SUPPLEMENTAL INFORMATION

Regulation of Mitochondrial Morphology through Proteolytic Cleavage of OPA1

Naotada Ishihara, Yuu Fujita, Toshihiko Oka, and Katsuyoshi Mihara

Supplemental Materials and Methods

Materials

Antibodies against HA (BabCO), FLAG (M2; Sigma Chemical Co.), mHsp70 (SPS-825; Stressgen), cytochrome c (7H8.2C12; BD Bioscience) and PARP fragments (Promega) were purchased from the companies indicated. Antibodies against Tim23 (Ishihara and Mihara, 1998), Tom22, Tom40 (Suzuki et al., 2000), and AIF (Otera et al., 2005) were described previously. Rabbit polyclonal antibodies against rat OPA1 were prepared using recombinant protein of the N-terminal half of rat OPA1 (1-500). Purified TEV protease (AcTEV protease; Invitrogen) was treated with Biobeads SM-2 (BioRad) to remove detergent before use. HeLa cells were treated with 20 μM actinomycin D (Sigma Chemical Co.) for induction of apoptosis.

Construction of mammalian expression plasmids

To obtain rat OPA1 cDNA, a λgt10 rat liver cDNA library (Mihara, 1990) was screened using the RT-PCR fragment of rat OPA1 as the probe. All rat OPA1 constructs were prepared by PCR, and the fragments were cloned into the p3xFLAG-CMV-14 vector (Sigma Chemical Co.) for expression of the C-terminal FLAG-tagged proteins. The cDNAs of human paraplegin and human AFG3L2 were amplified by PCR from the human kidney cDNA library (Clontech). The PCR fragments were subcloned into pcDNA3.1-HA or pRcCMV-HA for expression of C-terminal HA tagged protein. To determine the

membrane topology of OPA1, the recognition sequence for TEV protease (ENLYFQ-SR) was substituted with residues 170-200 of rat OPA1. The matrix targeting signal (1-69) of *N. crassa* F_0 ATPase subunit 9 (Eura et al., 2003) and the HA-tag were fused on the DNA level to 27-kDa protease subunit of TEV protease (Faber et al., 2001) at the N-terminus and C-terminus, respectively.

siRNA

For RNAi assay, siRNAs, comprising 19 nucleotides and a dTdT overhang at each 3'-terminus were chemically synthesized. For RNAi for paraplegin or PARL, a cocktail of three siRNAs was used. The target sequences for paraplegin were: 308-326: 5'-CCTCAAGGTTGAAGCAGAA-3', 740-758: 5'-GAACAGGATTCTTTGGAAA-3', and 825-843: 5'-CGGGATGACTGGAAGGGAA-3'. The target sequences for PARL were: 177-195: 5'-GGTTGAACCTCGAAGATCA-3', 203-221: 5'-GGACAAGTGGTGAAGCATA-3', and 1081-1099: 5'-GTGAAAATCTGGCATGAAA-3'. The target sequence of human AFG3L2 was: 1941-1959: 5'-AGATGACTTGAGAAAAGTA-3'. The target sequence of human OPA1 was: 1707-1728: 5'-CACGTTTTAACCTTGAAAC-3'.

Processing of OPA1 variants in yeast mutants

The cDNA of rat OPA1 was subcloned into yeast expression plasmid, and transformed to the wild-type (BY4741) or deletion strains; *Δyta10/afg3* (yer017c), *Δyta12/rca1* (ymr089c), *Δyme1* (ypr024w), *Δrbd1/pcp1/mdm37* (ygr101w), or *Δrbd2* (ypl246c). These yeast cells were cultured to early log phase (OD_{600} = ~1.0) in SD(-Trp), and the total cell lysates were analyzed by immunoblotting.

Mitochondrial import of preprotein and MPP processing

The reticurocyte lysate synthesized proteins were analyzed for *in vitro* mitochondrial import reaction, or MPP processing reaction using 50 μg/ml purified MPP, as reported previously (Otera et al., 2005).

Supplemental Figure Legends

Fig S1. Processing of OPA1 variant 7. OPA1(1-90)-DHFR and su9(1-69)-DHFR were synthesized in reticulocute lysates in the presence of ^{35}S-methionine. The translation products were subjected to mitochondrial import ("import"), or MPP (50μg/ml) processing ("MPP"), then analyzed by autoradiography. Note that the processed or imported mature forms of OPA1(1-90)-DHFR or su9(1-69)-DHFR had almost similar mobility as DHFR.

Fig S2. Intracellular localization and membrane topology of OPA1. (A) The total cell lysate (total), cytosol (cyt), and mitochondria (mt) were fractionated from HeLa cells expressing FLAG-tagged OPA1 variants. The mitochondria were treated with or without 50 μg/ml proteinase K (ProK) under the indicated conditions. TX: 0.5 % TritonX-100. The reaction mixtures were subjected to SDS-PAGE and subsequent immunoblotting using antibodies against FLAG or against the indicated mitochondrial marker proteins. Tom22, outer membrane marker; AIF, IMS marker; mHsp70, matrix marker. **(B)** Schematic representation of constructs used to analyze the N-terminal topology of the OPA1 variant 7. Residues 170-200 of FLAG-tagged OPA1 variant 7 was substituted with the TEV recognition sequence "(Δ170-200)-*tev*". HA-tagged 27 kDa protease subunit of TEV protease was fused with mitochondria matrix-targeting signal "mit-TEV-HA". **(C)** FLAG-tagged OPA1 variant 7 or (Δ170-200)-*tev* was co-transfected with or without mit-TEV-HA to HeLa cells. The mitochondrial fractions were analyzed by immunoblotting using anti-FLAG antibodies. **(D)** Isolated mitochondria from the HeLa cells expressing (Δ170-200)-*tev* were treated with 100 U/ml purified TEV protease or 20 μg/ml ProK in the absence or presence of 0.01% Triton X-100 (TX) on ice for 60 min. **(E)** Schematic drawing of OPA1 membrane topology.

Fig S3. Processing of OPA1 by induction of apoptosis. (A) HeLa cells were treated with or without 20 μM actinomycin D for the indicated time periods and analyzed by fluorescence microscopy as in Fig. 2A. Scale bar; 10 μm. **(B)** HeLa cells were treated with 20 μM actinomycin D for the indicated time periods, and endogenous OPA1, Tom40 as mitochondrial outer membrane marker, and the 85-kDa fragment of PARP as an apoptosis marker were detected by immunoblotting. **(C)** HeLa cells expressing OPA1-FLAG variants were treated with actinomycin D as in (B) and analyzed by immunoblotting using anti-FLAG antibodies.

Fig. S4. Exogenous expression of processing-defect mutants of OPA1 and their effects on mitochondrial morphology. (**A**) The indicated constructs were transfected to HeLa cells, and after 36h-culture, the cells were labeled with MitoTracker and subjected to immunofluorescence microscopy using anti-FLAG antibodies. Scale bar; 10 μm. (**B**) HeLa cells with filamentous-network, intermediate, or completely fragmented mitochondrial structures in (A) were counted. At least 100 cells were counted from three different optical fields.

Fig S5. Processing of OPA1 variants in yeast protease-deficient mutants. (A) Wild-type yeast cells harboring the expression plasmids for OPA1 variant1 or variant 7 were cultured in SD(-Trp), and the cell lysates were analyzed by immunoblotting using anti-OPA1 antibodies. The HeLa cell lysates expressing these variants were also analyzed as a reference. **(B)** Wild-type yeast cells (BY4741: WT) or mitochondrial protease-deficient yeast cells (*Δyta10*, *Δtya12*, *Δpcp1*, *Δrbd2*, or *Δyme1*) harboring the expression plasmid of OPA1 variants were cultured in SD(-Trp), and the cell lysates were analyzed by

immunoblotting using anti-OPA1 or anti-Mgm1 antibodies. **(C)** Wild-type (WT) or *Δyta10* yeast cells with or without the multicopy expression plasmid for YTA10 were transfected with expression plasmid for rat OPA1 variants, cultured in SD(-TrpLeu), and analyzed as in (B).

Fig S6. Effect of exogenously expressed m-AAA protease on OPA1 processing. (A) The expression vectors for the indicated proteases were co-transfected with OPA1 variant 1-FLAG into HeLa cells. These cells were labeled with ^{35}S-Met/Cys mix for 30 min, then chased for the indicated time periods. The cell lysates were subjected to immunoprecipitation using anti-FLAG antibodies as in Fig. 1D and E. **(B)** HeLa cells transfected with the plasmid harboring the indicated proteases were subjected to pulse-chase analysis with ^{35}S-Met/Cys mix. The cell lysates were subjected to immunoprecipitation using anti-OPA1 antibodies and analyzed as in (A).

SUPPLIMENTAL REFERENCES

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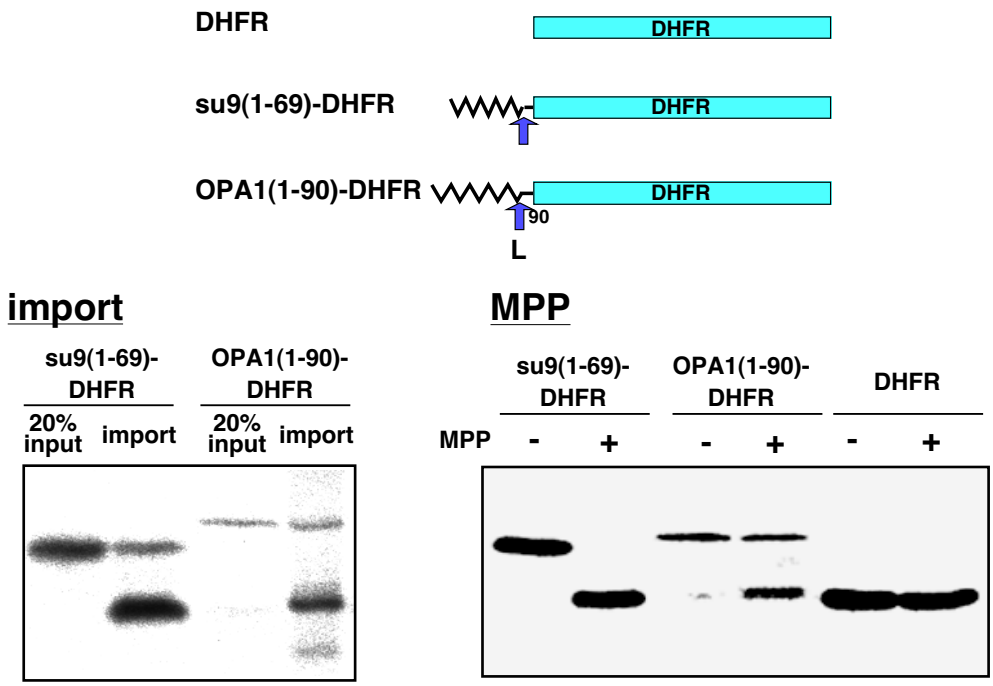
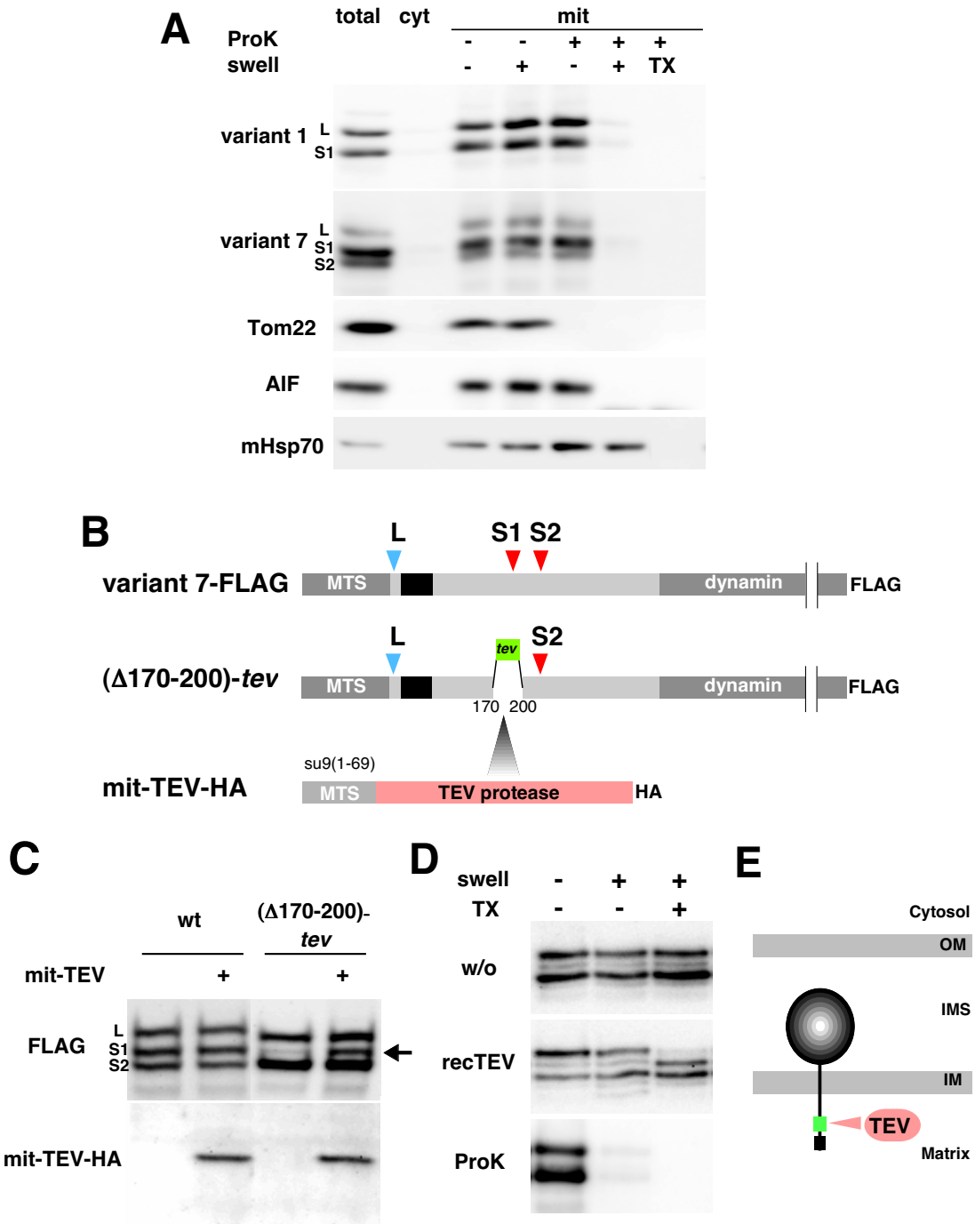
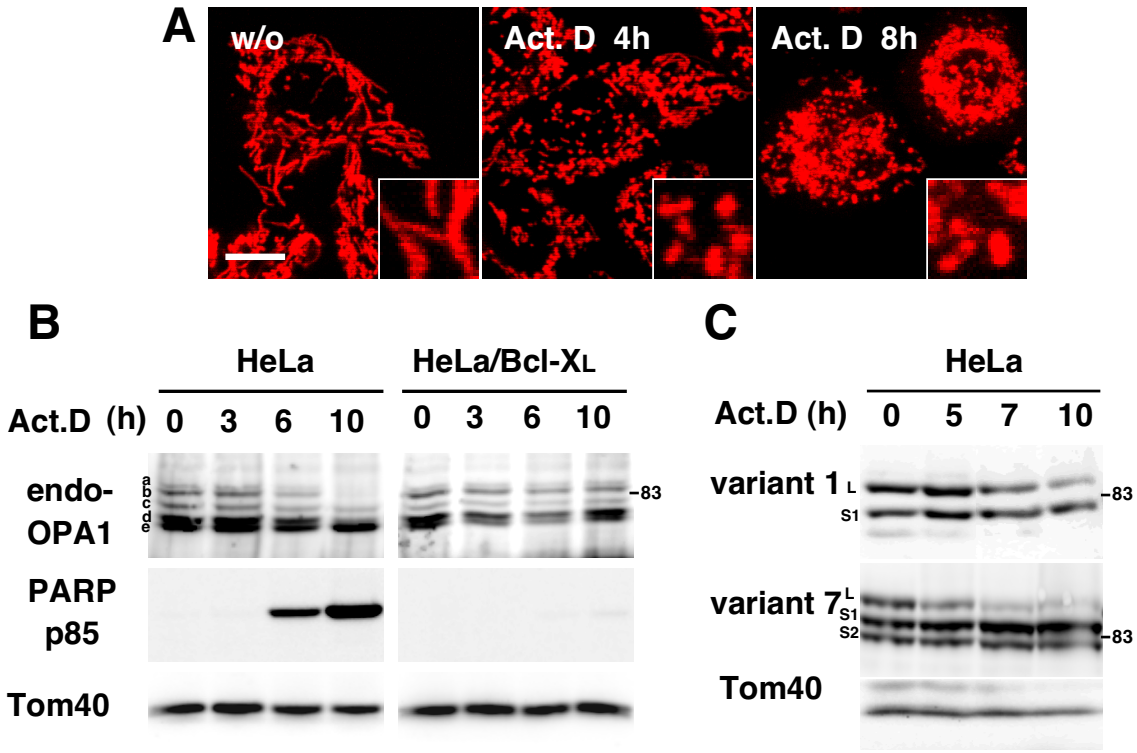


Supplemental Figure 1 (Ishihara N. et al.)



Supplemental Figure 2 (Ishihara N. et al.)



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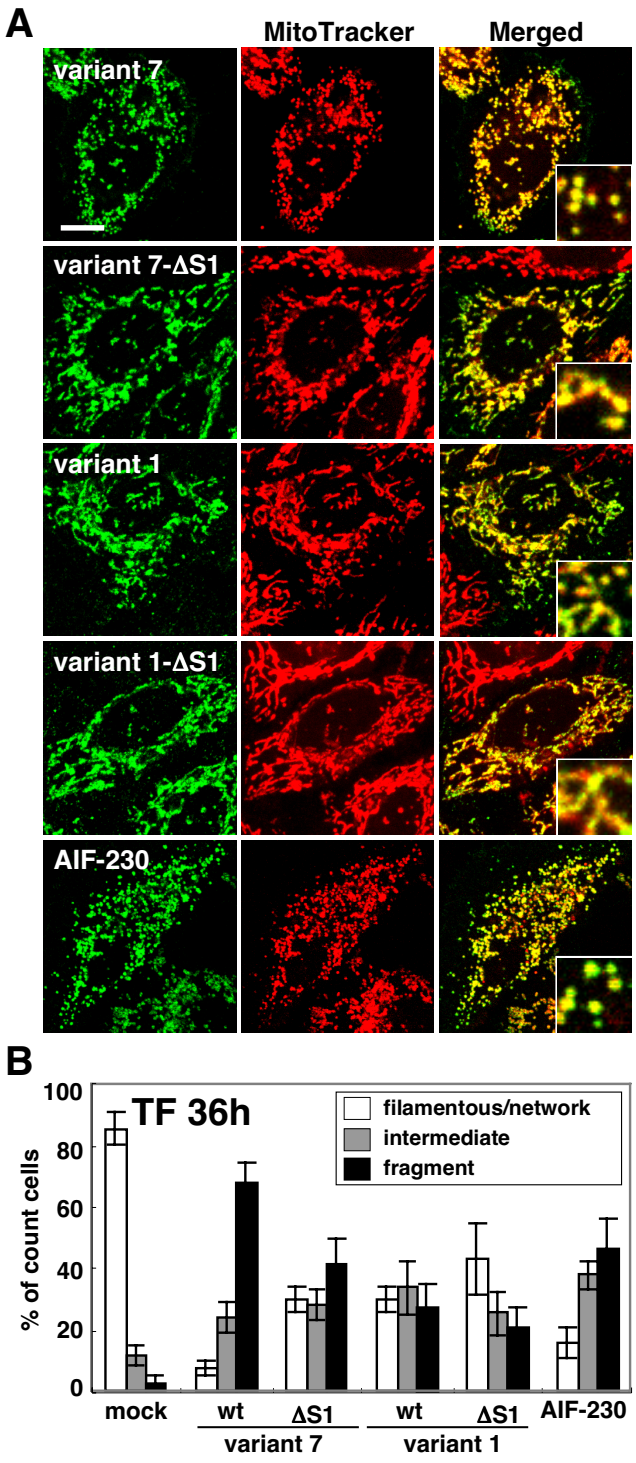

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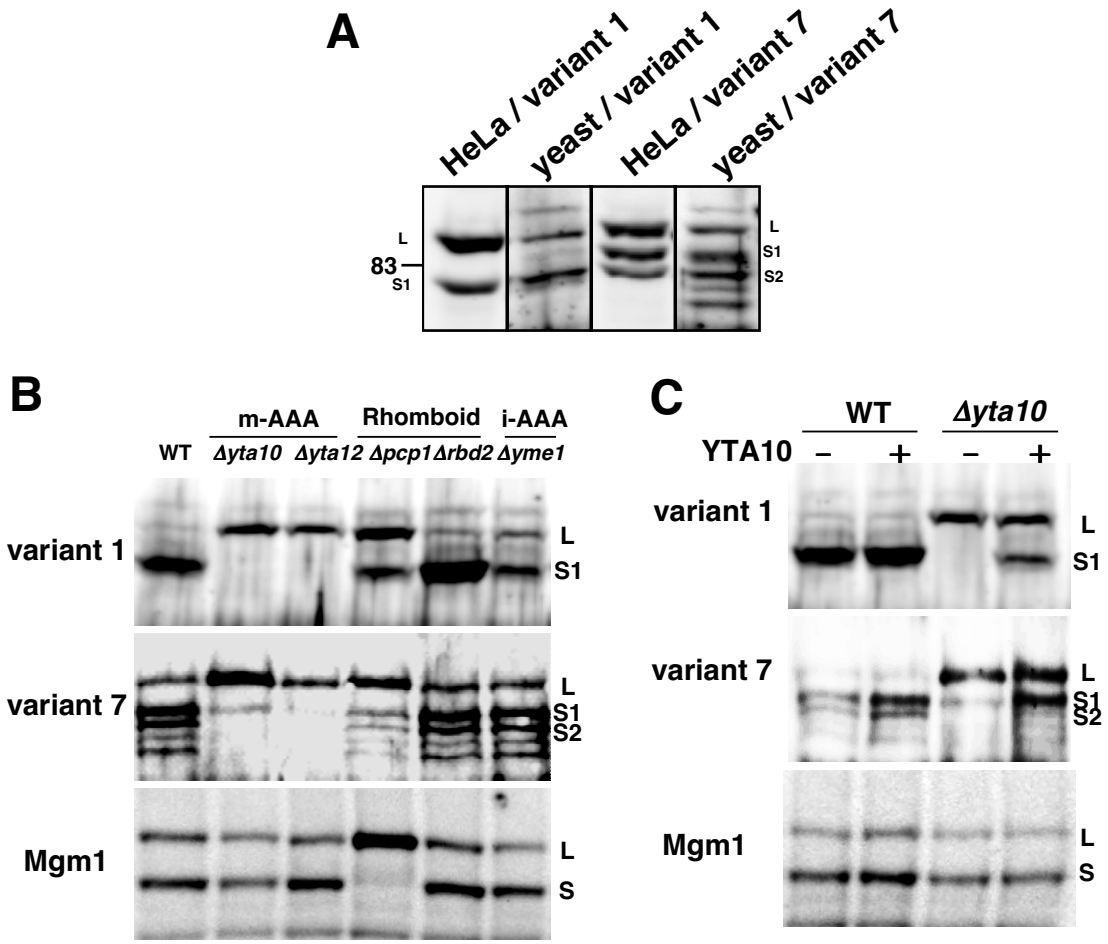
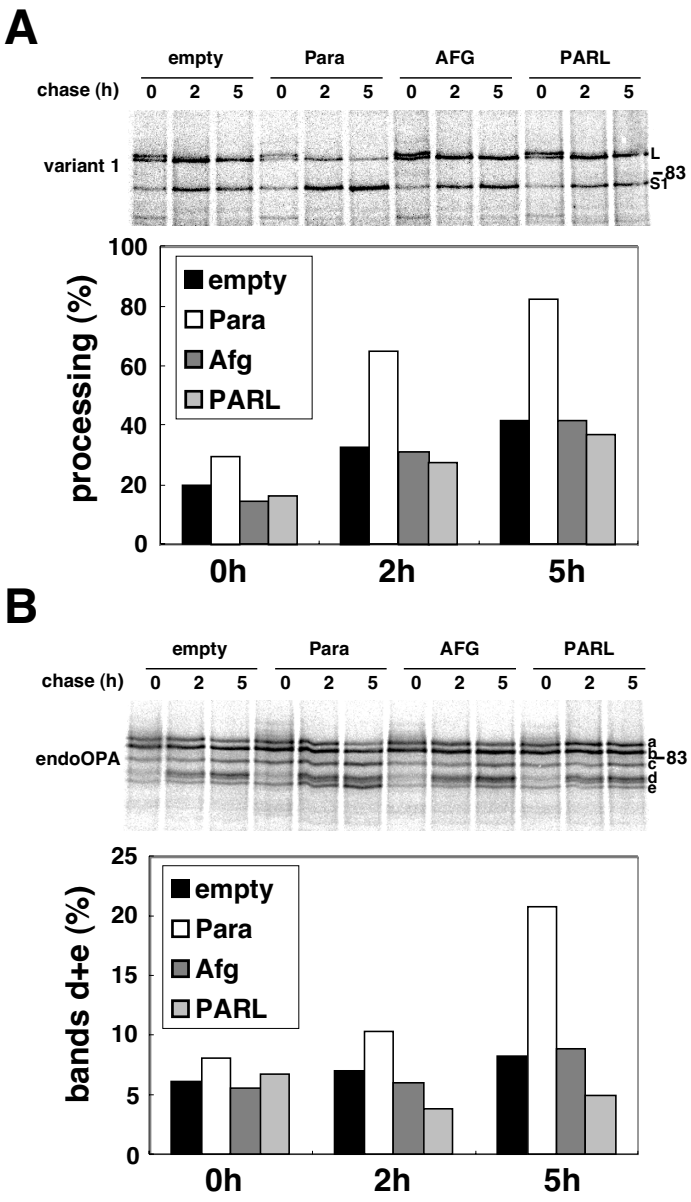


Supplemental Figure 5 (Ishihara N. et al.)



Supplemental Figure 6 (Ishihara N. et al.)