ade6-M210 leu1-32 ura4-D18 his3-D1 kanMX6-mCherry(VS)-myo1 pSGP-573-3xPnmt1-mGFP-L-SpMyo1*	This study
SB108	*h^+ ade6-M210 leu1-32 ura4-D18 his3-D1 kanMX6-mCherry(VS)-myo1 pSGP-573-3xPnmt1-mGFP-L-SpMyo1ΔTH1*	This study

Table S2. Primers used in this study.

Name	Sequence[a]
subM1utr5r	**ACGTGTAGGTTTCTT**CTAATAC
subM1utr3d	**GCAATAATGAATGGC**TTGTGAC
m1Myo1Ed	**AAGAAACCTACACGT**AAGTGCGGACTTGTCGTTAACCTATCTCTTGTTC GGCCATAAGTGAATAGATTTTATGGGAAGCAAAGGTGTCTAC
m1Myo1Er	**GCCATTCATTATTGC**AAATTAAGAATCAAGCAATGTACTGGCCGTATTAT ATATACATCATAATGAAAGCTCAGATCTTGGTCACATAGTTG
TEDs site mut m1e F[b]	GGGGAGGCAAATCC**TCC**TCCATCCACGTGAC
TEDs site mut m1e R[b]	GTCACGTGGATGGA**GGA**GGATTTGCCTCCCC
m1e-GFP-int-F	TGGACGGGTCGACTACGAGGCAAGCAGGGCCTGTTCCCCAACAACTAT GTGACCAAGATCCGGATCCCCGGGTTAATTAA
m1-Kxr	GCCATTCATTATTGCAAATTAAGAATCAAGCAATGTACTGGCCGTATTAT ATATACATCATAATGAAAGCGAATTCGAGCTCGTTTAAAC
SpHs1p1	**GTTCGTAATGAGGGT**ACTAAAC
SpHs1hA	**ACCCTCATTACGAAC**TTGAACGTATTTCTTCCGGGC
SpHs2p2	**AAGTTTTGGGATACA**ATGGCAACC
SpHs2hB	**TGTATCCCAAAACTT**CTCTCTCATCTCTTCTAAAAG
SpHs3p3	**ACGTTCAAGTTCCAT**GTTAAC
SpHs3hC	**ATGGAACTTGAACGT**ATGAGAGAAGAAGCCTCAGACC
SpHs4p4	**ATCACGCATGTCTTC**GAGC
SpHs4hD	**GAAGACATGCGTGAT**AGAAAGTATGATGGGTATGC
SpHs5p5	**TCTGAAGCTGCTGCT**TGTATTCAG
SpHs5hE	**AGCAGCAGCTTCAGA**TTGAACGTATTTCTTCCGGGC
Myo1 F[c]	CA***GCTAGC****GGTACTGGATCC*ATGGCCATCCTTAAGAGAACAAAC
SpTail R[c]	CA***GCGGCCGC***TCACCAATCTTCTTCTTCATCA
Myo1e F[c]	CA***GCTAGC****GGTACTGGATCC*ATGGGAAGCAAAGGTGTCTAC
Myo1e R[c]	CA***GCGGCCGC***TCAGATCTTGGTCACATAGTTG
Chimera6	**TATGTTAGAAGAAGA**ATGAGAGAAGAAGCCTCAGACCTCTTA
m1D1IQ2L	**TCTTCTTCTAACATAA**GAACGC
pSPG-m1etail-F	**AGCGGTACTGGATCC**ATGAGAGAAGAAGCCTCAGACCTC
pSPG-m1etail-R	**GGATCCAGTACCGCT**AGCA
SpMyo1deltaTH1	**ATGGAACTTGAACGT**AAGAGTAAGCCTTTCCCTCGCTTAGCA
IQ2rev	**ACGTTCAAGTTCCAT**GTTAAC
SpMyo1delta23CA	**TTGCCTCCTACCAGT**TGAGCGGCCGCTCTAGGTCGACAGATC
SpMyo1TH1rev	**ACTGGTAGGAGGCAA**GCCAGT
CHIM3deltaTH1	**ATGGAACTTGAACGT**CTGCCCAAGAACTCCCGTCCTACCAGA
Sp1m-m1e-TH1-F	**AGCATCGGACCTGGA**TGAGCGGCCGCTCTAGGTCGACAGATC
hmyo1edelTH2	**TCCAGGTCCGATGCT**GACCTGCAG

[a]15-nt overlap regions required for In-Fusion cloning are bolded. HsMyo1e sequences are shaded grey.

[b]Mutated codon corresponding to HsMyo1e E337S TEDS site mutation is bolded and underlined.

[c]NheI and NotI cloning sites are bolded and italicized and the sequence of a portion of the linker is italicized.

Table S3. Plasmids for expression of mGFP-tagged proteins used in this study and templates and primers used for their construction.

Construct		**Hs template**		**Sp template**		**Subcloning primers**
SpMyo1				pBS-SpMyo1		Myo1 F SpTail R
HsMyo1e		pBS-HsMyo1e				
HsMyo1e (E337S)		pBS-HsMyo1e(E337S)				
Construct	**Description**	**Hs template**	**Hs primers**	**Sp template**	**Sp primers**	**Subcloning primers**
HsM-SpT-1	HsMyo1e motor-IQ SpMyo1 tail	pEGFP-C1-myo1e-EcoR1-	m1Myo1Ed SpHs1hA	pBS-SpMyo1	subM1utr5r SpHs1p1	Myo1e F SpTail R
HsM-SpT-2	HsMyo1e motor SpMyo1 IQ1-IQ2-tail	pEGFP-C1-myo1e-EcoR1-	m1Myo1Ed SpHs2hB	pBS-SpMyo1	subM1utr5r SpHs2p2	Myo1e F SpTail R
SpM-HsT-1	SpMyo1 motor-IQ1-IQ2 HsMyo1e tail	pEGFP-C1-myo1e-EcoR1-	SpHs3hC m1Myo1Er	pBS-SpMyo1	SpHs3p3 subM1utr3d	Myo1 F Myo1e R
SpM-HsT-2	SpMyo1 motor HsMyo1e IQ-tail	pEGFP-C1-myo1e-EcoR1-	SpHs4hD m1Myo1Er	pBS-SpMyo1	SpHs4p4 subM1utr3d	Myo1 F Myo1e R
HsM-SpT-3	HsMyo1e motor-IQ SpMyo1 IQ2-tail	pEGFP-C1-myo1e-EcoR1-	m1Myo1Ed SpHs5hE	pBS-SpMyo1	subM1utr5r SpHs5p5	Myo1e F SpTail R
Construct	**Description**	**Template**	**Primers**			
SpM-HsT-3	SpMyo1 motor-IQ1 HsMyo1e tail	SpM-HsT-1	Chimera6 m1D1IQ2L			
HsMyo1e tail	HsMyo1e tail	HsMyo1e	pSPG-m1etail-F pSPG-m1etail-R			
SpMyo1ΔTH1	SpMyo1ΔTH1	SpMyo1	SpMyo1deltaTH1 IQ2rev			
SpMyo1ΔTH2-SH3-CA	SpMyo1ΔTH2-SH3-CA	SpMyo1	SpMyo1delta23CA SpMyo1TH1rev			
SpM-HsT-1 ΔTH1	SpM-HsT-1ΔTH1	SpM-HsT-1	CHIM3deltaTH1 IQ2rev			
SpM-HsT-1 ΔTH2-SH3	SpM-HsT-1 ΔTH2-SH3	SpM-HsT-1	Sp1m-m1e-TH1-F hmyo1edelTH2			
GFP	Modified pSGP-573					

SpMyo1, HsMyo1e, HsMyo1e(E337S), and chimeras were first made in pBluescript, then amplified by PCR and cloned into pCR-BluntII-TOPO followed by subcloning into modified pSGP-573 vector, in which GFP sequence was replaced with mGFP sequence and N-terminal mGFP is separated from C-terminal portion of the fusion protein by 7-aa linker with the sequence GASGTGS. The ΔTH1 and ΔTH2-SH3-CA mutants of SpMyo1 were derived from SpMyo1 and SpM-HsT-3, SpM-HsT-1ΔTH1 and SpM-HsT-1ΔTH2-SH3 were derived from SpM-HsT-1 by In-Fusion cloning. HsMyo1e tail construct was similarly derived from HsMyo1e construct.