Supplementary information

Supplementary methods

Immunoprecipitations and pull downs

For the myc-tagged Myo5 immunoprecipitations from PM and cytosolic fractions, samples were processed as described for the IgG pull-downs except that 50 µl of 50% anti-myc-Agarose (Roche) was used instead of IgG-Sepharose.

For the pull down experiments (Fig. S3), 1 µg of bead-bound GST, GST-C or GST-Cmd1 were incubated with protein extracts of the strains expressing PA-C, PA-H, PA-Hn, PA-HT or PA-HnT prepared in IP buffer 1% Triton X-100 in the presence of PIs and in the presence of either 1 mM EGTA or 5 mM CaCl₂ when indicated, to induce Cmd1 dissociation. After incubation for 1 hour at 4°C, beads were recovered, washed 3 times with IP buffer 1% Triton X-100 in the presence of either 1 mM EGTA or 5 mM CaCl₂ as appropriate, and twice with IP buffer in the presence of either 1 mM EGTA or 5 mM CaCl₂ as appropriate. Beads were resuspended in 30 µl of SDS-sample buffer and extracted proteins were separated by SDS-PAGE and transferred to a nitrocellulose filter. GST-C and GST were detected by staining with Ponceau red and the PA-Myo5 constructs were detected using PAP. 1/125 of the total protein extracts were loaded as input.

Trypsin digestion

PA-Myo5 precipitated with IgG-Sepharose was washed with IP buffer containing 1 M salt and with trypsin digestion buffer (50 mM TrisHCl, pH7.5, 100 mM NaCl). Each pellet was divided into 2 equal aliquots and incubated with 20 µl 0.2 µg/ml trypsin (prepared in trypsin digestion buffer; Sigma-Aldrich, from bovine pancreas) for 2.5 min at room temperature. A zero time point was obtained by addition of 20 µl Trypsin digestion buffer alone. The digestion was stopped by addition of 20 µl 2x Laemmli sample buffer supplemented with protease inhibitors followed by incubation at 95°C for 3 minutes.

Purification of yeast PM and cytosol

The isolation of PM and cytosolic fractions were performed based on (Serrano, 1988). Briefly, a 4 x 10⁹ cell pellet was glass bead-lysed in LB in the presence of PIs. The extract was diluted with LB, 0.5 mM PMSF to 12.5 ml. Unbroken cells were eliminated by centrifugation at 700 g for 10 min at 4°C. The supernatant was recovered (sA) and the pellet was resuspended in 12.5 ml of BB, 0.5 mM PMSF. After centrifugation at 700 g for 10 min at 4°C, the supernatant (sB) was mixed with the same volume of sA. The combined supernatants (total) were centrifuged in a JS-13.1 Beckman rotor at 20,000 g for 20 min at 4°C. The supernatant was collected
(precytosol) and the pellet was resuspended in 10 ml of BB, 0.5 mM PMSF. To eliminate non-solubilized material, the membrane suspension was centrifuged at 700 g for 10 min at 4°C. The supernatant was then applied to a discontinuous sucrose gradient made of 9 ml of 53% and 18 ml of 43% sucrose in BB (w/w) in an Ultra-Clear ultracentrifuge tube (#344058, Beckman). Centrifugation was carried out in a SW-28 Beckman rotor at 100,000 g for 3 h at 4°C. The 43% / 53% sucrose interphase was recovered, diluted with 6 volumes of water and the PM was recovered by centrifugation at 80,000 g for 20 min at 4°C. The PM pellet was dissolved in 1.3 ml LB:BB (1:1), 0.5 mM PMSF. To obtain the cytosol, the precytosol was centrifuged in a SW-28 Beckman rotor at 100,000 g for 3 h at 4°C. The supernatant was recovered.

**In vitro actin polymerization assay**

The rhodamine-actin polymerization assay on Sepharose beads was performed as described (Idrissi et al, 2002) except that IgG-Sepharose beads coated with PA-tagged Myo5 constructs purified from yeast were used instead of the GST construct purified from *E. coli*. For further detail, see supplementary data. Briefly, 7 µl of a yeast extract (Idrissi et al, 2002) prepared in XB (10 mM HEPES pH 7.7, 100 mM KCl, 2 mM MgCl₂, 1 mM DTT, 1 mM ATP, 0.1 mM CaCl₂, 5 mM EGTA and 200 mM Sucrose) were mixed with 1 µl of ARS (10 mg/ml creatine kinase, 10 mM ATP, 10 mM MgCl₂, 400 mM creatine phosphate), 1 µl of 10 mM rhodamine-actin (APHR-C, Cytoskeleton, Inc.) and 1 µl of 50% IgG-Sepharose beads coated with the indicated PA-tagged constructs purified from yeast (see below). Samples were incubated at 26°C for 5 min. Beads were pipetted on glass slides, covered with a coverslip and visualized with an Axiophot fluorescence microscope (Zeiss). For preparation of IgG-Sepharose beads coated with the PA-tagged Myo5 constructs, 2 x 10¹⁰ SCMIG275 cells expressing the appropriate construct were glass bead-lysed in IP buffer (50 mM Tris HCl 7.3, 5 mM EDTA, 150 mM NaCl) containing either 5 mM CaCl₂ or 1 mM EGTA plus PIs. Extracts were diluted with the corresponding IP to 1.5 ml and Triton was adjusted to 1%. Extracts were clarified by centrifugation at 10,000 g for 10 minutes at 4°C. The extract was incubated with the appropriate amount of IgG-Sepharose to obtain beads coated with equimolar amounts of the different constructs. Samples were then washed with the corresponding IP buffer and resuspended in 5 µl of XB for the actin polymerization assay.

**Supplementary figure legends**
Figure S1. A. Co-localization of GFP-Myo5 with endocytic markers. Fluorescence micrographs of live myo5Δ cells RFP-tagged ABP1 (SCMIG880) expressing the indicated GFP-Myo5 fusion proteins from centromeric plasmids under the control of the MYO5 promoter. Cells were grown to mid-log phase at 25°C and directly observed by fluorescence microscopy. Arrowheads indicate endocytic cortical patches. B. Immunoblot showing the expression level of the different GFP-tagged Myo5 constructs. 50 µg of total protein extracts from indicated strains were separated by SDS-PAGE and transferred to a nitrocellulose filter. GFP-tagged constructs were detected using an anti-GFP antibody and the appropriate peroxidase-conjugated secondary antibody. Bar = 1 µm.

Figure S2. Deletion of individual IQ motifs does not have a major influence in Vrp1 binding or recruitment of Myo5 to cortical patches. A. Immunoblots of IgG-Sepharose pull-downs from myo5Δ vrp1Δ cells (SCMIG304) expressing the indicated PA-Myo5 constructs and Vrp1-HA. PAP, a peroxidase-conjugated anti-HA antibody and an anti-Cmd1 serum, combined with the adequate peroxidase-conjugated secondary antibody, were used for detection of the PA-Myo5 constructs, Vrp1 and Cmd1, respectively. All PA-Myo5 constructs and Vrp1-HA were expressed from centromeric plasmids under the control of their own promoters. B. Fluorescence micrographs of live myo5Δ cells RFP-tagged ABP1 (SCMIG880) expressing the indicated GFP-Myo5 constructs from centromeric plasmids under the control of the MYO5 promoter. Cells were grown to mid-log phase at 25°C and directly observed by fluorescence microscopy. Arrowheads indicate endocytic cortical patches.

Figure S3. The Myo5 TH1 domains binds to the Myo5 Cext and free Cmd1 does not bind to the Myo5 Cext. A. Immunoblot of glutathione-Sepharose pull-downs of GST-C or GST, incubated with extracts of a myo5Δ strain (SCMIG275) expressing the indicated PA-Myo5 constructs from centromeric plasmids under the MYO5 promoter in the absence (-) or presence (+) of 5 mM CaCl₂ to trigger Cmd1 dissociation. Ponceau red and PAP were used to detect GST-C and GST or the PA-Myo5 constructs, respectively. B. Immunoblots of IgG-Sepharose pull-downs from myo5Δ cells (SCMIG275) expressing the indicated PA-Myo5 constructs or native Myo5. PAP and an anti-Cmd1 serum, combined with the adequate peroxidase-conjugated secondary antibody, were used for detection of the PA-Myo5 constructs and Cmd1, respectively. All PA-Myo5 constructs were expressed from centromeric plasmids under the control of their own promoters. C. Immunoblot of glutathione-Sepharose pull-downs of GST-Cmd1 or GST, incubated with extracts of a
myo5Δ strain (SCMIG275) expressing the indicated PA-Myo5 constructs from centromeric plasmids under the MYO5 promoter. Ponceau red and PAP were used to detect GST-C and GST or the PA-Myo5 constructs, respectively.

**Figure S4. Cmd1-mCherry does not localize at cortical patches.** Fluorescence micrographs of live myo5Δ cells bearing the chromosomal copy of CMD1 tagged with mCherry (Cmd1-mCh) (SCMIG1080) expressing GFP-Myo5 from centromeric plasmids under the control of the MYO5 promoter. Cells were grown to mid-log phase at 25°C and directly observed by fluorescence microscopy. Bar = 1 μm.

**Figure S5. Cytosolic or PM-associated Myo5 do not oligomerize under the same conditions that show myosin binding to Cmd1 or Vrp1 A.** Immunoblot of 10 μg of a total protein yeast extract (T) or of yeast PM (PM) or cytosolic (C) fractions prepared from myo5Δ strains (SCMIG275) expressing myc-tagged Myo5 (MYC-Myo5) and either untagged Myo5 (Myo5) or HA-tagged Myo5 (Myo5-HA) from centromeric plasmids under the control of the MYO5 promoter. Nitrocellulose membranes were decorated with antibodies against the PM marker Gas1 (α-Gas1) and the cytosolic marker hexokinase (α-Hxk1). Myc-Myo5 and Myo5-HA were detected with α-MYC or α-HA antibodies, respectively. **B.** Immunoblots of immunoprecipitations (α-MYC IP) with anti-MYC agarose beads from yeast PM (PM) and cytosolic (C) fractions of the strains described in A. MYC-Myo5 and Myo5-HA were detected as described in A.

**Figure S6. Cmd1 dissociation exposes a Trypsin digestion site within the TH1 domain of Myo5.** Immunoblot of the indicated PA-Myo5 constructs purified from yeast, and incubated for the indicated time with Trypsin. PAP was used for detection of the PA-Myo5 fragments.
Fig. S1

A  
GFP-Myo5  Abp1-RFP  overlay

Myo5

nTC

HnC

C

HC

B

1. GFP-Myo5
2. GFP-HnT
3. GFP-Hn
4. GFP-H
5. GFP-nTC
6. GFP-TC
7. GFP-C
8. GFP-HTC
9. GFP-HnC

Fig. S1
Fig. S2

A

B

GFP-Myo5  Abp1-RFP  overlay

Myo5

Myo5-iq1Δ

Myo5-iq2Δ

Myo5-iqΔ
Fig. S3

A

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B

1. PA-Myo5
2. Myo5
3. PA-nT
4. PA-C

C

input pull down

PA-H PA-Hn PA-C PA-H PA-Hn PA-C

PAP

GST-Cmd1

α-Cmd1

Ca^2+ Ponceau

97 PAP

66

45

31

GST

Ponceau
Fig. S4

A  GFP-Myo5 Cmd1-mCh overlay
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Fig. S5
**Fig. S6**

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**Legend:**
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