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A hierarchical cascade activated by non-canonical Notch signaling and the mTOR–Rictor complex regulates neglect-induced death in mammalian cells

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The regulation of cellular metabolism and survival by trophic factors is not completely understood. Here, we describe a signaling cascade activated by the developmental regulator Notch, which inhibits apoptosis triggered by neglect in mammalian cells. In this pathway, the Notch intracellular domain (NIC), which is released after interaction with ligand, converges on the kinase mammalian target of rapamycin (mTOR) and the substrate-defining protein rapamycin independent companion of mTOR (Rictor), culminating in the activation of the kinase Akt/PKB. Biochemical and molecular approaches using site-directed mutants identified AktS473 as a key downstream target in the antiapoptotic pathway activated by NIC. Despite the demonstrated requirement for Notch processing and its predominant nuclear localization, NIC function was independent of CBF1/RBP-J, an essential DNA-binding component required for canonical signaling. In experiments that placed spatial constraints on NIC, enforced nuclear retention abrogated antiapoptotic activity and a membrane-anchored form of NIC-blocked apoptosis through mTOR, Rictor and Akt-dependent signaling. We show that the NIC-mTORC2-Akt cascade blocks the apoptotic response triggered by removal of medium or serum deprivation. Consistently, membrane-tethered NIC, and AktS473 inhibited apoptosis triggered by cytokine deprivation in activated T cells. Thus, this study identifies a non-canonical signaling cascade wherein NIC integrates with multiple pathways to regulate cell survival.
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Cellular dependence on extrinsic factors for survival is developmentally programed and continues through the life of adult metazoans.[1] In their absence, cells undergo death by the intrinsic pathway of apoptosis that converges on, and is regulated by mitochondria.[2] Although it is increasingly evident that pathways regulating metabolic activity and cell growth also regulate survival, the underlying mechanisms and molecular correlates of these interactions are not completely understood. Thus, mechanisms by which growth factors regulate survival and intersect with the nutrient-sensing machinery are of interest as core elements of these signaling pathways are conserved across phyla.

Signaling through the Notch family of transmembrane receptors is initiated by interactions with ligands that trigger proteolytic cleavage of the receptor. The final proteolytic step involves the γ-secretase-mediated release of the Notch intracellular domain (NIC), which localizes to the nucleus functioning as a cofactor to initiate transcription with members of the Mastermind family of proteins and CSL (CBF1/Su(H)/LAG-1).[3] It is now well established that the core pathway of Notch signaling intersects with pathways controlling division,

proliferation, and apoptosis.[4] We and others have described a role for Notch signaling in the regulation of cell survival,[5,6] with Notch activity converging on the kinase Akt to inhibit death in several cell types.[6–10]

Akt regulates processes related to proliferation, metabolism, growth, and survival.[11] The kinase modulates cellular responses to environmental cues by regulating the localization of glucose transporters[12] and impinges on mitochondrial integrity and function.[13,14] Akt couples phosphatidylinositol-3 kinase (PI3K) signaling to the nutrient-sensing mammalian target of rapamycin complex-1 (mTORC1) pathway through the phosphorylation of tuberous sclerosis complex-2 (TSC2),[15,16] and is a target of mTOR when the latter is associated with the proteins Rictor, mLST8, and SIN1 to form mTORC2.[17–19] Thus, Akt signaling has important functional consequences for cells.

We show that Notch signaling converges on the activation of Akt to inhibit apoptosis triggered by neglect, that is, the withdrawal of nutritional cues, and describe the molecular intermediates in this cascade. Our experiments reveal integral roles for the nutrient sensor kinase mTOR and its

substrate-defining protein Rictor, which regulates the activation of Akt for Notch-mediated survival. Both biochemical approaches and the analysis of site-directed mutants identify AktS473 activity as the downstream target of the NIC-mTORC2 signaling cascade. We present evidence that Notch processing was required for the activation of the signal transduction cascade. However, dominant-negative (DN) and RNA interference approaches, as well as the use of a deletion mutant, showed that NIC activity did not require CBF1/RBP-J-mediated transcription. Reduced nuclear retention did not inhibit the antiapoptotic activity of NIC. Furthermore, the signaling cascade recapitulated by a modified membrane-anchored form of the Notch intracellular domain, supported the cytoplasmic localization of NIC for its antiapoptotic function. This cascade blocked death triggered by the withdrawal of medium or serum in cell lines, and membrane-tethered NIC, and AktS473 activity inhibited apoptosis triggered by cytokine deprivation in activated T cells. Thus, the functional interactions with mTOR-Rictor and Akt identify a novel Notch-mediated signaling cascade that favors cell survival.

Results

Characterization of deprivation (neglect)-induced death. If growth medium is replaced by buffered saline, HeLa cells in culture undergo apoptosis within 6 h (neglect-induced death). Apoptotic nuclear damage was determined using Hoechst 33 342 to reveal changes in morphology and the TUNEL assay to detect double-stranded breaks in cells (Figure 1a). The TUNEL assay gave comparable results to that obtained with Hoechst 33 342 (Figure 1b), and in all subsequent analysis, the assessment of nuclear morphology using Hoechst has been presented as the readout of damage. Dying cells were also characterized by the externalization of phosphatidylserine (Figure 1c). Induction of apoptotic damage was detected after 3 h of deprivation and increased substantially by 6 h (Supplementary Figure 1A). Furthermore, apoptosis was abrogated in cells following the siRNA-mediated depletion of the proapoptotic Bcl-2 family protein, Bax (Figure 1d).

Ligand-dependent activation of Notch inhibited neglect-induced apoptosis. Apoptosis induced by the withdrawal of medium was inhibited by the antiapoptotic protein, Bcl-2, or by full-length Notch1/Notch FL (Figure 2a), providing a system to explore the signaling cascade downstream of Notch. NFL-mediated inhibition was abrogated by the coexpression of soluble Jagged1 (sJag1; Figure 2a), which inhibited Notch processing and release of the intracellular domain (Supplementary Figure 1B). Consistently, apoptosis was prevented by the ectopic expression of the recombinant processed form of the NIC (Figure 2b and c and Supplementary Figure 1C), which protected from apoptosis for an extended period (Supplementary Figure ID). The requirement for ligand-dependent processing for Notch activity was verified using mutants disabled for interactions with ligand. The recombinant Notch1[LNG], which lacks the epidermal growth factor (EGF)-like repeats in its extracellular domain,[20] did not block death (Figure 2b), whereas a construct corrected for this defect (Notch1[LNG CC≫SS]) imparted
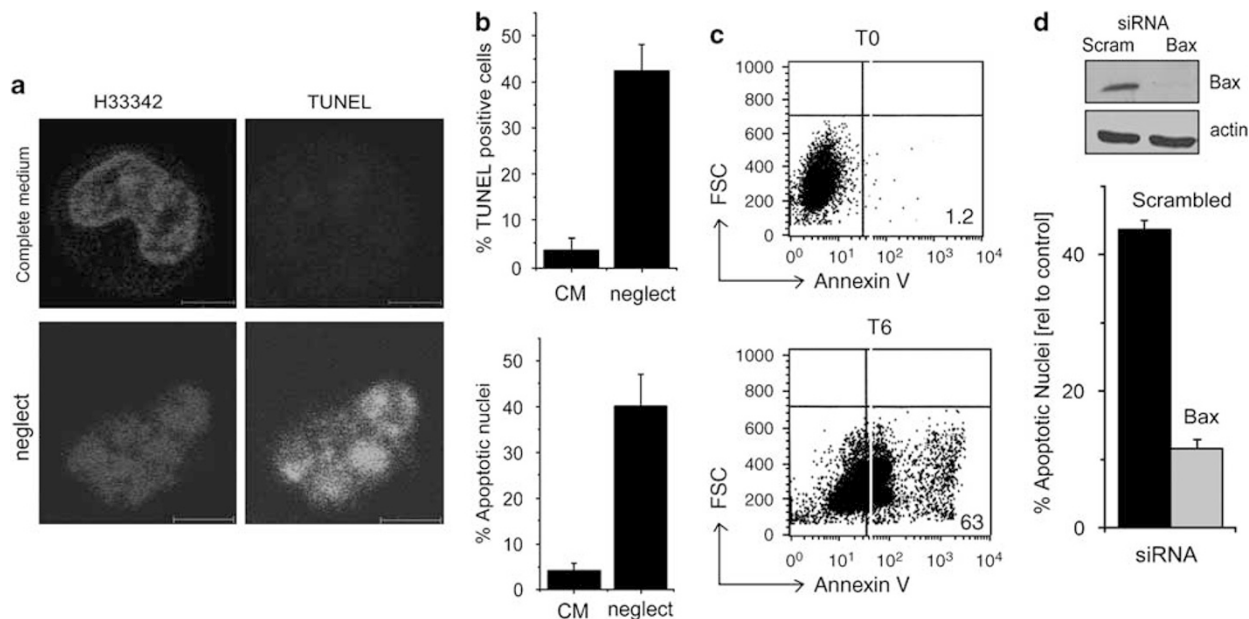

Figure 1 Characterization of the apoptotic response to neglect in HeLa cells. (**a**) Confocal image of HeLa cells cultured in complete medium (CM; nutrient replete) or buffered saline (neglect). After 6 h, cells were harvested and assessed for apoptotic changes using Hoechst 33 342 or by the TUNEL assay; scale bar: 5 μm. (**b**) Quantitation of apoptotic nuclear damage as assessed by the two readouts, described in panel a, from three separate experiments. (**c**) Phosphatidylserine exposure assessed using Annexin-V FITC in HeLa cells at the onset of the assay ($t = 0$) and after culture in buffered saline for 6 h (neglect). (**d**) HeLa cells were treated with siRNA to Bax, or a scrambled control for 48 h. Cells were continued in CM or buffered saline, and apoptotic nuclear damage was assessed after 6 h. The western blot shows the levels of Bax protein in siRNA-treated groups. Data from three independent experiments are plotted
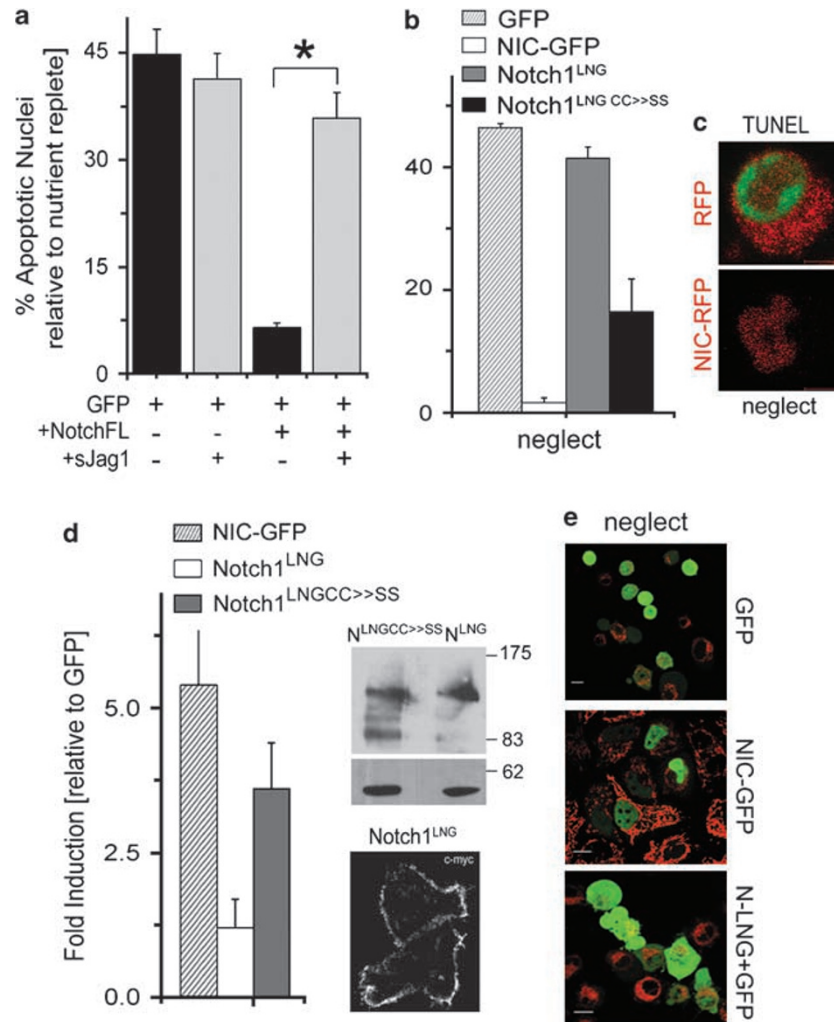
Figure 2 Notch activity inhibits neglect-induced death in mammalian cells. (**a** and **b**) HeLa cells were transfected with the indicated plasmids and cultured overnight. Culture medium was replaced with complete medium (CM; nutrient replete) or buffered saline (neglect) for each transfected plasmid. Apoptotic nuclear damage was assessed in GFP + cells using Hoechst 33 342 after 6 h . The data are corrected to the nutrient-replete condition and represent mean ± S.D. from three separate experiments. *$P < 0.001$. (**c**) HeLa cells transfected with RFP or NIC-RFP were cultured as described in panel a. After 6 h, apoptotic damage was analyzed using the TUNEL assay (green), and representative cells from the condition of neglect are shown; scale bar: 5 μm. (**d**) Hes1 promoter activity assessed in a luciferase assay in HeLa cells transfected with the constructs shown in the panel; inset: upper panel shows an immunoblot analysis of cells expressing the myc-tagged Notch1 constructs. Lower panel shows central plane of a confocal image of HeLa cells expressing Notch1$^{LNG-myc}$ stained with an antibody to myc. (**e**) HeLa cells were transfected with the indicated constructs, cultured in the presence or absence of medium for 6 h, and stained with Mitotracker Red to mark the mitochondria, and field views imaged at confocal resolution. Images of cultures in the neglect condition are shown. NIC-GFP predominantly marks the nucleus, and in the other two conditions, free GFP is detected in the nucleus and cytoplasm; scale bar: 10 μm

protection from death (Figure 2b). As expected, only Notch1$^{LNG~CC \gg SS}$ activated the Hes1 promoter (a readout of transcriptional activity) in reporter assays (Figure 2d). Immunofluorescence analysis confirmed that myc-tagged Notch1^{LNG} was detected at the membrane as expected (Figure 2d, inset). Furthermore, by immunoblot analysis, both myc-tagged constructs were expressed at comparable levels with increased amounts of processed NICD ($\sim$ 85 kDa) generated in Notch1$^{LNG~CC \gg SS}$-transfected cells (Figure 2d inset). Cells undergoing neglect-induced apoptosis showed a loss of adherence to substrate, which was apparent in the rounded morphology of cells undergoing neglect and disorganization of mitochondria (Figure 2e, GFP). Both these changes were prevented in cells expressing NIC, but not in cells expressing Notch1^{LNG} (Figure 2e). Thus, the inhibition

of nuclear damage by NIC activity correlated with adherence to matrix in cells deprived of nutrients.

Subsequent experiments were designed to characterize molecular intermediates downstream of NIC that coordinated the antiapoptotic response. Notch signaling activates Akt in several contexts,[7–10,21] and we assessed whether Akt was required for Notch activity in the regulation of neglect-induced death.

Akt/PKB is an intermediate in the antiapoptotic pathway activated by NIC. The status of Akt activation in cells experiencing neglect in the presence or absence of Notch FL/NFL was determined. Phosphatidylinositol 3,4,5-triphosphates generated through PI3K activity bind the PH domain of Akt enabling its translocation to the plasma membrane
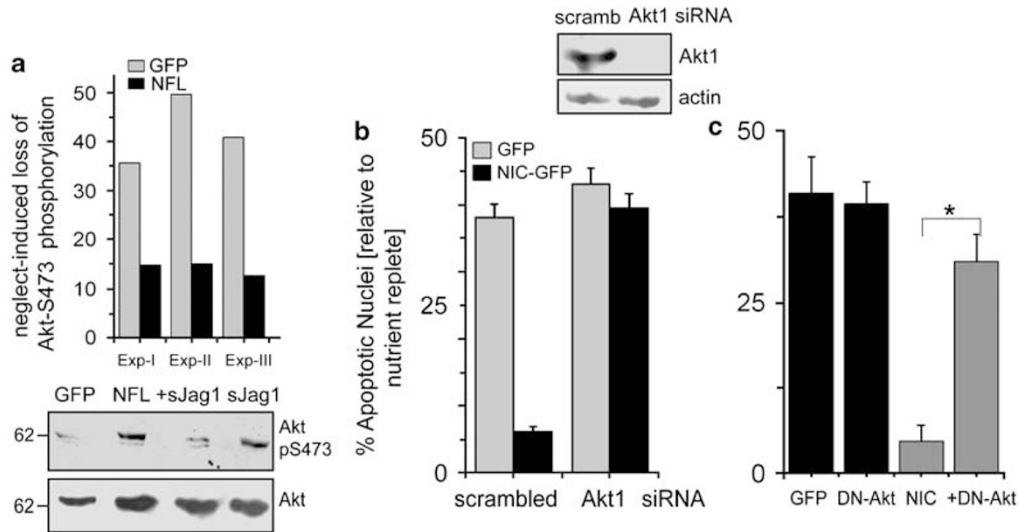
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Figure 3 NIC activates Akt signaling to block neglect-induced death. (**a**) Cells were transfected with the plasmids indicated and cultured overnight. Cells were shifted to buffered saline, and AktS473 phosphorylation was assessed by western blot analysis in cell extracts after 3 h incubation in buffered saline. The graph shows data derived by densitometry analysis of the western blots from three separate experiments. The representative western blot shows the phosphorylation of AktS473 in the conditions described in the various lanes. (**b**) HeLa cells were transfected with GFP or NIC-GFP in cells that were pretreated with siRNA to Akt1 or a scrambled control for 48 h. Cultures were continued with complete medium (CM) or buffered saline as described in Figure 2a. Apoptotic nuclear damage was assessed in GFP + cells after 6 h; inset: levels of Akt1 protein in cells treated with siRNA. (**c**) HeLa cells transfected with GFP or NIC-GFP with or without DN-Akt as indicated were cultured in CM or buffered saline. Apoptotic nuclear damage was assessed in GFP + cells after 6 h. *$P < 0.001$; data represent mean ± S.D. from a minimum of three separate experiments. In panels b and c, the data are normalized to the CM/nutrient-replete condition

and subsequent phosphorylations on T308 and S473.[11] These experiments were initiated because of the observation made in the HEK cell line that ectopically expressed NIC modulated the phosphorylation of Akt, specifically on S473 (Supplementary Figure 2A and B). Neglect triggered a loss of (endogenous) AktS473 phosphorylation, which was prevented in HeLa cells expressing NFL (Figure 3a, upper panel). NFL activity was attenuated by the coexpression of sJag1 (Figure 3a, western blot; compare lanes 2 and 3), consistent with the requirement for ligand-dependent interactions for Notch function.

NIC-GFP-mediated inhibition of apoptosis was abrogated in cells where Akt1 protein was depleted using siRNA (Figure 3b), or following the cotransfection of a DN (defective ATP-binding mutant) construct of Akt (DN-Akt; Figure 3c), positioning Akt as an intermediate in the pathway activated by Notch. As Notch lacks intrinsic kinase activity, we speculated that the phosphorylation of Akt in cells expressing Notch was indicative of another signaling intermediate in this transduction cascade. Recent work has characterized the nutritional sensor mTOR in association with Rictor, mLST8, and SIN1 (mTORC2) as the complex regulating the phosphorylation of Akt on S473.[17,22–26] Pathways upstream of mTORC2 are not well characterized, which prompted us to test the involvement of mTOR and Rictor in Notch-mediated antiapoptotic activity.

Notch activity converges on mTOR and Rictor to regulate neglect-induced death. The depletion of mTOR attenuated NIC-mediated inhibition of apoptosis (Figure 4a), positioning mTOR as an intermediate in the pathway. The two complexes of mTOR – mTORC1 and mTORC2 – are distinguished by dependence on the proteins Raptor and

Rictor, respectively. To investigate the roles of mTORC1 and mTORC2 in this pathway, the requirement for Raptor or Rictor in the antiapoptotic function of Notch was assessed. Cellular pools of Rictor (mTORC2) or Raptor (mTORC1) were independently depleted using siRNA (Figure 4c). Depletion of Rictor abrogated NIC-GFP function (Figure 4b), whereas reducing the levels of Raptor or the scrambled siRNA (included as a control in all experiments) had no effect on NIC-mediated inhibition of death (Figure 4b). Although the functional interaction between mTORC2 and Notch signaling revealed by these experiments was consistent with the demonstrated requirement for Akt and its phosphorylation on S473, the requirement for mTORC2 for Notch-mediated phosphorylation of Akt remained to be established. This was again monitored in conditions where the cellular levels of mTOR, Rictor, or Raptor were reduced by siRNA. Depletion of mTOR or Rictor inhibited the phosphorylation of Akt on S473 in NIC-expressing cells (Figure 4d and f), whereas the depletion of Raptor was without effect (Figure 4e). As mTOR and Rictor dependence of a signaling cascade is consistent with the activation of Akt on S473,[19] site-directed mutants were employed to test whether AktS473 was a functional effector of the NIC–mTOR–Rictor signaling cascade in the regulation of cell survival.

Akt/PKB-S473-dependent activity is required for NIC function. We generated site-directed mutants of Akt where the residues T308 or S473 were independently substituted by the neutral amino-acid alanine. The substitutions were made on the backbone of a myristoylated form of the kinase, which is referred to as constitutively active (CA) Akt
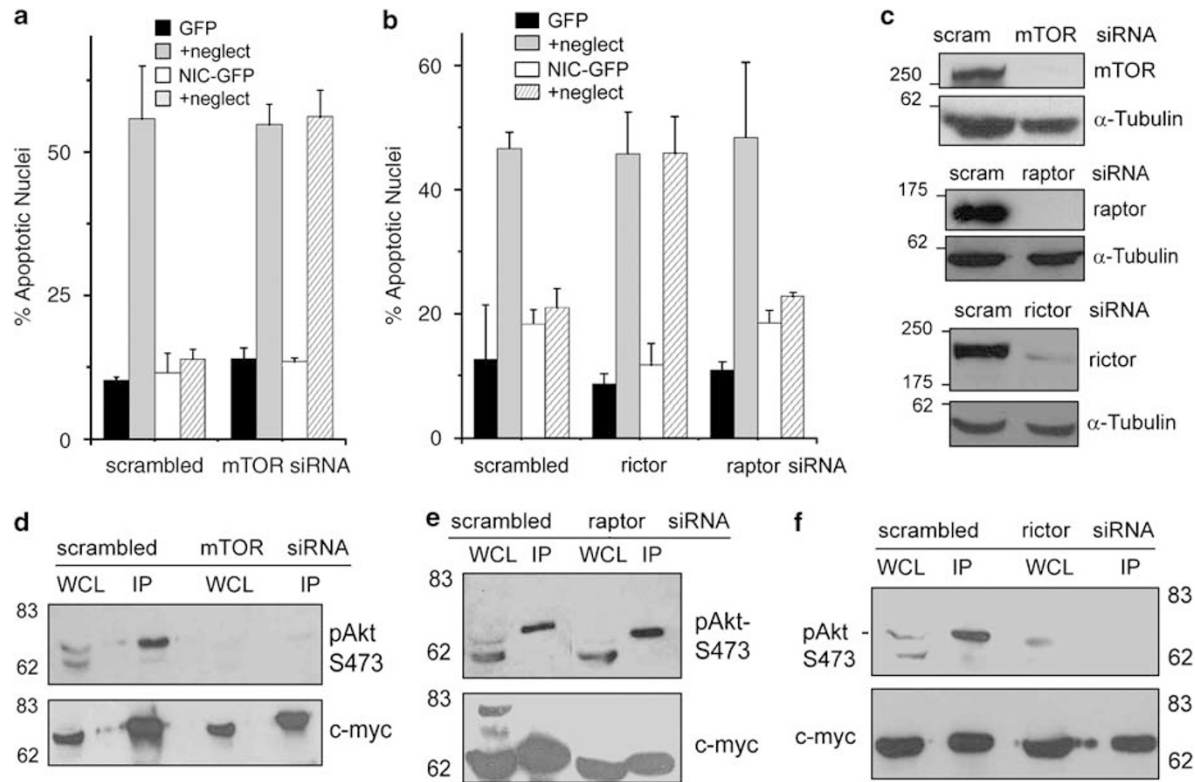
Figure 4 mTOR and Rictor regulate NIC-mediated antiapoptotic activity. (**a** and **b**) Apoptotic nuclear damage triggered by neglect in HeLa cells expressing GFP or NIC-GFP in cells pretreated with siRNA to mTOR (**a**), Rictor or Raptor (**b**) for 48 h. A scrambled control siRNA was included in all assays. Data are plotted as mean ± S.D. from three independent experiments. (**c**) Representative immunoblots showing the levels of proteins in cells treated with the indicated siRNA. (**d**–**f**) Immunoblots showing the phosphorylation of AktS473 in HeLa cells transfected with NIC-GFP + Akt-myc in conditions where the cells have been treated with siRNA to mTOR (**d**), Raptor (**e**) or Rictor (**f**)

and is the nomenclature followed henceforth. Myristoylation decouples the requirement for PI3K-driven membrane translocation of Akt from the dependence on endogenous kinases for the phosphorylation of T308 or S473. In the constructs generated, T308 and S473 were phosphorylated independent of each other, permitting their use to dissect the differential contributions of these residues in regulating neglect-induced death (Figure 5a).

As expected, the unmodified CA-Akt construct blocked apoptosis triggered by neglect (Figure 5c). The CA-Akt[S473A] mutant (S473 substituted by Ala) did not block apoptosis, whereas CA-Akt[T308A] displayed antiapoptotic activity comparable to that of CA-Akt (Figure 5c). The phosphorylation and activity of T308 has been reported to depend on the phosphorylation of S473.[27] However, phosphorylation of S6 Kinase 1, a readout of T308 activity,[15,23] was substantially reduced only in cells expressing CA-Akt[T308A], but not in the CA-Akt[S473A] mutant (Figure 5b). This suggested that T308-dependent functions were not compromised in our experimental conditions and were independent of S473 in the CA-Akt[S473A] recombinant.

As observed in NIC, the antiapoptotic activity of the CA-Akt[T308A] mutant was abrogated by siRNA to mTOR and Rictor (Figure 5d), but was unchanged in cells treated with siRNA to Raptor or a scrambled control (Figure 5d). The siRNA depletions were comparable with those shown in Figure 4, and are therefore not shown again. Further, the depletion of Notch1 (Figure 5g inset) attenuated the anti-apoptotic activity of this recombinant (Figure 5e). To more directly address whether AktS473 activity suffices to block death, we generated a phosphomimetic for the site by substitution with the negatively charged amino acid, glutamic acid. When patterns of phosphorylated substrates of Akt were compared, CA-Akt[T308A-S473E] expression resulted in increased kinase activity compared with cells transfected with GFP alone (Figure 5f). CA-Akt[S473E] was dominantly active, bypassing proximal events of Notch signaling to inhibit apoptosis independent of Rictor, mTOR, or Notch1 (Figure 5g), confirming the functional outputs of this signaling cascade.

Once released from the membrane, NIC translocates to the nucleus to initiate transcription. The requirement for canonical signaling for NIC-mediated inhibition of apoptosis was assessed in the experiments that follow.

Notch activity is CBF1/RBP-J independent. In the canonical pathway, NIC interacts with the DNA-binding factor, CBF-1, to form a ternary complex, which includes members of the MAML family of proteins. Coexpressing DN-CBF1, which binds NIC, but cannot interact with DNA, abrogated NIC-mediated Hes1 promoter activation (Figure 6a), but not the antiapoptotic function of NIC (Figure 6b). Similarly, pretreatment of cells with siRNA to deplete endogenous RBP-J did not abrogate NIC-mediated antiapoptotic activity (Figure 6c), although it blocked NIC
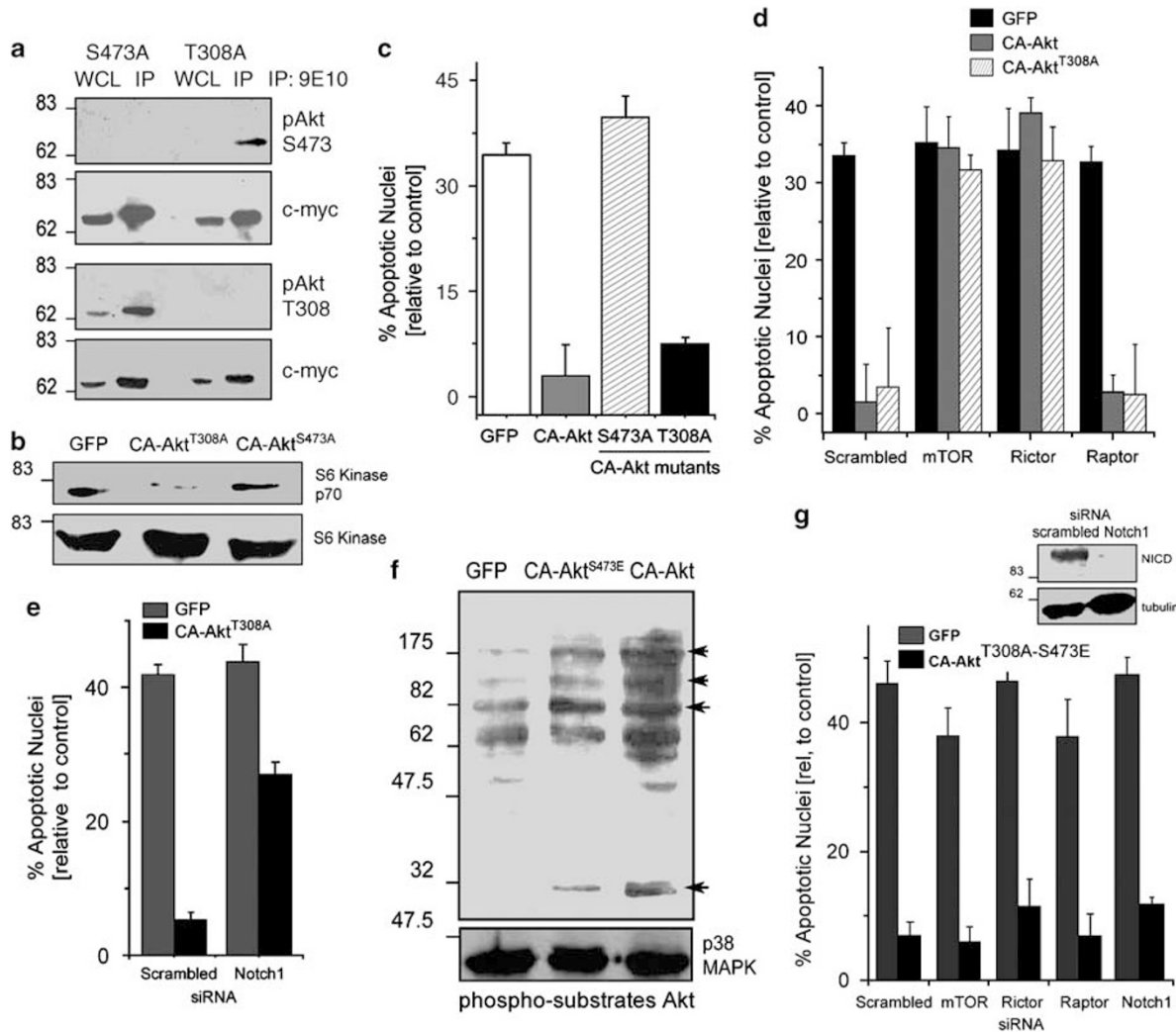
Figure 5 AktS473 activated through NIC, mTOR, and Rictor-dependent signaling inhibits neglect-induced death. (**a**) Western blot analysis of the phosphorylation of Akt on S473 or T308 assessed using phospho-specific antibodies in HeLa cells transfected with the CA-Akt mutants, such as CA-Akt^{S473A} or CA-Akt^{T308A}. (**b**) Phosphorylation of S6 kinase in HeLa cell lysates transfected with GFP, CA-Akt^{T308A}, or CA-Akt^{S473A} assessed by the western blot analysis. Membranes were stripped and reprobed for total S6K. (**c**) HeLa cells were transfected with GFP + empty vector or GFP with CA-Akt, or CA-Akt^{S473A} or CA-Akt^{T308A}. After an overnight culture, each transfection group (generated in duplicate) was continued in the complete medium (CM) or buffered saline. Apoptotic nuclear damage was assessed in GFP + cells after 6 h. (**d** and **e**) HeLa cells were transfected with GFP + empty vector or GFP + CA-Akt, or CA-Akt^{T308A} after 48 h of treatment with the siRNA indicated. After an overnight incubation, transfection groups were handled as described in panel c. (**f**) Western blot analysis with an antibody recognizing phosphorylated substrates of Akt in whole cell lysates of HeLa cells expressing GFP or CA-Akt$^{T308A-S473E}$ (CA-Akt^{S473E}) or CA-Akt. (**g**) HeLa cells transfected with the GFP + empty vector, or GFP + CA-Akt$^{T308A-S473E}$ after pretreatment with the siRNA indicated. Cultures were set up as described in panel c; inset shows a western blot analysis for endogenous Notch1 in cell extracts treated with siRNA to Notch1 or with a scrambled control. In panels c, d, e, and g, data shown are mean ± S.D. from three independent experiments and are normalized to the CM (control) condition

activation of the Hes1 promoter in reporter assays (Figure 6c, inset). Furthermore, an NIC construct modified by a deletion of the RBP-Jκ/CBF1-associated module (RAM) domain also inhibited neglect-induced death (Figure 6d). Thus, these experiments ruled out a role for the canonical (CBF1-dependent) pathway of Notch signaling in the inhibition of neglect-induced death.

The molecules mTOR, Rictor, and Akt have very well-characterized functions in the cytoplasm. The dependence of NIC signaling on mTOR and Rictor, coupled with the activation of CBF1-independent signaling, prompted us to investigate the subcellular localization of NIC activity in this paradigm.

Nuclear retention is not required for NIC activity.
Subsequent experiments assessed the consequences of placing spatial constraints on NIC. Thus, we used NIC constructs modified for nuclear retention by an additional nuclear localization sequence (GFP-N1^{IC-NLS}) or a nuclear export signal (GFP-N1^{IC-NES}) to increase or decrease nuclear retention, respectively.[28,29] An additional NLS (GFP-N1^{IC-NLS}) resulted in a loss of NIC-mediated antiapoptotic activity (Figure 7a) compared with cells expressing NIC ($P < 0.001$). However, the GFP-N1^{IC-NES} construct tested in the same experiments was as effective as NIC ($P = 0.64$) in protecting cells from neglect-induced death (Figure 7a). These data suggest that the antiapoptotic signaling cascade was
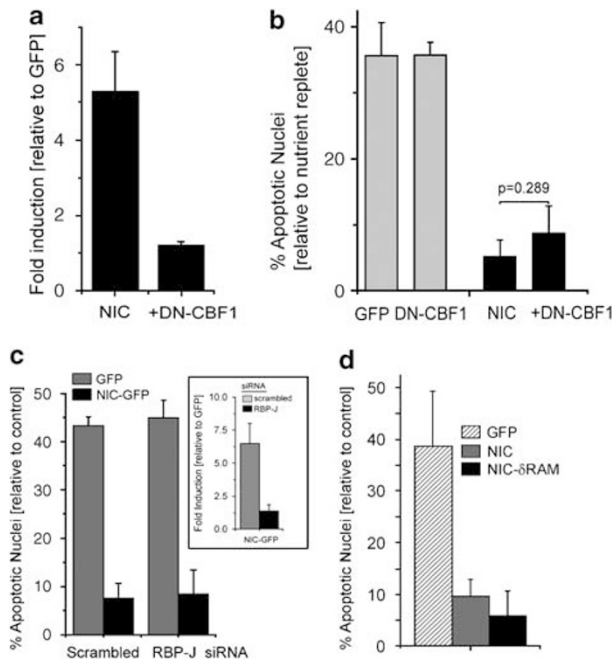
Figure 6 Notch activity is CBF1/RBP-J independent. (**a**) Hes1 promoter activity measured in a luciferase assay in cells expressing the indicated constructs. (**b**) Induction of apoptotic nuclear damage in HeLa cells transfected with GFP or NIC-GFP with and without DN-CBF1. Cells were cultured overnight and assessed for apoptotic damage after culturing for 6 h in the complete medium (CM) or buffered saline. Values in graph are corrected for the CM condition. (**c**) HeLa cells were transfected with GFP or NIC-GFP in cells pretreated with siRNA to RBP-J or with a scrambled control. After an overnight culture, each transfection group was continued with CM or buffered saline. Apoptotic nuclear damage was assessed in GFP + cells after 6 h. Values in the graph are corrected for the CM condition; inset shows Hes1 promoter activity as measured in a luciferase assay for the same conditions. (**d**) HeLa cells were transfected with GFP + empty vector, NIC-GFP, or GFP + NIC-δRAM, and cultured overnight. Cultures were continued with CM or buffered saline, and apoptotic nuclear damage was assessed in GFP + cells after 6 h. Values plotted in the graph are corrected for the CM (control) condition. In all panels, data are shown as mean ± S.D. derived from 3–5 independent experiments

activated in the cytoplasm as prolonged nuclear residence curtailed NIC function, prompting us to test for cytoplasmic pools of NIC-GFP. In confocal images of NIC-GFP-transfected cells, although enriched in the nucleus, at appropriate laser power, a GFP signal was also discerned in the cytoplasm (Figure 7b, inset). To discriminate between free GFP and NIC-GFP, we used fluorescence correlation spectroscopy to test for a cytoplasmic pool, if any, of NIC-GFP in live cells. The diffusion coefficient for NIC-GFP ($D = 2.9 \pm 0.7 \, \mu m^2/s$) was expectedly lower than that for GFP ($D = 20.8 \pm 1.8 \, \mu m^2/s$) commensurate with the higher molecular weight of the former. However, the autocorrelation curves for NIC-GFP in the nucleus and cytoplasm overlapped, consistent with a cytoplasmic pool of NIC-GFP (Figure 7b).

To confirm non-nuclear localization, we tested a membrane-tethered construct, modified to facilitate membrane sequestration by replacing the intracellular domain of the CD8 transmembrane protein with NIC-GFP to generate the CD8-NIC-GFP recombinant. Confocal microscopy established that unlike NIC-GFP, the CD8-NIC-GFP chimera was not detected

in the nucleus (Figure 7c). The chimera did not activate the Hes1 promoter in luciferase assays (Figure 7d), but protected from neglect-induced death (Figure 7e). Further, CD8-NIC-GFP recapitulated the signaling pathway activated by NIC-GFP. Antiapoptotic activity of CD8-NIC-GFP was abrogated by coexpressing DN-Akt (Figure 7e). The siRNA to mTOR or Rictor, but not to Raptor, abolished CD8-NIC-GFP-mediated antiapoptotic activity (Figure 7f and g). NIC-GFP was also included for comparison in this analysis, but the data are not shown to avoid repetition. Thus, the experiments with the CD8-NIC-GFP and the GFP-N1[IC-NES] constructs argued that nuclear residence was not essential for the inhibition of apoptosis or for the activation of mTOR, Rictor, and Akt in this paradigm.

Notch activity is shown in multiple cellular systems. Finally, we tested whether Notch inhibited neglect-induced apoptosis in other cell systems. Recapitulating the observations made in the HeLa cell line, serum deprivation-induced apoptosis in HEK cells was inhibited by NIC through an mTOR–Rictor-dependent cascade, as indicated by the analysis of cells selectively depleted of components of the cascade (Figure 8a and Supplementary Figure 3A). CA-Akt[T308A], but not CA-Akt[S473A] (data not shown), inhibited apoptosis through the mTORC2-dependent signaling pathway again assessed by selectively knocking down intermediates. The CA-Akt[T308A-S473E] recombinant was dominantly active in these cells as well (Figure 8a). NIC also blocked the apoptotic response to serum deprivation or removal of medium in two other cell lines, CaSki (Figure 8b and c) and LTG (Supplementary Figure 3B). Activated T cells are dependent on cytokines for the assimilation of extracellular nutritional cues undergoing apoptosis in the absence of cytokines even in nutrient-rich environments. Extending an earlier result, we now show that membrane-tethered CD8-NIC-GFP blocked the neglect-induced death of activated T cells (Figure 8d). Further, activated T-cell apoptosis was inhibited by CA-Akt[T308A], supporting a role for AktS473 in this pathway (Figure 8e and f). These data suggest that the molecular correlates of the Notch-activated survival pathway may also operate in primary cells.

Discussion

Here, we show that Notch activity coordinated survival in response to nutritional cues in mammalian cells. Several unusual features of Notch signaling were revealed in this study. Strikingly, nuclear functions of NIC were not essential for this pathway and were specifically shown to be independent of the transcription factor, CBF1. NIC activity could be initiated by a membrane-anchored form of NIC, and integrated with Rictor and mTOR triggering the activation of Akt, and consequently cell survival. In support of this, independently inhibiting mTOR, Rictor, or the effector kinase Akt compromised Notch function (Figures 3 and 4). Consistent with the requirement of mTORC2, ectopically expressed CA-Akt[T308A] (a functional AktS473 mutant dependent on mTORC2 activity), but not CA-Akt[S473A] (functional T308), inhibited apoptosis. Thus, the antiapoptotic activity of NIC, CA-Akt, or CA-Akt[T308A] was regulated by Rictor and mTOR, but not by
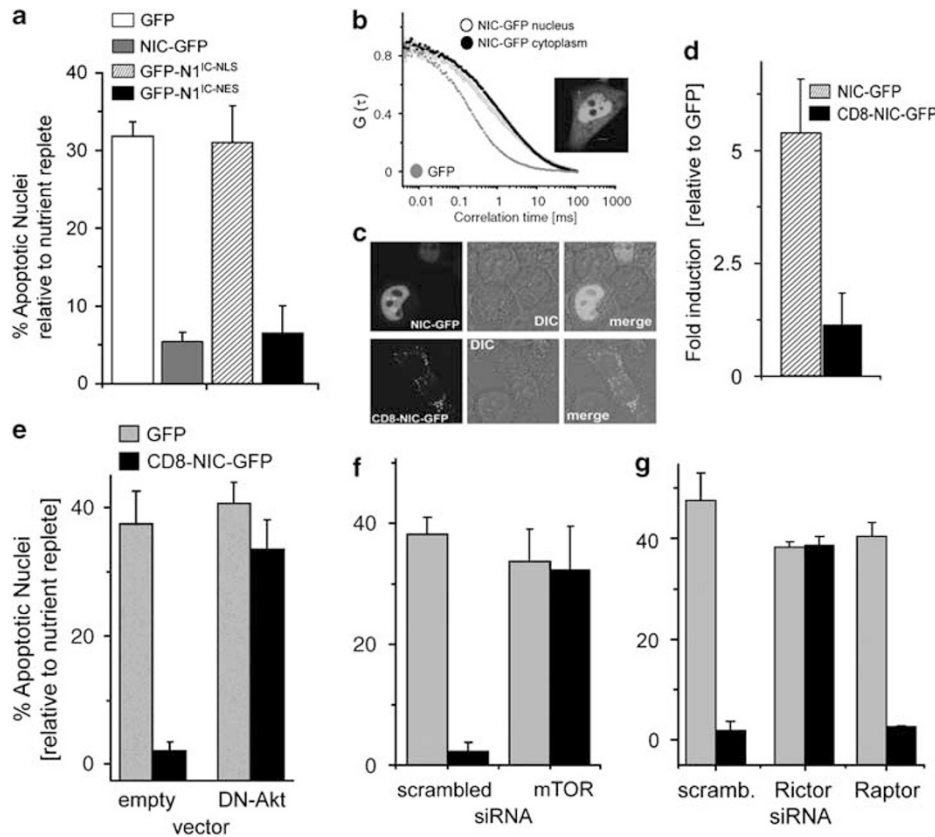
Figure 7 CD8-NIC-GFP blocks death through the mTORC2 pathway. (**a**) HeLa cells were transfected with the indicated plasmids and cultured overnight. Subsequently, culture medium was replaced with the complete medium (CM; nutrient replete) or buffered saline (neglect). Apoptotic nuclear damage was assessed after 6 h. (**b**) Autocorrelation curves for GFP (red) and NIC-GFP in the cytosol (black) and nucleus (gray); inset shows the fluorescence image of an NIC-GFP-transfected cell; scale bar: 5 μm. (**c**) Confocal images (central plane) of cells expressing NIC-GFP or CD8-NIC-GFP, and the corresponding images in DIC setting; scale bar: 5 μm. (**d**) Hes1 promoter activity measured using the luciferase assay in cells expressing the indicated constructs. (**e**) HeLa cells transfected with the indicated plasmids were cultured overnight. Subsequently, culture medium was replaced with CM (nutrient replete) or buffered saline (neglect). Apoptotic nuclear damage was assessed after 6 h. HeLa cells (**f** and **g**) were pretreated with the indicated siRNA. After 48 h, cells were transfected with GFP or CD8-NIC-GFP. Each transfection group was cultured in CM and buffered saline as described in panel a. Apoptotic nuclear damage was assessed after 6 h. In panels a, e, f and g, data are normalized to the CM (nutrient replete) condition and plotted as mean ± S.D. from three independent experiments

Raptor (Figures 4 and 5). Furthermore, the CA-Akt^{S473E} recombinant was dominantly active and inhibited apoptosis independent of proximal events in NIC signaling. We show that this cascade regulated the apoptotic response to serum deprivation or removal of medium. Further, non-nuclear localized recombinant CD8-NIC and AktS473 activity independently blocked the apoptotic response triggered by cytokine deprivation in activated T cells, supporting the key observations made in the cell lines.

Genetic and molecular studies have established that despite the reiterative use of common signaling cascades, varied outcomes result from context-dependent cross-talk between pathways.[30] The Notch pathway is one of the relatively small number of signal transduction cascades coordinating diverse outcomes associated with metazoan development.[4] Both mTOR and Akt activate evolutionarily conserved pathways, which serve as nutrient sensors, promoting cell survival, growth, and division.[31,32] Akt positively regulates mTOR function through the phosphorylation of TSC2,[15] and can also function as a downstream target of

mTOR–Rictor activity through a pathway conserved across species.[17–19,22] In agreement with our data positioning mTOR and Rictor as regulators of survival, defects in responses to growth factor (IGF and PDGF)-mediated signaling have been reported in Rictor-null primary fibroblasts.[25]

Positioning Akt as the principal effector of Notch-mediated survival is consistent with the established functions of Akt in regulating cellular bioenergetics and mitochondrial integrity.[13,14] Phosphorylation of Akt on both S473 and T308 are required for Akt activity.[33,34] Notably, there is evidence suggesting otherwise, with differences in substrate preferences associated with phosphorylation on the serine or threonine residue of the kinase.[23,25,35] In our experiments, Ala substitution of S473 abrogated antiapoptotic activity, although this was not the case with T308. We do not rule out contributions of endogenous Akt to the function of the recombinant proteins in the experiments. Nonetheless, the loss of S6K1 activity in cells expressing Akt^{T308A}, but not Akt^{S473A}, indicated that endogenous Akt activity may not fully account for the phenotypes observed. The modulation of Akt
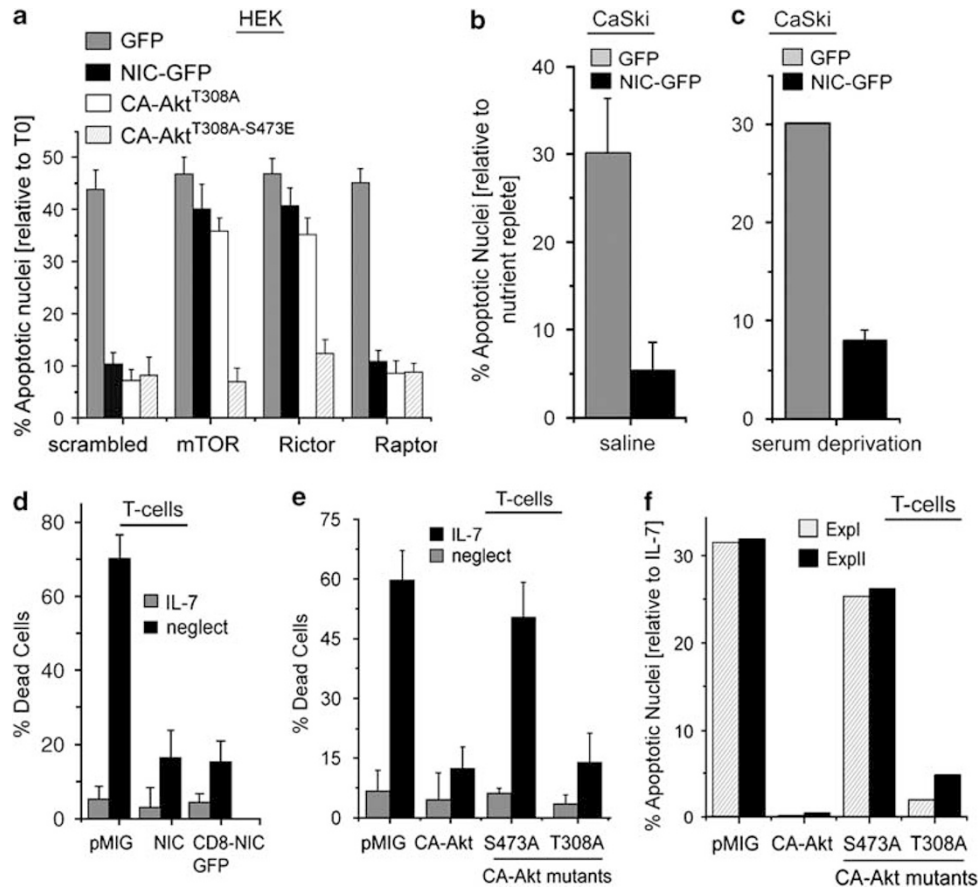
Figure 8 NIC blocks neglect-induced death in multiple cellular systems. (**a**) HEK cells were pretreated with the indicated siRNA for 48 h, and then transfected with the plasmids shown. Cells were cultured without serum for 90 h before assessing apoptotic damage. Data are plotted relative to residual apoptotic damage at the onset of the assay (T0). CaSki cells (**b** and **c**) were transfected with GFP or NIC-GFP and cultured overnight. Subsequently, culture medium was replaced with the complete medium (CM; nutrient replete) or buffered saline (**b**), or medium without serum (**c**). Apoptotic nuclear damage was assessed in GFP + cells using Hoechst 33 342. The data are corrected to the nutrient replete condition and are the mean ± S.D. from three independent experiments. The error bar in the GFP condition in panel c is very low, and hence not visible. (**d**) T cells retrovirally infected with the indicated constructs were cultured with or without the cytokine IL-7 for 18 h. Cell death was assessed as described in Materials and Methods and represent mean ± S.D. from three independent experiments. Cell death in response to cytokine deprivation in activated T cells retrovirally infected with pMIG or the CA-Akt mutants indicated. (**e** and **f**) . In panel f, cells were sorted and nuclear damage was assessed using Hoechst 33 342. In panel e, the data represent mean ± S.D. of three separate experiments, and in panel f, data from two independent experiments are plotted

function by disruption of mTOR or Rictor was corroborated by the analysis of the Ala mutants and the phosphomimetic. These data suggest that AktS473-dependent activity is a necessary step for the antiapoptotic cascade activated by Notch.

As mentioned earlier, NIC function was independent of the DNA-binding factor, CBF1, an activator of canonical Notch signaling. Non-canonical Notch signaling is reported in flies[36] and mammalian cells.[37] The demonstrated requirement for Notch processing, but CBF1 independent signaling, and functional interactions with molecules, such as mTOR and Rictor, prompted an assessment of the subcellular localization of NIC for the activation of this cascade. The experiments with the GFP-N1[IC-NES] recombinant supported the cytoplasmic localization of the signaling cascade activated by NIC. These data were strengthened by experiments with CD8-NIC-GFP, which also recapitulated the requirement for mTOR, Rictor, and Akt for NIC function. Furthermore, lending credence to the cytoplasmic localization of NIC activity, FCS analysis supported a cytoplasmic pool of NIC-GFP in transfected cells.

Ongoing experiments are attempting to identify the compartments where NIC activity is localized. At this time, we can only speculate that NIC may be actively sequestered in the cytosol for the functional outputs described by us. We propose a novel, non-canonical signaling cascade activated by NIC, which may indeed be membrane proximal in localization (Figures 6–8). The cascade suggests a possible conserved mechanism by which extracellular cues transmitted through the Notch receptor to the cellular nutrient sensor mTOR are coupled with survival through mTORC2-dependent activation of Akt. The physical organization and functional outputs of the Notch–mTOR–Rictor signaling complex remain to be established. These are being assessed in primary cells and by live cell imaging to address issues of recruitment dynamics as well as the spatiotemporal regulation of Notch, Rictor, and other interacting proteins.

Materials and Methods

Cells. The HeLa, HEK, CaSki, and LTG cell lines were maintained in culture medium supplemented with antibiotics and 5% fetal calf serum (Hyclone, Logan,

UT). T cells were isolated (using commercially available reagents) from spleens of 6- to 8-week-old C57BL/6 mice, and stimulated with beads coated with antibodies to CD3–CD28 (Dynal, Norway) for 48 h. At the end of this culture period, beads were removed by magnetic separation, and activated T cells were washed and continued in culture with the cytokine, IL-2 (R&D Systems), if required, or used in assays of neglect. All experiments involving animals were performed with the approval of the Institutional Animal Ethics Committee at NCBS, Bangalore.

Reagents. Antibodies were procured from the following sources: myc (clone 9E10), Notch (6014), and Akt from Santa Cruz (Santa Cruz, CA, USA); α-tubulin, actin from Neomarker (Fremont, CA, USA); Bax, pAkt-T308, and pAkt-S473, phosphorylated substrates of Akt, phospho-S6K, S6K, cleaved Notch1 (Val-1744), c-myc, mTOR, Raptor, and Rictor from Cell Signaling Technology (Beverly, MA, USA). Protein-G Agarose beads were from Pierce Biotechnology (Rockford, IL, USA). siRNA to Bax, mTOR, Rictor, Raptor, Akt1, RBP-J, Notch1, and the scrambled control were obtained from Dharmacon Inc (Chicago, IL, USA). The TUNEL kit was obtained from Roche (Basel, Switzerland). Mitotracker Red 580 was from Invitrogen, CA, USA. The Dual luciferase kit was from Promega (MD, WI, USA). Annexin-V was from eBioscience, CA, USA.

Plasmids. The NIC-GFP construct has been described earlier.[8] NIC-RFP and CD8-NIC-GFP were generated for this study using standard procedures for subcloning. CA-Akt (myristoylated), DN-Akt, wild-type Akt1 cDNA (Akt-myc), and sJag were from Upstate Biotechnology (Lake Placid, NY, USA). DN-CBF1 was from J Aster, the Notch1[LNG] and Notch1[LNG CC≫SS] constructs were from R Kopan, the pMIG and pMIG-NIC constructs were from A Rangarajan, and the GFP-N1[IC-NLS] and GFP-N1[IC-NES] constructs were from B Osborne. The CA-Akt point mutations, CA-Akt[S473A] and CA-Akt[T308A], and CA-Akt[T308A-S473E] were generated by site-directed mutagenesis, and were also subcloned into the retroviral construct-pMIG. The sequences were verified by automated sequencing (BioServe Biotechnologies India Pvt. Ltd and MWG, India).

Transfections. Cells were routinely transfected at 70–80 percent confluency using lipofectamine 2000 (Invitrogen). Plasmid concentrations were as follows: GFP (1 μg), Notch or Akt constructs (3 μg), and pcDNA3 was used as the empty vector to equalize the concentration of DNA across transfection groups. HeLa or HEK cells were transfected with 100 nM siRNA as per the manufacturer's instructions. Loss of protein was confirmed by western blot analysis, and cells were used for functional or biochemical analysis after 48 h of siRNA transfection. For the luciferase assays, cells were transfected with 1 μg Hes1-Luc and 100 ng Renilla-Luc in combination with the other constructs. The luciferase assay was performed according to the manufacturer's instructions, 36 h post-transfection. Retroviruses were generated and infections of activated T cells were performed as described earlier.[6]

Assays of apoptosis. Growth medium was replaced with PBS (saline buffer) for 6 h or cells were cultured in medium without serum (90 h) to trigger neglect-induced death. In cells transfected with GFP-tagged proteins, nuclear morphology was scored only in GFP + cells. Cells were stained for 5–10 min at ambient temperature using the DNA-binding dye Hoechst 33 342 (1 μg/ml), and scored for apoptotic nuclear morphology by microscopy. For analyzing mitochondrial morphology, HeLa cells in dishes were incubated with MitoTracker Red 580 (100 ng/ml) in complete medium (CM) at 37°C in a CO_2 incubator for 30–45 min. Cells were washed twice with PBS before analysis. Images were obtained using Zeiss 510 Meta and 63X NA 1.4 objective lens. The TUNEL reagent and Annexin-V were used as per the manufacturer's instructions.

Retrovirally infected T cells were identified on the basis of GFP expression. Following infections, all groups were assessed for cell death using the following two assays. In one set, the percentage of residual GFP + cells was scored at the end of culture duration in different assays. Cell death/loss was estimated from the change in the percentage of GFP + cells relative to the percentage in the input population. To assess cell death using nuclear morphology as a readout, GFP + cells in the different infection groups were enriched by fluorescence-based cell sorting. Sort efficiencies were > 90%, and the GFP + sorted cells were used in various assays of neglect and apoptotic nuclear damage scored using Hoechst 33 342.

Immunostaining and confocal imaging. Cells were permeabilized with 0.2% CHAPS, and the anti-myc antibody was used at 1 : 100. Secondary antibody was used at a 1 : 500 dilution. Images were acquired using Zeiss LSM 510 Meta and 63X NA 1.4 objective lens.

Assay of Akt phosphorylation. HEK and HeLa (two million) cells were cotransfected with Akt1-myc and appropriate vectors, maintaining total DNA constant across transfection groups with the empty vector pcDNA 3.0. After 24 h of transfection, cells were lysed with MPER buffer (Pierce Technology) supplemented with protease and phosphatase inhibitors. In all, 10 μg of antibody to c-myc (9E10 clone) was used to immunoprecipitate recombinant Akt. The immunoprecipitated bead-bound complex was divided into two and resolved on reducing gels. Proteins were electrophoretically transferred to membranes and detected by western blot analysis. Routinely, one membrane was probed with antibodies specific for either phospho-form of Akt, whereas the other was probed with the c-myc antibody to estimate recombinant Akt protein. Membranes were developed together to keep the exposure time constant. Antibodies specific to phospho-forms of Akt reveal two bands in the whole cell lysate corresponding to endogenous and recombinant Akt protein. Lanes loaded with the immunoprecipitate revealed a single band indicating the recombinant protein.

Fluorescence correlation spectroscopy. For FCS experiments, cells were examined with C-Apochromat 40X, 1.2 NA water- corrected objective using a previously published protocol.[38] EGFP fusion protein was excited with the 488-nm line of an argon–ion laser and the emission collected with a 500-530 nm band pass filter. The data were collected for a period of 10-s intervals and averaged over 10 runs to get the autocorrelation function and the corresponding fits. All FCS data are an average of more than eight cells.

Statistical analysis. All data are presented as mean ± S.D. derived from a minimum of three independent experiments unless stated otherwise. Statistical significance was calculated using F-test and a two-population Student's t-test set at $P < 0.001$. In all experiments, apoptotic damage is normalized to the values obtained for cells cultured in nutrient replete conditions.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)