

Expanded View Figures

Figure EV1. FRAP analysis and TIRFM image processing pipeline.

- A FRAP analysis of Mup1-GFP in the presence and absence of 100 μ M LatB. Times are given in min relative to localized bleaching of fluorescence. The kymograph was drawn around the indicated cell periphery (red dashed lines) and shows fluorescence recovery in the bleached area. Mup1-GFP fluorescence recovery was determined in the bleached area at t = 30 min. Time arrow represents 10 min. Values are means \pm SD, n = 10 cells. The green line indicates significantly different data sets. An overview of the performed statistics can be found in Table EV3.
- B Representative raw, deconvolved (deconv), and thresholded (binary) TIRFM images generated by an automated MATLAB algorithm to determine the degree of colocalization of GFP and RFP signals (Pearson Mean) and to quantify the fraction of the GFP signal present in the RFPlabeled structure (GFP overlap).

Data information: Scale bars: 2 $\mu m.$ Values are listed in Table EV1.



Figure EV2. Cellular regulation of Mup1 patterning.

- A Myriocin—but not aureobasidin A—induced SIm1 and Mup1 MCC exit is reversed upon treatment with phytosphingosine (PHS). Pil1-RFP was used to determine the degree of concentration within the MCC.
- B, C Requirements of TORC1 and TORC2 for the delivery of Mup1-GFP to the PM upon methionine starvation (B) and for Mup1 endocytosis upon addition of methionine (C). In control cells, only TORC1 is rapamycin (Rap) sensitive, in the $avo3\Delta$ C mutant, both TORC1 and TORC2 are sensitive to rapamycin, and in the $tor1-1/avo3\Delta$ C mutant, only TORC2 is rapamycin-sensitive. Representative equatorial images are shown.
- D Influence of sphingolipid stress on the lateral segregation of the tetraspanners Nce102 and Sur7 under Met starvation. Pil1-RFP was used to determine the degree of concentration within the MCC.
- E Representative two-color TIRFM images from the experiments summarized in (D).
- F Nce102 leaves the MCC upon TORC2 inhibition.
- G Representative two-color TIRFM images from the experiments summarized in (F).

Data information: All values plotted are means \pm SD, n = 20-150 cells. In (B, C and G), light gray labels indicate inactive TOR complexes, and bold labels indicate active TOR complexes. Green and red lines indicate significantly different or non-significant data sets, respectively. An overview of the performed statistics can be found in Table EV3. Scale bars: 2 μ m. All measured values are listed in Table EV1.

A S. cerevisiae TMD1		тмдз	¹⁵ ² g g 1 1 □ uptake rate (1) (1) (1) (1) (1) (1) (1) (1) (1) (1)
Mup1 63 ILSCIGLICNRMLGT Bap2 97 SRHVVMMSLGTGIGT Can1 90 QRHIGMIALGGTIGT Gap1 92 NRHLQMIAIGGAIGT Gnp1 151 PRHTVMMSLGTGIGT Hip1 92 VRHLLTLAVGGAIGT Lyp1 111 QRHIGMIALGGTIGT Tat2 84 PRHLIMIAIGGSIGT E. coli AdiC 12 LIPVTLMVSGNIMGS	VFAVSS 84 149 YI GLLVANA 118 179 WL GLFIGLS 111 171 YM GLLVGSG 113 173 FN GLLVGNS 172 233 WL GLYVNTG 113 173 LN GLFVGIS 132 192 YM GLFVGSG 105 165 TI GVFLLPA 33	FFLG AAGNSI 161 YCFQWLTVLPL 191 YWFSWAITFAL 183 YMLQWLVVLPL 185 FCLQWLCVCPL 245 YLAQWLVLLPL 185 YWFNWAITYAV 204 YVLQWFFVLPL 177	equatorial TIRE with Carl and
CadB 9 LFACTGVVAGNMMGS PotE 11 VVQLTILTMVNMMGS	GIALLPA 30 GIIMLPT 32		0
B G7 W1 C-p FW	s 55 lug RV	D/E K/R/H C-plug	
E 520 530	540 550 I 550	560 570 I I I	
TMD12 AQLLPRWGHYKLVSKDVLGEE HHHSSSSSSSSS AC A533 A537 A543 A565 wt	GEWRVKIAKVYDDIIGDVDTQEI SSSSSSSSSHHF	DGVIETNIIEHYKSEQEKSL ИННННННННННННННН	
F S. cerevisiae 513 QLLP S. kudriavzevii 513 QLLP N. dairenensis 511 QLLP N. castellii 514 QILP C. glabrata 500 KLLP K. naganishii 516 QLLP K. africana 509 QILP T. phaffii 509 QILP T. blattae 518 RLIP T. delbrueckii 505 QLLP K. lactis 514 VVLP E. gossypii 499 RLLP C. albicans 449 QLLP	RWGHYK LVSKDVLGED GF KWGHYK LVTKDVLGED GF RIFHYK LDTKDILGED GF RIYHYK LETKDILGDD GF RWGKYK LVTKDVLGED GF RYGGYK LDTKDVLGED GF RIFHYK LVTKDVLGED GF RIGHYE LVSKEVLGDD GF RIGHYE LVSKEVLGED GF KWNNYE LVHSEVLGED GF	RVKIAKV YDDTIGDV RVKIAKV YDSSNSDV RVQIVKV YDKGHGNE RVQIVKV YDDESKNV RMQIIKV PESEVGDD RVKIVKV PNATSEDD KVQIIKK YHDEVDED KISIIKV YDKDVSEK RKQIIKV FKDKKDDG RTEISRM YSDAATNT RQKIAKR KINEANAE RNKIVKK YAGEDSVS RNKIYKV SKDSTEEE	Hydrophobic large (MIV) Hydrophobic aromatic (WFY) Hydrophobic small (AG) Charged negative (ED) Charged positive (KRH)

Figure EV3. Mup1 sequence analysis and structure prediction.

- A Sequence alignments of predicted TMD1 and TMD3 of Mup1 with those of other yeast and *Escherichia coli* transporters. Conserved residues (dark gray), conservative replacements (light gray), and selected Mup1 mutations (G78 and W155) are highlighted.
- B Structural organization of Mup1 as predicted by the Phyre2 software. High (red) and low (white) confidence predictions are highlighted (left panel). Mutations affecting Met transport (green and light blue), the C-terminal "plug" oriented toward the substrate-binding pocket (yellow), the conserved C-terminal motif required for Mup1 localization and function (purple), as well as negatively (dark blue) and positively (red) charged residues around the binding pocket and the "C-plug" are indicated (middle and right panels).
- C ¹⁴C-methionine uptake rates and PM expression of truncations and GFP-fused Mup1 mutants. Values are means \pm SD, n = 2 experiments (uptake) and n > 70 cells (PM expression).
- D Representative equatorial and TIRFM images of Mup1 Δ N/ Δ C-GFP in the absence of Met, showing its delivery to the PM and partitioning into the MCC (colocalization with Pil1-RFP). Scale bars: 2 µm.
- E Sequence of the Mup1 C-terminal region. Degrees of conservation among Mup1 homologues (from ClustalW) calculated from various other fungi as shown in (F) (u: predicted ubiquitination site, p: predicted phosphorylation site) are indicated above the sequence, with secondary structure predictions (H: alpha helix, S: beta sheet) below it. The predicted "C-plug" is indicated in yellow. The different C-terminal truncations analyzed are shown.
- F Sequence alignment of the Mup1 C-terminal segment (513–550) with homologues from various other fungi. Gray shading indicates conserved region.



Figure EV4. Effects of truncations on function, segregation, and turnover of Mup1.

- A, B Function of different Mup1 mutants as measured by direct quantification of ¹⁴C-Met uptake (A) and growth sensitivity to the toxic Met analog selenomethionine (SeMet, B). Growth assay is shown from top to bottom as a fivefold dilution series.
- C, D Endocytic internalization of different Mup1 mutants. Ratios of PM to cytosolic fluorescence intensities (C) and representative images at equatorial planes (D) are shown.
- E, F Lateral PM segregation shown in representative TIRFM images and quantification of the Network factor (E) or the colocalization with Pil1-RFP (F). Mutants utilized: wt (wild-type Mup1), ΔC (deletion of C terminus after aa519), W155A and G78N (respective point mutants), Δ565/543/537/533 (deletion of C terminus beyond the indicated position). All strains refer to Mup1 variants fused to C-terminal GFP.

Data information: Values are means \pm SD, n = 2-4 experiments (A) and n = 50-200 cells (C, E, F). n.d.: not determined. Scale bars: 2 µm. All values are listed in Table EV1.

Α			- Met			- 1	/let + 2 µM SeMet				
	control	۲	۲	-							
	Pma1-GB		•	0 :		•					
	ΔC Pma1-GB			*	90-	٠		•	*	i.	
в	0.45										
	ptake	<u> </u>				Т	1				
	C-Met u nmol/mir										
		ontro	ol Δpi	11 co	ntrol	∆pil1	-				
c		١	wt		ΔC						
Č		- Arg	g + 1.5	µM C	anava	anine	- Arg	g + 4	µM Ca	anava	nine
	Can1-GFP		•								
	∆can1		•	•	P	\$1	۲	•	•	1	-
	∆pil1	۲	۲	۲	3	154	۲	۲		N.	14
	Can1-GFP Sur7-GB	•									
C	Can1-GFP Pma1-GB		•	0		17		•		1	10

Figure EV5. Mup1 uptake upon MCC disruption and Can1 sensitivity to canavanine.

- A Growth assay of indicated mutants in the absence of methionine and in the absence or presence of 2 μ m selenomethionine (SeMet).
- presence of 2 μ m selenomethionine (SeMet). B ¹⁴C-methionine uptake rate measured for Mup1 and Δ C in the wt and the $\Delta pil1$ background. Values are means \pm SD, n = 2-4 experiments. All values are listed in Table EV1.
- C Growth assay of indicated mutants in the absence of arginine (–Arg) and the presence of indicated concentrations of the toxic arginine analog canavanine. Growth assays are shown from left to right as a fivefold dilution series. White separator lines indicate borders to lanes that were removed from the original plate images.