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Novel Interactome of Saccharomyces cerevisiae Myosin Type II Identified by a Modified Integrated Membrane Yeast Two-Hybrid (iMYTH) Screen

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ABSTRACT Nonmuscle myosin type II (Myo1p) is required for cytokinesis in the budding yeast Saccharomyces cerevisiae. Loss of Myo1p activity has been associated with growth abnormalities and enhanced sensitivity to osmotic stress, making it an appealing antifungal therapeutic target. The Myo1p tail-only domain was previously reported to have functional activity equivalent to the full-length Myo1p whereas the head-only domain did not. Since Myo1p tail-only constructs are biologically active, the tail domain must have additional functions beyond its previously described role in myosin dimerization or trimerization. The identification of new Myo1p-interacting proteins may shed light on the other functions of the Myo1p tail domain. To identify novel Myo1p-interacting proteins, and determine if Myo1p can serve as a scaffold to recruit proteins to the bud neck during cytokinesis, we used the integrated split-ubiquitin membrane yeast two-hybrid (iMYTH) system. Myo1p was iMYTH-tagged at its C-terminus, and screened against both cDNA and genomic prey libraries to identify interacting proteins. Control experiments showed that the Myo1p-bait construct was appropriately expressed, and that the protein colocalized to the yeast bud neck. Thirty novel Myo1p-interacting proteins were identified by iMYTH. Eight proteins were confirmed by coprecipitation (Ape2, Bzz1, Fba1, Pdi1, Rpl5, Tah11, and Trx2) or mass spectrometry (AP-MS) (Abp1). The novel Myo1p-interacting proteins identified come from a range of different processes, including cellular organization and protein synthesis. Actin assembly/disassembly factors such as the SH3 domain protein Abp1 represent likely Myo1p interactions during cytokinesis.

KEYWORDS Myo1p proteomics interactome cytokinesis yeast

The Saccharomyces cerevisiae myosin type II (Myo1p) is found in the contractile ring that contributes to its function in cell division coupled with chitin synthase 2 driven membrane ingression (Bi et al. 1998; VerPlank and Li 2005; Schmidt et al. 2002). Myo1p is a large protein (223.6 kDa) that consists of a globular N-terminal head domain [amino acids (aa) 1–800], and a long tail (aa 850–1928). The globular head binds to filamentous actin and ATP. The tail domain has been predicted to adopt a coiled-coil conformation with breaks (May et al. 1998). Unexpectedly, Myo1p was found to function in cytokinesis as a tail-only domain (Tolliday et al. 2003; Lord et al. 2005; Lister et al. 2006), suggesting that the N-terminal domain power-stroke function is not an essential feature of Myo1p. Furthermore, a minimum localization domain...
(MLD) identified in the terminal 1000 aa, which was previously associated with Myo1p oligomerization, appears to provide additional biological activity, and possibly serves as a site for the recruitment of the cytokinesis machinery and/or to signal for cell division (Lister et al. 2006). Previous yeast two-hybrid experiments (Drees et al. 2001; Wang et al. 2012), and TAP-tag protein copurification experiments (Gavin et al. 2002) identified multiple putative Myo1p-interacting proteins, but shed little light on the role of the Myo1p tail as a potential recruitment of proteins that may regulate cytokinesis. Earlier studies relied heavily on the recovery of soluble proteins, which the Myo1p-protein interactions were likely biased against. In this study, we used a modified integrated split-ubiquitin membrane yeast two-hybrid (iMYTH) technique to search for Myo1p interactions with associated proteins (Stagljar et al. 1998; Paumi et al. 2007; Snider et al. 2010, 2013) that may support the function of the C-terminal region of Myo1p at the bud neck during cytokinesis.

MATERIALS AND METHODS

Yeast strains and culture conditions

All yeast strains were grown at 30° in YPD broth while shaking at 225 rpm (Table 1). Artificial bait L40, Myo1 L40 L2, and Myo1 L40 L3 strains were maintained on YPD or YPD +200 µg/mL of G418 with 2% agar medium, and were transferred to synthetic dropout medium lacking histidine. Strains used in this study are available upon request.

Bait construction

To construct the bait strain for iMYTH experiments, we followed the protocol described by Snider et al. 2010. Briefly, to generate Myo1 L40 L2 and Myo1 L40 L3-containing strains (Figure 1A), the Myo1p-MLD 5' (CGAAAAATATTGATAGTAAACTTCAGACAGGAAAAATTTCCATGATGGGAGATCCCTCC) and Myo1p-MLD 3' (GGTGAAGAGTTCATGACTATATATACGGCTGTTCTGACTATAGGGGAGACCGGCGAG) primers were used to PCR-amplify a Cub-LexA-VP16 KanMX, or Cub-YFP-LexA-VP16 KanMX, cassette from L2 or L3 plasmids, respectively (Snider et al. 2010). The L40 yeast reporter strain was transformed with the cassette, and transformants were selected with YPD + 200 µg/mL G418. For bait validation, microscopy fluorescence (Figure 1B) and NubG/I self-activation tests (Figure 2) were performed.

Library transformation, bait-dependency test, and interactome generation

Myo1 L40 was transformed with NubG-X genomic and cDNA libraries provided by the Stagljar laboratory. Positive clones were selected using synthetic dropout medium without tryptophan (SD–W). The plasmids recovered from the transformed yeast cells were partially sequenced (~300 bp) to identify the encoded gene, and their interaction with the bait protein was reconfirmed using a bait dependency test under selection using synthetic dropout medium without tryptophan and histidine (SD–WH) (Snider et al. 2010). The exact size of the prey cDNAs was not determined. The A0287 strain used as a negative control in the bait dependency test expresses a Matx signal sequence and the transmembrane domain of human T-cell surface glycoprotein CD4 fused to the C-terminal ubiquitin domain plus transcription factors LexA and VP16. The positive interactors were classified according to their Gene Ontology (GO;http://geneontology.org/) functions and previously reported physical and genetic interactions. The Biological General Repository for Interaction Datasets (BioGRID;http://thebiogrid.org/), a curated database of yeast protein–protein and genetic interactions, was used to identify previously reported interactions. Cytoscape (http://www.cytoscape.org/) was used to generate two-dimensional interaction maps for visualization of these interactions.

In vitro validation of the Myo1p iMYTH interactors by coimmunoprecipitation

To capture the Myo1p-interacting proteins for in vitro validation of the physical interactions identified by iMYTH, and for their subsequent identification by coimmunoprecipitation (coIP), we employed a modified pull-down approach (Babu et al. 2009) using a TAP epitope fused
to the Myo1p C-terminus (Myo1-TAP). The Myo1-TAP bait strain was transformed with a full-length prey protein in expression vector BG1805, a URA3 multicopy 2 μ plasmid containing a GAL1 promoter (Gelperin et al. 2005), using the standard lithium acetate procedure (Gietz and Woods 2006). The Myo1p protein was captured using the calmodulin-binding peptide in the TAP epitope. Briefly, 40 ml of culture was grown for 2 d in YPD at 30°C at 200 rpm. Subsequently, the cells were centrifuged at 3000 rpm for 3 min, and washed twice with 20 ml IPLB buffer (20 mM Hepes KOH, pH 7.4, 150 mM KOAc, 2 mM Mg(Ac)2, 2 mM CaCl2, and 10% glycerol). For plasmid expression, cells were resuspended in YP-GAL, and incubated for 2–4 hr. The yeast cells were lysed with breaking buffer (IPLB buffer plus 1% Triton-X100 and 1× Protease inhibitor cocktail 1 from Calbiochem (EMD, #599131), and disrupted with glass beads by vortexing at maximum speed for 5 min. Cell lysates were cleared by centrifugation at 4000 rpm for 4 min. For protein isolation, 50 μL of calmodulin beads were mixed with 700 μL of the cell lysate in a microcentrifuge tube, and placed in a rotator for 2 hr at 4°C. After incubation, the beads were centrifuged at 5000 rpm for 2 min at 4°C. The resulting supernatant

Table 2 Myo1p-interacting proteins validated by communoprecipitation

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Systematic Name</th>
<th>Description (According to SGD, <a href="http://www.yeastgenome.org/">http://www.yeastgenome.org/</a>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APE2</td>
<td>YKL157W</td>
<td>Aminopeptidase ysc1; may have a role in obtaining leucine from dipeptide substrates; APE2 has a paralog, AAP1, that arose from whole genome duplication</td>
</tr>
<tr>
<td>BZZ1</td>
<td>YHR114W</td>
<td>SH3 domain protein implicated in regulating actin polymerization; able to recruit actin polymerization machinery through its SH3 domains; colocalizes with cortical actin patches and Las17p; interacts with type I myosins</td>
</tr>
<tr>
<td>FBA1</td>
<td>YKL060C</td>
<td>Fructose 1,6-bisphosphate aldolase; required for glycolysis and gluconeogenesis; catalyzes conversion of fructose 1,6 bisphosphate to glyceraldehyde-3-P and dihydroxyacetone-P; locates to mitochondrial outer surface upon oxidative stress; N-terminally propionylated in vivo</td>
</tr>
<tr>
<td>PDI1</td>
<td>YCL043C</td>
<td>Protein disulfide isomerase; multifunctional protein of ER lumen, essential for formation of disulfide bonds in secretory and cell-surface proteins, unscrambles non-native disulfide bonds; key regulator of Ero1p; forms complex with Mnl1p, which has exomannosidase activity, processing unfolded protein-bound Man8GlcNAc2 oligosaccharides to Man7GlcNAc2, promoting degradation in unfolded protein response; PDI1 has a paralog, EUG1, that arose from whole genome duplication</td>
</tr>
<tr>
<td>RPL5</td>
<td>YPL131W</td>
<td>Ribosomal 60S subunit protein L5; nascent Rpl5p is bound by specific chaperone Sso1p during translation; homologous to mammalian ribosomal protein L5 and bacterial L18; binds SS rRNA and is required for 60S subunit assembly</td>
</tr>
<tr>
<td>TAH11</td>
<td>YJR046W</td>
<td>DNA replication licensing factor; required for prereplication complex assembly</td>
</tr>
<tr>
<td>TRX2</td>
<td>YGR209C</td>
<td>Cytoplasmic thioredoxin isoenzyme; part of thioredoxin system that protects cells against oxidative and reductive stress; forms LMA1 complex with Pbi2p; acts as a cofactor for Tsa1p; required for ER-Golgi transport and vacuole inheritance; with Ttx1p, facilitates mitochondrial import of small Tims (Tim9p, Tim10p, and Tim13p) by maintaining them in reduced form; abundance increases under DNA replication stress; TRX2 has a paralog, TRX1, that arose from whole genome duplication</td>
</tr>
</tbody>
</table>

SGD, Saccharomyces genome database; ER, endoplasmic reticulum.
The iMYTH system is a powerful tool for screening protein interactions, both in vivo and in the natural cellular environment where these interactions are expected to occur (Paumi et al., 2007; Snider et al., 2010). The advantage of iMYTH is that it may detect weak protein-protein interactions, and/or temporally regulated interactions, which may not be detected by conventional pull-down methods. The *S. cerevisiae* wild-type myosin type II (Myo1p) localizes to the bud-neck during cell division (Bi et al., 1998); the Myo1p fusion protein used as the bait in this study was also shown to localize precisely to this site (Figure 1B). Another advantage of iMYTH is that the bait, in this case the Myo1 fusion protein, is localized in its natural cellular environment instead of an artificial one, such as the nucleus, as in other classical yeast two-hybrid systems (Drees et al., 2001). Another valuable feature of the iMYTH system is that the bait protein is expressed by its native promoter so that the protein levels are not artificially elevated. Furthermore, the system is monitored for self-activation of the bait modules through the use of positive and negative control preys (Figure 2), helping reduce the incidence of false positive results.

In this study, we used a modified iMYTH screen to identify Myo1p-interacting proteins. A total of 30 initial hits were identified (Supplemental Material, Figure S1), all of which passed a secondary bait dependency test. Eight of these hits were confirmed by colP or AP-MS (Figure 3, Table 2, and Table 3, respectively), and were therefore considered as highly reliable Myo1p-interacting proteins. A null mutation of *ABP1* did not visibly alter the bud neck localization of a Myo1p-YFP fusion protein (data not shown). Haploid null mutations of *FBA1*, *PDI1*, *RPL5*, and *TAH11* are lethal and were not tested. Null strains of *TRX2*, *APE2*, and *BZZ1* were not available for testing.

The high number of validated hits identified (26.7%) demonstrated the value of iMYTH as a robust high-throughput screening tool for the discovery of novel target proteins with important regulatory functions. None of the 30 Myo1p iMYTH interactors identified in this study (Figure S1) were previously reported in the Saccharomyces Genome Database (SGD; http://www.yeastgenome.org/) (see summary in Figure 4). Furthermore, we did not identify other expected interactors in this screen that were previously reported in the SGD, such as actin (*ACT1*), or the myosin light chains (*MLC1* and *MLC2*) (Drees et al., 2001; Gavin et al., 2002; Krogan et al., 2006). This may have been due to the physical constraints of the iMYTH construct, where *ACT1* interacts with the actin-binding domain at the N-terminal region of Myo1p, while *MLC1* and *MLC2* bind to the neck region proximal to the ATPase domain of Myo1p. Nonetheless, when the *ACT1* gene was cloned in the prey plasmid BG1805, and coexpressed with the Myo1p bait, it was confirmed to interact with Myo1p in colP assays (Figure S2A). Therefore, the absence of clones encoding Act1p, and possibly other known

### RESULTS AND DISCUSSION

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### Table 3 Myo1p-interacting proteins validated by affinity purification-mass spectrometry (AP-MS)

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Systematic name</th>
<th>Negative control</th>
<th>MYO1 Replicate 1</th>
<th>MYO1 Replicate 2</th>
<th>MYO1 Replicate 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABP1</td>
<td>YCR088W</td>
<td>2 98.97</td>
<td>3 99.58</td>
<td>5 99.58</td>
<td>4 99.58</td>
</tr>
</tbody>
</table>

Exclusive unique peptide count for new Myo1p interactors identified by iMYTH and validated by affinity purification-mass spectrometry.

Figure 4 Venn diagram showing the 30 novel Myo1p-interacting partners identified by iMYTH, AP-MS, and colP. These 30 Myo1p-interacting proteins (purple) have not been reported previously among the 103 Myo1p interactors listed in the SGD. Seven of the 30 novel iMYTH-positive interactors were validated by colP (teal); one of the 30 was validated by AP-MS experiments (green). None of these Myo1p-interacting proteins were validated by all three methods. Therefore, a total of eight were classified as confirmed positive Myo1p interactors.
Myo1p-interacting proteins, in our iMYTH screen may have been caused by a low representation of these clones in the prey library. Cloning and expression of the individual genes in a prey vector is therefore the recommended approach to validate these and other reported Myo1p-interacting proteins. On the other hand, other known interactors of Myo1p that were missed, such as Mlc1p, Mlc2p, Bni5p, and Kar2p, as well as Act1p, were reconfirmed by our AP-MS experiments (Table 4). Therefore, the iMYTH method was capable of identifying different types of protein–protein interactions that are not stabilized under the conditions employed in traditional capture methods such as AP-MS. In this regard, the coIP assay strategy used here, coupled with detection by Western blot, proved to be a more effective method for confirmation of iMYTH hits.

AP-MS analysis of Myo1p-GFP pull downs—a method capable of pulling down large protein complexes attached to Myo1p irrespective of their site of interaction within the protein—generated 49 Myo1p physical interactors with >2 unique (present at least in one of the three replicate purifications), statistically significant peptides (>90%) (Table S1). Bni5, a septin protein required for cytokinesis in yeast (Lee et al. 2002) that was previously reported to interact with Myo1p (Schneider et al. 2013), is among the five proteins reconfirmed by our study (Table 4). To summarize our findings, a proposed interaction network incorporating the eight novel Myo1p-interacting proteins confirmed in this study is presented in Figure 5.

Abp1p is an actin-binding protein of the cortical actin cytoskeleton that is important for activation of the Arp2/3 complex that plays a key role in actin cytoskeleton organization and inhibits barbed-end actin filament elongation (Michelot et al. 2013). Given its known function in organization of the actin cortical cytoskeleton, we propose that Abp1p could be associated with the process of disassembly of the actin ring as it contracts during cytokinesis. The confirmation of Bzz1p as a Myo1p-interacting protein by both iMYTH and coIP places another protein implicated in regulating actin polymerization (Soulard et al. 2002), together with Myo1p potentially at the bud neck. These results support the possibility that Abp1p and Bzz1p with Bni5p (as reported previously by Schneider et al. 2013) may function together with Myo1p as part of a complex to regulate actin filament dynamics at the cytokerinetic ring. Going forward, it will be valuable to map the sites of iMYTH interaction between Myo1p and the recovered protein set, and to investigate the functional consequences of direct and indirect associations revealed by this study.

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Table 4 Exclusive unique peptide count for Myo1p physical interactors previously reported in the SGD and confirmed by affinity purification–mass spectrometry in this study

<table>
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<tr>
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<th>MYO1 Replicate 2</th>
<th>MYO1 Replicate 3</th>
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<tr>
<td>MLC1</td>
<td>YGL106W</td>
<td>0 0</td>
<td>15 99.58</td>
<td>6 99.58</td>
<td>4 86.03</td>
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<tr>
<td>MLC2</td>
<td>YPR188C</td>
<td>0 0</td>
<td>33 99.58</td>
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<td>32 99.58</td>
</tr>
<tr>
<td>BNI5</td>
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<td>5 99.58</td>
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<tr>
<td>KAR2</td>
<td>YJL034W</td>
<td>2 96.57</td>
<td>2 96.12</td>
<td>5 99.58</td>
<td>3 98.17</td>
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<tr>
<td>ACT1</td>
<td>YFL039C</td>
<td>5 99.58</td>
<td>34 99.58</td>
<td>17 99.58</td>
<td>14 99.58</td>
</tr>
</tbody>
</table>

Figure 5 Proposed network of Myo1p-interacting proteins identified by iMYTH, coIP, and AP-MS. A Myo1-TAP construct was used as the bait protein to confirm seven novel Myo1p-interacting proteins by subsequent coIP assays. Abp1p, also a novel interactor, was confirmed by AP-MS as listed in Table 2 and Table 3, respectively. Previously reported physical interactors of Myo1p: Mlc1p, Mlc2p, Bni5p, Kar2p, and Act1p were confirmed by AP-MS (see Table 4).
LITERATURE CITED


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