A novel role for Tpm2 in regulating formin-mediated actin assembly
Senior Thesis

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by
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ABSTRACT
Tropomyosins are a family of ubiquitously expressed actin binding proteins that bind along the length of actin filaments, and have established roles in stabilizing their dynamics and governing myosin motor activity. Although Tropomyosin was one of the first actin associated proteins discovered, its role in regulating actin filament dynamics is still poorly understood. *Saccharomyces cerevisiae* expresses two Tropomyosin isoforms, Tpm1 and Tpm2, which have traditionally been thought to play redundant roles in stabilizing actin cables. However, strong genetic evidence collected by our lab shows that *TPM2*, but not *TPM1*, may destabilize rather than stabilize actin networks *in vivo*. In light of this evidence, I performed complementary *in vivo* and *in vitro* tests of Tpm2 function designed to elucidate its specific roles in regulating actin filament dynamics. My *in vivo* analysis confirmed that *TPM2* helps prevent overgrowth of actin cables, consistent with a role in either destabilizing actin filaments or suppressing actin filament growth. However, *in vitro* analysis showed that purified Tpm2 has no effect on actin filament stability, and in fact appears to weakly protect against cofillin-mediated severing. Instead, Tpm2 showed potent, formin-specific effects on actin filament nucleation that may explain its *in vivo* roles. Specifically, Tpm2 strongly inhibited nucleation by the formin Bnr1, yet enhanced nucleation by the formin Bni1. These results suggest a novel isoform-specific activity of Tpm2 in regulating actin cable formation *in vivo*, and provide the first evidence of a Tropomyosin controlling actin nucleation, and the first evidence for Tropomyosins differentially regulating the functions of formins.
INTRODUCTION

**Actin cytoskeleton dynamics**

From yeast to humans, the actin cytoskeleton is a highly dynamic structure essential for numerous fundamental cellular processes including cell division, endocytosis, cytokinesis, cell polarity, and cell morphogenesis (Moseley, 2006). This high degree of dynamicity is governed by changes in nucleotide state of actin monomers in addition to a vast array of actin binding proteins that bind filaments and regulate the dynamics of their assembly and disassembly. The stability of actin filaments can be altered dramatically by small changes in F-actin conformation brought about by changes in the nucleotide state of the individual actin subunits in a filament, e.g., those bound to ATP and ADP + P$_i$ actin are more stable, and those bound to ADP are less stable (Pollard, 2009). As ATP bound actin subunits are added to the barbed (or ‘plus’) ends of filaments, they are gradually converted to the ADP state via ATP hydrolysis on actin, followed by phosphate release. However, changes in nucleotide state alone do not account for the high rate of actin turnover. High rates of actin filament turnover also require additional actin binding proteins, such as Cofilin, Aip1, and Coronin, which sever and depolymerize filaments in the ADP-bound state. Still other actin binding proteins stabilize and/or spatially organize filaments by binding to their sides and coating them (e.g., Tropomyosins) or crosslinking them into parallel bundles or orthogonal arrays (e.g., Fimbrin, Fascin, and α-actinin) (Pollard, 2009). The mechanisms by which these actin-binding proteins collectively control the dynamics of cellular actin structures are a subject of intense study, and budding yeast has provided an excellent model for addressing this problem.

In yeast, bundles of linear actin filaments known as ‘cables’ serve as polarized tracks for myosin-mediated transport of cellular cargo during asymmetric growth (to form a bud) and later
during cytokinesis (to form a septum that divides the cell in two). Actin cables are assembled at the bud tip and bud neck, and extend into the mother cell along the mother-bud axis (Moseley, 2006). Cables are highly dynamic and require on-going assembly and disassembly. Based on cable extension rates, and other experimental observations, cables are estimated to turnover within 10 seconds (Yang, 2001; Yu, 2011). Additionally, the majority of the filaments in the cable are believed to be oriented with their barbed ends pointed toward the bud, which is essential for barbed-end directed myosin motors to transport secretory vesicles, organelles, and other cargo to the bud tip to enable daughter cell growth. The formation and orientation of these filaments within actin cables are dependent on the nucleating and elongating activity of formins. In yeast, there are two formins, Bni1 and Bnr1, which localize to the bud tip and bud neck respectively, and are required for actin cable formation (Pruyne, 2004; Evangelista, 2002). In vitro, Bni1 and Bnr1 have been shown to directly nucleate actin filament formation, and remain associated with the barbed ends of the growing filaments, accelerating the elongation of the filaments by 3-4 fold compared to control filaments without formins (Chesarone, 2010). Despite major advances in our understanding of formin protein structure and function, the mechanisms by which formin activities are regulated in vivo by other factors to control the formation of elaborate and correctly sized actin structures such as cables are only beginning to be understood.

**Tropomyosin functional diversity**

Tropomyosins, like formins and actin, are required for cable formation in yeast (Pruyne 1998). Tropomyosins are dimeric alpha helical coiled coils—approximately 20 angstroms in diameter and 200 angstroms in length—that bind cooperatively end-to-end along the sides of actin filaments (Stewart, 2001; Greenfield, 2006). In yeast, are two Tropomyosin isoforms—
Tpm1 and Tpm2—with Tpm1 expression approximately 6-fold higher than Tpm2 (Drees, 1995). Tpm1 was first identified in 1989 and exhibited the same hallmark biochemical properties as vertebrate Tropomyosins (Liu, 1989). In 1995, Tpm2 was identified as the second yeast Tropomyosin isoform, showing 64.5% sequence homology overall to Tpm1 (Figure 1; Drees, 1995). Analysis of the genetic sequence of the two isoforms revealed that while Tpm1 contains a 38 amino acid pseudo repeat, Tpm2 lacks exactly one of these 38 amino acid motifs (Figure 1; Drees, 1995). Like Tpm1, Tpm2 exhibited the hallmark properties of the Tropomyosin family, including dimerization, divalent cation-dependent F-actin binding, and resistance to denaturation at high temperatures (Drees, 1995).

In yeast, it was traditionally believed that both Tropomyosin isoforms stabilized F-actin structures in vivo. Three primary of pieces of evidence supported this hypothesis: (1) *TPM1* and *TPM2* are synthetic lethal, suggesting that they have at least partially overlapping or redundant functions in vivo (Drees, 1995), (2) immunofluorescence microscopy revealed that Tropomyosins localize primarily to actin cables in vivo (Liu, 1989), and (3) Tropomyosin is required for cable formation—upon shift to the restrictive temperature, a *tpm1-2 tpm2Δ* temperature sensitive mutant loses all cables within 60 seconds and the wild type phenotype is restored within fives minutes of switching back to the permissive temperature (Pruyne 1998). Together, these results provided strong evidence that Tpm1 and Tpm2 play at least partially redundant roles in the formation of actin cables.

Despite the observations above, other studies have suggested that Tpm1 and Tpm2 may in fact have distinct functions. Despite their structural similarity, the two isoforms exhibit some differences in biochemical properties. For instance, Tpm1 and Tpm2 exhibit different F-actin binding stoichiometries, with Tpm1 dimers spanning 4.6 actin subunits and Tpm2 dimers
spanning 3.6 actin subunits (Drees, 1995). Further, co-sedimentation assays demonstrate that Tpm1 is unable to displace Tpm2 from F-actin, while Tpm2 is effective in displacing Tpm1, suggesting that Tpm2 may have a higher affinity for F-actin (Drees, 1995). Further, while tpm1Δ cells exhibit almost a complete loss of actin cables (and therefore are sick), tpm2Δ cells show no loss of cables (Drees, 1995). In addition, while Tpm1 expression is about 6-fold higher that that of Tpm2, overexpression of TPM2 to similar levels fails to restore cables or proper cell growth in tpm1Δ cells (Drees, 1995). Tpm1 and Tpm2 also have been shown to differentially regulate myosin activity on F-actin in vivo. Tpm2 inhibits retrograde actin flow by inhibiting binding of type II myosin (Myo1) to F-actin, whereas Tpm1 does not have this affect (Huckaba, 2006), providing strong evidence that Tpm1 and Tpm2 have distinct functions in actin cable formation and myosin-mediated transport of cellular cargo.

**Further genetic evidence of Tropomyosin functional diversity**

Additional work from our lab in 2005 extends the view of Tpm1 and Tpm2 performing distinct functions in vivo based on their highly distinct profiles of genetic interactions with a large collection of mutant actin (act1) alleles. In this screen, 17 different actin-binding proteins were overexpressed on plasmids in yeast strains expressing 25 different nonlethal act1 alleles in order to identify functional relationships between these actin binding proteins and the act1 alleles. Categories of genetic interactions included: (i) the ability of the act1 allele to suppress the growth defects caused by overexpression of the actin binding protein, (ii) the ability of the overexpressed actin binding protein to suppress growth defects caused by the act1 allele, or (iii) mutual suppression. The first piece of evidence from this screen that indicated functional differences between Tpm1 and Tpm2 was the characterization of each gene’s overexpression
phenotype in wild type cells. TPM2 overexpression caused severe growth defects, while TPM1 overexpression caused mild to no defects (Figure 2; Hendries, 2005). The differences in these phenotypes suggest functional dissimilarity between these two isoforms. Further, TPM2, unlike TPM1, exhibited a similar pattern of genetic interactions with a set of act1 alleles as three actin disassembly factors—TWF1, AIP1, and CRN1—suggesting a possible functional similarity between TPM2 and the disassembly factors (Figure 2; Hendries, 2005). Notably, one of the act1 alleles that these proteins exhibited interactions with, act1-159, is a well-studied allele that significantly stabilizes filaments by causing them to maintain the ADP + Pi conformation even after phosphate is released, resulting in hyper-stable filaments that are resistant to latrunculin A treatment (Belmont, 1998). Together, these results suggested not only that Tpm2 may have distinct functions from Tpm1, but more specifically that Tpm2 may be involved in the destabilization—as opposed to stabilization—of actin networks in vivo.

**Elucidating Tpm2 function**

In light of the observations above, I have investigated in this thesis the role of Tpm2 in destabilizing actin dynamics both in vivo and in vitro and asked whether these activities were distinct from those of Tpm1. Defining functional differences amongst Tropomyosin isoforms has direct applications to human health. Both muscle and non-muscle Tropomyosin genes are critical for cell/organism viability, from yeast to mammals, and mutations in human Tropomyosin genes are linked to cardiac defects such as Familial Hypertrophic Cardiomyopathy and Dilated Cardiomyopathy (Redwood, 2013). In mammals, addressing Tropomyosin functional diversity is more difficult, because of there are four different genes that are alternatively spliced to generate >40 different isoforms, each with potentially different
localization patterns and functions (Gunning, 2005). On the other hand, budding yeast has only two Tropomyosin genes, *TPM1* and *TPM2*, and is highly amenable to both genetic and biochemical analyses.

Based on the genetic observations described above, I used fluorescence microscopy to quantitatively compare actin cable length in wild type and *tpm2Δ* cells, and to compare cable resistance to latrunculin-B (which sequesters actin monomers), thereby testing whether *tpm2Δ* causes cable hyper-stabilization or overgrowth. In parallel, I purified Tpm2 and determined in bulk kinetic assays and in single filament TIRF microscopy assays its abilities to: (1) directly regulate F-actin stability, (2) influence cofilin-mediated severing of filaments, or (3) influence Bni1- and Bnr1-mediated actin nucleation and filament elongation. This analysis has revealed a novel isoform-specific activity of Tpm2 in regulating formin-mediated actin assembly.
RESULTS

Loss of TPM2 alters actin cable morphology and dynamics in vivo

In order to investigate the role of Tpm2 in destabilizing actin cables in vivo, I first wanted to determine how the deletion of TPM2 affects actin cable morphology. I hypothesized that the loss of a destabilization factor would produce increased actin cable assembly, possibly producing longer or more numerous actin cables. To test this hypothesis, I quantitatively compared actin cable number and length in wild type, tpm2 Δ, aip1 Δ, and crn1 Δ cells. I found that the deletion of each of these genes had no effect on cable number, but in each case increased the average cable length as compared to wild type cells (Figure 3, A-C). Thus, tpm1Δ and tpm2Δ have opposite effects on cables, causing severe loss of cables versus cable overgrowth, respectively.

To complement these observations I measured the sensitivity of actin cables to Latrunculin-B (LatB), which sequesters actin monomers and thus prevents new actin assembly (Wakatuski, 2001). Previous studies have shown that mutations in actin disassembly factors such as COF1, AIP1, and CRN1 give rise to cables that are more resistant to Latrunculin than cables in wild type cells (Okada, 2006; Gandhi, 2009). I fixed cells at various time points after LatB treatment and scored cells with mid-sized buds for visible cables in the mother cell. I found that both tpm2Δ and cof1-22 cells exhibited cells with a population of Latrunculin resistant cables even after 10 minutes of treatment, whereas wild-type cells lost all visible cables by 5 minutes (Figure 4, A-B). These results demonstrate that cables in tpm2Δ cells are resistant to LatB, further confirming the observation that tpm2Δ cells have abnormally long cables and the hypothesis that Tpm2 and Tpm1 have distinct roles in regulating cable formation and/or stability in vivo.
**Tpm2 weakly protects actin filaments against Cof1-mediated severing**

Next, I purified untagged, overexpressed Tpm2 from *tpm1 Δ* yeast cells, and tested its effects on F-actin stability in bulk pyrene-F-actin disassembly kinetic assays, both in the presence and absence of purified yeast coflin (Cof1). This analysis showed that Tpm2 (2.5 µM) had little if any effect on the rate of F-actin disassembly in the absence of Cof1, and in fact weakly protected filaments against Cof1-mediated disassembly (Figure 5A).

In addition, I quantified the effects of Tpm2 on Cof1-mediated actin filament severing by TIRF microscopy. In these assays, actin filaments (10% Oregon green labeled actin, 0.5% biotin-actin) are first polymerized and anchored to the surface, then free monomers are washed out, proteins and/or buffer are flowed in, and filaments are monitored for 10 sec. Severing events are quantified for at least 10 filaments in each of at least 2 experiments and the average severing rate is calculated, expressed as number of filament breaks per second per micron of filament. This analysis showed that filament severing by Cof1 was reduced in the presence Tpm2 (Figure 5, B-C), consistent with the effects observed in my bulk kinetic assays. Further, Tpm2 alone did not induce detectable severing or depolymerization of filaments. Thus, Tpm2 does not promote F-actin disassembly on its own or in the presence of Cof1.

**Tpm2 inhibits Bnr1-mediated actin filament nucleation**

I next tested an alternative hypothesis to explain the in vivo results, which is that rather than promoting the disassembly of actin cables, Tpm2 instead inhibits the assembly of cables. To test this model, I used a combination of bulk and TIRF assays to compare the effects of Tpm2 on the rate of actin filament nucleation (i.e., new filament formation) and rate of filament elongation (barbed end polymerization speed), both with and without the formins Bni1 and Bnr1 present. In
TIRF assays in the absence of formins, Tpm2 did not appreciably change the rates of actin nucleation or elongation (Figure 6, A-B). However, in bulk assays with Bnr1 present, Tpm2 caused a robust concentration-dependent inhibition of Bnr1-mediated actin assembly (Figure 7). To study these inhibitory effects in TIRF assays, I used biotinylated, anchored SNAP-tagged Bnr1 molecules, such that the barbed end of the Bnr1-polymerized filaments stays fixed in place (with the pointed end moving away at the speed of barbed end polymerization), while the barbed ends of control filaments (no formin on the end) freely extend, allowing me to distinguish between Bnr1-bound and unbound filaments. All experiments including controls were performed in the presence of yeast profilin (Pfy1). From this analysis, I found that Tpm2 had no effect on the rate of Bnr1-mediated filament elongation (Figure 8A). However, quantification of the number of filaments formed after 6 minutes in these reactions showed that Tpm2 decreased Bnr1-mediated nucleation by ~10 fold (Figure 8, B-C), consistent with the effects we observed in bulk assays. This result supports the alternative hypothesis that Tpm2 may destabilize actin cables \textit{in vivo} and prevent their overgrowth by negatively regulating formin-mediated actin cable assembly.

\textit{Tpm2 enhances Bni1-mediated actin filament nucleation}

In stark contrast to its effects on Bnr1, Tpm2 caused a slight increase in the rate of Bni1-mediated actin assembly in bulk assays (Figure 9). Further analysis by TIRF microscopy showed that Tpm2 had no effect on Bni1-mediated actin filament elongation (Figure 10A), but enhanced Bni1-mediated actin filament nucleation by ~4.5 fold (Figure 10, B-C). These results in the presence of formins are both striking and unanticipated, and reveal a highly novel role for Tpm2 in differentially regulating actin nucleation by the two yeast formins. Work from Sal Alioto in
the lab, has shown that Tpm1, unlike Tpm2, enhances actin nucleation by both Bni1 and Bnr1. Thus, Tpm1 and Tpm2 differ markedly in their regulatory effects on Bnr1. How the unique activities of Tpm2 may be used to regulate cable formation in vivo is discussed below.
DISCUSSION

*Insight into the role of Tpm2 in the destabilization of actin networks*

While it has long been known that Tpm1 and Tpm2 share an essential role in actin cable formation, the mechanism(s) underlying this function have remained elusive. In the absence of direct experimental evidence, it had been assumed that the function of the Tropomyosins was to stabilize cables and protect them from disassembly factors. Further, it was assumed that Tpm1 had the dominant genetic role in this function because it was 6-fold more abundant in cells than Tpm2. However, the work presented in this thesis directly challenges the earlier view and provides new biochemical and genetic evidence for an isoform-specific role of Tpm2 in controlling cable formation by differentially regulating Bni1- and Bnr1-mediated actin nucleation.

Because the genetic profile of Tpm2 with act1 alleles was highly similar to several actin disassembly factors (Figure 1; Hendries, 2005), I originally hypothesized that Tpm2 may directly promote the disassembly of actin filaments, alone or working together with cofilin. My *in vivo* work supported this hypothesis, showing that *tpm2Δ* cells display hyper-elongated and LatB-resistant cables, similar to mutants in disassembly factors. However, my *in vitro* analysis of the biochemical effects of purified Tpm2 on filament disassembly showed that Tpm2 has no effect on filament dynamics in the absence of cofilin, and in fact weakly protects filaments against severing by cofilin. These observations are consistent with the view that Tropomyosins stabilize filaments in part by sterically hindering cofilin-binding sites on actin and increasing the strength of the interaction between actin monomers (Prulière, 1986).

The strongest *in vitro* effects of Tpm2 that I observed were its 4.5-fold stimulatory and 10-fold inhibitory effects on Bni1 and Bnr1-mediated actin nucleation, respectively. These
results are consistent with my *in vivo* observations, because the loss of a negative regulator of actin assembly could produce cable overgrowth in a similar manner to the loss of a disassembly factor. Further, this activity may also account for the dosage suppression interaction between TPM2 and the stabilizing act1-159 allele. This allele, which causes actin to mimic the ADP + Pi state even when its phosphate is lost, produces hyper-stable filaments that are Latrunculin-resistant (Belmont, 1998). If overexpression of Tpm2 reduces levels of actin nucleation, this could help limit the build up of stabilized actin filaments in act1-159 cells.

The structure of the donut-shaped FH2 dimer in both yeast formins suggests a possible mechanism by which Tpm2 could regulate formin-mediated actin nucleation. The donut-shaped FH2 domain dimer is both necessary and sufficient for actin nucleation (Pruyne, 2002). It is approximately 11 nm in diameter, whereas the actin filament it encircles is approximately 8 nm in diameter, leaving ample room to accommodate the narrow Tropomyosin molecule between the FH2 and actin. Tpm2 dimers bound to a few actin subunits might promote nucleation (in case of Bni1) by creating a nucleus that is captured at the barbed end by the Bni1 FH2, or instead block nucleation (in the case of Bnr1) by disrupting contacts between the Bnr1 FH2 domain and actin.

Why might it be important for yeast cells to express a specific TPM isoform, Tpm2, that both promotes nucleation by one formin (Bni1) but inhibits nucleation by the other (Bnr1)? Previous work has demonstrated that Bnr1 is 10-15 fold more potent in stimulating actin nucleation than Bni1 (Moseley, 2005). Thus, Tpm2 may play a role in maintaining the balance between actin cable nucleation by Bni1 versus Bnr1 in vivo. This model can next be tested experimentally by comparing the effects of Tpm2 overexpression on actin cable levels in *bni1Δ* versus *bnr1Δ* cells (should have opposite effects), and by comparing cable levels in *bnr1Δ* versus
bnr1Δ tpm2Δ cells (should be lower in the double mutant), and in bni1Δ versus bni1Δ tpm2Δ cells (should be higher in the double mutant). Overall, my current model is that Tpm1 and Tpm2 have an antagonistic relationship in regulating formin-mediated actin cable assembly. However, it will also be important to test whether the relative levels of Tpm1 and Tpm2 in cells changes over the cell cycle, possibly to balance Bni1 and Bnr1 activities as the bud size increases, since Bni1 makes cables in the bud and Bnr1 makes cables specifically in the mother.

**Future Directions**

While this work demonstrates a novel formin regulatory activity of Tpm2 that may play a role in limiting actin cable assembly *in vivo*, there are other possible mechanisms by which Tpm2 might function. For instance, Tpm2 might regulate actin distribution between cortical actin patches versus actin cables. Tpm2 has never been localized, and it is only assumed that it resides on cables; however, it is equally possible that it resides on actin patches instead of, or in addition to, cables. In fact, Tpm1 antibodies, which cross-react with Tpm2 on western blots, show Tropomyosin staining on cables as well as patches *in vivo*, although the patch staining is never considered (Liu, 1989). If Tpm2 does localize to patches, it may play a role in regulating actin dynamics within the patch. By tilting the balance of actin monomers in favor of patches, Tpm2 could serve to indirectly destabilize actin cables. To address this, an experiment I will be performing in the near future is to repeat the anti-TPM immunostaining in wild-type, tpm1Δ, and tpm2Δ cells (using anti-TPM antibody generously provided by Professor Anthony Bretscher at Cornell University). If the patch staining disappears specifically in tpm2Δ cells, this would be strong evidence that Tpm2 does indeed localize to patches, though it would not rule out additional localization on cables.
Another possibility is that Tpm2’s ability to regulate myosin V activity promotes the destabilization of actin cables *in vivo*. It has been shown that increased tension on filaments makes them less susceptible to disassembly by cofilin (Hayakawa, 2011). Since Tpm2 decoration of filaments inhibits myosin binding, whereas Tpm1 does not (Huckaba, 2006), cables in tpm2Δ cells may be under increased tension and therefore grow too long and turnover more slowly. One way this could be tested in the future is to develop an in vitro TIRF assay in which the actin filaments are attached to surface-anchored myosin molecules (similar to classic coverslip assays in which tethered myosins cause filaments to ‘glide’), and compare cofilin severing efficiency in the presence and absence of Tpm2.

In addition, future experiments are needed to test whether the Tpm2 regulatory effects I have observed on formins are similar or different using N-terminally acetylated Tpm2, which is believed to be an important post-translational modification (PTM) in vivo, and can promote Tpm2 binding to F-actin (Maytum, 2000). In this PTM, the N-terminal methionine (on both Tpm1 and Tpm2) is acetylated by NatB acetyltransferase, increasing the affinity of Tropomyosins for F-actin (Polevoda, 2003). Because my Tpm2 construct was purified from an overexpression vector, it is unclear whether the purified Tpm2 protein I used in this study is acetylated at all, or to the same extent as endogenous Tpm2. Thus, key experiments should be repeated using a Tpm2 ‘acetylation mimic’ construct, which has at its N-terminus an added tripeptide that has been shown to mimic the effects of acetylation (Frye, 2010). We have in fact recently purified the acetylation-mimic versions of Tpm1 and Tpm2, and preliminary experiments indicate that the Tpm2 acetylation mimic is more effective in protecting actin filaments from cofilin-mediated severing. Thus, further *in vitro* work with this construct may reveal that the activities of Tpm2 are more extensive than described here, e.g., regulating formin-
mediated actin nucleation as well as resistance to cofillin-mediated severing, to better explain the functional roles of TPM2 \textit{in vivo}.

\textbf{Biomedical Implications}

Elucidating the functional diversity of Tropomyosins has important implications for human health. In humans, mutations in multiple Tropomyosin isoforms have been linked to Familial Hypertrophic Cardiomyopathy (HCM) and Dilated Cardiomyopathy (DCM) (Wieczorek et al. 2008). However, the molecular and cellular basis for the defects are not understood, in part because the different roles of Tropomyosins in cells are not clear. This question would be difficult to address in mammalian systems, because after alternative splicing there are more than 40 Tropomyosin isoforms, which are likely to perform a diverse range of cellular functions. On the other hand, this question is approachable in budding yeast where there are only two Tropomyosin isoforms and all of the genetic and biochemical tools are in place to perform such an analysis. This work demonstrates that Tpm1 and Tpm2 perform distinct roles in \textit{S. cerevisiae}, making this system ideal for studying Tropomyosin isoform diversity. With cardiovascular disease being the leading cause of death worldwide, it is imperative to find an efficient approach to gaining a better understanding of the basic mechanisms underlying these diseases.

In addition to improving our understanding of the mechanisms underlying cardiovascular disease, Tropomyosin functional diversity offers a promising target for developing cancer treatments. Misregulation of the actin cytoskeleton is a critical step in the transformation of almost all cancer cells, yet there are currently no anti-actin compounds used to fight cancer. This is primarily due to difficult nature of finding a compound that is able to discriminate between
actin functions in healthy versus cancerous cells. The high degree of isoform diversity in the Tropomyosin family may offer a solution to this problem, enabling the development of drugs that target specific Tropomyosin isoforms predominantly expressed in cancerous cells. Indeed, a recently-developed anti-Tm compound, TR100, preferentially targets a Tropomyosin isoform enriched in cancer cells (Stehn, 2013), and has proven effective in reducing tumor size in melanoma and neuroblastoma mouse xenograft models without effecting cardiac actin function (Stehn, 2013). These developments demonstrate that increasing our understanding of Tropomyosin functional diversity holds promise for the development of targeted treatments.
MATERIALS AND METHODS

Yeast Strains and Plasmids

BGY3683 (WT), BGY206 (tpm1Δ), BGY480 (tpm2Δ) were generated in previous studies.

SAY42 (WT), SAY44 (tpm1Δ), SAY45 (tpm2Δ), BGY3727 (aip1Δ), and BGY3738 (crn1Δ)
were generated by transformation of a Longtine PCR product with 50 bp sequence homology to
the target gene. Deletions were confirmed by genomic PCR.

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Protein Purification

Tpm2 was overexpressed from a 2µ GAL-TPM2 plasmid in S. cerevisiae strain BGY206 (tpm1Δ). Cell culture was grown to log phase in minimal media with galactose, then pelleted and resuspended in 1/4 volume water. Resuspended culture was added dropwise to liquid nitrogen to freeze and was stored at -80°C. Frozen cells were lysed using a coffee grinder before resuspension in 1/5 volume 5x HE buffer (100 mM HEPES, pH 7.5, 5 mM EDTA, pH 8.0) and allowed to thaw on ice. Once thawed, the solution was boiled for 20 minutes in a water bath and then centrifuged for 20 minutes at 11,000 rpm at room temperature. The supernatant was harvested, and brought to 30% ammonium sulfate. Precipitated proteins were pelleted by 10 minutes of centrifugation at 8,000 rpm at room temperature. The supernatant was harvested,
ammonium sulfate added to 65%. Precipitated proteins were pelleted by 20 minutes of centrifugation at 11,000 rpm at room temperature. The supernatant was discarded, and the pellet was resuspended in 5mL HE buffer (20 mM HEPES, pH 7.5, 1 mM EDTA). The resuspended protein was then dialyzed against 2L HEK buffer (20mM HEPES, pH 7.5, 1 mM EDTA, 50 mM KCl) at 4°C for 3 hours. The buffer was then exchanged for a fresh 2L and the solution was dialyzed at 4°C overnight. Sample was loaded onto MonoQ ion exchange column equilibrated with HEK buffer and eluted with a 20mL linear gradient (300-800mM KCl), collecting 0.5 mL fractions. Peak fractions were pooled and concentrated to 500 µL. Concentrated product was loaded onto Superdex75 size exclusion column equilibrated with HEK buffer and collected in 1.5 mL fractions. Peak fractions were pooled, concentrated, and stored at 4°C.

**Pyrene Assembly Assays**

Gel filtered monomeric actin was cleared by centrifugation at 90,000 rpm for 1 hour at 4°C and the top 75% of the supernatant was recovered. Each assembly reaction contained 2µM G-actin (5% pyrene labeled). G-actin was converted to Mg2+-ATP-actin 2 min before use, and 42 uL of converted G-actin was mixed with 15uL proteins/buffer mixture containing and 3uL of initiation mix (40 mM MgCl2, 10mM ATP, 1M KCl) at the start of reactions. Pyrene actin fluorescence was monitored measured by a Tecan plate reader at excitation and emission wavelengths of 365 and 407 nm, respectively. All reactions were performed at 25°C. For each reaction, maximum fluorescence was normalized to 100% of total actin polymerization.
**Pyrene Disassembly Assays**

3μM gel-filtered monomeric actin (10% pyrene labeled) was polymerized in F-buffer (10mM Tris-HCl pH 7.5, 0.2 mM DTT, 0.2 mM CaCl2, 50 mM KCl, 2 mM MgCl2, and 0.7 mM ATP) for 2 hours in the dark. 40 uL pre-formed F-actin (2 μL final concentration) was incubated with 5uL F-buffer for 200 seconds while pyrene signal was monitored in a fluorescence spectrophotometer at excitation and emission wavelengths of 365 and 407 nm, respectively. At 200 seconds, 15 uL of protein-buffer mix was added and disassembly was induced by slowly mixing sample with a gel-loading tip. Pyrene signal was monitored for an additional 1800 seconds. For each reaction, maximum fluorescence was normalized to 0% disassembly.

**Cell Imaging**

Cells were grown to saturation, diluted to OD$_{600}$ ~0.1, and allowed to grow to log phase. Cells were fixed with 5% formaldehyde and incubated at 25°C for 1 hour. Cells were then pelleted, washed twice with 1 mL PBS, resuspended in 100 μL PBS, and stored at 4°C. 10 μL of fixed cells were then combined with 1 μL phalloidin and 39 μL of PBS. Cells were incubated for ~4 days at 4°C in the dark. In preparation for imaging, cells were pelleted, washed twice with 100uL PBS, and resuspended in 3 μL PBS. 1 μL of cells was mixed with 1 μL mounting media and 1μL of this mixture was applied to glass cover slip. Images were taken at 350 ms exposure time. All images were acquired on a Zeiss Axioskop epifluorescence microscope using a Hamamatsu ORCA-ER digital CCD camera running OpenLab software.
**TIRF Microscopy**

TIRF microscopy slides were prepared by first sonicating glass coverslips in 2% micro-90 detergent for 1 hour in a slide holder suspended in a heated bath sonicator. Slides were then sonicated in 100% ethanol for 1 hour, 0.1 M KOH for 30 minutes, and MilliQ water for 10 minutes. Between each sonication, slides were rinsed thoroughly with MilliQ water. Slides were then dried with filtered nitrogen in a laminar flow hood and coated with PEG 2000/Bio-PEG 3350 solution (2 mg/mL Peg-2k, 20 µg/mL Biotin-Peg). Coated slides were incubated overnight at 70°C. Slides were adhered to flow cells prior to imaging using double-sided tape and epoxy. For experiments requiring biotin-streptavidin surface tethering, flow cells were first incubated with streptavidin (17 µg/mL in HEK) for ~1 min, followed by the standard incubation with HBSA (20 mM HEPES, 1 mM EDTA, 50 mM KCl, and 1% bovine serum albumin) for ~1 min and equilibration with TIRF buffer (10 mM imidazole, 50 mM KCl, 1 mM MgCl₂, 1 mM ethylene glycol tetraacetic acid [EGTA], 0.2 mM ATP, 10 mM DTT, 15 mM glucose, 20 µg/ml glucose oxidase, and 0.5% methylcellulose [4000 cP], pH 7.4) for ~1 min. All reactions contained 1 µM G-actin (10% Oregon green labeled), 3 µM yeast profilin, and varying concentrations of Tpm1, Tpm2, Cof1, Bnr1, and Bni1 in 1x TIRF buffer as described. Movies were recorded for 10 minutes on a Nikon Ti2000 microscope (Nikon Corporation, Tokyo, Japan), using 200 ms exposures every 10 s. Image analysis was performed using ImageJ. Nucleation was quantified by counting the number of filaments present in the field of view following 6 minutes of polymerization. Elongation rates were quantified by measuring filament lengths every 10 seconds over 5 minutes of elongation. Disassembly rates were quantified by measuring the length of a filament and recording the number of severing events over a 5-minute time frame.
**Latrunculin Treatment**

Cells were grown to saturation, diluted to OD~0.1, and allowed to grow to log phase. Cells were then pelleted and resuspended in YPD. Latrunculin B was added to 100 µM and samples were fixed at 0, 1, 2, 5, and 10 min time points. After 1 hour of incubation at 25°C cells were washed 2x with 1 mL PBS, resuspended in 100µL PBS, and stored at 4°C. Cells were then stained and imaged as described above.
**FIGURES**

![Diagram of TPM1 and TPM2 regions with sequence identity percentages](image)

**Figure 1.** Sequence identity between Tpm1 and Tpm2 in *S. cerevisiae* (adapted from Drees et al., 1995). Tpm1 is divided into four regions, with B and B' representing a 38-amino acid pseudo-repeat. Tpm2 lacks exactly one of these repeats.
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Figure 2. Summary of dosage suppression interactions (adapted from Hendries et al., 2005).
Figure 3. Actin cable defects in tpm2Δ cells. (A) Representative images of Alexa488 phalloidin stained cells. (B) Average cable length in given strains was quantified in budding cells (n>10 cells/strain). **p < 0.01, ****p < 0.0001, Student’s t-test. (C) Average cable number for given strains was quantified in budding cells. (n>10 cells/strain), ANOVA.
Figure 4. (A) Representative images from experiments in which shown strains were treated with 100 μM LatB, fixed at indicated time points, and stained with Alexa488 phalloidin. (B) The proportion of cells with mid-sized buds containing cables in the mother cell was quantified (50 cells total). Data were quantified from 3 experiments. Error bars represent standard error of the mean (SEM).
Figure 5. Effects of Tpm2 on filament disassembly (A) Pyrene-actin disassembly assay containing Vitamin D binding protein, 2 μM F-actin (5% labeled), and 2.5 μM Tpm2 and/or 200 nM Cof1 where indicated. (B) Severing events for given reactions visualized using TIRF microscopy. 0.5 μM 10% OG-labeled monomeric actin was polymerized for 10 minutes in the presence of 2.5 μM Tpm2 when indicated. After 10 minutes, either buffer, 100 nM yeast Cof1 and/or 2.5 μM Tpm2 were flowed into the reaction chamber as indicated. Filament lengths were measured following flow in and severing events were quantified over a 5 minute time period (n=10 filaments/reaction). Data were quantified from two experiments. **p < 0.01, Student’s t-test. (C) Representative montages of entire fields of view of given conditions visualized by TIRF microscopy at indicated times after completion of flow-in of given proteins.
Figure 6. TIRF microscopy analysis of the effects of Tpm2 alone on actin filament assembly. (A) 1.0 μM, 10% Oregon green labeled monomeric actin was polymerized in the presence/absence of 2.5 μM Tpm2 as indicated. (A) Elongation rates were obtained by measuring filament length every 30 seconds for 5 minutes. Average rates are shown (n ≥ 8 filaments/reaction). Data were quantified from 3 experiments. Student’s t-test. (B) Nucleation was quantified by counting the number of filaments present in the field of view after 6 minutes of polymerization. Average number of filaments/FOV are shown (n> 3 FOV/reaction). Data were quantified from 3 experiments. Student’s t-test.
Figure 7. Effects of Tpm2 on Bnr1-mediated actin filament assembly. Pyrene-actin assembly assay containing 2.0 μM monomeric actin (10% labeled), 100 pM Bnr1, and the indicated concentrations of Tpm2. Data were quantified from 3 experiments.
**Figure 8.** TIRF microscopy analysis of the effects of Tpm2 on Bnr1-mediated actin filament assembly. (A) 1.0 μM, 10% Oregon green labeled monomeric actin was polymerized in the presence of 2.5 μM Tpm2 and/or 200 pM biotinylated Bnr1 as indicated. All reactions contain 3 μM yeast Pfyl. Elongation rates were obtained by measuring filament length every 30 seconds for 5 minutes. Average rates are shown (n ≥ 5 filaments/reaction). Data were quantified from 2 experiments. Student’s t-test. (B) 1.0 μM, 10% Oregon green labeled monomeric actin was polymerized in the presence of 2.5 μM Tpm2 and/or 400 pM Bnr1 as indicated. All reactions contain 3 μM yeast Pfyl. Nucleation was quantified by counting the number of filaments present in the field of view after 6 minutes of polymerization. Average number of filaments/FOV are shown (n ≥ 6 FOV/experiment). Data were quantified for 3 experiments. ***p < 0.0001, Student’s t-test. (C) Representative images of actin filaments under given conditions after six minutes of polymerization visualized using TIRF microscopy.
Figure 9. Effects of Tpm2 on Bni1-mediated actin filament assembly. Pyrene-actin assembly assay containing 2.0 μM monomeric actin (10% labeled), 100 pM Bnr1, and the indicated concentration of Tpm2. Data were quantified from 3 experiments.
**Figure 10.** TIRF microscopy analysis of the effects of Tpm2 on Bni1-mediated actin filament assembly. (A) 1.0 µM, 10% Oregon green labeled monomeric actin was polymerized in the presence of 2.5 µM Tpm2 and/or 600 pM Bni1 as indicated. Data were quantified from 2 experiments. All reactions contain 3 µM yeast Py1. Elongation rates were obtained by measuring filament length every 30 seconds for 5 minutes. Data were quantified for 2 experiments. Average rates are shown (n ≥ 10 filaments reaction). Student’s t-test. (B) Nucleation was quantified by counting the number of filaments present in the field of view after 6 minutes of polymerization. Data were quantified from 3 experiments. Average number of filaments/FOV are shown (n ≥ 3 FOV/reaction). **p < 0.01, Student’s t-test. (C) Representative images of actin filaments in given conditions after six minutes of polymerization visualized using TIRF microscopy.
REFERENCES


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