## Supplementary methods

### Cell culture and transfection and infection

WT, VDAC isoforms specific knockouts, bax/bak$^{-/-}$ (kindly provided by late Dr. Stanley J Korsmeyer, Dana Farber Cancer Institute), bak$^{-/-}$ and bax$^{-/-}$ (kindly provided by Dr. Craig B Thompson, Univ. of Pennsylvania) MEFs were cultured in DMEM supplemented with 10% fetal bovine serum as described before (Roy et al., 2009). For imaging experiments cells were plated onto poly-D-lysine-treated glass coverslips at a density of 20,000−25,000/cm$^2$ and were grown for 3−4 days. For cell suspension studies, cells were cultured for 4−6 days in 75 cm$^2$ flasks. Cells were transfected with plasmid DNA (cyto c-GFP, VDAC2 and mitochondrial matrix targeted DsRed; mtDsRed) by means of electroporation in suspensions (4.5x10$^6$ cells + 20 µg of each cDNA in 250 µl medium). Electroporation was carried out in a BTX-830 square-pulse generator in a 4mm gap cuvette using a single 275V 13ms pulse. For FACS sorting (MoFlo FACS sorter equipped with a 488nm laser), 8.5-12.5x10$^6$ cells transfected with cytochrome c-GFP and either mtDsRed or VDAC2 and were cultured for 24h. tBid adenoviruses (kindly provided by Dr. Atan Gross, Weizmann Institute) were produced and used as previously described (Sarig et al., 2003). VDAC2 adenovirus infection was done for 36-48 hrs using 2,000 viral particles per cell.

### $\Delta\Psi_m$ and cyto c release assay in suspension of permeabilized cells

Measurement of $\Delta\Psi_m$ in permeabilized MEFs was carried out as described previously (Madesh et al., 2002; Roy et al., 2009). Briefly, equal aliquots of cells (2.4 mg protein each) were resuspended and permeabilized with 20-40 µg/ml digitonin in 1.5 ml of an intracellular medium (ICM) composed of 120 mM KCl, 10 mM NaCl, 1 mM $KH_2PO_4$, Hepes/Tris 20 mM, pH 7.2 supplemented with 1 µg/ml of each antipain, leupeptin, pepstatin. For washout protocols, cells were permeabilized with 20-40 µg/ml digitonin in 1.5 ml of ICM supplemented with 1 µg/ml of each antipain, leupeptin, pepstatin and 2 mM MgATP for 5 min. Cells were separated rapidly by centrifugation at 10,000*g* for 5 minutes and the pellet was washed once with fresh ICM. Measurements were carried out in the presence of 2 mM MgATP and 2 mM succinate and 5µg/ml oligomycin was also added to prevent reversed function of the mitochondrial $H^+$ ATPase. Under this condition, depletion cytochrome c causes mitochondrial depolarization. To detect $\Delta\Psi_m$, TMRE (1-2µM) or Rhodamine 1,2,3 (R-123, 2µM) was used. Fluorescence was monitored in a fluorometer (Delta RAM, PTI) using ex: 540nm and em: 580nm for TMRE and ex: 500nm and em: 530nm for R-123. Experiments were carried out at 35˚C under continuous stirring.

tBid and Bax (Antonsson et al., 2001; Desagher et al., 1999) were kindly provided by Dr. Bruno Antonsson (Merck SERONO) or were obtained from R&D Systems.

At the end of the fluorimetric measurements of $\Delta\Psi m$, cytosol was separated from the membranes by centrifugation at 10,000*g* for 5 minutes or by a rapid filtration technique (Madesh et al., 2002). Lysates from membrane fractions and whole cells were generated by using RIPA buffer supplemented with complete protease inhibitor (Roche).

**Fluorescence imaging**

Prior to use, the transfected cells were pre-incubated for 20 minutes in an extracellular medium containing 2% BSA at 37$^{o}$C. For imaging of mitochondria in skinned cells, the samples were washed with a $Ca^{2+}$ free extracellular buffer and then incubated in ICM supplemented with 20-40µg/ml of digitonin (Pacher and Hajnoczky, 2001). After permeabilization, the cells were washed into fresh buffer without digitonin and incubated in the imaging chamber at 35$^{o}$C. In some experiments, propidium iodide (PI) was included in the buffer to determine the time of the plasma membrane permeabilization.

Fluorescence imaging was performed using an inverted microscope (Leica DMIRE2) fitted with a 40x objective (Uapo340, NA 1.35) or a 100x objective (HCX PL APO, NA 1.4) and a cooled CCD camera (Photometrics Ltd). A computer controlled motorized turret allowed us to alternate among optical filter sets (Chroma) for fluorescein (cyto c-GFP and Bak-GFP) and for rhodamine (mtDSRed) with a 6 s acquisition delay. All image analysis was done using custom designed software (Spectralyzer).

**Subcellular fractionation, mitochondrial cyto c release assay and Bax insertion**

Cells were harvested using trypsin/EDTA and washed with Na-Hepes/EGTA. All further steps were carried out at 4Cº. Cells were resuspended in a hyposmotic buffer (water (80%), ICM (20%), 5mM $MgCl_2$, 200µM EGTA and 1X complete protease inhibitors (Roche)) on ice for 10-15 min. Subsequently, the cells were homogenized in a dounce glass/glass homogenizer (30-35 strokes, tight pestle). To restore osmolarity 3 volume of 100% ICM supplemented with sucrose 125 mM, EGTA 200µM and $MgCl_2$ 5 mM was added. To eliminate unbroken cells and nuclei the homogenate was centrifuged at 750g (10 min). The supernatant was further centrifuged at 10,000g (15 min) and the pellet (mitochondrial fraction) was resuspended in 200-250µl ICM, protease inhibitors and EGTA (10µM) and stored on ice. Cyto c release assay was done using 0.5mg/ml mitochondria resuspended in ICM supplemented with 2 mM MgATP and 2 mM succinate and 5µg/ml oligomycin. Incubations were carried out at 35˚C under continuous stirring for 10 min. After the assay supernatant was separated from the pellet by centrifugation at 10,000*g* for 5

min. Pellets were lysed in RIPA buffer supplemented with complete protease inhibitor. For Bax insertion analysis, isolated mitochondria from control and tBid adenovirus infected cells or membrane fractions from permeabilized control and tBid treated cells were incubated for 30 min at 4°C in 0.1 M $Na_2CO_3$ (pH 11.5). The mitochondrial membranes, containing the integrated proteins, were pelleted by ultracentrifugation at 100,000 *g* for 60 min. Pellets were dissolved in SDS sample buffer, boiled and kept in -20°C for further use.

**Protein oligomerization analysis:**

For protein Bak/Bax oligomerization studies, the membrane fractions isolated after fluorimetric measurements of permeabilized cells were resuspended in PBS and then 10mM (final concentration) of the freshly prepared crosslinker BMH (Pierce) was added. The mixture was rotated for 30 min in room temp. and was quenched with 1M Tris, pH 7.5 with a final concentration 20mM. After quenching, RIPA buffer was added to the samples and rotated for 30 min in 4˚C followed by centrifugation at 12,000 rpm for 15 min. Supernatant was collected for immunoblot analysis.

**Treatment of cells with Cycloheximide and Proteasome inhibitors:**

MEFs were plated on 6 cm culture plates (Nunc) until 80% confluency. For proteasome inhibitor studies cells were treated either with 40uM of MG132 (Biomol) or 0.5mM of o-Phenanthroline (Sigma) for 8hr. For Cycloheximide (CHX, Sigma) studies, cells were incubated for 6hr or 12hr in presence of 1mM CHX. Respective vehicle controls were run simultaneously. After incubation cells were harvested and permeabilized with digitonin for 5min as mentioned in the analysis of suspended permeabilized cells. Membrane fractions were separated out by centrifugation after permeabilization and lysates from membrane fractions were generated by using RIPA buffer supplemented with complete protease inhibitor (Roche).

**Immunoblot analysis:**

For Western blot analysis, proteins 25–30 µg (from cytosol and membrane fractions) were loaded in each lane of a 12% SDS-PAGE and electrophoretically transferred to nitrocellulose filters. Filters were blocked with blocking buffer (Pierce) overnight, followed by incubation with primary antibodies according to the dilutions recommended by the manufacturers. Primary antibodies were anti–cyto c (clone 7H8.2C12; Pharmingen), anti-Bid (R&D Systems), anti-Bak NT (Upstate), anti-Bax N20 (Santa Cruz), anti-VDAC2, anti-VDAC1, anti-Prohibitin, anti-Tom40 (all from Abcam), anti-OPA1 and anti-Actin (from

BD Transduction). After incubation with the primary antibody, bound antibodies were visualized using horseradish peroxidase–coupled secondary antibody (GE Healthcare) and West Pico chemiluminescence–developing kit (Pierce).

**Fas induction and assessment of Caspase-3 activity:**

MEFs were plated on 35 mm dishes and treated with 2ug/ml CHX (sigma) and 500ng/ml Fas antibody (clone Jo2, BD Pharmingen). Cells were harvested in specific time points and cell lysates were generated by using RIPA buffer supplemented with complete protease inhibitor (Roche).

Caspas3 activities of equal amount of protein of different samples were measured by using Ac-DEVD-AMC (Alexis) according to the manufacturer's protocol. Assay was done in a fluorescence plate reader (Molecular Devices) at 37˚C for 60min using an excitation wavelength 380nm and an emission wavelength 460nm.

**Flow cytometry**

WT and $V2^{-/-}$ MEFs (80-85% confluency) were grown on 35mm dishes in normal serum containing medium and infected with tBid adenovirus for 16h. Then the cells (both attached and detached) were harvested and washed once with PBS. Cell pellets were incubated with Annexin-V Alexa Fluor 488 conjugate (1:40 dilution; Molecular Probes) and PI (2.5µg/ml) at room temperature in dark for 15 min. Samples were analyzed within 1hr by a flow cytometer (Beckman Coulter Epics, 488nm and 568nm excitation).

**Statistics**

Experiments were carried out with ≥3 different cell preparations. Data are presented as means ± S.E.M. Significance of differences from the relevant controls was calculated by Student's *t* test.

## Supplementary figure legends:

**FigS1.** Analogue traces showed $\Delta\Psi m$ as monitored in permeabilized WT, $V1^{-/-}$, $V3^{-/-}$ and $V1/3^{-/-}$ MEFs treated with 3.7nM of recombinant tBid for 5 min. FCCP (5μM) was added at the end of each run to depolarize the entire mitochondrial population. Immunoblot of rapidly separated supernatants (cytosol) showed the release of cytochrome c upon tBid (3.7nM) treatment.

**FigS2.** Immunoblot of the mitochondrial protein OPA1 showed the purity of the isolated mitochondrial preparations used in the cyto c release assay.

**FigS3.** Attenuated Caspase-3 activition evoked by Fas antibody treatment (Jo2 for 18hrs) in $V2^{-/-}$ MEFs.

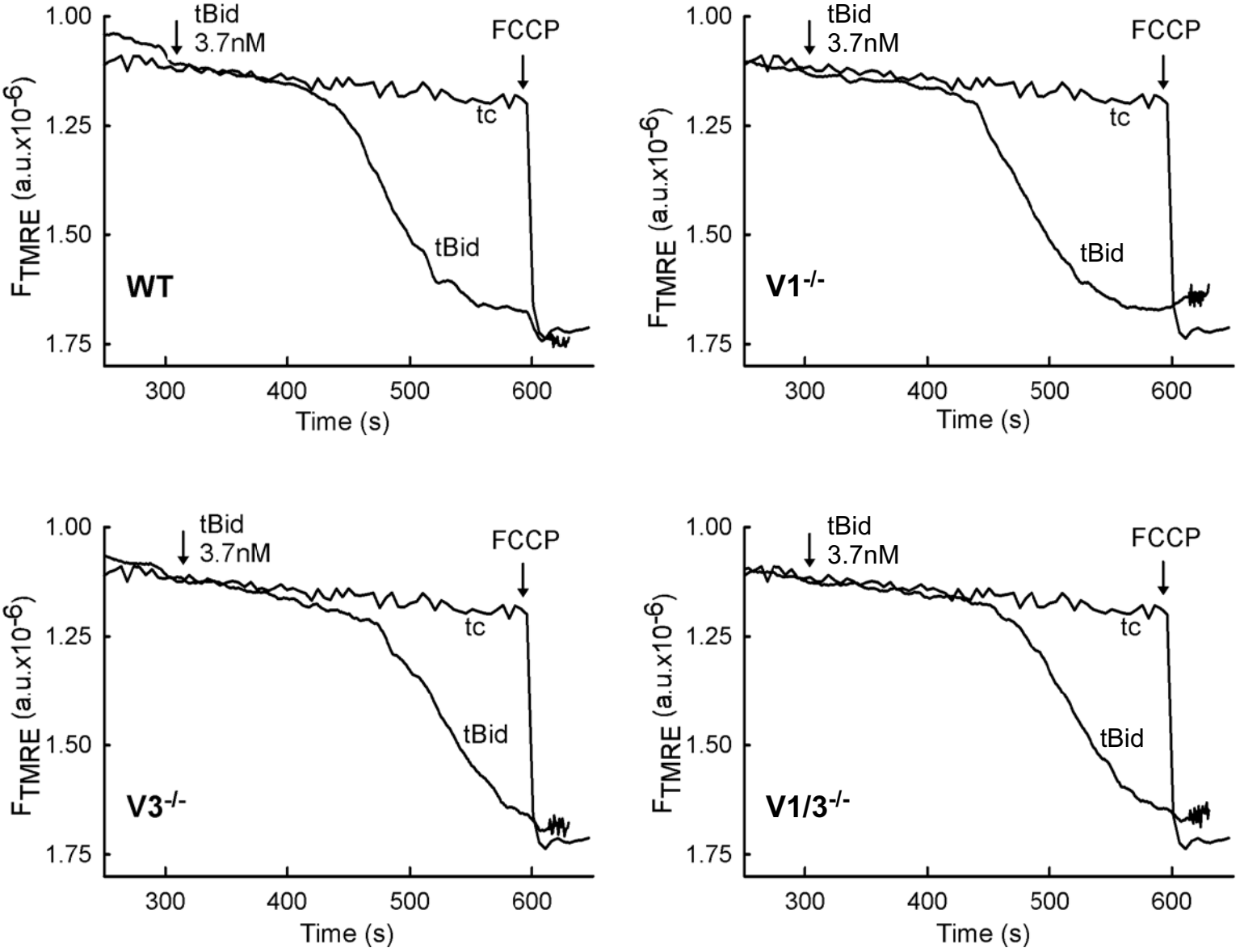
**FigS4. (A)** Longer exposed film of the immunoblot showing the level of Bak in the membrane fractions of permeabilized WT and different VDAC KO MEFs. **(B)** Immunoblot showed the level of VDAC1 in the whole cell lysates and the cytosolic fractions of permeabilized WT and $V2^{-/-}$ MEFs. Partial permeabilization by digitonin did not cause the extraction of the membrane bound proteins. Actin was used as a loading control. **(C)** Immunoblot showed the level of Bak and Tom40 in the membrane fractions of $V2^{-/-}$ MEFs treated with MG132 and o-Phenanthroline for 8hr. There was no obvious change at the level of Bak which indicated the lack of rapid turnover of this protein. **(D)** Immunoblot showed the level of Bak in the membrane fractions of permeabilized WT and $V2^{-/-}$ MEFs treated with Cycloheximide for two different time points. There was no obvious change in the level of Bak which indicates the lack of decreased stability of this protein in $V2^{-/-}$ MEFs. Prohibitin was used as a loading control. **(E)** Bak insertion to the mitochondria was studied in WT, $V2^{-/-}$ and rescued $V2^{-/-}$ MEFs transfected with Bak-GFP and mtDsRed. Images of intact cells show that Bak-GFP was localized to mitochondria and caused mitochondrial derangement in WT MEFs, whereas it distributed in the entire cytoplasm and caused no obvious mitochondrial morphology change in $V2^{-/-}$ MEFs (images in the left). Scalebar is 10um. For quantitative analysis of the membrane insertion of Bak-GFP, green and red fluorescence images were taken before and after plasma membrane permeabilization and the fluorescence retained after permeabilization was normalized to the pre-permeabilization value in each cell. Mean±SE values were calculated for the single cells (n=30-50cells) and were plotted in the bar charts (right). The results show that a substantial fraction of Bak-GFP fluorescence was released by plasma membrane permeabilization only in $V2^{-/-}$ MEFS ($p<0.02$), indicating the lack of membrane integration. The mitochondria-targeted DsRed fluorescence was insensitive to plasma membrane permeabilization in every cell type.

**FigS5. (A)** Isolated membrane fractions obtained from tBid-treated permeabilized cell suspensions of $V2^{-/-}$ and rescued $V2^{-/-}$ MEFs were treated with BMH crosslinker. Another aliquot of the membrane fractions was treated with 0.1M $Na_2CO_3$ instead of crosslinker. Immunoblots showed clear membrane localization of Bak in rescued $V2^{-/-}$ MEFs and Bak oligomerization in tBid-treated samples. However, membrane insertion and oligomerization of Bax was not detectable under similar conditions. **(B)** Mitochondria were isolated from control and ad-tBid infected (16 hrs) WT and $V2^{-/-}$ MEFs. Isolated mitochondria were alkali (0.1M $Na_2CO_3$) treated and ultracentrifuged to harvest the pellet which contains membrane inserted proteins. Immunoblot of the pellet lysate showed higher Bax insertion in the mitochondria of the tBid expressing WT and $V2^{-/-}$ MEFs. Prohibitin was used as loading control.

**FigS6. (A)** Comparison of the Caspase-3 activity of WT, $bak^{-/-}$ and $bax^{-/-}$ MEFs after 5 and 10 hrs of treatment with an anti-Fas antibody, respectively. $Bak^{-/-}$ MEFs showed less Caspase-3 activation than WT and $bax^{-/-}$ MEFs. **(B)** Immunoblot showed similar level of VDAC2 in the membrane fractions of permeabilized WT, $bak^{-/-}$ and $bax^{-/-}$ MEFs. **(C)** Immunoblot showing the level of Bax and Bak in the whole cell lysates of WT, $bak^{-/-}$ and $bax^{-/-}$ MEFs. Bax was overexpressed in normal $bak^{-/-}$ MEFs. Actin was used as a loading control. **(D)** Immunoblot of the cytosol of permeabilized cell and permeabilized washed cells showed the washout-caused removal of the cytosolic Bax in WT, $bak^{-/-}$ and $bax^{-/-}$ MEFs.

**FigS7.** Interplay between VDAC2, Bak and Bax in the tBid induced OMM permeabilization.
Schematic presentation of VDAC2, Bak and Bax in the OMM of WT, $V2^{-/-}$ and $bak^{-/-}$ MEFs.

Fig S1


tBid
3.7nM
FCCP
tc
tBid
WT
V1-/-
V3-/-
V1/3-/-
FTMRE (a.u.x10-6)
1.00
1.25
1.50
1.75
300
400
500
600
Time (s)

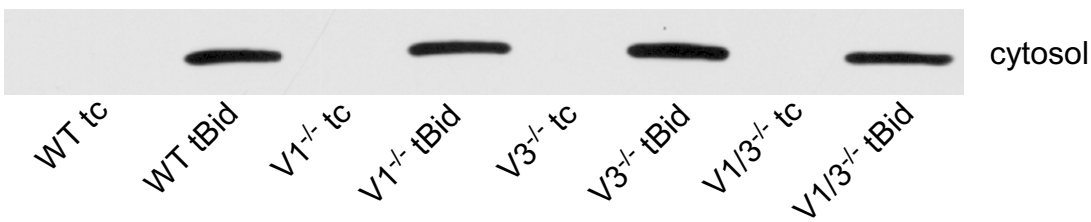

Cyto C
cytosol
WT tc
WT tBid
V1-/- tc
V1-/- tBid
V3-/- tc
V3-/- tBid
V1/3-/- tc
V1/3-/- tBid

Fig S2

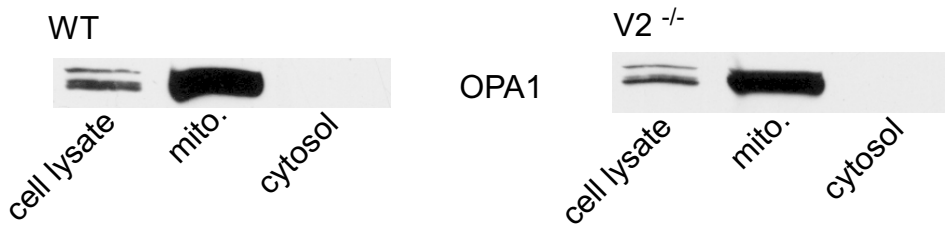

Fraction purity controls
WT
V2 -/-
OPA1
cell lysate
mito.
cytosol
cell lysate
mito.
cytosol

Fig S3

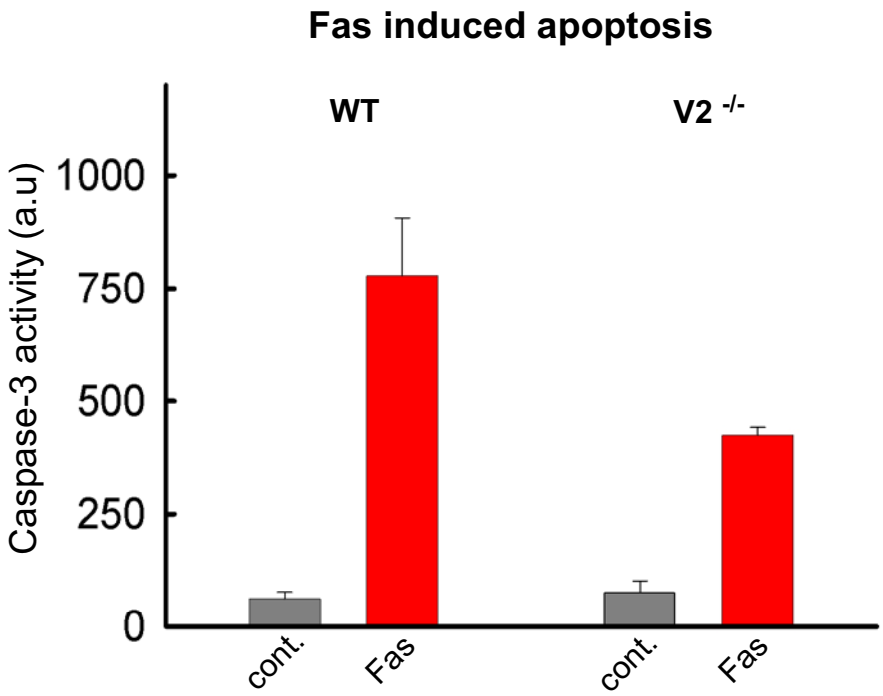
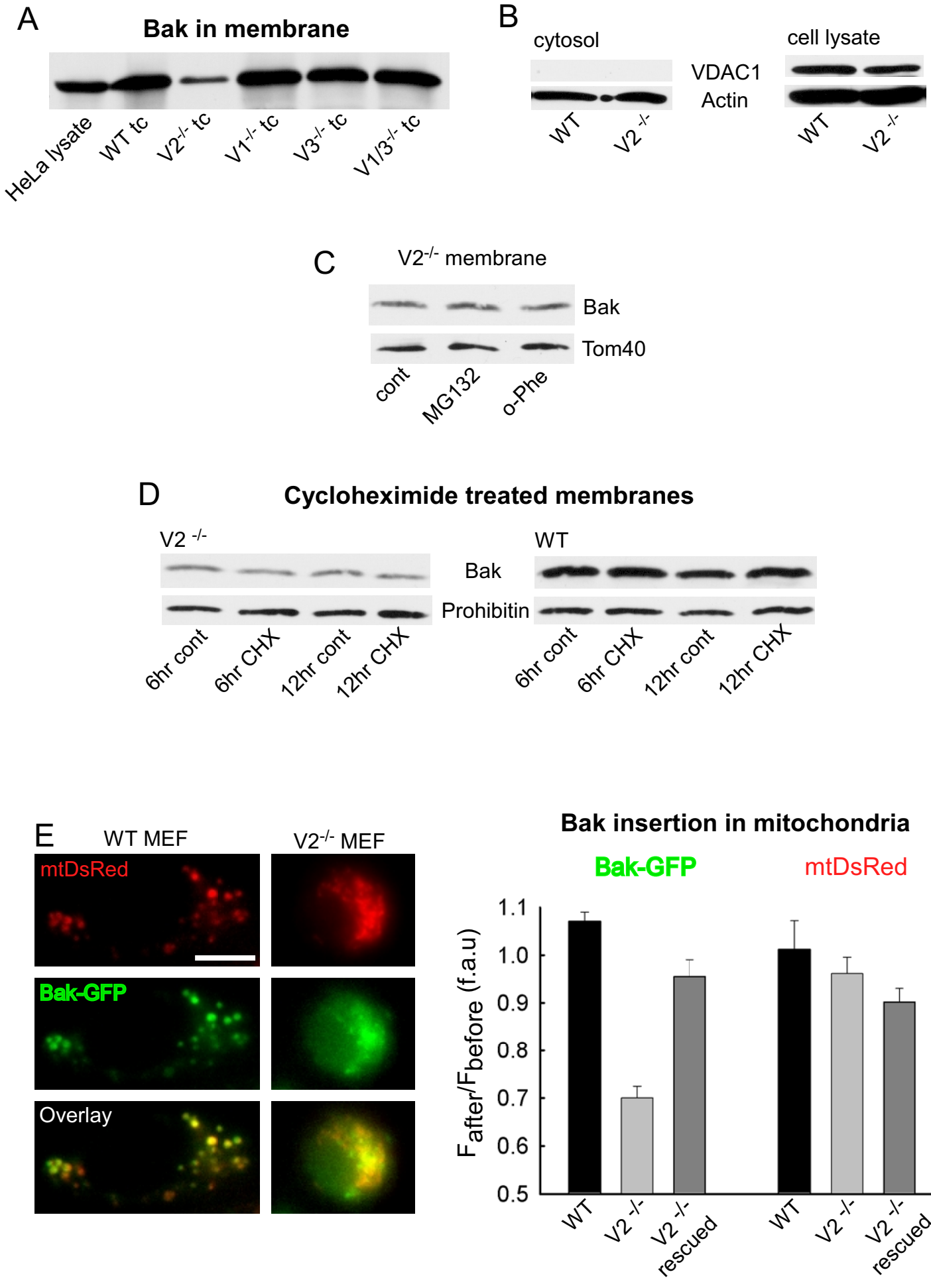
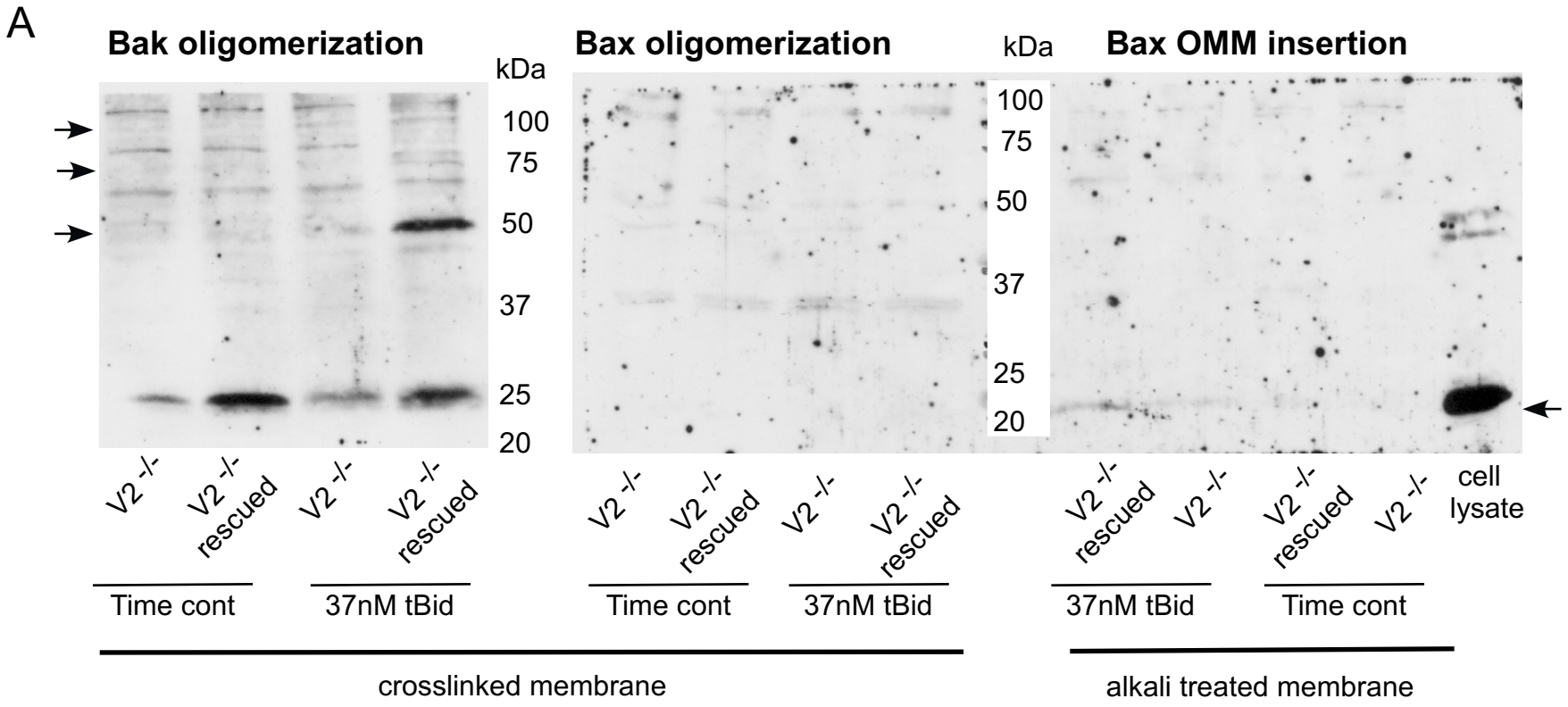

Fas induced apoptosis
WT
V2 -/-
Caspase-3 activity (a.u)
1000
750
500
250
0
cont.
Fas
cont.
Fas

Fig S4

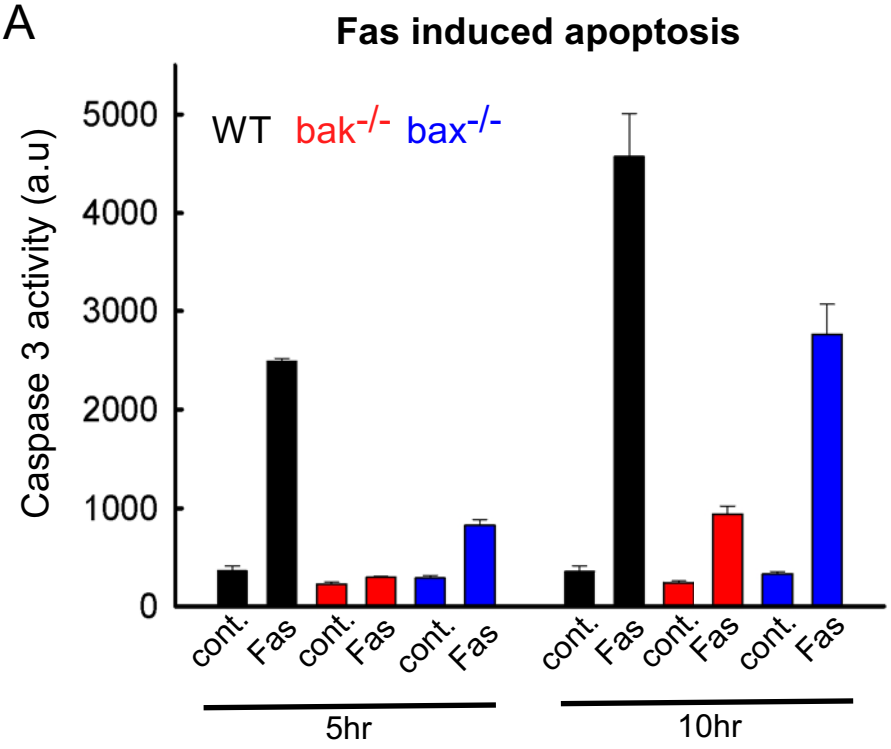

A
Bak in membrane
HeLa lysate
WT tc
V2-/- tc
V1-/- tc
V3-/- tc
V1/3-/- tc
B
cytosol
cell lysate
VDAC1
Actin
WT
V2 -/-
WT
V2 -/-
C
V2-/- membrane
Bak
Tom40
cont
MG132
o-Phe
D
Cycloheximide treated membranes
V2 -/-
WT
Bak
Prohibitin
6hr cont
6hr CHX
12hr cont
12hr CHX
6hr cont
6hr CHX
12hr cont
12hr CHX
E
WT MEF
V2-/- MEF
mtDsRed
Bak-GFP
Overlay
Bak insertion in mitochondria
Bak-GFP
mtDsRed
Fafter/Fbefore (f.a.u)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
WT
V2 -/-
V2 -/- rescued
WT
V2 -/-
V2 -/- rescued


A
Bak oligomerization
kDa
100
75
50
37
25
20
Bax oligomerization
kDa
100
75
50
37
25
20
Bax OMM insertion
V2 -/-
V2 -/- rescued
V2 -/-
V2 -/- rescued
V2 -/-
V2 -/- rescued
V2 -/-
V2 -/- rescued
V2 -/- rescued
V2 -/-
V2 -/- rescued
V2 -/-
cell lysate
Time cont
37nM tBid
Time cont
37nM tBid
37nM tBid
Time cont
crosslinked membrane
alkali treated membrane

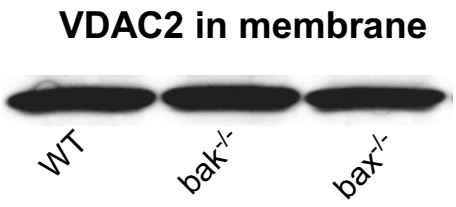

B
Bax insertion in mito. membrane
WT
V2 -/-
Bax
Prohibitin
cont
tBid
cont
tBid

Fig S6

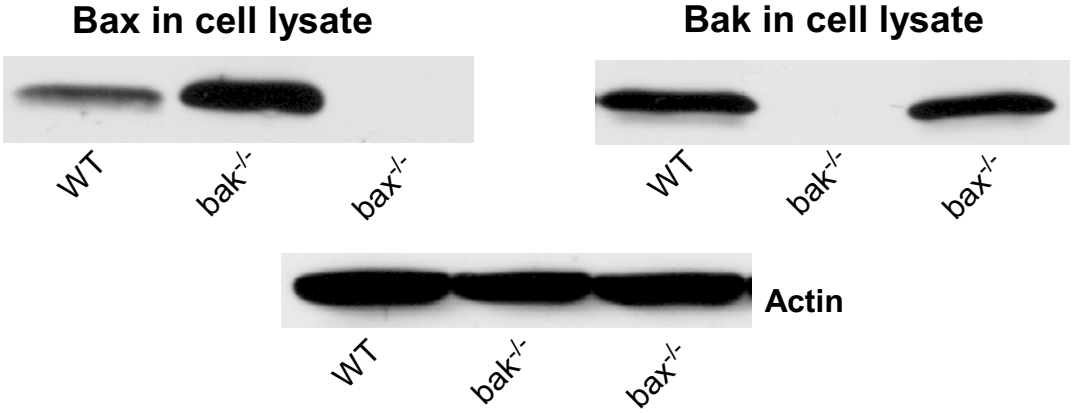
A


Fas induced apoptosis
Caspase 3 activity (a.u)
5000
4000
3000
2000
1000
0
WT
bak-/-
bax-/-
cont.
Fas
5hr
10hr

B


VDAC2 in membrane
WT
bak-/-
bax-/-

C


Bax in cell lysate
Bak in cell lysate
WT
bak-/-
bax-/-
Actin

D

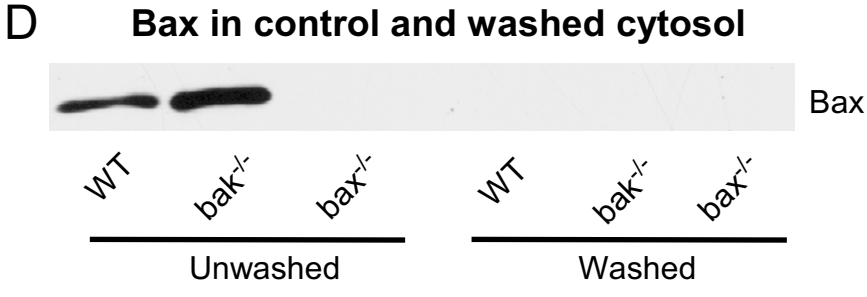
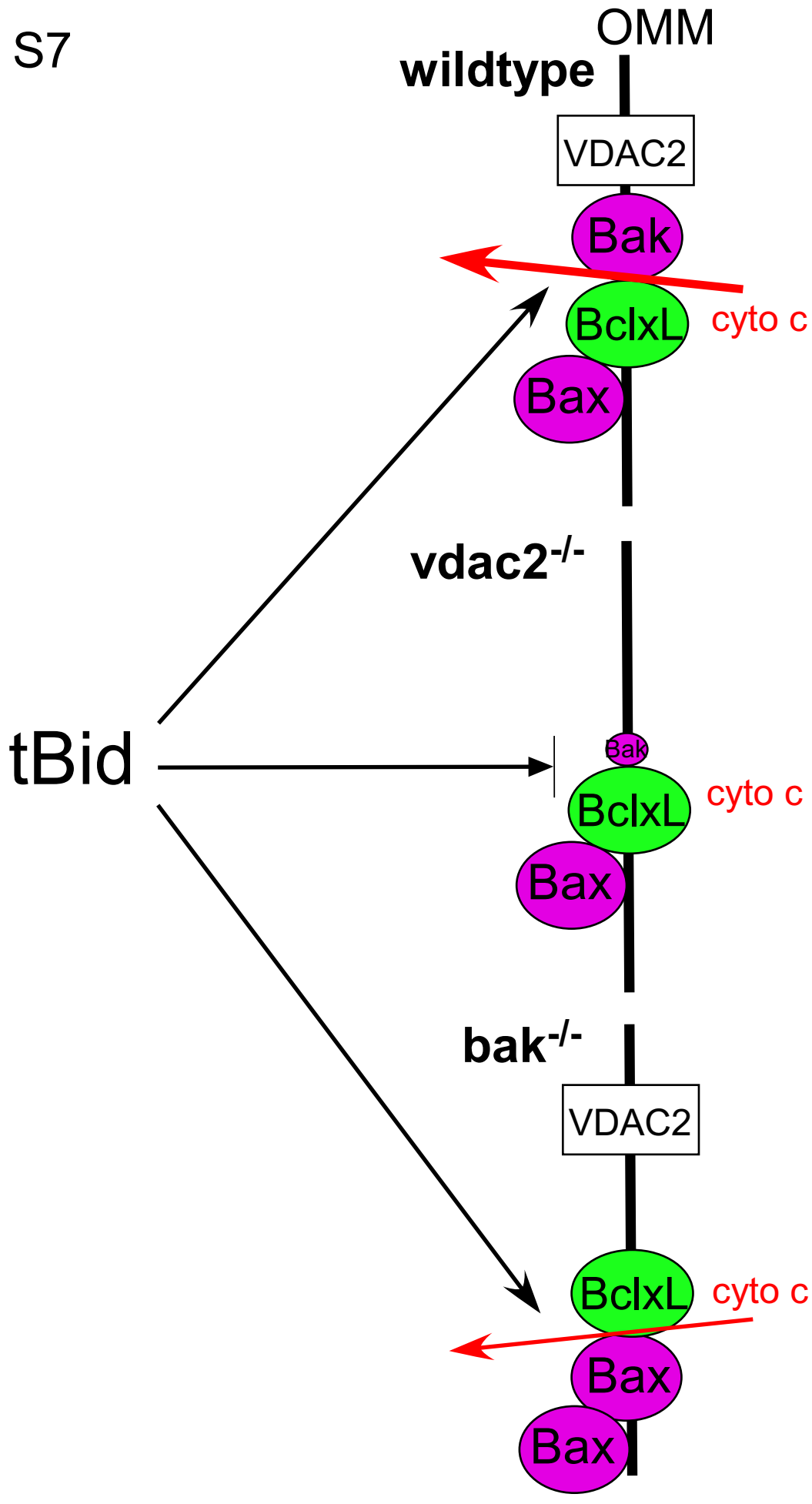

Bax in control and washed cytosol
Bax
WT
bak-/-
bax-/-
Unwashed
Washed

Fig S7


OMM
wildtype
VDAC2
Bak
BclxL
cyto c
Bax
vdac2-/-
tBid
Bak
BclxL
cyto c
Bax
bak-/-
VDAC2
BclxL
cyto c
Bax
Bax