UCS Protein Rng3p Is Essential for Myosin-II Motor Activity during Cytokinesis in Fission Yeast

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Abstract

UCS proteins have been proposed to operate as co-chaperones that work with Hsp90 in the de novo folding of myosin motors. The fission yeast UCS protein Rng3p is essential for actomyosin ring assembly and cytokinesis. Here we investigated the role of Rng3p in fission yeast myosin-II (Myo2p) motor activity. Myo2p isolated from an arrested rng3-65 mutant was capable of binding actin, yet lacked stability and activity based on its expression levels and inactivity in ATPase and actin filament gliding assays. Myo2p isolated from a myo2-E1 mutant (a mutant hyper-sensitive to perturbation of Rng3p function) showed similar behavior in the same assays and exhibited an altered motor conformation based on limited proteolysis experiments. We propose that Rng3p is not required for the folding of motors per se, but instead works to ensure the activity of intrinsically unstable myosin-II motors. Rng3p is specific to conventional myosin-II and the actomyosin ring, and is not required for unconventional myosin motor function at other actin structures. However, artificial destabilization of myosin-I motors at endocytic actin patches (using a myo1-E1 mutant) led to recruitment of Rng3p to patches. Thus, while Rng3p is specific to myosin-II, UCS proteins are adaptable and can respond to changes in the stability of other myosin motors.

Introduction

Unc-45/Cro1/She4 (UCS) proteins are a family of myosin motor regulators that are conserved across eukaryotes [1]. UCS proteins typically contain a C-terminal UCS domain that binds myosin motors, a variable central domain that may play a role in oligomerization [2], and an N-terminal tetratricopeptide repeat (TPR) domain that interacts with the Hsp90 chaperone (Figure 1) [3]. Interaction between the UCS protein and Hsp90 has been shown to be important for the latter stages of the de novo folding of muscle myosin-II motors [4,5]. Here the UCS protein may act as a co-chaperone that accelerates motor folding by bringing motors bound at the UCS domain into close contact with Hsp90 bound by the TPR domain [1,2,4].

Defects in UCS protein function have been associated with various diseases including arteriovenous malformation, cardiomyopathy, and cancer [6–9]. Studies from C. elegans have shown that misregulated levels of UCS protein (Unc-45) expression result in improper myofibril organization in body wall muscle, where either lack or over-expression of Unc-45 results in a decrease in thick filament assembly and paralysis [10,11]. Similar defects are seen when UCS protein function is attenuated in zebrafish and Drosophila [9,12–14].

Interestingly, fungal UCS proteins lack a TPR domain (Figure 1), suggesting an alternative role for the UCS family beyond acting as co-chaperones for Hsp90. Any such role is unlikely unique to fungi given that C. elegans Unc-45 does not depend on its TPR domain for function in the cell [15]. An Hsp90-independent role may well define the major role of UCS proteins in the regulation of myosin. Indeed, Hsp90 was recently proposed to be inhibitory to C. elegans Unc-45 as the two proteins compete for binding to myosin-II [15]. Furthermore, UCS proteins alone can limit myosin motor aggregation in vitro [3,16,17] consistent with an Hsp90-independent role in motor stabilization.

Fission yeast (Schizosaccharomyces pombe) provides a versatile and tractable model to study myosin-II and its role in actomyosin ring function and cytokinesis [18,19]. Myo2p motor activity is required to drive contractile ring assembly following the accumulation of ring pre-cursors (nodes) at the medial division site in early mitosis [20,21]. Previous work on the fission yeast UCS protein (Rng3p) has shown that it is essential for actomyosin ring formation [22] and is capable of enhancing the in vitro motility and apparent actin affinity of purified Myo2p [23,24]. Use of different mutant alleles has shown that Rng3p works in the same genetic pathway as fission yeast Myo2p and Hsp90 [22,25], suggesting a role in Myo2p folding or stabilization.

Studies from budding yeast (Saccharomyces cerevisiae) and fission yeast have shown that the UCS proteins from both yeasts associate with all the myosin motors found in their respective system [26,27]. Nevertheless the budding yeast UCS protein is only required for the function of three of the five myosins: the type-I myosins Myo3p and Myo5p and the type-V myosin Myo4p [27,28]. While previous studies have implicated Rng3p in Myo2p

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Citation: Stark BC, James ML, Pollard LW, Sirotkin V, Lord M (2013) UCS Protein Rng3p Is Essential for Myosin-II Motor Activity during Cytokinesis in Fission Yeast. PLoS ONE 8(11): e79593. doi:10.1371/journal.pone.0079593

Editor: Takashi Toda, Cancer Research UK London Research Institute, United Kingdom

Received July 23, 2013; Accepted September 27, 2013; Published November 14, 2013

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Funding: This work was supported by National Institutes of Health-National Institute of General Medical Sciences NIH-GMS ROI: GM097193 (http://www.nigms.nih.gov/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.
myo1 mutagenized 5 kb EcoR1 fragment was transformed into a
This plasmid was generated by transferring the
Not plus light chain-binding domains: amino acids 1–815, bp 1–2445).
C-terminal GFP-tagged sub-fragment 1 form of Myo2p (motor
samples. The pMyo2p-S1-GFP plasmid was used to over-express a
P. anserina

Figure 1. Schematic showing relative size and domain organi-
zation of a sample of UCS proteins. UCS proteins are a highly
conserved family of proteins by virtue of the C-terminal myosin motor-
binding UCS domain (green). Higher eukaryotes have a TPR domain
(pink) at the N-terminus, whereas fungal homologs (e.g. S. cerevisiae
She4p, S. pombe Rng3p, and P. anserina Cro1p) lack this TPR domain.
The central domain (yellow) is the most variable domain between
family members. The H. sapiens UCS protein included here is the
striated muscle isoform.
doi:10.1371/journal.pone.0079593.g001

Materials and Methods

Yeast Strains and Plasmids

Yeast strains used in this study are listed in Table S1. All strains
were constructed by genomic integrations using homologous
recombination or back-crossing following established protocols
[29,30]. The myo1-E1 strain was constructed by introducing the
G308R mutation into the 5 kb EcoR1-fragment of the
myo1 gene (containing 619 nucleotides of 5′ UTR, ORF, and 646 nucleotides
of 3′ UTR) in pBluescript [31] by PCR-based mutagenesis. The
mutagenized 5 kb EcoR1 fragment was transformed into a
myo1Δura4+ strain, followed by 5-FOA selection to isolate
ura4+ transformants which integrated the mutated
myo1-E1 marker. Isolated transformants were verified
by PCR and sequencing.

Plasmids pGST-LEU2-cdc4, pGST-LEU2-rol1, and pGST-ura4-
rol1 [23] were employed to facilitate purification of Myo2p
samples. The pMyo2p-S1-GFP plasmid was used to over-express a
C-terminal GFP-tagged sub-fragment 1 form of Myo2p (motor
plus light chain-binding domains: amino acids 1–815, bp 1–2445).
This plasmid was generated by transferring the
Not1/Not1 myo2 fragment from pDS472a-Myo2head [24] into pDS572a.
The pMyo2p-S1-FLAG plasmid was used to over-express a C-terminal
FLAG-tagged S1 form of Myo2p. This plasmid was generated by first
amplifying a 3′ portion of the S1 coding region (bp 2128–2445) from pDS472a-Myo2head using a 5′ myo2 primer (centered
on a native KpnI site: CCAACAGGTACCTATGTGGAATC) and
a 3′ primer in which DNA encoding the FLAG tag (underlined)
and a stop codon was included upstream of the
Not1 cloning site at the
3′ end of the myo2 S1 sequence (5′ SalI ACGCTGCACT-
TCATTCGATCGATCGTCTTGTAGCTGCTCCGCCGGCC-
GGGGGTTGATAGAT): The KpnI/ SalI S1-FLAG fragment was
ligated into pDS472a-Myo2head in place of the
myo2-FLAG FL

Figure 2. Isolating Myo2p in the absence of functional Rng3p.
Full-length over-expressed Myo2p motors were purified from wild-type
(rng3– nmt41prom-myo2) and mutant (rng3–65 nmt41prom-myo2) cells
following growth at the permissive (25°C) or restrictive (37°C)
temperature. A) Western blot depicting Myo2p levels (upper panel)
from whole cell lysates following induction of Myo2p over-expression
(upon removal of thiamine, time zero) in rng3–65 cells. Cells were shifted
to the restrictive temperature at 16 hours post-induction, before any
appreciable Myo2p expression is detected by antibody (upper blot). The
lower blot shows loading controls (Hsp90 levels) for each sample.
Protein samples were normalized based on total protein concentration
of the lysates. The plot shown below tracks relative levels of Myo2p
detected versus time (post-induction). Relative density values for the
Myo2p bands were corrected using densities from accompanying
Hsp90 bands; final values were normalized whereby 1 is equal to the
maximum value. B) Western blots to determine the relative levels of
Myo2p produced in the different isolations. Blots were employed
(instead of Coomassie staining) to clearly distinguish the Myo2p
heavy chain bands in these crude samples. Upper blot: α-Myo2p depicting
the relative levels of Myo2p 1-step purified from cells shifted to 37°C
(or retained at the permissive growth temperature in accompanying
controls) at 16 hours post-induction. The levels shown are from
samples harvested at 28 hours post-induction. Samples were diluted
100-fold prior to loading on gels. Lower blot: loading control showing
a non-specific band detected by α-Hsp90 antibodies in undiluted samples.
Myo2p concentrations were equalized based on the relative
levels observed in these blots (prior to testing of the different samples
in motility assays; Table 1).
doi:10.1371/journal.pone.0079593.g002

Site-directed mutagenesis by overlap extension [33] was
performed on the
myo2 S1 sequence to incorporate the
-E1 mutation (G345R). Flanking primers utilized the native
NheI (bp 571) and KpnI (bp 2128) sites within the
myo2 S1 sequence (5′ NheI-
myo2: CT-TGCCAGGAAATTCGATGC; 3′ KpnI-my2:

The chimera and control constructs were transformed and used to test whether the Myp2p motor relied on Rng3p function, along with empty vector and GFP-temperature (36°C growth.  

The mutant Nhe1/kmp1 fragment was transferred into pDS472a-myo2-head and subsequently modified (to generate GFP- and FLAG-tagged constructs) as described above. The fidelity of all constructs was confirmed by DNA sequencing.

The LEU2 GFP-myp2-head/myp2-tail chimera plasmid [34], along with empty vector and GFP-mg3 control plasmids [24] were used to test whether the Myp2p motor relied on Rng3p function. The chimera and control constructs were transformed and expressed in temperature-sensitive mg3-63 cells grown at the permissive temperature (25°C) followed by a shift to the restrictive temperature (36°C) to test their ability to rescue function and cell growth.

### Microscopy

Myo1p-GFP patch dynamics were tracked using time-lapse imaging of cells. Cells were mounted on a flat media pad (30 μl +1% agarose) on a slide surface and a coverslip was then sealed above with a 1:1:1 ratio of petroleum jelly, lanolin, and paraffin. Each frame consisted of a z-stack (0.3 μm for seven sections) collected on an Applied Precision Delta Vision imaging station constructed on an Olympus IX-70 inverted microscope base with a 1006 oil immersion lens (1.4 NA) and immersion oil with a refractive index of 1.518. Images were deconvolved using the point-spread functions and software supplied by the manufacturer. Maximum intensity projections of the deconvolved 3D stacks were then used to measure patch lifetimes. Subsequent analysis employed ImageJ (http://rsbweb.nih.gov/ij/), Microsoft Excel (Redmond, WA), and GraphPad Prism software (La Jolla, CA).

Myo2p-3xGFP, Rng3p-3xGFP, Cam2p-mCherry, Myo1p-GFP (single plane), and Sad1p-CFP localization was imaged in cells using a Nikon TE2000-E2 inverted microscope. A motorized fluorescence filter turret and a Plan Apo 60X (1.45 NA) objective was used to capture DIC and epifluorescence cell images (Melville, NY) with an EXFO X-CITE 120 illuminator (Nikon). NIS elements software (Nikon) was used to control the microscope, two Uniblitz shutters (Vincent Associates, Rochester, NY), and the cameras. A Photometrics CoolSNAP HQ2 14-bit camera (Tucson, AZ) was employed for Rng3p-3xGFP, Cam2p-mCherry, and Myo1p-GFP (single planes) and Sad1p-CFP (z-stack, 0.3 μm for seven sections), and an Andor iXon 897 EMCCD camera (Belfast, Northern Ireland) was used for time-lapse tracking of Myo52p-3xGFP particles (single planes). Cells were mounted as described above.

### Calculating Doubling Time

Cells grown in YE5S media at 25°C until saturation were back-diluted to an OD595 nm of 0.035 in fresh YE5S media. Cells were then allowed to continue to grow at 25°C with cell density measured every 60–90 minutes until an OD595 nm of 1.4 or greater was reached. Calculation of the doubling time was done in GraphPad Prism using the linear (exponential) phase of the growth curve.

### Purification of Motors

Full-length and S1 forms of Myo2p (and -E1) plus light chains were purified as previously described [23,24]. For motility assays
with S1 forms, S1-GFP fusions were employed following one-step purification on glutathione Sepharose (GE Healthcare) via the GST-tagged light chains. S1-FLAG forms were purified in two steps via the GST-tagged light chains and by a second step on anti-FLAG resin (Sigma).

Rng3p is essential for *S. pombe* growth, and as such cannot be deleted from the genome. We therefore performed isolations of full-length Myo2p in temperature shift assays to assay its dependence on Rng3p function. One-liter cultures of wild-type and temperature-sensitive rng3-65 cells were induced to over-express Myo2p heavy and light chains grown at the permissive (25°C) temperature for 16 hours. The cultures were then split evenly into two 500 ml cultures and then brought to a final volume of one liter using temperature-equilibrated media, one remained at 25°C and the other was grown at the restrictive temperature (37°C) to eliminate Rng3p function. The cells were then harvested 12 hours post-shift and Myo2p 1-step purified via GST-tagged light chains. Control samples in which Rng3p function was attenuated following the initiation of Myo2p over-expression were also performed in which cultures were split and shifted 22 hours post-induction [24].

**In vitro Motility Assays**

Motility assays were based on an established protocol [35] with ~40 µg/ml of myosin applied to the motility chambers. Fluorescent actin stocks were prepared at 5 µM from chicken skeletal muscle G-actin stocks purified from acetone powder as previously described [36] and polymerized with the addition of 50 mM KCl and 1 mM MgCl₂ in the presence of 5 µM rhodamine phalloidin (Invitrogen) for 30 min. Motors were adhered to the surface of a nitrocellulose-coated coverslip for 10 min and the chamber was then washed as follows: (a) three times with motility buffer (25 mM imidazole pH 7.4, 50 mM KCl, 1 mM EGTA, 4 mM MgCl₂, 2 mM DTT) plus 0.5 mg/ml BSA; (b) three times with motility buffer; (c) twice with motility buffer containing 1 µM vortexed (1 min) unlabelled actin filaments; (d) three times with motility buffer plus 1 mM ATP; (e) twice with motility buffer plus 25 mM rhodamine phalloidin-labeled actin filaments and oxygen scavengers (50 µg/ml catalase, 130 µg/ml glucose oxidase, and 3 mg/ml glucose); (f) twice with motility buffer plus 20 mM DTT, 0.5% methyl-cellulose and oxygen scavengers; and (g) twice with motility buffer plus 20 mM DTT, 0.5% methyl-cellulose, 1.5 mM ATP, and oxygen scavengers. S1-GFP fusions were indirectly attached to the motility surface using monoclonal GFP antibodies (50 µg/ml in motility buffer) [37]. Excess antibodies were removed from the nitrocellullose by the BSA wash (a) prior to 10 min incubation with the S1 samples and subsequent washes (b-g). Filaments were observed at room temperature and recorded by time-lapse imaging at 2 s intervals. ImageJ software with the MTrackJ plug-in was used to calculate average filament velocities.

**High-salt ATPase Assays**

High-salt ATPase assays were carried out at room temperature in 2 mM Tris-HCl pH 7.2, 10 mM imidazole, 500 mM KCl, 2 mM ATP, 1 mM DTT and either 10 mM CaCl₂ or 10 mM MgCl₂ with 0.2–0.8 µg/ml myosin motors. Malachite green was used to quantitate Pᵢ release [38]. Background Pᵢ was subtracted from all values using controls lacking myosin. Pᵢ release was calculated using Microsoft Excel and GraphPad Prism software.

Figure 4. Myo2-E1p motors lack activity and stability. The function of S1 fragments of wild-type Myo2p and -E1 motors were compared in vitro following their over-expression and isolation from fission yeast. A) Histogram comparing the distribution of motility rates of S1-GFP constructs in actin filament gliding assays. Average values are shown inset (0.43±0.05 µm/s for wild-type; 0.01±0.02 µm/s for -E1). B) The ATPase activity of S1-FLAG constructs was assayed and compared over a range of temperatures in the absence of actin and the presence of high salt (0.5 M KCl) with 10 mM CaCl₂. (n = 3). C) S1-FLAG proteins were subjected to limited proteolysis by trypsin and then run on SDS-PAGE gels. The results of three independent proteolysis experiments are shown. Samples were taken at 0, 1, 2, and 5 minutes following the addition of trypsin before proteolysis was stopped by the addition of SDS-PAGE sample buffer. The far left lane in the top gel shows the running position of 75 and 50 KDa molecular weight standards, bands which run at the same position as the undigested S1 heavy chain band and its primary breakdown product respectively. The intensity of the Coomassie-stained undigested S1 (75 KDa band) and its breakdown product (50 KDa band) were quantified by densitometry. Signals were normalized by setting the intensity of the 75 KDa band to 1.0 and the 50 KDa band to 0 at the 0 min time point. The plot shows the average densitometry values for both the 75 KDa and 50 KDa bands from the three experiments.

doi:10.1371/journal.pone.0079593.g004
Limited Proteolysis

Purified Myo2p and -E1 S1 samples were incubated with 200 ng Trypsin (Sigma) buffered in Tris-HCl pH 8.0 in a total reaction volume of 100 µl. For each time point, 20 µl was removed from the reaction and added to 8 µl 5X SDS loading buffer followed by boiling at 95°C for 5 min to stop proteolysis. After all time points had been taken, each sample was boiled again at 95°C for 5 min then loaded onto a 12% SDS-PAGE gel. The gel was then Coomassie-stained and scanned; densitometry analysis was performed using ImageJ. Data was analyzed using GraphPad Prism.

Supporting Information

Figures S1 (Myo2p versus mutant Myo2-E1p ATPase data), S2 (Rng3p and Myo2p over-expression tests), S3 (Myo1p localization in wild-type versus myo1-E1 cells), and Table S1 (fission yeast strains) can be found in this section. Movie S1 (motility activity of Myo2p purified from wild-type versus mg3-65 cells), Movie S2 (motility activity of wild-type Myo2p S1 versus mutant Myo2-E1p S1 forms), Movie S3 (Myo1p patch dynamics in wild-type versus rng3-65 cells), and Movie S4 (Myo52p particle movement in wild-type versus rng3-65 cells) can also be found here.

Results

Myo2p Motor Activity Depends on Rng3p

Previous work in yeast and higher eukaryotes has shown that UCS proteins and myosin motors associate with one another [3–5,17,23,27,28]. To further understand the impact of Myo2p’s interaction with Rng3p, we purified myosin motors in the presence and absence of functional Rng3p. Rng3p is essential for cell viability, which precludes isolation of Myo2p from a mg3Δ strain. In order to circumvent this, we utilized the temperature-sensitive mg3-65 mutant. Cells were induced to over-express Myo2p in both mg3-65 and wild type backgrounds, followed by growth at both
Figure 6. Rng3p is required for the function of non-essential myosin-II Myp2p. A Myp2p-head/Myo2p-tail chimera construct was tested for its ability to rescue the growth of temperature-sensitive rng3-65 mutant cells under restrictive growth conditions (36°C). Plasmid transformants were grown under permissive conditions (25°C) on EMM-Leu Ura minimal media plates before re-streaking and subsequent incubation on plates at 36°C. Double drop-out plates were employed to accommodate the differing markers of the pGFP-myp2-head/myo2-tail (LEU2) and pGFP-myp2-head/myo2-tail (ura4) plasmids. The vector alone transformant (left, negative control) carried empty LEU2 and ura4 plasmids; the Rng3p transformant (center, positive control) carried an empty LEU2 vector and pGFP-myg3; and the Myp2p-head/Myo2p-tail transformant (right) carried pGFP-myp2-head/myo2-tail and an empty ura4 vector. doi:10.1371/journal.pone.0079593.g006

permissive and restrictive temperatures. Given the long lag in expression from the sua1 promoter, cells were shifted to the restrictive temperature (37°C) 16 hours after induction. This allowed sufficient time to establish an appropriate cell density before attenuating Rng3p function (prior to the start of Myo2p over-expression at ~20 hours post-induction) (Figure 2A). We included a control experiment where Myo2p over-expression was initiated before attenuating Rng3p function. This was achieved by shifting the cells to the restrictive temperature 22 hours after induction. In all experiments cells were harvested 28 hours post-induction before rapid isolation of Myo2p by one-step purification [23].

In the experiment where cells were shifted 16 hours post-induction, we observed no difference in the rate of Myo2p in vitro motility in actin filament gliding assays following its isolation from wild-type cells at 25°C (control) versus those shifted to 37°C (Table 1; Movie S1). Myo2p purified from the mg3-65 background following growth under permissive conditions (25°C) showed a significantly slower rate of filament gliding (Table 1). More strikingly, the rate of filament gliding was reduced ~10-fold (Table 1; Movie S1) when Myo2p was expressed and purified from the mutant following the shift to restrictive conditions (37°C). In this case most filaments bound to Myo2p on the cover-slip surface lacked any observable motility. It is worth noting that this form of Myo2p is unstable and prone to aggregation, which has limited further analysis in other types of assays. Our data indicates that Rng3p is required to establish active Myo2p motors.

The control experiments (where cells were shifted to 37°C at 22 hours post-induction) indicated that Rng3p was not essential for maintaining Myo2p motility once an active population of motors had been synthesized, as previously reported [24]. Myo2p isolated at either temperature from mg3-65 cells shifted 22 hours post-induction showed no obvious difference in rates of actin filament gliding (Table 1).

While lacking in motility activity, soluble Myo2p was still expressed and isolated from mg3-65 cells shifted at 16 hours post-induction (Figure 2B). However, the levels of over-expressed Myo2p obtained from mg3-65 cells were lower than their wild-type counter-parts (Figure 2B). Collectively our findings suggest that Rng3p is required to generate an active and stable population of Myo2p motors.

A Mutant Form of Myo2p Hyper-sensitive to altered Rng3p Function Lacks Motor Activity

One specific point mutation in the motor domain (myo2-E1; G345R) makes Myo2p particularly sensitive to changes in Rng3p function [22]. Unlike other temperature-sensitive myo2 motor mutants tested, myo2-E1 exhibits synthetic lethality when combined with temperature-sensitive rng3 mutants [22]. Furthermore, compared with other motor mutants, myo2-E1 cells recruit increased levels of Rng3p to the contractile ring [22,24]. In summary, Myo2p’s reliance on Rng3p and the prevalence of Rng3p-Myo2p associations appear to be exaggerated in myo2-E1 cells.

We first used homology modeling to examine the structure of a myo2-E1 motor to gain some insight. The E1 mutation is defined by an amino acid substitution (G345R) at a highly conserved glycine (Figure 3A) that is found in every myosin sequence we have so far examined. Modeling of the Gly 345 residue from Myo2p localizes it to the end of a helix near the ATP-binding pocket (Figure 3B). The model suggests that the longer side chain of the Arg 345 in Myo2p-E1p introduces a steric clash with a conserved tyrosine (Tyr 297) (Figure 3B), potentially propagating destabilization within the motor domain.

We over-expressed and isolated full-length forms of the wild type and mutant -E1 motors from fission yeast to compare their function in vitro. Previous analysis of full-length -E1 in actin filament gliding assays failed to detect any actin binding or motility [23]. Thus, we included methyl-cellulose in our running buffers to favor actin-binding by minimizing diffusion of actin filaments away from motors at the cover-slip surface in the motility chambers. This approach facilitated actin binding in these assays. However, most filaments (99%) bound by -E1 were non-motile, while most filaments bound by wild-type Myo2p were motile (Movie S2). We next assayed the ATPase activity of the motors in the presence of high salt (0.5 M KCl) and the absence of actin. Typical of myosins under such conditions, wild-type motors exhibited no detectable ATPase activity in the presence of MgCl2, while showing healthy activity in the presence of CaCl2 (Figure S1). However, -E1 motors exhibited relatively low activity under either condition (Figure S1). These experiments performed in the absence of actin suggest that defects in -E1 motors are not specific to actin displacement and motility, and probably reflect a general defect in conformation and function.

The predicted steric clash within the -E1 motor (Figure 3B) may lead to a more labile motor conformation. To examine this further, we over-expressed and purified S1-forms (sub-fragment 1 forms: motor domain plus lever arm and associated light chains) of both wild-type and -E1 motors from fission yeast. The first thing we noted was that yields were typically ~10-fold lower for S1-E1. Secondly, as with the full-length proteins, we observed similar defects in actin filament gliding (Figure 4A) and high salt ATPase activity (Figure 4B) with the S1-E1 samples. Thirdly, S1-E1 motors were more sensitive to trypsin digestion in limited proteolysis experiments. As shown in Figure 4C, while both S1 heavy chains are degraded at a similar rate, wild-type motors breakdown into a relatively stable lower molecular weight form. However, the lower molecular weight form of -E1 failed to accumulate over time following protease addition (Figure 4C), suggesting an altered conformation more sensitive to proteolysis. In summary, all of our
in vitro experiments with full-length and S1 forms of the -E1 protein suggest that this mutant form lacks activity due to defects in stability and activity, defects which presumably dictate increased Rng3p recruitment and regulation in vivo.

We also attempted to correlate our in vitro findings with experiments further examining the inter-dependence of Rng3p and Myo2p function in vivo. We tested whether over-expression of Rng3p could rescue the myo2-E1 mutant given the increased abundance of Rng3p observed at actomyosin rings in this mutant [22]. Rng3p over-expression did not rescue the myo2-E1 mutant (Figure S2); likewise over-expression of Myo2p did not rescue the cytokinesis defects of a rng3-65 mutant (Figure S2). However, such experiments are difficult to interpret because over-expression of either protein has previously been shown to be toxic in wild-type backgrounds [23,39,40].

Rng3p is Specific to Myosin-II
rng3-65 cells show obvious defects in contractile ring formation and cell morphology reflecting a role for Rng3p in Myo2p function during cytokinesis [22]. It was recently proposed that Rng3p works with all the myosins in fission yeast [26]. We turned to live cell imaging of wild-type and rng3-65 cells to assess myosin-I (Myo1p) and myosin-V (Myo52p) function in vivo. We first compared the lifetime of Myo1p at endocytic actin patches. Previous work in budding yeast and fission yeast has shown that defects in myosin-I function lead to an increase in the lifetime of myosin-I at actin patches [41–43]. We found that attenuating Rng3p function (by shifting cells to 37°C for 5 hours) had no effect on the lifetime of Myo1p at actin patches.

Table 2. Growth rates for wild-type, rng3-65, myo1-E1, and rng3-65 myo1-E1 cells.

| Strain         | Doubling time (min) |
|----------------|---------------------|
| wild-type      | 186 ± 32            |
| rng3-65        | 274 ± 21            |
| myo1-E1        | 341 ± 18            |
| rng3-65 myo1-E1| 415 ± 45            |

All values are the mean ± SEM.
Cells were grown at 25°C in YE5S media.
doi:10.1371/journal.pone.0079593.t002
on Myo1p patch dynamics (Movie S3). Myo1p patches showed similar signal intensities and average lifetimes in wild-type and rng3-65 cells (Figure 5A-B).

We next assayed the type-V myosin Myo52p to see whether its function relied on Rng3p. Myo52p is involved in intracellular transport and can be visualized moving along actin cables within the cell [42,44]. Firstly, we observed no defects in the intensity or pattern of Myo52p localization in rng3-65 cells grown at 37°C (Figure 5C). To assess motor function directly, we measured Myo52p particle velocity in vivo but found no significant differences in speeds (Figure 5D; Movie S4) or event frequency (Figure 5E) irrespective of whether Rng3p function is perturbed. Taken together, our in vivo findings suggest that Rng3p is specific for Myo2p and cytokinesis, and is not required for myosin-I and myosin-V function at other actin structures.

Use of a chimera construct allowed us to test whether Rng3p is also required for the function of the non-essential fission yeast myosin-II Myp2p (Figure 6). Like Myo2p, Myp2p functions in cytokinesis and localizes to contractile rings [45–47]. However, unlike Myo2p, loss of Myp2p does not prevent cytokinesis given myp2Δ cells grow normally and only display mild cytokinesis defects under most conditions [45,47]. It was previously shown that the Myp2p motor of the myp2-head/myo2-tail chimera rescues loss of Myo2p motor function and cell growth [34]. The fact that Myo2p motor activity is essential for function and cytokinesis [48] tells us that the Myp2p head in the chimera is a functional motor that can work in place of the Myo2p motor. Since Myo2p motor activity relies on Rng3p one would assume that the chimera could bypass the need for Rng3p and rescue the growth of rng3-65 mutants if Myp2p functioned independently of Rng3p. However, the myp2-head/myo2-tail chimera failed to restore viability to arrested rng3-65 cells (Figure 6). This result suggests that Rng3p is also required for Myp2p motor function. We did not test whether Rng3p was required for the function of Myo1p, the myosin-V found at the contractile ring [49,50]. While we cannot rule out a role in Myo51p function, Rng3p's critical role likely lies with myosin-II and myosin-V, since deletion of the myo51 gene does not markedly affect cytokinesis [49,50].

Rng3p can be Artificially Targeted to Myosin-I Patches in a myo1-E1 Mutant

While Rng3p is specific to myosin-II in fission yeast, the budding yeast UCS protein functions with myosin-I and myosin-V. We therefore wished to determine whether Rng3p function was specific to the intrinsic properties of Myo2p motors, or whether it is capable of responding to instability or inactivity inherent to any myosin. In order to do this, we artificially destabilized Myo1p by constructing a myo1-E1 mutant. Figure 3B highlights the conserved glycine residue that defines the myo2-E1 mutation and we introduced this same mutation into the motor domain of Myo1p (G308R).

myo1-E1 cells appeared rounded (Figure 7A) and grew much slower than wild-type cells (Table 2), morphological and growth defects reminiscent of a myo1Δ mutant [31,52]. Double mutants containing both the myo1-E1 and rng3-65 mutations were isolated and exhibited a morphology that was intermediate between the rounded myo1-E1 phenotype and the elongated rng3-65 phenotype (Figure 7A). Unlike the synthetic lethality of myo2-E1 rng3-65 double mutants, any additive phenotype associated with myo1-E1 rng3-65 cells was not obvious. This presumably reflects the fact that the myo1-E1 mutant essentially pheno-posed a myo1 null, and suggests that any role for Rng3p in rescuing the function of Myo1-E1p motors is negligible. That being said, myo1-E1 rng3-65 double mutants exhibited a slower growth rate than the single mutants (Table 2).

Comparing Myo1p levels in wild-type versus myo1-E1 cells revealed decreased signal intensity at patches in the mutant (Figure S3). Thus, while Myo1-E1p motors may have some residual function at patches their stability is significantly compromised in vivo. Our previous work indicated that Rng3p localizes faintly to mature contractile rings in wild-type cells [23], and is not detectable with Myo1p at endocytic actin patches (Figure 7B). However, when we visualized Rng3p in myo1-E1 cells we found that it co-localized with Myo1-E1p in patch-like structures (Figure 7B). Admittedly, complete co-localization at these dynamic patches was impossible to resolve given the exposure times needed to sequentially generate the images (from the relatively faint Rng3p-3xGFP and Cam2p-Cherry signals) versus the short lifetimes of patches. Nevertheless, it is clear that Rng3p is recruited to patch-like structures in a Myo1-E1p-dependent fashion, a sub-population of which were captured co-localizing with Myo1-E1p patches (Figure 7B). This redistribution in localization indicates that the UCS protein is not strictly exclusive to myosin-II in fission yeast. Our data suggests that UCS proteins are capable of providing general surveillance of myosin motor function which may be unnecessary, minor, or significant depending on the myosin involved or perhaps the requirement of a cellular system to adapt to changes in its environment.

Discussion

UCS proteins are conserved throughout eukaryotes and have been proposed to act as co-chaperones that work with Hsp90 during the de novo folding of myosin motor domains. While lacking the conserved N-terminal Hsp90-binding TPR domain, fungal UCS proteins have been implicated in the function of multiple classes of myosins [22,26–28]. In this study we investigated the mode of action of the fission yeast UCS protein, Rng3p. We propose a chaperone-like role for Rng3p in the regulation of myosin-II motor stability and activity.

Rng3p Activates Myo2p Motors

We propose that Rng3p stabilizes active motor conformations that support actin filament motility and cytokinesis. Rng3p may also play a role in repairing Myo2p motors that are damaged from forces generated during ring assembly and constriction. This may explain how Rng3p enhances the in vitro motility of purified Myo2p [23]. More notably, our new biochemical data provides a direct link between Rng3p function and Myo2p motor activity. The loss of actin filament gliding when Myo2p motors are generated in the absence of functional Rng3p and the associated drop in yield highlight the importance of Rng3p in Myo2p activity and stability. Similarly, destabilized Myo2p (Myo2-E1p) motors that show an increased dependency on Rng3p function in vivo [22], bound actin without supporting ATPase or motility activity, exhibited an altered conformation, and showed a reduced yield following over-expression and purification from fission yeast. Taken together our findings indicate that Rng3p is the key regulator in establishing Myo2p motor activity.

Rng3p is Specific to Myosin-II and the Actomyosin Ring

Previous work revealed that Rng3p associates co-translationally with all five of the fission yeast myosins, suggesting that Rng3p functions in the general folding of these myosins [26]. We examined the importance of Rng3p in the function of other myosins that operate throughout the cell cycle at other actin structures. Loss of Rng3p function had no effect on the localization
and dynamics of Myo1p or Myo52p. Thus, while Rng3p may be capable of interacting with these unconventional myosins, it is not necessary for their function in the cell. Rng3p function appears to have evolved to establish and maintain Myo2p activity during actomyosin ring assembly and constriction at cytokinesis. Rng3p was also found to be critical for the non-essential myosin-II Myo2p which is also found at the ring. Destabilizing Myo1p via the myo1-E1 mutation presumably strengthens any transient interactions between this motor and Rng3p, leading to aberrant localization of Rng3p to Myo1p patches where it is not normally found or needed. The ability of Rng3p to associate co-translationally with Myo1p (and its targeting to Myo1-E1p patches in cells) suggests that the UCS protein is not strictly exclusive to myosin-II. Rather, Rng3p probably responds to the intrinsic properties of all myosin classes and can provide surveillance (and regulation) depending on the health or requirement of the motor.

The budding yeast UCS protein (She4p) appears to function somewhat differently to Rng3p. As opposed to working with conventional myosin, She4p is required for unconventional myosin (myosin-I and myosin-V) function [27,28]. Furthermore, our previous work indicated that while She4p was not required for myosin-4 activity, it was required to maintain myosin-I levels in the cell [24]. This may reflect another fundamental difference between the roles of budding and fission yeast UCS proteins: She4p is required to maintain the steady state levels of motors in the cell, whereas Rng3p functions to establish both the activity and levels of motors.

An Alternative Role for UCS Proteins Besides Functioning as Hsp90 Co-chaperones?

UCS proteins are thought to act as co-chaperones that link unfolded myosin motors bound at their UCS domains to Hsp90 [1,2,4]. UCS proteins interact with Hsp90 through their TPR domain [1,3], yet the fungal UCS proteins lack this domain. While previous work in fission yeast has provided a link between Hsp90, Rng3p, and Myo2p function during cytokinesis [25], UCS proteins can chaperone myosin motors in the absence of Hsp90 [3,16,17]. Hsp90 may function upstream of Rng3p in the de novo folding of Myo2p motors. In this case, a defect in Hsp90 function may compromise the stability of Myo2p motors, much the same way the myo2-E1 mutant does. Alternatively, Rng3p and Hsp90 may act at the same time, functioning as independent chaperones that establish and maintain active motor conformations.

Our work does not argue against a role for the UCS protein as a Hsp90 co-chaperone. After all, UCS proteins from higher eukaryotes possess a TPR domain. How this TPR domain influences Hsp90 and myosin motor function may vary depending on the UCS protein, the myosin substrate, or cellular process. While the UCS protein can participate in the Hsp90-dependent folding of muscle myosin-II motors [4,5], the C. elegans UCS protein works antagonistically to Hsp90 as the two proteins compete for myosin-II binding [15]. Our work from fission yeast, combined with recent findings showing that the C. elegans UCS domain alone can support full function in vivo [15] suggest an additional role for UCS proteins independent of Hsp90 and de novo folding.

Supporting Information

Figure S1  Comparing the ATPase activity of Myo2p and Myo2-E1p. The ATPase activity of full-length Myo2p and Myo2-E1p was assayed in the absence of actin and the presence of high salt (0.5 M KCl) with 10 mM MgCl2 or 10 mM CaCl2. (TIF)

Figure S2  Over-expression of Rng3p or Myo2p does not rescue the lethality of myo2-E1 or rng3-63 mutants. A) Temperature-sensitive myo2-E1 cells were transformed with pD8573a-LEU2 (empty vector control), pYFP-myo2 (positive control expressing YFP-Myo2p from the myo2 promoter), and pGST-rng3-FL (over-expression construct expressing GST-Rng3p from the high-strength 3xmyt1 inducible promoter). Transforms were isolated on EMM-Leu+ (+thiamine) plates at 25°C. Left: cells were re-streaked onto EMM-Leu+ plates (lacking thiamine) and grown at 36°C to induce over-expression of Rng3p and attenuate Myo2-E1p function. B) Temperature-sensitive rng3-63 cells were transformed with pD8573a (empty vector control) and pGFP-rng3 (positive control expressing GFP-Rng3p from the low-strength 81xmyt1 inducible promoter). A rng3-63 strain carrying an integrated medium-strength 4xmyt1 inducible promoter (in place of the myo2 promoter) and pGST-rng3 was included to over-express Myo2p. Transforms were isolated on EMM-Ura+ (+thiamine) plates at 25°C. Left: cells were re-streaked onto EMM-Ura+ plates lacking thiamine and grown at 36°C to induce over-expression of Myo2p and attenuate Rng3-63p function. Images on right: representative cells from the two plates imaged by DIC microscopy. Bars: 4 μm. (TIF)

Figure S3  Myo1p levels are reduced in the myo1-E1 mutant. Merged GFP and CFP fluorescence image of a mixed population of wild-type myo1-GFP (with sad1-CFP) and mutant myo1-E1-GFP cells. Sad1-CFP was colored in red to distinguish wild-type cells from the myo1-E1 cells (marked with asterisks). Bar: 4 μm. (TIF)

Table S1  S. pombe strains used in this study. (DOC)

Movie S1  Actin-filament gliding rate is decreased when full-length Myo2p motors are purified in the absence of Rng3p function. Myo2p motors were expressed and purified from either wild-type or temperature-sensitive mg3-63 fission yeast cells following 12 hours of growth at the restrictive temperature (37°C). Motors were assayed in vitro in actin filament gliding assays. Movie images captured every 2s and played at 10 frames/s (sped up 20×). (MOV)

Movie S2  Mutant Myo2-E1p motors bind actin filaments but do not support filament gliding. Full-length Myo2p and Myo2-E1p motors were overexpressed and purified from fission yeast cells and tested in actin filament gliding assays. Movie images captured every 2s and played at 10 frames/s (sped up 20×). (MOV)

Movie S3  Myo1p patch dynamics are not altered when Rng3p function is perturbed. Myo1p-GFP patch dynamics in wild-type and rng3-63 cells grown under restrictive conditions (37°C for 5 hours) to perturb Rng3p function. Movies were generated from maximum projection images (7 z-sections over the depth of the cell) collected every 3s. Movie played at 10 frames/s (sped up 30×). (MOV)

Movie S4  Myo52p particle motility is still observed when Rng3p function is perturbed. Myo52p-3xGFP motile particles in wild-type and rng3-63 cells grown under restrictive conditions (37°C for 5 hours) to perturb Rng3p function. Movies...
were generated from single plane images captured every 1.5s. Movie played at 10 frames/s (sped up 15×).

(MOV)

Acknowledgments

We are grateful to John Allingham (Queens University, Kingston, Canada) for the homology models of the Myo2p and Myo2p-E1 motor domains.

We acknowledge Mohan Balasubramanian (Temasek Life Sciences Laboratory, Singapore) for providing the myo2-E1 and mrg3-65 mutants, and Susan Forssburg (University of Southern California, Los Angeles, CA) for the pDS73a plasmid. We thank Aoife Heslip and Keven Juaire for assistance with fluorescence microscopy and live cell imaging analysis.

Author Contributions

Conceived and designed the experiments: BCS VS ML. Performed the experiments: BCS MJL LWPS VS ML. Analyzed the data: BCS ML. Contributed reagents/materials/analysis tools: BCS LWPS. Wrote the paper: BCS ML.

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