RESEARCH ARTICLE

Figures and figure supplements

A nuclear-based quality control pathway for non-imported mitochondrial proteins

Viplendra PS Shakya *et al*

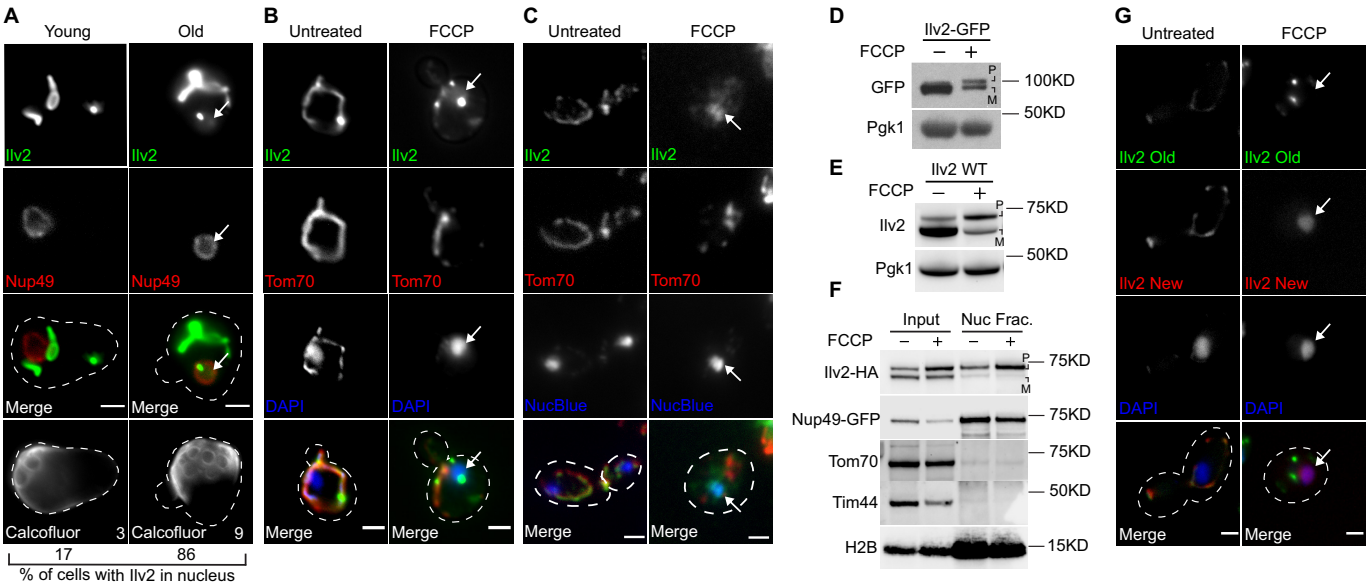

Figure 1. Non-imported mitochondrial protein Ilv2 localizes to the nucleus upon import failure. (**A**) Representative images of old and young yeast expressing the indicated Ilv2-GFP and nuclear marker Nup49-mCherry. Percentage of cells with Ilv2 in the nucleus (n = 30 cells) and age of representative cell (determined by bud scar counting) are indicated in bottom panels. Bud scars stained with calcofluor. (**B**) Yeast expressing Ilv2-GFP and Tom70-mCherry ± FCCP. (**C**) Indirect immunofluorescence for endogenous Ilv2 of yeast expressing Tom70-mCherry. (**D**) Western blots of yeast expressing Ilv2-GFP ± FCCP. (**E**) Western blots of endogenous Ilv2 in yeast treated ± FCCP. Pgk1 = loading control in all instances. (**F**) Western blot showing enrichment of the precursor form of Ilv2-HA in the nuclear fraction. Nup49-GFP and H2B = nuclear markers, Tom70 and Tim44 = mitochondrial markers. (**G**) RITE-tagged cells treated with β-estradiol at time of FCCP addition to initiate Cre/lox switching of Ilv2 epitope tag from GFP (old) to RFP (new). (**B**, **C**, and **G**) Nucleus stained with DAPI. P = precursor, M = mature. All scale bars = 2 μm. Arrows denote nucleus.

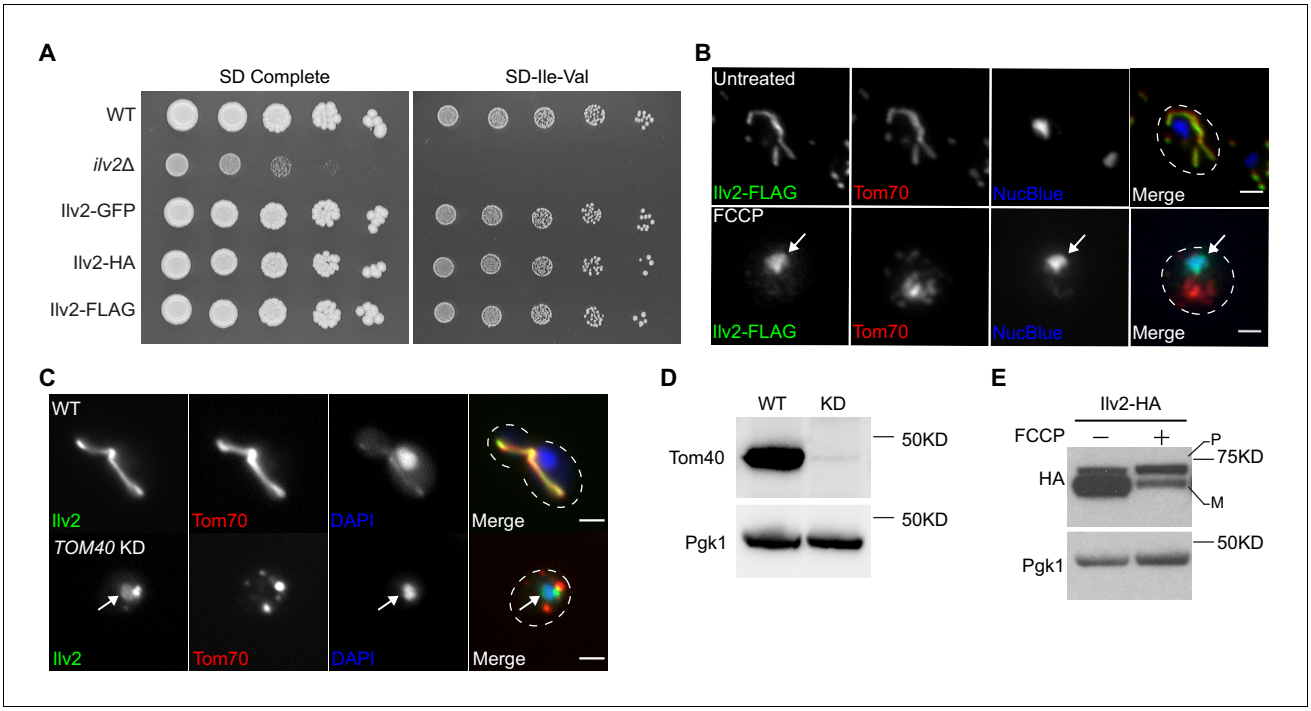

Figure 1—figure supplement 1. Mitochondrial protein Ilv2 localizes to the nucleus upon import failure. (**A**) Fivefold serial dilutions of WT, Ilv2 KO, and endogenous C-terminally GFP, HA, or FLAG-tagged Ilv2 expressing yeast strains on SD complete or isoleucine and valine dropout agar plates. (**B**) Indirect immunofluorescence of yeast expressing Ilv2-FLAG and Tom70-mCherry. Nucleus in (**B**, **C**) stained with NucBlue and DAPI, respectively. Arrows indicate nucleus. (**C**) Tom70-mCherry wild type (WT) and *tet$_p$-TOM40* (*TOM40 KD*) yeast expressing Ilv2-GFP in the presence of doxycycline. (**D**) Western blot for Tom40 in wild-type (WT) and *tet$_p$-TOM40* (*KD*) strains in the presence of doxycycline. (**E**) Western blot of yeast expressing Ilv2-HA ± FCCP. P = precursor and M = mature. Scale bars = 2 μm.

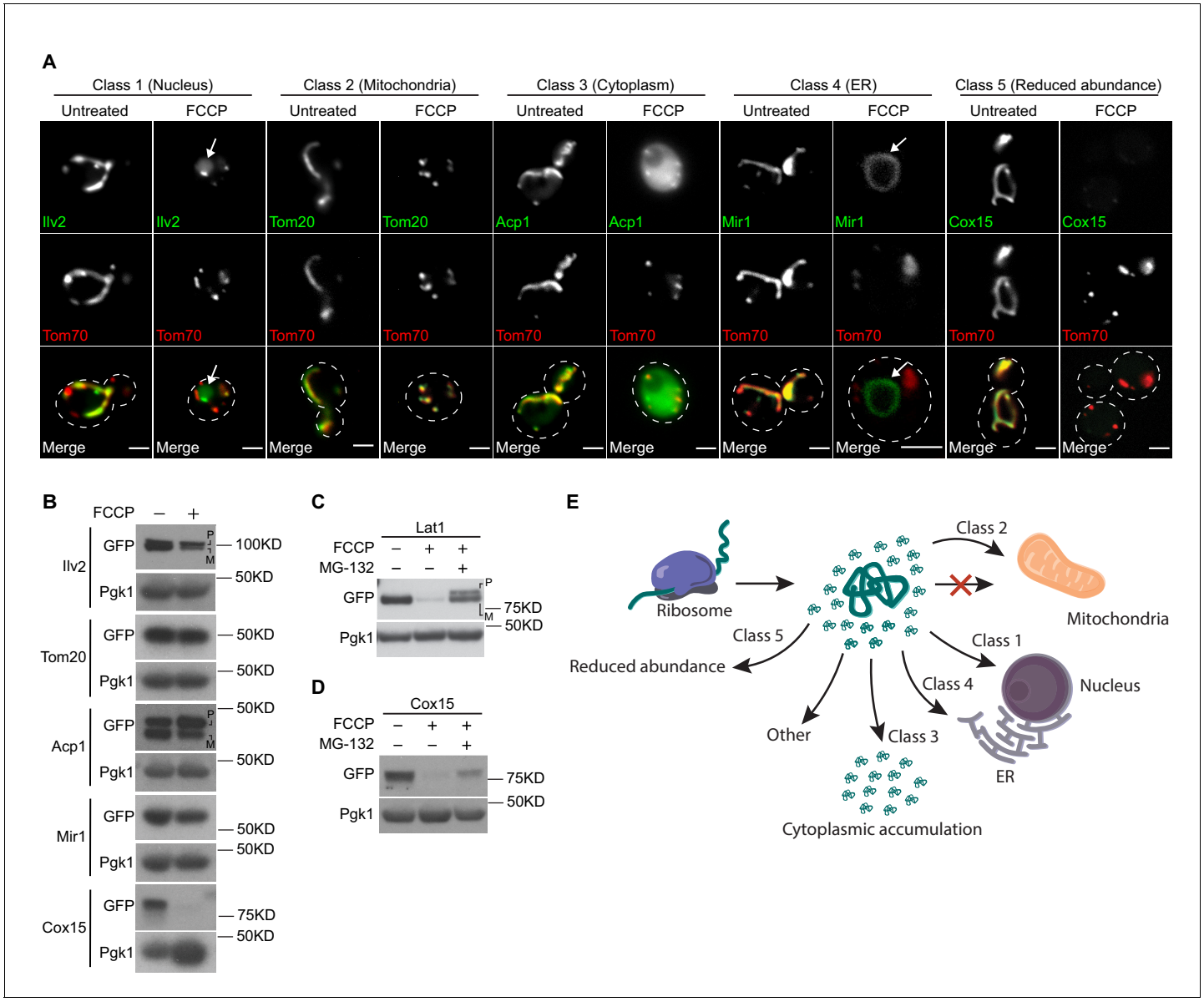

Figure 2. The nucleus in one of several fates for non-imported mitochondrial proteins. (**A**) Yeast expressing the indicated mCherry or GFP-tagged mitochondrial proteins ± FCCP. (**B–D**) Western blots of yeast expressing the indicated GFP-tagged mitochondrial proteins ± FCCP or ± FCCP ± MG-132. (**E**) Summary of non-imported mitochondrial protein fates. All scale bars = 2 μm. Arrows denote nucleus (**A**, class 1) or ER (**A**, class 4). P = precursor and M = mature.

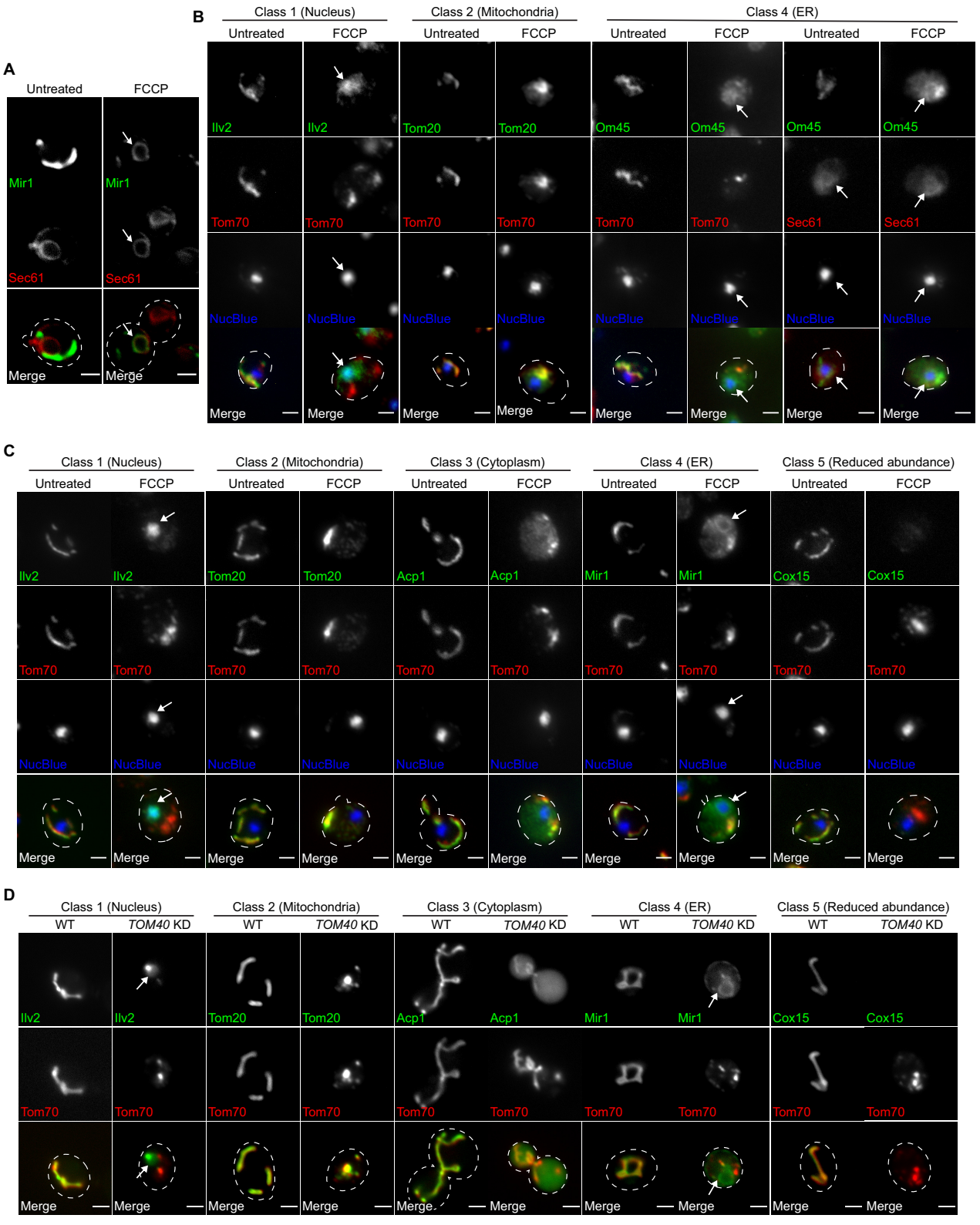

Figure 2—figure supplement 1. Non-imported mitochondrial proteins have several distinct fates. (**A**) Yeast expressing ER marker Sec61-mCherry and Mir1-GFP ± FCCP. (**B**) Indirect immunofluorescence of Ilv2, Tom20, and OM45 in yeast expressing Tom70-mCherry or Sec61-mCherry. (**C**) Indirect

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immunofluorescence of yeast expressing the indicated FLAG-tagged proteins and Tom70-mCherry. (**D**) Tom70-mCherry wild-type (WT) and tet_p-*TOM40* (*TOM40 KD*) yeast expressing the indicated GFP-tagged mitochondrial proteins in the presence of doxycycline. All bars = 2 μm. Nucleus in (**B**, **C**) stained with NucBlue. Arrows indicate nucleus (**B–D**, class 1) or ER (**A–D**, class 4).

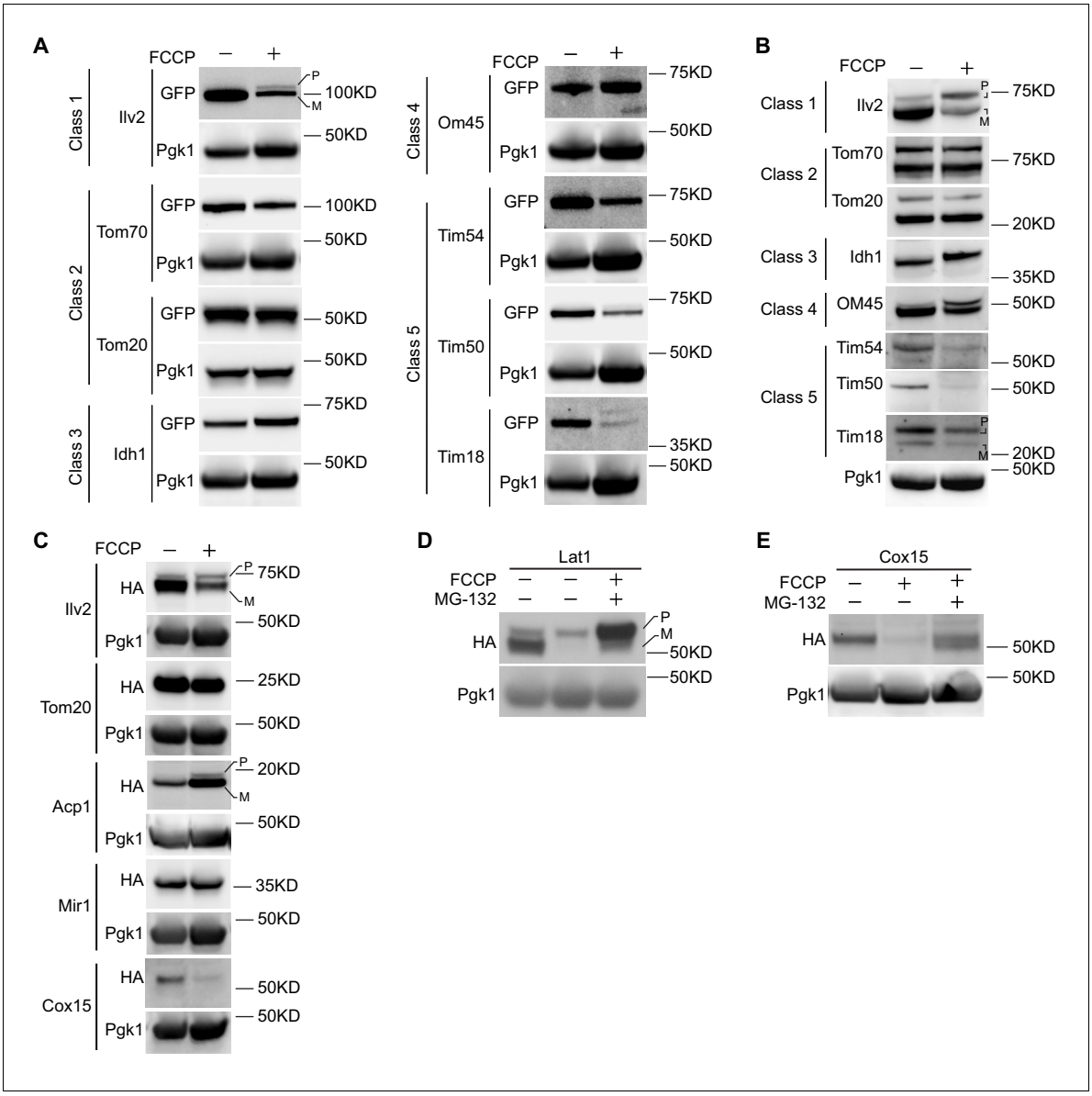

Figure 2—figure supplement 2. Non-imported mitochondrial proteins have numerous fates. (**A**) Western blots of yeast expressing the indicated GFP-tagged proteins ± FCCP. (**B**) Western blots against indicated native protein in yeast ± FCCP. (**C–E**) Western blots of yeast expressing the indicated HA-tagged mitochondrial proteins ± FCCP (**C**) or ± FCCP ± MG-132 (**D, E**). P = precursor form, M = mature form. Pgk1 = loading control.

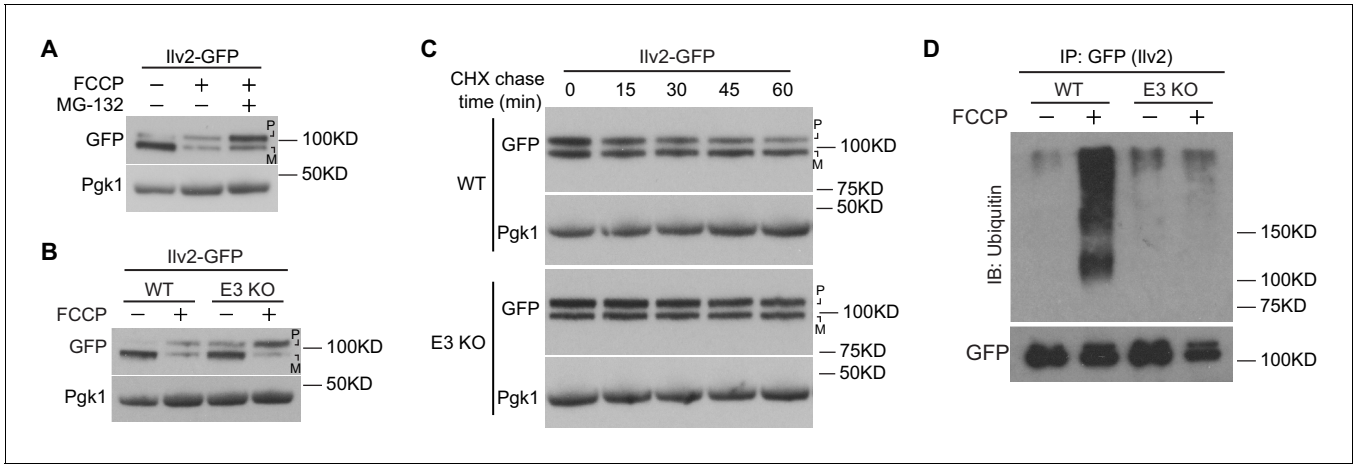

Figure 3. Nuclear protein quality control degrades non-imported mitochondrial proteins. (**A**) Western blot of yeast expressing Ilv2-GFP ± FCCP ± MG-132. (**B**) Western blot of yeast expressing Ilv2-GFP ± FCCP in wild-type (WT) and E3 KO strains. (**C**) Western blots showing cycloheximide (CHX) chase of Ilv2-GFP in WT and E3 KO strains in the presence of FCCP. (**D**) Western blot showing ubiquitylation of immunoprecipitated Ilv2-GFP ± FCCP in WT and E3 KO strains. Pgk1 = loading control. E3 KO = *san1*Δ *ubr1*Δ *doa10*Δ. P = precursor and M = mature.

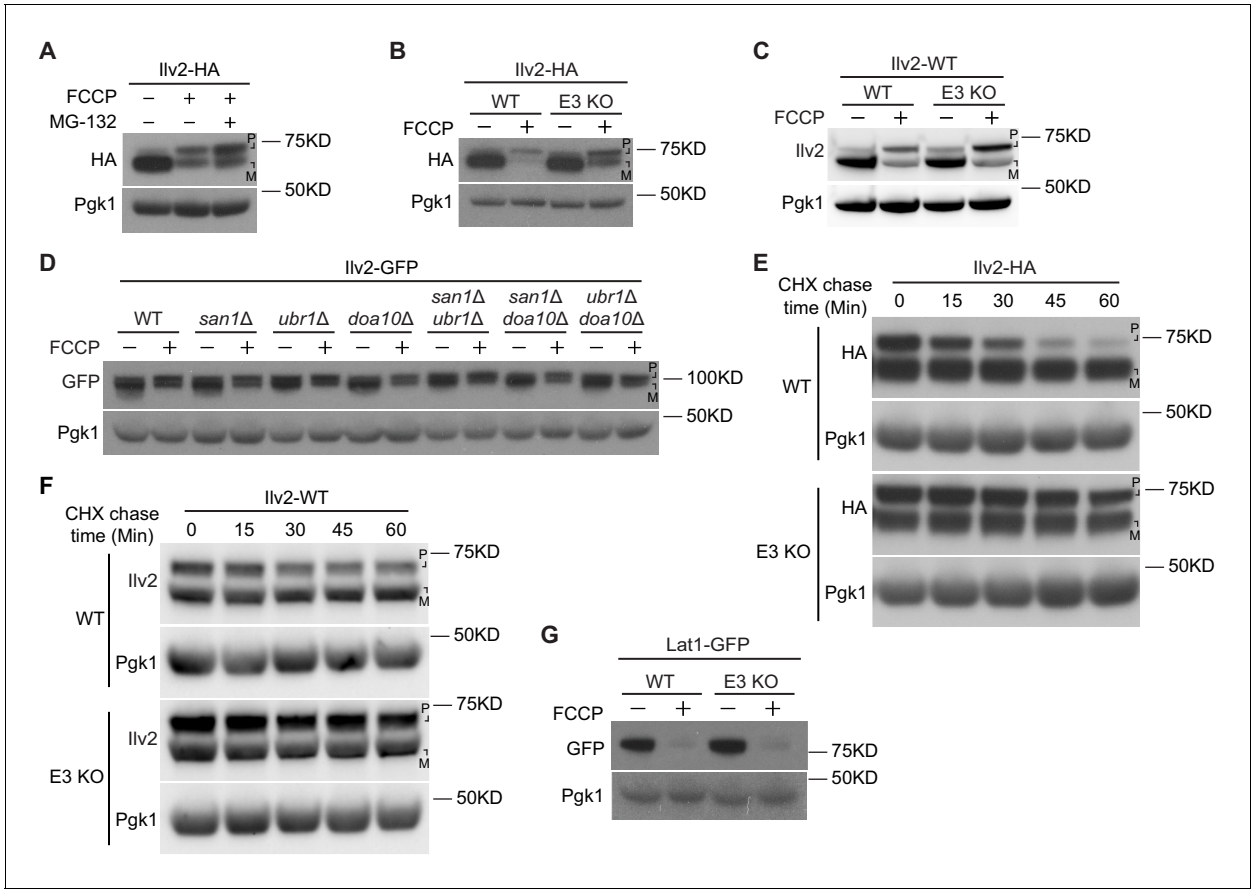

Figure 3—figure supplement 1. Nuclear protein quality control promotes non-imported mitochondrial protein degradation. (**A**) Western blot of yeast expressing Ilv2-HA ± FCCP ± MG-132. (**B, C**) Western blots of yeast expressing the Ilv2-HA (**B**) or endogenous Ilv2 (**C**) ± FCCP in WT and E3 KO strains. (**D**) Western blots of yeast expressing Ilv2-GFP ± FCCP in WT and the indicated mutant yeast strains. (**E, F**) Western blots showing the CHX chase of Ilv2-HA (**E**) or endogenous Ilv2 (**F**) in WT and E3 KO strains in the presence of FCCP. (**G**) Western blots of yeast expressing the Lat1-GFP ± FCCP in WT and E3 KO strains. P = precursor form, M = mature form. Pgk1 = loading control.

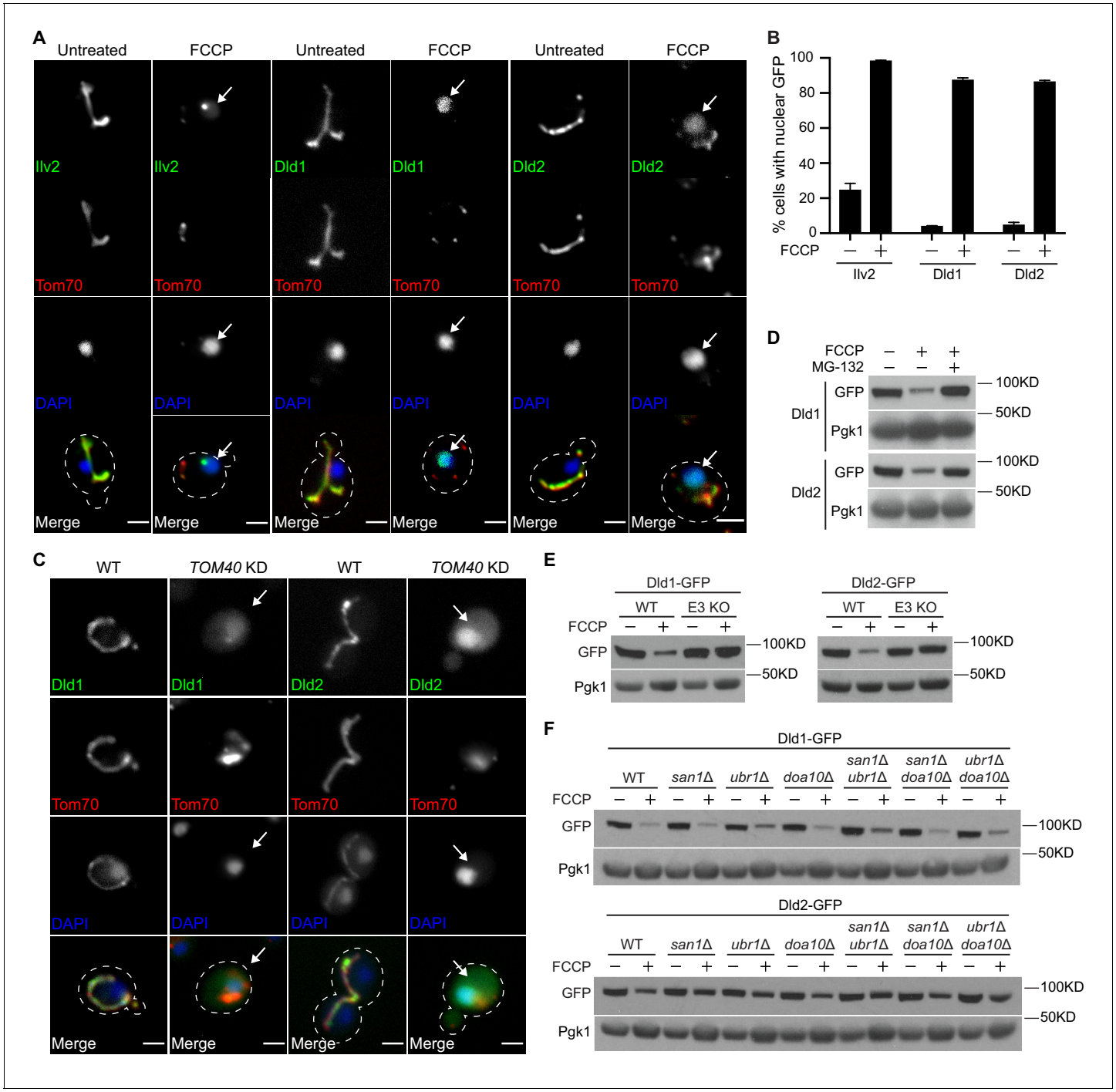

Figure 3—figure supplement 2. Nuclear protein quality control promotes non-imported mitochondrial protein degradation. (**A**) Yeast expressing the indicated GFP and mCherry tagged mitochondrial proteins ± FCCP. (**B**) Quantification of (**A**). N > 99 cells per replicate, error bars = SEM of three replicates. (**C**) Tom70-mCherry wild-type (WT) and *tet$_p$-TOM40* (*TOM40 KD*) yeast expressing the indicated GFP-tagged mitochondrial proteins in the presence of doxycycline. (**A, C**) Nucleus stained with DAPI, Arrows = nucleus. Bar = 2 µm. (**D**) Western blots of yeast strains expressing indicated GFP-tagged mitochondrial proteins ± FCCP ± MG-132. (**E**) Western blots of yeast expressing the indicated GFP-tagged mitochondrial proteins ± FCCP in WT and E3 KO strains. (**F**) Western blot of yeast expressing indicated GFP-tagged mitochondrial proteins ± FCCP in WT and the indicated mutant yeast strains. Pgk1 = loading control. E3 KO = san1Δ ubr1Δ doa10Δ. P = precursor and M = mature.

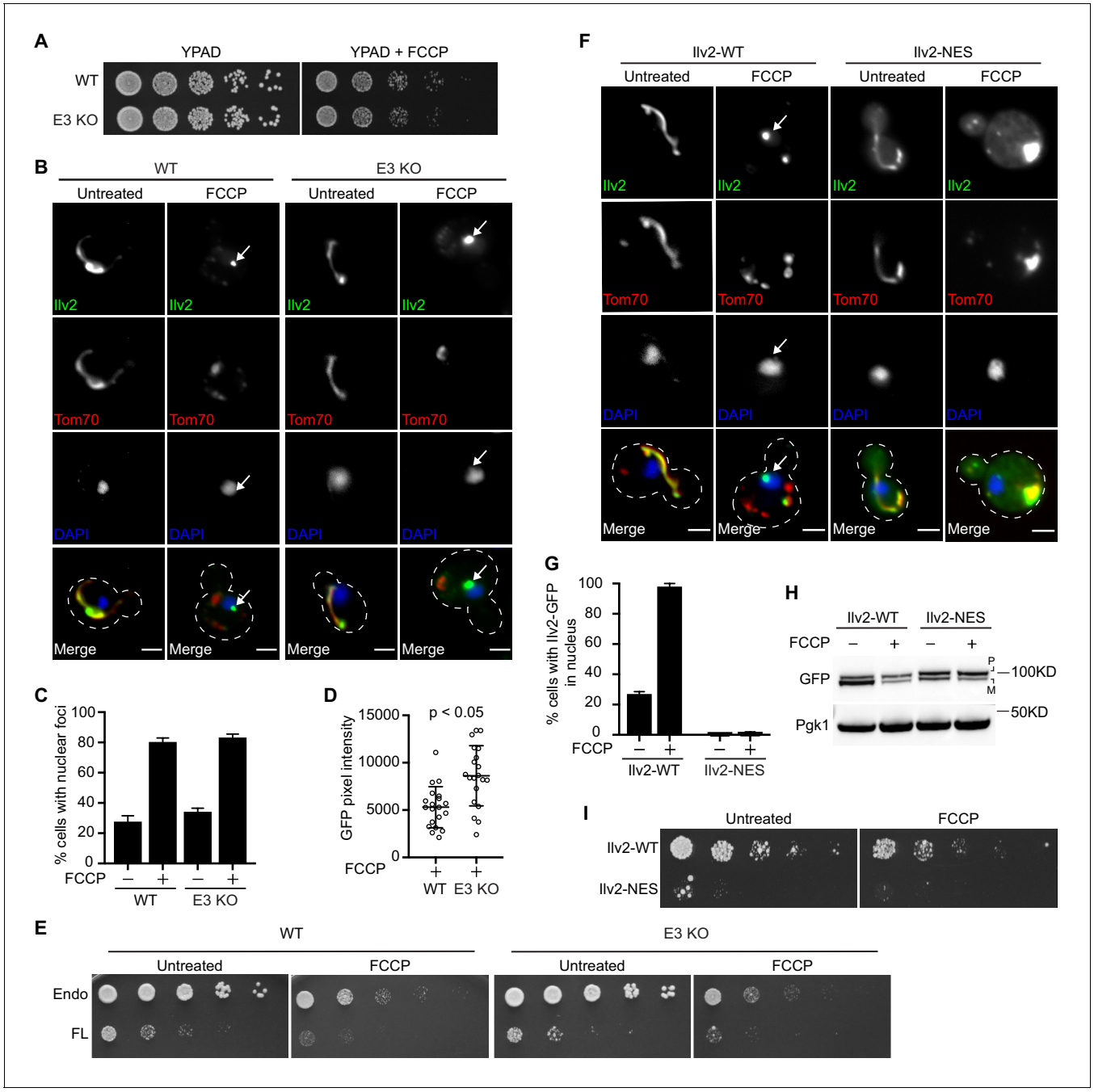

Figure 4. Non-imported mitochondrial precursors are toxic and localize to nuclear-associated foci when clearance is impaired. (**A**) Fivefold serial dilutions of WT and E3 KO strains on YPAD ± FCCP agar plates. (**B**) WT and E3 KO yeast expressing Ilv2-GFP and Tom70-mCherry ± FCCP. Nucleus stained with DAPI. Arrows = nuclear associated foci. Bar = 2 µm. (**C**) Quantification of (**B**). N > 99 cells per replicate, error bars = SEM of three replicates. (**D**) Quantification of average pixel intensity of Ilv2-GFP nuclear foci from (**B**). N = 20 cells, error bars = SD, p-value=0.0005. (**E**) Fivefold serial dilutions of WT and E3 KO strains expressing endogenous Ilv2-GFP (endo) ± mild overexpression of full-length Ilv2-GFP (FL) from pRS413-Ilv2-GFP on SD-His ± FCCP agar plates. (**F**) Yeast strain expressing WT Ilv2-GFP or Ilv2-NES-GFP and Tom70-mCherry ± FCCP. Nucleus stained with DAPI. Arrows = nucleus. Bar = 2 µm. (**G**) Quantification of (**F**). (**H**) Western blot of yeast expressing Ilv2-GFP or Ilv2-NES-GFP ± FCCP. (**I**) Fivefold serial dilutions of endogenously tagged WT Ilv2-GFP and Ilv2-NES-GFP strains on YPAD ± FCCP agar plates. P = precursor and M = mature.

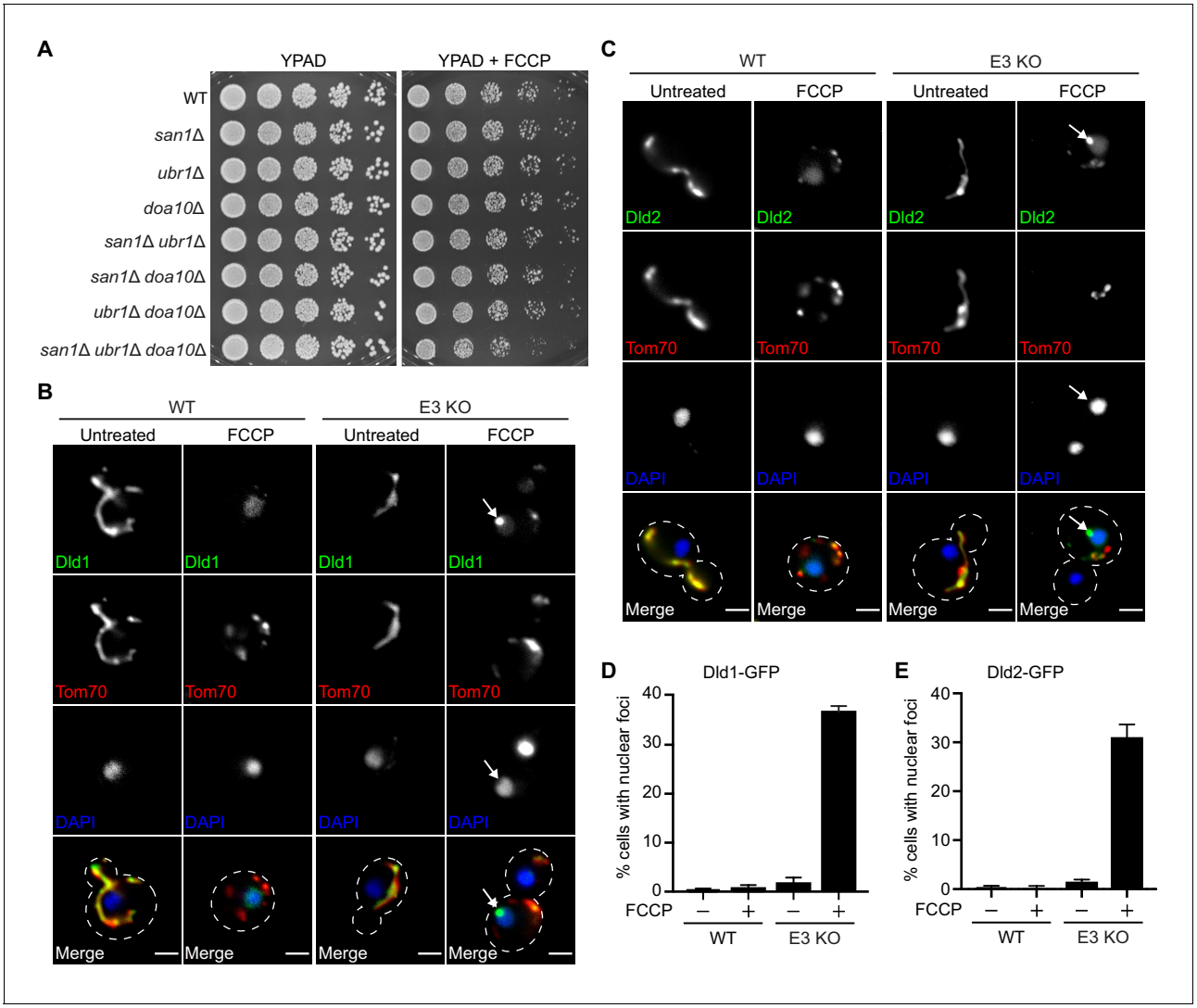

Figure 4—figure supplement 1. Impaired clearance of non-imported mitochondrial proteins targets them to nuclear-associated foci. (**A**) Fivefold serial dilutions of WT and the indicated mutant strains on YPAD ± FCCP agar plates. (**B, C**) WT and E3 KO (*san1Δ ubr1Δ doa10Δ*) yeast expressing Dld1-GFP or Dld2-GFP and Tom70-mCherry ± FCCP. Nucleus stained with DAPI, arrows = nuclear associated foci, and bar = 2 µm. (**D, E**) Quantification of (**B**) and (**C**), respectively. N > 99 cells per replicate, error bars = SEM of three replicates.

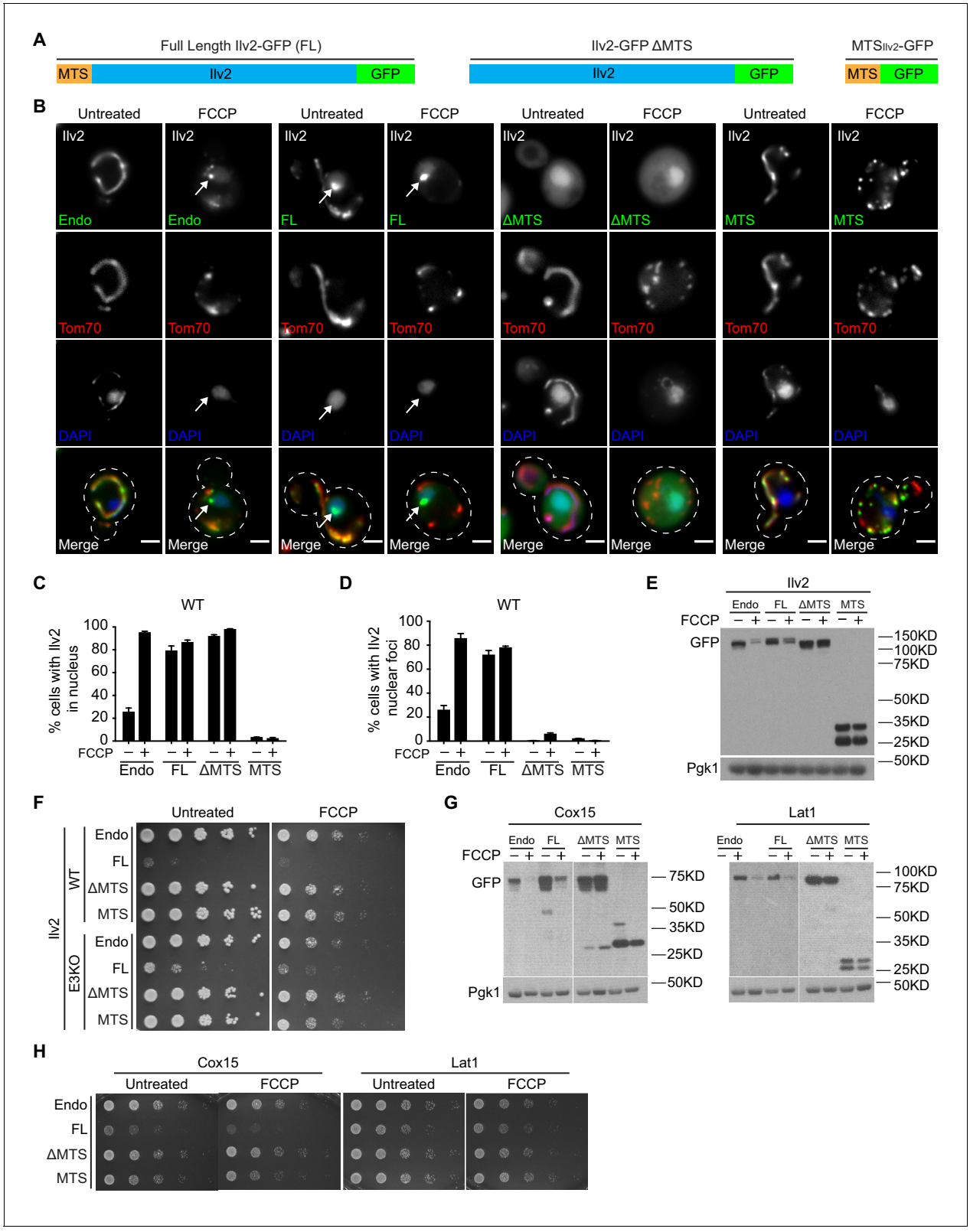

Figure 5. The mitochondrial targeting sequence (MTS) is required for non-imported precursor toxicity and quality control. (**A**) Schematic of full-length GFP-tagged Ilv2 (FL), mitochondrial targeting sequence deleted (ΔMTS) GFP-tagged Ilv2, and MTS_{Ilv2} GFP only (MTS). (**B**) Tom70-mCherry yeast expressing endogenous Ilv2-GFP ± the indicated Ilv2 variant ± FCCP. Nucleus stained with DAPI. Arrows = nuclear-associated foci. Bars = 2 µm. (**C, D**) Quantification of cells with diffuse Ilv2 nuclear localization (**C**) or Ilv2 nuclear foci (**D**) from (**B**). For (**C, D**), N > 99 cells per replicate, error bars = SEM of

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three replicates. (**E**) Western blot of strains expressing indicated Ilv2-GFP variants ± FCCP. Pgk1 = loading control. (**F**) Fivefold serial dilutions of WT and E3 KO strains expressing endogenous Ilv2-GFP (endo) ± mild overexpression of the indicated Ilv2-GFP variants on SD-His ± FCCP agar plates. (**G**) Western blot of strains expressing indicated Cox15-GFP or Lat1-GFP variants, respectively, ± FCCP. Pgk1 = loading control. (**H**) Fivefold serial dilutions of WT strains expressing endogenous Cox15-GFP or Lat1-GFP (endo) ± mild overexpression of the indicated variants on SD-His ± FCCP agar plates. P = precursor and M = mature.

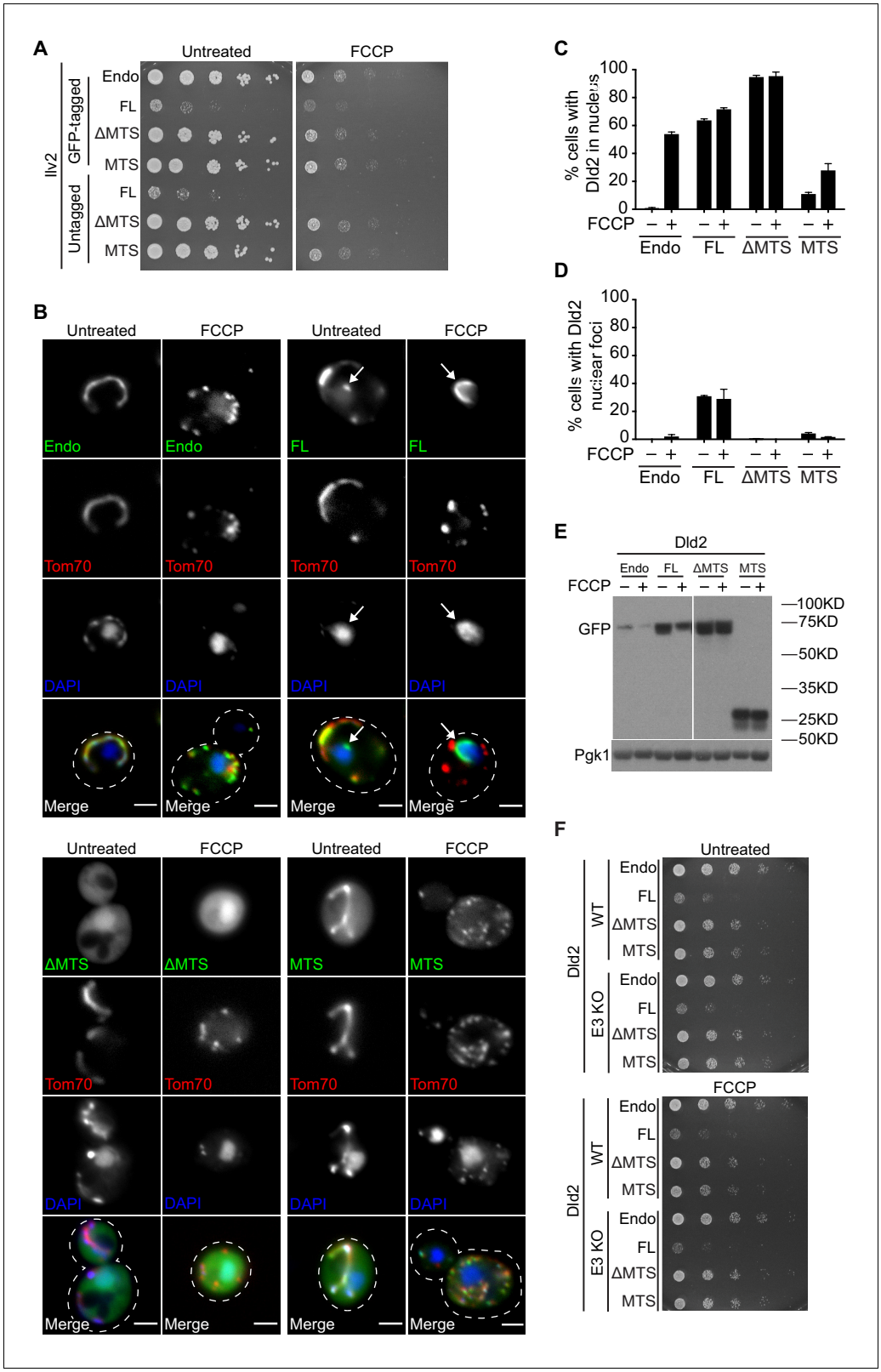

Figure 5—figure supplement 1. The MTS is required for non-imported precursor toxicity and degradation. (**A**) Fivefold serial dilutions of strains expressing endogenous Ilv2-GFP (endo) ± mild overexpression of the indicated Ilv2-GFP variants or untagged Ilv2 variants on SD-His ± FCCP agar plates. (**B**) Tom70-mCherry yeast expressing endogenous Dld2-GFP ± the indicated Dld2 plasmid-expressed variants ± FCCP. Nucleus stained with DAPI.

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Arrows = nuclear associated foci. Bars = 2 μm. (**C, D**) Quantification of cells with diffuse Dld2-GFP nuclear localization (**C**) or Dld2-GFP nuclear foci (**D**) from (**B**). For (**C and D**), N > 99 cells per replicate, error bars = SEM of three replicates. (**E**) Western blot of strains expressing indicated Dld2-GFP variants ± FCCP. Pgk1 = loading control. (**F**) Fivefold serial dilutions of WT and E3 KO strains expressing endogenous Dld2-GFP (endo) ± mild overexpression of the indicated Dld2-GFP variants on SD-His ± FCCP agar plates.

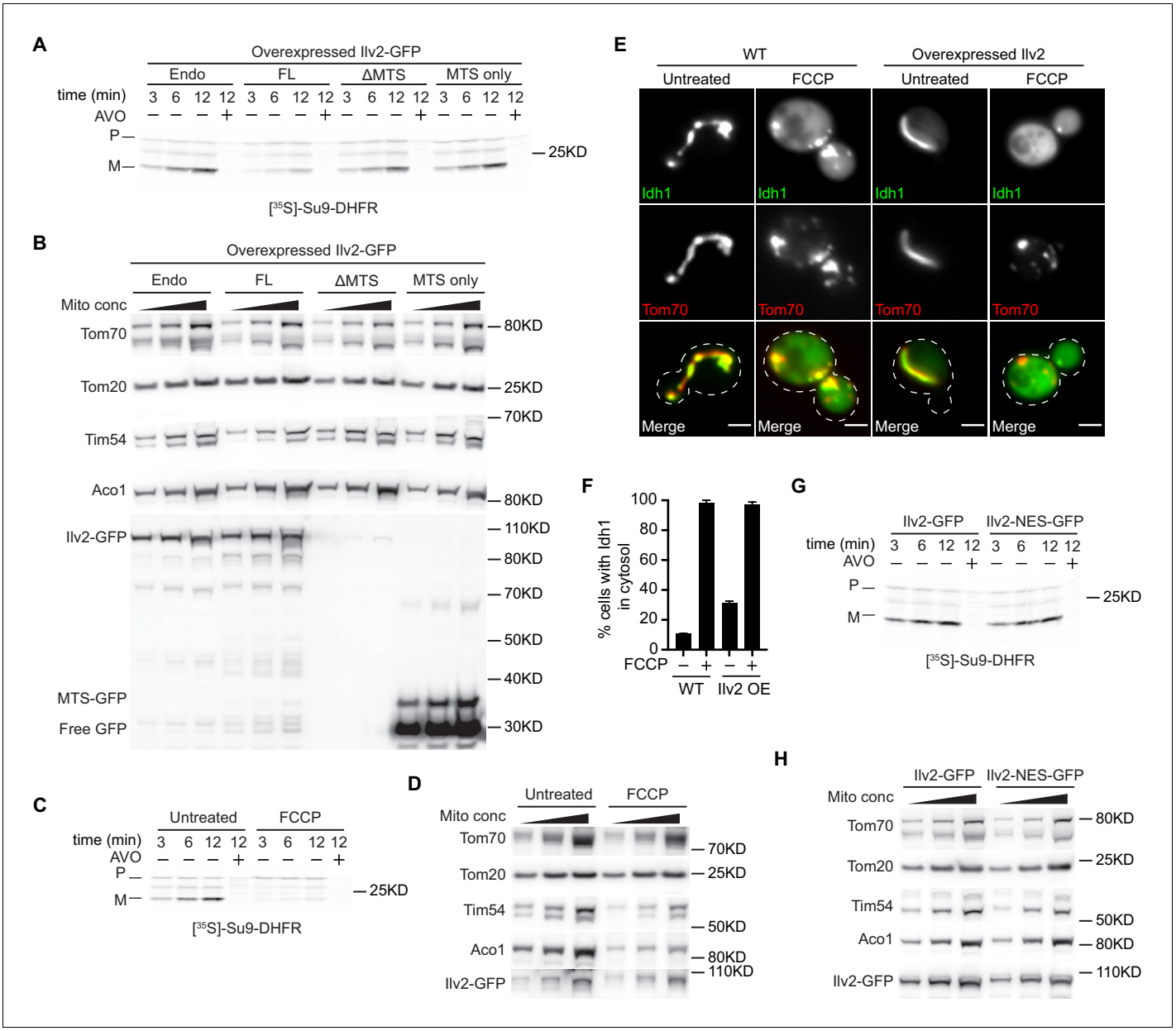

Figure 6. MTS-mediated toxicity of Ilv2 is not entirely linked to mitochondrial import clogging. (**A**) ^{35}S-labeled Su9-DHFR was imported into mitochondria isolated from yeast overexpressing the indicated Ilv2-GFP variants. (**B**) Western blot showing protein levels in mitochondria isolated from yeast overexpressing the indicated Ilv2-GFP variants. (**C**) ^{35}S-labeled Su9-DHFR was imported into mitochondria isolated from yeast expressing endogenous Ilv2-GFP ± FCCP. (**D**) Western blot showing protein levels in mitochondria isolated from yeast expressing endogenous Ilv2-GFP ± FCCP. (**E**) Yeast expressing Idh1-GFP and Tom70-mCherry in WT or Ilv2 (untagged) overexpressing strains ± FCCP. Bars = 2 µm. (**F**) Quantification of (**E**). (**G**) ^{35}S-labeled Su9-DHFR was imported into mitochondria isolated from yeast expressing endogenous Ilv2-GFP or Ilv2-NES-GFP ± FCCP. (**H**) Western blot showing protein levels in mitochondria isolated from yeast expressing endogenous Ilv2-GFP or Ilv2-NES-GFP. For (**A**), (**C**), and (**G**), a mixture of antimycin A, oligomycin, and valinomycin (AVO) was used to dissipate the membrane potential. Non-imported proteins were proteolytically removed with proteinase K and the import was analyzed by SDS–PAGE and autoradiography. P = precursor and M = mature.