Expanded View Figures

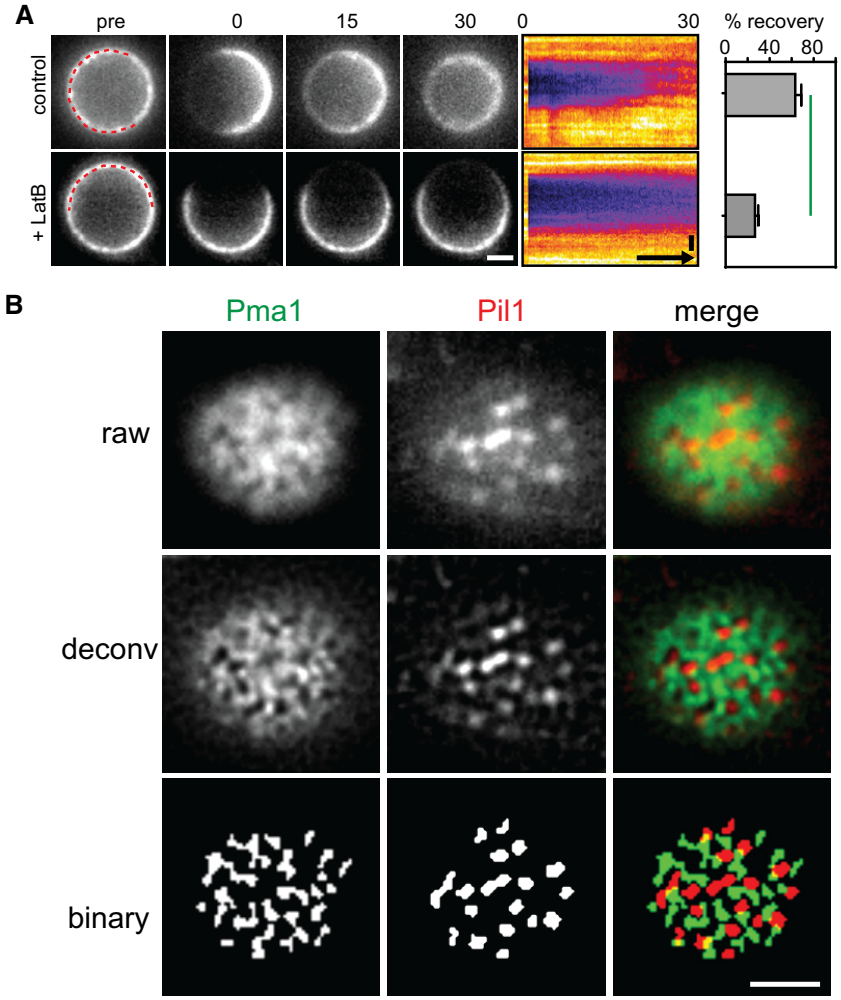

Figure EV1. FRAP analysis and TIRFM image processing pipeline.

A FRAP analysis of Mup1-GFP in the presence and absence of 100 μM 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 ± 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 RFP-labeled structure (GFP overlap).

Data information: Scale bars: 2 μm. Values are listed in Table EV1.

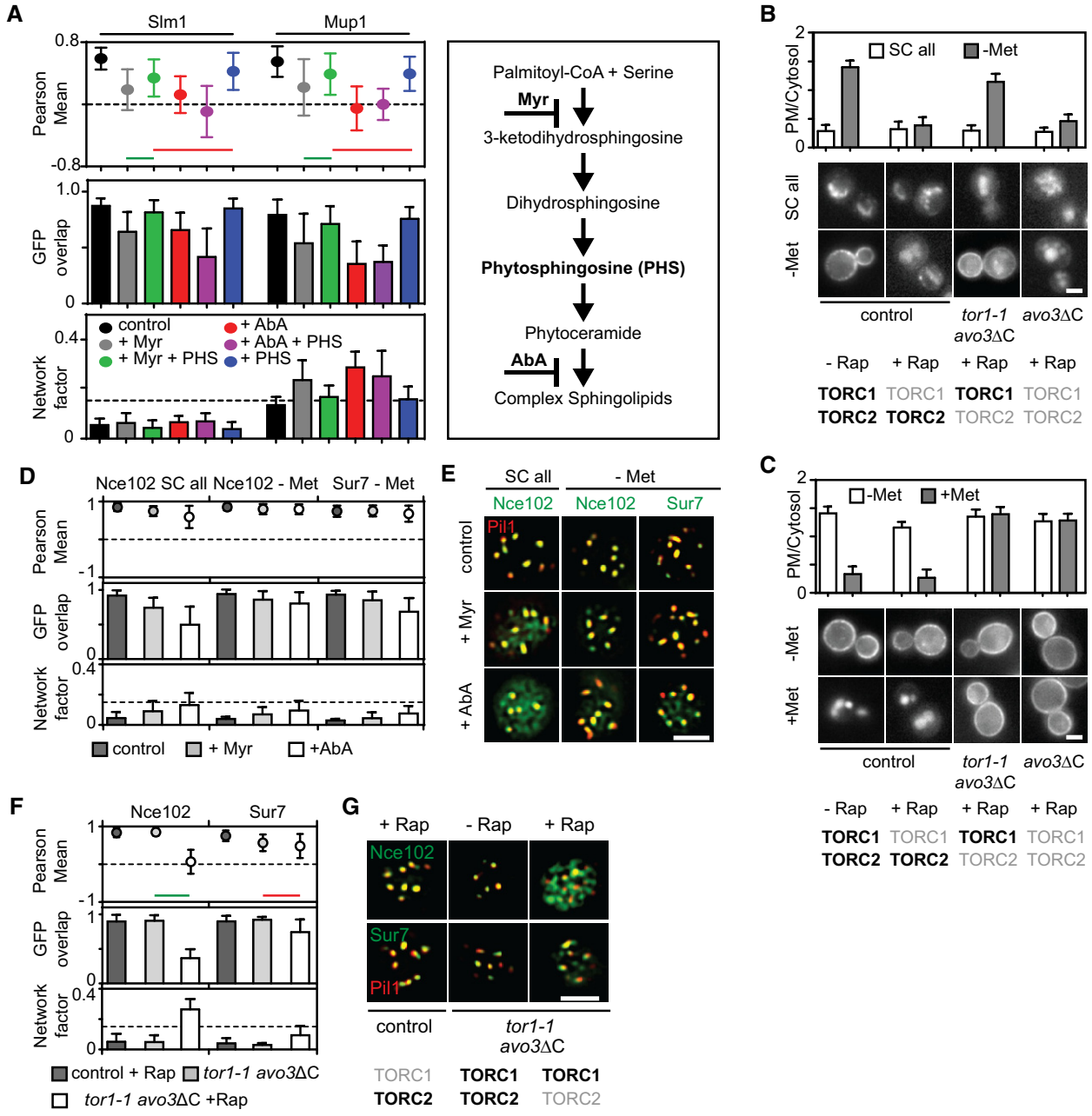

Figure EV2. Cellular regulation of Mup1 patterning.

A Myriocin—but not aureobasidin A—induced Slm1 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*ΔC mutant, both TORC1 and TORC2 are sensitive to rapamycin, and in the *tor1-1/avo3*ΔC 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 ± 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 μm. All measured values are listed in Table EV1.

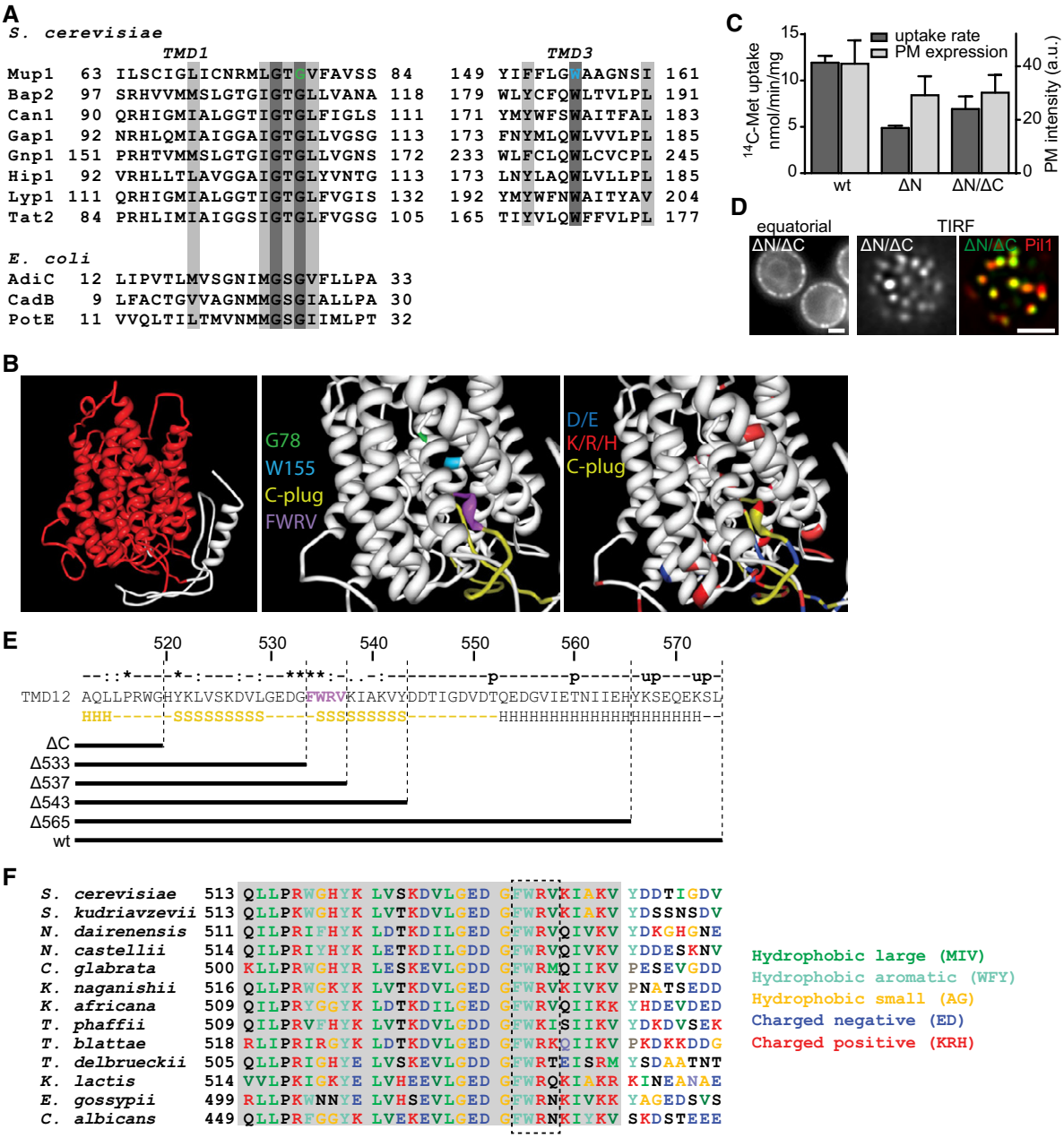

Figure EV3. Mup1 sequence analysis and structure prediction.

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 ^{14}C-methionine uptake rates and PM expression of truncations and GFP-fused Mup1 mutants. Values are means ± SD, n = 2 experiments (uptake) and n > 70 cells (PM expression).

D Representative equatorial and TIRFM images of Mup1ΔN/ΔC-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.

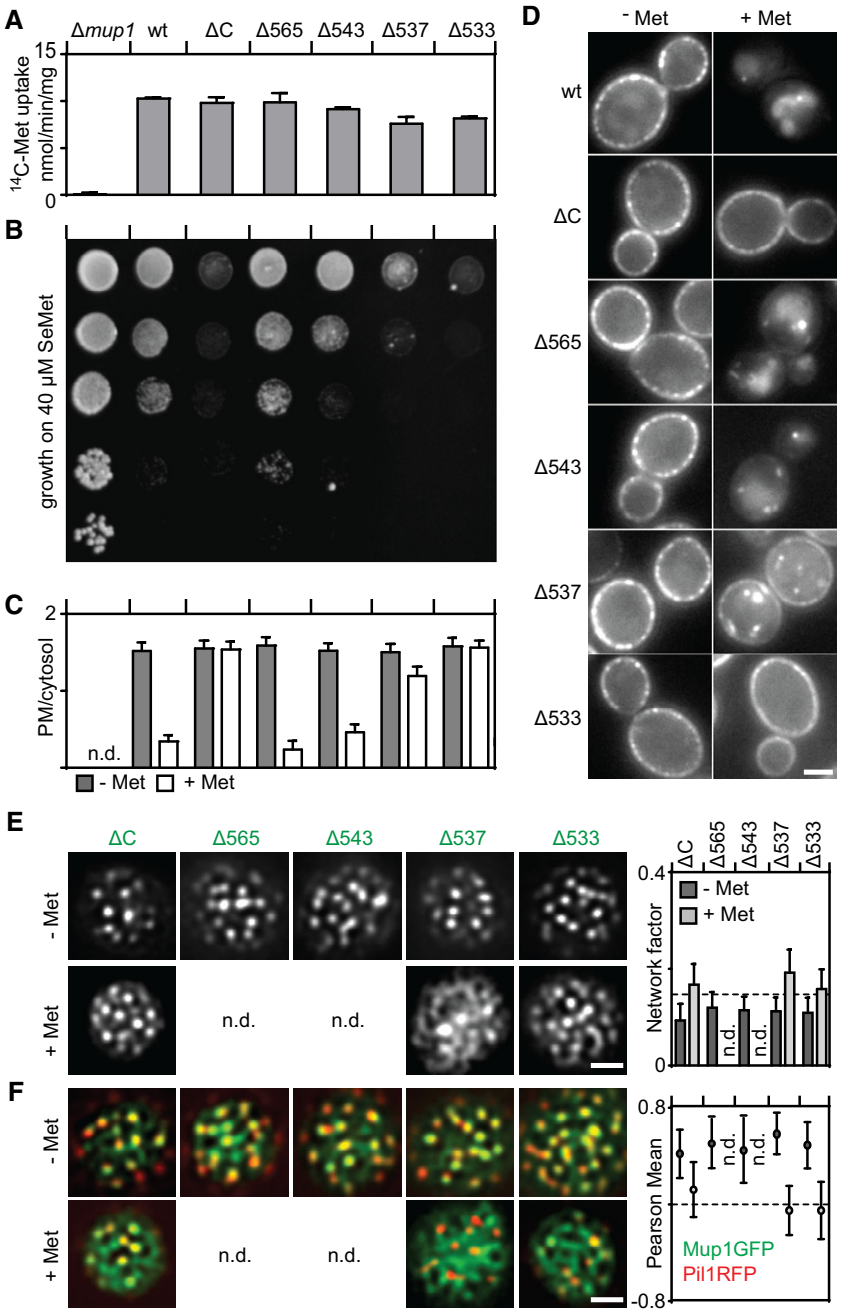

Figure EV4. Effects of truncations on function, segregation, and turnover of Mup1.

A, B Function of different Mup1 mutants as measured by direct quantification of ^{14}C-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 ± 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.

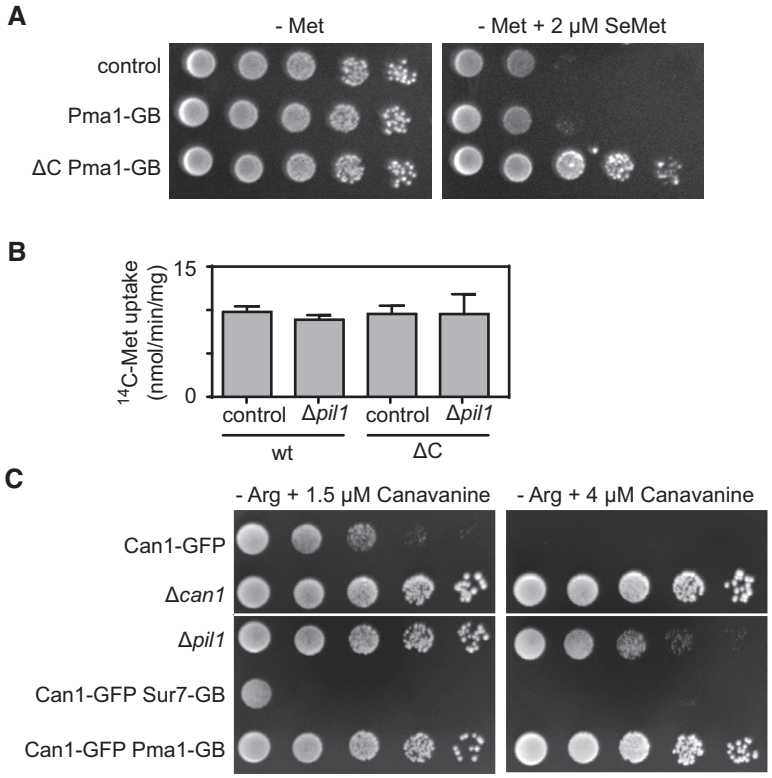

Figure EV5. Mup1 uptake upon MCC disruption and Can1 sensitivity to canavanine.

A Growth assay of indicated mutants in the absence of methionine and in the absence or presence of 2 µm selenomethionine (SeMet).

B ^{14}C-methionine uptake rate measured for Mup1 and ΔC in the wt and the Δ*pil1* background. Values are means ± 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.