Supplemental Information

**Supplemental Methods:**

GST-pulldown experiments (GGA3 for active Arf1) were performed as previously described (27). Briefly, pGEX5T-GGA3(1-313) was expressed in bacteria, purified using GST-bind resin (Novagen) and then used to pull down active Arf1 from native S. pombe cell lysates. After transfer to PVDF (Immobilon, Millipore), the lysates and bead-bound proteins were probed with anti-Arf1 (polyclonal PA5-2227, Thermo) or streptavidin for visualization of Arf1 and ubiquitin, respectively.

**Supplementary Figure S1.** S. pombe 5DUB delete cell morphology A) Images of wildtype (WT) and 5DUB delete cells grown asynchronously. Scale bar is 5 μm. B) Septation index of WT and 5DUB delete cells (inset numbers indicate number of septa found within cells). C) Box and whiskers plot (quartiles are indicated) of vacuolar volume of WT and 5DUB delete cells. See Experimental Procedures for details.

**Supplementary Figure S2.** Biochemical confirmation of Ub’n of membrane trafficking DUB substrates. A) Yeast strains overexpressing pREP1-Flag-Ub and containing endogenously tagged genes producing HBH-tagged proteins (putative substrates) were used to assay Ub’n in WT(+) and DUB delete strains (Δ = ubp4Δ1 ubp5Δ ubp9Δ sst2Δ) using medium scale two step denaturing purifications followed by Western blotting. B) GGA3-GST (on GST-bind beads) was used to capture active, GTP-bound Arf1 from native cell lysates of WT or 5DUB delete strains overexpressing pREP1-HBH-Ub. After pull down, the beads were boiled and proteins were detected by Western blot as indicated (see Supplemental Information for details).

**Supplementary Figure S3.** Annotated spectra for each unique peptide containing a ubiquitination site with an Ascore>13. Spectra are in order of ORF, as in Supplemental Table S6.

**Supplementary Figure S4.** Di-Ub cleavage reactions. A) Western blot showing purification of DUB-TAPs. DUBs were purified using rabbit IgG dynabeads and blots were probed with rabbit IgG. B) Representative image of time course di-Ub cleavage reactions monitored by silver staining. C) Negative control di-Ub cleavage reactions using an untagged strain to control for non-specific background cleavage. The middle lane (SB) is SeeBlue Plus2 protein markers (from bottom: 6, 14 and 17 kD). D) Graphs indicating the relative preference of each membrane-trafficking DUB for various Ub chain types. Note that cleavage efficiency is *not* normalized.
**Supplementary Figure S5.** Sensitivities of single DUB deletion strains to cellular stress. 10-fold dilution series of strains as indicated. The indicated drugs were added to YE agar at concentrations as indicated in Experimental Procedures.

**Supplementary Figure S6.** Sensitivities of quadruple DUB deletion strains to cellular stress. 10-fold dilution series of strains as indicated. The indicated drugs were added to YE agar at concentrations as indicated in Experimental Procedures.

**Supplementary Figure S7.** Degeneracy and connectivity of membrane trafficking DUB substrates. Each node represents one putative DUB substrate or DUB. Edges (lines) indicate potential targeting of the node (substrate) by a specific DUB (larger colored nodes). Nodes are color-coded to indicate degree (number of edges connecting the node, i.e. how many DUBs target that specific substrate) with yellow nodes being targeted by the fewest DUBs and dark orange nodes being targeted by all five DUBs. Edges (lines between nodes) are color-coded to indicate which DUB the node is connected to (i.e. which DUB targets a specific substrate). Image generated in Cytoscape (v 2.8.3, (Shannon et al, 2003)).

**Supplementary Table S1.** List of *S. pombe* strains used in this study.

**Supplementary Table S2.** List of proteins (background, BG) identified in control HBH purifications (related to Figs. 2 and 3).

**Supplementary Table S3.** List of proteins identified in WT and single DUB delete HBH-Ub purifications (related to Fig. 2).

**Supplementary Table S4.** List of putative DUB delete substrates from comparative analysis of HBH-Ub purifications of single delete strains (related to Fig. 2).

**Supplementary Table S5.** List of proteins identified in all WT and 5DUB delete HBH-Ub purifications (related to Fig. 3).

**Supplementary Table S6.** List of putative 5DUB delete substrates from comparative analysis of HBH-Ub purifications (related to Fig. 3).

**Supplementary Table S7.** Ub’n sites identified in HBH-Ub purifications (related to Fig. 3).

**Supplementary Table S8.** Gene ontology details for shared 5DUB substrates (related to Fig. 3).

**Supplementary Table S9.** Details of quantitative MS analysis for ubiquitin chain analysis (related to Fig. 4).