The recruitment of acetylated and unacetylated tropomyosin to distinct actin polymers permits the discrete regulation of specific myosins in fission yeast

Arthur T. Coulton[1], Daniel A. East[1], Agnieszka Galinska-Rakoczy[2,*], William Lehman[2] and Daniel P. Mulvihill[1,‡]

[1]School of Biosciences, University of Kent, Canterbury, Kent, CT2 7NJ, UK
[2]Department of Physiology and Biophysics, Boston University School of Medicine, Boston, MA 02118, USA
*Present Address: Department of Biology, University of Pennsylvania, 415 South University Avenue, 222 Leidy Labs, Philadelphia, PA 19104, USA
‡Author for correspondence (d.p.mulvihill@kent.ac.uk)

Accepted 21 June 2010
Journal of Cell Science 123, 3235-3243

doi:10.1242/jcs.069971

Summary

Tropomyosin (Tm) is a conserved dimeric coiled-coil protein, which forms polymers that curl around actin filaments in order to regulate actomyosin function. Acetylation of the Tm N-terminal methionine strengthens end-to-end bonds, which enhances actin binding as well as the ability of Tm to regulate myosin motor activity in both muscle and non-muscle cells. In this study we explore the function of each Tm form within fission yeast cells. Electron microscopy and live cell imaging revealed that acetylated and unacetylated Tm associate with distinct actin structures within the cell, and that each form has a profound effect upon the shape and integrity of the polymeric actin filament. We show that, whereas Tm acetylation is required to regulate the in vivo motility of class II myosins, acetylated Tm had no effect on the motility of class I and V myosins. These findings illustrate a novel Tm-acetylation-state-dependent mechanism for regulating specific actomyosin cytoskeletal interactions.

Key words: Tropomyosin, Acetylation, Fission yeast, *Schizosaccharomyces pombe*, Myosin, Cdc8, NatB

Introduction

Tropomyosin (Tm) is an evolutionarily conserved α-helical, coiled-coil protein, which associates along the length of each actin filament strand in muscle and non-muscle cells (Lees-Miller and Helfman, 1991; Perry, 2001). Tm polymerisation on actin stabilises actin filaments, which is essential for a wide range of cellular processes to occur, including cytokinesis, organelle transport and endocytosis (Gunning et al., 2005). In more specialised cellular tissue such as muscle, Tm-stabilised actin filaments provide a means of regulating myosin binding to actin which is essential for efficient muscle contraction to occur (Lehman et al., 1995; McKillop and Geeves, 1991; McKillop and Geeves, 1993). Studies have shown that N-terminal acetylation is essential for Tm to bind actin with high affinity (Monteiro et al., 1994). Unacetylated skeletal Tm is unable to bind actin in the absence of troponin (Hitchcock-DeGregori and Heald, 1987; Urbancikova and Hitchcock-DeGregori, 1994) and although unacetylated α-smooth muscle Tm (SmTm) is able to associate with actin, it has ~100 times weaker affinity for actin than acetylation-mimic SmTm mutants (Coulton et al., 2006). This difference in affinity between unacetylated and acetylated Tm isoforms is also observed for the high molecular mass fibroblast Tm isoforms, but not for the shorter fibroblast isoforms (Pittenger and Helfman, 1992). It is thought that end to end Tm–Tm contacts are strengthened by Tm acetylation (Brown et al., 2001; Holmes and Lehman, 2008), but the precise mechanism by which acetylation mediates high affinity actin binding is not fully understood.

Single celled organisms such as yeast provide an excellent system in which to study tropomyosin regulation and function. The genome of the budding yeast *Saccharomyces cerevisiae* encodes for two short tropomyosins, Tpm1 and Tpm2, each having distinct cellular functions. Although deletion of the *TPM1* gene is lethal to the cell, deletion of *TPM2* is not, and *TPM2* overexpression is not sufficient to complement the *TPM1* deletion (Drees et al., 1995). Tpm1 is required to maintain actin filament integrity which is necessary for directed vesicle transport within the cell (Liu and Bretscher, 1992; Liu and Bretscher, 1989). Tpm2 by contrast, has a specific role in the maintenance of myosin-II-dependent retrograde actin flow (Huckaba et al., 2006). TPM1 and TPM2 are N-terminally acetylated in vivo through the action of the NatB complex, which is composed of the catalytic and the regulatory protein subunits, Nat3 (Naa20) and Mdm20 (Naa25) (Polevoda et al., 2009; Polevoda et al., 2003; Singer and Shaw, 2003).

Cdc8, the sole Tm of the fission yeast, *Schizosaccharomyces pombe*, is essential for the formation and maintenance of actin filaments (Balasubramanian et al., 1992; Kurahashi et al., 2002; Pelham and Chang, 2001) and is present in both acetylated and unacetylated forms within the fission yeast cell (Skoumpla et al., 2007). Acetylation increases the affinity of Cdc8 for actin five fold, and dramatically enhances the ability of this conserved coiled-coil protein to regulate myosin activity (Skoumpla et al., 2007). The significance of the presence of both acetylated and unacetylated forms of Cdc8 within the yeast cell is currently unknown.

We show that acetylated Tm is largely enriched at the cytokinetic actomyosin ring (CAR) during mitosis, whereas unacetylated Tm is only seen on actin filaments during interphase. We show how the cytokinetic defect, which is associated with *S. pombe* cells lacking the NatB *N*-α-acetyltrasferase regulatory subunit, Naa25, is due to a lack of Tm acetylation. We go on to show that although Cdc8 acetylation has no effect upon the motility of class I or class V myosins on actin polymers, it plays a crucial role in regulating

both fission yeast class II myosins during cell division. These data indicate the presence of a novel Tm-acetylation-state-dependent mechanism for regulating specific actomyosin cytoskeletal interactions.

Results

In fission yeast acetylation of the tropomyosin, Cdc8, has a significant impact upon the ability of the protein to regulate the interaction between actin and myosin in vitro (Skoumpla et al., 2007). Within *S. pombe* cells a constant proportion of Cdc8 (~80%) is acetylated, while 20% is unacetylated, however, it has not been possible to determine how each of these subpopulations contribute to the cellular function of this Tm. In an attempt to address this we generated a strain in which the gene encoding for the *S. pombe* NatB N-terminal α-acetyltransferase complex regulatory subunit, Naa25 (Polevoda et al., 2009; Polevoda et al., 2003; Singer et al., 2000), had been deleted from the genome. The Naa25 protein was found to be non-essential for vegetative growth, but *naa25Δ* cells were not only temperature sensitive (Fig. 1A), but also grew slowly (Fig. 1A) and had cytokinetic (Fig. 1B,C) defects with 58% of *naa25Δ* cells possessing 1 (35%) or more (23%) septa. This is in dramatic contrast to an equivalent *naa25*$^{+}$ cells in which 15% of cells possessed a single septa. These septation defects were reminiscent of *myo2-E1* or *myp2Δ* cells (Balasubramanian et al., 1998; Bezanilla et al., 1997; Naqvi et al., 1999), as many of these cells (14%) acquired a branched phenotype at sites of failed cytokinesis during subsequent growth cycles (Fig. 1, arrows).

We initially attempted to visualise actin filament dynamics within live *naa25Δ* cells. However, the expression of each actin polymer labelling fusion protein we tested (i.e. GFP–CHD$_{Rng2}$, GFP–Cdc8, For3–GFP) (Doyle et al., 2009; Karagiannis et al., 2005; Martin and Chang, 2006; Skoumpla et al., 2007) rescued the *naa25Δ* strain cytokinenetic phenotypes (not shown). This not only indicates that each GFP fusion protein stabilises actin filaments and affects normal actin polymer dynamics, but suggests that the *naa25Δ* defect is brought about by instability of the actin ring. Rhodamine–phalloidin staining of cortical actin patches in fixed *naa25Δ* revealed the proportion of interphase cells undergoing either monopolar (23%) or bipolar growth (77%) were equivalent in wild-type and *naa25Δ* cells (Fig. 1D). This F-actin staining also indicated that actin cables were either absent or more fragile in *naa25Δ* cells than in the equivalent wild-type cells (Fig. 1D). We went on to use the more sensitive (in our hands) anti-Cdc8 immunofluorescence to study actin cable distribution within *naa25Δ* cells. This revealed that, compared with wild-type cells, less Tm was bound to fewer than normal actin polymers, which also appeared more fragile in *naa25Δ* cells especially at the CAR during mitosis (Fig. 2A).

We have previously reported that mid-log phase wild-type fission yeast cells contain both unacetylated and acetylated Cdc8 (Skoumpla et al., 2007). Mass spectroscopic analysis of Cdc8, purified from *naa25Δ* cells confirmed Tm acetylation is dependent upon Naa25 and illustrated that the less distinct filaments coincided with an absence of acetylated Cdc8 within the cell. To test whether lack of Cdc8 acetylation is the cause of *naa25Δ*-associated growth defects, a plasmid encoding for smooth muscle Tm with an N-terminal alanine–serine acetylation mimic dipeptide extension (SmTm-AS), capable of complementing Cdc8 function (Skoumpla et al., 2007) was introduced into *naa25Δ* fission yeast cells.

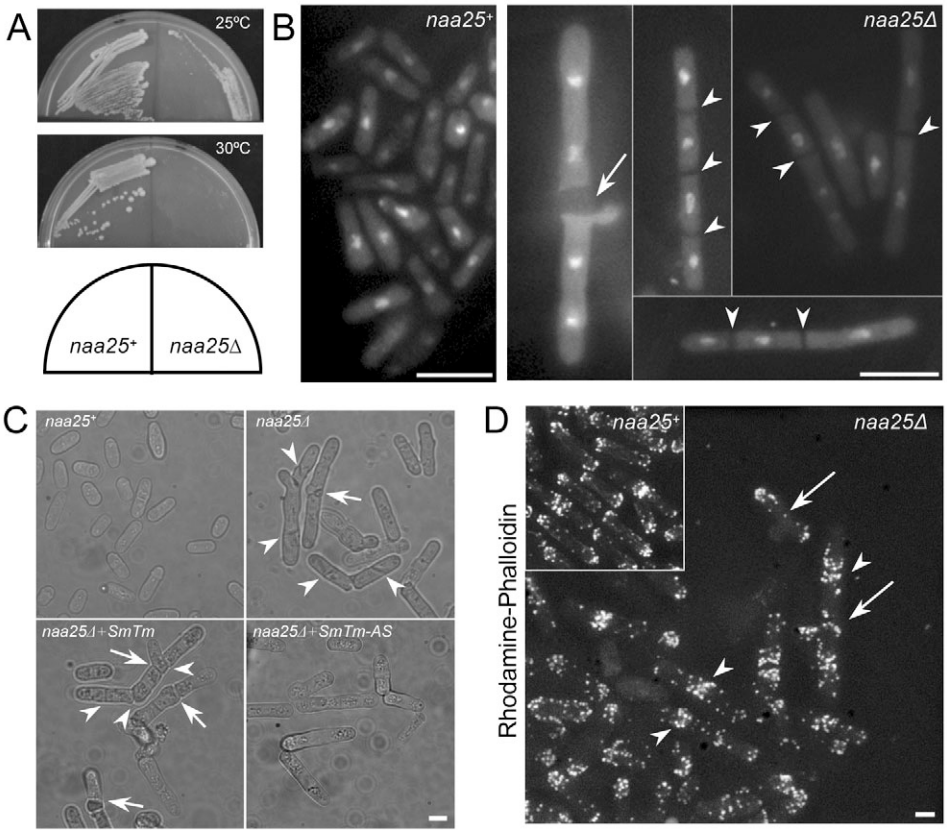

Fig. 1. Growth and division of naa25Δ *S. pombe* cells. (**A**) Plates showing growth of *naa25*$^{+}$ (left) and *naa25Δ* (right) cells when incubated at 25°C (top panel) or 30°C (middle panel). (**B**) Micrographs of DAPI-stained *naa25*$^{+}$ (left) and *naa25Δ* (right) cells grown at 25°C. *naa25Δ* cells frequently fail to complete septation (arrowheads) and these cells often continue to grow at the septum and form branches during subsequent growth cycles (arrows). Scale bars: 10 μm. (**C**) Phase-contrast images of *naa25*$^{+}$, *naa25Δ*, *naa25Δ pREP41*αSmTm and *naa25Δ pREP41*αSmTm-AS cells grown on minimal medium at 25°C. Scale bar: 5 μm. (**D**) Rhodamine-phalloidin staining of actin in *naa25Δ* cells grown in EMM2 minimal medium at 25°C compared with *naa25*$^{+}$ cells grown under equivalent conditions (inset). Scale bar: 5 μm.

Expression of SmTm-AS but not wild-type SmTm rescued the *naa25Δ*-associated cyokinesis defects (Fig. 1C). However growth rates indicated that neither protein was able to rescue the slow growth phenotype seen in cells lacking Naa25 (doubling times in EMM2 at 25°C were: *naa25*$^{+}$, 4.3±0.3 hours; *naa25Δ*, 9.2±1.2 hours; *naa25Δ*+*SmTm*, 9.25±0.8 hours; *naa25Δ*+*SmTm-AS*, 9.8±0.9 hours). These data indicate that whereas the cytokinesis defects observed in *naa25Δ* cells are probably due to a defect in Cdc8 acetylation and subsequent actin filament stability at the CAR, the slow growth phenotype was a consequence of a lack of acetylation of proteins other than Tm.

Unacetylated-Cdc8 associates with and affects actin polymer structure.

To confirm the above observation that unacetylated Cdc8 could associate with actin filaments we examined electron micrographs of negatively stained reconstituted filaments consisting of F-actin associated with either acetylated Cdc8, unmodified Cdc8, or cardiac muscle Tm. Tm strands could be easily identified in electron micrographs of negatively stained F-actin; however, each form of the Tm filaments (Fig. 2B–D), which coiled around the actin filaments with a characteristic pattern, had a very different effect upon the physical appearance of the polymer. In contrast to the regular and straight filaments composed of actin in complex with cardiac Tm (Fig. 2D), acetylated Cdc8 caused the actin polymers to have a more jagged and irregular appearance (Fig. 2B). This irregular appearance was even more pronounced in actin–unacetylated Cdc8 filaments (Fig. 2C), which not only bundled together (arrows) but also had a propensity to break into shorter lengths (arrowheads). This suggests the actin–unacetylated Cdc8 filaments are relatively rigid, but are less stable than the other actin-Tm polymers examined here, possibly because of the interruption or enhanced fragility of tropomyosin end-to-end linkages. Although unacetylated Cdc8 was seen to decorate actin, a large number of Tm dimers were visible in the background, illustrating the low affinity this Tm has for actin (Fig. 2C). These data show unacetylated Cdc8 is capable of associating with actin polymers (in vivo and in vitro) and also illustrates that the association of each Cdc8 form affects the physical nature of actin–Tm filaments in distinct ways.

Characterisation of acetylation-state-specific anti-Cdc8 antibodies

To further explore the significance of the finding that both unacetylated and acetylated Cdc8 are capable of associating with actin filaments within the cell, we generated two acetylation-state-specific anti-Cdc8 antibodies. These were either raised and purified against an unmodified (anti-Cdc8^{UNACE}) or acetyl-conjugated (anti-Cdc8^{ACE}) peptide encompassing the N-terminus of the Cdc8 protein. Western blot analysis revealed that the anti-Cdc8^{UNACE} antibodies specifically recognised unmodified Cdc8 purified from *E. coli* but not an equivalent quantity of acetylated Cdc8 purified from wild-type *S. pombe* cells (Fig. 3A). By contrast, anti-Cdc8^{ACE} antibodies only detected acetylated Cdc8 but not the unacetylated form (Fig. 3A). In addition, whereas anti-Cdc8 serum recognised Cdc8 in extracts of wild-type and *naa25Δ* cells, the anti-Cdc8^{ACE} antibodies only recognised Cdc8 from wild-type cell extracts (Fig. 3B). These results confirm the specificity of these antibodies and our observation that Cdc8 remains unacetylated in *naa25Δ* cells.

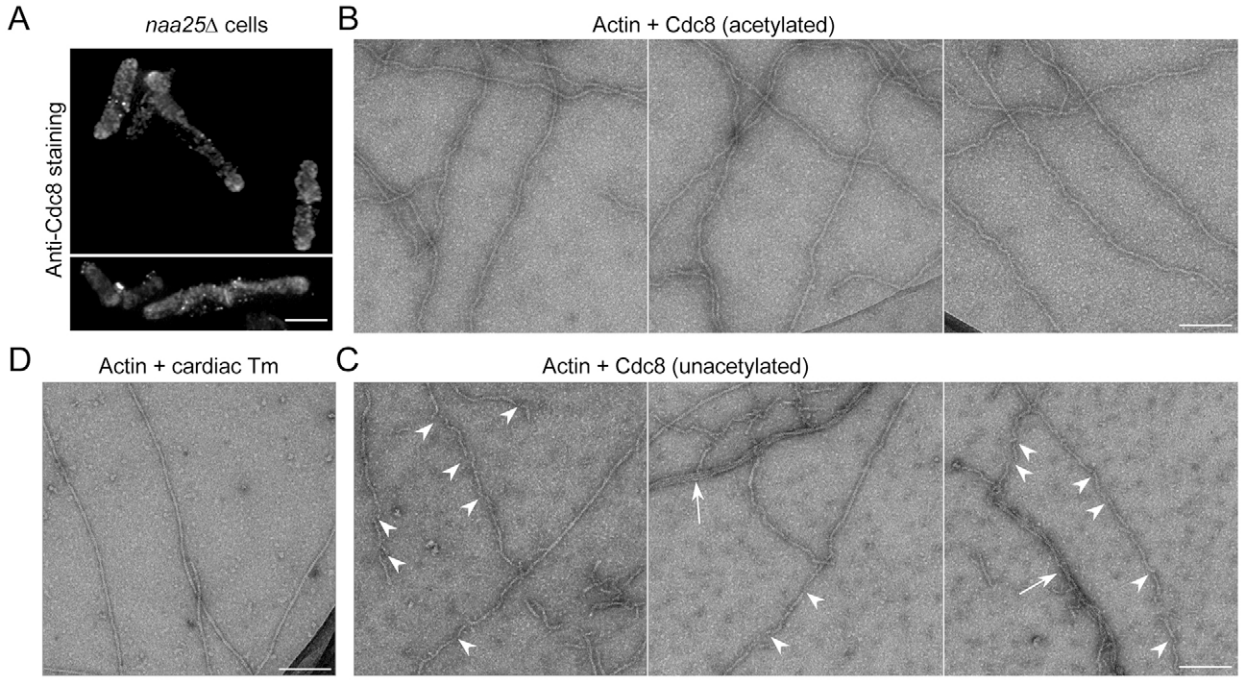

Fig. 2. Unmodified Cdc8 associates with and affects the appearance of Cdc8–actin polymers in vivo and in vitro. (**A**) Cdc8 immunofluorescence staining of the *naa25Δ* strain using Cdc8 antiserum. Scale bar: 5 μm. (**B–D**) Negative staining of (B) F-actin–acetylated Cdc8, (C) actin–unmodified Cdc8, or (D) actin–cardiac Tm. Obliquely oriented strands characteristic of tropomyosin (Lehman et al., 1994) are observed in each sample. In contrast to cardiac Tm and acetylated-Cdc8-labelled actin filaments (B,D), breaks (arrowheads) are evident in the unacetylated Cdc8–actin polymers (C), which also often formed bundles (arrows). Samples were prepared using identical methods. Scale bars: 40 nm.

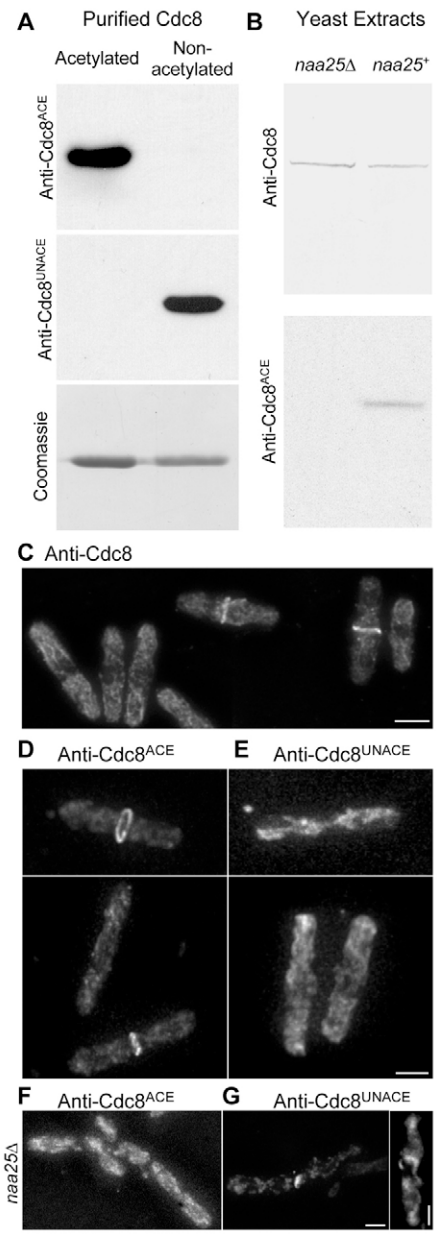

Fig. 3. Acetylation-state-specific anti-Cdc8 antibodies reveal that only acetylated Cdc8 localises to the cytokinetic actomyosin ring. (**A**) Purified endogenous and recombinant Cdc8 proteins were used to characterise the anti-Cdc8^{ACE} antibody and the anti-Cdc8^{UNACE} antibody. Protein samples were run on a Coomassie Blue-stained gel to demonstrate equal loading. (**B**) Anti-Cdc8 (upper panel) and anti-Cdc8^{ACE} (lower panel) antibodies were used to probe *naa25Δ* and *naa25*$^{+}$ cell extracts. The anti-Cdc8^{ACE} antibody only recognises Cdc8 from *naa25*$^{+}$ cells. (**C–G**) Immunofluorescence of *naa25*$^{+}$ (C,D,E) and *naa25Δ* (F,G) cells using anti-Cdc8 (C), anti-Cdc8^{ACE} (D,F) or anti-Cdc8^{UNACE} (E,G) acetylation-specific antibodies. Scale bars: 5 μm.

Acetylated and unacetylated Cdc8 localise to distinct actin polymers in yeast cells

The acetylation-state-specific antibodies were then used to determine whether unacetylated and acetylated Cdc8 have distinct localisation patterns within fission yeast. The same fixed sample of wild-type cells were subjected to indirect immunofluorescence using either anti-Cdc8 (Fig. 3C), anti-Cdc8^{ACE} (Fig. 3D) or anti-Cdc8^{UNACE} (Fig. 3E) antibodies. Although anti-Cdc8^{ACE} antibodies associated strongly with actin filaments within the CAR (Fig. 3D), very little bound to interphase filaments compared with the anti-Cdc8 sera staining (Fig. 3C). By contrast, anti-Cdc8^{UNACE} antibodies were never seen associated with actin filaments incorporated into the CAR during mitosis (Fig. 3E) in wild-type cells, and instead were seen exclusively associated with actin-Cdc8 polymers extending throughout the cell during interphase and at the end of cytokinesis (Fig. 3E). Anti-Cdc8^{ACE} immunofluorescence of *naa25Δ* cells showed no discrete localisation pattern (Fig. 3F); however, anti-Cdc8^{UNACE} antibodies were capable of recognising Cdc8 associated with the CAR in *naa25Δ* cells containing only unmodified protein (Fig. 3G). This not only illustrates that acetylated Cdc8-actin polymers are normally incorporated into the CAR during wild-type mitosis but in the absence of acetylated Tm, unmodified Cdc8 is capable of associating with CAR-associated actin filaments and partially stabilise this organelle. These data indicate that there is a distinct cellular distribution of each Cdc8 form, and although the CAR normally only contains acetylated Tm, the interphase filaments are enriched with the unmodified Cdc8, and this may reflect the different functional demands upon each cytoskeletal structure.

Cellular distribution and dynamics of class I and V myosin appear normal in *naa25Δ* cells

Previous data has shown that Cdc8 acetylation has the potential to provide a regulatory mechanism for modulating myosin function within the cell (Skoumpla et al., 2007). We next determined whether this was the case in vivo by exploring how Cdc8 acetylation affects the motility of each class of myosin present in yeast (classes I, II and V) by generating *naa25Δ* strains, each expressing a separate class of fluorophore-tagged myosin.

The fission yeast class I myosin, Myo1, localises to dynamic foci at sites of cell growth, from which they seed the polymerisation of Arp2/3-dependent actin patches and promote endocytosis (Attanapola et al., 2009; Codlin et al., 2008; Lee et al., 2000). As these actin structures lack Cdc8 it was unsurprising that lack of Tm acetylation had no significant affect upon the cellular distribution of this motor protein, which continued to be recruited to foci at sites of growth in *naa25Δ* cells (Fig. 4A,B; Table 1). In addition,

Table 1. Localisation of myosins in *naa25*$^{+}$ and *naa25Δ* cells

Myosin	Location	*naa25*$^{+}$ (% of total)	*naa25Δ* (% of total)
Myo1	Endosomes alone	0.0	8.4
	Cell poles	72.3	80.6
	Cell equator	8.7	5.0
	Septum	18.3	4.7
	Cell poles and septum	0.7	0.3
	Multiple septa	0.0	1.0
Myo2	Functional CAR	11.7	4.1
	Aberant CAR	0.0	9.1
	Functional CAR + filaments	0.0	2.2
	Aberant CAR + filaments	0.0	1.6
	Interphase filaments	0.0	26.1
	Diffuse localisation	88.3	59.9
Myo52	Cell poles	86.2	79.9
	Cell equator	13.8	1.9
	Poles and equator	0.0	10.4
	Multiple septa	0.0	4.2
	Only motile foci	0.0	3.5

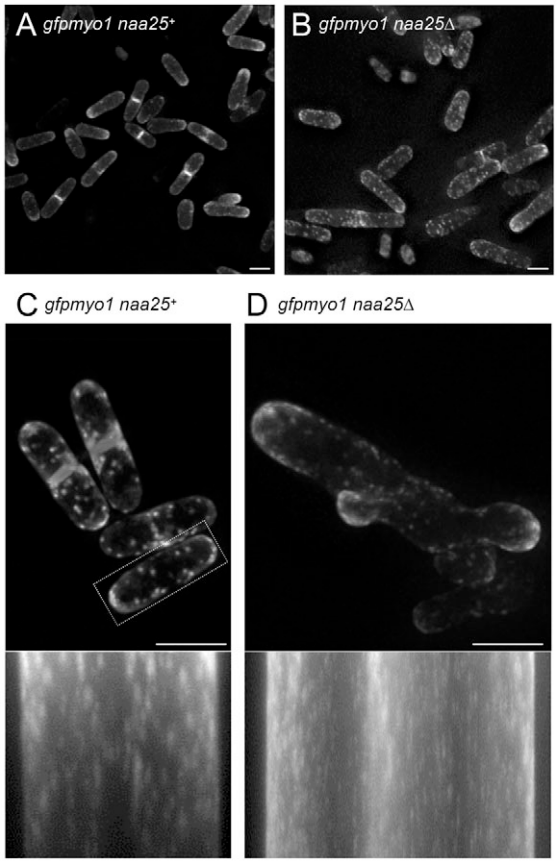

Fig. 4. Class I myosin dynamics do not require acetylated Cdc8. (**A**,**B**) GFP signal from *gfp-myo1 naa25*$^{+}$ (A) and *gfp-myo1 naa25Δ* (B) cells reveal Myo1 is recruited to the cell poles and septum in both strains. (**C**,**D**) Kymograph analysis of GFP-Myo1 in vivo dynamics in *S. pombe naa25*$^{+}$ (C) and *naa25Δ* (D) strains. Kymographs are maximum projections of 100 five-z-slice stacks at different time points, each 250 mseconds apart. Scale bars: 5 µm.

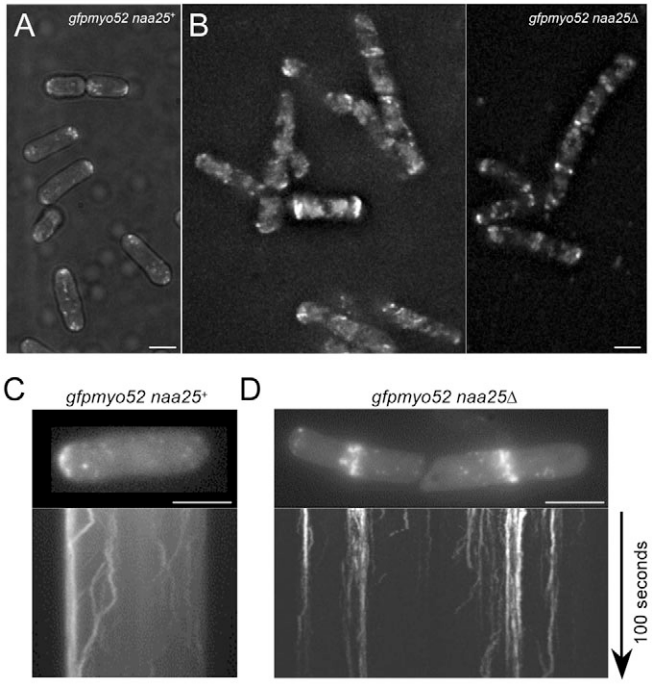

Fig. 5. Class V myosin movements do not require acetylated Cdc8. (**A**,**B**) GFP signal from *gfp-myo52 naa25*$^{+}$ (A) and *gfp-myo52 naa25Δ* (B) cells reveal Myo52 is recruited to motile foci that concentrate at sites of cell growth in each strain. (**C**,**D**) Kymograph analysis of GFP-Myo52 in vivo motility in *S. pombe naa25*$^{+}$ (C) and *naa25Δ* (D) strains. Kymographs are maximum projections of 100 five-z-slice stacks at different time points, each 250 mseconds apart. Scale bars: 5 µm.

comparison of kymographs generated from time-lapse movies of *gfp-myo1 naa25*$^{+}$ and *gfp-myo1 naa25Δ* cells (Fig. 4C,D; supplementary material Movies 1 and 2) revealed no difference in the frequency or duration of Myo1 foci signal, which confirmed that Cdc8 acetylation does not affect the Cdc8-independent actin association of this class I myosin.

Fission yeast cells possess two type V myosins, Myo51 and Myo52, but only the latter has any discernible function or motility during the vegetative life cycle (Win et al., 2001). Myo52 moves along Cdc8–actin polymers within the *S. pombe* cell and concentrates at actin-rich sites of cell growth (Grallert et al., 2007). Not only was the ability of Myo52 to localise to actin filaments or concentrate at regions of growth unaffected in the *naa25Δ* cells (Fig. 5A,B; Table 1), but lack of Cdc8 acetylation had no effect upon the movement and velocity of this motor protein upon actin–Cdc8 polymers (Fig. 5C,D; Table 1; supplementary material Movies 3 and 4). Fewer Myo52 movements were observed in cells lacking Naa25 when compared with wild-type cells, which was likely to be a consequence of the reduced number of stable Cdc8–actin polymers observed within these cells (Fig. 2A). These data indicate that acetylated Cdc8 has no role in regulating the normal actin-dependent in vivo motility of fission yeast myosin V.

Localisation of class II myosins and CAR function are disrupted in *naa25Δ* cells

As in many eukaryote non-muscle cells, the two fission yeast class II myosins, Myo2 and Myp2, only localise to the CAR during mitosis (Bezanilla et al., 1997; Kitayama et al., 1997). These motor proteins provide the force for the constriction of this specialised actin structure, which precedes deposition of the septum (Mulvihill and Hyams, 2003). A strain expressing *myo2-mCherry* was used to examine the effect that lack of Cdc8 acetylation had upon Myo2 localisation and CAR constriction. In contrast to wild-type cells, in which Myo2 was recruited to functional contractile rings (Fig. 6A,C; supplementary material Movie 5), Myo2 was not only seen to localise to nonfunctional actin rings in *naa25Δ* cells (Fig. 6B,D; supplementary material Movie 6; Table 1), but also to actin filaments not associated with the CAR during interphase and mitosis (Fig. 6B; supplementary material Movie 7; Table 1) and aberrant contractile structures extending the length of the cell (supplementary material Movie 8).

To confirm that the abnormal Myo2 structures were not a consequence of the fluorophore tag, anti-Myo2 and anti-Myp2 antibodies were used to examine the localisation of both endogenous fission yeast class II myosins in the absence of acetylated Cdc8. Anti-Myo2 and anti-Myp2 immunofluorescence revealed that both class II myosins were only recruited to actin filaments to form a complete CAR structure in wild-type cells, as reported previously (Bezanilla et al., 1997; Kitayama et al., 1997) (Fig. 6E,F, insets). In cells lacking Naa25, however, Myo2 and Myp2 localised not only to filaments not associated with the CAR (Fig. 6E,F arrowheads) but also to abnormal contractile structures

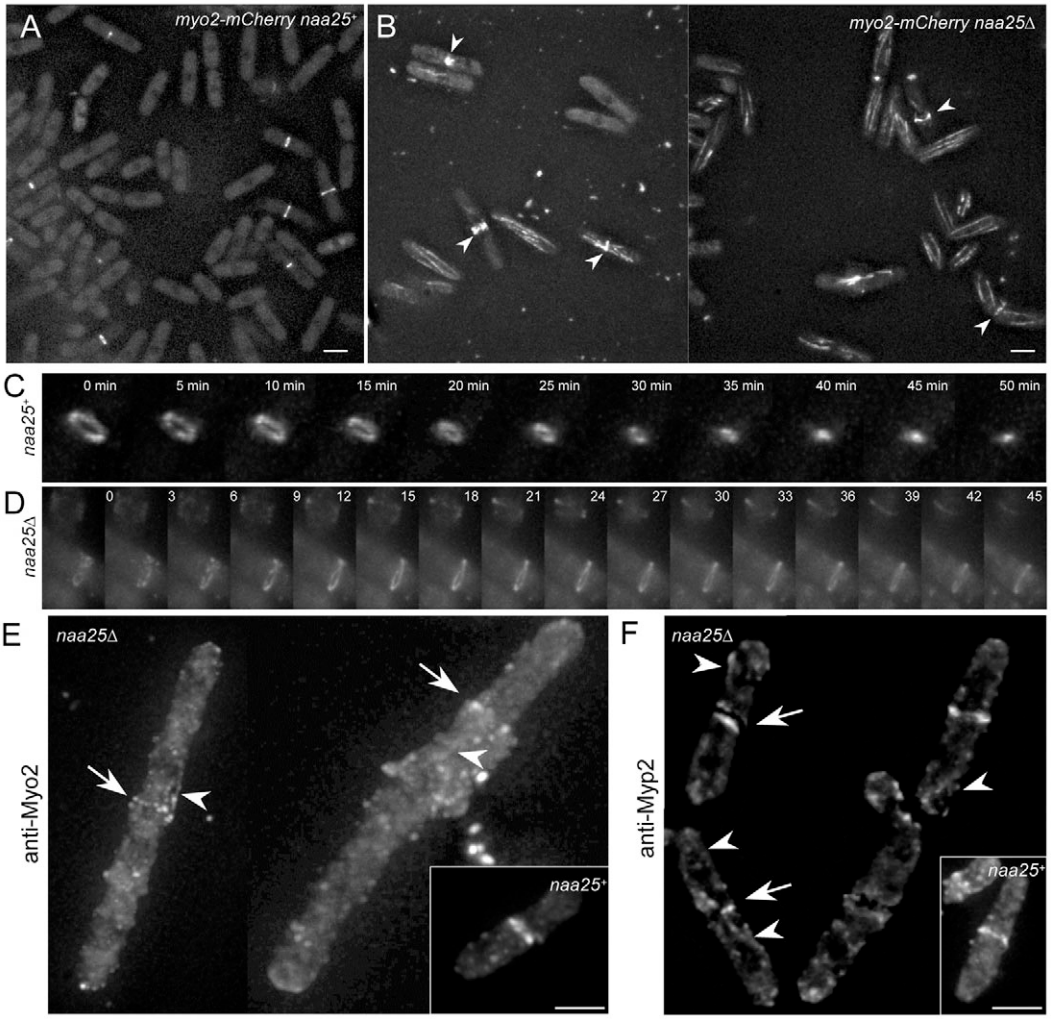

Fig. 6. Function of both *S. pombe* class II myosins require acetylated Cdc8. In vivo localisation of Myo2 in *S. pombe* *naa25*$^{+}$ (**A**) and *naa25Δ* (**B**) cells. Myo2 was seen localised to abnormal contractile structures (arrowheads) and actin filaments not associated with the CAR in cells lacking Naa25. (**C**) Myo2 rings constricted within an hour of forming in *naa25*$^{+}$ cells. (**D**) Myo2 often localised to contractile rings which failed to form properly or constrict in *naa25Δ* cells. (**E**,**F**) Anti-Myo2 (E) and anti-Myp2 (F) immunofluorescence of each endogenous class II myosin confirmed the live cell imaging data. Although each myosin only localised to distinct CAR structures during mitosis (insets), both proteins were seen associated with disrupted CAR structures (arrows) and interphase actin filaments (arrowheads) in *naa25Δ* cells. Scale bars: 5 μm.

which either did not contract properly or had a punctate and ill-defined appearance (Fig. 6E,F arrows). These data indicate that acetylated Cdc8 plays an important role in regulating the localisation and function of class II myosins in yeast.

Discussion

N-terminal acetylation of proteins is a ubiquitous post-translational modification crucial for regulating the stability, structure and function of a large number of proteins. Acetylation of the N-terminus occurs in up to 90% of eukaryote proteins and is carried out co-translationally at the ribosome by a group of enzymes called N-terminal α-acetyltransferases (NATs), which transfer an acetyl group from acetyl coenzyme A to the N-terminal amino acid of a protein (Polevoda and Sherman, 2003). Acetylation of the N-terminal methionine of the actin cytoskeleton component tropomyosin enhances the end-to-end interactions between Tm dimers (Brown et al., 2001; Urbancikova and Hitchcock-DeGregori, 1994), which increases the integrity of actin–Tm filaments and stabilises the position of the Tm polymer on actin.

During this study we generated a strain of *S. pombe* lacking the gene encoding for Naa25, the regulatory subunit of the NatB N-terminal acetyltransferase complex. This allowed us to examine how acetylation affects the in vivo function of the essential fission yeast tropomyosin, Cdc8. Consistent with the observation that acetylation enhances the ability of Cdc8 to bind and associate with actin (Skoumpla et al., 2007), cells lacking Naa25 (and therefore lack acetylated Cdc8) had perturbed CAR function, and had a phenotype reminiscent of that seen in cells lacking a functional copy of either of the *S. pombe* type II myosins, Myo2 or Myp2 (Balasubramanian et al., 1998; Bezanilla et al., 1997; Kitayama et al., 1997). Significantly, this cytokinesis defect was rescued by expression of SmTm with a dipeptide acetylation mimic N-terminal extension, whereas overexpression of unmodified Tm did not (Fig. 1C). This implies lack of Tm acetylation is the major contributory factor in the appearance of a septation phenotype in this mutant.

Surprisingly, electron microscopy revealed that the acetylation state of Cdc8 has a dramatic effect on Cdc8–actin filaments. Although acetylated Cdc8–actin filaments had a slightly wavy appearance (when compared with actin alone or actin decorated with cardiac Tm), the filaments appeared stable and failed to bundle. By contrast, actin filaments decorated with unacetylated Tm were not only considerably more wavy, but also bundled and were significantly more fragile than the acetylated form. These findings may indicate that as well as modulating the stability of the actin polymer, the acetylation state of this Tm may play a previously unpredicted and active role in regulating F-actin dynamics within the cell. This is intriguing when considered with the finding that each form of Cdc8 associated with distinct actin filaments within

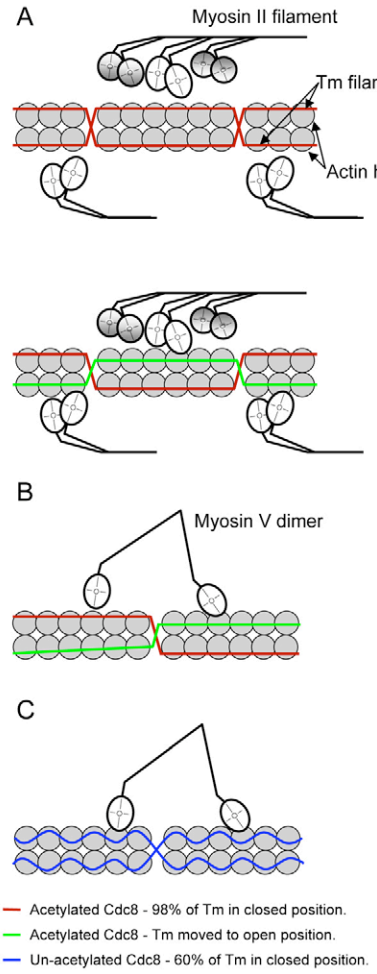

Fig. 7. Model comparing the effect that Tm acetylation has upon actin-based motility of class II and class V myosins. (**A**) Acetylation of Cdc8 permits the actomyosin interaction-driven cooperative movement of the Tm filament from its normal residency in the closed (red lines) into the open position (green lines) on an individual actin strand (grey circles). This facilitates the regulated cooperative binding of multiple motor domains of myosin II heavy chain filaments along the actin strand. (**B**) The 36 nm step taken by myosin V dimers results in each motor domain interacting with a separate actin strand. As 98% of acetylated Tm filaments occupy the closed or 'off' position on actin, each actomyosin interaction would be regulated by Tm and would therefore affect this dimeric motor's motility. (**C**) By contrast, significantly less (32%) of the of unacetylated Tm filaments (blue lines) occupy the closed position and therefore have negligible regulatory effect on myosin.

the cell. The acetylated Cdc8 was found enriched on actin filaments associated with the CAR during mitosis, with which class II myosins associate. By contrast, unacetylated Cdc8 was seen to localise exclusively to actin filaments that extend throughout the cell, not associated with the CAR. The mechanism by which the localisation of each Tm form to discrete actin filaments is regulated is currently unclear, however, several regulators of the cell cycle and actin cytoskeleton are likely to play direct roles in this.

These distinct localisation patterns suggested the possibility that each form of Cdc8 plays a role in differential regulation of each myosin, depending upon their cellular location. We went on to provide evidence that Tm acetylation plays a crucial role in regulating distinct classes of myosin. Consistent with these observations we discovered that myosin V and myosin I movement are unaffected by lack of Tm acetylation. Although Myo1 normally only associates with short polymers of cortical actin, and is prevented from associating with actin filaments labelled with forms of Cdc8 (Attanapola et al., 2009; Sirotkin et al., 2005), Tm acetylation has no effect upon this inhibition. Similarly our findings show that Myo52 motility not being affected by Cdc8 acetylation is consistent with the observations that Tm does not affect the motility of mammalian myosin Va in vitro (Stark et al., 2010). By contrast, our data suggest the acetylated form of the fission yeast Tm plays a crucial role in regulating the myosin II motors at the CAR structure, as the absence of acetylated Cdc8 has a profound effect upon the structural integrity of this myosin-II-associated actin structure.

Why should the acetylation state of Tm specifically affect class II myosin motor activity and not that of myosin V? Acetylation enhances the end-to-end interactions between Tm dimers (Brown et al., 2001; Urbancikova and Hitchcock-DeGregori, 1994), which increases the integrity of actin–Tm filaments and stabilises the position of the Tm polymer on actin. This has been shown to be the case in fission yeast, where 98% of the acetylated Cdc8 sits firmly in the closed position of the actin helix and thereby enhances the regulation of myosin motor activity (Skoumpla et al., 2007) by modulating the actin affinity and duty cycle of myosin II in vitro (Stark et al., 2010). However, the position of non-acetylated Tm polymers are less constrained upon actin, and will therefore have no significant effect upon actomyosin interactions. These differences in the physical properties of the Tm–actin filaments are therefore crucial only in affecting a specific class of myosins, as each myosin class interacts with actin in fundamentally different ways.

Multiple motor domains of myosin II dimer thick filaments interact simultaneously with adjacent actin monomers at the CAR. This working in concert allows the myosins to exert significantly more force or stress on the actin polymer than a single head alone. In addition the cooperative nature of the Cdc8^{ACE} filament (Skoumpla et al., 2007) means that the interaction of each myosin II with actin moves the Tm polymer on adjacent actin molecules of the same actin helix strand from a position that only allows myosin to bind in the weak state (closed position) to one that permits binding in the strong state and contraction (open position) (McKillop and Geeves, 1993), thus enhancing subsequent myosin–actin interactions in these positions (Fig. 7). Importantly, CAR assembly and disassembly are tightly coordinated processes that occur once every cell cycle, with no obvious growth or shrinkage of actin filaments once they are incorporated within the structure (Pollard and Wu, 2010; Wu et al., 2003). Therefore, a trade off is required between the need to have more dynamic actin polymers and the need to produce a more stable actin structure capable of withstanding the forces generated by a number of myosin II molecules working in concert along the same actin–Cdc8 cable (Lord and Pollard, 2004; Stark et al., 2010). By contrast, a single myosin V dimer normally takes large 36 nm steps, which span between adjacent strands of the actin helix. Therefore each myosin-head-to-actin contact will not affect the position of the Tm on actin during subsequent binding events. The more flexible actin–unacetylated Cdc8 filament would not inhibit the processive nature of myosin V movements. As the myosin V dimer does not exert undue stress on the actin–Tm complex when compared with simultaneous interactions from multiple myosin II dimers at the

CAR, the ability of Tm to increase the stability or integral strength of the actin polymer is likely to be of less significance for actin cables which only act as tracks for individual myosin V dimers (Veigel et al., 2005). Therefore we propose that concerted action of multiple myosin II heavy chain displaces the acetylated Cdc8 filament from the closed to the open position, so allowing myosin to generate force without challenging the integrity of the actin cable.

Materials and Methods

Yeast cell culture and strains

Cell culture and maintenance were carried out as described elsewhere (Moreno et al., 1991). Cells were grown in Edinburgh minimal medium supplemented with the appropriate amino acids. Genetic crosses were undertaken on MSA plates (Egel et al., 1994). Strains used in this study are listed in supplementary material Table S1.

Molecular biology

The *naa25*$^{+}$ gene corresponds to the designated coding sequence SPBC1215.02c within the *S. pombe* genome. *myo2-mCherry:hphMX6* and *naa25::kanMX6* strains were created as described previously using appropriate templates and primers (Bahler et al., 1998; Tanaka et al., 2005).

Immunological techniques

Standard immunological methods were used as described previously (Harlow and Lane, 1988). Acetylation-state-specific anti-Cdc8 antibodies were raised against either an unmodified or acetyl-conjugated peptide encompassing the N-terminus of the Cdc8 protein in SPF rabbits (Eurogentec, Seraing, Belgium), and were subsequently affinity purified. Anti-Myo2 and anti-Myp2 antibodies were raised against the KLH-conjugated polypeptides LKEHRPSGKENNIPA and LLEDVPNNTRNQIKG, respectively (Myo2 residues 1370–1384; Myp2 residues 1517–1531) in SPF rabbits (Eurogentec, Seraing, Belgium).

Analysis of yeast extracts

Protein extracts were prepared and analysed as described elsewhere (Skoumpla et al., 2007). For western blot analysis anti-Cdc8 serum was diluted 1:1000, and affinity purified acetylated-state-specific anti-Cdc8 antibodies were diluted 1:100.

Microscopy

Samples were visualised as described elsewhere (Martin-Garcia and Mulvihill, 2009). Statistical analyses were carried out on averages of individual samples of more than 300 cells. Immunofluorescence microscopy was performed as described previously (Hagan and Hyams, 1988) except that glutaraldehyde was omitted. Anti-Cdc8, Myo2 and Myp2 sera were used at a dilution of 1:100 and acetylation-state-specific anti-Cdc8 antibodies were used at a dilution of 1:1000. Electron microscopy was undertaken as described previously (Skoumpla et al., 2007) mixing 20 μM F-actin with 5.7 μM cardiac Tm or 50 μM of either acetylated or unmodified Cdc8.

We thank Jerry Hyams (Toulouse) for the generous gift of anti-Myo2 and anti-Myp2 antibodies and Mike Regnier (Washington), Kay Barr, Mike Geeves and Armenius Centurion (Kent) for discussions. This work was funded by a BBSRC project grant (BB/F011784/1) to D.P.M. and NIH grants (HL86655 and HL36153) to W.L. Deposited in PMC for release after 12 months.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/19/3235/DC1

References

Attanapola, S. L., Alexander, C. J. and Mulvihill, D. P. (2009). Ste20-kinase-dependent TEDS-site phosphorylation modulates the dynamic localisation and endocytic function of the fission yeast class I myosin, Myo1. *J. Cell Sci.* **122**, 3856-3861.

Bahler, J., Wu, J. Q., Longtine, M. S., Shah, N. G., McKenzie, A., 3rd, Steever, A. B., Wach, A., Philippsen, P. and Pringle, J. R. (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. *Yeast* **14**, 943-951.

Balasubramanian, M. K., Helfman, D. M. and Hemmingsen, S. M. (1992). A new tropomyosin essential for cytokinesis in the fission yeast S. pombe. *Nature* **360**, 84-87.

Balasubramanian, M. K., McCollum, D., Chang, L., Wong, K. C., Naqvi, N. I., He, X., Sazer, S. and Gould, K. L. (1998). Isolation and characterization of new fission yeast cytokinesis mutants. *Genetics* **149**, 1265-1275.

Bezanilla, M., Forsburg, S. L. and Pollard, T. D. (1997). Identification of a second myosin-II in Schizosaccharomyces pombe: Myp2p is conditionally required for cytokinesis. *Mol. Biol. Cell* **8**, 2693-2705.

Brown, J. H., Kim, K. H., Jun, G., Greenfield, N. J., Dominguez, R., Volkmann, N., Hitchcock-DeGregori, S. E. and Cohen, C. (2001). Deciphering the design of the tropomyosin molecule. *Proc. Natl. Acad. Sci. USA* **98**, 8496-8501.

Codlin, S., Haines, R. L. and Mole, S. E. (2008). btn1 affects endocytosis, polarization of sterol-rich membrane domains and polarized growth in Schizosaccharomyces pombe. *Traffic* **9**, 936-950.

Coulton, A., Lehrer, S. S. and Geeves, M. A. (2006). Functional homodimers and heterodimers of recombinant smooth muscle tropomyosin. *Biochemistry* **45**, 12853-12858.

Doyle, A., Martin-Garcia, R., Coulton, A. T., Bagley, S. and Mulvihill, D. P. (2009). The fission yeast type V myosin Myo51, is a meiotic spindle pole body component, with discrete roles during cell fusion and spore formation. *J. Cell Sci.* **122**, 4330-4340.

Drees, B., Brown, C., Barrell, B. G. and Bretscher, A. (1995). Tropomyosin is essential in yeast, yet the TPM1 and TPM2 products perform distinct functions. *J. Cell Biol.* **128**, 383-392.

Egel, R., Willer, M., Kjaerulff, S., Davey, J. and Nielsen, O. (1994). Assessment of pheromone production and response in fission yeast by a halo test of induced sporulation. *Yeast* **10**, 1347-1354.

Grallert, A., Martin-Garcia, R., Bagley, S. and Mulvihill, D. P. (2007). In vivo movement of the type V myosin Myo52 requires dimerisation but is independent of the neck domain. *J. Cell Sci.* **120**, 4093-4098.

Gunning, P. W., Schevzov, G., Kee, A. J. and Hardeman, E. C. (2005). Tropomyosin isoforms: divining rods for actin cytoskeleton function. *Trends Cell. Biol.* **15**, 333-341.

Hagan, I. M. and Hyams, J. S. (1988). The use of cell division cycle mutants to investigate the control of microtubule distribution in the fission yeast Schizosaccharomyces pombe. *J. Cell Sci.* **89**, 343-357.

Harlow, E. and Lane, D. (1988). *Antibodies: A Laboratory Manual.* Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Hitchcock-DeGregori, S. E. and Heald, R. W. (1987). Altered actin and troponin binding of amino-terminal variants of chicken striated muscle alpha-tropomyosin expressed in Escherichia coli. *J. Biol. Chem.* **262**, 9730-9735.

Holmes, K. C. and Lehman, W. (2008). Gestalt-binding of tropomyosin to actin filaments. *J. Muscle Res. Cell Motil.* **29**, 213-219.

Huckaba, T. M., Lipkin, T. and Pon, L. A. (2006). Roles of type II myosin and a tropomyosin isoform in retrograde actin flow in budding yeast. *J. Cell Biol.* **175**, 957-969.

Karagiannis, J., Bimbo, A., Rajagopalan, S., Liu, J. and Balasubramanian, M. K. (2005). The nuclear kinase Lsk1p positively regulates the septation initiation network and promotes the successful completion of cytokinesis in response to perturbation of the actomyosin ring in Schizosaccharomyces pombe. *Mol. Biol. Cell* **16**, 358-371.

Kitayama, C., Sugimoto, A. and Yamamoto, M. (1997). Type II myosin heavy chain encoded by the myo2 gene composes the contractile ring during cytokinesis in Schizosaccharomyces pombe. *J. Cell Biol.* **137**, 1309-1319.

Kurahashi, H., Imai, Y. and Yamamoto, M. (2002). Tropomyosin is required for the cell fusion process during conjugation in fission yeast. *Genes Cells* **7**, 375-384.

Lee, W. L., Bezanilla, M. and Pollard, T. D. (2000). Fission yeast myosin-I, Myo1p, stimulates actin assembly by Arp2/3 complex and shares functions with WASp. *J. Cell Biol.* **151**, 789-800.

Lees-Miller, J. P. and Helfman, D. M. (1991). The molecular basis for tropomyosin isoform diversity. *BioEssays* **13**, 429-437.

Lehman, W., Craig, R. and Vibert, P. (1994). Ca(2+)-induced tropomyosin movement in Limulus thin filaments revealed by three-dimensional reconstruction. *Nature* **368**, 65-67.

Lehman, W., Vibert, P., Uman, P. and Craig, R. (1995). Steric-blocking by tropomyosin visualized in relaxed vertebrate muscle thin filaments. *J. Mol. Biol.* **251**, 191-196.

Liu, H. P. and Bretscher, A. (1989). Disruption of the single tropomyosin gene in yeast results in the disappearance of actin cables from the cytoskeleton. *Cell* **57**, 233-242.

Liu, H. and Bretscher, A. (1992). Characterization of TPM1 disrupted yeast cells indicates an involvement of tropomyosin in directed vesicular transport. *J. Cell Biol.* **118**, 285-299.

Lord, M. and Pollard, T. D. (2004). UCS protein Rng3p activates actin filament gliding by fission yeast myosin-II. *J. Cell Biol.* **167**, 315-325.

Martin, S. G. and Chang, F. (2006). Dynamics of the formin for3p in actin cable assembly. *Curr. Biol.* **16**, 1161-1170.

Martin-Garcia, R. and Mulvihill, D. P. (2009). Myosin V spatially regulates microtubule dynamics and promotes the ubiquitin-dependent degradation of the fission yeast CLIP-170 homologue, Tip1. *J. Cell Sci.* **122**, 3862-3872.

McKillop, D. F. and Geeves, M. A. (1991). Regulation of the acto.myosin subfragment 1 interaction by troponin/tropomyosin. Evidence for control of a specific isomerization between two acto.myosin subfragment 1 states. *Biochem. J.* **279**, 711-718.

McKillop, D. F. and Geeves, M. A. (1993). Regulation of the interaction between actin and myosin subfragment **1**, evidence for three states of the thin filament. *Biophys. J.* **65**, 693-701.

Monteiro, P. B., Lataro, R. C., Ferro, J. A. and Reinach Fde, C. (1994). Functional alpha-tropomyosin produced in Escherichia coli. A dipeptide extension can substitute the amino-terminal acetyl group. *J. Biol. Chem.* **269**, 10461-10466.

Moreno, S., Klar, A. and Nurse, P. (1991). Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. *Methods Enzymol.* **194**, 795-823.

Mulvihill, D. P. and Hyams, J. S. (2003). Role of the two type II myosins, Myo2 and Myp2, in cytokinetic actomyosin ring formation and function in fission yeast. *Cell Motil. Cytoskeleton* **54**, 208-216.

Naqvi, N. I., Eng, K., Gould, K. L. and Balasubramanian, M. K. (1999). Evidence for F-actin-dependent and -independent mechanisms involved in assembly and stability of the medial actomyosin ring in fission yeast. *EMBO J.* **18**, 854-862.

Pelham, R. J., Jr and Chang, F. (2001). Role of actin polymerization and actin cables in actin-patch movement in Schizosaccharomyces pombe. *Nat. Cell Biol.* **3**, 235-244.

Perry, S. V. (2001). Vertebrate tropomyosin: distribution, properties and function. *J. Muscle Res. Cell Motil.* **22**, 5-49.

Pittenger, M. F. and Helfman, D. M. (1992). In vitro and in vivo characterization of four fibroblast tropomyosins produced in bacteria: TM-2, TM-3, TM-5a, and TM-5b are co-localized in interphase fibroblasts. *J. Cell Biol.* **118**, 841-858.

Polevoda, B. and Sherman, F. (2003). N-terminal acetyltransferases and sequence requirements for N-terminal acetylation of eukaryotic proteins. *J. Mol. Biol.* **325**, 595-622.

Polevoda, B., Cardillo, T. S., Doyle, T. C., Bedi, G. S. and Sherman, F. (2003). Nat3p and Mdm20p are required for function of yeast NatB Nalpha-terminal acetyltransferase and of actin and tropomyosin. *J. Biol. Chem.* **278**, 30686-30697.

Polevoda, B., Arnesen, T. and Sherman, F. (2009). A synopsis of eukaryotic Nalpha-terminal acetyltransferases: nomenclature, subunits and substrates. *BMC Proc.* **3**, S2.

Pollard, T. D. and Wu, J. Q. (2010). Understanding cytokinesis: lessons from fission yeast. *Nat. Rev. Mol. Cell Biol.* **11**, 149-155.

Singer, J. M. and Shaw, J. M. (2003). Mdm20 protein functions with Nat3 protein to acetylate Tpm1 protein and regulate tropomyosin-actin interactions in budding yeast. *Proc. Natl. Acad. Sci. USA* **100**, 7644-7649.

Singer, J. M., Hermann, G. J. and Shaw, J. M. (2000). Suppressors of mdm20 in yeast identify new alleles of ACT1 and TPM1 predicted to enhance actin-tropomyosin interactions. *Genetics* **156**, 523-534.

Sirotkin, V., Beltzner, C. C., Marchand, J. B. and Pollard, T. D. (2005). Interactions of WASp, myosin-I, and verprolin with Arp2/3 complex during actin patch assembly in fission yeast. *J. Cell Biol.* **170**, 637-648.

Skoumpla, K., Coulton, A. T., Lehman, W., Geeves, M. A. and Mulvihill, D. P. (2007). Acetylation regulates tropomyosin function in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **120**, 1635-1645.

Stark, B. C., Sladewski, T. E., Pollard, L. W. and Lord, M. (2010). Tropomyosin and myosin-II cellular levels promote actomyosin ring assembly in fission yeast. *Mol. Biol. Cell*. **21**, 989-1000.

Tanaka, K., Kohda, T., Yamashita, A., Nonaka, N. and Yamamoto, M. (2005). Hrs1p/Mcp6p on the meiotic SPB organizes astral microtubule arrays for oscillatory nuclear movement. *Curr. Biol.* **15**, 1479-1486.

Urbancikova, M. and Hitchcock-DeGregori, S. E. (1994). Requirement of amino-terminal modification for striated muscle alpha-tropomyosin function. *J. Biol. Chem.* **269**, 24310-24315.

Veigel, C., Schmitz, S., Wang, F. and Sellers, J. R. (2005). Load-dependent kinetics of myosin-V can explain its high processivity. *Nat. Cell Biol.* **7**, 861-869.

Win, T. Z., Gachet, Y., Mulvihill, D. P., May, K. M. and Hyams, J. S. (2001). Two type V myosins with non-overlapping functions in the fission yeast Schizosaccharomyces pombe: Myo52 is concerned with growth polarity and cytokinesis, Myo51 is a component of the cytokinetic actin ring. *J. Cell Sci.* **114**, 69-79.

Wu, J. Q., Kuhn, J. R., Kovar, D. R. and Pollard, T. D. (2003). Spatial and temporal pathway for assembly and constriction of the contractile ring in fission yeast cytokinesis. *Dev. Cell* **5**, 723-734.