Supporting Information

Perumalsamy et al. 10.1073/pnas.0910060107

SI Methods

Immunoprecipitation. 10×10^6 cells were lysed for 10 min at room temperature (RT) using MPER buffer (Pierce) supplemented with protease inhibitors (Sigma-Aldrich). Cell lysates were incubated with 10 μg indicated antibodies (Mfn2, Akt from Santa Cruz and Isotype controls from Sigma) for 1 h at RT on a rotational cell mixer. Immune complexes were precipitated for 2 h at 4 °C using Sepharose G plus beads on a rotational cell mixer. Beads bound to complexes were washed five times with ice-cold PBS at 1,700 rpm. Finally, beads were boiled in SDS lysis buffer for 10 min before Western blot analysis.

TMRM Staining. TMRM (tetramethyl rhodamine methyl ester) from Sigma was used for the assessment of mitochondrial transmembrane potential. HeLa cells in the different experimental conditions were incubated with 50 nM TMRM in complete media for 15 min at 37 °C, followed by two washes with PBS. Cells were imaged by confocal microscopy using Zeiss LSM 510 Meta, Plan-Apochromat 63× NA 1.4 oil with sequential excitation and emission conditions for EGFP or TMRM. TMRM fluorescence intensity in the mitochondria was quantitated in EGFP-positive cells by determining average pixel intensity in the red channel using Image J software.

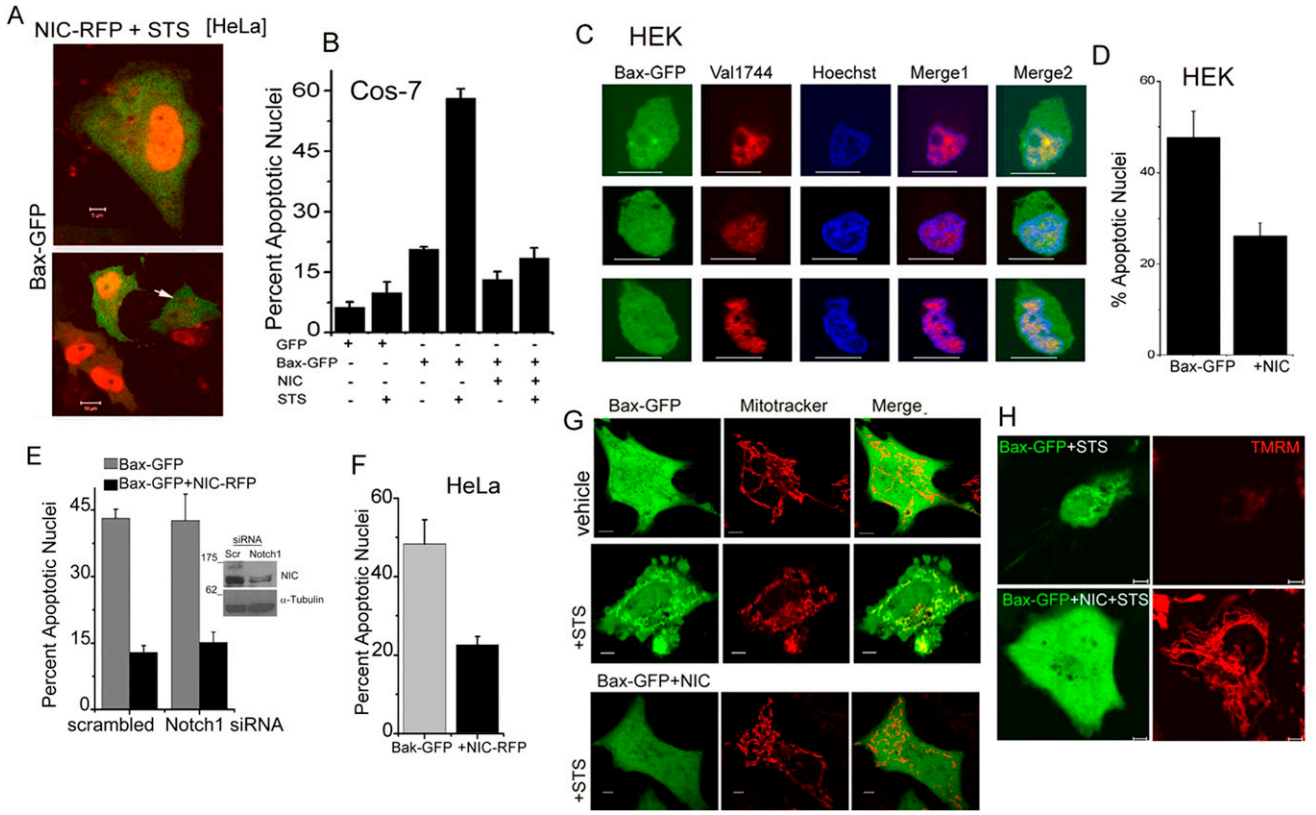


Fig. S1. (*A*) Confocal images of HeLa cells expressing Bax-GFP (0.5 μg) + NIC-RFP (3 μg), treated with 1 μM STS for 1 h. (Scale bar: 5 μm.) Arrowhead shows Bax puncta formation in a cell not expressing NIC-RFP. (*B*) COS-7 cells transfected with Bax-GFP (0.5 μg) + pcDNA (3 μg) or Bax-GFP (0.5 μg) + NIC (3 μg); following overnight incubation, cells were treated with 1 μM STS for 1 h and nuclear damage was scored in GFP positive cells Data plotted are mean ± SD from three individual experiments. (*C*) Cellular localization of NIC detected using Val-1744 primary antibody (red) in HEK cells transfected with Bax-GFP (1 μg) and NIC (3 μg). Nuclei were stained with Hoechst 33342 (blue). Merge 1 is of Val-1744 (red) with the nucleus (blue) and Merge 2 is of Bax-GFP (green) with NIC (red) and nucleus (blue). (Scale bar: 10 μm.) (*D*) HEK cells transfected with Bax-GFP (1 μg) with or without NIC (3 μg) were cultured for 20 h and nuclear damage was scored in GFP positive cells. Data plotted are mean ± SD from three individual experiments. (*E*) HeLa cells pretreated with scrambled or Notch1 siRNA for 48 h were transfected with Bax-GFP or Bax-GFP+NIC, cultured overnight, and scored for STS-induced apoptotic damage. (*Inset*) Immunoblot (representative of two experiments) confirming the loss of Notch1 protein in cells treated with siRNA to Notch1. (*F*) HeLa cells transfected with GFP, Bak-GFP, or GFP+NIC-RFP or Bak-GFP+NIC-RFP were cultured overnight. In all cases, the amount of DNA was held constant by the addition of the RFP empty vector control. Apoptotic nuclear damage was estimated in cells positive for both GFP and RFP. The data are normalized to apoptotic damage in the GFP group and the mean ± SD from three individual experiments plotted. (*G*) Cells expressing Bax-GFP with or without NIC were stained with Mitotracker Red and either treated with a vehicle control or STS for 1 h and imaged by confocal microscopy. $n > 50$ cells in each case. (Scale bar: 5 μm.) (*H*) HeLa cells transfected with Bax-GFP or Bax-GFP + NIC, cultured overnight, and then treated with STS for 1 h were stained with TMRM and imaged. Confocal images of central planes of cells are shown. (Scale bar: 5 μm.)

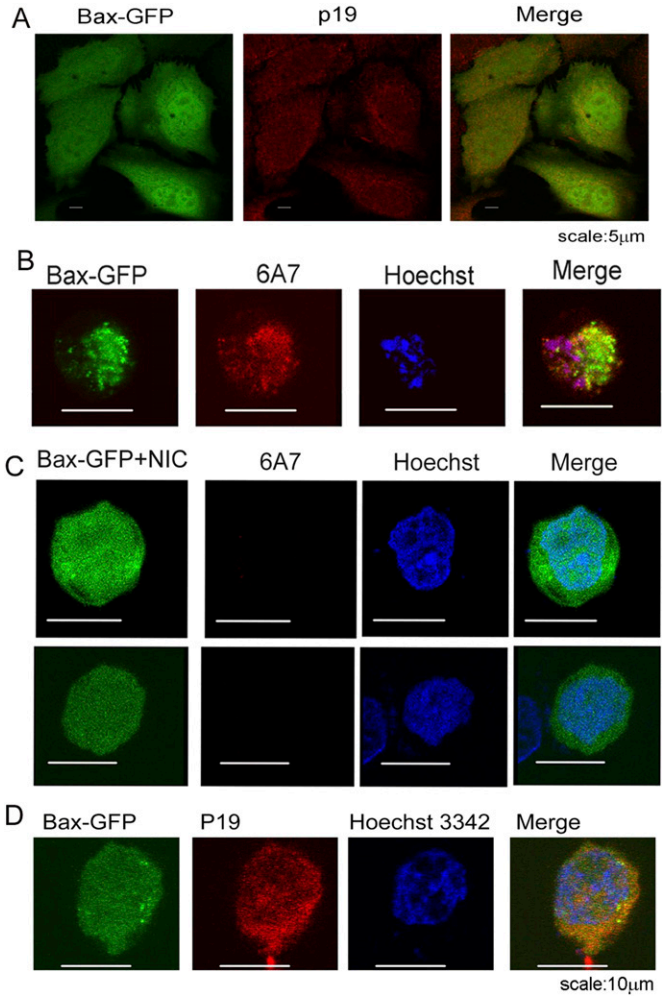


Fig. S2. (*A*) HeLa cells transfected with Bax-GFP (0.5 μg) and stained for total Bax distribution with clone P-19. (Scale bar: 5 μm.) (*B* and *C*) HEK cells transfected with Bax-GFP (1 μg) (B) or Bax-GFP (1 μg) +NIC (3 μg). (*C*) were harvested after 20 h and stained with clone 6A7. Cells were counter stained with Hoechst 33342. (Scale bar: 10 μm.) (*D*) p-19 staining in Bax-GFP expressing HEK cells counter stained with Hoechst 33342. (Scale bar: 10 μm.)

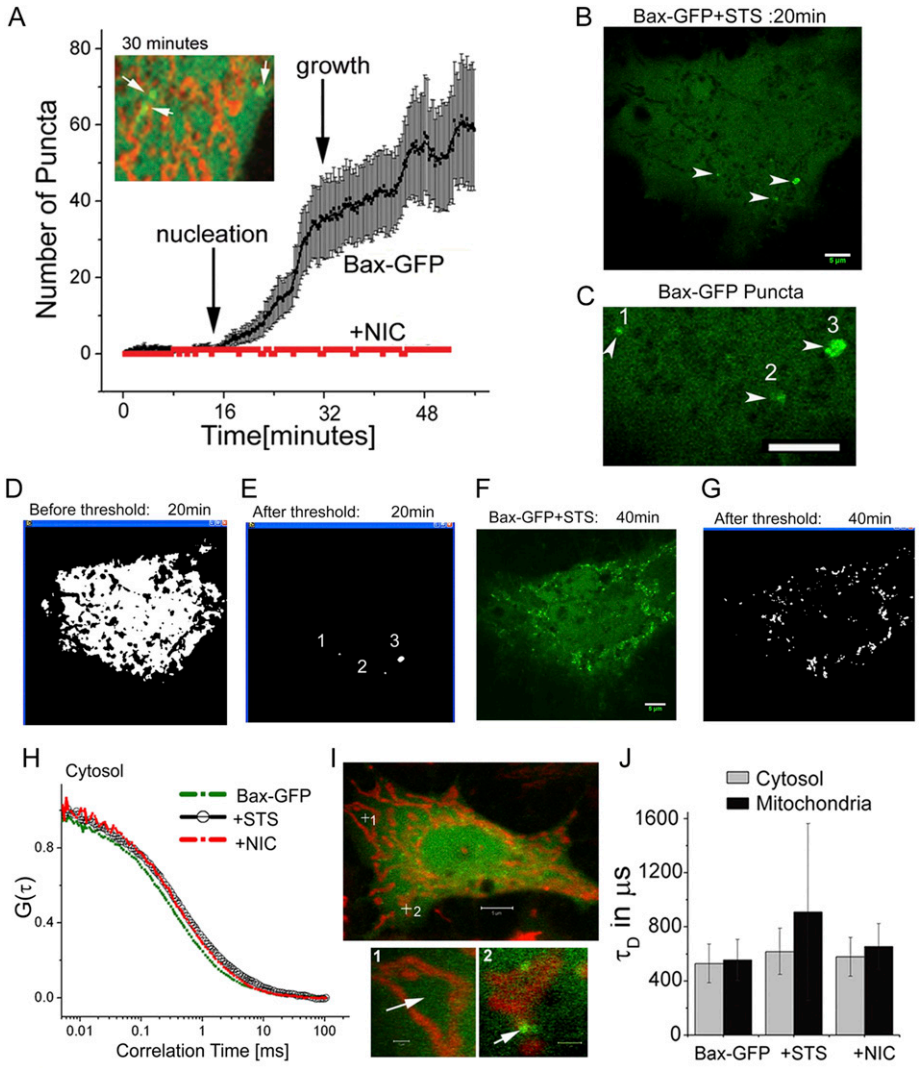


Fig. S3. (*A*) Averaged Bax puncta analysis as detailed in methods, showing the number of Bax-GFP puncta as a function of time in cells expressing Bax-GFP or Bax-GFP+NIC, following the addition of STS. The curve is plotted after a 5-point smoothening. Data represent mean ± SEM of the recordings of 16 cells. (*Inset*) enlarged section of an STS-treated Bax-GFP expressing cell (from Movie S2 at 30 min), which has been counterstained with Mitotracker Red. Arrowheads indicate Bax puncta. Bax-GFP puncta were analyzed with a custom-made Labview program, which calculated the number of Bax-GFP puncta in each frame. Bax-GFP puncta were identified by setting a threshold above the background of diffuse Bax-GFP. (*B*) Movie still (from Movie S1) of a Bax-GFP expressing cell 20 min after the addition of STS. (*C*) Enlarged section of a portion of the cell in *B*. Arrowheads mark Bax-GFP puncta. (*D*) Corresponding image of *B* taken from the Labview program before setting a threshold. (*E*) Image of the cell in *B* after threshold revealing the three puncta also seen in *B*. (*F*) Movie still (from Movie S1) of a Bax-GFP expressing cell 40 min after the addition of STS. (*G*) Corresponding image of *D* from the Labview program following thresholding. (Scale bar: 5 μm.) (*H*) HeLa cells expressing Bax-GFP or Bax-GFP+NIC were stained with Mitotracker Red and treated with vehicle or STS for half an hour before analysis by FCS. The associated autocorrelation curves of Bax-GFP in cytosol are shown (n = 8). (*I*) Confocal image of STS-treated Bax-GFP expressing cell showing the positions of recordings in the cytosol (1) and mitochondria (2) in the FCS analysis. (Scale bar: 5 μm.) Detail micrographs enlarged from the images to show the positions (arrow) for FCS reading in cytosol (1) and on mitochondria (2). (Scale bar: 2 μm.) (*J*) The correlation time (τ_D in microseconds) of Bax-GFP in the cytosol and near mitochondria in the conditions indicated (n = 15 cells for Bax-GFP, 54 cells for Bax-GFP+STS and 15 cells for Bax-GFP+NIC+STS).

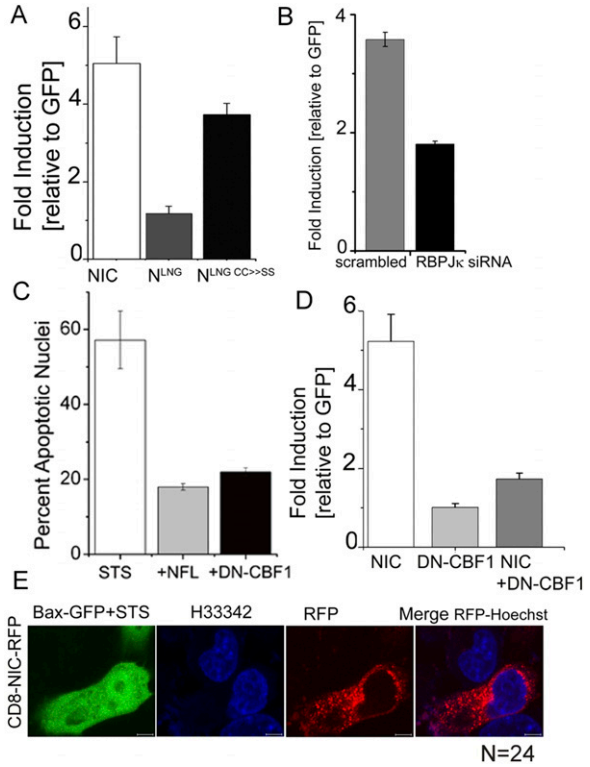


Fig. S4. (*A*) Luciferase activity assessed 20 h after cells were cotransfected with GFP (1 μg) or N^{LNG} or $N^{LNG\ CC>>SS}$ and the Hes1 promoter driven luciferase construct. The *Renilla* luciferase construct was used as transfection control. Data are presented as fold induction in activity with respect to GFP. (*B*) Cells were pretreated with siRNA to RBP-Jκ or a scrambled control for 48 h. Cells were transfected with GFP (1 μg) or NIC and the Hes1 promoter driven luciferase construct and luciferase activity assessed 20 h later as described in *A*. (*C*) HeLa cells transfected with Bax-GFP or Bax-GFP + NFL or Bax-GFP+NFL+ DN-CBF1 were cultured overnight and scored for STS-induced apoptotic damage as described in methods. (*D*) Luciferase activity assessed 20 h after cells were cotransfected with GFP (1 μg) or NIC or DN-CBF1 or NIC+DN-CBF1 (3 μg) and the Hes1 promoter driven luciferase construct as described in *A*. (*A–D*) Data plotted are the mean± SD of three separate experiments. (*E*) Cells were transfected with Bax-GFP and CD8-NIC-RFP. After 14 h cultures were treated with STS for 1 h, counterstained with Hoechst 33342 to mark nuclei and imaged. Confocal images of central planes of cells are shown. (Scale bar: 5 μm.)

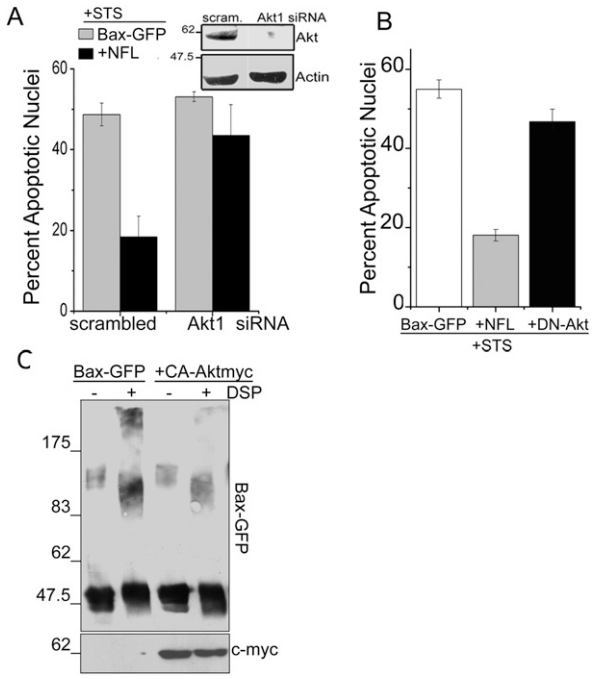


Fig. S5. Akt is required for Notch-mediated inhibition of apoptosis. (*A*) HeLa cells pretreated with siRNA to Akt1 or a scrambled control for 48 h were transfected with Bax-GFP or Bax-GFP+NFL. Cultures were scored for STS-induced apoptotic damage as described in *Methods*. Data are mean ± SD of three independent experiments. (*Inset*) Immunoblot (representative of two experiments) confirming the loss of Akt protein in cells treated with siRNA to Akt1. Actin established parity of loading. (*B*) HeLa cells transfected with Bax-GFP or Bax-GFP+NFL or Bax-GFP+NFL+ DN-Akt were cultured overnight and scored for STS-induced apoptotic damage as described in *Methods*. Data plotted are the mean ± SD of three separate experiments. (*C*) HEK cells were transfected with Bax-GFP $^{+/-}$ CA-Akt-myc. At 20 h post transfection, cell lysates were processed as in Fig. 2*D*. Immunoblots were probed with antibodies to GFP and c-myc to detect Bax-GFP and CA-Akt, respectively. One of two experiments is shown.

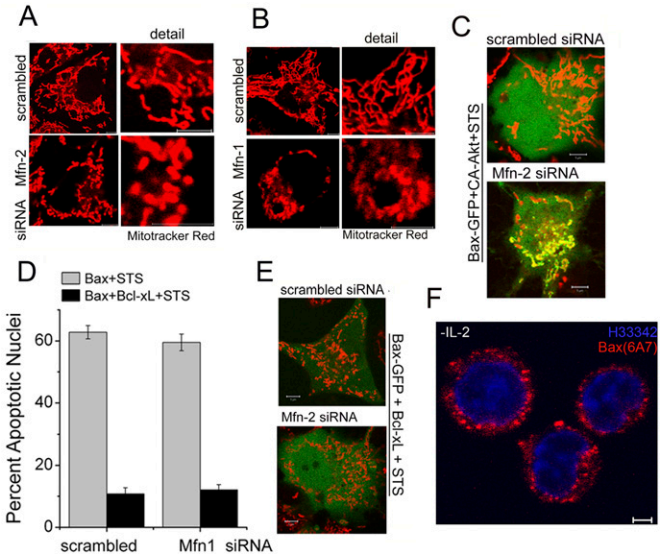
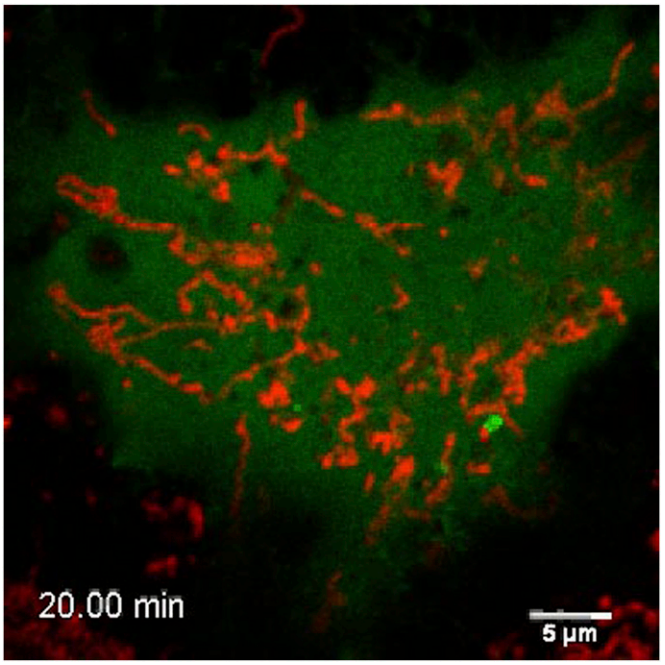
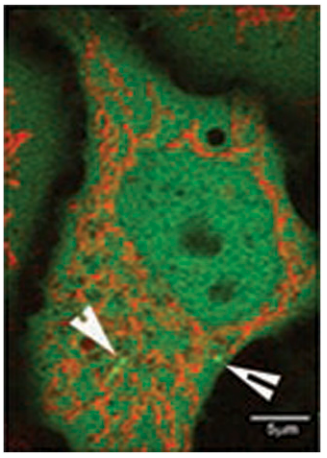


Fig. S6. Mitofusins are intermediates in the antiapoptotic cascade. **(***A*–*B*) Confocal images of cells pretreated with siRNA to Mfn-2 (*A*), Mfn-1 (*B*), or scrambled control for 48 h and imaged after loading with Mitotracker Red. The micrographs show details from the images. (Scale bar: 5 μm.) (*C*) Confocal images of cells (*n* = 45) pretreated with Mfn-2 or scrambled siRNA and transfected with the indicated constructs counterstained with Mitotracker Red. (*D*) HeLa cells pretreated with scrambled or Mfn1 siRNA were transfected with Bax-GFP or Bax-GFP+Bcl-xL. Cells were cultured overnight and then scored for STS-induced apoptotic damage. Data plotted are mean ± SD derived from three independent experiments. (*E*) Confocal images (central plane) of scrambled or Mfn2 siRNA pretreated cells transfected with Bax-GFP + Bcl-x_L (3 μg) and treated with 1 μM STS for 1 hour. Cells were counterstained with Mitotracker Red. (Scale bar: 5 μm.) Representative of 20 cells. (*F*) Representative field-view showing 6A7 reactivity in activated T cells cultured without cytokine.



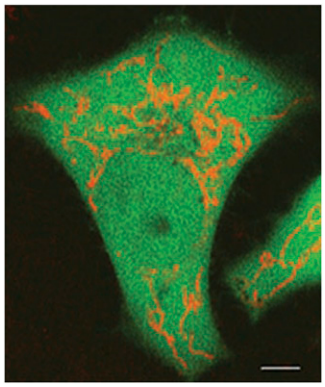
Movie S1. Appearance of Bax-GFP puncta in live HeLa cells expressing Bax-GFP, 20 min after the addition of STS.

Movie S1



Movie S2. Appearance of Bax-GFP puncta in live HeLa cells expressing Bax-GFP, 10 min after the addition of STS.

Movie S2



Movie S3. Live imaging of HeLa cells expressing Bax-GFP and NIC from 10 min after the addition of STS.

Movie S3