Tropomyosin and myosin-II cellular levels promote actomyosin ring assembly in fission yeast

Benjamin C. Stark*, Thomas E. Sladewski*, Luther W. Pollard, & Matthew Lord†

Department of Molecular Physiology & Biophysics
University of Vermont
Burlington, VT 05405.

*These authors contributed equally to the work
†Corresponding author: matthew.lord@uvm.edu; Tel. 802-656-9898; Fax. 802-656-0747.

Running Head: Tropomyosin regulates S. pombe myosin-II
Materials and Methods

Fission yeast strains and genetic methods
The strains employed in this study are listed in Supplementary Table S1. Strains were constructed by back-crossing or genomic integrations using homologous recombination (Bahler et al., 1998; Moreno, 1991). To integrate two copies of YFP-myo2 into a haploid a myo2+/myo2Δ::YFP-ura4+ heterozygote diploid (Sladewski et al., 2009) was transformed with the LEU2+ pYFP-myo2 plasmid (Sladewski et al., 2009), sporulated, and haploid segregants isolated by random spore analysis. myo2Δ::YFP-ura4+ haploids harboring pYFP-myo2 were isolated by screening segregants for growth on EMM-Ura–Leu– plates. A YFP-myo2 fragment (including the myo2 terminator) was then amplified from pYFP-myo2 using 5’ YFP and 3’ myo2 terminator cloning primers. The fragment was transformed and integrated into the myo2Δ::YFP-ura4+ haploid (harboring pYFP-myo2) in place of ura4+. Cells were grown over-night on YE5S plates and then replica-plated to minimal media plates containing 0.1 % 5-fluoroorotic acid (5-FOA) to isolate ura– colonies. Most ura– colonies no longer carried the pYFP-myo2 plasmid and possessed an integrated form of YFP-myo2 (as confirmed by visualization of YFP-labeled contractile rings by fluorescence microscopy and diagnostic PCRs of genomic DNA). A sub-population of the ura4+ colonies were LEU2+. However, of all LEU2+ isolates tested, none harbored pYFP-myo2 since cells lacked the cytokinesis defects associated with elevated Myo2p expression from this multi-copy plasmid, and yielded 2:2 leu+LEU2+ tetrad progeny upon back-crossing with a wild-type leu– haploid. leu+ progeny from these crosses lacked YFP-labeled contractile rings, whereas the LEU2+ progeny exhibited YFP-labeled rings that were much brighter than those in ura– leu– YFP-myo2 cells. This pattern of segregation indicated that the integrated LEU2+ marker was linked to the myo2 locus. The integration of a second copy of YFP-myo2 originating from the pYFP-myo2 LEU2+ plasmid was confirmed by diagnostic PCR of genomic DNA.

Plasmids
The plasmids employed in this study are listed in Supplementary Table S2. To construct the pGST-cdc8 plasmid the cdc8 ORF was PCR-amplified from a genomic DNA library using 5’ NotI-cdc8: GCGGCCGCATGGATAAGCTTAGAGAGGTATGAG and 3’ SalI-cdc8: GTCGACTCTACAATTCCTCAAGAGGTTGTAAC and the iproof DNA polymerase. The fragment was sub-cloned into a TOPO vector, cut with NotI and SalI and then ligated into NotI/SalI lineraized pDS473a (nmt1 inducible promoter, LEU2 marker). The fidelity of the construct was confirmed by DNA sequencing. prlc-ura4+ is a genomic clone spanning the rlc1 promoter and ORF from a pUR19 genomic DNA library.

Protein purification
Myo2p was purified as previously described (Lord and Pollard, 2004; Sladewski et al., 2009). Cdc8p was purified following over-expression in fission yeast as a GST-fusion using the maximum-strength nmt1 inducible promoter. Liquid cultures were grown to saturation in EMM Leu– with 5 µg/ml of thiamine. Cells were harvested and washed three times in EMM Leu– medium and diluted to an optical density at 595 nm (OD595) of ~0.05 in 4 liters of the same medium. Over-expression was achieved by 24–28 hours of growth at 32°C, by which time the OD had reached ~3. Cells were harvested and washed once in water and once in ice-cold lysis buffer (750 mM KCl, 25 mM Tris-HCl, pH 7.4, 4 mM MgCl2, 20 mM Na3P2O7, 2 mM EGTA, and 0.1% Triton X-100). Pellets were resuspended in an equal volume of ice-cold lysis buffer.
with additives consisting of 1 mM DTT, 4 mM ATP, 2 mM PMSF, and complete EDTA-free protease inhibitors (Roche). Cells were lysed by glass bead beating with a Fastprep bead beater (MP Biochemicals). The lysate was centrifuged at 500 g for 5 min to remove unlysed cells and beads and further centrifuged at 100,000 g for 45 min to remove insoluble matter. The supernatant was batch incubated with 1.5 ml of glutathione-Sepharose (Amersham Biosciences) for 90 min, and then transferred to a 20 ml column. The bound sample was washed with lysis buffer with additives (4 × 15 ml) and eluted in 5 ml of lysis buffer plus additives and 10 mM glutathione. Following thorough dialysis into A15 buffer (0.5 M KCl, 10 mM imidazole, pH 7.0, 10 mM EDTA, 1 mM DTT, and 0.3 mM NaN₃) the sample was treated with thrombin to detach GST. GST was removed by overnight batch incubation with glutathione-Sepharose followed by 3-4 slow passages of the sample over the same resin after packing it into a column. Flow-through consisted of Cdc8p which was purified then passed over 0.2 ml of benzamidine Sepharose to remove thrombin. Actin was purified from rabbit skeletal muscle acetone powder (Spudich and Watt, 1971). Actin filaments were formed by addition of 50 mM KCl and 1 mM MgCl₂.
Supplemental Figures and Legends

**Figure S1. Contractile ring dynamics in cdc8-27 and myo2-E1 mutants.**
Time-lapse images of cells captured every 2 minutes by fluorescence microscopy. The montages compare Rlc1p-GFP/YFP-Myo2p ring dynamics in myo2-E1, cdc8-27, and cdc8-27 2xYFP-my2 strains. Bar: 4 μm. Rings from these series were used to generate kymographs presented in Figure 5A.

**Figure S2. cdc8-27 and myo2-E1 mutations delay the timing of contractile ring assembly and constriction.** Time-lapse series of single cells (A) and their accompanying plots (B) charting ring dynamics (dwell and constriction phases) in relationship to spindle elongation and breakdown during mitosis. A) Time-lapse images of wild-type, cdc8-27, and myo2-E1 cells (captured every 3 minutes for 1 hour in these particular examples) are shown following spindle pole body (SPB) separation. In each case contractile rings are marked by Rlc1p-GFP and SPBs by Sad1p-GFP. B) For each of the representative cells shown in A the timing of contractile ring assembly and constriction is monitored by overlaying ring diameter (filled circles) with corresponding SPB separation distances (diamonds).

**Figure S3. Purification of functional fission yeast tropomyosin.**
Fission yeast purified Cdc8p binds actin filaments. Lane 1: molecular weight markers. Cdc8p remains in the supernatant following ultracentrifugation in the absence of actin filaments (left lanes) and co-pellets with actin filaments (right lanes).
Supplementary Movie Legends

**Movie 1.** Myo2p-driven filament gliding from *in vitro* motility assays using bare actin filaments (*left*) versus Cdc8p-actin filaments (*right*). Myo2p was attached to nitrocellulose-coated coverslips at a concentration 32 μg/ml. Filaments are labeled with rhodamine-phalloidin. The movies were generated from time-lapse images recorded at 2 s intervals (sped up x20).

**Movie 2.** As in Movie 1, only Myo2p was attached to cover-slips at 0.8 μg/ml.
### Table S1. Fission yeast strains.

| Strain | Genotype | Source |
|--------|----------|--------|
| MLY 29 | *h'*:*leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-216 his3-D1/his3-D1 myo2/myo2Δ:*"YFP-ura4* | (Sladewski et al., 2009) |
| MLY 66 | *h'*:*leu1-32 ura4-D18 ade6 his3-D1 *"YFP-myo2* | (Sladewski et al., 2009) |
| MLY 140 | *h'*:*leu1-32 ura4-D18 ade6 his3-D1 *"YFP-myo2 sad1-*"GFP:kan* | This study |
| MLY 273 | *h'*:*leu1-32 ura4-D18 ade6 his3-D1 2X"YFP-myo2* | This study |
| MLY 275 | *h'*:*leu1-32 ura4-D18 ade6 his3-D1 2X"YFP-myo2 sad1-*"GFP:kan* | This study |
| MLY 402 | *h'*:*leu1-32 ura4-D18 ade6 his3-D1 myp2Δ:*"his7* "YFP-myo2 | (Sladewski et al., 2009) |
| MLY 400 | *h'*:*leu1-32 ura4-D18 ade6 his3-D1 myp2Δ:*"his7* 2X"YFP-myo2* | This study |
| MLY 463 | *h'*:*leu1-32 ura4-D18 ade6 his3-D1 *"YFP-myo2 rlc1-*"CFP: kan* | This study |
| MLY 464 | *h'*:*leu1-32 ura4-D18 ade6 his3-D1 cdc8-27 *"YFP-myo2* | This study |
| MLY 466 | *h'*:*leu1-32 ura4-D18 ade6 his3-D1 cdc8-27 2X"YFP-myo2* | This study |
| MLP 319 | *h'*:*leu1-32 ura4-D18 ade6-M216 his3-D1 myo2-E1 rlc1-*"GFP:kan* | This study |
| MLY 572 | *h'*:*leu1-32 ura4-D18 ade6-M216 his3-D1 rlc1-*"GFP:kan* sad1-*"GFP:kan* | This study |
| MLY 580 | *h'*:*leu1-32 ura4-D18 ade6-M216 his3-D1 cdc8-27 rlc1-*"GFP:kan* sad1-*"GFP:kan* | This study |
| MLY 570 | *h'*:*leu1-32 ura4-D18 ade6-M216 his3-D1 myo2-E1 rlc1-*"GFP:kan* sad1-*"GFP:kan* | This study |
| MLY 590 | *h'*:*leu1-32 ura4-D18 ade6 his3-D1 cdc8-27 2X"YFP-myo2 sad1-*"GFP:kan* | This study |
| MLY 639 | *h'*:*leu1-32 ura4-D18 ade6 his3-D1 2X"YFP-myo2 rlc1-*"CFP: kan* sad1-*"GFP:kan* | This study |
| TP 150 | *h'*:*leu1-32 | M. Yanagida |
| MLY 83 | *h'*:*leu1-32 ura4-D18 nat*:"41nmt1 prom-FLAG-myo2* | (Sladewski et al., 2009) |
| Plasmid               | Comment                                                        | Source                        |
|----------------------|----------------------------------------------------------------|-------------------------------|
| pGST-cdc4            | pDS473a-based (nmt1 promoter, LEU2 marker)                      | (Lord and Pollard, 2004)      |
| pGST-rlc1            | pDS473a-based (nmt1 promoter, ura4+ marker)                     | (Lord and Pollard, 2004)      |
| pGST-cdc8            | pDS473a-based (nmt1 promoter, LEU2 marker)                      | This study                    |
| pFA6a-nat8-nmt41prom-FLAG | pFA-nat8-nmt41prom with N-terminal FLAG tag                   | (Sladewski et al., 2009)      |
| KS-ura4              | template for gene replacement with ura4+                        | (Bahler et al., 1998)         |
| pYFP-myo2            | YFP-myo2 fusion in pDS473a-based vector (LEU2 marker); includes myo2 promoter and terminator | (Sladewski et al., 2009)      |
| pYFP-ura4+           | myo2 promoter-YFP-ura4+-myo2 terminator cassette in pDS473a-LEU2 vector; for YFP-myo2 integrations | (Sladewski et al., 2009)      |
| prlc1-ura4+          | rlc1 genomic clone on a ura4+ multi-copy plasmid                | This study                    |
References

Bahler, J., J.Q. Wu, M.S. Longtine, N.G. Shah, A. McKenzie, 3rd, A.B. Steever, A. Wach, P. Philippsen, and J.R. Pringle. 1998. Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. *Yeast*. 14:943-51.

Lord, M., and T.D. Pollard. 2004. UCS protein Rng3p activates actin filament gliding by fission yeast myosin-II. *J Cell Biol*. 167:315-25.

Moreno, S., Klar, A., Nurse, P. 1991. Molecular Genetic Analysis of Fission Yeast *Schizosaccharomyces pombe*. *Methods Enzymol*. 194:795-823.

Sladewski, T.E., M.J. Previs, and M. Lord. 2009. Regulation of fission yeast myosin-II function and contractile ring dynamics by regulatory light-chain and heavy-chain phosphorylation. *Mol Biol Cell*. 20:3941-52.

Spudich, J.A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J Biol Chem*. 246:4866-71.
myo2-E1  rlc1-GFP

cdc8-27  1xYFP-myo2

cdc8-27  2xYFP-myo2
Stark et al.
Figure S2.

A

wild-type

cdc8-27

myo2-E1

B

wild-type

cdc8-27

myo2-E1
Stark et al.
Figure S3.