Supporting Information

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SI Methods

Yeast Strains, Growth Conditions, Cell, and Genetic Methods. We used standard yeast growth conditions, genetic methods, and DNA staining procedures (1, 2). Table S4 lists fission and budding yeast strains used in this study. A *ura4* deletion (MLP 479) was made in fission yeast strains TP 150 and MLP 569 as described in ref. 3. *GFP*, *GST*, and *GAL1* promoter kan^R/*HIS5* cassettes were integrated at the budding yeast *MYO5* locus (4).

For temperature-shift experiments with the fission yeast *sid2-250 ts* strain and wild-type controls, cells growing exponentially in rich liquid media to an OD_{595} of 0.1 at 25°C were transferred to 36°C for 3–4 h of growth. Complementation of the fission yeast *rng3-65 ts* strain using *rng3* constructs was tested by streaking ura^+ plasmid transformants on EMM-Ura^- minimal media plates, which were then grown at 25°C. Cells were restreaked and grown at the restrictive condition (36°C). For localization experiments *rng3-65* transformants were grown exponentially at 25°C in EMM-Ura^- minimal media.

GFP fusion proteins and stains were imaged in cells as described in refs. 3 and 5. Images were acquired and processed using MetaMorph, UltraView RS, Image J, and PhotoShop software. Subcellular Myo5p-GFP fluorescence intensities and lifetimes (in patches) were measured in ImageJ by analysis of single and time-lapse micrographs recorded at intervals of 2 sec.

Plasmids. Table S1 lists fission and budding yeast plasmids. The fidelity of all DNA sequences was confirmed by automated sequencing. Where necessary, the orientation of inserts was confirmed by diagnostic restriction analysis.

p3xGFP. DNA encoding triple GFP was amplified from pFA6a-3xGFP-kanMX6 with the primers: 5′ XhoI *CTCGAG*GGAACAAAAGCTGGAGGATCC and 3′ NotI *GCGGCCGC*AGATATTAAAGAATACAGATCTTTGTTTGTACA. The DNA was ligated into pDS573a (harboring the weak-strength *81-nmt1* inducible promoter) at the XhoI and NotI sites yielding a construct with an N-terminal 3xGFP tag.

p3xGFP-rng3/UCS/central. The *rng3* ORF and DNA encoding the Rng3p UCS domain were liberated from pGST-*rng3*-FL and pGST-*rng3*-UCS (3) respectively and ligated into p3xGFP at the NotI site. DNA encoding the N-terminal "central" domain (bp 1–996) of Rng3p was amplified using the primers: 5′ NotI *GCGGCCGC*ATGACCCACGAGCTTTCCTCAAC and 3′ NotI *GCGGCCGC*TCAAATAACTTGAGAAAGCTGGTACTG. The DNA fragment was ligated into p3xGFP at the NotI site.

pDS473-LEU2-rlc1. The *rlc1* ORF was liberated from pDS473-*rlc1* (3) by *SalI* digestion and transferred to pDS473-LEU2 cut with SalI.

pDS472a-myo2-head. Base pairs 1–2445 of *myo2* were amplified from genomic DNA using the primers: 5′ NotI *GCGGCCGC*GGCGGTGGAATGACAGAAGTAATATCTAATAAAATAACTGC and 3′ NotI *GCGGCCGC*CGGGCCTTAGATTGAAAAATAACTTAGC. The fragment was ligated into pDS472a at the NotI site to generate an overexpression construct encoding the motor and light chain-binding domain of Myo2p fused at its C terminus to GST (lacking a thrombin cleavage site).

pPGAL1-GST-TRP1. The *MYO1* promoter fragment from pPGT (5) was liberated by digestion with KpnI and XhoI and replaced with a fragment containing the budding yeast *GAL1* promoter, which was amplified from pFA6a-kanMX6-PGAL1with primers 5′ KpnI *GGTACC*GTAAAGAGCCCCATTATCTTAGC and 3′ XhoI *CTCGAG*TTTGAGATCCGGGTTTTTTCTCCTTGAC. The XhoI/NotI GFP-encoding fragment was then replaced with the XhoI/NotI GST-encoding fragment taken from pDS473a.

pPGAL1-GST-URA3. The *GAL1* promoter-GST-*MYO1* terminator cassette from pPGAL1-GST-TRP1 was liberated by KpnI/SacI digestion and ligated into pRS316 at the KpnI and SacI sites.

pPGAL1-GST-CMD1-TRP1, pPGAL1-GST-CMD1-URA3. The budding yeast calmodulin ORF (*CMD1*) was amplified from genomic DNA using the primers: 5′ NotI *GCGGCCGC*GGTGGAGGTATGTCCTCCAATCTTACCGAAG and 3′ SalI *GTCGAC*CTATTTAGATAACAAAGCAGCGAATTG. *CMD1* was then ligated into both pPGAL1-GST-TRP1 and pPGAL1-GST-URA3 using NotI and SalI sites.

Protein Purification. ***Myo2/Myo2-head/Rng3p/Rng3p UCS domain.*** Myo2 (full-length Myo2p and its light chains, Cdc4p and Rlc1p), GST-Rng3p, and GST-Rng3p UCS domain (GST-UCSp) were purified as described in ref. 3. We used a strategy similar to that used for Myo2 to purify Myo2-head-GST, a truncated form of Myo2p (amino acids 1–815) with a C-terminal GST tag. We co-overexpressed Myo2-head-GST with GST-tagged light chains, using the *nmt1* promoter in MLP 479 cotransformed with either (*i*) pDS472a-*myo2-head* and pDS473-LEU2-*cdc4* or (*ii*) pDS472a-*myo2-head* and pDS473-LEU2-*rlc1*. Transformants were grown to saturation in liquid EMM Ura^- Leu^- medium (plus thiamine) and harvested. Cells were washed three times in EMM Ura^- Leu^- medium (lacking thiamine) before resuspending in 4 liters of the same medium yielding a starting OD_{595} of 0.05. Myo2-head-GST and light chain overexpression were induced during growth at 32°C for 24 h. Cultures (a and b) were combined and harvested. Pellets were resuspended 1:1 in Lysis Buffer [750 mM KCl, 25 mM Tris·HCl (pH 7.4), 4 mM $MgCl_2$, 4 mM ATP, 20 mM sodium pyrophosphate, 2 mM EGTA, 1 mM DTT, 2 mM PMSF, 0.1% Triton X-100, plus EDTA-free protease inhibitors (Roche)] and lysed with a Microfluidizer (model M-110S; Microfluidics). Myo2p-head-GST/GST-Cdc4p/GST-Rlc1p was recovered from lysates by enriching on glutathione-Sepharose, followed by thrombin cleavage to remove GST from the light chains. The sample was dialyzed into S1 Buffer [2 mM Tris·HCl (pH 7.0), 0.1 mM $CaCl_2$, 10 mM imidazole, 50 mM KCl, 1 mM $MgCl_2$, and 1 mM DTT] and further purified by two actin pelleting steps: (*i*) Myo2-head-GST was incubated (≈1:5) with hexokinase-treated actin filaments (3) and centrifuged at 100,000 × *g* for 2 h; and (*ii*) the pellet was resuspended in S1 buffer plus 2 mM ATP and spun again to separate actin in the pellet from dissociated Myo2-head-GST in the supernatant. Contaminating actin was removed by a second affinity step with glutathione Sepharose to yield purified Myo2-head-GST. The concentration of Myo2-head-GST was estimated by densitometry of Coomassie-stained bands after SDS/PAGE, using rabbit skeletal muscle myosin as the standard.

Myo5p. Haploid budding yeast strains in which genomic *MYO5* gene was replaced with *MYO5-GST* (MLY 697, 720) were grown in 2-liter of YPDa to an OD_{595} of 3. A control strain (JC 1284) in which *MYO5* was untagged was cultured in a similar fashion. To overexpress Myo5p, MLY 745 and MLY 758 (strains with the genomic *MYO5* promoter replaced with the *GAL1* promoter) were transformed with pPGAL1-GST-URA3-*CMD1* and pPGAL1-GST-TRP1-*CMD1* respectively. A control lacking a *GAL1* promoter upstream of *MYO5* (Y 258 transformed with pPGAL1-GST-URA3-*CMD1*) was included. Cells were grown in 2-liter of CSM-Ura^- (MLY 745, Y 258) or CSM-Trp^- (MLY

758) with 1% raffinose as the sole carbon source. Once cultures reached an OD595 of 0.3, overexpression was induced by addition of 2% galactose and further growth for 12 h. Cells were harvested and lysed as described in the Myo2-head-GST purification. Native Myo5-GST and overexpressed GST-Cmd1p (and accompanying Myo5p) were partially purified by binding to and elution from glutathione-Sepharose and then dialyzed vs. 0.5 M KCl, 10 mM imidazole, pH 7.0, and 1 mM DTT. The concentration of Myo5p generated by overexpression was estimated by densitometry of Coomassie-stained bands after SDS/PAGE using rabbit skeletal muscle myosin as the standard.

Skeletal muscle actin and myosin. Actin was purified from rabbit skeletal muscle acetone powder (6). Actin filaments were formed by addition of 50 mM KCl and 1 mM $MgCl_2$. Fluorescent actin filaments were prepared for motility assays by polymerizing 5 μM actin in 5 μM rhodamine-phalloidin. Rabbit skeletal muscle myosin was purified as described in ref. 7.

ATPase and *in Vitro* Motility Assays. Actin-activated myosin ATPase assays were carried out in 2 mM Tris·HCl (pH 7.2), 10 mM imidazole, 100 mM KCl, 0.1 mM $CaCl_2$, 3 mM $MgCl_2$, 2 mM ATP, and 1 mM DTT (3) with the following concentrations of proteins: 200 nM Myo2; 200 nM Myo2-head-GST; 800 nM GST-Rng3p or GST-UCSp; 12–18 μg/ml partially purified Myo5-GST (expressed from its native promoter); 50–75 nM overexpressed Myo5p; 0.5–120 μM actin filaments. Malachite green was used to quantitate P_i release (8). Controls omitting myosin were used and basal activity (detected in controls lacking actin) was subtracted to derive actin-activated ATPase rates.

High salt ATPase assays in 0.5 M KCl, 10 mM imidazole (pH 7.2), 2 mM ATP with either 10 mM $CaCl_2$, 10 mM $MgCl_2$, or 2 mM EDTA were carried out with the same concentrations of native (Myo5-GST) and overexpressed Myo5p as those used in actin-activated assays. All ATPase experiments with Myo5p included control samples derived from cells lacking an integrated GST tag (native Myo5p-GST assays), or lacking a *GAL1* promoter in front of genomic *MYO5* (overexpressed Myo5p assays).

We used *in vitro* motility assays based on the method of Kron and Spudich (9) with 50–1,500 nM Myo2 or Myo2-head-GST delivered into motility chambers along with 500–2,000 nM GST-Rng3p or GST-UCSp as indicated. After myosin adhered to the cover-slip surface for 10 min, the chamber was washed successively with 20 μl of (*i*) running buffer [25 mM imidazole (pH 7.4), 25 mM KCl, 4 mM $MgCl_2$, 1 mM ATP, and 100 mM DTT], (*ii*) running buffer plus 1 mg/ml BSA for 2 min, and (*iii*) two successive washes with running buffer with 10 nM rhodamine-phalloidin labeled actin filaments. Filaments were observed by epifluorescence microscopy with a 60× objective and recorded at intervals of 2 s with an Orca-ER (Hamamatsu) cooled CCD camera. Image J software was used to calculate filament velocities of 30 filaments in each time-lapse series. Overexpressed Myo5p was applied to chambers at a concentration of 400 nM. At this concentration Myo5p-driven motility was only apparent when 0.5% methyl-cellulose was included in the running buffer.

Immunoblots. Overexpressed Myo2 was enriched from wild-type (MLP 509) and *rng3-65* (MLP 586) cell extracts. Native Myo5-GST was enriched from JC 1284 (control), MLY 697, and MLY 720 cell extracts (as described above). After SDS/PAGE proteins were transferred to nitrocellulose (10). The Myo2p heavy chain was detected using rabbit anti-Myo2p tail antibodies [diluted 1:1,000 in PBS containing 0.1% Tween-20 (Sigma–Aldrich)]. Ubiquitinated Myo2p was detected using rabbit anti-ubiquitin antibodies (Sigma–Aldrich) diluted 1:100. Myo5p-GST was detected using a rabbit anti-GST primary antibody (Clontech) diluted 1:500. The secondary antibody was mouse anti-rabbit HRP-conjugated antibody (Sigma–Aldrich; diluted 1:4,000). To judge the solubility of Myo5p-GST in the MLY 697 and MLY 720 cell extracts, supernatant and pellet samples (resuspended in the same volume) were taken before enrichment with glutathione Sepharose. Samples of ≈20 μg of protein were mixed with heated SDS sample buffer, boiled for 10 min, separated by SDS/PAGE, and blotted.

In pulse–chase experiments Myo5p-GST expression was induced in MLY 801 and 802 strains by addition of 2% galactose to cells grown to an OD_{595} of 0.8 at 25°C in YP media containing 1% raffinose. Cells were harvested 1 h after addition of galactose and a sample (time 0) was taken. Cells were then washed once in YPDa media and then diluted in YPDa to yield an OD_{595} of 1. Cells were subsequently harvested at 10- to 90-min time points (recorded after transfer of YPDa cultures into the incubator) to generate soluble cell extracts for SDS/PAGE and immunoblotting with α-GST antibodies.

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Table S1. ATPase and *in vitro* motility activities of Myo2-head-GST and Myo2

Property	Myo2-head-GST	Myo2
K^+ Ca^{2+} ATPase, s^{-1}	3.9 ± 0.5	0.8 ± 0.1
Basal ATPase, s^{-1}	0.09 ± 0.03	0.06 ± 0.01
Actin-activated Mg^{2+} ATPase		
V_{max},* s^{-1}	1.05	0.38
K_{ATPase},* μM	30.8	10.7
+Rng3p V_{max},† s^{-1}	0.99 (1.10)	0.36 (0.34)
+Rng3p K_{ATPase},† μM	13.9 (26.9)	5.2 (7.8)
+Rng3p UCS V_{max},† s^{-1}	1.11 (1.03)	0.35 (0.33)
+Rng3p UCS K_{ATPase},† μM	42.6 (35.0)	13.9 (11.7)
In vitro motility, μm s^{-1}	0.22 ± 0.05	0.40 ± 0.05
rng3$^+$,‡ *25°C*	—	0.45 ± 0.05
rng3$^+$,‡ *36°C*	—	0.44 ± 0.06
rng3-65,‡ 25°C	—	0.34 ± 0.12
rng3-65,‡ 36°C	—	0.32 ± 0.10

ATPase activities: molecules of ATP hydrolyzed per myosin head per second (s^{-1}).

*The average maximal rate and K_{ATPase} (actin filament concentration required for half-maximal rate) were derived from five separate experiments used to generate the curves presented in Fig. 2*B*.

†V_{max} and K_{ATPase} values were derived as averages from curves used in Fig. 3 *A–D*. Three separate datasets were averaged to derive each curve. Activities measured in the presence of GST-Rng3p (or GST-UCSp) were recorded simultaneously with control measurements (given in parentheses) lacking GST-Rng3p or GST-UCSp.

‡Motility rates for Myo2 generated from wild-type and *rng3-65* strains after induction of overexpression at permissive (25°C) and restrictive (36°C) growth conditions (Fig. 4).

Table S2. ATPase activities for native budding yeast myosin-I (Myo5p-GST)

Property	Myo5p activity, nmol/min/mg		
	Control (no tag)	*MYO5-GST*	*MYO5-GST she4Δ*
K^+ Mg^{2+} ATPase	0	10	0 (0)
K^+ EDTA ATPase	0	191	26 (182)
Actin-Mg^{2+} ATPase*	0	87	14 (98)

ATPase activities are shown as nanomoles of ATP hydrolyzed per minute per milligram of protein ($nmol \cdot min^{-1} \cdot mg^{-1}$). Samples were generated by enriching Myo5-GST from soluble cell extracts of JC 1284 (wild type), MLY 697 (*MYO5-GST*), and MLY 720 (*she4Δ MYO5-GST*). Values reflect averages generated from two independent experiments using different segregants. Numbers given in parentheses represent normalized activities accounting for the relative levels of soluble Myo5-GST enriched from wild-type and *she4Δ* strains (see Fig. 5*F*).

*Activity measured using a final concentration of 15 μ M actin filaments.

Table S3. ATPase and *in vitro* motility activities for budding yeast myosin-I (Myo5p)

Property	p*GAL1-GST-CMD1* Control	p*GAL1-GST-CMD1* *GAL1-MYO5*	p*GAL1-GST-CMD1* *GAL1-MYO5 she4Δ*
K^+ Mg^{2+} ATPase, s^{-1}	ND	0.17 ± 0.10	0.15 ± 0.10
nmol·min^{-1}·mg^{-1}	0.00	19.20 ± 11.41	16.2 ± 10.2
K^+ Ca^{2+} ATPase, s^{-1}	ND	1.31 ± 0.09	1.98 ± 0.16
nmol·min^{-1}·mg^{-1}	2.34 ± 1.2	150.0 ± 0.06	226.8 ± 0.11
K^+ EDTA ATPase, s^{-1}	ND	7.59 ± 0.46	11.84 ± 1.34
nmol·min^{-1}·mg^{-1}	95.2 ± 12.0	868.8 ± 52.2	1355.4 ± 152.9
Basal ATPase (s^{-1})	ND	0.32 ± 0.03	0.48 ± 0.04
nmol·min^{-1}·mg^{-1}	10.8 ± 1.8	36.0 ± 4.2	55.2 ± 4.8
Actin-Mg^{2+} ATPase, s^{-1}*	ND	2.84 ± 0.15	3.87 ± 0.16
nmol·min^{-1}·mg^{-1}*	72.6 ± 12.0	355.2 ± 19.2	489.0 ± 20.4
V_{max}, s^{-1}	ND	3.87	4.99
K_{ATPase}, μM	ND	13.5	10.0
Motility, μm·s^{-1}	ND	0.30 ± 0.06	0.46 ± 0.11

ATPase activities are shown as molecules of ATP hydrolyzed per myosin head per second (s^{-1}), and nanomoles of ATP hydrolyzed per minute per mg of protein nmol·min^{-1}·mg^{-1}. V_{max} and K_{ATPase} (actin filament concentration required for half-maximal velocity) values were derived from curves in Fig. 6*B*. ND, not determined.
*Activity measured using a final concentration of 40 μM actin filaments.

Table S4. Yeast strains employed in this study

Strain	Genotype	Source
	Fission yeast	
TP 150	*h⁻ leu1-32*	M. Yanagida
MLP 374	*h⁻ leu1-32 ura4-D18 kan^R:3nmt1prom-myo2*	1
MLP 479	*h⁻ leu1-32 ura4Δ:kan^R*	This study
MLP 509	*h⁻ leu1-32 ura4Δ:kan^R nat^R:41nmt1prom-myo2*	1
MLP 569	*h⁻ leu1-32 ade6-216 rng3-65*	M. Balasubramanian
MLP 570	*h⁻ leu1-32 ade6-216 rng3-65 ura4Δ:kan^R*	This study
MLP 586	*h⁺ leu1-32 his7-366 ura4Δ:kan^R ade2-M216 rng3-65 nat^R:41nmt1prom-myo2*	1
MLP 660	*h⁻ leu1-32 rng3-3xYFP:kan^R*	1
MLP 665	*h⁻ leu1-32 ade6-M210 rng3-3xYFP:kan^R kan^R:CFP-myo2*	1
TP 60	*h⁺ leu1-32 ade6-M216 ura4-D18 myo2-E1*	M. Balasubramanian
MLP 683	*h⁺ leu1-32 ura4-D18 myo2-E1 rng3-3xYFP:kan^R*	This study
YDM 429	*h⁺ leu1-32 ade6-M210 ura4-D18 sid2-250*	D. McCollum
MLP 770	*h⁺ leu1-32 ura4-D18 rng3-3xYFP:kan^R kan^R:CFP-myo2 sid2-250*	This study
	Budding yeast	
JC 1284	*MATα leu2-3 his3-11 ura3-52 lys2 trp1-1*	J. Chant
JC 1401	*MATα leu2-3 his3-11 ura3-52 ade2-1 trp1-1 she4Δ::URA3*	J. Chant
Y 258	*MATa leu2-3 his4-580 ura3-52 pep4-3*	D. Gelperin
MLY 697	*MATα leu2-3 his3-11 ura3-52 lys2 trp1-1 MYO5-GST:kan^R*	This study
MLY 701	*MATα leu2-3 his3-11 ura3-52 lys2 trp1-1 MYO5-GFP:kan^R*	This study
MLY 720	*MATα leu2-3 his3-11 ura3-52 trp1-1 she4Δ::URA3 MYO5-GST:kan^R*	This study
MLY 724	*MATa leu2-3 his3-11 ura3-52 ade2-1 trp1-1 she4Δ::URA3 MYO5-GFP:kan^R*	This study
MLY 745	*MATa leu2-3 his4-580 ura3-52 pep4-3 kan^R:GAL1prom-MYO5*	This study
MLY 758	*MATα leu2-3 his3-11 ura3-52 ade2-1 trp1-1 she4Δ::URA3 kan^R:GAL1prom-MYO5*	This study
MLY 801	*MATα leu2-3 his3-11 ura3-52 lys2 trp1-1 HIS5:GAL1prom-MYO5-GST:kan^R*	This study
MLY 802	*MATα leu2-3 his3-11 ura3-52 trp1-1 she4Δ::URA3 HIS5:GAL1prom-MYO5-GST:kan^R*	This study

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Table S5. Plasmids employed in this study

Plasmid	Comment	Source
pFA6a-GFP-kanMX6	—	1
pFA6a-GST-kanMX6	—	1
pFA6a-kanMX6-PGAL1	—	1
pFA6a-3xGFP-kanMX6	—	W.-L. Lee and J. Cooper
	Fission yeast constructs	
pDS573a	*3-nmt1* promoter, N-terminal *GFP*, *ura4*	S. Forsburg
pDS573a-*81-nmt1*	*81nmt1* promoter in pDS573a	This study
p3xGFP	pDS573a-*81-nmt1* with N-terminal *3xGFP*	This study
p3xGFP-*rng3*	Encoding full-length Rng3p (amino acids 1–747)	This study
p3xGFP-*central*	Encoding "central" domain of Rng3p (bp 1–996; amino acids 1–332)	This study
p3xGFP-*UCS*	Encoding UCS domain of Rng3p (bp 997-2241; amino acids 333–747)	This study
pDS473-LEU2	*3nmt1* promoter, N-terminal *GST*, *LEU2*	2
pDS473-LEU2-*cdc4*	*cdc4* in pDS473-LEU2	2
pDS473-LEU2-*rlc1*	*rlc1* in pDS473-LEU2	This study
pDS472a	*3nmt1* promoter, C-terminal *GST*, *ura4*	S. Forsburg
pDS472a-*myo2-head*	Encoding the Myo2p head (bp 1–2445; amino acids 1–815)	This study
	Budding yeast constructs	
pRS-314, −316	*TRP1*, *URA3*, low copy, centromeric vectors	3
pPGAL1-GST-TRP1, -URA3	*GAL1* promoter-*GST* in *pRS-314*, *-316*	This study
pPGAL1-GST-*CMD1*-TRP1	*GAL1* promoter-*GST-CMD1* in *pRS-314*	This study
pPGAL1-GST-*CMD1*-URA3	*GAL1* promoter-*GST-CMD1* in *pRS-316*	This study

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2. Lord M, Pollard TD (2004) UCS protein Rng3p activates actin filament gliding by fission yeast myosin-II. *J Cell Biol* 167:315–325.
3. Sikorski RS, Hieter P (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics* 122:19–27.