Ste20-kinase-dependent TEDS-site phosphorylation modulates the dynamic localisation and endocytic function of the fission yeast class I myosin, Myo1

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Summary

Type I myosins are monomeric motors involved in a range of motile and sensory activities in different cell types. In simple unicellular eukaryotes, motor activity of class I myosins is regulated by phosphorylation of a conserved 'TEDS site' residue within the motor domain. The mechanism by which this phosphorylation event affects the cellular function of each myosin I remains unclear. The fission yeast myosin I, Myo1, activates Arp2/3-dependent polymerisation of cortical actin patches and also regulates endocytosis. Using mutants and Myo1-specific antibodies, we show that the phosphorylation of the Myo1 TEDS site (serine 361) plays a crucial role in regulating this protein's dynamic localisation and cellular function. We conclude that although phosphorylation of serine 361 does not affect the ability of this motor protein to promote actin polymerisation, it is required for Myo1 to recruit to sites of endocytosis and function during this process.

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Key words: *Schizosaccharomyces pombe*, Type I myosin, TEDS site, Endocytosis, Myo1, Fission yeast

Introduction

Class I myosins are a family of highly conserved single-headed motor proteins that are involved in a range of motile and sensory activities in cells (Kim and Flavell, 2008). Within the N-terminal catalytic motor domain of myosin I from lower eukaryotes is a phosphorylation site, designated the TEDS-rule phosphorylation site, that regulates motor activity (Bement and Mooseker, 1995). This site resides in a surface loop that forms part of the actin-binding site and its phosphorylation stabilises the actomyosin complex by increasing the affinity of myosin for actin. In *Dictyostelium discoideum*, absence of phosphorylation of the TEDS site of myosin I abolishes its motility and reduces actin affinity and ATPase activity (Fujita-Becker et al., 2005). Phosphorylation therefore provides an important mechanism for modulating motor activity.

Saccharomyces cerevisiae has two class I myosins, Myo3 and Myo5 (Geli and Riezman, 1996; Goodson and Spudich, 1995), whereas the fission yeast, *Schizosaccharomyces pombe*, possesses only one, Myo1 (Lee et al., 2000). In addition to the defects in polarised growth and F-actin patch polarisation brought about by deletion of *myo1*$^+$, deletion of both budding yeast myosin genes also results in defects in fluid-phase endocytosis (Codlin et al., 2008; Geli and Riezman, 1996; Lee et al., 2000). This is consistent with recent work that has shown the formation and polymerisation of cortical F-actin patches to be intimately linked to the process of endocytosis (Kaksonen et al., 2006). However, the precise roles of myosin-I motility and TEDS-site phosphorylation in this process remain unresolved.

Here, we show that phosphorylation of the conserved TEDS site in the fission yeast MyoI plays a crucial role in regulating the dynamic properties and cellular function of Myo1. Although mutating the TEDS site has no effect on actin organisation or growth dynamics, it has a dramatic effect on Myo1 localisation and affinity for actin. We provide evidence that Myo1 plays a crucial role during fission yeast endocytosis and that phosphorylation of this TEDS site is key in regulating this process.

Results and Discussion

To investigate the mechanisms by which the fission yeast class I myosin, Myo1, is regulated in vivo a strain expressing an integrated *gfp-myo1* allele under the control of the repressible *nmt41* promoter was generated. This allele complemented the function of the wild-type *myo1*$^+$ gene, as observed by its ability to rescue the growth and morphology defects associated with strains lacking Myo1 (Fig. 1A) (Lee et al., 2000; Toya et al., 2001). Western blot analysis using anti-Myo1-specific antibodies confirmed that expression levels were equivalent between Myo1 proteins expressed from either its endogenous or the de-repressed *nmt41* promoter (Fig. 1B).

The localisation and dynamics of GFP-Myo1 were equivalent to those reported previously for other *gfp-myo1* alleles (Lee et al., 2000; Sirotkin et al., 2005; Toya et al., 2001). Myo1 transiently localised to cell membrane associated foci, which concentrated to sites of cell growth (supplementary material Movie 1). These foci demonstrated no significant lateral movement even at the single molecule level (data not shown), and had an average lifetime of 15.4 seconds. Expression of *gfp-myo1* from an integrant allele under the control of the full-strength *nmt1* promoter resulted in a large proportion of cells failing to undergo cytokinesis (Fig. 1C), illustrating the importance of modulating expression levels, but there was no observable effect on the dynamics of the dynamic foci. Overexpression of *gfp-myo1* from a multicopy pREP1 plasmid resulted in localisation of Myo1 to non-polar cell wall membranes, an absence of dynamic foci, gross morphological defects, and a failure to complete cytokinesis (Fig. 1D). To explore whether cell-cycle variations in *myo1*$^+$ expression regulated Myo1 dynamics, cells were synchronised with respect to

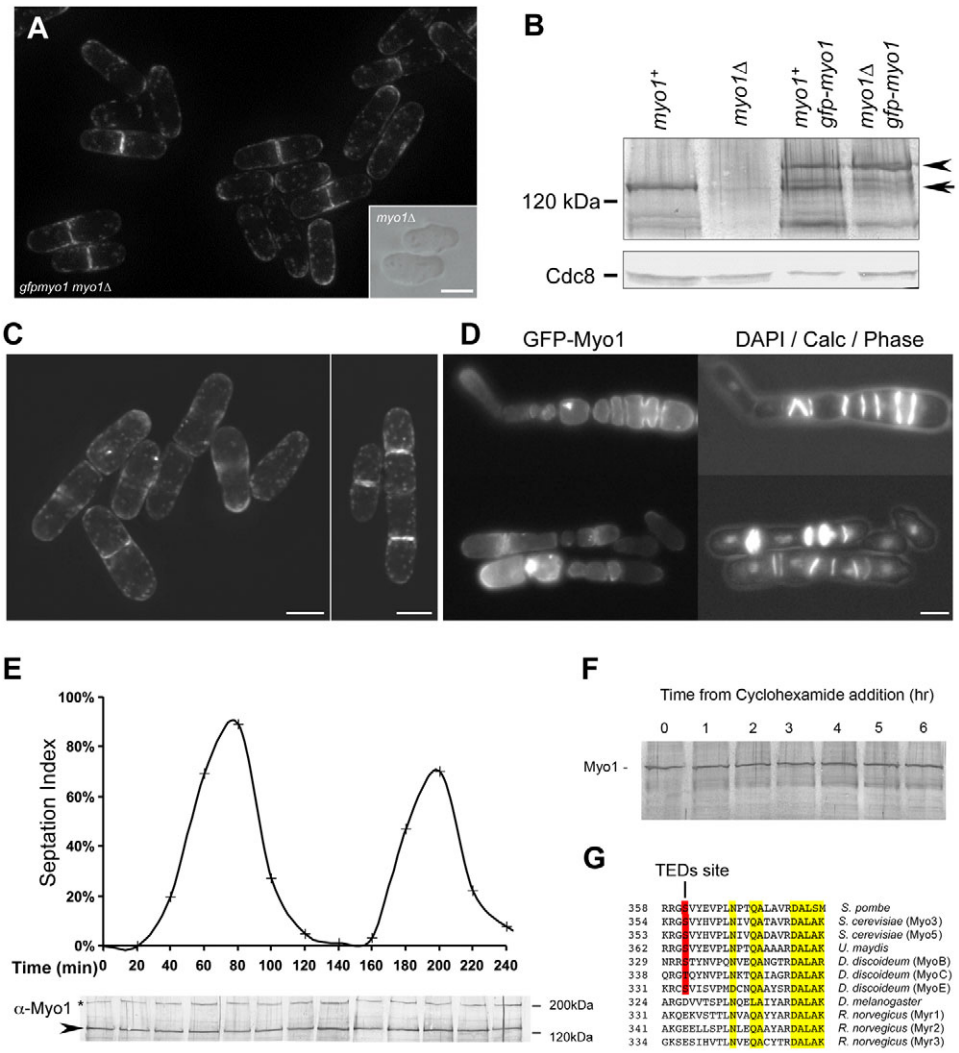

Fig. 1. Fission yeast Myo1 is subjected to post-translational modification. (A) GFP signal from *leu1::nmt41gfp-myo1*$^+$ *myo1Δ* cells concentrates to dynamic foci enriched to sites of cell growth. Inset: DIC image of *myo1Δ* cells for comparison. (B) Anti-Myo1 (upper panel) western blots of *myo1*$^+$, *myo1Δ*, *leu1::nmt41gfp-myo1*$^+$ and *leu1::nmt41gfp-myo1*$^+$ *myo1Δ* cell extracts. Anti-Myo1 sera recognise a single *myo1*$^+$ band that migrates at ~135 kDa (arrow) and a *gfp-myo1* allele-specific band corresponding to ~160 kDa (arrowhead), the intensity of which was comparable to the endogenous Myo1 band. Equal loading was confirmed using anti-Cdc8 sera (lower panel). (C,D) Cellular localisation of GFP-Myo1 is disrupted in wild-type cells bearing either the *leu1::nmt1gfp-myo1*$^+$ allele (C) or the plasmid pREP1*myo1*$^+$ (D, left), grown in the absence of thiamine. (D) Right panel shows corresponding DAPI and calcofluor (Calc) staining. (E) Myo1 protein levels (arrowhead) did not fluctuate during two cell cycles. A culture synchronised with respect to cell-cycle, was generated by transient arrest of the *cdc25-22* mutant. Samples were subjected to anti-Myo1 western blot analysis and septation index determination. Equal loading was confirmed by ponceau staining (not shown) and non-specific cross-reacting band (*). (F) Anti-Myo1 western blot of extracts from a time-course of cycloheximide-treated cells suggests that Myo1 is not subject to proteolysis. Equal loading was determined by ponceau staining (not shown). (G) Alignment of conserved TEDS phosphorylation sites (red) in myosin I. Numbers denote residue at start of sequences. Scale bars: 5 μm.

cell-cycle timing and revealed that Myo1 levels did not fluctuate over two subsequent cell cycles (Fig. 1E). In addition, when protein synthesis was inhibited with cyclohexamide, Myo1 levels did not diminish over three cell cycles (Fig. 1F). These data suggest that Myo1 is not regulated by cell-cycle-dependent changes in protein levels during vegetative growth.

Myo1 Ser361 is phosphorylated in vivo

We were keen to establish whether Myo1 function and its dynamic movements were regulated by an as-yet-undetermined post-translational modification. Phosphorylation is known to regulate several myosins by modulating their motor activity, ability to form filaments, or interactions with other molecules. The activity of myosin I in unicellular eukaryotes is regulated by phosphorylation of a conserved residue, called the TEDS site, within the motor domain (Bement and Mooseker, 1995) (Fig. 1G). Phosphospecific antibodies were generated that specifically recognised Myo1 that had been phosphorylated at the predicted TEDS site (Ser361). These phosphospecific anti-Ser361-*P* antibodies recognised bands corresponding to Myo1 and GFP-Myo1 in anti-Myo1 immunoprecipitates (Fig. 2A). The ability to recognise Myo1 was abolished when anti-Myo1 immunoprecipitates were incubated with λ-phosphatase (Fig. 2B), illustrating the phosphospecificity of this antibody. This anti-Ser361-*P* antibody failed to recognise Myo1 protein in which Ser361 had been mutated to alanine (Fig. 2C), illustrating its specificity to the Myo1 TEDS site phosphoserine. Together these data illustrate that the Myo1 TEDS site (Ser361) is phosphorylated in vivo.

TEDS site phosphorylation modulates Myo1 dynamics

Having established that Myo1 Ser361 is phosphorylated, we determined the effect this post-translational modification had on its distribution. Strains were generated in which Myo1 Ser361 had been replaced with either alanine or aspartic acid (which acts as a phosphomimic). Expression from these mutant alleles was confirmed to be equivalent to that of wild-type *myo1*$^+$ (Fig. 2D). Kymographs generated from time-lapse movies revealed that, unlike the native protein, Myo1-S361A failed to recruit to dynamic Myo1 foci at the cell cortex (Fig. 2E,F; supplementary material Movie 2). Although the cytoplasmic signal was higher than normal, Myo1-S361A protein still concentrated to sites of cell growth (Fig. 2E,F; supplementary material Movie 2).

By contrast, the Myo1-S361D phosphomimic localised to filaments that extended throughout the cell and around the cell equator of mitotic cells (Fig. 2G). These filaments disappeared after addition of Latrunculin A and in the absence of functional tropomyosin (not shown). As for the wild-type protein (Lee et al., 2000; Sirotkin et al., 2005), Myo1-S361D also required its tail

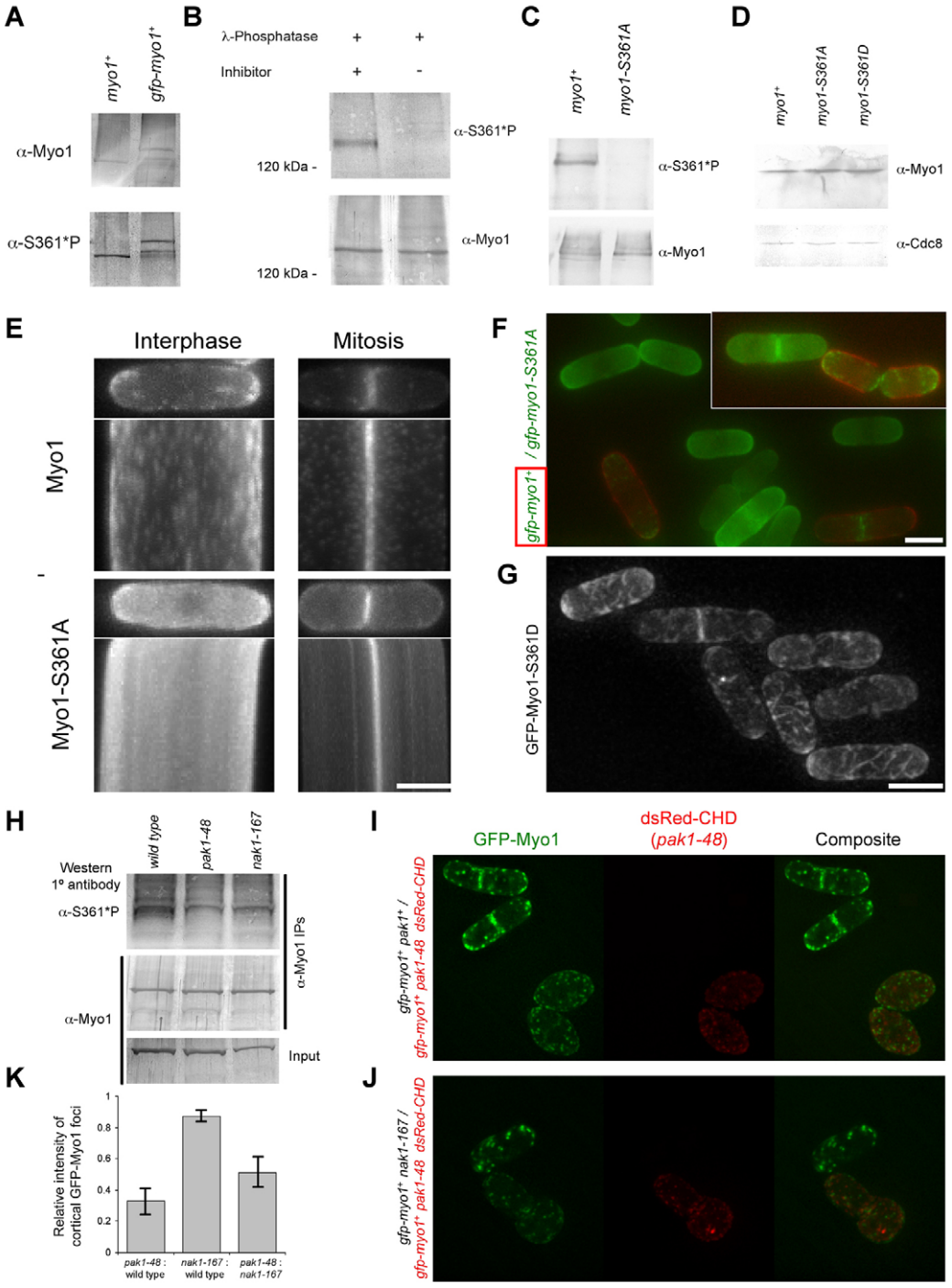

Fig. 2. Pak1-dependent phosphorylation of Ser361 regulates Myo1 dynamics. (A-C) Western blots of anti-Myo1 immunoprecipitates were probed with anti-Myo1Ser361-*P* (upper panels) antibodies and anti-Myo1 sera (lower panels) to confirm that Myo1 purification was equivalent. (A) Western blots of anti-Myo1 immunoprecipitates from *myo1*$^{+}$ and *myo1Δ leu1::nmt41gfp-myo1*$^{+}$ cells confirm that Ser361 is phosphorylated in this strain. (B) Western blots of anti-Myo1 immunoprecipitates that had been incubated with λ-phosphatase in the absence (left lane) or presence (right lane) of sodium vanadate confirm the phosphospecificity of the antibody. (C) Western blots of anti-Myo1 immunoprecipitates from *myo1*$^{+}$ and *myo1-S361A* cells confirm that anti-Myo1Ser361-*P* antibody only associates with Myo1 phosphorylated at Ser361. (D) Anti-Myo1 (upper panel) and anti-Cdc8 (lower panel) western blots of extracts from *myo1*$^{+}$, *myo1-S361A* and *myo1-S361D* cells confirm that equivalent levels of protein are expressed from each allele. (E) Micrographs and kymographs from time-lapse movies of *gfp-myo1*$^{+}$ (upper panels) and *gfp-myo1-S361A* (lower panels) cells. Unlike the wild-type protein, Myo1-S361A does not localise to dynamic foci. (F) Simultaneous observation of GFP signal (green) from TRITC-lectin-coated *gfp-myo1*$^{+}$ (red) and *gfp-S361A* cells confirm the aberrant localisation of the mutant protein. (G) GFP-Myo1-S361D associates with actin filaments and concentrates to sites of cell growth. (H) Anti-Myo1Ser361-*P* (upper panel) and anti-Myo1 (middle and bottom panels) western blots of anti-Myo1 immunoprecipitates (upper and middle panels) and extract inputs (bottom panel) from wild-type *pak1-48* or *nak1-167* cells that had been incubated at 36°C for 3 hours. Analysis of band intensities revealed that Myo1 Ser361 phosphorylation in wild-type cells relative to *pak1-48* and *nak1-167* cells was 1:0.52:0.84, respectively, when normalised against total Myo1 present in immunoprecipitates. (I,J) GFP-Myo1 (green) and dsRed-CHD (red) signals from simultaneous observation of *pak1-48* cells bearing the plasmid pREP82dsRed-CHD (Martin and Chang, 2006) together with either (I) wild-type or (J) *nak1-167* cells. (K) Average relative intensities of >50 GFP from samples described in I and J. Scale bars: 5 μm.

domain to localise. Together, these data indicate that Myo1-S361D localises to tropomyosin-associated actin filaments, to which the wild-type protein is not seen to associate (Lee et al., 2000; Toya et al., 2001) (S.L.A. and D.P.M., unpublished data).

Ser361 phosphorylation is Pak1/Orb2 dependent

Ste20 kinases have been implicated as being responsible for phosphorylation of myosin I in a number of organisms (Brzeska et al., 1997; Wu et al., 1996; Wu et al., 1997). We therefore wished to determine whether a member of this class of protein kinases phosphorylates *S. pombe* Myo1. Fission yeast contains four members of this conserved class of protein kinases (Gilbreth et al., 1996; Guertin et al., 2000; Huang et al., 2003; Yang et al., 1998), mutations in two of which (Nak1/Orb3 and Pak1/Orb2) bring about growth morphology defects reminiscent of *myo1Δ* cells (Verde et al., 1995). Myo1 Ser361 phosphorylation was significantly reduced in temperature-sensitive *pak1-48* cells as compared with wild-type cells (Myo1 Ser361 phosphorylation in *pak1-48* cells relative to *pak1*$^{+}$ was 1:0.52), whereas inactivation of Nak1 had less of an effect on Ser361 phosphorylation (Myo1 Ser361 phosphorylation in *nak1-167* cells relative to *nak1*$^{+}$ was 1:0.84) (Fig. 2H). In addition, reduced levels of Myo1 were recruited to cortical foci in *pak1-48* cells as compared with wild-type or temperature-sensitive *nak1-167* cells (Fig. 2I-K), consistent with the observed localisation pattern for the mutant Myo1-S361A protein. Together, these data indicate that Pak1 phosphorylates Myo1 Ser361 to modulate its ability to associate with cortical actin structures. However, residual Myo1 phosphorylation and localisation in cells lacking functional Pak1 suggests a level of overlapping functional redundancy between Pak1 and Nak1, which is consistent with the synthetic lethal relationship observed between these mutant alleles (not shown).

Myo1 TEDS site phosphorylation has no effect on cell growth and actin organisation

Myo1-S361A and Myo1-S361D mutant proteins were used to examine the role of Myo1 phosphorylation in regulating its cellular function. Each mutant allele was able to complement the growth

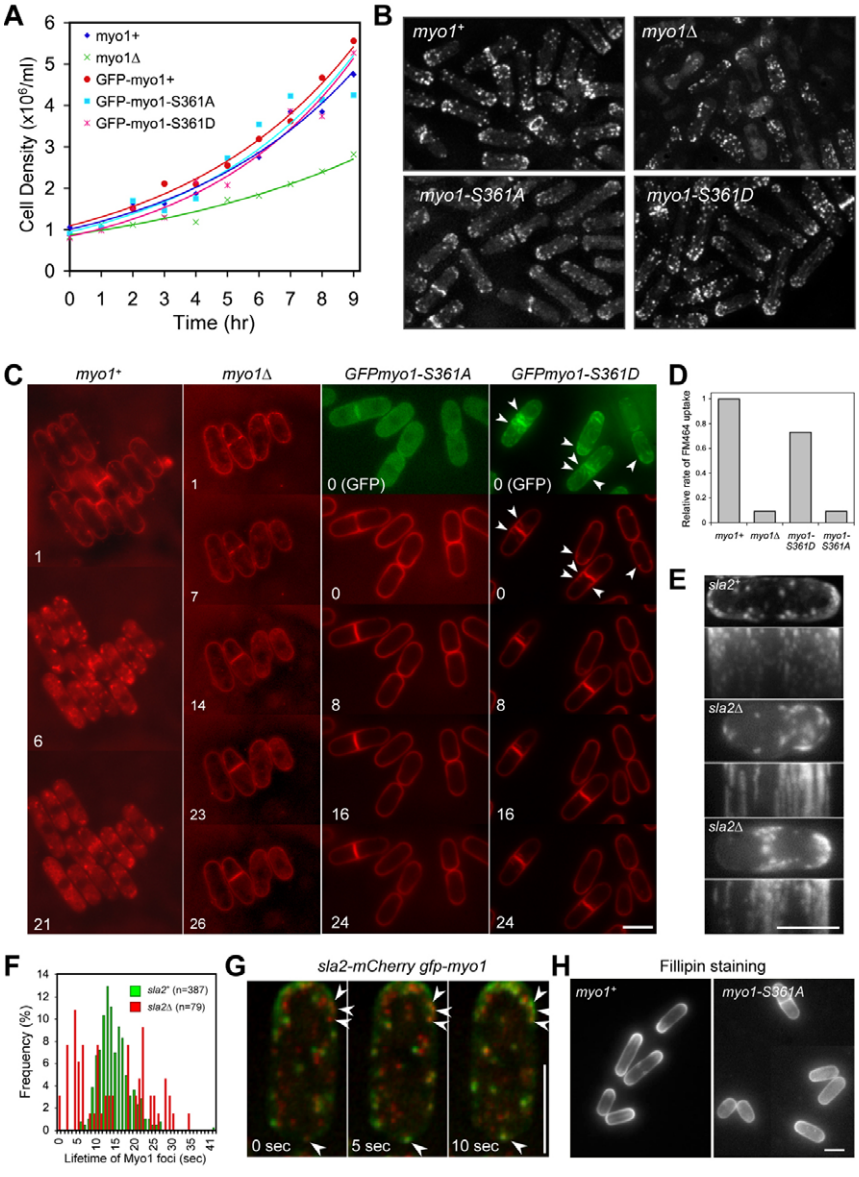

Fig. 3. Myo1 Ser361 phosphorylation regulates endocytosis in fission yeast. (A) Growth curves of *S. pombe* cells possessing different *myo1*$^{+}$ alleles. (B) Rhodamine-phalloidin staining of *myo1*$^{+}$, *myo1Δ*, *myo1-S361A* and *myo1-S361D* cells. (C) Images from time-course (in minutes) of FM4-64 uptake (red) in *myo1*$^{+}$, *myo1Δ*, *gfpmyo1-S361A* and *gfpmyo1-S361D* cells illustrate that Myo1 and TEDS-site phosphorylation are required for endocytosis. GFP fluorescence of *gfpmyo1-S361A* and *gfpmyo1-S361D* cells at the start of the experiment (green) illustrate where Myo1-S361D localisation coincides with FM4-64 uptake (arrowheads). (D) Histogram of relative rates of FM4-64 uptake in *myo1*$^{+}$, *myo1Δ*, *myo1-S361A* and *myo1-S361D* cells illustrates that Ser361 phosphorylation is required for Myo1 endocytic function. (E) Micrographs and kymographs from equivalent time-lapse movies of *gfp-myo1*$^{+}$ *sla2*$^{+}$ and *gfp-myo1*$^{+}$ *sla2Δ* cells. (F) Distributions of Myo1 foci lifetimes in *sla2*$^{+}$ (green bars) and *sla2Δ* (red bars) cells. (G) Images from time-course of *gfp-myo1*$^{+}$ *sla2-mCherry* cells illustrate that Myo1 foci localise to sites of endocytosis (e.g. arrowheads). (H) Micrographs of fillipin-stained *myo1*$^{+}$ and *myo1-S361A* cells reveal that Ser361 phosphorylation is required for proper organisation of sterol-rich lipid membranes. Scale bars: 5 μm.

defects associated with the *myo1* deletion (Fig. 3A) and the distributions of cell lengths for each mutant strain were equivalent to that of the wild type. Actin patches and filaments appear normal when Myo1-S361A and Myo1-S361D are expressed, although more cytoplasmic actin staining was observed in *myo1-S361A* cells (Fig. 3B). These data support the idea that fungal myosin-I tails alone are capable of activating the Arp2/3 complex, thereby promoting polymerisation of cortical F-actin to promote cell growth (Evangelista et al., 2000; Lee et al., 2000; Sirotkin et al., 2005), and illustrate that this process can occur in an apparently normal manner in the absence of myosin-I motor activity.

Myo1 Ser361 phosphorylation is required for endocytosis

We next determined whether Myo1 Ser361 phosphorylation played a role in regulating Myo1 function during endocytosis (Codlin et al., 2008). To confirm the role of Myo1 during endocytosis, the uptake of the lyphophylic dye FM4-64 was monitored in *myo1*$^{+}$, *myo1Δ myo1-S361A* and *myo1-S361D* cells, and FM4-64 internalisation from the moment of its addition to the cells. In agreement with Codlin and co-workers, we observed a significant disruption in FM4-64 uptake (i.e. endocytosis) in *myo1Δ* cells (Fig. 3C). As confirmation, we examined the dependency relationship between Myo1 and the conserved early endocytic marker, Sla2 (Alvarez-Tabares et al., 2007). Not only was the dynamic nature of Myo1 foci significantly disrupted in cells lacking Sla2 protein (Fig. 3E,F; supplementary material Movie 3), but Myo1 recruited to Sla2-labelled sites of endocytosis (Fig. 3G; supplementary material Movie 4). These data indicate that Myo1 plays an important role during endocytosis and further refute the findings reported by Takeda and Chang (Takeda and Chang, 2005).

We next examined the role Ser361 phosphorylation plays in this process by monitoring FM4-64 uptake in each *myo1-Ser361* mutant strain (Fig. 3C). Comparison of the rates of FM4-64 internalisation illustrates that the *myo1-S361D* mutation only partially complements the *myo1Δ*-associated endocytosis defects (Fig. 3D). Interestingly, Myo1-S361D filaments coincided precisely with sites of FM4-64 internalisation (Fig. 3C, arrowheads). By contrast, endocytosis was almost abolished in cells expressing Myo1-S361A. Together, these data suggest that careful modulation of Myo1 Ser361 phosphorylation plays a crucial role in regulating the internalisation of membranes during endocytosis.

As endocytosis is important in defining the sites of membrane dynamics and lipid organisation (Codlin et al., 2008), we decided to confirm the above finding by examining the effect the *myo1-S361A* mutation had on lipid raft organisation. The sterol-rich lipid stain fillipin revealed that, in contrast to the *myo1*$^{+}$ cells in which lipid rafts concentrated at sites of cell growth, this was abolished in *myo1-S361A* cells (Fig. 3H). This confirms the role that Ser361 phosphorylation plays in regulating Myo1 endocytic function in fission yeast.

The intimate relationship between actin patch dynamics and endocytosis has already been established (Kaksonen et al., 2005; Kaksonen et al., 2006). In budding yeast, myosin-I motor activity makes an important contribution during the later internalisation stages of this process (Sun et al., 2006). However, expression of myosin-I tail domains alone (i.e. myosin I lacking the motor domain) partially complements the endocytosis defects associated with the absence of full-length myosin I, suggesting that motor activity is not essential for this process to occur (Galletta et al., 2008). In fission yeast, the Myo1 tail recruits the protein to the cell cortex and promotes actin assembly through activation of the Arp2/3 complex (Sirotkin et al., 2005), a process that we show does not require motor activity. However, Myo1 dynamics and perhaps motor activity are required for the recruitment and function of this myosin at sites of endocytosis, which is reflected in the different phenotypes of the *myo1-S361A* and *myo1-S361D* alleles. Both proteins possess an Arp2/3 activating tail that is capable of promoting actin polymerisation. However, whereas Myo1-S361A does not associate with actin patches and is not seen at sites of endocytosis, Myo1-S361D is capable of associating with tropomyosin-dependent actin filaments, which is likely to reflect a change in actin affinity. Therefore, whereas the former is unable to remain associated with actin at sites of endocytosis, Myo1-S361D seems to have the actin affinity and subsequent motor activity required for endocytosis to occur. It remains unclear whether myosin-I motility pushes vesicles into the cell or impinges off the vesicle from the cell membrane. However, single-molecule analysis of Myo1 movement together with kinetic analysis of yeast myosin-I motor activity are likely to provide important insights into the nature and role of myosin-I motor activity during endocytosis.

Materials and Methods

Yeast cell culture and strains

The strains used in this study are listed in supplementary material Table S1. Cell culture and maintenance were carried out according to (Moreno et al., 1991). Cells were grown in supplemented minimal medium (EMM2) at 25°C. Expression from *nmt* promoters was repressed by the addition of 4 μM thiamine.

Molecular biology

S. pombe myo1^{+} cDNA was amplified from fission yeast genomic DNA using the primers oDM15 and oDM18 and cloned into plasmids to generate p*INT1GFPmyo1*$^{+}$, p*INT41GFPmyo1*$^{+}$ and p*REP1GFPmyo1*$^{+}$. *myo1-Ser361* mutant constructs were generated using primers oDM241 and oDM242 (p*INT41GFPmyo1-S361A*) or oDM243 and oDM244 (p*INT41GFPmyo1-S361D*) with p*INT41GFPmyo1*$^{+}$ template. All constructs were sequenced on completion. Oligonucleotides used in this study are listed in supplementary material Table S2.

Immunology

Standard immunological methods were used as described (Harlow and Lane, 1988). Myo1 antibodies were raised against a conjugated polypeptide of Myo1 residues 972-986. Phosphospecific anti-Myo1-Ser361-*P* antibodies were raised and affinity-purified against a phosphoserine modified conjugated peptide encompassing Ser361 (Eurogentec, Seraing, Belgium).

Yeast protein extracts

Protein extracts were prepared and analysed as described elsewhere (Skoumpla et al., 2007). Myo1 sera was used at a concentration of 1:100; anti-Myo1-Ser361-*P* antibodies were used at 1:200.

Microscopy

Samples were visualised as described previously (Skoumpla et al., 2007). Endocytosis was monitored by following FM4-64 uptake in cells mounted on open petri dishes. Endocytosis rates were averages calculated from >30 cells.

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