**Supplementary materials**

Fission yeast Myo2: molecular organization and diffusion in the cytoplasm

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**Supplemental experimental methods**

*In vivo labeling*. The wild-type strain (TP 150) or strains over expressing Myo2p (ML 386 or 387) were transformed with pGST-Rlc1 or pGST-Cdc4 plasmids (Lord and Pollard 2004) to over express tagged light chains and grown to log phase in phosphate-deficient media. Cells were washed and resuspended to $OD_{595}$ of 1 in phosphate-deficient EMM medium (EMMP), labeled 4 h with 1 mCi of $^{32}PO_4$ (Amersham), harvested by filtration and lysed with glass beads. Lysate was cleared by centrifugation at 14,000 x g for 15 min; Myo2 was affinity selected on glutathione-Sepharose (GE Healthcare) and eluted at 95°C with SDS-PAGE denaturing sample buffer. Samples were run on a 10% SDS-PAGE gel and $^{32}P$ was visualized by autoradiography.
*Phosphospecific antibody*. 21st Century Biochemicals (Boston, MA) immunized 2 rabbits with 6 injection cycles of peptides CLKGAEV[phosphoS]PQPTGQS and LKGAEV[phosphoS]PQPTGQSC. Sera from the final two bleeds were depleted with dephosphorylated peptides and then affinity purified with the phosphorylated peptides. For Western blots, proteins were transferred from SDS gels to nitrocellulose membranes, blocked with 5% BSA in Tris-buffered saline with 1% TWEEN-20 with 50 mM NaF and 100 μM Na $NaVO_4$ and incubated 1 h with antibody diluted 1:1500 in 1% BSA Tris-buffered saline. The blots were washed and reacted with secondary antibody (HRP-conjugated goat α-rabbit A6154, Sigma) before visualizing by chemiluminescence.

*Mass spectrometry*. Purified phosphorylated Myo2 was dialyzed into 50 mM $NH_4HCO_3$ pH 8.0, and then reduced with 5 mM DTT and alkylated with 15 mM iodoacetamide. Samples were digested overnight at 37°C with one of three proteases: (i) 1:20 ratio of trypsin (Promega Gold) to Myo2 in 60% methanol; (ii) 1:25 ratio of LysC or (iii) 1:25 ratio of AspN (Promega) in 1 M urea and 10 mM methylamine. Peptides were analyzed on an Orbitrap LTQ LC/MS/MS at the Yale Proteomics Facility. Overall coverage exceeded 75%.

**Supplemental results**

*Myo2 is phosphorylated in vivo*

As reported previously (Loo and Balasubramanian 2008; Lord and Pollard 2004; McCollum et al. 1999; Sladewski et al. 2009) the Myo2p heavy chain and both light chains are phosphorylated when Myo2 is isolated from unsynchronized *S. pombe* cells grown in radioactive $^{32}PO_4$ (Figure 2A). We used mass spectrometry to confirm that Myo2 is phosphorylated on S1444 (Figure S2), as reported by Sladewski et al. (Sladewski et al. 2009). Five separate experiments using LC/MS/MS to analyze peptides produced by tryptic, LysC and AspN digestion conclusively identified S1444 as a phosphorylation site, with significant peptide identifications ($p < 0.005$). Other than S1444 no Myo2p heavy chain phosphopeptides were observed consistently. In particular we did not detect phosphorylation of S1505 or S1518, which were suggested to be possible phosphorylation sites (Motegi et al. 2004; Mulvihill et al. 2001b).

When isolated under conditions that protect from dephosphorylation (20 mM sodium phosphate, 50 mM NaF, 100 μM $NaVO_4$ and 20 mM β-glycerophosphate), the Myo2p heavy chain of reacted with a phosphospecific dye (ProQ Diamond, Molecular Probes) and with purified rabbit polyclonal antibodies specific for phosphorylated S1444 in a peptide from Myo2p (Figure S3B lane 1). We refer to these preparations of Myo2 as "phosphorylated", although the available material did not allow for chemical measurement of the phosphate content. Myo2 isolated without precautions to prevent dephosphorylation did not react with the phosphospecific dye on blots and reacted weaker with the anti-phospho-S1444 antibody (Figure S3B lane 2). We call these preparations "dephosphorylated Myo2". We confirmed the specificity of the antibodies for phosphorylated S1444 using unphosphorylated recombinant Myo2p tail protein fragments produced in *E. coli* as a negative control.

Figure S1

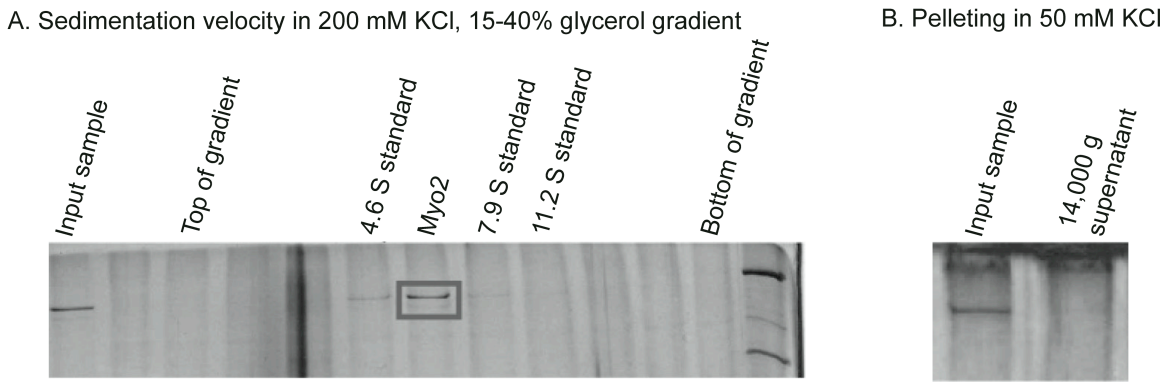


Figure S1. A. Fractions from a sedimentation velocity experiment analyzed by SDS-PAGE and silver staining. A sample of 2 $\mu$M Myo2 in 200 mM KCl, 10 mM imidazole pH 7.0 was applied to the top of a linear gradient of 15-40% glycerol and centrifuged at 48,000 rpm for 18 h in an SW55Ti swinging bucket rotor. The input sample is shown in the first lane followed by fractions from the gradient. Standards were run in separate tubes under identical conditions. B. Pelleting experiment with 2 μM Myo2 in 50 mM KCl, 10 mM imidazole pH 7.0 centrifuged at 14,000xg for 20 min. Lane 1 is the starting sample. Lane 2 shows the absence of Myo2 in the supernatant.

Figure S2

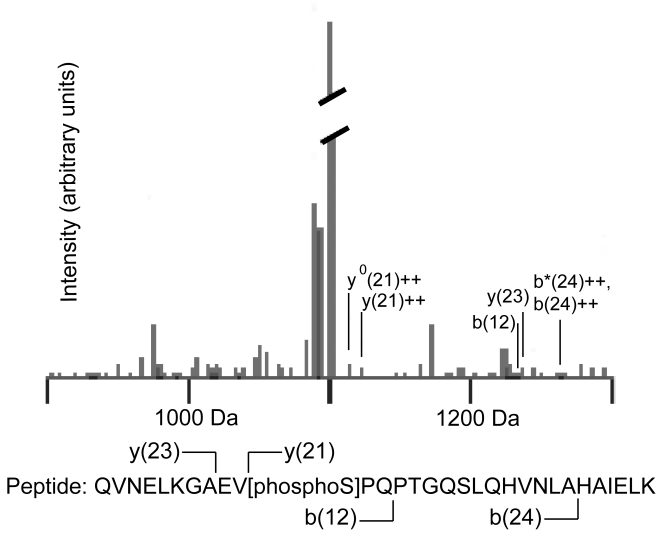


Figure S2. Identification of S1444 as a phosphorylation site on the Myo2 heavy chain. A. Excerpt from a LC/MS/MS spectrum of fragments a Myo2p peptide created by AspN digestion and including phosphorylated S1444. The sequence of the peptide is given below with labels (for example y(23)) corresponding to the observed MS/MS fragments. High-probability ($p < 0.005$) identifications of S1444 phosphorylation were obtained on five separate runs; overall coverage exceeded 75% of the entire sequence.

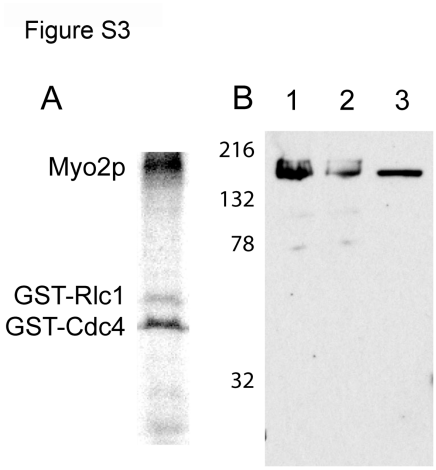


Figure S3. Phosphorylation of Myo2. All samples were prepared by SDS polyacrylamide gel electrophoresis. A. Autoradiogram of Myo2 with GST-tagged light chains isolated by affinity pull down from cells grown in $^{32}P$. Myo2p is the heavy chain, GST-Rlc1 is the regulatory light chain tagged with GST and GST-Cdc4 is the essential light chain tagged with GST. B Immunoblot of equal amounts of purified Myo2 reacted with antibodies specific for phosphorylated S1444. Lane 1: Myo2 purified under conditions that protect from dephosphorylation; Lane 2: Myo2 purified under dephosphorylating conditions; Lane 3: Myo2 incubated with 50 nM SpCmk1, ATP and $Ca^{2+}$/calmodulin.