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Notch4 Signaling Confers Susceptibility to TRAIL-Induced Apoptosis in Breast Cancer Cells

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ABSTRACT

Notch signaling has been established as a key regulator of cell fate in development, differentiation, and homeostasis. In breast cancers, increased Notch1 and Notch4 activity have been implicated in tumor progression and, accumulation of the intracellular domain of Notch4 (ICN4), reported in basal breast cancer cells. While, TNF-related apoptosis-inducing ligand (TRAIL) receptor agonists have demonstrated selectively in targeting tumor cells, the majority of primary tumors are resistant to TRAIL. This necessitates the identification of factors that might regulate TRAIL sensitivity. Here we investigate TRAIL sensitivity in tumor cells following the modulation of Notch (1 and 4) activity using siRNA-mediated depletions or ectopic expression of GFP-tagged constructs of the intracellular domains of Notch1 (ICN1) or Notch4 (ICN4). Our findings suggest that Notch4, but not Notch1 signaling, sensitizes breast tumor cells to TRAIL-induced apoptosis. ICN4-induced sensitization to TRAIL is characterized by CBF1-dependence. Apoptosis was mediated via caspase-8 activation and regulated by the Bcl-2 family pro-apoptotic proteins Bak and Bid. Finally, we present evidence that endogenous Notch4 activity regulates susceptibility to TRAIL in basal-like breast cancer cells but not in cell lines of luminal origin. These experiments reveal a hitherto unexplored Notch4-TRAIL signaling axis in breast cancer cells. J. Cell. Biochem. 116: 1371–1380, 2015.

KEY WORDS: NOTCH4; TRAIL; SIGNALING; APOPTOSIS; BREAST CANCER

Deregulation of key signaling pathways is a hallmark of cancer development and modulation of Notch activity can directly influence tumor progression [Korkaya and Wicha, 2009]. The four transmembrane Notch receptors in humans Notch1–Notch4 are activated by the binding of any of the five ligands, Delta-Like (DLL) 1, 3, and 4 and Jagged1 and 2. Upon ligand binding, Notch receptor undergoes a series of proteolytic cleavages [Schroeter et al., 1998; Brou et al., 2000; Mumm et al., 2000], releasing the intracellular domain of Notch (ICN), which translocates to the nucleus and regulates transcription. In addition to this canonical signaling pathway, Notch has also been reported to mediate biological processes in a ligand or transcription-independent manner [Perumalsamy et al., 2009; Sanders et al., 2009; Kwon et al., 2011]. Through both canonical and non-canonical signaling, Notch can integrate with multiple pathways involved in regulating cell fate decisions and consequently, impaired Notch activity has been implied as a contributing factor in carcinogenesis.

Signaling to apoptosis using TNF-related apoptosis-inducing ligand (TRAIL) [Wiley et al., 1995; Pitti et al., 1996] has garnered interest, primarily because of its unique ability to target tumor cells while sparing normal cells [Hao et al., 2004]. There are four transmembrane TRAIL receptors expressed on the cell surface; TRAIL-R1–TRAIL-R4 and one soluble receptor, osteoprotergerin [MacFarlane, 2003]. However, upon binding of TRAIL, only TRAIL-R1 or TRAIL-R2 can form the death-inducing signaling complex (DISC) via the recruitment of Fas-associated death domain (FADD) and procaspase-8 [Kischkel et al., 2000; Sprick et al., 2000]. Within the TRAIL–DISC, activation of caspase-8 leads to processing and activation of the

Abbreviations used: TRAIL, TNF-related apoptosis-inducing ligand; GSI, γ-secretase inhibitor; DISC, death-inducing signaling complex; ICN, intracellular domain of Notch; MAML, mastermind-like.

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effector caspase, caspase-3 or the BH3-only protein, Bid. Truncated Bid (tBid) translocates to the mitochondria where it activates Bax/Bak, leading to loss of mitochondrial membrane potential and release of cytochrome *c*, eventually culminating in cell death [Luo et al., 1998].

Despite accumulating reports that most cancer cell lines are sensitive to TRAIL-induced apoptosis, it is becoming evident that a majority of primary human tumors are resistant to TRAIL [Ehrhardt et al., 2003; MacFarlane et al., 2005a; Herbst et al., 2010]. Although aberrant Notch signaling has been reported in breast cancers, little has been studied regarding the Notch-TRAIL signaling axis. Notch activity is known to induce chemo-resistance in several contexts; however, one study has demonstrated that in hepatocellular carcinoma cells, Notch1 ectopic expression sensitizes cells to TRAIL-induced apoptosis [Wang et al., 2009]. Here, we investigate whether Notch signaling influences the sensitivity of breast cancer cells to TRAIL-induced apoptosis.

In the experiments in this study, we employed GFP-tagged versions of recombinant intracellular domains of Notch1 (ICN1) or Notch4 (ICN4) or RNA interference approaches to modulate Notch signaling in breast cancer cell lines and assess the consequences to TRAIL-induced apoptosis. For this purpose, we chose four different breast tumor cell lines as model systems. The cell lines BT474 and T47D belong to the luminal subtype of breast cancers and express both—estrogen and progesterone—receptors [Neve et al., 2006]. Additionally BT474 cells also over-express the Her2 receptor. The cell lines MDA-MB-231 and HCC1806 represent the basal subtype of breast cancers and show a triple-negative phenotype. Our results demonstrate that crosstalk between Notch4 and TRAIL signaling culminates in cell death coordinated by caspase-8-Bid-Bak activity. We also show that in the triple-negative breast cancer cells, TRAIL sensitivity is regulated by endogenous Notch 4 activity.

MATERIALS AND METHODS

CELL CULTURE

MDA-MB-231 and HeLa cells were obtained from American Typed Cell Collection (Manassas, VA) and T47D cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). BT474 and HCC1806 cells were a kind gift from A. Rangarajan (Indian Institute of Science, Bangalore, India). All breast cancer cell lines were maintained in RPMI 1640 (HeLa cells were cultured in DMEM), supplemented with 5% fetal calf serum (Thermo Scientific Hyclone™, Waltham, MA) and antibiotics.

REAGENTS

Human recombinant wild-type TRAIL and mutant ligands of TRAIL, specific to TRAIL-R1 (R1L) or TRAIL-R2 (R2L), were generated in-house at the MRC Toxicology Unit, Leicester, UK as described previously [MacFarlane et al., 2005b].

CELL SURFAE TRAIL RECEPTOR EXPRESSION

Cells were re-suspended in blocking buffer (10% normal goat serum in PBS) and following 30 min on ice were incubated with anti-TRAIL receptor antibodies or an isotype-matched control antibody (Insight Biotechnology, Wembley, UK) for 1 h on ice and then analyzed on FACSCalibur™ (BD Biosciences, Franklin, NJ).

PLASMIDS AND siRNA

The generation of the GFP-tagged intracellular domain of Notch1 (ICN1-GFP) construct has been described previously [Sade et al., 2004]. ICN4-GFP was a kind gift from A. Rangarajan (IISc, Bangalore, India); dominant negative CBF1 was gifted by J. Aster (Harvard Medical School, Boston, MA); dominant negative caspase-9 and (bv)p35 were gifted by C. Vincenz (University of Michigan Medical School, Ann Arbor, MI) and C. Zacharchuk (National Cancer Institute, National Institute of Health, Bethesda, MD), respectively. Bcl-xL-RFP [Parikh et al., 2007] and Bak-RFP (Bak plasmid obtained from Origene [Rockville, MD]) were generated in-house. Bcl-2 was purchased from Millipore-Upstate (Charlottesville, VA). Empty vector pcDNA3 was used to equalize DNA concentrations across experimental groups. siRNA oligonucleotides for scrambled (D-001810-10-05), caspase-8, RBPjK (L-007772-00-0005), Bid (L-004387-00-0005), Bak (L-003305-00-0005), Notch1 (L-007771-00-0005), and Notch4 (L-011883-00-0005) are from Dharmacon, Thermo Scientific.

TRANSFECTIONS

MDA-MB-231 and T47D cells were seeded at 0.5×10^5 cells/well in 6-well plates and after 12–16 h were transfected with plasmids using Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA). ICN1-GFP and ICN4-GFP were used at final concentrations of 1 μg/well; Bak-RFP, Baculoviral (bv)p35, dominant negative caspase-9 (DN-C9), Bcl-2, Bcl-xL-RFP, and dominant negative-CBF1 were used at 2 μg/well. Alternatively, T47D cells were plated at a density of 3×10^5 cells in 35 mm dishes and after overnight incubation, were transfected with siRNA oligonucleotides using Lipofectamine RNAiMax as per manufacturer's instructions. siRNA oligonucleotides were used at a final concentration of 100 nM. Loss of protein was confirmed by SDS–PAGE/Western blotting.

ASSAYS OF CELLULAR DAMAGE

For assessment of mitochondrial transmembrane potential, cells were harvested and re-suspended in 500 μl media. After a 20 min recovery period, cells were incubated with TMRM (Molecular Probes, Eugene, OR; 50 nM) for 20 min at 37°C, washed once to remove excess dye and analyzed using a FACSCalibur™ at excitation/emission wavelengths of 549/574 nm, respectively.

To detect apoptotic nuclear damage, cells were stained for 5 min at room temperature with Hoechst 33342 (1 μg/ml) and nuclear morphology was scored only in GFP-positive cells by fluorescence microscopy. In cells where both RFP- and GFP-tagged plasmids were expressed, only cells positive for both tags were scored for nuclear damage.

SDS–PAGE AND WESTERN BLOT ANALYSIS

Lysates of whole cells were prepared in Lamelli buffer and stored at −80°C till further use. Samples were run for SDS–PAGE and probed for proteins using the primary antibodies described below, followed by HRP-labeled secondary antibodies and detected using ImageQuant LAS 4000 (GE Healthcare Life Sciences, Little Chalfont, UK).

Antibodies were sourced as follows: anti-Hes1 from Millipore, anti-Bak from BD Pharmingen, anti-caspase-8, anti-STAT5 and anti-Bid from Cell Signaling Technology (CST, Danvers, MA), anti-Bcl2 and anti-Bcl-xL from Santa Cruz Biotechnology, Inc. (Dallas, TX), anti-actin and anti-tubulin from Thermo Scientific. Secondary anti-mouse antibody and anti-rabbit was obtained from CST. For visualizing the Bcl-xL Western blot, the secondary anti-rabbit antibody was pre-cleared with untreated whole cell lysate (4°C for 30 min) before use.

CLONOGENIC ASSAY

Cells were seeded at 4×10^4 cells/well in 6-well plate and transfected with GFP or ICN4. Twenty-four hours post-transfection, cells were treated with TRAIL (500 ng/ml) for 6 h before replacing media. Cells were stained with staining solution (0.5% crystal violet, 50% ethanol, 0.25% NaCl, and 1.57% formaldehyde) for 30 min after 6 days of culture. The stain was solubilized using 1% SDS and intensity read using a spectrophotometer (Metertech UV/VIS, Taipei, Taiwan) at an OD of 590 nm.

STATISTICAL ANALYSIS

All data are represented as mean ± standard deviation (mean ± SD) for three or more independent experiments. Statistical significance was measured using a two-tailed Student's *t*-test.

RESULTS

ECTOPIC EXPRESSION OF ICN4 SENSITIZES CELLS TO TRAIL-INDUCED APOPTOSIS

To test if Notch4 signaling modulates TRAIL sensitivity, we employed the cell line T47D, which is resistant to TRAIL-induced apoptosis (Fig. 1a, i) despite expressing both TRAIL-R1 and TRAIL-R2 receptors on the cell surface (Fig. 1a, i, inset). We ectopically expressed the intracellular domain of either Notch1 (ICN1)—previously demonstrated to sensitize hepatocellular carcinoma cells to TRAIL [Wang et al., 2009]—or Notch4 (ICN4). In contrast to the published observation in T47D cells, the expression of ICN4, but not ICN1, induced TRAIL sensitivity despite equivalent expression patterns of the recombinant proteins (Fig. 1a, i and ii and Supplementary Fig. S1a). Furthermore, siRNA-mediated depletion of Notch1 did not modulate TRAIL-induced apoptosis in ICN4-expressing cells indicating that Notch1 is not involved in Notch4-mediated sensitization to TRAIL (Supplementary Fig. S1b). As another readout of cellular damage, mitochondrial trans-membrane potential (MTP) was also assessed in treated and control cells. The loss of MTP in ICN4-positive T47D cells treated with TRAIL confirmed (ICN4-induced) susceptibility to TRAIL-induced apoptosis (Fig. 1b, i and ii). Finally, in the clonogenic assays, crystal violet staining demonstrated the presence of fewer surviving colonies in cells expressing ICN4 and treated with TRAIL as compared with untreated control (Fig. 1c).

We then expressed ICN1 or ICN4 in another ER-positive breast cancer cell line, BT474 to study if Notch signaling could also modulate sensitivity in inherently TRAIL sensitive cells. In the BT474 cells, ectopic expression of ICN4 but not ICN1 (Fig. 1d, ii) enhanced sensitivity of cells to TRAIL-induced apoptosis as in indicated assays of apoptotic nuclear damage (Fig. 1d, i) or loss in MTP (Fig. 1d, iii). Further to determine if ICN4 regulation of TRAIL sensitivity was restricted to breast cancer cell lines, we expressed ICN1 or ICN4 in the cervical cancer cell line HeLa. Consistent with the data in the breast cancer cell lines, ICN4 but not ICN1 modulated sensitivity of HeLa cells to TRAIL (Fig. 1e). Taken together, these data indicate that ICN4 has an intrinsic ability to modulate TRAIL sensitivity in multiple cell lines.

ICN4-MEDIATED TRAIL SENSITIZATION IS DEPENDENT ON RBPj-κ

We next investigated if sensitization was dependent on nuclear outputs of Notch signaling using a dominant negative form of CBF1 (DN-CBF1), which blocks transcriptional outcomes of Notch activity (Supplementary Fig. S2a). DN-CBF1 significantly attenuated apoptosis induced by TRAIL in ICN4 expressing T47D and BT474 cells, suggesting that transcriptional regulation by Notch4 is necessary for sensitization to TRAIL (Fig. 2a,b). Further, depletion of RBPj-κ in the T47D cells abrogated sensitization of cells to TRAIL-induced apoptosis, confirming a dependence of ICN4 on interaction with RBPj-κ to facilitate sensitization (Fig. 2c). Knockdown of RBPj-κ was confirmed by decrease in the Notch target protein, Hes-1 (Fig. 2c, inset and Supplementary Fig. S2b). Consistent with these data, ectopic expression of dominant negative mastermind-like 1 (DN-MAML) also blocked ICN4-mediated sensitization of T47D cells to TRAIL (Fig. 2d). These results demonstrate that Notch4-mediated modulation of TRAIL sensitivity is dependent on the engagement of ICN4 with its co-factors that regulate transcriptional outcomes.

Next we characterized the TRAIL cell death pathway, which was elicited in response to modulation of sensitivity by ICN4 expression. We employed the T47D cell line for this purpose, as there was no endogenous sensitivity to TRAIL, which would interfere with the characterization of the induced sensitivity.

APOPTOSIS INDUCED BY ICN4 AND TRAIL REQUIRES THE MITOCHONDRIAL AMPLIFICATION ARM

To verify if the cell death pathway activated by TRAIL in ICN4-expressing cells was caspase-dependent, the pan-caspase inhibitor baculoviral (bv)p35 was expressed in T47D and BT474 cells. The expression of bvp35 rescued T47D cells from ICN4-driven TRAIL sensitization (Fig. 3a). In BT474 cells, bvp35 protected cells from TRAIL in GFP or ICN4 expressing cells, suggesting that both inherent and enhanced TRAIL sensitivity is caspase-dependent (Fig. 3b). Next, we depleted caspase-8 in T47D cells, prior to ectopic expression of ICN4 (Fig. 3c, inset and Supplementary Fig. S3b), which abrogated Notch4-mediated sensitization of T47D cells to TRAIL-induced apoptosis (Fig. 3c). Further, ablation of the Bcl-2 family protein Bid (Fig. 3d, inset and Supplementary Fig. S3c) inhibited apoptosis caused by TRAIL in ICN4-positive T47D cells (Fig. 3d). The requirement of Bid for TRAIL-induced apoptosis suggests that, in T47D cells, TRAIL requires the mitochondrial amplification arm to facilitate apoptosis [Luo et al., 1998]. The co-expression of dominant negative caspase-9 (DN-C9) also protected the cells from TRAIL-induced apoptosis (Supplementary Fig. S3), consistent with a possible role for cytochrome *c*-dependent signaling during TRAIL-mediated apoptosis.

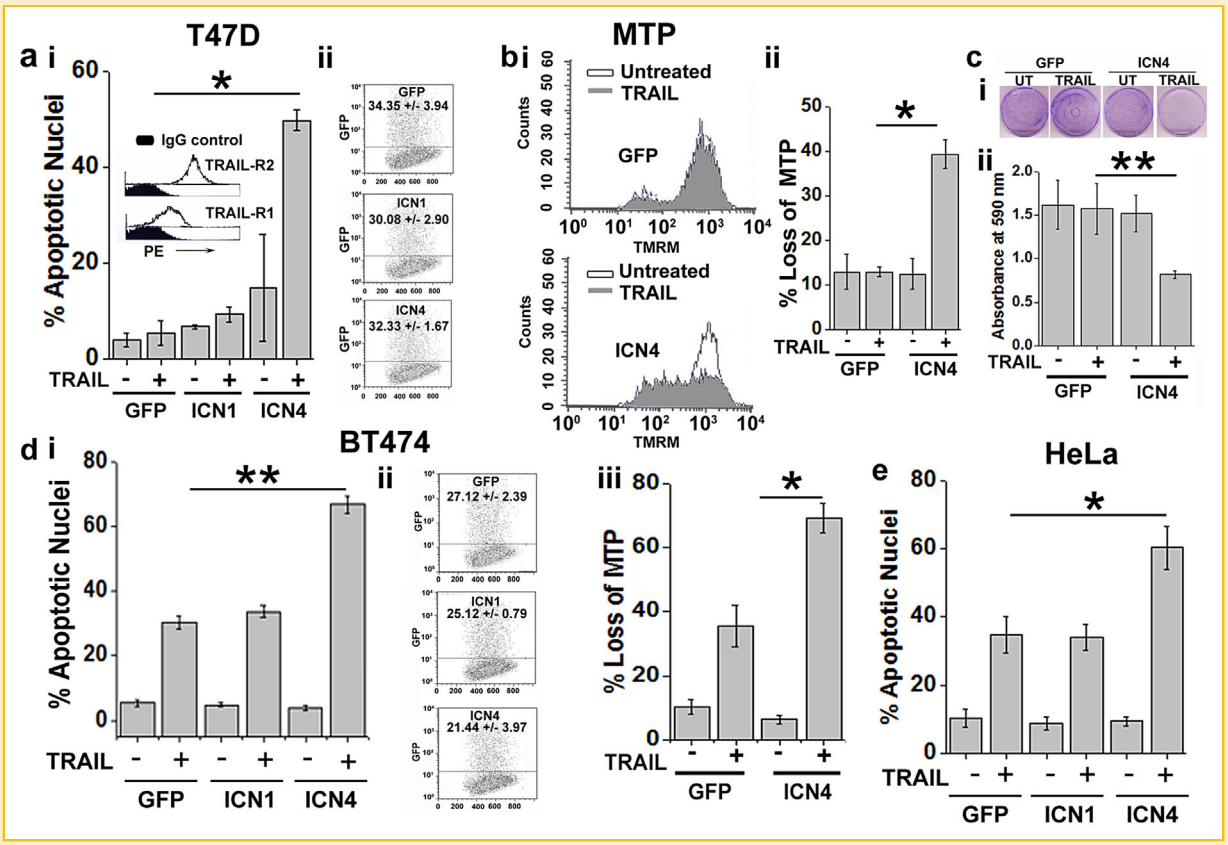

Fig. 1. Ectopic expression of ICN4 sensitizes tumor cells to TRAIL-induced apoptosis (a) ICN1 or ICN4 was ectopically expressed in T47D cells (ii—representative dot plots of transfection) and (i) apoptotic nuclei were assessed post 6 h of TRAIL (500 ng/ml) addition. i: Inset shows representative histograms of relative surface expression levels of TRAIL-R1 or TRAIL-R2 to IgG isotype control. b: Representative histograms of T47D cells expressing either GFP or ICN4 and treated with TRAIL (500 ng/ml for 3 h; (i)) and (ii) quantification of the loss of mitochondrial transmembrane potential (MTP) in ICN4-expressing cells. c: T47D cells transfected with GFP or ICN4 were treated with TRAIL and stained with crystal violet after 6 days culture; (i) representative images of stained cells and (ii) crystal violet was solubilized and quantitated as mentioned in Materials and Methods section (d) BT474 cells were transfected with GFP, ICN1 or ICN4 (ii—representative dot plots of transfection) and treated with TRAIL at 100 ng/ml for 4 h and apoptosis was assessed by (i) counting apoptotic nuclei or (iii) loss in MTP. e: HeLa cells, transfected with GFP, ICN1 or ICN4 were treated with 500 ng/ml TRAIL for 4 h and apoptosis was measured. Values in all panels are the mean ± SD of separate experiments ($n = 3$) with significance $^{*}P < 0.05$, $^{**}P < 0.001$ (Student's t-test).

In the experiments that follow, we characterized the molecular intermediates involved in activation of the mitochondrial amplification arm in TRAIL-induced apoptosis in ICN4-positive cells.

ICN4 SENSITIZATION TO TRAIL-INDUCED APOPTOSIS IS BAK-DEPENDENT

Ectopically expressed Bcl-xL-RFP completely abrogated TRAIL-induced apoptosis in ICN4-positive T47D cells (Fig. 4a and Supplementary Fig. S4a). Similarly, recombinant Bcl-2 significantly attenuated ICN4-mediated TRAIL sensitization of T47D cells (Fig. 4b and Supplementary Fig. S4b), thus verifying a key role for mitochondrial integration of TRAIL-induced apoptotic signaling in ICN4-positive cells. Bax and Bak are members of the Bcl-2 family implicated in pore formation and subsequent loss of mitochondrial membrane potential [Wei et al., 2001]. Hence, we performed knockdown experiments and the ablation of Bak (Fig. 4c inset and Supplementary Fig. S4c) abrogated cell death caused by TRAIL in ICN4-positive T47D cells (Fig. 4c), suggesting that Bak is required for apoptosis induction by TRAIL in these cells. Consistently, when ICN4 and Bak-RFP were co-expressed in T47D cells, ICN4 failed to rescue T47D cells from Bak-RFP-induced apoptosis (Supplementary Fig. S4d), indicating that ICN4 cannot protect cells from apoptotic signaling proceeding through Bak. Based on these data, we suggest that in ICN4-positive cells, TRAIL induces loss of mitochondrial membrane potential via caspase-8-Bid-Bak-dependent signaling.

Since we demonstrate that ectopic expression of ICN4 could enhance sensitivity to TRAIL-induced apoptosis, we next assessed if endogenous Notch4 activity conferred TRAIL sensitivity in breast cancer cells.

NOTCH4 BUT NOT NOTCH1 MODULATES TRAIL-INDUCED APOPTOSIS IN TRIPLE-NEGATIVE BREAST CANCER CELL LINES

We tested if inherent Notch4 activity could regulate TRAIL sensitivity in the MDA-MB-231 and HCC1806 cell lines of basal-cell origin. In MDA-MB-231 cells, which are sensitive to TRAIL (Fig. 5a), depletion of ICN4 inhibited inherent TRAIL sensitivity (Fig. 5b, i and ii and Supplementary Fig. S5a). Similarly in HCC1806 cells, TRAIL induced apoptosis in the cells transfected with scrambled siRNA, but was

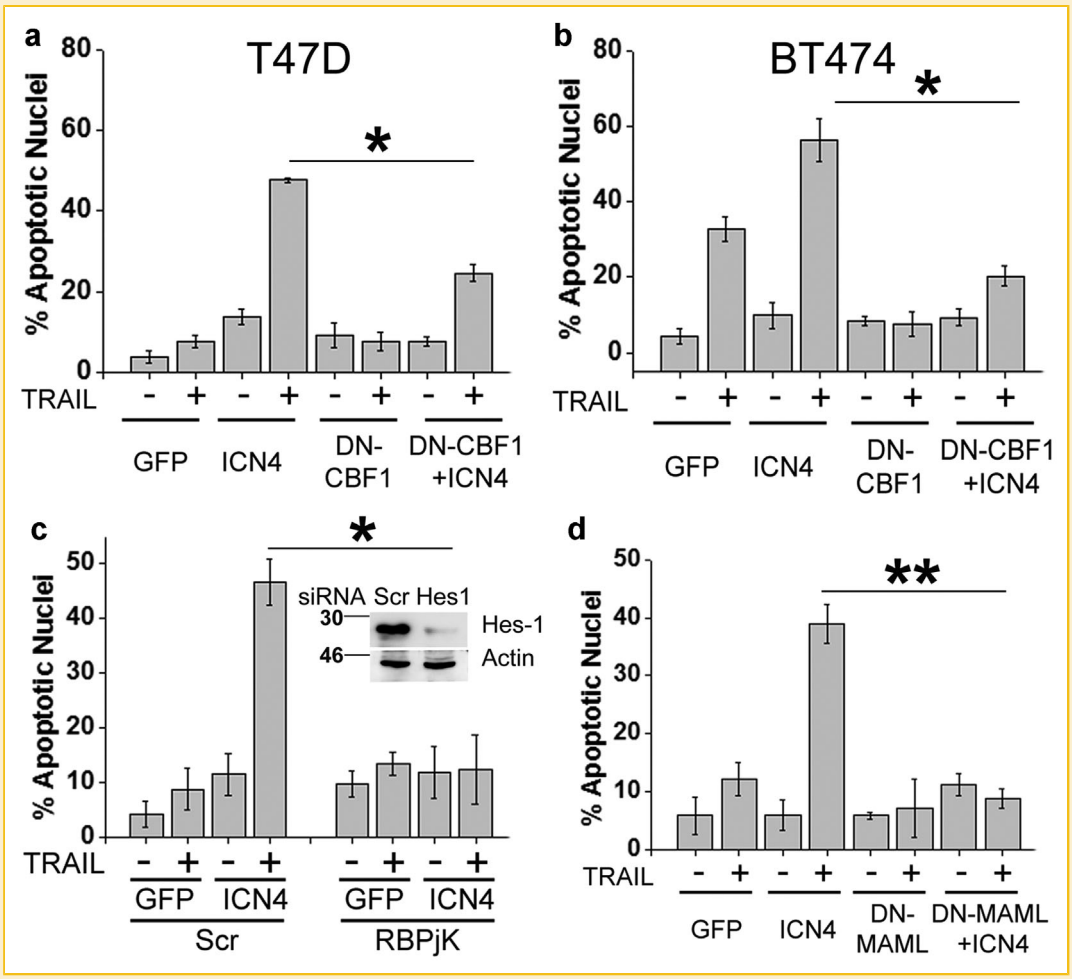

Fig. 2. ICN4–mediated TRAIL sensitization is dependent on RBPjK. a: T47D or (b) BT474 transfected with GFP or ICN4 ± DN-CBF1 were treated with TRAIL (500 ng/ml; 6 h or 100 ng/ml; 4 h, respectively) and apoptotic nuclei were counted. c: GFP or ICN4 was expressed in T47D cells transfected with either scrambled (Scr) or RBPjκ siRNA before treatment with TRAIL (500 ng/ml; 6 h) and assessment of apoptotic nuclei. Inset: immunoblot for Hes1 in cells treated with RBPjκ or scrambled siRNA. d: T47D cells transfected with GFP or ICN4 ± DN-MAML were treated with TRAIL (500 ng/ml; 6 h or 100 ng/ml; 4 h, respectively) and apoptotic nuclei were counted.

without effect in Notch4-depleted groups (Fig. 5c, i and ii and Supplementary Fig. S5b). It should be observed that there are differences in the molecular weight of Notch4 between the MDA-MB-231 and HCC1806 cell lines; this may be due to different post-translational modifications as the molecular weight predicted for Notch4 varies from 40, 61, and 80 kDa in different cells. Together these observations verify the results with recombinant ICN4, suggesting that in these cells endogenous Notch4 signaling regulates sensitivity to TRAIL. Further, as seen in the luminal cancer cell lines, ectopic expression of ICN4, but not ICN1, increased the sensitivity of MDA-MB-231 cells to TRAIL-induced apoptosis, indicating that Notch4 modulates TRAIL sensitivity in these cells (Fig. 5d, i and ii represents transfection efficiency). We assessed if signaling through either TRAIL-R1 or -R2 specifically regulated Notch4-dependent sensitization to TRAIL. In both TRAIL-resistant T47D and TRAIL-sensitive MDA-MB-231 cells, ICN4 induced a significant increase in the extent of apoptosis triggered by a mutant variant of TRAIL, which specifically activates TRAIL-R1 (R1L), but not a variant that activates TRAIL-R2 (R2L; Fig. 5e) [MacFarlane et al., 2005b].

Finally, to test if Notch4 predominantly sensitizes cells to apoptotic stimuli, we treated ICN4-expressing cells with thapsigargin, which triggers apoptosis by perturbing calcium homeostasis [Lytton et al., 1991]. ICN4 inhibited thapsigargin-induced cell death in both the T47D and MDA-MB-231 cells (Fig. 5f), implying that the function of Notch4 in promoting or inhibiting cell death is context dependent. Importantly, ICN4 also inhibited cell death induced by the genotoxic agent etoposide, in the MDA-MB-231 cells, confirming specific sensitization to TRAIL-induced apoptosis (Supplementary Fig. S5c).

DISCUSSION

The data presented in this study show that in the triple-negative breast cancer cell lines, MDA-MB-231 and HCC1806 depletion of Notch4 results in resistance to TRAIL-induced apoptosis. While this data in itself is not sufficient to directly correlate Notch4 signaling with TRAIL sensitivity, evidence from Notch4 modulation in these

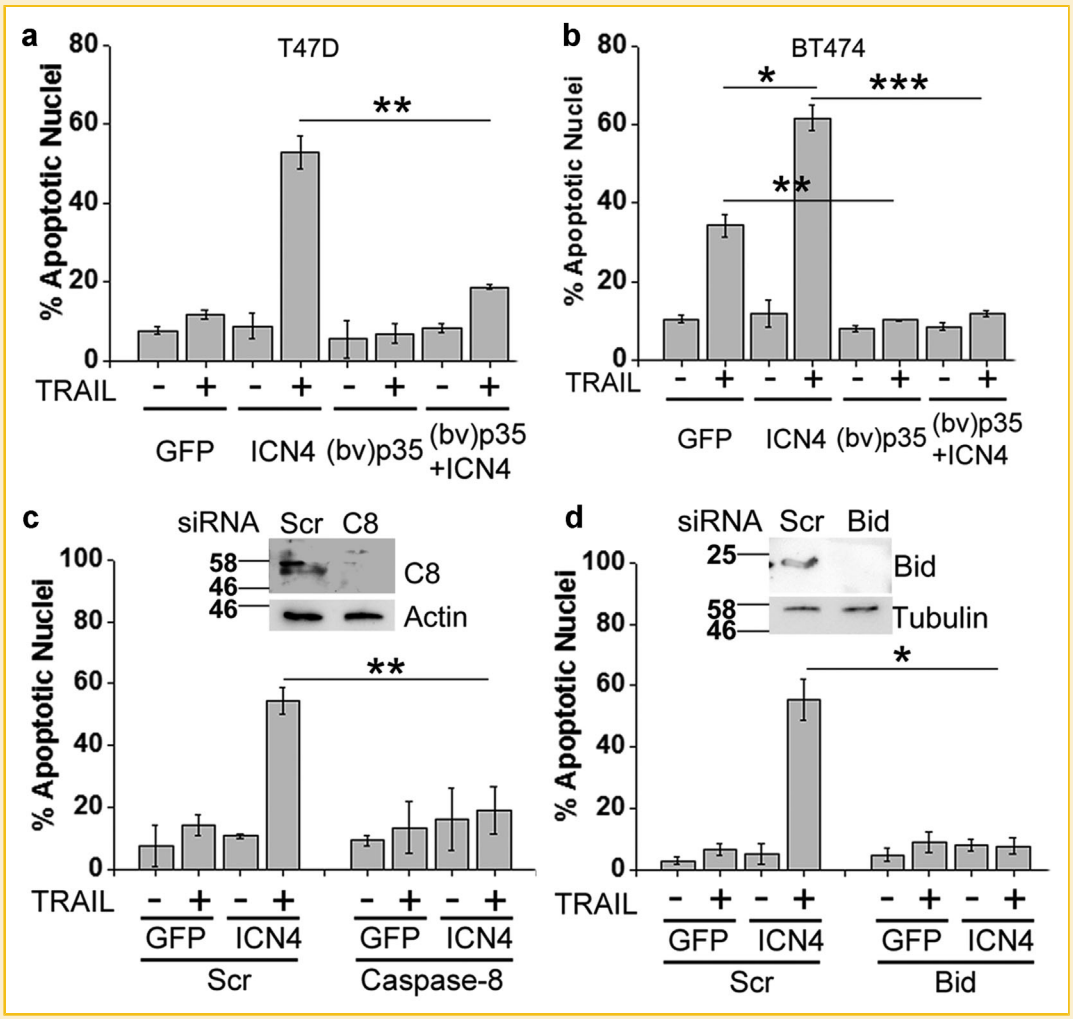

Fig. 3. TRAIL-induced apoptosis in cancer cells is dependent on activation of mitochondrial apoptotic arm. a: T47D or (b) BT474 cells were transfected with ICN4 ± (bv)p35 for 24 h before treatment with TRAIL (500 ng/ml; 6 h or 100 ng/ml; 4 h, respectively) and apoptotic nuclei were scored. c–d: Alternatively, cells depleted of caspase-8 (c; inset) or Bid (d; inset) were transfected with GFP or ICN4 and TRAIL-induced apoptotic nuclear damage was determined. Values in all panels are the mean ± SD of separate experiments ($n = 3$) with significance $^{*}P < 0.05$, $^{**}P < 0.001$ (Student's t-test).

and the estrogen receptor positive cell lines, T47D and BT474, supports the existence of a Notch4-TRAIL signaling axis. Thus, ectopic expression of Notch4 activity enhanced TRAIL sensitivity of the cell lines, MDA-MB-231, BT474, and HeLa possibly by driving Notch4 signaling. Additionally, ectopic expression of ICN4 sensitized the TRAIL-resistant ER positive cell line, T47D to TRAIL-induced apoptosis. Signaling through estrogen receptor is known to block Notch activity by preventing Notch cleavage [Rizzo et al., 2008]; however the introduction of ICN4 into these cells modulated TRAIL sensitivity. Indeed inhibition of estrogen signaling also synergizes with TRAIL to induce cell death in breast cancer cells [Lagadec et al., 2008]. TRAIL reduced the number of colonies formed by T47D cells transfected with ICN4 as compared with untreated control.

Based on our results, we postulate that enhanced endogenous Notch4 activity confers increased sensitivity of tumor cells to TRAIL-R1-induced apoptosis. Notably, ICN4 activity conferred protection to apoptosis induced by the genotoxic agent, etoposide, in the same cells.

Increased Notch4 activity is reported in more than 30% of triple-negative breast cancers [Andre et al., 2009] and in a majority of invasive cancers [Rizzo et al., 2008; Yao et al., 2011]. Since tumor cells inherently possess enhanced Notch4 signaling, these data reveal a possible window of difference between normal and tumor cells. ICN4 inhibited apoptosis induced by thapsigargin and etoposide in breast cancer cells, indicating that Notch4-mediated sensitization to apoptotic stimuli is not the result of an ectopic expression system, but is indeed stimulus-specific within the same cellular system. Since TRAIL sensitivity is not only dependent on Notch4 signaling, therefore a correlation between TRAIL sensitivity and Notch4 activity is difficult to establish. However, the data demonstrates that irrespective of inherent sensitivity, the expression of ICN4 enhances/sensitizes breast cancer cells to TRAIL-induced apoptosis. In this context, it should be noted that an anti-apoptotic function of Notch4 has been reported in endothelial cells where basal Notch4 expression maintains cell quiescence thereby blocking apoptosis [Quillard et al., 2010].

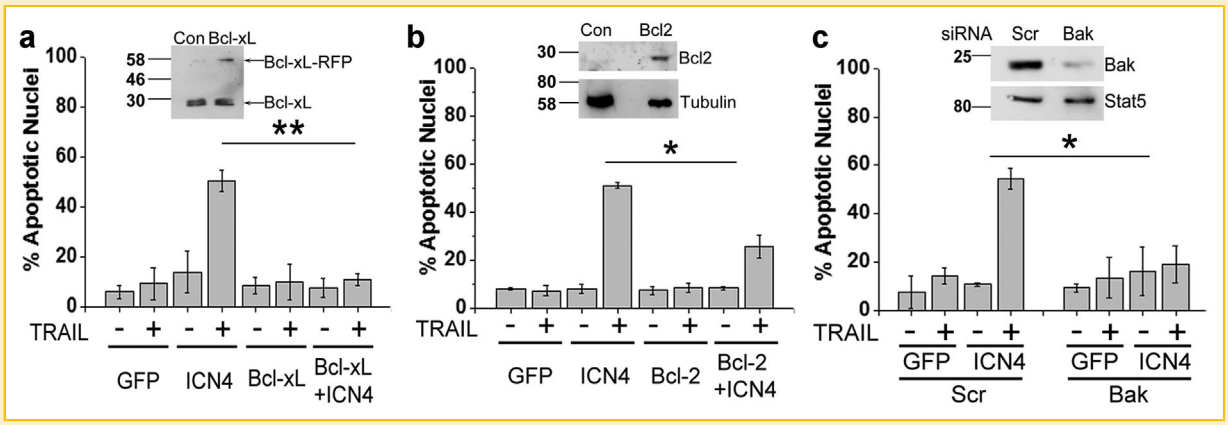

Fig. 4. TRAIL-induced apoptosis in ICN4-GFP expressing cells is dependent on Bak. a and b: T47D cells were transfected with ICN4-GFP ± (a) Bcl-xL or (b) Bcl-2 for 24 h before treatment with TRAIL (500 ng/ml) for 6 h and apoptotic nuclei were scored. Insets show the immunoblots to demonstrate the expression of indicated proteins. c: Cells depleted of Bak (c; inset) were transfected with GFP or ICN4 and TRAIL-induced apoptotic nuclei were determined. Values in all panels are mean ± SD of separate experiments ($n = 3$) with significance $^{*}P < 0.05$, $^{**}P < 0.001$ (Student's *t*-test).

Notch4-facilitated TRAIL-induced apoptosis proceeds via the caspase-8-Bid-dependent pathway and is integrated by Bak activation. This is in concert with recent data, which demonstrate that Bid preferentially activates Bak during apoptotic signaling [Sarosiek et al., 2013] and reiterates previous results showing a preferential engagement of Bak in TRAIL-induced apoptosis [Neise et al., 2008]. The loss of mitochondrial membrane integrity is critical for engagement of apoptosis as both Bcl-xL and Bcl-2 inhibited

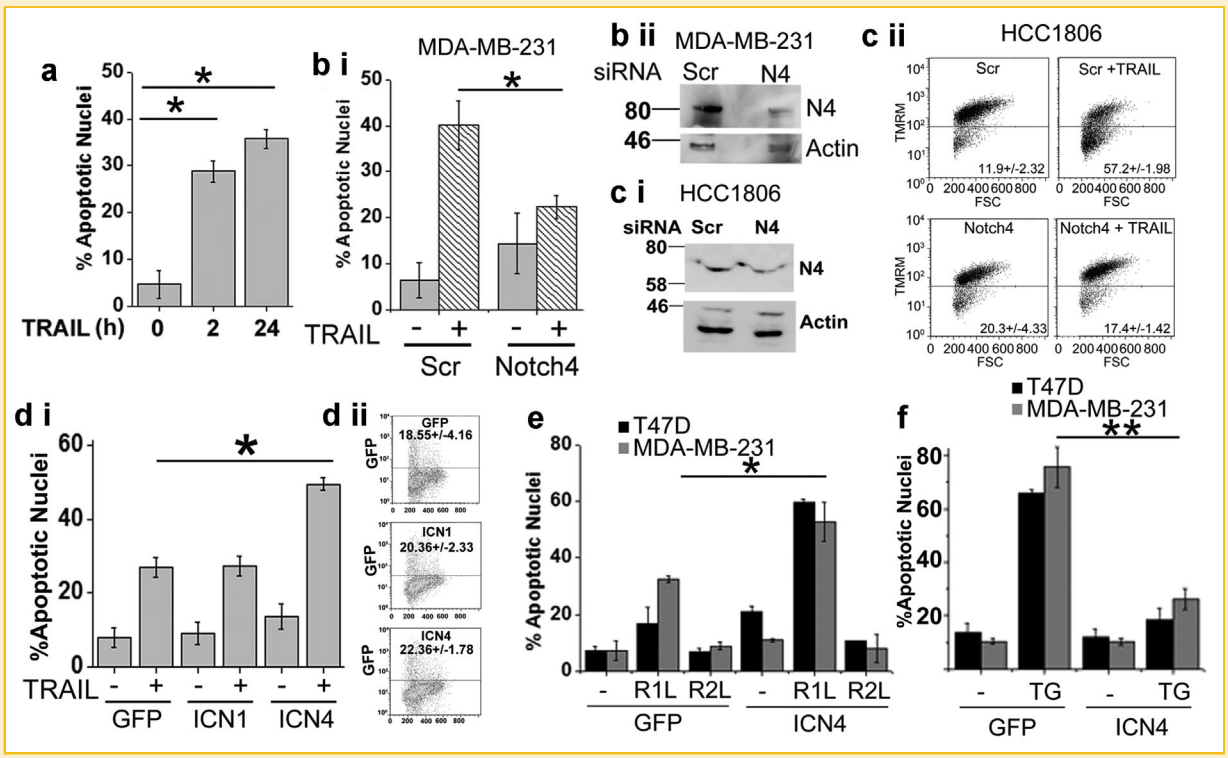

Fig. 5. Endogenous Notch4 modulates TRAIL sensitivity in MDA-MB-231 cells. a: MDA-MB-231 cells were treated with 500 ng/ml TRAIL for indicated time and apoptotic nuclei were measured. b: Notch4 was depleted in MDA-MB-231 cells using (ii) siRNA for 48 h and treated with TRAIL for 2 h prior to (i) detection of apoptosis. c: (i) Scrambled or Notch-4 transfected HCC1806 cells were treated with TRAIL for 4 h before assessment of apoptosis using (ii) loss in mitochondrial transmembrane potential (MTP). d: MDA-MB-231 cells transfected with GFP, ICN1 or ICN4 (ii shows transfection efficiency) were treated with wild-type TRAIL and apoptosis was measured post 2 h. e: Cells transfected with GFP or ICN4 were treated with agonists specific to TRAIL-R1 (R1L) or TRAIL-R2 (R2L) and apoptotic nuclei were counted. f: GFP or ICN4 transfected cells were treated with thapsigargin (TG; 10 μM) for 24 h and apoptotic nuclei were measured. Values in all panels are the mean ± SD of separate experiments ($n = 3$) with significance $^{*}P < 0.05$, $^{**}P < 0.001$ (Student's *t*-test).

TRAIL-induced apoptosis, possibly by inhibiting Bak and Bid activation respectively [Yi et al., 2003; Willis et al., 2005]. The attenuation of Notch4-mediated sensitization to TRAIL in cells ectopically expressing a dominant negative variant of CBF1 argues that CBF1-dependent transcriptional changes are essential for the sensitization of cells to TRAIL. The inability of ICN1 to recapitulate the effects of ICN4 suggests differential targets of ICN1 and ICN4 and indicates that canonical Notch1 targets might not be involved in the sensitization event. Although we focus on Notch1 and Notch4 in this study, we cannot rule out roles for Notch 2 and/or Notch3 in the signaling pathway. Both Notch2 and Notch3 are implicated in malignancy and susceptibility to apoptosis in diverse contexts [Quillard et al., 2009; Wang et al., 2010; Xiao et al., 2011; Li et al., 2013; Shi et al., 2014] but a possible role in modulating susceptibility to TRAIL mediated-apoptosis remains to be investigated in breast cancer cells.

In our experiments, Notch4 sensitized to TRAIL-induced apoptosis when triggered through TRAIL-R1 but not TRAIL-R2. However, it is important to note that the TRAIL ligands used in this study have not been cross-linked and TRAIL-R2 is an effective trigger of apoptosis, following cross-linking, in some contexts [Kelley et al., 2005; Natoni et al., 2007; Rahman et al., 2009]. Hence, TRAIL-R2 involvement in this pathway cannot be ruled out. The results obtained using the ligands is consistent with previous data indicating that in some types of tumors, TRAIL-R1 is the predominant apoptosis-signaling TRAIL death receptor [Leverkus et al., 2000; Lemke et al., 2010]. While the assessment of the Notch4-TRAIL signaling axis and the involvement of the receptors in multiple primary human breast cancer cells remains to be undertaken, our experiments suggest a role for Notch4 signaling in regulating TRAIL sensitivity.

Aberrant Notch signaling underlies the development of certain tumors and thus, inhibition of Notch signaling is being pursued as a therapeutic strategy against these tumors. Specifically in breast cancer, Jagged1 and Notch1 mRNA levels have been reported to be up-regulated in high grade breast cancers and these patients show a significantly poor overall survival as compared with patients displaying low mRNA levels for these genes [Reedijk et al., 2005; Dickson et al., 2007]. Incidentally, elevated Notch ligand expression has been detected in the triple-negative/basal subset of breast cancer, which is characterized by poor prognosis [Reedijk et al., 2005; Lee et al., 2008; Reedijk et al., 2008]. About 50% of breast tumors also report a loss of Numb, a negative regulator of Notch activity and this loss correlates with high-grade tumors and poor prognosis [Pece et al., 2004; Colaluca et al., 2008]. Alternatively it has also been shown that a Notch activating peptide can significantly enhance breast tumor self-renewal, which is abrogated by the addition of a Notch4 blocking antibody [Dontu et al., 2004; Harrison et al., 2010]. An anti-Notch4 monoclonal antibody has also been reported to inhibit the efficiency of mammosphere formation from primary breast ductal carcinoma in situ [Farnie et al., 2007]. The observed inhibition of etoposide-induced apoptosis in cells expressing ICN4 in our experiments is consistent with reports that Notch inhibition sensitizes various tumors to chemotherapy [Schott et al., 2013]. Particularly combining Notch inhibition with other chemotherapeutics has been reported to improve treatment efficacy as enhanced Notch signaling confers chemo-resistance to cancer cells [Zang et al., 2010].

Conversely, increased Notch activity has been demonstrated to enhance sensitivity of hepatocellular carcinoma cells to TRAIL-induced apoptosis [Wang et al., 2009]. Consistently, we report a Notch4-TRAIL signaling axis and demonstrate that the processed form of Notch4, but not Notch1, confers sensitivity to TRAIL-induced apoptosis in multiple breast cancer cell lines irrespective of their origin. Thus, TRAIL may prove an effective bio-therapeutic in Notch4 over-expressing tumors, particularly in the triple-negative subset of breast tumors.

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