Expanded View Figures

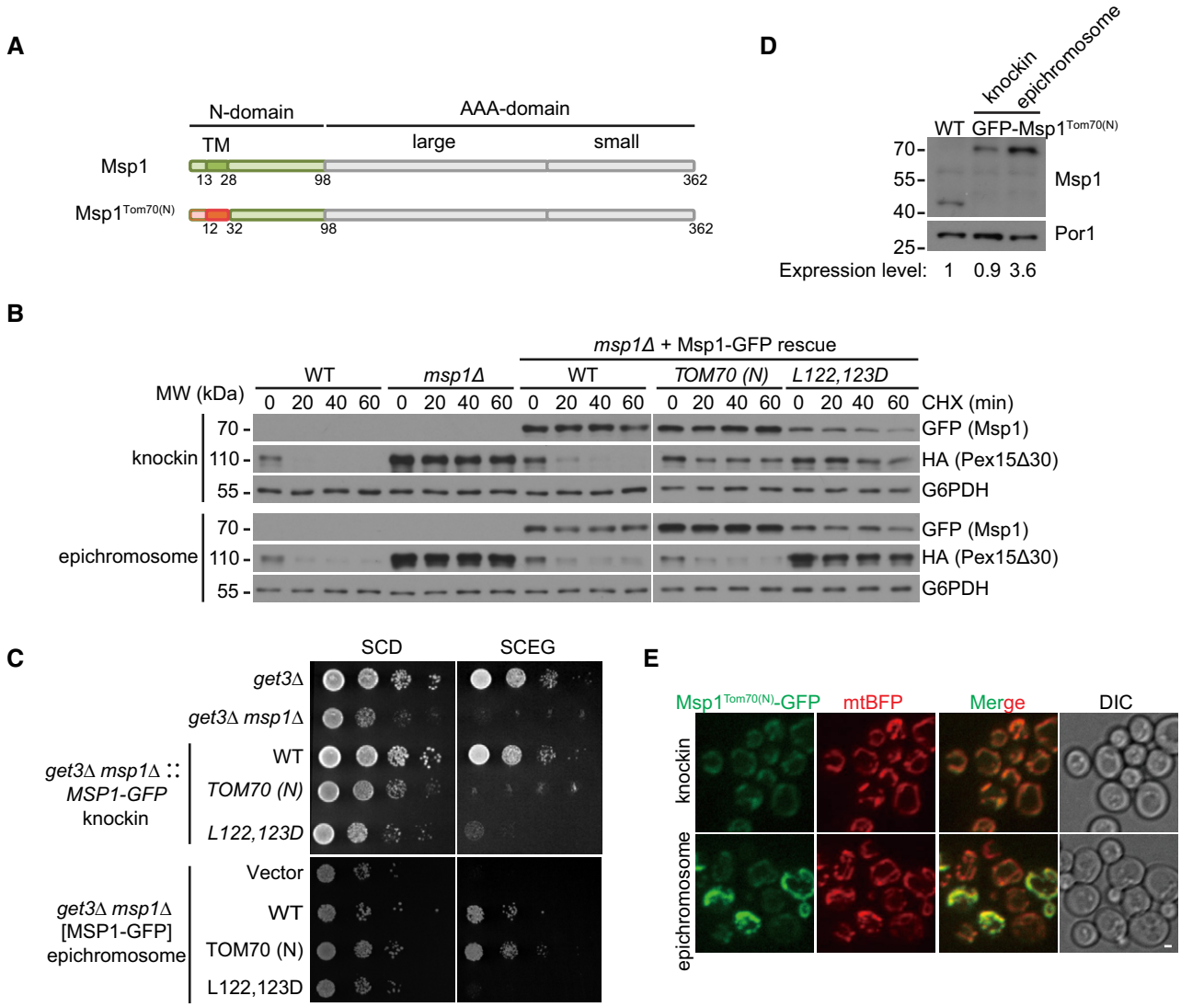

Figure EV1. N-domain is essential for Msp1 function under endogenous expression level (associated with Fig 1).

A Domain structure of Msp1 and Msp1$^{Tom70(N)}$. Msp1$^{Tom70(N)}$ was constructed by replacing the first 32 amino acids of Msp1 with that of Tom70.

B Msp1$^{Tom70(N)}$-GFP, when expressed at endogenous level by the knockin method, failed to restore mRFP-HA-Pex15Δ30 degradation. mRFP-6xHA-tagged Pex15Δ30 was expressed from a centromeric plasmid under the control of *TEF1* promoter. The gene cassettes expressing WT or mutant forms of Msp1 under the control of *MSP1* endogenous promoter were either integrated at the endogenous locus (knockin) or cloned into a centromeric plasmid (epichromosome). The indicated strains were grown in synthetic glucose media to log phase and then treated with CHX and collected at the indicated time points.

C Msp1$^{Tom70(N)}$-GFP, when expressed at endogenous level by the knockin method, failed to rescue the growth defect of *get3Δ msp1Δ* cells. The indicated strains constructed similarly as in (B) were grown in glucose media to log phase and then spotted on SCD or SCEG plates in a 10-fold serial dilution, and then incubated for 2–5 days.

D The relative protein level of chromosomally and epichromosomally expressed Msp1$^{TOM70(N)}$-GFP. The intensity ratios of Msp1/Por1 were calculated using ImageJ software, and the value in WT was set to 1.

E Heterogeneous levels of Msp1$^{TOM70(N)}$-GFP expressed from a centromeric plasmid. Z projections and DIC images are shown. Scale bar represents 1 μm.

Figure EV2. The localization of Msp1 N-domain mutants and their effects on GFP-Pex15Δ30 degradation (associated with Fig 1).

A The normal localization of Msp1 N-domain mutants. Z projections and DIC images are shown. Mitochondrial Msp1-GFP colocalizes with mtBFP. Peroxisomal Msp1 appears as extra-mitochondrial dots. Scale bar represents 1 μm.

B Degradation of GFP-Pex15Δ30 in WT and Msp1 N-domain mutant cells. Mutants displaying defective degradation are highlighted in red.

C Degradation of GFP-Pex15Δ30 in untagged Msp1 N-domain mutant cells. *msp1^{E193Q}-FLAG* strain is used as a control for Msp1 loss of function.

Data information: In this figure, WT and mutant forms of Msp1-GFP and Msp1-FLAG were expressed from the endogenous chromosomal locus, and GFP-Pex15Δ30 was expressed from a centromeric plasmid under the control of *TEF1* promoter.

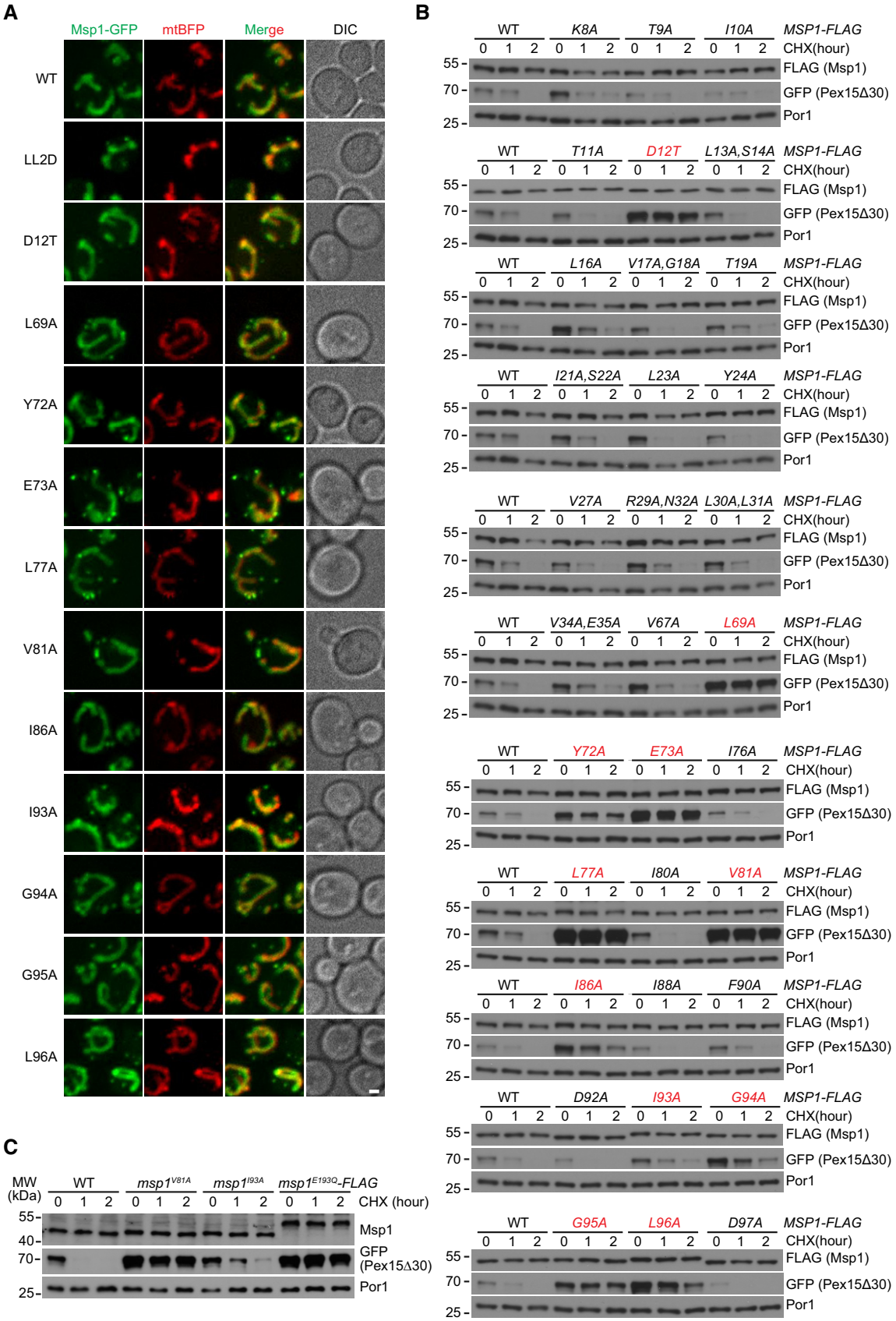

Figure EV2.

Figure EV3. Localization of WT or mutant forms of GFP-Pex15Δ30, GFP-Gem1, and GFP-Fis1 (associated with Figs 3 and 4).

A Localization of GFP-Pex15Δ30 carrying the indicated Outer mitochondrial membrane Targeting Sequence (OTS). Mutations in the IMS tail are highlighted in red.
B Localization of GFP-Pex15Δ30 carrying indicated OTS in *msp1Δ* cells.
C Localization of GFP-Pex15Δ30 truncation mutants in $msp1^{E193Q}$ cells.
D Localization of GFP-Pex15Δ30 hydrophobic patch mutants in $msp1^{E193Q}$ cells.
E Localization of GFP-Gem1 and its insertion mutants in $msp1^{E193Q}$ cells.
F Localization of GFP-Fis1 and its insertion mutants in $msp1^{E193Q}$ cells.

Data information: In this figure, GFP-tagged TA proteins and their mutants were expressed from a centromeric plasmid under the control of *TEF1* promoter. Z projections and DIC images are shown. Scale bars represent 1 μm.

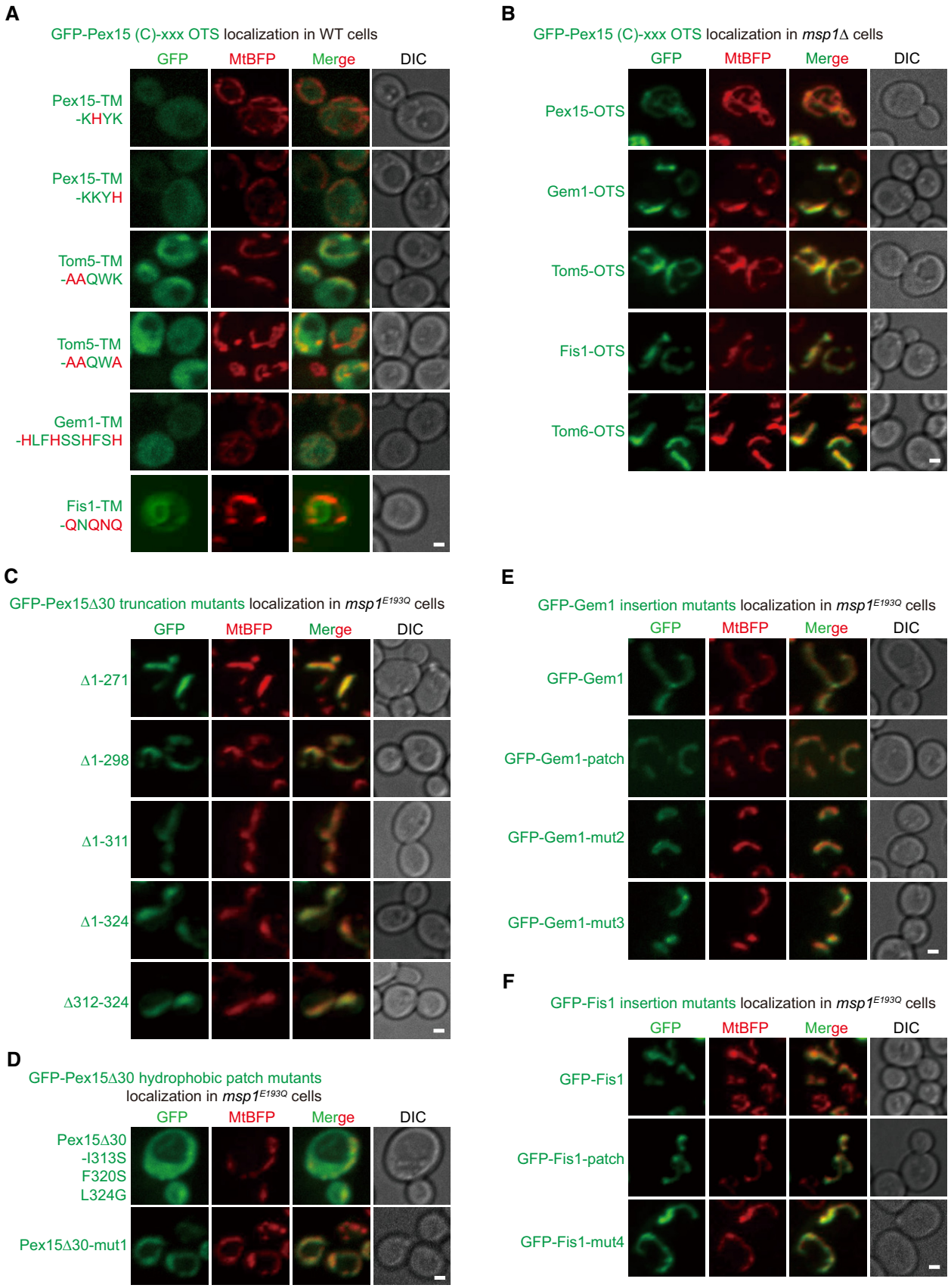

Figure EV3.

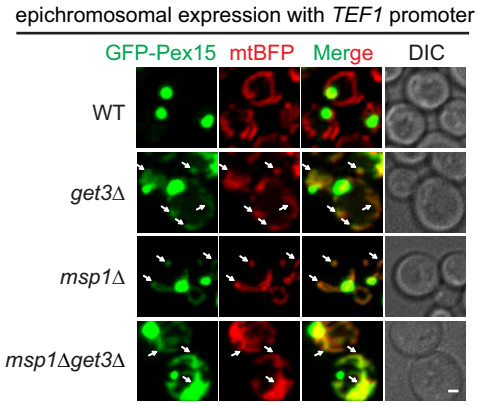

Figure EV4. Localization of GFP-Pex15 overexpressed by the *TEF1* promoter in the indicated strains (associated with Fig 5).
Arrows point to mitochondrial mislocalization of GFP-Pex15. Scale bar represents 1 μm.

Figure EV5. Imaging analysis of non-mitochondrial TA proteins in *msp1Δ get3Δ* cells (associated with Fig 6).

A–C GFP-tagged TA proteins were expressed from a centromeric plasmid under the control of *TEF1* promoter in *msp1Δ get3Δ* cells. (A) TA proteins with non-mitochondrial localization. (B) TA proteins with undetectable GFP signals. (C) TA proteins with mitochondrial localization. Z projections and DIC images are shown. Scale bars represent 1 μm.

D Scatter plots depicting tail charge (*y*-axis) and TM GRAVY (*x*-axis) for each non-mitochondrial TA protein in yeast. Their mitochondrial mislocalization in *msp1Δget3Δ* cells is color-encoded: Gray means no mitochondrial mislocalization; green indicates mitochondrial mislocalization when epichromosomally overexpressed; red indicates mitochondrial mislocalization under both over- and physiological expression levels. Proteins in the bracelets are non-mislocalized proteins having overlapping scores with mislocalized ones.

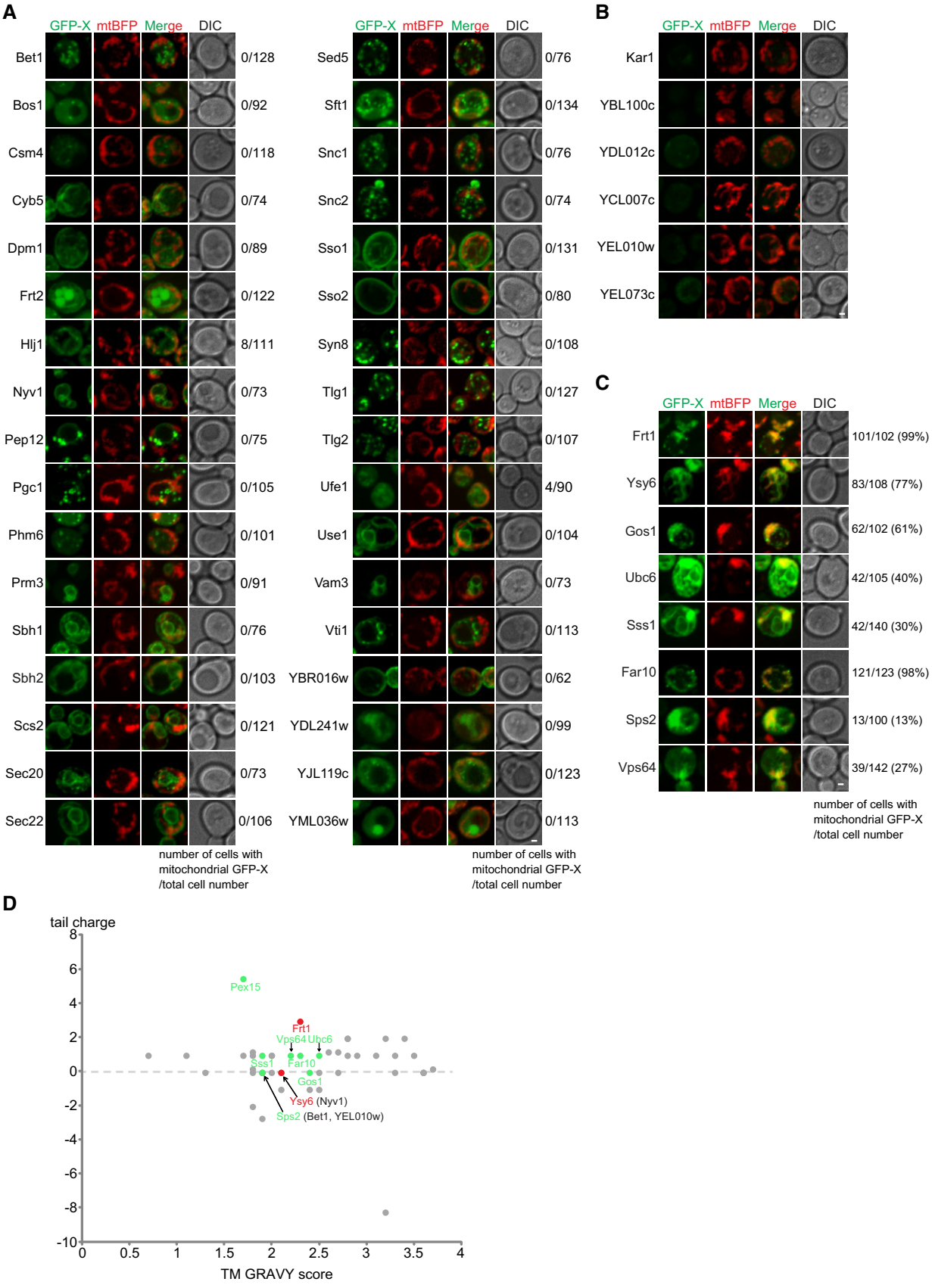

Figure EV5.