

Notch-activated signaling cascade interacts with mitochondrial remodeling proteins to regulate cell survival

Lakshmi R. Perumalsamy, Manjula Nagala, and Apurva Sarin¹

National Centre for Biological Sciences, Bangalore, Karnataka 560065, India

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Survival of differentiated cells is one of several processes regulated by Notch activity, although the general principles underlying this function remain to be characterized. Here, we probe the mechanism underlying Notch-mediated survival, building on emerging evidence that apoptotic responses coordinated by specialized intermediates converge on mitochondria, identifying a core event in death pathways. The Bcl-2 family protein Bax is one such intermediate, which in a unifying response to diverse apoptotic stimuli nucleates multiprotein assemblies on mitochondria, committing cells to irrevocable damage. Using Bax as the prototype stimulus, we analyze Notch signaling for potential interactions with mitochondria, probe intrinsic properties of the Notch receptor, and describe key intermediates in the Notch-activated signaling cascade. Ligand-dependent processing was necessary to generate the Notch intracellular domain (NIC) although signaling was independent of canonical interactions with nuclear factors. Notably, antiapoptotic activity was recapitulated by NIC recombinants, localized outside the nucleus, and compromised by enforced nuclear sequestration. NIC signaled via the kinase Akt to prevent the loss of mitochondrial function, contiguity, and consequent nuclear damage, outcomes critically depend on mitochondrial remodeling proteins Mitofusins-(Mfn)-1 and 2. Thus, the NIC-Akt-Mfn signaling cascade identifies a pathway regulating cell-survival, independent of canonical functions associated with NIC activity.

apoptosis | Bax | mitofusins | signaling

Notch signaling, which regulates a variety of processes in different developmental contexts in metazoans (1), is initiated by interactions with ligands that trigger sequential, proteolytic cleavage of the transmembrane receptor. Typically, processing culminates in the release of the Notch intracellular domain (NIC), which localizes to the nucleus to initiate transcription (2). The pleiotropic outcomes of Notch signaling arise from its interactions with other signaling networks, best understood in the context of nuclear functions of NIC (3). The more recently appreciated antiapoptotic functions of Notch are linked to interactions with molecules such as Akt and NF- κ B (4–7), positioned as nodes across multiple signaling pathways, suggesting that Notch may function as a key molecular sensor regulating survival. Here, we probe the mechanism underlying Notch-mediated cell survival in an effort to reveal general principles underlying this function.

Bcl-2 family proteins determine mitochondrial involvement in death cascades, regulating commitment to apoptosis to diverse stimuli, which include developmental cues, nutrient-deprivation, and intrinsic cytotoxic stressors (8). Because Notch-mediated survival has been reported in several of these contexts, we build on previous characterizations to probe the mechanism underlying Notch-mediated antiapoptotic activity vis-à-vis intersections with mitochondrial events. Thus, we interrogated Notch-mediated survival using the proapoptotic protein Bax, which defines a core conserved event—mitochondria coordination—in mammalian death cascades (8, 9). Our results suggest a model for NIC function in the cytoplasm, which is distinct from its canonical roles. Further,

we demonstrate functional interactions with proteins regulating mitochondrial fusion (10) revealing a mitochondrial link for NIC activity. These observations lay the foundations for detailed analysis of a noncanonical Notch pathway operating to regulate cell survival.

Results

Notch Activity Inhibited Bax-Induced Apoptosis. We first investigated if Notch signaling inhibited Bax induced apoptosis. Bax tagged to enhanced GFP (Bax-GFP) was expressed in the HeLa cell line and apoptotic damage triggered within 60–80 min by the addition of 1 μ M of the broad spectrum kinase inhibitor Staurosporine (STS) (Fig. 1 *A* and *B*). Coexpressing untagged or RFP-tagged NIC recombinants inhibited nuclear damage in HeLa (Fig. 1 *C* and *D* and Fig. S1*A*), COS-7 (Fig. S1*B*) and the HEK cell line, where Bax triggered apoptosis without the addition of STS (Fig. S1 *C* and *D*). NIC activity was independent of endogenous Notch1 (Fig. S1*E*) and inhibited apoptosis triggered by Bak another proapoptotic member of the Bcl-2 family (Fig. S1*F*).

Fluorescence recovery after photobleaching (FRAP) measures rate of refilling of a bleached area from connected neighboring fluorophore (such as Mitotracker Red) filled areas over a period and reflects organelle luminal contiguity. Mitochondrial fragmentation in STS-treated Bax-GFP cells resulted in dramatically reduced FRAP recoveries as compared to the untreated group (Fig. 1*E*), whereas recoveries were comparable to control untreated cultures in STS-treated Bax-GFP+NIC cells (Fig. 1*E*). These results were confirmed by visualizing mitochondrial organization in all three conditions (Fig. 1*F*). The mitochondrial network of live Bax-GFP expressing cells is rendered fragmented during apoptosis but is tubular and interconnected in cells coexpressing NIC (Fig. S1*G*). Additionally, partitioning of the fluorescent dye TMRM is dependent on mitochondrial transmembrane potential (MTP) and reduced uptake indicates a loss of outer mitochondrial membrane (OMM) integrity, which was inhibited in cells expressing NIC (Fig. S1*H*). At the onset of apoptosis, the OMM is rendered permissive to molecules, which irrevocably compromise organelle integrity. One signature is the change in Bax conformation and consequent multimerization at the MOM (11). Subsequent experiments assessed the consequences of NIC activity on these events.

NIC Inhibits Bax Multimerization. Changes in Bax-GFP organization associated with onset of apoptosis were followed by live imaging

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¹To whom correspondence may be addressed. E-mail: sarina@ncbs.res.in or apurva.sarin@gmail.com.

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time scales, at nascent puncta near mitochondria (Fig. 2E, filled black circles, and Fig. S3J). Thus, although there is no STS-induced change in Bax-GFP mobilities in the cytoplasm (Fig. S3H), slower diffusion time scales at mitochondria in these cells is consistent with the formation of higher order Bax complexes and were recorded in a substantial (60–65%) number of the total cells ($n = 40$) analyzed (Fig. S3J, +STS). We detect no STS-induced changes in mobilities in NIC+Bax-GFP cells (Fig. 2E). Overlapping autocorrelation curves of GFP in cells in the presence or absence of STS (Fig. 2E *Inset*) argued against changes in viscosity as the underlying cause. These approaches, suggest that NIC signaling intersects with early events in the apoptotic process, reflected in changes in Bax dynamics and conformational transitions in apoptotic cells.

Because NIC is a predominantly nuclear localized protein with emerging evidence of cytoplasmic functions (6, 17, 18), subsequent experiments dissected the spatial coordinates of NIC and the intrinsic properties of the receptor determining this function. Various modified Notch/NIC recombinants were employed to assess the dependence on processing, canonical intermediates, and sustained nuclear localization of NIC for antiapoptotic activity.

Nuclear Sequestration Abrogates NIC-Mediated Antiapoptotic Activity.

A processing resistant Notch recombinant (19) disabled for interactions with ligand (N^{LNG}), did not block apoptosis (Fig. 3A and *Inset*), whereas a recombinant ($N^{LNG} CC>>SS$) corrected for this defect was active (Fig. 3A and *Inset*). The Notch target *hes1* promoter activation by these constructs (Fig. S4A) was consistent with published reports (19). Antiapoptotic activity of full-length Notch (NFL) was abrogated in cells coexpressing an inhibitory form of its ligand—soluble Jagged-1 (sJag1) (Fig. 3B).

Deletion of the RBP-Jk/CBF1-associated module (RAM) domain, which mediates NIC-RBP-Jk interactions (20), did not abrogate NIC function (Fig. 3C). siRNA-mediated ablation of

RBP-Jk or DN-CBF1 prevented NIC-mediated activation of the *hes-1* promoter (Fig. S4B and D), but did not suppress NFL-mediated antiapoptotic activity (Fig. 3D and Fig. S4C) suggesting that canonical interactors were dispensable for Notch activity. A recombinant where the transmembrane domain of the CD8 receptor was incorporated to generate CD8-NIC-GFP/CD8-NIC-RFP is predominantly excluded from the nucleus (Fig. S4E), compromised for *hes-1* transcription, (17) but inhibited apoptosis with an efficacy comparable to other active recombinants (Fig. 3E). Further, inclusion of an additional NLS (GFP-N1^{IC-NLS}) compromised antiapoptotic activity (Fig. 3F). Collectively, these data suggest that canonical interactions in the nucleus or a sustained nuclear presence were dispensable for NIC functioning in its capacity as a regulator of cell survival. Building on previous observations (5, 17, 21, 22) and using a combination of siRNA and dominant negative constructs we established that the kinase Akt is an intermediate in the Notch-activated antiapoptotic pathway (Fig. 4A and B and Fig. S5A–C). In subsequent experiments we tried to elucidate the mechanism by which NIC activity impinged on mitochondrial integrity.

Distinctively, unlike Bcl-x_L, a well-characterized Bax antagonist, NIC-mediated antiapoptotic activity restored mitochondrial connectivity, suggesting a mechanism that may integrate with the machinery regulating organelle morphology for NIC function. Mitochondrial morphology and dynamics are the outcome of a balance between the opposing processes of organelle fusion and fission (10). The cellular roles of mitochondria including energy conversion, maintenance of transmembrane potential, and calcium homeostasis are thought to be intimately linked to molecules that regulate mitochondrial remodeling (23, 24), although the mechanisms remain to be elucidated. Mitofusins are core conserved components of the fusion machinery, and we first asked if NIC-Akt signaling converged on these molecules.

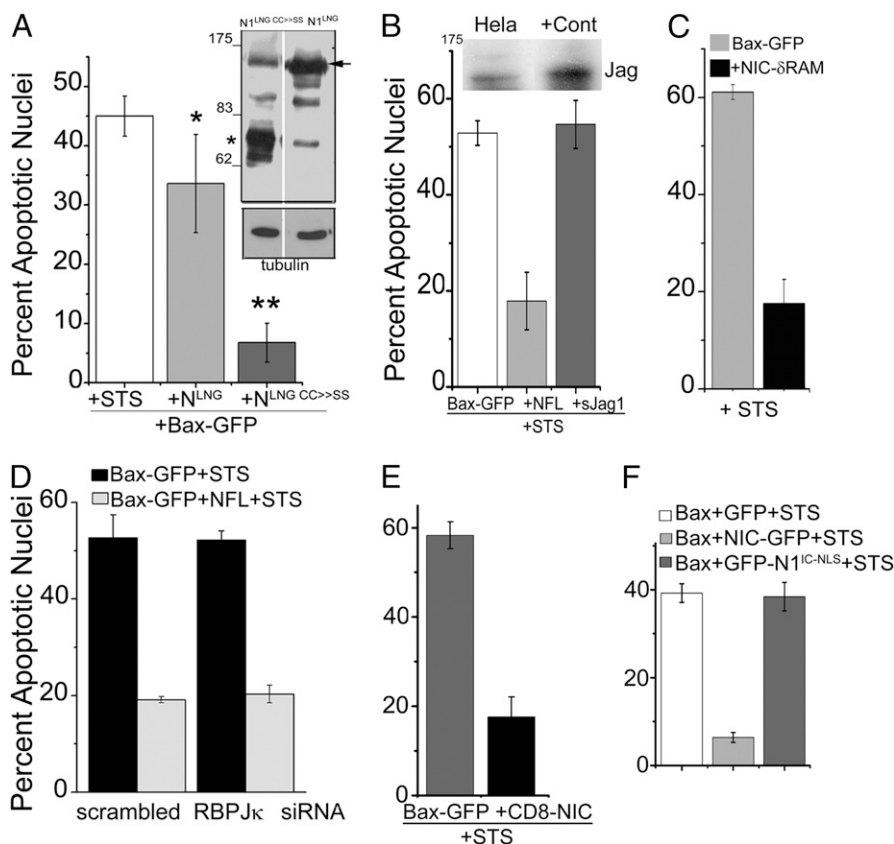


Fig. 3. Nuclear localization is not essential for NIC activity. (A) HeLa cells transfected with Bax-GFP or Bax-GFP+ N^{LNG} or $N^{LNG} CC>>SS$ were cultured overnight. Cultures were processed and scored for STS-induced apoptotic nuclear damage. * $P < 0.01$; ** $P < 0.001$. (*Inset*) The recombinants detected by an antibody to myc in immunoblots. Arrow and the star indicate the unprocessed and processed form of the constructs, respectively. Representative of two experiments (B–E), HeLa cells transfected with different combinations of constructs shown in the panels were cultured overnight and then scored for STS-induced apoptotic damage as described in *Methods*. (*Inset*) The immunoblot shows Jag expression in HeLa cells. In (D) cells were pretreated with siRNA to RBP-Jk or a scrambled control for 48 h before transfection. (F) HeLa cells transfected with Bax (2 μ g) with and without NIC-GFP (1 μ g) or GFP-N1^{IC-NLS} (1 μ g) were cultured overnight and scored for STS-induced apoptotic damage. (A–F) The data plotted are mean \pm SD of three separate experiments.

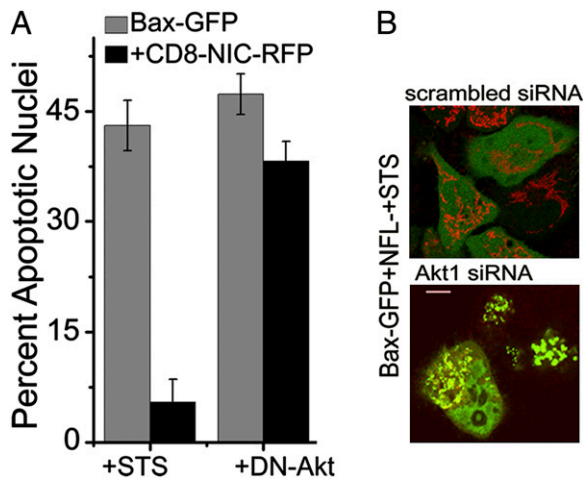


Fig. 4. Akt is required for NIC activity (A) HeLa cells transfected with Bax-GFP or Bax-GFP+CD8-NIC-RFP^{+/−} DN-Akt were cultured overnight and scored for STS-induced apoptotic damage. Data plotted are the mean \pm SD of three separate experiments. (B) Representative image of cells ($n = 30$) pretreated with siRNA to Akt1 or a scrambled control for 48 h, cotransfected with Bax-GFP+NFL. Cells counterstained with Mitotracker were imaged 60 min after the addition of STS. (Scale bar: 5 μ m).

Notch-Akt-Mediated Antiapoptotic Activity Requires Mitofusins. Mitofusins (Mfn)-1 and Mfn-2, are mitochondrial outer membrane GTPases regulating the coordinated and sequential fusion of mitochondrial membranes (10). Depletion of Mfn1 or Mfn2 (using siRNA) resulted in punctate mitochondria (Fig. S6A and B) and abrogated NIC-mediated inhibition of nuclear damage and restoration of MTP (Fig. 5A–C). CD8-NIC and CA-Akt demonstrated a similar dependence on Mfns for activity (Fig. 5D and E and Fig. S6C) positioning Mfns as intermediates in the NIC-Akt signaling module. Both NIC-GFP and CD8-NIC-GFP were detected in cellular fractions enriched for the mitochondrial resident cytochrome *c* (Fig. 5F), indicating a possible (temporally regulated) association with mitochondria. Distinctively, Bcl-x_L-mediated antiapoptotic activity was independent of Mfns (Fig. 5G and Fig. S6E and D).

To identify a possible physiological correlate of the interaction between NIC and Bax function described by these experiments we turned to the analysis of activated T cell apoptosis in response to cytokine deprivation.

Notch Regulates Bax Activation in Primary T Cells. Bax is one of three proapoptotic molecules of the Bcl-2 family implicated in activated T-cell apoptosis for the down-regulation of the immune response (25). Key aspects of this deletion can be recapitulated in vitro in T cells activated using surrogate antigens. Because Notch signaling inhibited activated T cell apoptosis following cytokine deprivation (26), we explored interactions between NIC and Bax in activated T cells. 6A7 reactivity, a signature of Bax activation, was not detected in live cells cultured in cytokine (Fig. 6A Upper) but, as seen in T cells deprived of cytokine (Fig. S6F), was induced following treatment with a gamma secretase inhibitor (GSI), which abrogates Notch processing (Fig. 6A Lower), triggering T cell apoptosis (Fig. 6C). Similarly, Bax dimers stabilized using cross-linkers were detected in cells following cytokine deprivation or treatment with GSI (Fig. 6B). Thus, inhibition of NIC activity recapitulated apoptotic features triggered by cytokine deprivation in T cells. Further, recombinant CD8-NIC, which blocks activated T cell apoptosis (17), also inhibited Bax activation (Fig. 6D). As seen in the cell lines, NIC and Akt were detected in the tubulin negative, mitochondrial fraction of T cells enriched for cytochrome *c* (Fig. 6E). However, immunoprecipitation analysis did not indicate stable associations between NIC, Mfn2, and Akt in T cells (Fig. 6F).

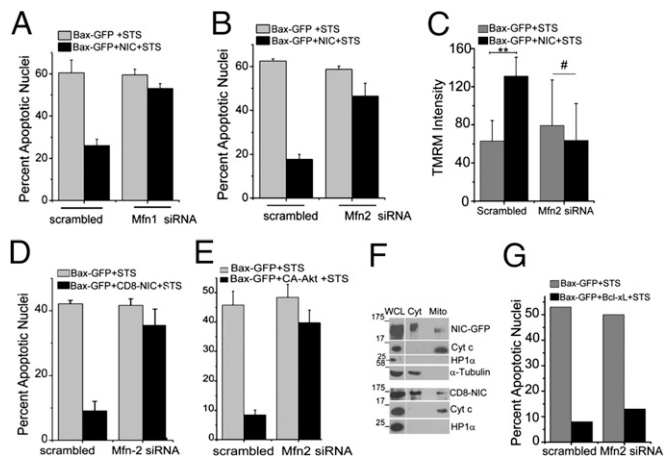


Fig. 5. Notch-mediated antiapoptotic activity requires Mitofusins. (A and B) Cells pretreated with Mfn1(A), Mfn2 (B) or scrambled siRNA for 48 h were transfected with Bax-GFP or Bax-GFP+NIC, cultured overnight, and scored for STS-induced apoptotic damage. (C) Cells pretreated with M or scrambled siRNA for 48 h, transfected with Bax-GFP or Bax-GFP+NIC, cultured overnight, treated with STS, and scored for TMRM dye intensity by confocal microscopy. Differences in TMRM intensities in Bax transfected scrambled and M siRNA-treated cells are not significant ($P = 0.91$). ** $P < 0.001$; # $P = 0.54$. (D) Cells pretreated with Mfn-2 or scrambled siRNA for 48 h were transfected with Bax-GFP or Bax-GFP+CD8-NIC-RFP. Cultures were processed as described in *Methods*. (E) Cells pretreated with Mfn2 or scrambled siRNA for 48 h were transfected with Bax-GFP or Bax-GFP+CA-Akt and processed as in A. (A–E) Data plotted are the mean \pm SD from three individual experiments. (F) Representative immunoblot analysis of NIC-GFP or CD8-NIC-GFP transfected cells fractionated and probed for GFP, Cytochrome *c*, tubulin, and HP1 α . (G) Cells pretreated with Mfn2 or scrambled siRNA for 48 h were transfected with Bax-GFP or Bax-GFP+Bcl-x_L. Cultures were processed for STS-induced apoptotic damage. The data in the graph shows one experiment representative of two separate trials.

Discussion

Emerging evidence indicates roles for mitochondria in the regulation of cellular signaling cascades, extending their functions beyond the primary, well-characterized role in the control of cell metabolism (24). This study positions the mitochondrion as an integral part of a cytoplasmic Notch-activated signaling cascade that regulates cell survival (Fig. 7).

NIC signaling was investigated using the Bcl-2 family protein Bax as a probe, which converges on the OMM for apoptotic output. Antiapoptotic activity was demonstrated using multiple approaches (in live and fixed cells) monitoring changes in Bax dynamics, conformation, and oligomerization in established cell lines and apoptosis induced by cytokine-deprivation (mediated by the mitochondrion), in ex vivo activated primary T cells, supporting the link between NIC signaling and mitochondrial function. Experiments designed to elucidate spatial coordinates positioned cytoplasmic NIC, as opposed to the nuclear localized form, as the active signaling intermediate. This was based on evidence that membrane-targeting or NES addressing tags did not compromise antiapoptotic activity and supported by the previous demonstration of cytoplasmic pools of the transgene (ascertained by FCS) in NIC-GFP expressing cells (17).

The mitochondrion is central to several apoptotic cascades, and the OMM is a primary target of antiapoptotic proteins. A possible link between NIC activity and the mitochondrion was suggested by the unexpected observation that the NIC-Akt signaling cascade, unlike Bcl-2 family antiapoptotic proteins (27), restored both organelle connectivity and integrity in cells where it blocked death, suggesting interactions with molecules that maintain organelle structure. The Mfns are required for maintenance of organelle morphology and architecture and linked to the regulation of the myriad metabolic activities of mitochondria (23, 24, 28). Depleting

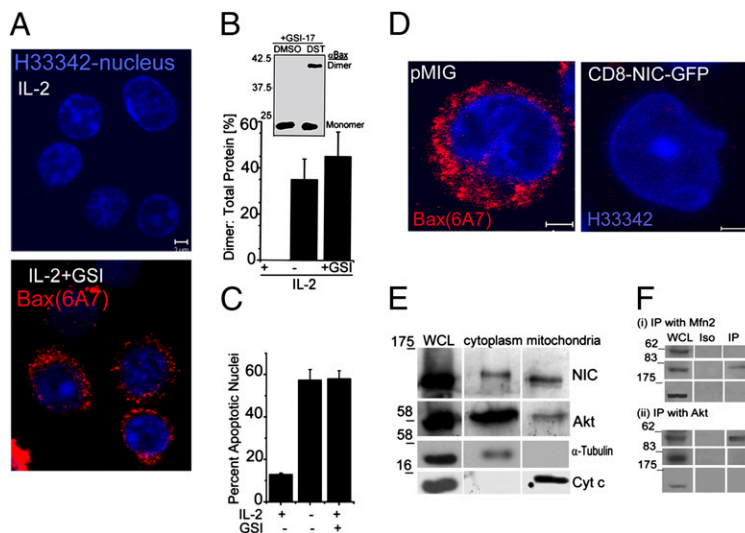


Fig. 6. NIC regulates Bax activation in T cells. (A) Activated T cells were cultured with IL-2 or IL-2 + 10 μ M gamma secretase inhibitor (GSI-17) for 12 h, stained with 6A7 and H33342, and imaged, (Scale bar: 2 μ m.) (B) The cross-linking assay was performed in cell lysates in the different conditions and assessed for Bax multimers by immunoblot analysis (*Inset*). The graph shows band intensities of dimer Bax relative to total Bax protein by densitometric analysis (mean \pm SD from three independent assays). (C) Cells treated as in A were scored for nuclear damage using H33342. Data are the mean \pm SD from three separate experiments. (D) Activated T cells expressing GFP or CD8NIC-GFP were cultured without IL-2 for 14 h and stained for 6A7 (red) and Hoechst 33342. (*n* = 40 cells in two experiments). (E) Representative immunoblot analysis of activated T cells fractionated and probed for NIC, Akt, cyt c, and tubulin in cytoplasmic and mitochondrial fractions and whole cell lysates (WCL). (F) Immunoblot of immunoprecipitation analysis of Akt, Mfn-2, and NIC in activated T cells. Iso: isotype control Ig. White spacers indicate empty lanes in the original membranes.

Mfns did not perturb Bcl-x_L activity but abrogated NIC and Akt function, identifying an essential role for these molecules in the cascade. Although NIC and Akt are detected in the cellular fractions enriched for mitochondria, we do not detect stable associations between NIC-Akt and Mfns, although the possibility of dynamic interactions underlying functional outputs remains to be addressed. The mitochondrial link suggested in this work is supported by the recent report that phosphorylated Akt-S473—an output of cytoplasmic NIC activity as demonstrated by our previous work (17)—promotes mitochondrial retention of the kinase (29).

Altered bioenergetic capabilities associated with loss of Notch activity have been previously reported in *Drosophila melanogaster* (30). However, the mechanism(s) translating changing mitochondrial dynamics into altered functional outputs is not understood, given instances where respiratory competency and maintenance of

MTP are decoupled from organelle shape regulating activities (31). Because Mfns are essential intermediates in the NIC-Akt signaling module, this cellular system provides both a molecular handle to explore NIC interactions with mitochondria and a means to explore mechanistic connects between mitochondrial function, morphology, and the coupling to extracellular cues. Apart from their well defined role in cellular bioenergetics, mitochondria are increasingly thought to function as hubs that coordinate signaling cascades in response to extracellular cues (24, 28). It is tempting to speculate that a signaling module comprising evolutionarily conserved intermediates—NIC, Akt, and Mfn—may constitute a generalized mechanism modulating mitochondrial function and differentiation (21, 32) in diverse biological contexts.

Methods

Antibodies. Antibodies to mNIA (NIC), GFP and cytochrome c were from BD Biosciences; Bax-Clone 6A7 and α -Tubulin from Neomarker; Bax (clone P-19), Notch1, Akt, Mfn-2, and Jagged from Santa Cruz, c-myc and HP1 α from Cell Signaling Technology or Millipore-Upstate.

Plasmids. The Bax-GFP, untagged Bax constructs NIC-GFP and NIC-RFP have been described (17, 33). CD8-NIC was cloned into the plasmid RFP-N3 between the BglIII and EcoRI sites to make the CD8-NIC-RFP recombinant and subcloned into pMIG as described elsewhere (17). The following plasmids were obtained as gifts: AcN1 (NIC) and DN-CBF1 from J. Aster (Harvard Medical School, Boston); Notch^{LNG} and N^{LNGCC>>SS} from R. Kopan (Washington University, St. Louis); GFP-N1^{IC-NL5} from B. A. Osborne (Massachusetts University Amherst, Amherst, MA), and Notch full-length (NFL) from A. Rangarajan (Indian Institute of Science, Bangalore, India). All other constructs were from Millipore-Upstate. Plasmids were used at the following concentrations unless specified otherwise: Bax-GFP, 0.5 μ g; NFL, NIC6RAM, and CD8-NIC-RFP, 2 μ g; NIC, N^{LNG}, N^{LNG CC>>SS}, sJag1, DN-Akt, CA-Akt, Bcl-2, and Bcl-xl, 3 μ g. Empty vector pcDNA was used to equalize DNA concentrations across experimental groups.

Assays of Cellular Damage. HeLa, HEK 293, and the COS-7 cells, maintained as described (17), were plated at a density of 3×10^5 cells per 35-mm dish 14–16 h before transfection with Lipofectamine 2000 (Invitrogen). All siRNA were from Dharmacon, Inc. and were used at 100 nM concentration as per the manufacturer's instructions. For HeLa and COS-7 cells 14 h posttransfection, cells were cultured for an additional hour with or without 1 μ M STS (Sigma) to induce apoptotic damage. The data are normalized to apoptotic damage in the group not treated with STS. HEK cells were analyzed for apoptotic nuclear damage 18–24 h posttransfection. Generation of activated T cells, treatment with GSI, transductions using retroviruses and isolation of transfected cells by flow-sorting were performed as described (26). To analyze nuclear morphology, cells were stained (for 5–7 min at room temperature) with the DNA binding dye Hoechst (H) 33342 (1 μ g/mL) and nuclear morphology in GFP

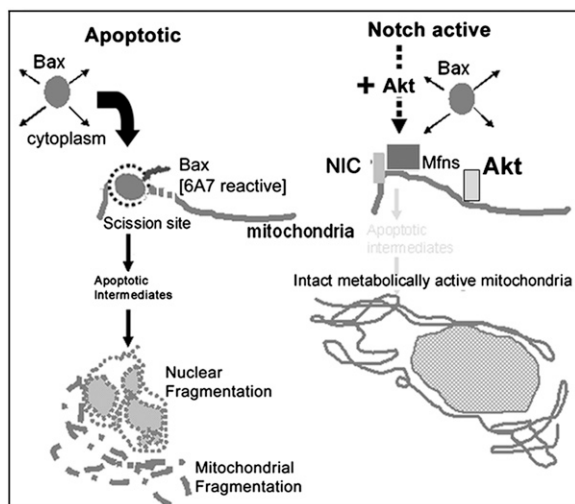


Fig. 7. Schematic summarizing key features of the NIC-activated anti-apoptotic cascade. Cytoplasmic NIC activity, inhibited apoptosis coordinated by mitochondria in cell lines (triggered using Bax), and primary T cells. The mitochondrial remodeling proteins—Mitofusins—are identified in this work as key intermediates in the antiapoptotic cascade, regulating mitochondrial function and connectivity downstream of the NIC-Akt signaling pathway. Despite evidence of mitochondrial pools of NIC and Akt, we do not detect stable, direct associations between NIC-MFN-AKT suggesting the possibility of dynamic interactions among these molecules regulating signaling outputs.

positive cells scored by microscopy. In cells expressing RFP- and GFP-tagged recombinants, only cells positive for both tags were scored. For the analysis of mitochondrial morphology, adherent cells were incubated with 100 ng/mL of MitoTracker Red 580 (Invitrogen) for 30–45 min at 37 °C. Cells were imaged by confocal microscopy after two PBS washes. Bax oligomerization was assessed in cell lysates as described (12). Cell fractionations used the mitochondria isolation kit for mammalian cells (Pierce) as per the manufacturer's instructions.

Quantitative Fluorescence Assays. Cells were maintained at 37 °C with a temperature and CO₂ stage controller and examined using Olympus Confocal FV1000, 60x, NA1.4 Objective Lens. FCS and FRAP analysis were performed as described (14). The analysis of Bax-GFP puncta number and kinetics was performed using a custom-made Labview program, which analyzed Bax-GFP puncta in each frame. Puncta were set as described (Fig. S3), the number of Bax-GFP puncta in each frame was plotted as a function of time and averaged for 16 to 20 cells.

Statistical Analysis. Data are presented as mean \pm SD derived minimally from three to five independent experiments unless stated otherwise. FRAP and

Bax-GFP puncta analyses are presented as mean \pm SEM. Statistical significance was calculated using the two population Student's *t* test.

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