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

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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 (Lee-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 tropinin (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-acetyltransferase 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 Cdc8 was deleted from the genome. The Naa25 protein was found to be non-essential for vegetative growth, but *naa25*Δ cells were not only temperature sensitive (Fig. 1A), but also grew slowly (Fig. 1A) and had cytokinetic 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) rescued the *naa25*Δ strain cytokinetic 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 *naa25*Δ defect is brought about by instability of the actin ring.

Rhodamine–phalloidin staining of cortical actin patches in fixed *naa25*Δ cells revealed the proportion of interphase cells undergoing either monopolar (23%) or bipolar growth (77%) were equivalent in wild-type and *naa25*Δ cells (Fig. 1D). This F-actin staining also indicated that actin cables were either absent or more fragile in *naa25*Δ 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 wild-type and *naa25*Δ cells. This revealed that, compared with wild-type cells, less Tm appeared more fragile in *naa25*Δ cells especially at the CAR during mitosis (Fig. 2A).

![Fig. 1. Growth and division of *naa25*Δ S. pombe cells.](image)

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 *naa25*Δ 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 *naa25*Δ-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 *naa25*Δ fission yeast cells.
Expression of SmTm-AS but not wild-type SmTm rescued the \textit{naa25Δ}-associated cytokinesis 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: \textit{naa25+}, 4.3±0.3 hours; \textit{naa25Δ}, 9.2±1.2 hours; \textit{naa25Δ+SmTm}, 9.25±0.8 hours; \textit{naa25Δ+SmTm-AS}, 9.8±0.9 hours). These data indicate that whereas the cytokinesis defects observed in \textit{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.

\textbf{Uncacylated-Cdc8 associates with and affects actin polymer structure.}

To confirm the above observation that unacylated 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–unacylated 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 unacylated 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 unacylated 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.

\textbf{Characterisation of acetylation-state-specific anti-Cdc8 antibodies}

To further explore the significance of the finding that both unacylated 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\textit{UNACE}) or acetyl-conjugated (anti-Cdc8\textit{ACE}) peptide encompassing the N-terminus of the Cdc8 protein. Western blot analysis revealed that the anti-Cdc8\textit{UNACE} antibodies specifically recognised unmodified Cdc8 purified from \textit{E. coli} but not an equivalent quantity of acetylated Cdc8 purified from wild-type \textit{S. pombe} (Fig. 3A). By contrast, anti-Cdc8\textit{ACE} antibodies only detected acetylated Cdc8 but not the unacylated form (Fig. 3A). In addition, whereas anti-Cdc8 serum recognised Cdc8 in extracts of wild-type and \textit{naa25Δ} cells, the anti-Cdc8\textit{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 unacylated in \textit{naa25Δ} cells.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig2}
\caption{Unmodified Cdc8 associates with and affects the appearance of Cdc8–actin polymers in vivo and in vitro. (A) Cdc8 immunofluorescence staining of the \textit{naa25Δ} strain using Cdc8 antiserum. Scale bar: 5 \textmu m. (B–D) Negative staining of (B) F-actin–acylated 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 unacylated Cdc8–actin polymers (C), which also often formed bundles (arrows). Samples were prepared using identical methods. Scale bars: 40 nm.}
\end{figure}
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 different 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\textsuperscript{ACE} (Fig. 3D) or anti-Cdc8\textsuperscript{UNACE} (Fig. 3E) antibodies. Although anti-Cdc8\textsuperscript{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\textsuperscript{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\textsuperscript{ACE} immunofluorescence of \textit{naa25}\textsuperscript{Δ} cells showed no discrete localisation pattern (Fig. 3F); however, anti-Cdc8\textsuperscript{UNACE} antibodies were capable of recognising Cdc8 associated with the CAR in \textit{naa25}\textsuperscript{Δ} 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 \textit{naa25}\textsuperscript{Δ} 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 \textit{naa25}\textsuperscript{Δ} 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 effect upon the cellular distribution of this motor protein, which continued to be recruited to foci at sites of growth in \textit{naa25}\textsuperscript{Δ} cells (Fig. 4A,B; Table 1). In addition, Cdc8\textsuperscript{UNACE} antibodies were capable of recognising Cdc8 associated with the CAR in \textit{naa25}\textsuperscript{Δ} 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.

Table 1. Localisation of myosins in \textit{naa25}\textsuperscript{Δ} and \textit{naa25}\textsuperscript{Δ} cells

| Myosin | Location | \textit{naa25}\textsuperscript{Δ} (%) of total | \textit{naa25}\textsuperscript{Δ} (%) 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 |
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 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 not associated with the CAR during interphase and mitosis (Fig. 6B; supplementary material Movie 6; Table 1) and aberrant contractile structures extending the length of the cell (supplementary material Movie 8).
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 unappreciated 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...
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 Hitecock-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 Cdc8ACF 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 Gheeves, 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

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.

Myosin-II-specific tropomyosin regulation
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::kanMX6 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 SRF rabbits (Eurogentec, Seraing, Belgium), and were subsequently affinity purified. Anti-Mya2 and anti-Myp2 antibodies were raised against the KHL-conjugated polyepitopes LKHEPSPGKENNIPA and LLEDVPNTRNKIQK, respectively (Mya2 residues 1370–1384; Myp2 residues 1517–1531) in SRF 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 sera 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, Mya2 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 mM F-actin with 5.7 mM cardiac Tm or 50 mM of either acetylated or unmodified Cdc8.

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