

## **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<sup>Tom70(N)</sup>. Msp1<sup>Tom70(N)</sup> was constructed by replacing the first 32 amino acids of Msp1 with that of Tom70.
- B Msp1<sup>Tom70(N)</sup>-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<sup>Tom70(N)</sup>-GFP, when expressed at endogenous level by the knockin method, failed to rescue the growth defect of *get3 msp1* 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<sup>TOM70(N)</sup>-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<sup>TOM70(N)</sup>-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-Pex15∆30 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  $\mu m.$
- 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<sup>E193Q</sup>-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.



55 <b>-</b>	V	VT 1	2	0	78A 1 2	0	<i>T9A</i> 1	2	0	<u>10A</u> 1 2	MSP1-FLAG CHX(hour) FLAG (Msp1)
70- 25-		-	_	-	-	-		_	_		GFP (Pex15∆30) Por1
55 - 70 - 25 -	V	VT 1	2		11A 1 2	0	<u>D127</u> 1	2	<u>L13</u> 0	A, S14A 1 2	MSP1-FLAG CHX(hour) FLAG (Msp1) GFP (Pex15∆30) Por1
55 - 70 -		VT 1	2	0	16A 1 2	<u>V1</u> 0	7 <i>A,G<sup>.</sup></i> 1	18A 2	0	7 <u>19A</u> 1 2	MSP1-FLAG CHX(hour) FLAG (Msp1) GFP (Pex15∆30)
25 - 55 - 70 -		<u>VT</u>	2	<u>1214</u> 0	12	0	<i>L23A</i> 1	2	) 0	<u>′24A</u> 12	MSP1-FLAG CHX(hour) FLAG (Msp1) GFP (Pex15Δ30)
25 - 55 - 70 -		VT 1	2	<u>V:</u> 0	27A 1 2	. <u>R2</u> : 0	9 <i>A,N</i> 3 1	<u>32A</u> 2	<u>L30</u> 0	A,L31A 1 2	MSP1-FLAG CHX(hour) FLAG (Msp1) GFP (Pex15Δ30) Por1
25 - 55 - 70 - 25 -		VT 1	2	<u>V34</u> /	1, <i>E35A</i> 12	0	<u>V67A</u> 1	2	0	<u>.69</u> 1 2	MSP1-FLAG CHX(hour) FLAG (Msp1) GFP (Pex15∆30) Por1
55 - 70 -	0	VT 1	2	<u>у</u> 0	72A 1 2	0	E734	2	0	176A 12	MSP1-FLAG CHX(hour) FLAG (Msp1) GFP (Pex15∆30) Por1
55 - 70 -		VT 1	2		<mark>77A</mark> 1 2	0	<i>180A</i> 1	2	0	V81A 1 2	<i>MSP1-FLAG</i> CHX(hour) FLAG (Msp1) GFP (Pex15Δ30) Por1
25 - 55 - 70 -		VT 1	2		86A 1 2	0	<i>1884</i> 1	2	0	F90A 1 2	<i>MSP1-FLAG</i> CHX(hour) FLAG (Msp1) GFP (Pex15Δ30) Por1
55 - 70 - 25 -		VT 1	2		092A 1 2	0	193A	2	0	G94A 1 2	MSP1-FLAG CHX(hour) FLAG (Msp1) GFP (Pex15Δ30) Por1
55 - 70 - 25 -	0	WT 1	2	0	95A 1 2	0	<u>L96</u> 1	2	0	D97A 1 2	MSP1-FLAG CHX(hour) FLAG (Msp1) GFP (Pex15∆30) Por1

MW	WT			n							)1 <sup>E19.</sup>	<sup>3Q</sup> -FI	AG
(kDa)	0	1	2	0	1	2	0	1	2	0	1	2	CHX (hour)
55- 40	-	-		-	-	-	-	-	-	-	-	-	Msp1
40- 70-	-			-	-	-	-	-	-	-	-	-	GFP (Pex15∆30)
25-	-	-	-	-	-	-		-	-	-	-	-	Por1

Figure EV2.

### Figure EV3. Localization of WT or mutant forms of GFP-Pex15A30, GFP-Gem1, and GFP-Fis1 (associated with Figs 3 and 4).

- A Localization of GFP-Pex15 $\Delta$ 30 carrying the indicated Outer mitochondrial membrane Targeting Sequence (OTS). Mutations in the IMS tail are highlighted in red.
- B Localization of GFP-Pex15 $\Delta$ 30 carrying indicated OTS in *msp1* $\Delta$  cells.
- C Localization of GFP-Pex15 $\Delta$ 30 truncation mutants in msp1<sup>E193Q</sup> cells.
- D Localization of GFP-Pex15 $\Delta$ 30 hydrophobic patch mutants in *msp1*<sup>E193Q</sup> cells.
- E Localization of GFP-Gem1 and its insertion mutants in *msp1*<sup>E193Q</sup> cells.
- F Localization of GFP-Fis1 and its insertion mutants in *msp1<sup>E193Q</sup>* 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.



## С

GFP-Pex15∆30 truncation mutants localization in *msp1*<sup>E193Q</sup> cells



D





Figure EV3.

GFP-Pex15 (C)-xxx OTS localization in msp1Δ cellsGFPMtBFPMergeDICPex15-OTSImageImageImageGem1-OTSImageImageImageImageTom5-OTSImageImageImageImageFis1-OTSImageImageImageImageTom6-OTSImageImageImageImageTom6-OTSImageImageImageImage

Е

В

GFP-Gem1 insertion mutants localization in *msp1*<sup>£1930</sup> cells GFP MtBFP Merge DIC

GFP-Gem1	P	2	2	8
GFP-Gem1-patch	Ç-C	2.0	2.0	Óo
GFP-Gem1-mut2	66.	5 7 3	53.5	00
GFP-Gem1-mut3	2	5.0	3. 6	8

F

GFP-Fis1 insertion mutants localization in msp1E193Q cells

	GFP	MtBFP	Merge	DIC
GFP-Fis1	2.	( 1 0 V	10	000
GFP-Fis1-patch	50.0	ż.	s. S	2
GFP-Fis1-mut4	50	5	33	P

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epichromosomal expression with TEF1 promoter

**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 msp1A get3A 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 nonmitochondrial 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 *msp1Aget3A* 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.



