# Supplementary data

## Materials and methods

### Materials

SP600125 was obtained from BIOMOL, anisomycin was from SIGMA, EGF was from UPSTATE, staurosporine was from ICN Biomedicals, Hoechst 33342 was from Molecular Probes, caspase-3/CPP32 fluorometric protease assay kit was from MBL and Z-VAD-$CH_2$DCB was from Phoenix Pharmaceuticals.

### Antibodies

Antibodies to Bax (N-20, Santa Cruz), to VDAC (31HL, Calbiochem), to Bim (Cat. #559685, BD PharMingen and Cat. #202000, Calbiochem), to 14-3-3ζ (C-16, Santa Cruz), to 14-3-3σ (C-18, Santa Cruz), to JNK1 (C-17, Santa Cruz) to c-Jun (Cat. #9162, Cell Signaling), or to phospho–c-Jun (Cat. #9261, Cell Signaling), to Akt (Cat. #9272, Cell Signaling), to phospho-Ser473 Akt (587F11, Cell Signaling), to GST (B-14, Santa Cruz), to T7 Tag (Cat. #69522-3, Novagen), to cytochrome c (7H8.2C12, BD PharMingen), to $F_0F_1$-ATPase subunit α (7H10, Molecular Probe), or to α-tubulin (DM1A, Sigma) were used for immunoblot analysis. Antibodies to the Flag epitope (M2, Sigma), to 14-3-3σ (C-18, Santa Cruz) or normal goat IgG (Santa Cruz) were used for immunoprecipitation. Antibodies to 14-3-3ζ phosphorylated on $Ser^{184}$ or to 14-3-3σ phosphorylated on $Ser^{186}$ were generated in rabbits by injection with keyhole limpet hemocyanin–conjugated phosphopeptides corresponding to human 14-3-3ζ (SVFYYEILNpSPEKA) or human 14-3-3σ (SVFHYEIANpSPEEA) sequences. Antibodies

were purified from the resulting antisera by column chromatography on phosphopeptide-conjugated affinity resin.

**Plasmid construction and cell transfection**

The constructs encoding GFP-Bax, DsRed-Mito and Akt were described previously (Tsuruta et al., 2002). The localization of GFP-Bax reflects the localization of endogenous Bax except for its aberrant nuclear distribution as reported before (Wolter et al., 1997). We obtained the constructs encoding p35 from Dr. M. Miura, human 14-3-3β from Dr. M. Yaffe, bovine 14-3-3ζ from Dr. H. Fu, human 14-3-3σ from Dr. B. Vogelstein, and dominant negative JNK, Flag-JNK and JBD from Dr. R Davis. The full-length MKK7 and JNK1 cDNAs have been described previously (Ito et al., 1999). The MKK7-JNK(WT) fragment was constructed by ligation of the Flag-JNK1 cDNA into the pEF-Flag-MKK7 plasmid, and was subcloned into the BamHI site of pcDNA3. Bcl-XL cDNA was amplified by the polymerase chain reaction and subcloned into pcDNA3. Human c-Jun and the N-terminal fragment of c-Jun (residues 1 to 79) cDNA were amplified by the polymerase chain reaction and cloned into the BamHI sites of pCS2 and pET28a, respectively. The cDNA for a dominant negative mutant of c-Jun (lacking residues 3 to 122 containing the $NH_2$-terminal transactivation domain) was amplified by the polymerase chain reaction from the c-Jun cDNA and cloned into the BamHI sites of pCS2. Site-directed mutagenesis was performed with a QuickChange kit (Stratagene) to generate the $Ser^{186}$ → Ala mutants of 14-3-3β and 14-3-3σ, the $Ser^{184}$ → Ala mutant of 14-3-3ζ, and the $Lys^{434}$-$Lys^{435}$ → Met-Met mutant (KN) of MKK7-JNK. Each 14-3-3 cDNA was cloned into the BamHI sites of pET28a (Novagen) or pGEX6P-1 (Amersham Biosciences).

COS-1 cells were transfected with plasmids by the use of FuGENE6 (Roche), HCT116 and HeLa cells were by Lipofectamine Plus (Invitrogen), according to the manufacturer's instructions.

**Protein purification**

The expression of GST-14-3-3ζ was induced in *Escherichia coli* BL21 cells by culture in the presence of 0.4 mM isopropyl-β-D-thiogalactopyranoside, and the recombinant protein was purified with the use of glutathione-Sepharose 4B (Amersham Biosciences). The expression of $His_6$-tagged c-Jun, 14-3-3β, 14-3-3ζ, 14-3-3σ, or MKK7-JNK was similarly induced in *E. coli* BL21 cells, and the recombinant proteins were purified with the use of ProBond resin (Invitrogen). His-tagged Bax was described previously (Narita et al., 1998).

**Immunoblot analysis**

Cells were washed with phosphate-buffered saline (PBS) and then lysed in an extraction buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium pyrophosphate, 5 mM NaF, 1 mM $Na_3VO_4$, 0.5% Triton X-100, 1 mM dithiothreitol] supplemented with protease inhibitors [1 mM phenylmethylsulfonyl fluoride, leupeptin (5 μg/ml), pepstatin A (5 μg/ml), aprotinin (5 μg/ml)]. The cell lysates were subjected to immunoblot analysis with specific antibodies and the immune complexes were detected with chemiluminescence reagent (PerkinElmer).

**Subcellular fractionation**

Cells were washed with PBS, scraped into an isotonic buffer [200 mM mannitol, 70 mM

sucrose, 1 mM EDTA, 10 mM Hepes-NaOH (pH 7.4), 1 mM dithiothreitol] supplemented with the protease inhibitors described above, and homogenized with a Potter-Elvehjem homogenizer. Nuclei and unbroken cells were removed by centrifugation at 500 × *g* for 10 min, and the supernatant was further centrifuged at 100,000 × *g* for 60 min. The resulting supernatant was saved as the cytosolic fraction, and the pellet was washed with the isotonic buffer, resuspended in extraction buffer supplemented with protease inhibitors, and centrifuged at 20,000 × *g* for 5 min to remove debris. The resulting supernatant was saved as the mitochondrial fraction.

**Luciferase reporter gene assay**

COS-1 cells were transfected with the indicated expression vectors as well as with the AP-1 Luc reporter plasmid (Promega), which contains the luciferase gene under the control of an AP-1–dependent promoter, and with an expression plasmid for β-galactosidase. Cell lysates were subsequently assayed for both luciferase and β-galactosidase activities with a kit (Promega and ICN, respectively), and the former activity was normalized on the basis of the latter.

**In vitro kinase assay**

COS-1 cells ($4.0 \times 10^5$) were transfected with the Flag-JNK expression vector, incubated with or without anisomycin, lysed in extraction buffer supplemented with protease inhibitors, and subjected to immunoprecipitation with antibodies to Flag. The kinase assay was performed for 30 min at 30°C in a reaction mixture (final volume of 50 μl) containing JNK immunoprecipitate, purified $His_6$–14-3-3 protein, $His_6$-c-Jun or His-Bax, 5 μCi of [γ-$^{32}$P]ATP,

100 μM unlabeled ATP, 20 mM Tris-HCl (pH 7.5), and 15 mM $MgCl_2$. Proteins were then resolved by SDS-polyacrylamide gel electrophoresis and subjected to autoradiography. Recombinant MKK7-JNK was used as the source of kinase activity in some experiments.

**Two-dimensional gel electrophoresis**

HCT116 cells were transfected with the MKK7-JNK(WT) expression vector or the corresponding empty vector, lysed in an extraction buffer supplemented with protease inhibitors, and subjected to isoelectric focusing using 24 cm Immobiline DryStrip (pH 4.0-5.0) and an IPGphor isoelectric unit (Amersham Biosciences), and subjected to immunoblot analysis with antibodies to 14-3-3σ.

**Co-immunoprecipitation analysis**

In our hands, the only available antibody which reacts with native 14-3-3 was the anti-14-3-3σ antibody from Santa Cruz (C-18). Therefore, we performed co-immunoprecipitation analysis by the use of this antibody and the cell line HCT116 which expresses 14-3-3σ isoform. Transfected HCT116 cells were washed with PBS, and then lysed in an extraction buffer containing 10 mM Hepes-NaOH (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaC, 1% CHAPS and protease inhibitors. The cell lysates were centrifuged at 15,000 × *g* for 15 min, and the resulting supernatant was subjected to immunoprecipitation with antibodies to 14-3-3σ. The immunoprecipitates were subjected to immunoblot analysis with antibodies to Bax or to 14-3-3σ.

**GST pull down assay**

Recombinant GST–14-3-3ζ was incubated with or without purified MKK7-JNK in the presence of 100 μM ATP, 20 mM Tris-HCl (pH 7.5), and 15 mM $MgCl_2$ for 30 min at 30°C. After the reaction, the GST-14-3-3ζ was precipitated with glutathione–Sepharose 4B, and the beads were washed with PBS to remove MKK7-JNK. HeLa cells ($3.0 \times 10^6$) were scraped into 300 μl of a solution containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.5% glycerol, and protease and phosphatase inhibitors, and homogenized with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 100,000 × *g* for 60 min, and the resulting supernatant was incubated overnight at 4°C with GST–14-3-3ζ, and the bead-bound proteins were subjected to immunoblot analysis with antibodies to Bax and to GST.

**Supplementary Reference**


Ito, M., Yoshioka, K., Akechi, M., Yamashita, S., Takamatsu, N., Sugiyama, K., Hibi, M., Nakabeppu, Y., Shiba, T. and Yamamoto, K.I. (1999) JSAP1, a novel jun N-terminal protein kinase (JNK)-binding protein that functions as a Scaffold factor in the JNK signaling pathway. *Mol. Cell. Biol.*, **19**, 7539-7548.

Narita, M., Shimizu, S., Ito, T., Chittenden, T., Lutz, R.J., Matsuda, H. and Tsujimoto, Y. (1998) Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. *Proc. Natl. Acad. Sci. USA*, **95**, 14681-14686.

Tsuruta, F., Masuyama, N. and Gotoh, Y. (2002) The phosphatidylinositol 3-kinase (PI3K)-Akt pathway suppresses Bax translocation to mitochondria. *J. Biol. Chem.*, **277**, 14040-14047.

Wolter, K.G., Hsu, Y.T., Smith, C.L., Nechushtan, A., Xi, X.G. and Youle, R.J. (1997) Movement of Bax from the cytosol to mitochondria during apoptosis. *J. Cell. Biol.*, **139**, 1281-1292.


**Figure Legends**

**Supplementary Figure 1**

COS-1 cells ($1.0 \times 10^5$) were transfected with expression vectors for 0.5 μg of MKK7-JNK(WT) or 1.0 μg of caspase-8 in the presence or absence of a vector for p35, and caspase-3 activity was measured by caspase-3/CPP32 fluorometric protease assay kit (MBL).

**Supplementary Figure 2**

COS-1 cells were incubated first for 30 min with or without 20 μM SP600125 and then for 6 h in the presence or absence of anisomycin (10 μg/ml). They were then subjected to subcellular fractionation, and the amounts of endogenous Bax and VDAC in the mitochondrial fraction were assessed by immunoblot analysis.

**Supplementary Figure 3**

COS-1 cells were transfected for 10 h with expression vectors for GFP-Bax and p35 together with a vector for a dominant negative (DN) form of JNK or the corresponding empty vector, as indicated. The cells were then incubated for 6 h in the presence or absence of 1 μM staurosporine, after which the percentage of cells exhibiting GFP-Bax localization to mitochondria was determined. Data are means ± SD of values obtained from five fields of 30-150 cells (*, P<0,005).

**Supplementary Figure 4**

Equal amounts of recombinant $His_6$-tagged c-Jun and Bax were incubated with or without recombinant MKK7-JNK(WT or KN) in the presence of [$\gamma$-$^{32}$P]ATP and then subjected to

electrophoresis and autoradiography.

**Supplementary Figure 5**

COS-1 cells were transfected for 20 h with an expression vector for GFP and with vectors for MKK7-JNK(WT) and DN c-Jun, as indicated, and were then subjected to subcellular fractionation. The amounts of endogenous Bax and VDAC in the mitochondrial fraction were assessed by immunoblot analysis.

**Supplementary Figure 6**

COS-1 cells were transfected for 15 h with expression vectors for MKK7-JNK(WT or KN), and then incubated for 30 min in the presence or absence of 50 ng/ml EGF. Cell lysates were subjected to immunoblot analysis with antibodies to phosphorylated Akt, to Akt, or to JNK.

**Supplementary Figure 7**

COS-1 cells were transfected for 17 h with expression vectors for GFP-Bax, p35 together with those for MKK7-JNK(WT) and either constitutively active Akt or dominant negative Akt. The percentage of cells in which GFP-Bax was localized to mitochondria was then determined (N.S., not significant).

**Supplementary Figure 8**

COS-1 cells were transfected for 15 h with expression vectors for MKK7-JNK(WT or KN), incubated for 30 min with or without 20 μM SP600125, and then for 30 min in the presence

or absence of anisomycin (10 μg/ml). Cell lysates were then subjected to immunoblot analysis with antibodies to Bim, to phosphorylated c-Jun, to c-Jun, or to tubulin.

**Supplementary Figure 9**

(**A**) COS-1 cells were transfected for the indicated times with expression vectors for GFP-Bax and p35 together with those for MKK7-JNK(WT) and either 14-3-3ζ(S184A) (left panel) or 14-3-3σ(S186A) (right panel). The percentage of cells exhibiting GFP-Bax localization to mitochondria was then determined. Data are means ± SD of values obtained from five fields of 30-150 cells in each of three independent experiments (*, $P<0.0005$ as compared with MKK7-JNK (WT) group). The amount of transfected 14-3-3 constructs was two times greater than that used in the experiments in Figure 6, which resulted in more robust attenuation of GFP-Bax translocation.

(**B**) COS-1 cells were transfected for 11 h with expression vectors for GFP-Bax, p35 and either 14-3-3ζ(S184A) (left panel) or 14-3-3σ(S186A) (right panel), and were incubated for the indicated times in the presence or absence of anisomycin (10 μg/ml). The percentage of cells in which GFP-Bax was localized to mitochondria was then determined and shown as in (**A**) (*, $P<0.0005$ and **, $P<0.005$ as compared with MKK7-JNK (WT) group).

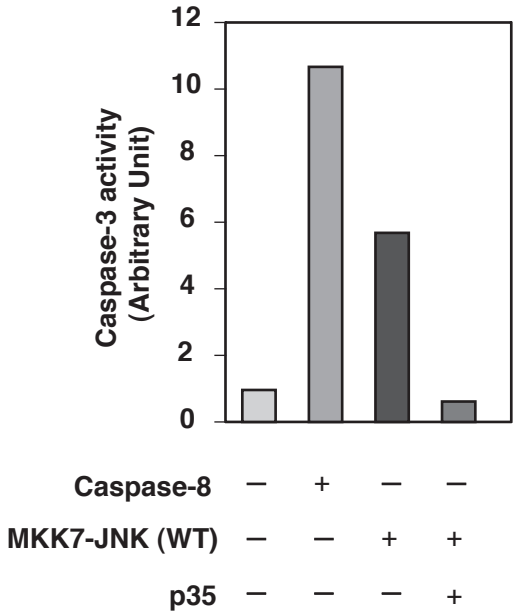
**Supplementary Figure 10**

COS-1 cells were transfected for 20 h with expression vectors for GFP, MKK7-JNK(WT), and either 14-3-3ζ S184A or 14-3-3σ S186A, as indicated, and were then subjected to subcellular fractionation. The amounts of endogenous Bax and VDAC in the mitochondrial fraction were determined by immunoblot analysis with specific antibodies.

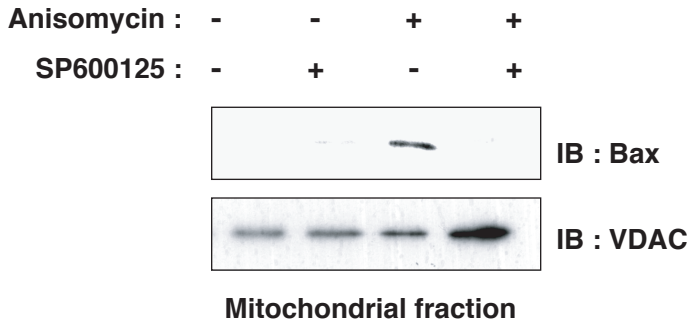
**Supplementary Figure 11**

HeLa cells were transfected with expression vectors for GFP and DN JNK and then for 3 h in the presence or absence of anisomycin (10 μg/ml), They were then stained with Hoechst 33342 (6.7 μg/ml) for 10 min, and the percentage of GFP-positive cells with pyknotic nuclei was determined and shown as in Figure 7(**C**) (*, P<0.0005).

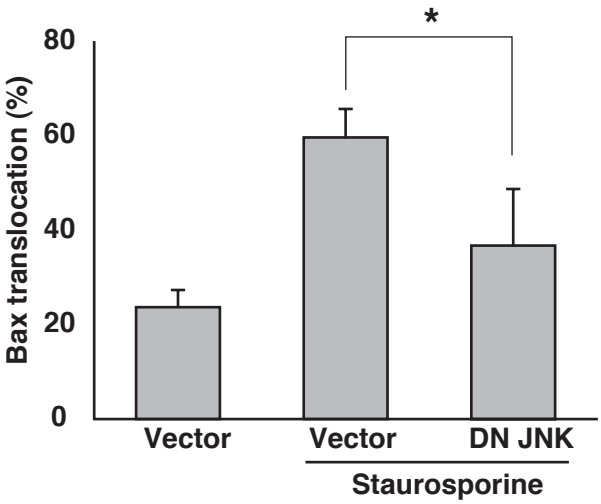
## Supplementary Figure 1



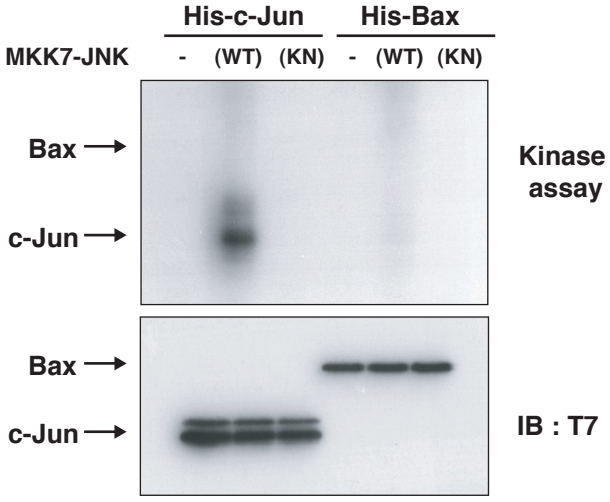

## Supplementary Figure 2




## Supplementary Figure 3




## Supplementary Figure 4



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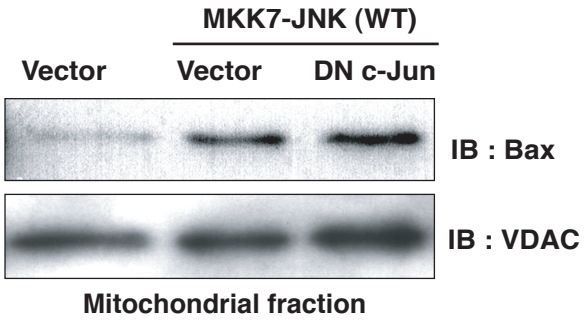
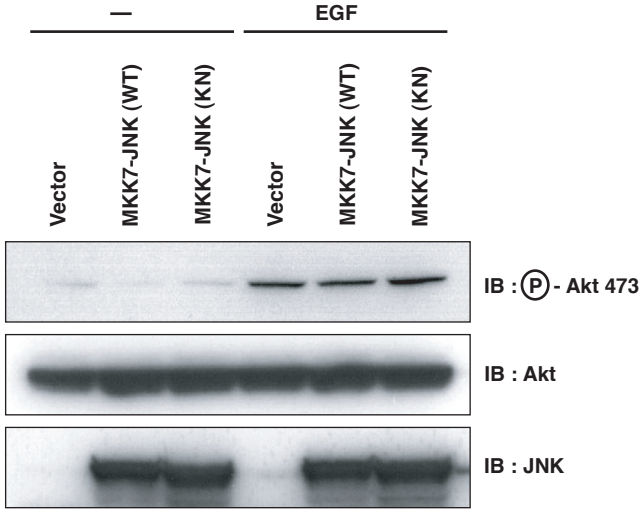
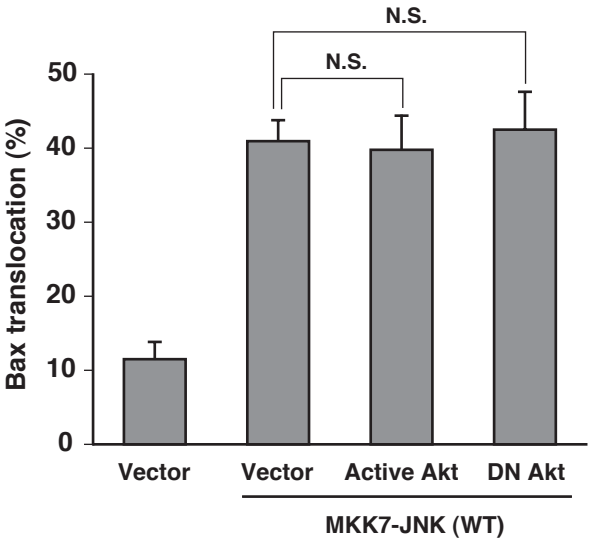


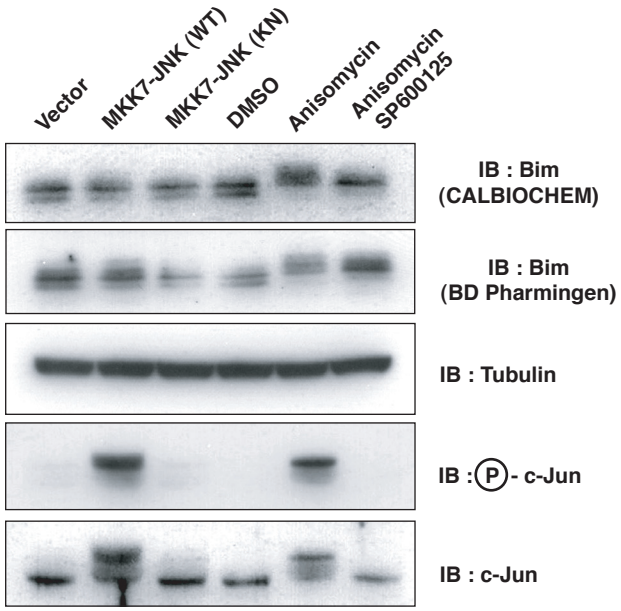

## Supplementary Figure 6



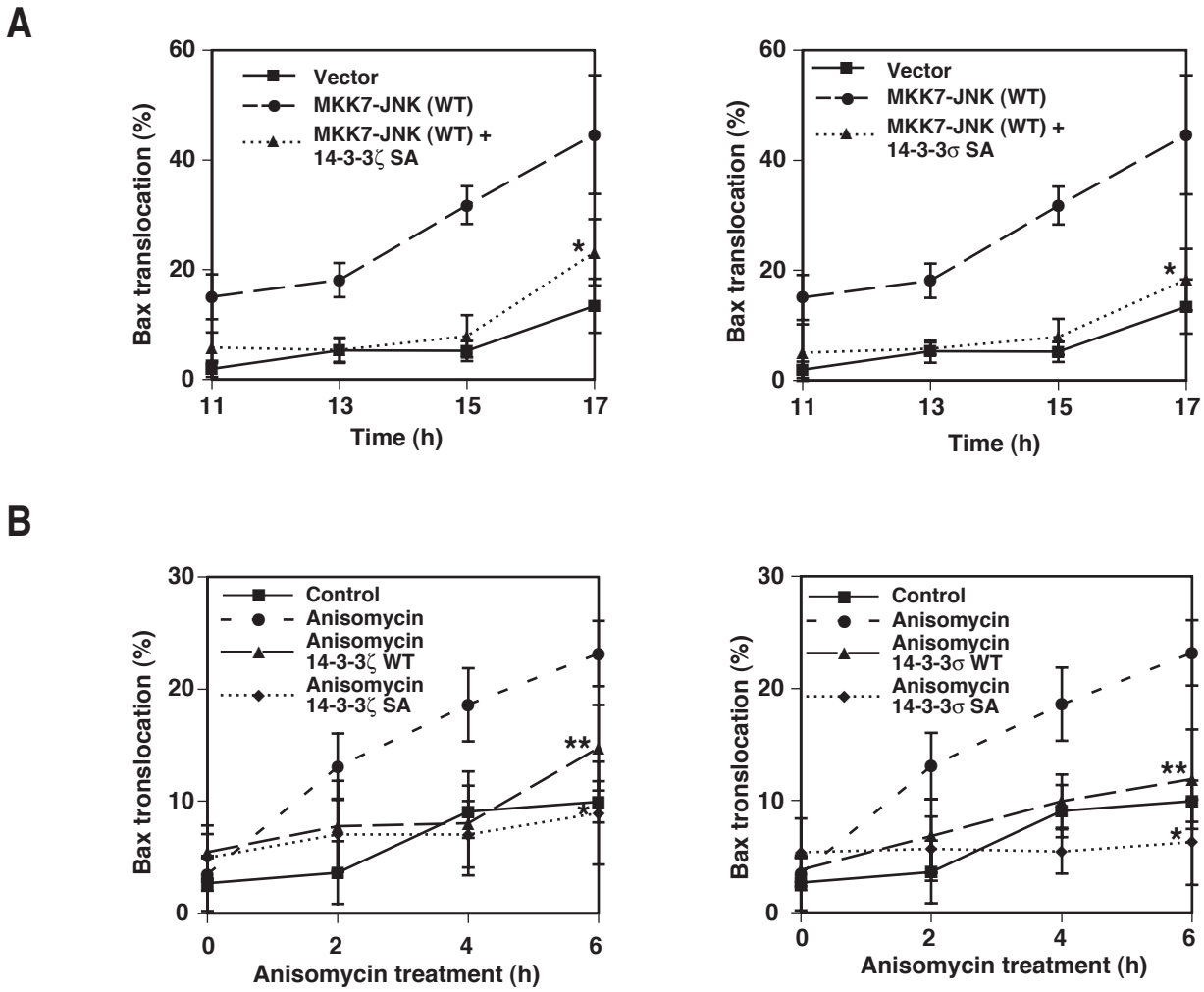

## Supplementary Figure7



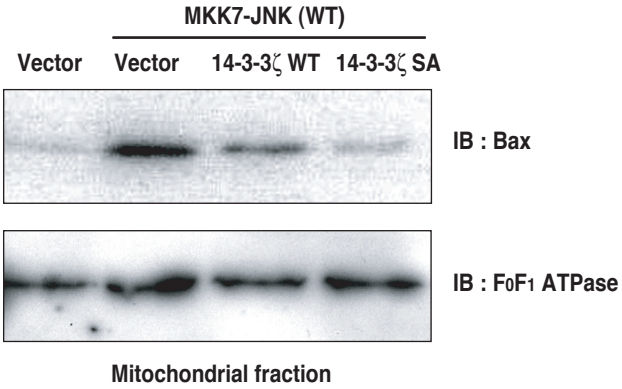

## Supplementary Figure 8



## Supplementary Figure 9




## Supplementary Figure 10




## Supplementary Figure 11