SUPPLEMENTARY INFORMATION

SUPPLEMENTARY MATERALS AND METHODS

Regents

Restriction enzymes and DNA-modifying enzymes were purchased from Nippon Gene and Takara BIO Inc. Digitonin was purchased from Wako Pure Chemical Industries. Fetal bovine serum and Dulbecco's modified Eagle' medium were from Sigma Chemical Co.

Construction of expression plasmids

C-tail anchored proteins and GFP fusion constructs were all created by polymerase chain reaction using the appropriate cDNAs as the templates and the appropriate combinations of forward and reverse oligonucleotide primers. All resulting polymerase chain reaction fragments were inserted into the appropriate restriction sites of the pcDNA3.1 vector (Invitrogen). All the constructs were confirmed by nucleotide sequencing and used for the assays. Information on the oligonucleotide sequences is available upon request.

siRNA treatment.

The following RNA oligonucleotide pairs (QIAGEN) were used to create siRNA duplexes according to the manufacturer's instructions.

Control:

5'-UUCUCCGAACGUGUCACGUdTdT-3',

5'-ACGUGACACGUUCGGAGAAdTdT-3',

VDAC1:

5'-GUAAUUAACAACACUUAAAdTdT-3',

5'-UUUAAGUGUUGUUAAUUACdAdG-3';

TOM22:

5’-GCACAUUGAUCUAUCUAAAdTdT-3’,

5’-UUUAGAUAGAUCAAUGUGCdCdA-3’.

For knockdown of VDAC2 (Catalog No. SI00302148), Tom70 (Catalog No. 1022528), Tom40 (Catalog No. SI00301966), and Tom20 (Catalog No. 1022527), specific siRNAs were purchased from QIAGEN as validated siRNAs (sequences not opened). siRNA transfection to HeLa cells was performed by Lipofectamine 2000 (Invitrogen). At 24 h after the initial treatment, the second siRNA transfection was performed and cells were grown for 48 h. At 72 h after initial treatment, cells were fixed with 4% paraformaldehyde for indirect immunofluorescence study or lysed with SDS-loading buffer for immunoblot study, respectively.

siRNA treatment and mitochondrial protein import assay

For assay of mitochondrial protein import in siRNA-treated cells (“in vivo import”), HeLa cells were transfected with the target siRNAs twice with a 24-h interval. At 72 h after the initial treatment, cells were transfected with the expression plasmids for Su9-GFP, 3FLAG-Bak, or HA-Tom5. Sixteen hours after the DNA transfection, cells were processed for double indirect immunofluorescence microscopy. For immunofluorescence-based *in vitro* import assay, at 72 h after the initial treatment, cells were permeabilized with 25 μg/ml digitonin and then incubated with *in vitro*-synthesized proteins for 60 min at 26℃.

Alkali extraction.

Cells were treated with 0.1 M Na2CO3 (pH 11.5) on ice for 30 min, and soluble and integral membrane proteins were separated by ultracentrifugation at 100,000 x *g* for 40 min. All the

fractions were analyzed by SDS-PAGE and subsequent immunoblotting.

SUPPLEMENTAL FIGURE LEGENDS

Supp. Figure 1. Characterization of mitochondrial import of C-TA proteins in semi-intact cells. (A) Import of 3FLAG-BakΔC or 3FLAG-Bak as described in Fig. 1. The imported substrates were immunostained by mouse monoclonal anti-FLAG antibodies (shown in green; a and b). Mitochondria were stained by rabbit anti-Tom22 antibodies (shown in red; c and d). Merged images are also shown (e and f). **(B)** The import reactions were performed using 3FLAG-Bak for 60 min at either 26℃ or 4℃. The cells were stained with anti-FLAG antibodies (shown in green; a and b) and anti-Tom22 antibodies (shown in red; c and d). Merged images are also shown (e and f). Other conditions were as in (A). **(C)** Reticulocyte lysate-synthesized Su9-DHFR-HA and 3FLAG-Bak were incubated with the semi-intact cells at 26°C. At the indicated time points, the cells were washed and immunostained with mouse monoclonal anti-HA antibodies (for Su9-DHFR-HA) or anti-FLAG antibodies (for 3FLAG-Bak). Mitochondria were counter-stained by rabbit anti-Tom22 antibodies (red). Bars = 20 μm**. (D)** The cells shown in (C) were washed and analyzed by SDS-PAGE and subsequent immunoblotting using anti-HA, anti-FLAG, and anti-Tom22 antibodies. P and m represent precursor protein and mature protein, respectively. The extent of import was quantified by the NIH image. The fluorescence intensities (mean±s.d.) were analyzed for three distinct fields containing at least 50 cells each within a representative experiment, and shown setting those at 60 min at 100%.

Supp. Figure 2. Membrane topology of in vitro imported 3FLAG-Bak and Su9-DHFR-HA. (A) Schematic representation of immunofluorescence analysis of submitochondrial localization of mitochondria-imported 3FLAG-Bak and Su9-DHFR-HA. Antibodies are shown in green. **(B)** Reticulocyte lysate-synthesized 3FLAG-Bak and Su9-DHFR-HA were separately incubated with the semi-intact cells at 26℃ for 60 min. After import, the semi-intact cells were washed and then stained with anti-FLAG or anti-HA antibodies before (b, d, f, h, j, and l) or after (a, c, e, g, i, and k) fixation and permeabilization with 1% Triton X-100 for immunofluorescence microscopy. The imported substrates were detected by anti-FLAG antibodies or anti-HA antibodies (green) under confocal microscopy. HtrA2 (IMS marker) and Tom20 (MOM marker) were also immunostained (red). Merged images are also shown. Magnification, x 630; bar = 20 μm. **(C)** 3FLAG-Bak was expressed in vivo, or reticulocyte lysate-synthesized 3FLAG-Bak was incubated with semi-intact cells as in (B). The semi-intact cells were washed and then treated with sodium carbonate (pH 11.5) to separate the supernatant (S) and membrane precipitates (P), which were then analyzed by SDS-PAGE and subsequent immunoblotting using the antibodies against FLAG, Tim23 (IM marker), and mtHsp70 (matrix marker).

Supp. Figure. 3. ATP-independent import of C-TA proteins. (A) Reticulocyte lysate synthesized import substrates were subjected to gel-filtration to deplete ATP. ATP-depleted import substrates were then incubated at 26°C for 60 min with the semi-intact cells in the absence or presence of 10 mM AMP-PNP. The cells were washed and then immunostained by anti-HA antibodies (Su9-DHFR-HA and HA-VDAC2; shown in green) or anti-FLAG

antibodies (3FLAG-Bak; shown in green). **(B)** After *in vitro* import reaction as in (A), the cell lysates were analyzed by SDS-PAGE and subsequent immunoblotting using antibodies against HA, FLAG or the indicated proteins. The band intensities were quantified by Fuji LAS1000 and shown setting band intensities of mock treated cells at 100%. Note that mock treated substrates were efficiently transported to mitochondria, probably because of the presence of residual ATP in the gel-filtrated lysates. **(C)** After in vitro import reaction in the absence or presence of AMP-PNP, cells were treated with 100 mM sodium carbonate (pH 11.5) and centrifuged to separate the supernatant (S) and membrane (P) fractions, which were analyzed by SDS-PAGE and subsequent immunoblotting with the antibodies against FLAG, cytochrome c and Tom22**.** **(D)** The indicated preproteins were subjected to mitochondrial import in the semi-intact cells in the presence and absence of 20 μM CCCP. Other conditions were as in Fig. 1. **(E)** The cells in (D) were subjected to sodium carbonate (pH 11.5) extraction and analyzed as in (C).

Supp. Figure. 4. Quantitative analysis of TOM-dependent or -independent mitochondrial protein import using semi-intact cell system. (A) HeLa cells were subjected to siRNA transfection for the indicated proteins, stained by MitoTracker (red), and used for the subsequent import in semi-intact cell system. The indicated preproteins were subjected to the import as in Fig. 3B. In a separate experiment, HeLa cells were cultured in the presence of 20μM CCCP for 2 h and then stained by MitoTracker (red) to use for the import assay as described above. After the import reaction, the semi-intact cells were stained with either anti-HA antibodies (green; for Su9-DHFR-HA and HA-VDAC2) or anti-FLAG antibodies (green; for 3FLAG-Bak). **(B)** The extent of import was quantified by the NIH image. The

fluorescence intensities (mean±s.d.) were measured for three distinct fields of at least 100 cells each within a representative experiment, and shown setting those of control cells at 100%.

Supp. Figure. 5. Mitochondrial import of C-TA proteins in vivo is unaffected by the TOM components depletion. HeLa cells were transfected with the indicated siRNAs. After 72 h, cells were transfected with expression plasmids for Su9-GFP, 3FLAG-Bak, or HA-human Tom5. After 16 h, the expressed proteins were detected by GFP fluorescence (green; Su9-GFP) or either anti-FLAG (shown in green; 3FLAG-Bak) or anti-HA antibodies (shown in green; HA-human Tom5) under confocal microscopy. Depletion of the target TOM components was confirmed by immunostaining with antibodies against the target proteins (shown in red). Merged images are also shown. The cells with a cytoplasmic localized (non-mitochondrial) pattern were counted for at least 300 cells each in three distinct experiments. Percentage (mean ±s.d.) of mitochondrial signal-positive cells is shown.

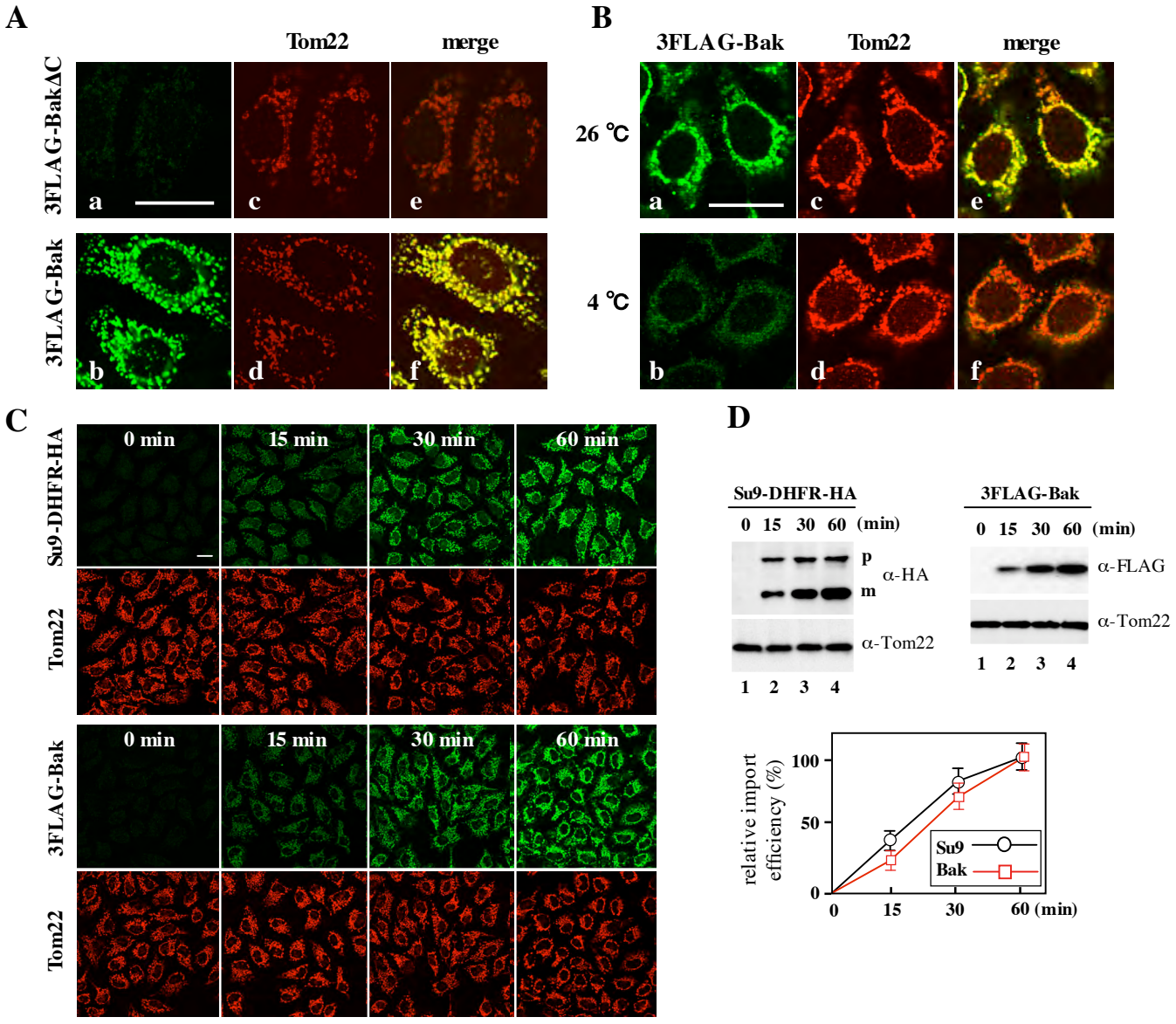
Supp. Figure. 6. VDAC2-dependency of MOM import is restricted only to the C-TA constructs carrying the cytoplasmic domain of Bak. (A) GFP-BakC or GFP-Bak was subjected to mitochondrial import in semi-intact VDAC2-depleted cells. RNAi and the in vitro import assay were performed as described in Fig. 3. Cells were processed for double indirect immunofluorescence microscopy with either anti-GFP antibodies (green; GFP-Bak and GFP-BakC) and anti-Tom22 antibodies (red). Merged image are shown. The extent of import was quantified by NIH Image. Each graph indicates mean±SD in 3 distinct fields of at least 50 cells within a representative experiment. **(B)** GFP-BakC or GFP-Bak was subjected to

mitochondrial import in semi-intact VDAC2-overexpressing cells. Cells were processed for double indirect immunofluorescence microscopy. The asterisks indicate cells overexpressing HA-VDAC2. **(C)** The cell lysates (equal protein amounts) used in (B) were analyzed by SDS-PAGE and subsequent immunoblotting using antibodies against the indicated proteins.

Supp. Figure. 7. Mitochondrial import of GFP-BakC in semi-intact cells prepared under stronger permeabilization condition. HeLa cells immobilized on coverslips were semi-permeabilized with 25 μg/ml digitonin (**a**) or 100 μg/ml digitonin (**b**) for 5 min and incubated at 26°C for 60 min with the PURESYSTEM-synthesized GFP-BakC. The semi-intact cells were then fixed and processed for indirect immunofluorescence microscopy using anti-GFP antibodies.

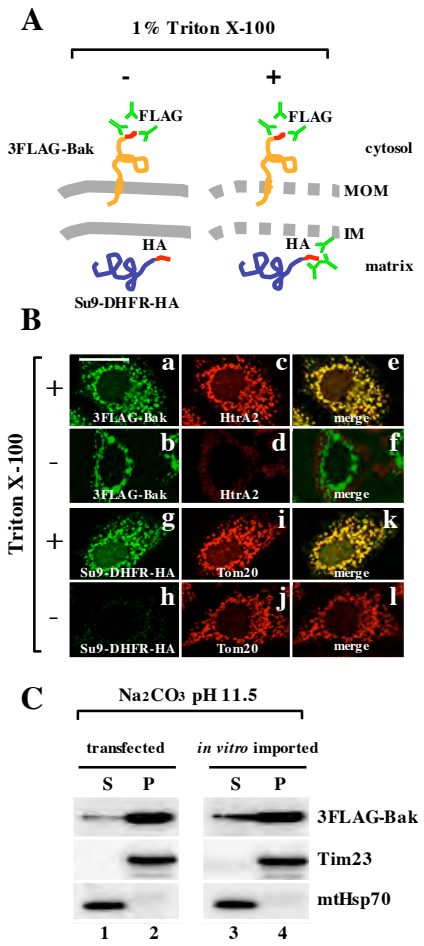
Supp. Figure 8. Cytosol requirement for the mitochondrial import of C-TA proteins depends on conformation of the reporter segments extruding to the cytoplasm. (A and B) Reticulocyte lysate-synthesized and immunopurified 3FLAG-Bak (A) and 2FLAG-GST-Bcl-XL (B) were subjected to mitochondrial import in semi-intact cells in the presence or absence of reticulocyte lysate. +RL: 20 mg/ml reticulocyte lysate; ++RL: 60 mg/ml reticulocyte lysate. After import, the cells were processed for double indirect immunofluorescence microscopy using anti-FLAG antibodies (green) and anti-Tom22 antibodies (red). The extent of import was quantified by NIH Image. Each graph indicates mean ±SD in three distinct fields (each contains at least 50 cells) in a representative experiment. Bar = 20μm. **(C, D)** PURESYSTEM-synthesized 3FLAG-Bak (C) or GFP-Bak (D) was subjected to

mitochondrial import with in semi-intact cells in the presence or absence of rabbit reticulocyte lysate. Other conditions are as in (A).



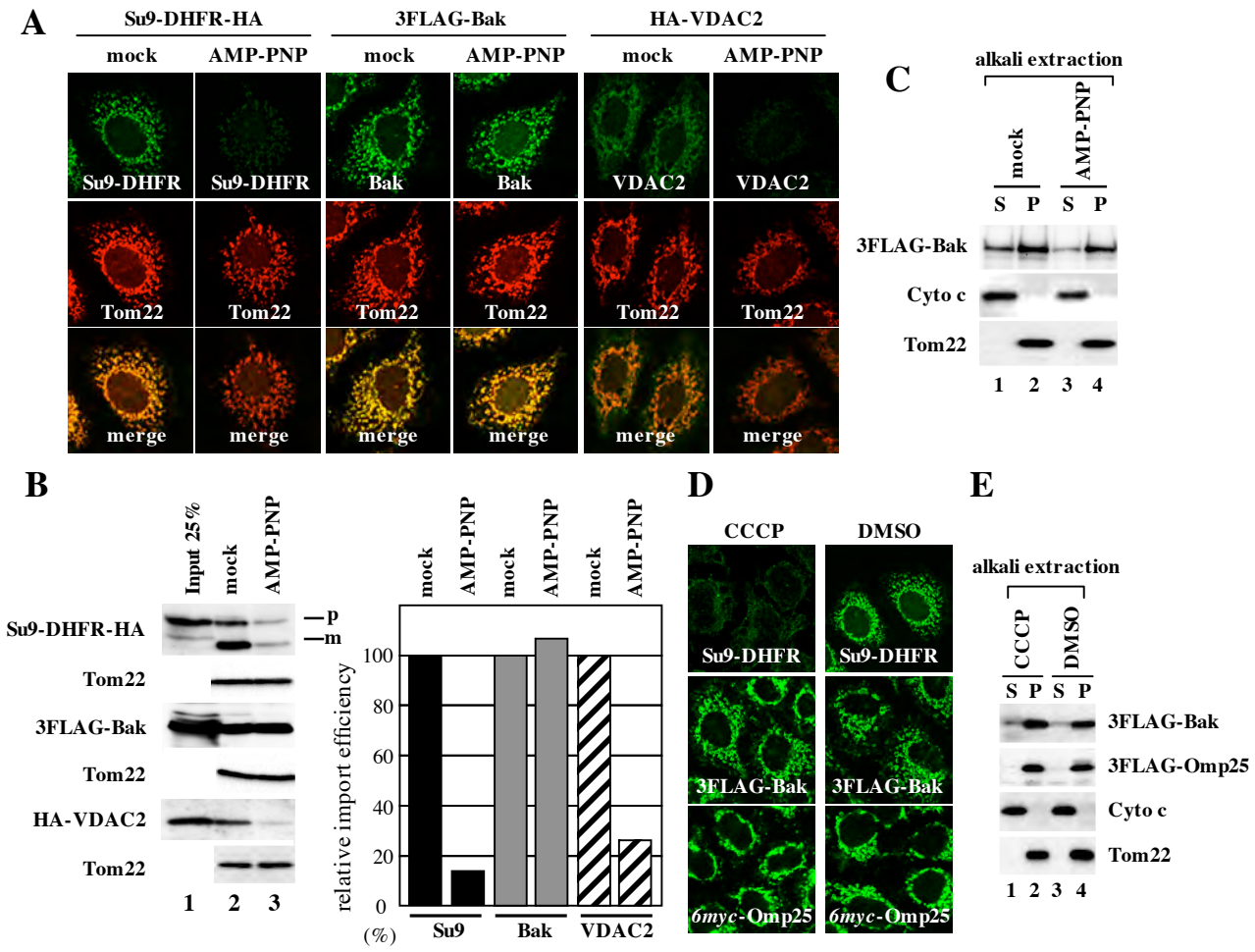
Supplemental figure 1

K. Setoguchi & H. Otera *et al*



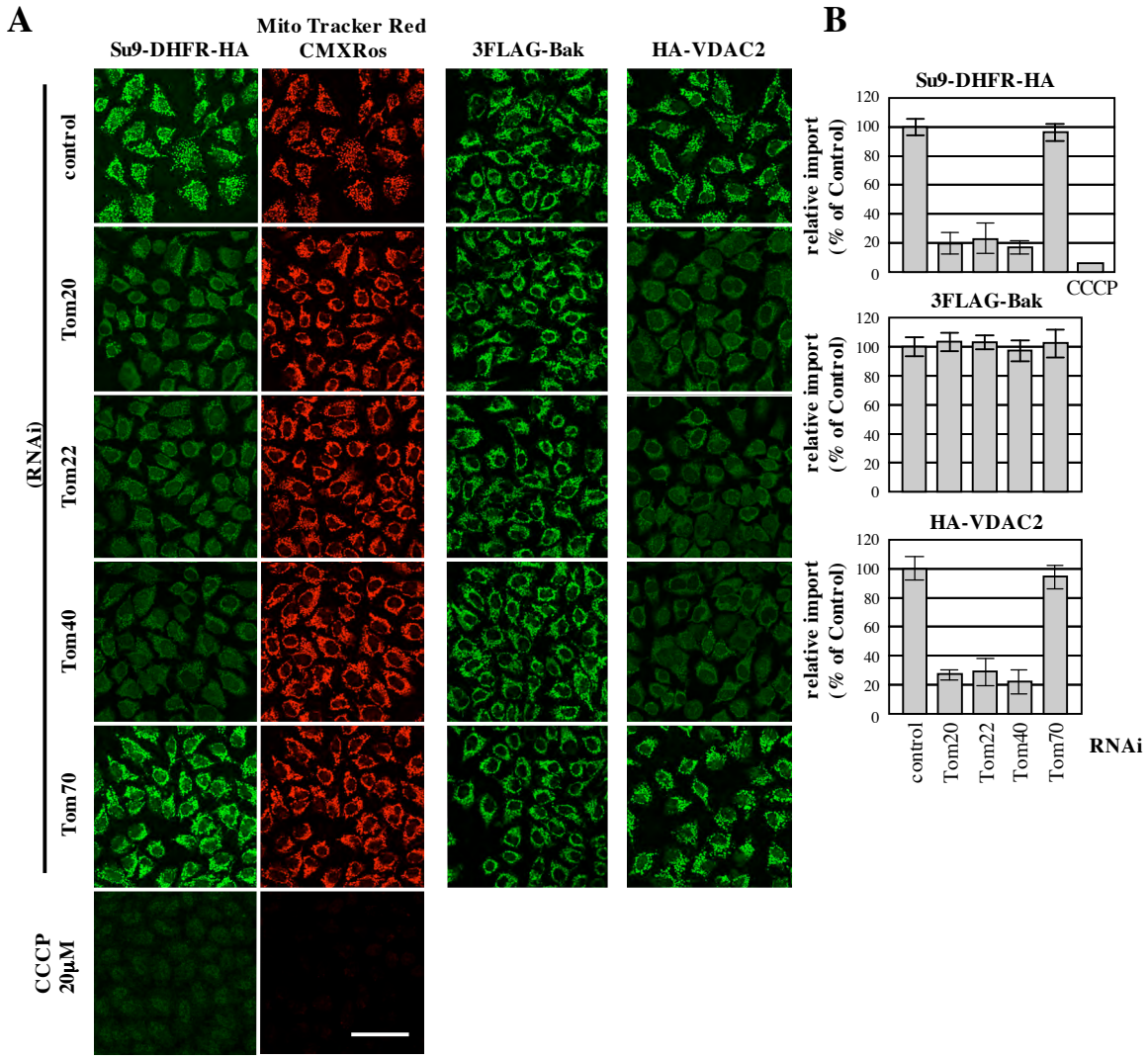
Supplemental figure 2

K. Setoguchi & H. Otera *et al*



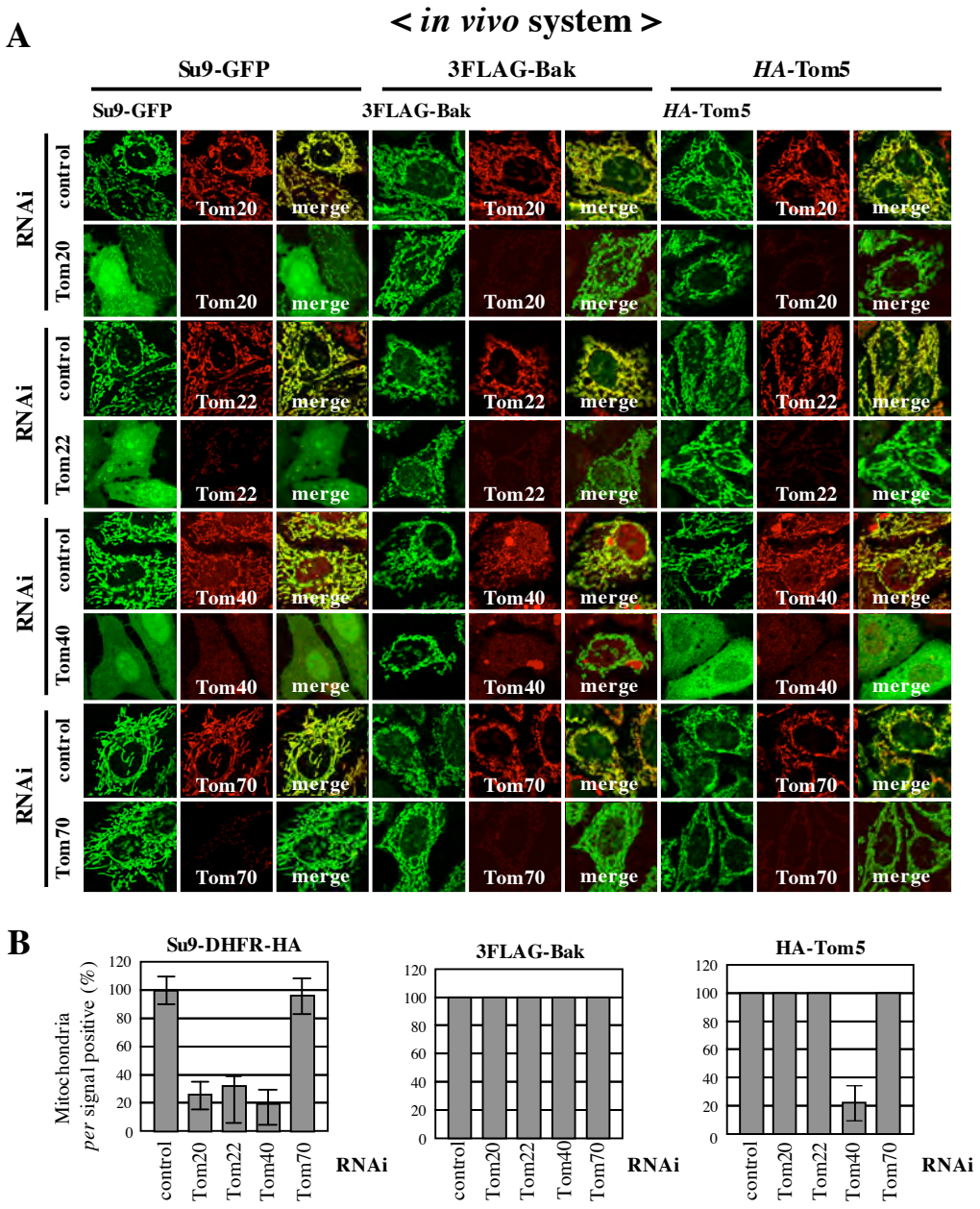
Supplemental figure 3

K. Setoguchi & H. Otera *et al*



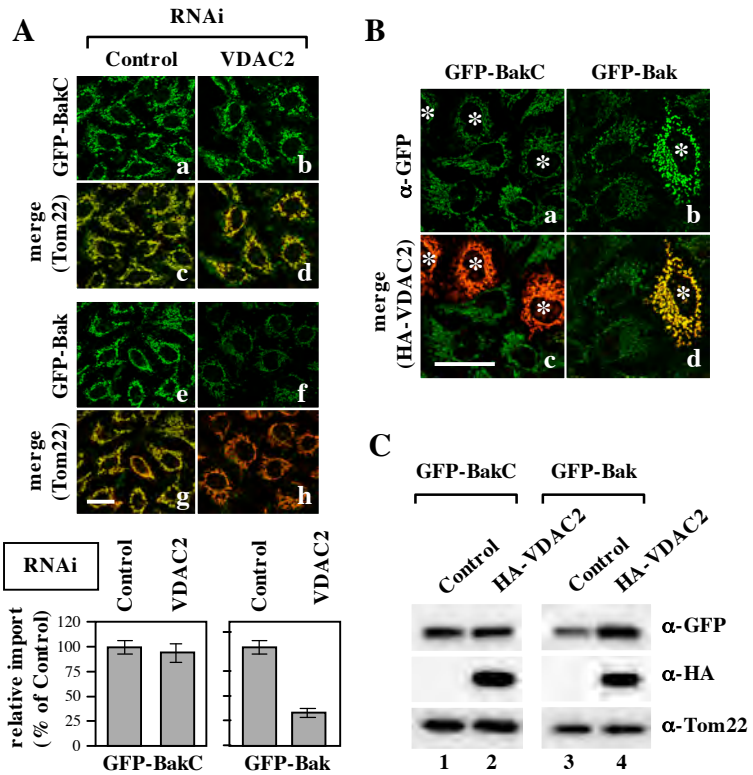
Supplemental figure 4

K. Setoguchi & H. Otera *et al*



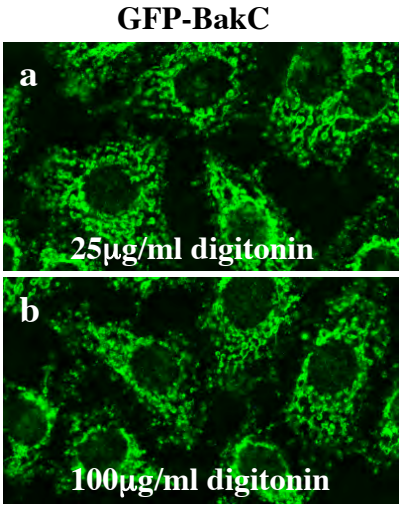
Supplemental figure 5

K. Setoguchi & H. Otera *et al*



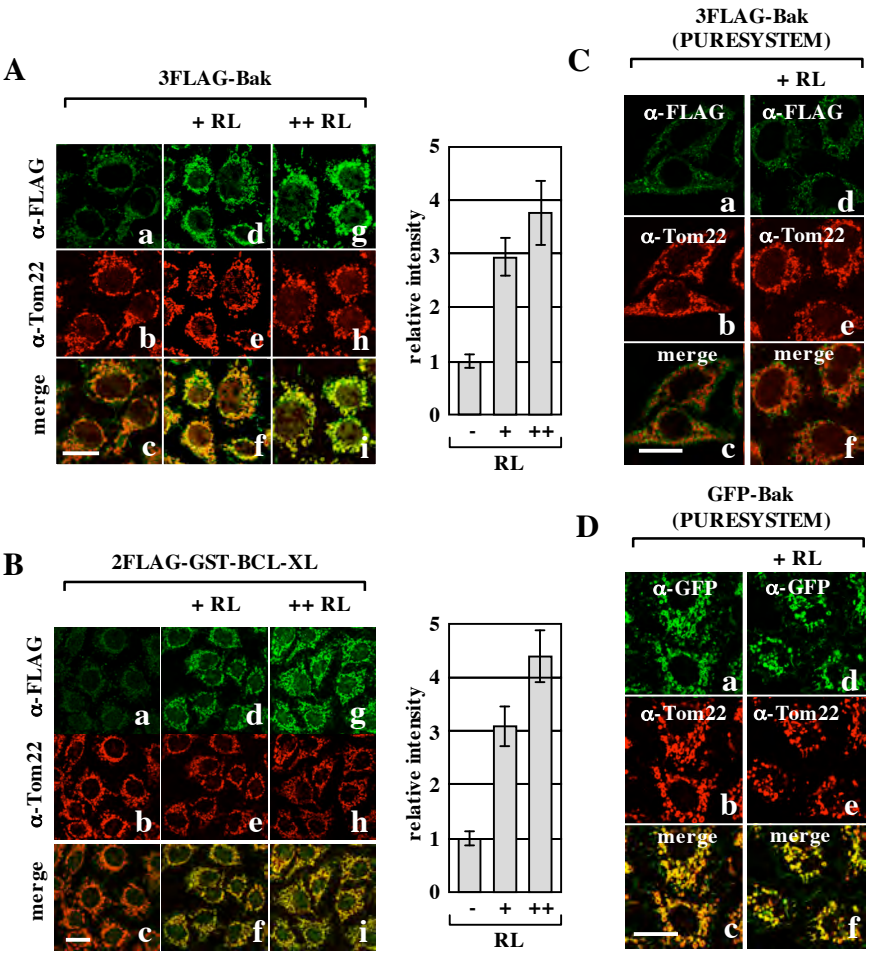
Supplemental figure 6

K. Setoguchi & H. Otera *et al*



Supplemental figure 7

K. Setoguchi & H. Otera *et al*



Supplemental figure 8

K. Setoguchi & H. Otera *et al*