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FKHRL1-mediated expression of Noxa and Bim induces apoptosis via the mitochondria in neuroblastoma cells

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Protein kinase-B (PKB) and its target, the forkhead transcription factor like 1 (FKHRL1)/FoxO3a, have been suggested as regulators of neurotrophin-mediated cell survival in neuronal cells. We analyzed human neuroblastoma cells and found that FKHRL1 was phosphorylated, suggesting its inactivation. To study FKHRL1 function, we infected SH-EP and NB15 cells with a 4OH-tamoxifen-regulated FKHRL1(A3)ER^{tm} transgene. Activation of FKHRL1 promoted cytochrome-*c* release and caspase-dependent apoptosis. FKHRL1 induced TRAIL and the BH3-only proteins Noxa and Bim, implicating both extrinsic and intrinsic death pathways. However, expression of dnFADD did not inhibit FKHRL1-induced cell death, whereas Bcl2 protected against apoptosis. This excluded the death-receptor pathway and suggested that cell death decision is regulated by Bcl2-rheostat. Importantly, RNAi knockdown of Noxa or Bim decreased apoptosis, indicating that Noxa and Bim cooperate to mediate FKHRL1-induced cell death. We conclude that Noxa and Bim establish a connection between FKHRL1 and mitochondria, and that both BH3-only proteins are critically involved in FKHRL1-induced apoptosis in neuroblastoma.
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The intracellular transduction of neuronal growth-factor signals involves the activation of the phosphatidylinositol-3-kinase (PI3K) – protein kinase-B (PKB) signaling cascade.[1] When activated, PKB phosphorylates a large set of target genes that control growth, cell cycle progression and apoptosis. Among them, PKB inactivates the forkhead transcription factor like 1 (FKHRL1)/FoxO3a through phosphorylation on the specific amino-acid residues threonine-32, serine-253, and serine-315.[2] This phosphorylation causes association of FKHRL1 with 14-3-3 proteins, export from the nucleus and as a consequence thereof repression of its target gene transcription. In the absence of growth factors, FKHRL1 regulates the expression of proteins that are involved in cell cycle arrest, cell division, and apoptosis in different cell types.[2–6]

Apoptosis is a process initiated by a large number of signals that either activate specific membrane death receptors ('extrinsic pathway') and/or intracellular pathways controlled by members of the Bcl2 family and the mitochondria ('intrinsic pathway').[7] Both pathways converge at the level of specific proteases, called effector caspases that are thought to be the executioners of most forms of apoptosis. Fas ligand and TRAIL, proapoptotic members of the TNF–family, mediate their apoptotic signal via the 'extrinsic pathway' by binding to their cognate receptors Fas/CD95 and TRAIL-R1 or TRAIL-R2, respectively. This induces the formation of a death-inducing signaling complex (DISC) that contains the adaptor molecule FADD and procaspase-8. As a consequence, autocatalytic cleavage of caspase-8 and activation of a downstream caspase cascade occurs that finally causes nuclear DNA fragmentation and apoptotic cell death. In some cells, caspase-8 also connects to the 'intrinsic pathway' through cleavage of the Bcl2 family member Bid, thereby providing a 'cross-talk' between 'extrinsic' and 'intrinsic' death pathways. The 'intrinsic pathway' is triggered by signals such as DNA damage, growth-factor withdrawal and anoikis. It is regulated at the level of mitochondria by the balance of pro- and antiapoptotic Bcl2-protein family members, which is also termed Bcl2-rheostat. Upon cell death decision, cytochrome *c* is released from mitochondria, it binds to Apaf1 that recruits procaspase-9 and activates this so-called 'apoptosome'. Caspase-9 and caspase-8 cleavage causes activation of the downstream effector caspase-3 and -7.

Depending on the cell type, apoptotic cell death induced by the transcription factor FKHRL1 has been ascribed to the induction of either death-receptor molecules such as Fas ligand in the Chinese hamster lung fibroblast cell line CCL39 and TRAIL in prostate cancer or key regulators of the 'intrinsic pathway', namely the BH3-only protein Bim in mouse pre-B cells.[2,5,6]

However, in cells of neuronal origin, only little is known about the function of FoxO transcription factors. AFX, FKHR and FKHRL1 have been suggested as critical downstream components of neurotrophin receptor signaling mediated by

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Abbreviations: BDNF, brain-derived neurotrophic factor; FKHRL1, forkhead transcription factor like 1; NGF, nerve growth factor; NT-3, neurotrophin-3; NT-4, neurotrophin-4; PI3K, phosphatidylinositol-3-kinase; PKB, protein kinase B; 4OHT, 4OH-tamoxifen
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the tyrosine kinase receptors (TrkA, TrkB and TrkC). In hippocampal, sympathetic and cortical neurons FoxO activation induces apoptotic cell death.[8–11] Activation of the PI3K pathway by nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4) and IGF-1 causes inactivation of FKHRL1 and provides neuronal survival.[1,11,12] Neuroblastoma tumors with high BDNF and TrkB expression show an unfavorable prognosis and in such tumors PKB is one of the key mediators of BDNF/TrkB survival signaling that protects neuroblastoma cells from chemotherapy-induced cell death.[13] Although BDNF has been shown to induce phosphorylation of FKHRL1 in human SH-SY5Y neuroblastoma cells, it is unclear whether this results in reduced cell death sensitivity in neuroblastoma cells.[14]

In this study, we specifically addressed the function of FKHRL1 in neuroblastoma cells by retroviral transduction of a conditional FKHRL1(A3)ER^{tm} transgene that can be activated by 4OH-tamoxifen (4OHT) and observed that cell death occurred independent of death receptors via induction of the proapoptotic BH3-only proteins Noxa and Bim.

Results

Activation of the PI3K–PKB–FKHRL1 pathway in neuroblastoma cells. PI3K, PKB and their downstream target FKHRL1 have been suggested as regulators of neurotrophin-mediated cell survival in neuroblastoma cells.[15] We therefore analyzed the protein levels of the regulatory PI3K subunit p85, of PKB1, PKB2, FKHRL1, phospho-PKB (serine-473) and phospho-FKHRL1 (threonine-32) by immunoblot in the neuroblastoma cell lines SH-EP, LAN-1 and NB15.[16] All proteins were expressed and PKB phosphorylation was detected with a pan-phospho-PKB antibody in all three cell lines. FKHRL1 was strongly phosphorylated on threonine-32 in SH-EP and NB15 and to a lesser extend in LAN-1 cells, suggesting inactivation of FKHRL1 by the highly active PI3K–PKB pathway in SH-EP and NB15 cells (Figure 1a).

4OHT-activated FKHRL1 promotes apoptotic cell death in SH-EP and NB15 cells. A retrovirus coding for a 4OHT-inducible, phosphorylation-independent FKHRL1-estrogen receptor (FKHRL1(A3)ER^{tm}) (Figure 1b) was retrovirally transduced into SH-EP and NB15 cells to analyze the function of the transcription factor FKHRL1.[5] The expression of the transgene was verified by Northern blot (Figure 1c) and immunoblot (Figure 1d). Neuroblastoma cells expressing the transgene were further termed SH-EP/FKHRL1 and NB15/FKHRL1. Activation of FKHRL1 by addition of 75 nM 4OHT induced apoptotic cell death within 24 h in SH-EP/FKHRL1 and 48 h in NB15/FKHRL1 cells, respectively, as determined by FACS analysis of propidium-iodide (PI)-stained nuclei (Figure 2a) and by microscopy (Figure 2b). FKHRL1 induced DNA fragmentation (sub-G1 DNA-peak) and characteristic morphologic changes of apoptotic cell death, such as cellular fragmentation and formation of DNA-containing apoptotic bodies. The viability of control cell lines infected with an empty vector was not affected by 4OHT (Figure 2a).

To further investigate FKHRL1-induced cell death in neuroblastoma cells, the mitochondrial activity was measured by CMX-Ros staining, and the subcellular localization of cytochrome *c* was assessed by fluorescence microscopy and immunoblot. Apoptotic cell death was associated with a gradual increase of CMX-Ros-negative cells, suggesting loss of mitochondrial membrane potential and involvement of mitochondria in the cell death pathway in SH-EP/FKHRL1 (Figure 3a, left panel) and NB15/FKHRL1 cells (Figure 3a, right panel), respectively. To assess, whether cytochrome *c* is released in the course of apoptosis induction, SH-EP/FKHRL1 cells were infected with GFP-tagged cytochrome *c*, treated with 75 nM 4OHT and analyzed by life-cell imaging.[17] Mitochondrial localization of cytochrome *c* disappeared in part

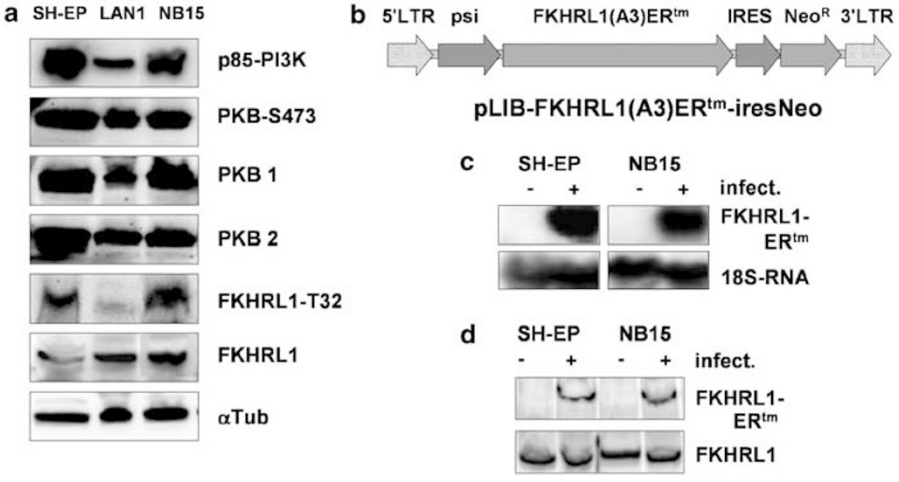

Figure 1 Expression of critical components of the PI3K–PKB-FKHRL1 pathway in neuroblastoma cells and generation of FKHRL1(A3)ER^{tm} transgenic SH-EP and NB15 cells. The expression of p85-PI3K, phospho-PKB-Ser473, PKB1, PKB2, phospho-FKHRL1-T32 and FKHRL1 was assessed in SH-EP, LAN-1 and NB15 neuroblastoma cells by immunoblot analysis. α-Tubulin was used as a loading control (**a**). The coding region of the FKHRL1(A3)ER^{tm} fusion protein was cloned into the pLIB expression vector (**b**) and retrovirally transferred into the cell lines SH-EP and NB15. Bulk-selected SH-EP/FKHRL1, NB15/FKHRL1 and control neuroblastoma cells were analyzed for transgenic FKHRL1 expression by Northern blot (**c**) and immunoblot (**d**)

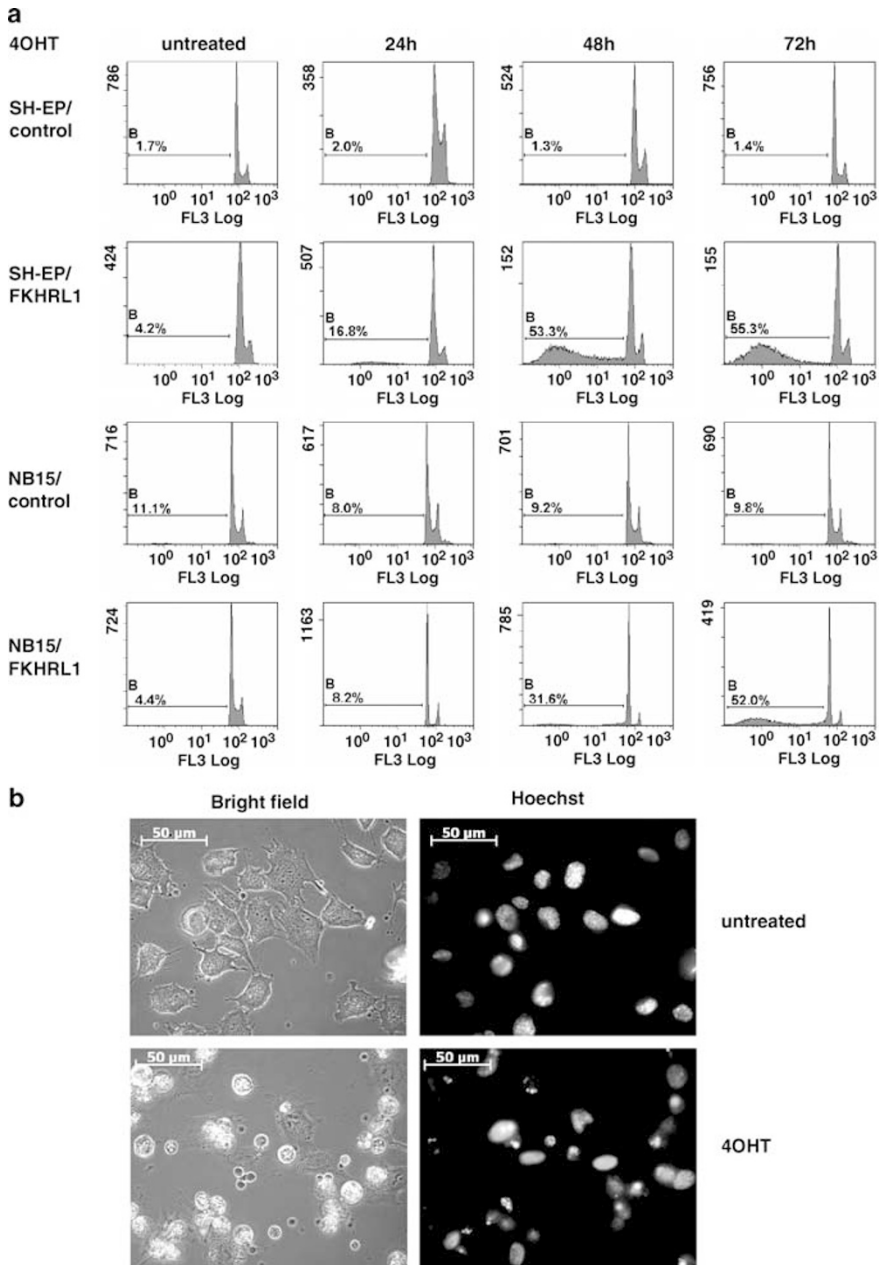

Figure 2 Activation of transgenic FKHRL1 induces apoptotic cell death in SH-EP and NB15 cells. SH-EP/FKHRL1, NB15/FKHRL1 cells and mock-transfected controls (infected with an empty pLIB-MCS2-iresNeo vector) were cultured for 0, 24, 48 and 72 h in presence of 75 nM 4OH-tamoxifen (4OHT) and then subjected to FACS analysis of PI-stained nuclei (**a**). Apoptosis induction by FKHRL1 was further demonstrated by microscopic analysis of SH-EP/FKHRL1 cells cultured with or without 75 nM 4OHT for 36 h. Chromosomal DNA was stained with Hoechst and analyzed by fluorescence microscopy (**b**)

of the cells after 24 h, suggesting release of cytochrome *c* from mitochondria during FKHRL1-induced apoptosis (Figure 3b). When analyzing mitochondrial and cytoplasmic fractions for cytochrome *c* by immunoblot, the amount of cytochrome *c* in the cytoplasmic fraction of 4OHT-treated SH-EP/FKHRL1 cells was significantly increased (Figure 3c).

FKHRL1-induced cell death pathways involve caspase-8, caspase-9 and caspase-3. To assess the initiation phase of cell death execution during FKHRL1 activation, caspase-8 and caspase-9 protein levels were analyzed by immunoblot. In SH-EP/FKHRL1 cells, we observed faint but characteristic cleavage products of caspase-8 and -9 already 8–12 h after FKHRL1 activation (Figure 4a). NB15/FKHRL1 cells lack caspase-8 expression, but showed cleavage of caspase-9 after 36 h in the presence of 4OHT. Caspase-10 was expressed in both cell lines (data not shown). Despite the expression of caspase-10, only SH-EP cells are sensitive to Fas-induced cell death (Figure 6c), suggesting that caspase-10 cannot substitute for the lack of caspase-8 in

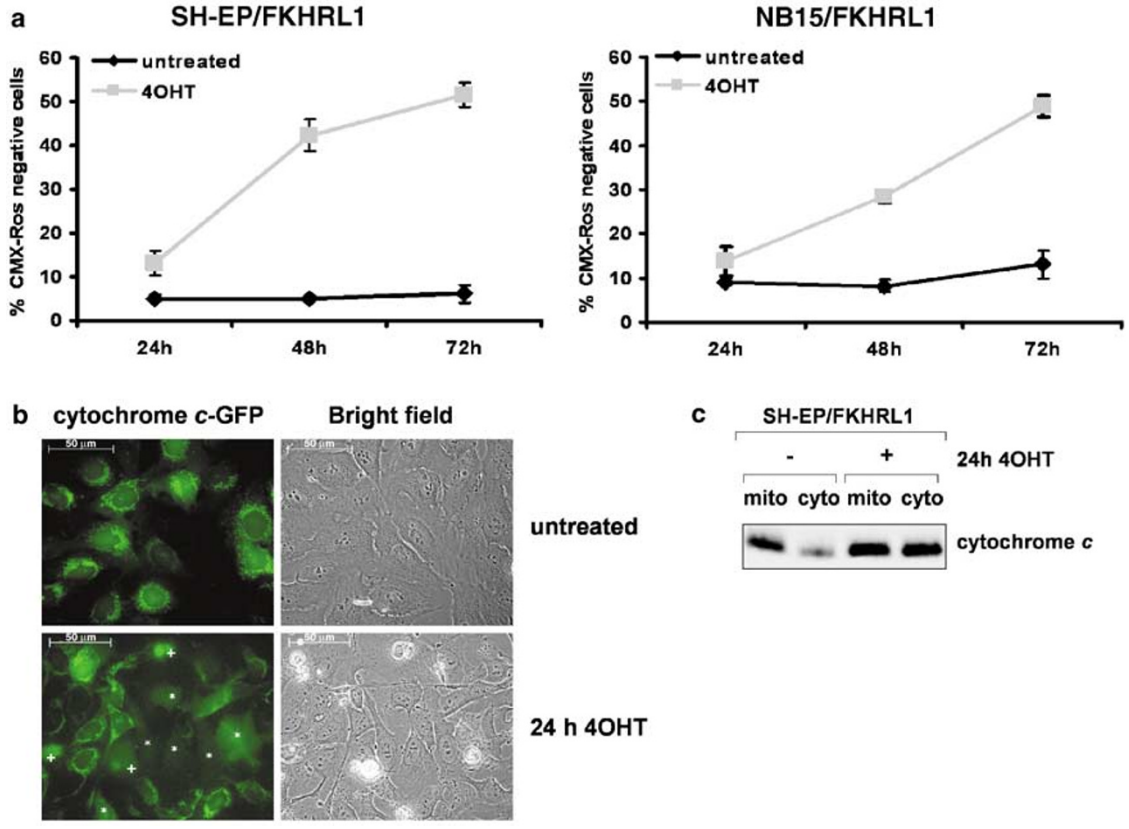

Figure 3 Loss of mitochondrial activity and release of cytochrome *c* during FKHRL1-induced cell death. Mitochondrial activity was assessed by CMX-Ros staining in untreated and 4OHT-treated (75 nM) SH-EP/FKHRL1 and NB15/FKHRL1 cells. The mean of three independent experiments is shown (**a**). SH-EP/FKHRL1-cytoC cells were incubated in the presence or absence 75 nM 4OHT for 24 h and analyzed by life-cell imaging using an Axiovert200M microscope (Zeiss). Mitochondrial localization of cytochrome *c*-GFP is lost during FKHRL1 activation indicating cytochrome *c* release. A proportion of cells with diffuse cytochrome *c* staining retain normal morphology (*), whereas others already show an apoptotic phenotype (+), suggesting early and late stages after cytochrome *c* release (**b**). The cytochrome *c* release was further demonstrated by immunoblot analysis of mitochondrial (mito) and cytoplasmic (cyto) fractions of untreated or 4OHT-treated (for 24 h) SH-EP/FKHRL1 cells. In untreated SH-EP/FKHRL1 cells, cytochrome *c* is mainly present in the mitochondrial fraction, whereas activation of FKHRL1 for 24 h causes cytochrome *c* accumulation in the cytoplasm (**c**)

NB15 cells (data not shown) at least in Fas-induced apoptosis.[18] The BH3-only protein Bid was not cleaved, either in SH-EP/FKHRL1 or in NB15/FKHRL1 cells. In addition, cells with active caspase-3 were quantified by a fluorometric caspase-activity assay after 48 and 72 h of 4OHT treatment (Figure 4b). A gradual increase in caspase-3-positive cells was observed that correlated with the formation of a sub-G1 DNA peak in Figure 2a. However, from these data, it was not clear whether caspases are essential for cell death induction by FKHRL1. Treatment of SH-EP/FKHRL1 cells with the broad-specificity caspase inhibitor z-VAD.fmk prevented the onset of apoptotic cell death for up to 72 h, proving that caspases are critical for the execution of apoptosis (Figure 4c). To specifically inhibit caspase-8 and caspase-9 activation, we either retrovirally expressed the caspase-8-inhibitory protein CrmA (Figure 4d) or treated the cells with the caspase-9-specific inhibitor LEHD.fmk (Figure 4e). CrmA and the peptide inhibitor LEHD.fmk decreased cell death after 48 h suggesting that both, caspase-8 and caspase-9 may be involved in either initiation or amplification of the caspase-cleavage cascade.

Quantitative RT-PCR analysis reveals induction of TRAIL and repression of TRAIL-R2 and TRAIL-R3 mRNA during FKHRL1 activation. To identify possible upstream regulators of inducer–caspases, we assessed the mRNA steady-state levels of death-receptor proteins at different time points *post* activation of transgenic FKHRL1(A3)ER^{tm}. By quantitative RT-PCR, we measured a 12-fold increase of TRAIL mRNA within 6 h in SH-EP/FKHRL1 (Figure 5a, left panel) and a 25-fold increase in NB15/FKHRL1 cells after 8 h (Figure 5a, right panel). Interestingly, in both cell lines, FKHRL1 activation caused a strong repression of TRAIL-R2 mRNA after 6 h of 4OHT treatment (Figure 5b). TRAIL-R3 was also three-fold repressed in both cell lines (Figure 5c), but a two-fold induction of TRAIL-R4 mRNA was detected in NB15/FKHRL1 cells (Figure 5d). Fas/CD95 mRNA steady-state levels were five-fold induced in SH-EP/FKHRL1 cells (Figure 5e, left panel), whereas in NB15/FKHRL1 cells Fas/CD95 mRNA was even repressed (Figure 5e, right panel). Fas ligand and TRAIL-R1 mRNA steady-state levels were below detection threshold in both cell lines and TRAIL-R4 mRNA was not detected in SH-EP/FKHRL1 cells (data not shown).

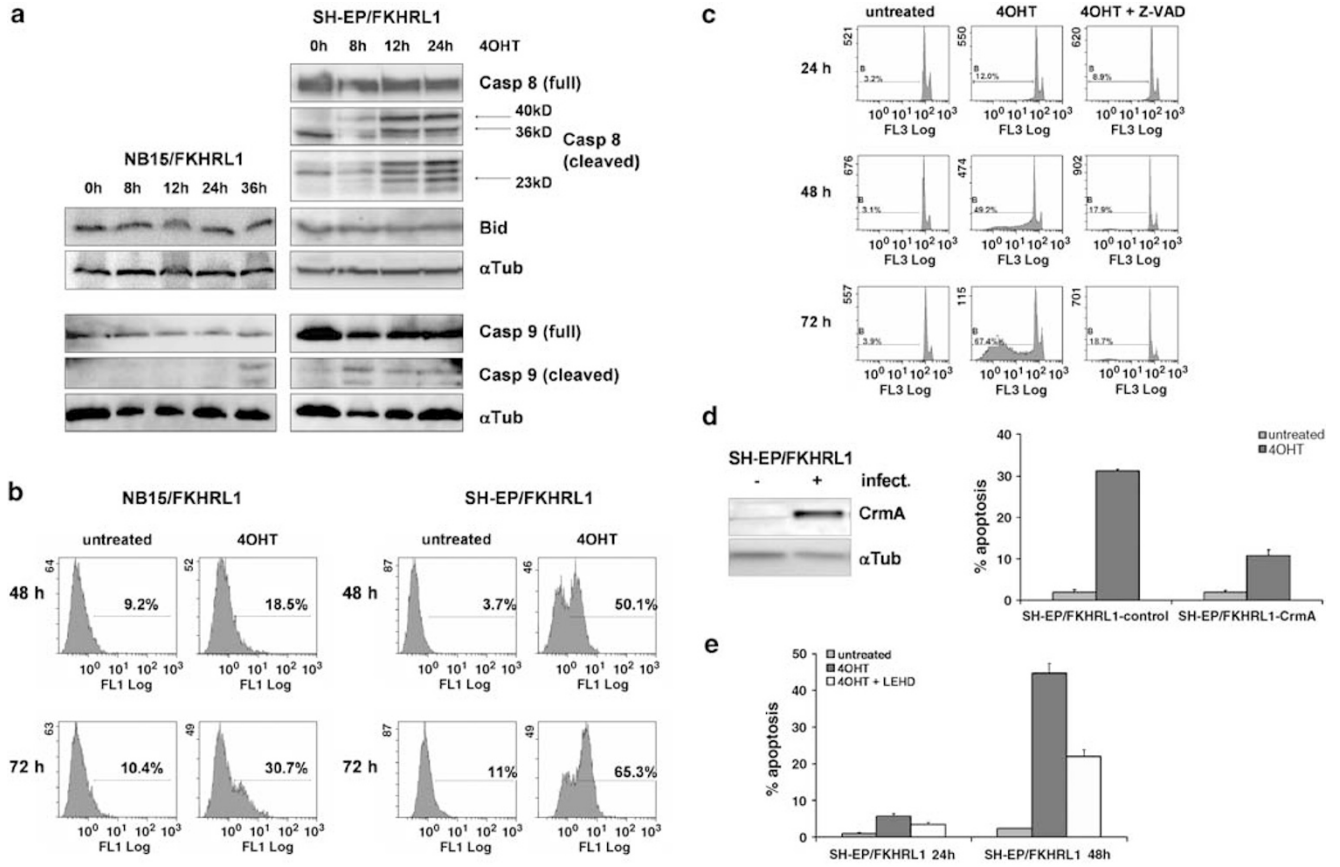

Figure 4 FKHRL1-induced cell death pathways involve caspase-8, caspase-9 and caspase-3. To analyze caspase activation, SH-EP/FKHRL1 and NB15/FKHRL1 cells were treated with 75 nM 4OHT for the times indicated. The lysates were subjected to immunoblotting using specific antibodies directed against caspase-8 (the antibody recognizes also the cleaved products of 40, 36 and 23 kDa), caspase-9 (cleaved products of 37 and 35 kDa are detected), Bid and α-Tubulin (**a**). Active caspase-3 was detected by a fluorogenic FITC-DEVD.fmk substrate in untreated and 4OHT-treated SH-EP/FKHRL1 as well as in NB15/FKHRL1 cells after 48 and 72 h. A representative experiment is shown (**b**). SH-EP/FKHRL1 cells were treated for 24, 48 and 72 h with 75 nM 4OHT alone or in combination with the pan-caspase-inhibitor z-VAD.fmk (20 μM) and then subjected to FACS analysis of PI-stained nuclei (**c**). The caspase-8-inhibitory protein CrmA was retrovirally infected into SH-EP/FKHRL1 cells and transgenic expression of CrmA was verified by immunoblot. α-Tubulin served as a loading control. PI-FACS analysis of SH-EP/FKHRL1-control (infected with the empty plasmid pLIB-MCS2-iresPuro) and SH-EP/FKHRL1-CrmA cells were performed after 48 h treatment with 4OHT (**d**). SH-EP/FKHRL1 cells were treated with 4OHT (75 nM) alone and in combination with the caspase-9 inhibitor LEHD.fmk (20 μM) for 24 and 48 h and subjected to FACS analysis of PI-stained nuclei (**e**)

In contrast to NB15 cells, SH-EP cells express caspase-8 and are sensitive to Fas/CD95-induced apoptosis (Figure 6c), implicating that the observed regulation of death ligands and their receptors might contribute to FKHRL1-induced cell death at least in SH-EP/FKHRL1 cells. However, the loss of mitochondrial activity (Figure 3a), the release of cytochrome *c* (Figure 3b and c) and the cleavage of caspase-9 in both cell lines (Figure 4a) suggest that mitochondria are involved and damaged during FKHRL1-induced apoptosis. This might be either initiated by death receptors or occur independently, for example, via the regulation of pro- and antiapoptotic proteins of the Bcl2 family.

Retroviral expression of dnFADD blocks Fas- but not FKHRL1-induced cell death. To functionally validate the involvement of death-receptor signaling and/or Bcl2 proteins, SH-EP/FKHRL1 and NB15/FKHRL1 cells were infected with retroviruses coding for a dominant negative mutant of human FADD (dnFADD) and human Bcl2. Mock-infected cells (pLIB-MCS2-iresPuro) served as controls. Transgenic expression of Bcl2 and dnFADD in bulk-selected SH-EP/FKHRL1 and NB15/FKHRL1 cells was verified by immunoblot analysis (Figure 6a and b). To test, whether the expression of dnFADD efficiently blocked death-receptor-mediated apoptosis, SH-EP cells were treated with a crosslinking anti-Fas antibody (clone CH11). dnFADD almost completely abrogated Fas-induced cell death, as shown in Figure 6c. We next investigated the effect of dnFADD on FKHRL1-induced apoptosis. However, different from Fas-mediated cell death, dnFADD did not affect apoptosis by FKHRL1 and caspase-3 cleavage in SH-EP/FKHRL1 (Figure 7a and c) and NB15/FKHRL1 cells (Figure 7b and d). This argues against the involvement of death receptors and their ligands, despite the fact that TRAIL was strongly induced by FKHRL1. In contrast, transgenic Bcl2 markedly reduced apoptotic cell death in SH-EP/FKHRL1 and NB15/FKHRL1 cells for up to 72 h, prevented the accumulation of CMX-Ros-negative cells (data not shown) and the activation of caspase-3 (Figure 7c and d). These data also supported the notion that cell death decision critically involves mitochondria and is initiated by proapoptotic members of the Bcl2 family.

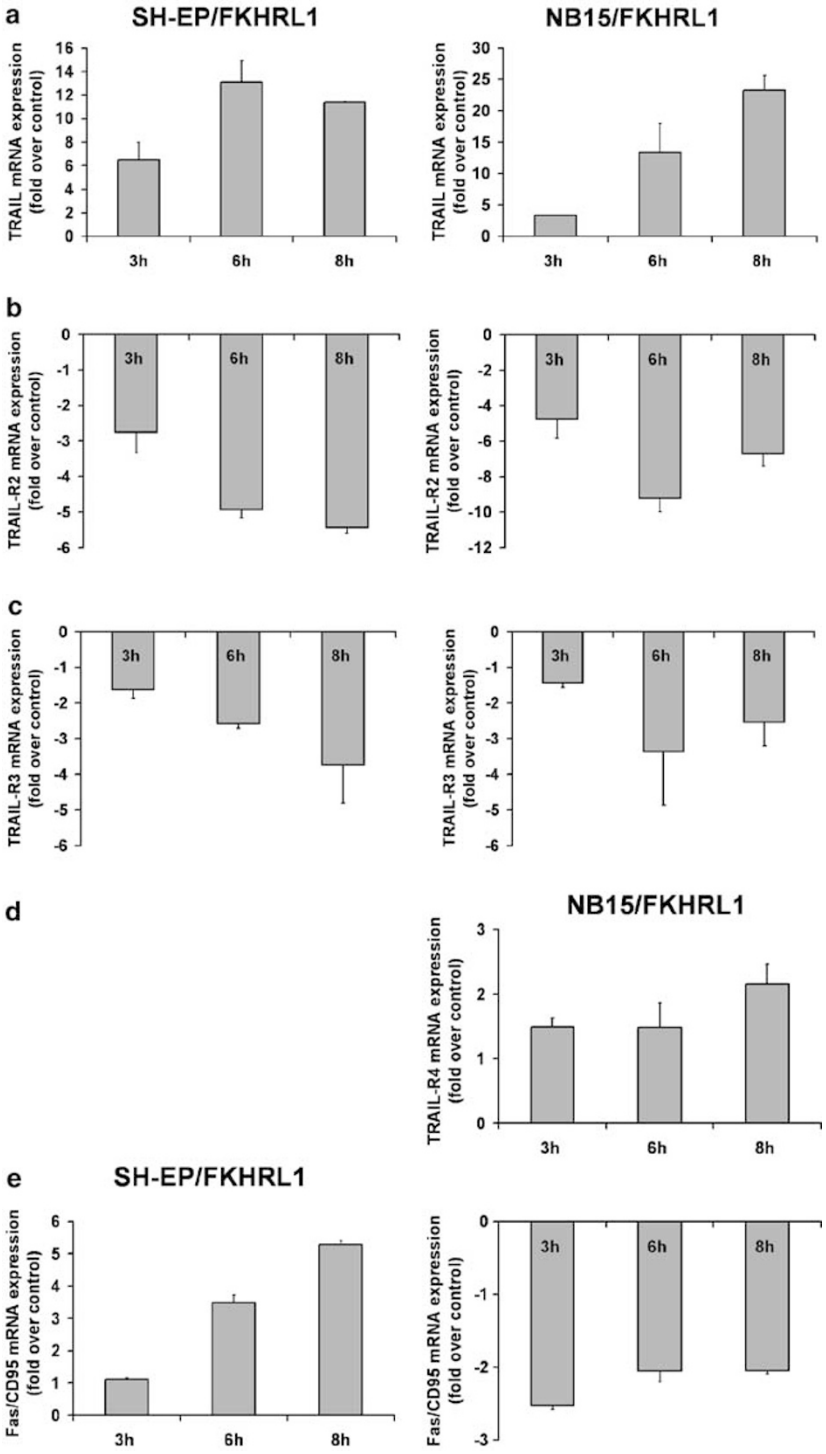

Figure 5 Activation of FKHRL1 induces TRAIL but represses TRAIL-R2 and TRAIL-R3 mRNA in SH-EP/FKHRL1 and NB15/FKHRL1 cells. For quantitative RT-PCR, SH-EP/FKHRL1 and NB15/FKHRL1 cells were incubated for 0, 3, 6 and 8 h with 4OHT (75 nM) and then subjected to mRNA preparation and cDNA synthesis. mRNA steady-state levels of TRAIL (**a**), TRAIL-R2 (**b**), TRAIL-R3 (**c**), TRAIL-R4 (**d**) and Fas/CD95 (**e**) were determined by quantitative RT-PCR. TRAIL-R4 is not expressed in SH-EP/FKHRL1 cells. The mean of three independent experiments, each performed in triplicate, is shown

FKHRL1 induces the proapoptotic BH3-only proteins Bim and Noxa. We next quantified mRNA steady-state levels of the proapoptotic BH3-only proteins Bim, Puma/bbc3 and Noxa/PMAIP1 by quantitative RT-PCR. We observed a strong induction of Bim mRNA within 3 h in both cell lines reaching an 18-fold induction after 8 h in SH-EP/FKHRL1 cells (Figure 8a, left panels) and a four-fold induction in NB15/FKHRL1 cells (Figure 8a, right panels). This elevation in mRNA was also reflected on protein level, as Bim expression significantly increased within 12 h *post* addition

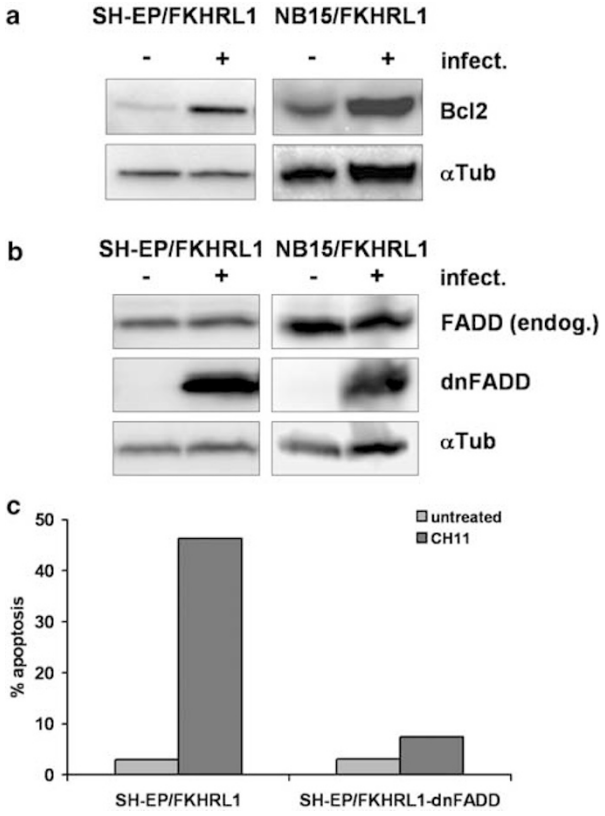

Figure 6 Generation of Bcl2- and dnFADD-transgenic SH-EP/FKHRL1 and NB15/FKHRL1 cells. Ectopic expression of Bcl2 in SH-EP/FKHRL1-Bcl2 and NB15/FKHRL1-Bcl2 neuroblastoma cells was verified by immunoblot analysis (**a**). A retroviral vector coding for dnFADD was constructed and used to infect SH-EP/FKHRL1 and NB15/FKHRL1 cells. Bulk-selected SH-EP/FKHRL1-dnFADD and NB15/FKHRL1-dnFADD cells and parental SH-EP/FKHRL1 as well as NB15/FKHRL1 cells were subjected to immunoblot analysis with a monoclonal anti-FADD antibody that detects both, the endogenous (endog.) FADD and the truncated dnFADD protein. α-Tubulin served as a loading control (**b**). SH-EP/FKHRL1 and SH-EP/FKHRL1-dnFADD cells were incubated with 500 ng/ml anti-Fas/CD95 (clone CH11) for 48 h. Apoptosis induction was measured by PI-FACS analysis (**c**)

of 4OHT. Most interestingly, the BH3-only protein Noxa was also markedly, although transiently, induced in both cell lines (Figure 8b). Noxa has been proposed as an apical regulator in apoptosis induction via the mitochondria due to its ability to almost exclusively bind and inactivate the antiapoptotic proteins Mcl-1 and A1.[19,20] In parallel, mRNA steady-state levels and protein expression of the BH3-only protein Puma were decreased in SH-EP/FKHRL1 and NB15/FKHRL1 cells, respectively (Figure 8c). Puma protein did not completely disappear, but remained attenuated up to 24 h (data not shown). The combined RT-PCR and immunoblot results show that Noxa and Bim are induced in response to FKHRL1 activation, whereas Puma steady-state protein levels were decreased. This suggests that these BH3-only proteins are direct transcriptional targets of FKHRL1 that trigger cell death via the mitochondrial pathway in neuroblastoma cells.

Stable gene knockdown of Noxa and Bim demonstrates their pivotal role during FKHRL1-induced cell death. To specifically investigate Noxa and Bim as FKHRL1-induced death regulators, we next functionally interfered with their induction by RNA-interference. For this purpose, oligonucleotides for the expression of short RNA hairpin loops were cloned downstream of the H1-polymerase III promoter into the retroviral vector pQ-tetH1-SV40-Puro (unpublished) and stably infected into SH-EP/FKHRL1 and NB15/FKHRL1 cells. Bulk-selected cells and individual clones were subjected to immunoblot analysis for Noxa and Bim, respectively. Cells infected with an empty vector served as controls. Noxa-shRNA strongly repressed basal Noxa expression and diminished Noxa induction by FKHRL1 compared to the protein levels of untreated cells in both SH-EP/FKHRL1 (Figure 9a) and NB15/FKHRL1 cells (Figure 9c). As shown in Figure 9b and d, Noxa-shRNA expression decreased the number of apoptotic cells by 30–50% in SH-EP/FKHRL1 and even to 50–60% in NB15/FKHRL1 cells compared to mock-transfected controls, which implies that Noxa is not only a transcriptional target of FKHRL1 but also critical for FKHRL1-induced cell death.

Bim-shRNA completely prevented the induction of Bim by FKHRL1 in an individual clone of SH-EP/FKHRL1 (Figure 10a) and still attenuated Bim increase in bulk-selected NB15/FKHRL1 cells (Figure 10c). Similar to Noxa-RNAi, Bim-specific shRNAs decreased FKHRL1-induced cell death by more than 40% in an individual SH-EP/FKHRL1 cell clone (Figure 10b) and by more than 60% in bulk-selected NB15/FKHRL1 cells (Figure 10d). This suggests that Noxa and Bim cooperate to promote FKHRL1-induced cell death most likely by inactivating different prosurvival proteins of the Bcl2 family in neuroblastoma cells.

The combined data clearly show that active FKHRL1 promotes apoptosis via the mitochondria in human neuroblastoma cells. We identified Noxa and Bim as transcriptional targets of FKHRL1 and provide evidence that these BH3-only proteins are both critical for FKHRL1-induced apoptosis.

Discussion

The PI3K–PKB signal transduction axis that is activated by neurotrophins such as BDNF, NT-3 and NT-4 via their cognate Trk receptors has been implicated as an essential pathway for the survival of neuronal cells.[13,21] Emerging evidence indicates that the downstream target of PI3K–PKB signaling, the forkhead transcription factor FKHRL1/FoxO3a is regulated by neurotrophins and that FKHRL1 can induce cell death in differentiated neurons.[1,9,14] In contrast to these studies on differentiated neuronal cells, almost nothing is known about the possible function of FKHRL1 in malignant neuroblastoma. In this report, we investigated the effect of FKHRL1 activation and the distinct pathways involved in FKHRL1-induced cell death in neuroblastoma cells. We found that in the three analyzed cell lines SH-EP, LAN-1 and NB15, the proteins p85-PI3K, PKB and FKHRL1 were expressed and PKB was phosphorylated suggesting hyperactivation of this survival pathway (Figure 1). Activation of a phosphorylation-independent, 4OHT-regulated FKHRL1(A3)ER^{tm} fusion protein promoted apoptotic cell death in the cell lines SH-EP/FKHRL1 and NB15/FKHRL1. Cell death was associated with characteristic morphologic changes, loss of mitochondrial membrane potential, cytochrome *c* release, caspase activa-

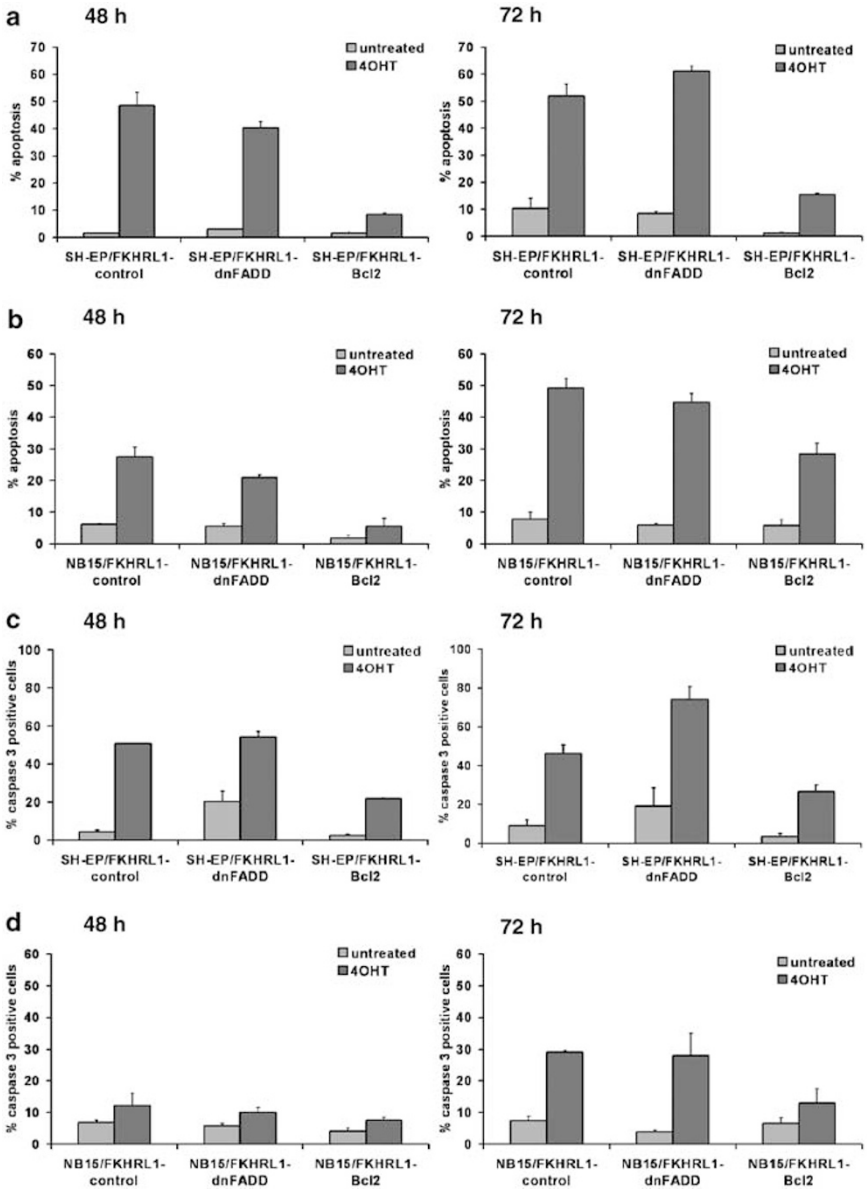

Figure 7 Dominant-negative FADD does not protect against FKHRL1-induced apoptosis, whereas transgenic expression of Bcl2 protects against FKHRL1-induced cell death for up to 72 h. Characteristic nuclear fragmentation during activation of transgenic FKHRL1 was measured after 48 and 72 h in the presence of 4OHT (75 nM) in SH-EP/FKHRL1-control, SH-EP/FKHRL1-dnFADD, SH-EP/FKHRL1-Bcl2, NB15/FKHRL1-control, NB15/FKHRL1-dnFADD and NB15/FKHRL1-Bcl2 neuroblastoma cells by FACS analysis of PI-stained nuclei, respectively (**a**, **b**). Caspase-3 activity in 4OHT-treated SH-EP/FKHRL1-control, SH-EP/FKHRL1-dnFADD, SH-EP/FKHRL1-Bcl2, NB15/FKHRL1-control, NB15/FKHRL1-dnFADD and NB15/FKHRL1-Bcl2 cells was determined by quantification of FITC-DEVD.fmk-positive cells (**c**, **d**). The mean of three independent experiments is shown

tion and DNA fragmentation (Figures 2, 3 and 4). By the administration of the peptide inhibitors z-VAD.fmk and LEHD.fmk or the retroviral expression of the caspase-8-inhibitor CrmA, we proved that caspases are essential for FKHRL1-induced cell death and that both, caspase-8 and caspase-9 (Figure 4c, d and e) may contribute to initiation or amplification of the caspase cascade. This implicated death receptors and/or mitochondrial death proteins as critical upstream regulators of caspase activation. Compared to the appearance of caspase-8 and caspase-9 cleavage bands, the onset of DNA fragmentation and caspase-3 activation as measured by flow cytometry (Figures 2a and 4b) was markedly delayed. However, this may be ascribed to the high expression of IAP proteins, such as survivin and XIAP in these cells that have to be downregulated during FKHRL1-induced cell death (unpublished observation).

In other cell types, FKHRL1 has been reported to initiate cell death either by inducing Fas ligand, TRAIL or by regulating the BH3-only protein Bim.[2,5,9] When analyzing the mRNA steady-state levels of TRAIL, TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, Fas ligand and Fas/CD95 in SH-EP/FKHRL1 and NB15/FKHRL1 cells, we found that FKHRL1 significantly

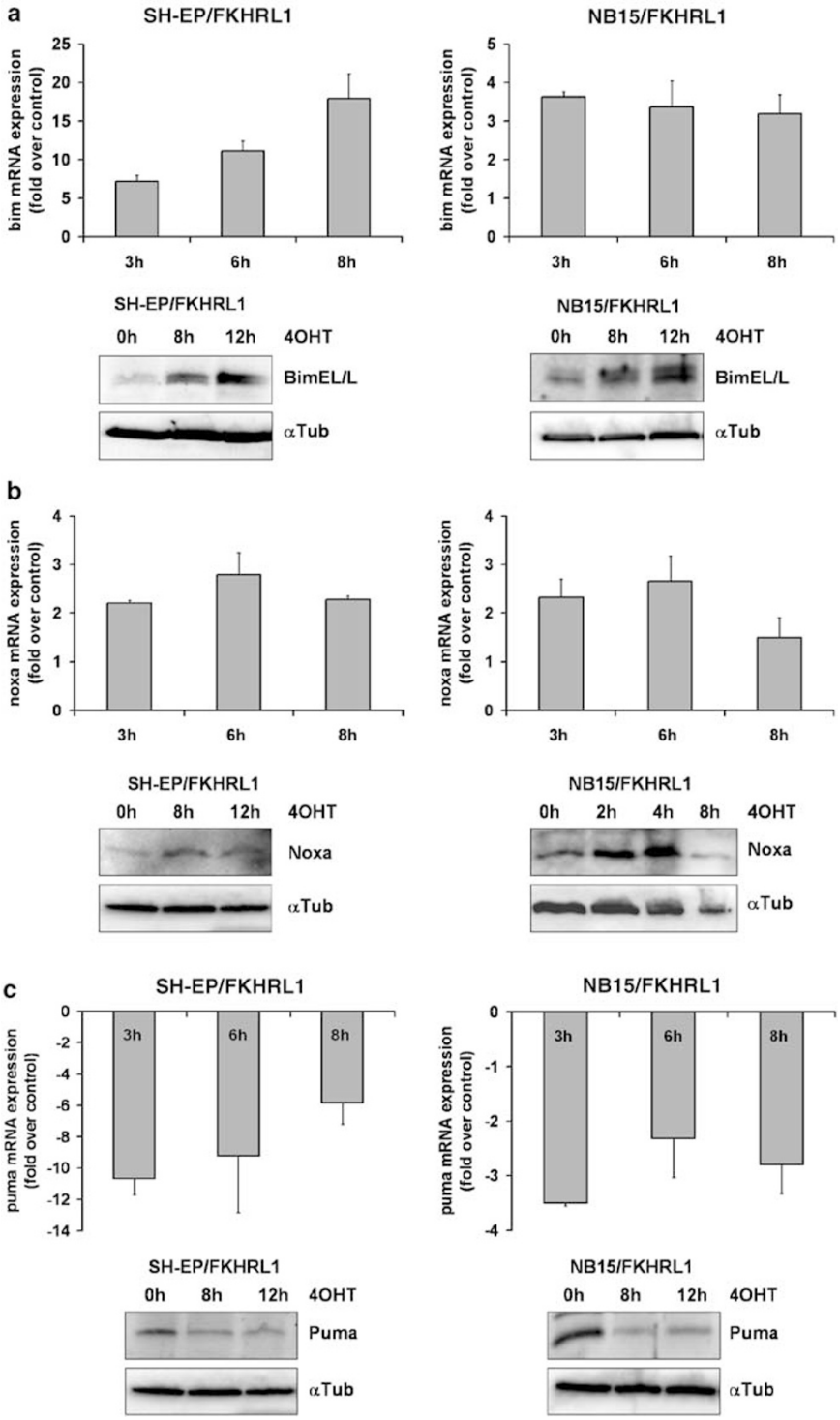

Figure 8 FKHRL1 induces the BH3-only proteins Bim and Noxa but represses Puma. Total RNA was prepared from SH-EP/FKHRL1 and NB15/FKHRL1 cells following 4OHT (75 nM) treatment for 0, 3, 6 and 8 h. The level of Bim (**a**), Noxa (**b**) and Puma (**c**) mRNA was assessed by quantitative RT-PCR. The bars represent fold-induction and repression over the control of three independent experiments each performed in triplicate. To measure protein expression, SH-EP/FKHRL1 and NB15/FKHRL1 cells were treated for the times indicated with 75 nM 4OHT and then subjected to immunoblot analysis using antibodies directed against human Bim (**a**), Noxa (**b**) and Puma (**c**). Equal protein loading was confirmed by α-tubulin staining

induced TRAIL mRNA and caused repression of TRAIL-R2 and TRAIL-R3 mRNA steady-state levels (Figure 5). This pointed towards a role of TRAIL/TRAIL-R2 during cell death signaling at least in caspase-8-expressing SH-EP/FKHRL1 cells. We also analyzed caspase-10 as a possible mediator of death-receptor signaling. Although caspase-10 was expressed in both cell lines, NB15 cells were insensitive to Fas treatment (data not shown). This suggests that caspase-

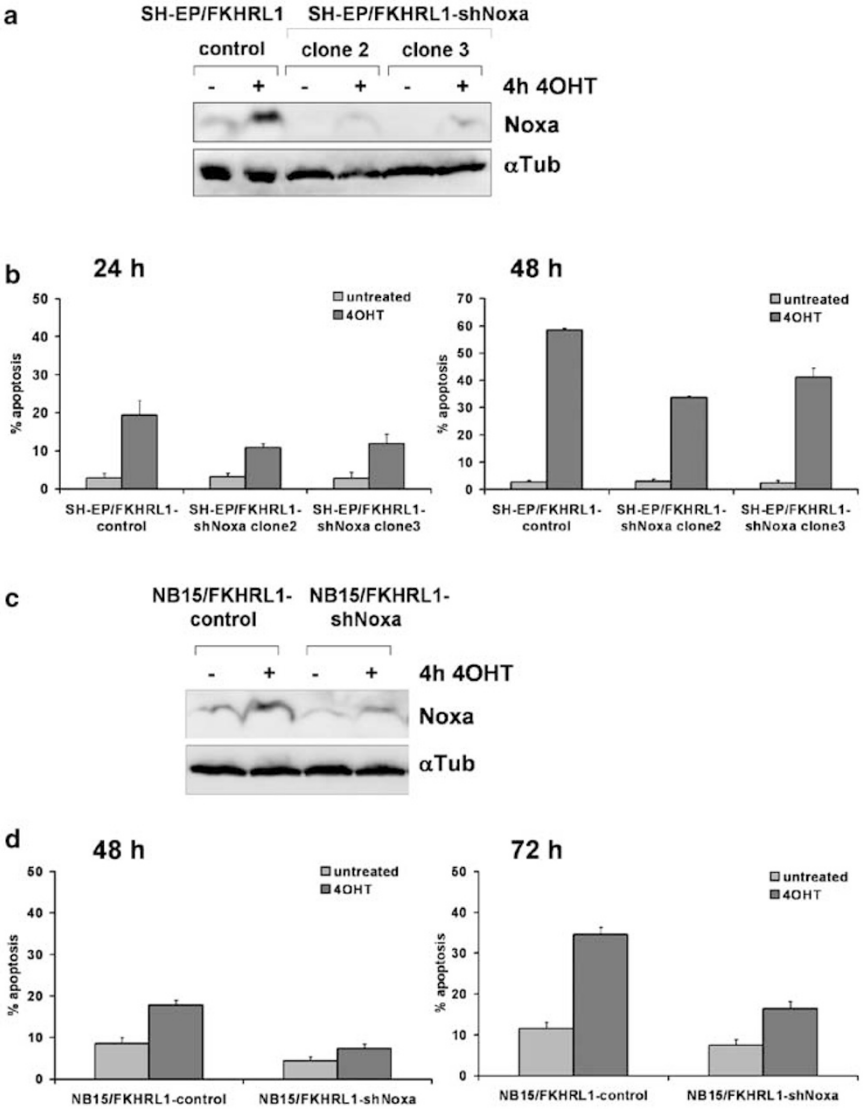

Figure 9 Gene knockdown of Noxa by short hairpin RNAs protects against FKHRL1-induced apoptosis in SH-EP/FKHRL1 and NB15/FKHRL1 cells. The pQ-tetH1-shNoxa-SV40-Puro retrovirus vector was infected into SH-EP/FKHRL1 and NB15/FKHRL1 cells. Individual clones were isolated by limiting dilution from bulk-selected SH-EP/FKHRL1-shNoxa cells. For NB15/FKHRL1-shNoxa experiments, bulk-selected cells were used. To determine the knockdown of endogenous Noxa, the cells were cultured in the presence or absence of 50 nM 4OHT for 4 h and subjected to immunoblot analysis (**a**, **c**). SH-EP/FKHRL1-control (infected with the empty plasmid pQ-tetH1-SV40-Puro) and SH-EP/FKHRL1-shNoxa clone 2 and 3 were treated with 50 nM 4OHT for 24 and 48 h (**b**), whereas mock-transfected NB15/FKHRL1-control and bulk-selected NB15/FKHRL1-shNoxa cells (**d**) were incubated with 4OHT for 48 and 72 h. Apoptosis induction was determined by FACS analysis of PI-stained nuclei

10 cannot substitute for caspase-8 in death-receptor signaling, which has also been reported for other cell types.[18] To exclude death-receptor signaling, dnFADD was introduced, which protected against Fas- but not FKHRL1-induced cell death (Figures 6 and 7). Although FKHRL1 induced TRAIL mRNA (Figure 5) and promoted cleavage of caspase-8 (Figure 4a), the initiating apoptotic signal was not mediated via the death receptor pathway. In contrast to dnFADD, Bcl2 significantly protected against FKHRL1-induced cell death up to 72 h *post* induction (Figure 7). This further supported the notion that FKHRL1-induced cell death is regulated by Bcl2-rheostat in neuroblastoma cells.

As a consequence thereof, we analyzed the expression of several BH3-only proteins that regulate cell death induction via the mitochondrial pathway.[7] The most striking induction was found in the expression of the BH3-only proteins Noxa and Bim (Figure 8a and b), whereas Puma, another member of this protein family, was even repressed (Figure 8c). Importantly, the transcriptional regulation of these BH3-only proteins occurred within the first 3 h of FKHRL1 activation, implicating that these genes are directly regulated by this transcription factor. Bim and Puma are rate limiting for death induced by γ-radiation and glucocorticoids in hematopoietic cells.[22] The BH3-only protein Bim is essential for death induced by cytokine withdrawal in cells as diverse as hematopoietic cells, neurons and granulocytes.[5,9,23] Experiments with bim-antisense oligonucleotides and neurons isolated from $bim^{-/-}$mice established that Bim may play an

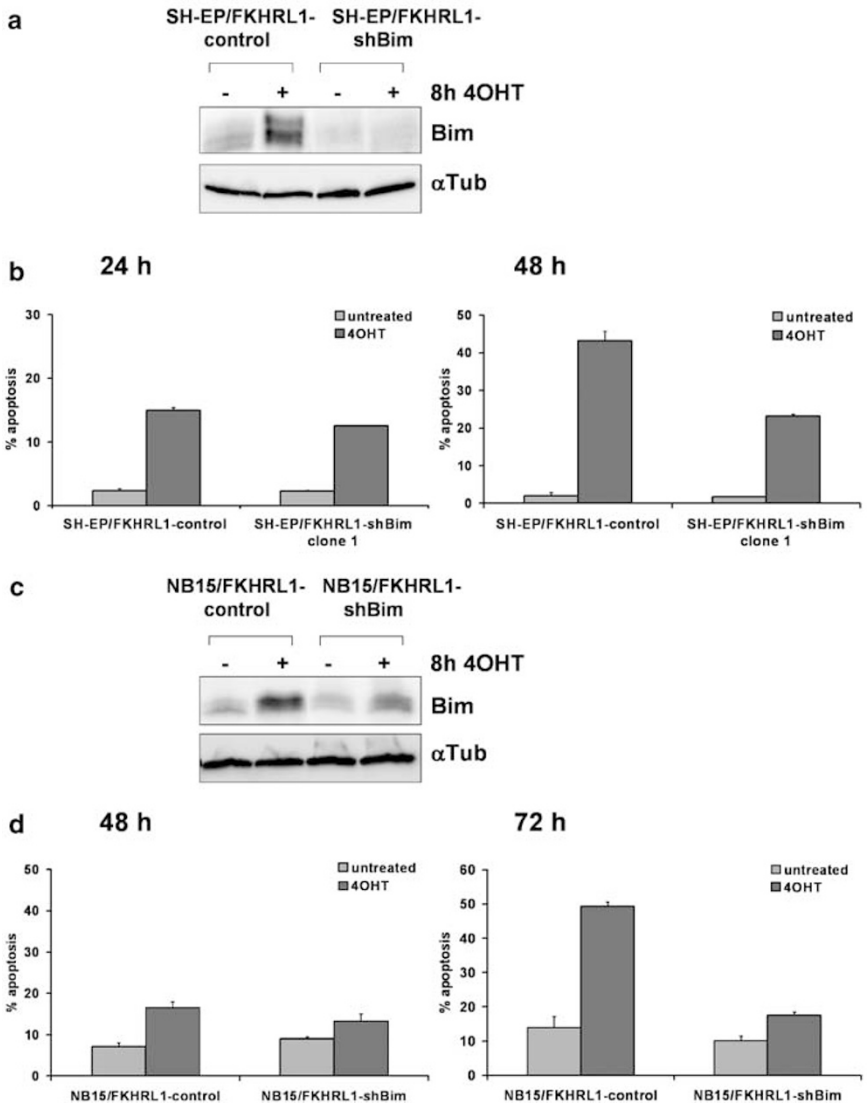

Figure 10 Bim short hairpin RNAs efficiently decrease FKHRL1-induced apoptosis. SH-EP/FKHRL1-shBim (clone 1), NB15/FKHRL1-shBim and mock-transfected control cells (infected with the empty plasmid pQ-tetH1-SV40-Puro) were treated for 8 h with 4OHT (50 nM) and subjected to immunoblot analysis. The induction of Bim by 4OHT in the control cells was prevented in SH-EP/FKHRL1-shBim and significantly attenuated in NB15/FKHRL1-shBim cells (**a**, **c**). SH-EP/FKHRL1-control and SH-EP/FKHRL1-shBim clone 1 cells were treated with 50 nM 4OHT for 24 and 48 h (**b**), NB15/FKHRL1-control and bulk-selected NB15/FKHRL1-shBim cells (**d**) were incubated with 4OHT for 48 and 72 h. Apoptosis induction was determined by FACS analysis of PI-stained nuclei

important role in NGF withdrawal-induced neuronal cell death and led to its identification as a transcriptional FKHRL1 target gene in differentiated neuronal cells.[9,24,25] Here we provide evidence that also in neuroblastoma cells Bim critically mediates FKHRL1-induced apoptotic cell death.

Puma and Bim have been reported to bind to the same subsets of prosurvival Bcl2 family members raising the question why, if Puma expression is lowered by FKHRL1, this does not compensate for the induction of Bim.[20] However, our experiments with conditional expression of Bim, Noxa and Puma suggest that Bim, but not Noxa or Puma induces apoptotic cell death *per se* in SH-EP cells, although not to the same extend as transgenic FKHRL1 (unpublished). This implies that Puma may not have the same apoptosis-inducing capacity as Bim in neuroblastoma cells and explain why attenuated expression of Puma did not abrogate apoptosis induction by Bim.

Noxa, like Puma was described as a *bona fide* p53 target gene, but also p53-independent mechanisms of Noxa induction have been reported.[26–30] However, the regulation of Noxa by FoxO family members downstream of the PKB survival signaling pathway has not been shown yet. The identification of Noxa as a transcriptional FKHRL1 target and critical regulator of FKHRL1-induced cell death now connects Noxa to survival signaling and may also explain why knockout of Bim only partially decreases growth-factor withdrawal-mediated cell death.[31]

By the expression of shRNAs specific for Noxa and Bim, we could show that knockdown of Noxa or Bim in FKHRL1-transgenic cells had a strong protective effect, although not to

the same extent as transgenic Bcl2. As Noxa was proposed to have a unique apical role in apoptosis induction by binding and inactivating its prosurvival targets Mcl-1 and A1, the early induction of Noxa may facilitate Bim-mediated inactivation of other antiapoptotic Bcl2 members, for example, Bcl2 or Bcl-X_L.[19,20] We found that both SH-EP/FKHRL1 and NB15/FKHRL1 cells express high levels of Mcl-1 (data not shown), suggesting that its inactivation may be necessary for apoptosis induction via Bcl2-rheostat. Noxa may displace Bak from Mcl-1, whereas Bim inactivates, for example, Bcl-X_L, thereby allowing the formation of proapoptotic Bak oligomers.[32] Thus, Bim and Noxa may cooperate in inducing FKHRL1-mediated cell death via the mitochondrial death pathway.

In this report we analyzed the function of the transcription factor FKHRL1 as a downstream regulator of the PI3K–PKB survival pathway in neuroblastoma cells. We found that activation of the transcription factor FKHRL1 promotes apoptotic cell death associated with the induction of TRAIL, loss of mitochondrial membrane potential, cytochrome *c* release and caspase activation. Despite TRAIL induction, cell death was independent of death-receptor signaling and strongly inhibited by transgenic Bcl2. In the search for candidate genes, we identified Noxa and Bim as FKHRL1-induced proapoptotic BH3-only proteins that were essentially involved in the initiation of FKHRL1-induced cell death (Figure 11). Thus, we established a new connection between survival signaling by the PI3K–PKB–FKHRL1 axis and cell death decision at the level of mitochondria and proved that different BH3-only proteins, namely Noxa and Bim, critically regulate Bcl2-rheostat during FKHRL1 activation in neuroblastoma cells. Our data further imply that inactivation of FKHRL1 in malignant neuroblastoma cells critically contributes to apoptosis resistance and that strategies to reactivate FKHRL1, for example, by small molecules that restore the function of a deregulated PKB-FKHRL1 pathway or block the nuclear export of FKHRL1, may significantly improve the therapy of this malignant disease.[33]

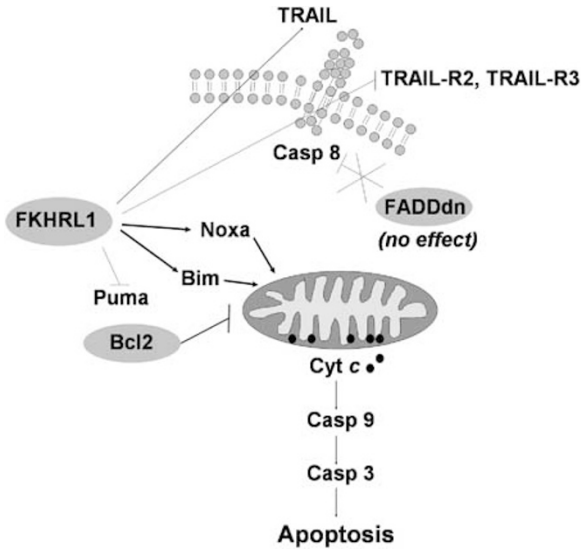

Figure 11 Model for FKHRL1-induced apoptosis in human neuroblastoma. Our data show that although FKHRL1 induces TRAIL and the BH3-only proteins, cell death is exclusively initiated via the mitochondria. FKHRL1-induced expression of Noxa and Bim and the protective effect of Noxa- and Bim-RNAi suggest that these BH3-only proteins inactivate different subsets of prosurvival Bcl2-family members (e.g. Mcl-1 and Bcl-X_L), thereby efficiently inducing cell death via loss of mitochondrial membrane integrity, cytochrome *c* release (Cyt c) and caspase (Casp 9, Casp 8, Casp 3) activation

Materials and Methods

Cell lines, culture conditions and reagents. The neuroblastoma cell lines SH-EP, LAN-1 (kindly provided by Dr. N Gross, Lausanne) and the neuroblastoma cell line STA-NB15 isolated at the St. Anna Children's Hospital, Vienna, further termed NB15[16] as well as Phoenix™ packaging cells for helper-free production of amphotropic retroviruses were cultured in RPMI 1640 (BioWhittaker, Belgium) containing 10% fetal calf serum (FCS; Gibco BRL, Paisley, GB), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Invitrogen, USA) at 5% CO_2 and 37°C in saturated humidity. All cultures were routinely tested for mycoplasma contamination. All reagents were purchased from Sigma-Aldrich unless indicated otherwise. For each experiment, mid-log-phase cultures were seeded in fresh medium.

Construction of retroviral vectors. pLIB-MCS2-iresPuro and pLIB-MCS2-iresNeo were constructed by inserting a multicloning site and a PCR-fusion product consisting of an encephalomyelitis virus (EMCV) IRES element and a puromycin/neomycin-resistance gene into the retroviral vector pLIB (BD-Clontech, USA). To obtain pLIB-FKHRL1(A3)ER^{tm}-iresNeo, the coding region of the estrogen-receptor-FKHRL1 fusion protein[34] was amplified from pCDNA3-FKHRL1(A3)ER^{tm}, the PCR product was digested with *BamH1* and cloned into pLIB-MCS2-iresNeo. For construction of pLIB-Bcl2-iresPuro, the coding region of Bcl2 was excised from pUHD-Bcl2[35] and inserted into the *EcoR1* site of pLIB-MCS2-iresPuro. Dominant negative human FADD was generated by amplifying the truncated coding region using the primers 5′-TATAGAATTCATGGAGCCCGGGCACAC (forward) and 5′-TATAGCGGCCGCTCAGGACGCTTCGGAGGTAG (reverse), thereby introducing a new start site at position 162 of the coding region. The mutated protein lacks 54 amino acids (the entire DD domain) and has a calculated size of 17 kDa. The PCR product was digested and cloned into the *Eco*R1–*Not*1 sites of the pLIB-MCS2-iresPuro plasmid. pLIB-CrmA-iresPuro was generated by PCR amplification of CrmA from pEF-CrmA.[36] The PCR-product was digested and inserted into the *Eco*R1–*Sal*1 sites of pLIB-MCS2-iresPuro.

For specific gene knockdown of Noxa and Bim, we used the self-inactivating retroviral vector pQCXIX (Clontech, USA) and inserted an SV40-puromycin-resistance cassette and an H1-polymerase III promoter. Oligonucleotides coding for shRNA double strands containing the Noxa-specific sequence GTCGAGTGTGC TACTCAACT or the Bim-specific sequence TGATGTAAGTTCTGAGTGTG were inserted into the *Bam*H1–*Mun*1 sites of pQ-tetH1-SV40-Puro.[37]

Production of retroviruses and retroviral infection. About 6×10^5 Phoenix™ packaging cells were transfected with 2 μg of retroviral vector and 1 μg of a plasmid coding for VSV-G protein using Lipofectamine2000 (Invitrogen, USA). After 48 h, the retrovirus-containing supernatants were filtered through 0.45 μm syringe filters (Sartorius, Germany) and the pLIB-FKHRL1(A3)ER^{tm}-iresNeo or empty control supernatants were used to infect SH-EP and NB15 cells. Bulk-selected cells were then infected with pLIB-Bcl2-iresPuro (SH-EP/FKHRL1-Bcl2, NB15/FKHRL1-Bcl2), pBABE-cyto.*c*-GFP (SH-EP/FKHRL1-cytoC), pLIB-dnFADD-iresPuro (SH-EP/FKHRL1-dnFADD, NB15/FKHRL1-dnFADD), pLIB-CrmA-iresPuro (SH-EP/FKHRL1-CrmA), pQ-tetH1-shNoxa-SV40-Puro, pQ-tetH1-shBim-SV40-Puro vectors (SH-EP/FKHRL1-shNoxa, NB15/FKHRL1-shNoxa, SH-EP/FKHRL1-shBim, NB15/FKHRL1-shBim) or the corresponding empty control vectors, respectively.

Northern blot analysis. Northern blot analysis was performed as described.[38] Briefly, 15 μg of total RNA was separated on a denaturing, formaldehyde-containing 1% agarose gel and blotted overnight onto Zetabind™ nylon membranes (Cuno, USA). After UV crosslinking, filters were prehybridized to block nonspecific binding at 65°C and hybridized for at least 12 h to ^{32}P-labeled, heat-denatured FKHRL1 and 18S-RNA cDNA probes, respectively. The washed blots were exposed to Agfa Curix X-ray films with an amplifying screen for several hours to days. Between hybridizations, the blots were stripped by boiling in 0.1% SDS.

Determination of apoptosis. Apoptosis was determined by quantification of propidium-iodide (PI) stained nuclei and forward/sideward scatter analysis using a CytomicsFC-500 (Beckman Coulter, USA). Briefly, 2×10^5 cells were centrifuged and resuspended in hypotonic PI solution containing 0.1% Triton X-100. Cellular debris and small particles were excluded from FACS analysis, and stained nuclei in the sub-G1 marker window were considered to represent apoptotic cells.[38] The activation of caspase-3 was determined by flow cytometry with a caspase-3-detection kit (FITC-DEVD-FMK from Oncogene Research Products, USA) according to the manufacturer's instructions. Mitochondrial activity was assessed by the fluorescence dye MitoTracker Red/CMX-Ros (Invitrogen, USA) according to the manufacturer's instructions.

Immunoblotting and cytochrome *c* release. Identical numbers of cells were lysed on ice in CelLytic™-M Mammalian Cell Lysis/Extraction Reagent containing a protease inhibitor cocktail. For analysis of cytochrome *c* release, we used the ApoAlert™ Cell Fractionation Kit (Clontech, USA) according to the manufacturer's instructions. The concentration of the protein was determined with 'protein reagent' (BioRad Laboratories, Germany). The supernatant was then mixed with $4\times$ SSB containing 20% β-mercaptoethanol and boiled. Samples were separated by SDS-PAGE on 7.5–15% polyacrylamide gels, and transferred to nitrocellulose membranes (Schleicher & Schuell, Germany) by a NOVEX blotter apparatus. The membranes were blocked with PBS blocking buffer containing 0.1% Tween20 and 5% nonfat dry milk, incubated with primary antibodies specific for human FKHRL1, phospho-FKHRL1-T32 (Upstate Biotechnology, USA), p85-PI3K, caspase-8, FADD, Bim (BD-Pharmingen, Germany), PKB1, PKB2 (Cell Signaling, USA), caspase-9, phospho-PKB-Ser473 (R&D Systems, USA), Noxa/PMAIP1 (Alexis Biochemicals, Switzerland), Puma/bbc3 (Sigma-Aldrich, USA), Bid (Biosource Int., USA), cytochrome *c* and α-Tubulin (Oncogene Research Products, USA), washed and incubated with anti-mouse, anti-rabbit or anti-rat horseradish-peroxidase-conjugated secondary antibodies (GE Healthcare, USA). The blots were developed by enhanced chemiluminescence (GE Healthcare, USA) according to the manufacturer's instructions and analyzed in an AutoChemi detection system (UVP, England).

Quantitative 'real-time' RT-PCR. To quantify Fas/CD95, TRAIL, TRAIL-R2, TRAIL-R3, TRAIL-R4, Bim, Noxa and Puma, mRNA levels, we designed 'real-time' RT-PCR assays, using GAPDH as reference gene. SH-EP/FKHRL1 and NB15/FKHRL1 cells were cultured in the presence of 75 nM 4OHT for 0, 3, 6 and 8 h, respectively. Total RNA was isolated from 5×10^6 cells using TRIzol™ Reagent (Invitrogen, USA) according to the manufacturer's instructions. Complementary DNA was synthesized from 1 μg of total RNA using the RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). The oligonucleotides used to amplify and detect Fas/CD95 (forward 5′-GCTCTTTCACTTCGGAGGATTGC and reverse 5′-GCCTTCCAAGTTCTGAGTCTCAAC), TRAIL (forward 5′-AAAGAGGTC CTCAGAGAGTAGCAGC and reverse 5′- GCTCAGGAATGAATGCCCACTC), TRAIL-R2 (forward 5′-GAAGGTGATCCCACTGAGACTCTG and reverse 5′-AGGGTGTGGACAGAGGCATCTC), TRAIL-R3 (forward 5′-CACAGCAACAGAGG CACAGCTTC and 5′-GGTTCATTGTTGGAAGCGTTGG), TRAIL-R4 (forward 5′-GTGGTTGGCTTTTCATGTCGGAAG and reverse 5′-TTACTCAGGGTCTCGTTGC GGG), Bim (forward 5′-AGCACCCATGAGTTGTGACAAATC and reverse 5′-CGTT AAACTCGTCTCCAATACGC), Noxa (forward 5′-AGCAGAGCTGGAAGTCGAGT GTG and reverse 5′-TGATGCAGTCAGGTTCCTGAGC), Puma (forward 5′-ACGACCTCAACGCACAGTACGAG and reverse 5′-TAATTGGGCTCCATCTC GGG) and GAPDH (forward 5′-TGTTCGTCATGGGTGTGAACC and reverse 5′-GCAGTGATGGCATGGACTGTG) were synthesized by MWG Biotech (Germany). Amplification efficiency was determined by serial log2 dilutions. All reactions were conducted in triplicates. Real-time RT-PCR was run on the iCycler instrument (BioRad Laboratories, Germany) using a thermal profile of an initial 3-min melting step at 95°C, followed by 40 cycles comprising 95°C for 20 s and 55°C (Bim, Noxa, Puma, TRAIL, TRAIL-R2, GAPDH), 65°C (Fas/CD95) or 68°C (TRAIL-R3, TRAIL-R4) for 45 s. To verify the presence of only one amplicon, a melting curve was processed after each run. After normalization on GAPDH expression, regulation was calculated between treated and untreated cells.

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