# FOXO3/FKHRL1 is activated by 5-aza-2-deoxycytidine and induces silenced caspase-8 in neuroblastoma

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**ABSTRACT** Forkhead box O (FOXO) transcription factors control diverse cellular functions, such as cell death, metabolism, and longevity. We analyzed FOXO3/FKHRL1 expression and subcellular localization in tumor sections of neuroblastoma patients and observed a correlation between nuclear FOXO3 and high caspase-8 expression. In neuroblastoma caspase-8 is frequently silenced by DNA methylation. Conditional FOXO3 activated caspase-8 gene expression but did not change the DNA-methylation pattern of regulatory sequences in the caspase-8 gene. Instead, FOXO3 induced phosphorylation of its binding partner ATM and of the ATM downstream target cAMP-responsive element binding protein (CREB), which was critical for FOXO3-mediated caspase-8 expression. Caspase-8 levels above a critical threshold sensitized neuroblastoma cells to tumor necrosis factor–related apoptosis-inducing ligand–induced cell death. The DNA-demethylating drug 5-Aza-2-deoxycytidine (5-azadC) induced rapid nuclear accumulation of FOXO3, ATM-dependent CREB phosphorylation, and caspase-8 expression in a FOXO3-dependent manner. This indicates that 5-azadC activates the FOXO3-ATM-CREB signaling pathway, which contributes to caspase-8 expression. The combined data suggest that FOXO3 is activated by 5-azadC treatment and triggers expression of caspase-8 in caspase-8–negative neuroblastoma, which may have important implication for metastasis, therapy, and death resistance of this childhood malignancy.

## INTRODUCTION

The members of the class O subfamily of forkhead box (FOXO) transcription factors FOXO1/FKHR, FOXO3/FKHRL1, FOXO4/AFX, and FOXO6 are regulators of cell cycle progression, apoptosis, longevity, and metabolism in mammalian cells (Calnan and Brunet, 2008;

Gross et al., 2008; Ho et al., 2008). Gene-knockout experiments revealed distinct functions of these transcription factors, that is, defects in developmental vascular formation by FOXO1 and a critical role of FOXO3 in fertility. Conditional triple-knockout animals provided evidence that FOXO1, FOXO3, and FOXO4 are involved in the protection of hematopoietic stem cells from oxidative stress and in the regulation of endothelial cell homeostasis (Arden, 2008).

The role of FOXOs in neuronal cells and tumors derived from neuronal precursor cells, such as neuroblastoma, is less well investigated. Neuroblastoma is a pediatric malignancy of neural crest origin (Park et al., 2008) and is characterized by clinical heterogeneity, including spontaneous regression or differentiation into benign ganglioneuromas (Yamamoto et al., 1998). One aspect of high-risk neuroblastoma is the lack of CASP8/caspase-8, which was ascribed to epigenetic gene silencing by methylation of gene-regulatory sequences in the second intron of the caspase-8 gene (Teitz et al., 2000; Hopkins-Donaldson et al., 2000; Fulda et al.,

**A**

Caspase 8 | FOXO3

Patient 1 — 400x, 400x

Patient 2 — 400x, 400x

**B**



FIGURE 1: Immunoperoxidase staining of FOXO3 and caspase-8 in tumor tissue of neuroblastoma patients postchemotherapy. (A) Caspase-8 expression correlates with subcellular localization of FOXO3. Shown are two exemplary patient biopsies qualified as low (top) or high (bottom) caspase-8–expressing cells. (B) Analyses of 27 different neuroblastoma patients (13 patients with preferential cytoplasmic FOXO3 and 14 samples with nuclear FOXO3) reveal a significant correlation between increased caspase-8 expression and nuclear FOXO3 localization ($p = 0.04$). Statistical analysis was performed using nonparametric Wilcoxon rank test calculated with GraphPad Prism software.

occurs by phosphorylation at serine 133 via protein kinase A (PKA) in response to cAMP or via ataxia-telangiectasia mutated (ATM) protein kinase during genotoxic stress. ATM is a member of the phosphoinositol-3-kinase–like kinase family that coordinates, together with other, related kinases, the cellular responses to DNA damage by activating DNA-repair and signaling pathways. ATM acts as critical sensor for DNA double-strand breaks but also for reactive oxygen species (ROS) in mammalian cells (reviewed in Ditch and Paull, 2012). ATM cooperates with FOXO3 in both DNA-damage (Tsai et al., 2008) and ROS response (Yalcin et al., 2008), suggesting that these two proteins are tightly interconnected during stress signaling.

Phosphorylation of FOXOs by protein kinase B (PKB) at distinct amino acids causes their export from the nucleus and, as a consequence, repression of target gene transcription (Burgering and Kops, 2002). In neuroblastoma, the hyperactivation of PKB contributes to therapy resistance (Li et al., 2005) and correlates with poor prognosis (Opel et al., 2007). We and others showed that hyperactive PKB is associated with FOXO3 phosphorylation in neuroblastoma cells (Schwab et al., 2005; Obexer et al., 2007). FOXO3 triggers apoptotic cell death by activating the two BH3-only proteins PMAIP1/Noxa and BCL2L11/Bim (Obexer et al., 2007) and sensitizes neuroblastoma cells to chemotherapy-induced cell death by repressing the IAP-family member BIRC5/Survivin (Obexer et al., 2009). Recently we also demonstrated that FOXO3 is activated by DNA-damaging agents and triggers ROS in response to genotoxic stress via activation of the BH3-only protein Bim (Hagenbuchner et al., 2012).

In the present study we analyzed tumor sections of neuroblastoma patients and determined how FOXO3 induces caspase-8 in caspase-8–negative neuroblastoma.

2001). In concordance with this assumption, the DNA-demethylating agent 5-aza-2-deoxycytidine (5-azadC) restored caspase-8 expression and sensitivity of neuroblastoma cells to chemotherapy- or death receptor–induced apoptosis (Fulda et al., 2001). Although there have been conflicting reports regarding the prognostic value of caspase-8 for neuroblastoma outcome (Fulda et al., 2006), it was shown that caspase-8 deficiency impairs cell death sensitivity (Eggert et al., 2000) and promotes metastasis of neuroblastoma (Stupack et al., 2006; Barbero et al., 2009).

Despite methylation of these regulatory sequences, caspase-8 expression can be induced by, for example, γ-interferon (Fulda and Debatin, 2002; Tekautz et al., 2006) or retinoic acid (Fernandes et al., 2007). In the latter case, the transcription factor cAMP-responsive element binding protein (CREB) is critical for the induction of silenced caspase-8 (Jiang et al., 2008). Activation of CREB

## RESULTS

### High expression of caspase-8 correlates with nuclear FOXO3 in neuroblastoma

To investigate caspase-8 and FOXO3 expression in human neuroblastoma tumors, we analyzed sections of 27 biopsies postchemotherapy by immunohistochemistry for the expression of caspase-8 and the expression and localization of FOXO3. Figure 1A shows exemplary samples of two patients. Neuroblastoma cells in biopsies of patient 1 expressed low levels of caspase-8, which correlated with cytoplasmic localization of FOXO3. In contrast, neuroblastoma cells from patient 2 presented with high caspase-8 expression (Figure 1A, bottom). In these cells, FOXO3 was localized almost exclusively to the nucleus. Analyses of biopsies from 27 neuroblastoma patients revealed that the expression level of caspase-8 correlated with the localization of FOXO3. As shown in Figure 1B, nuclear localization of
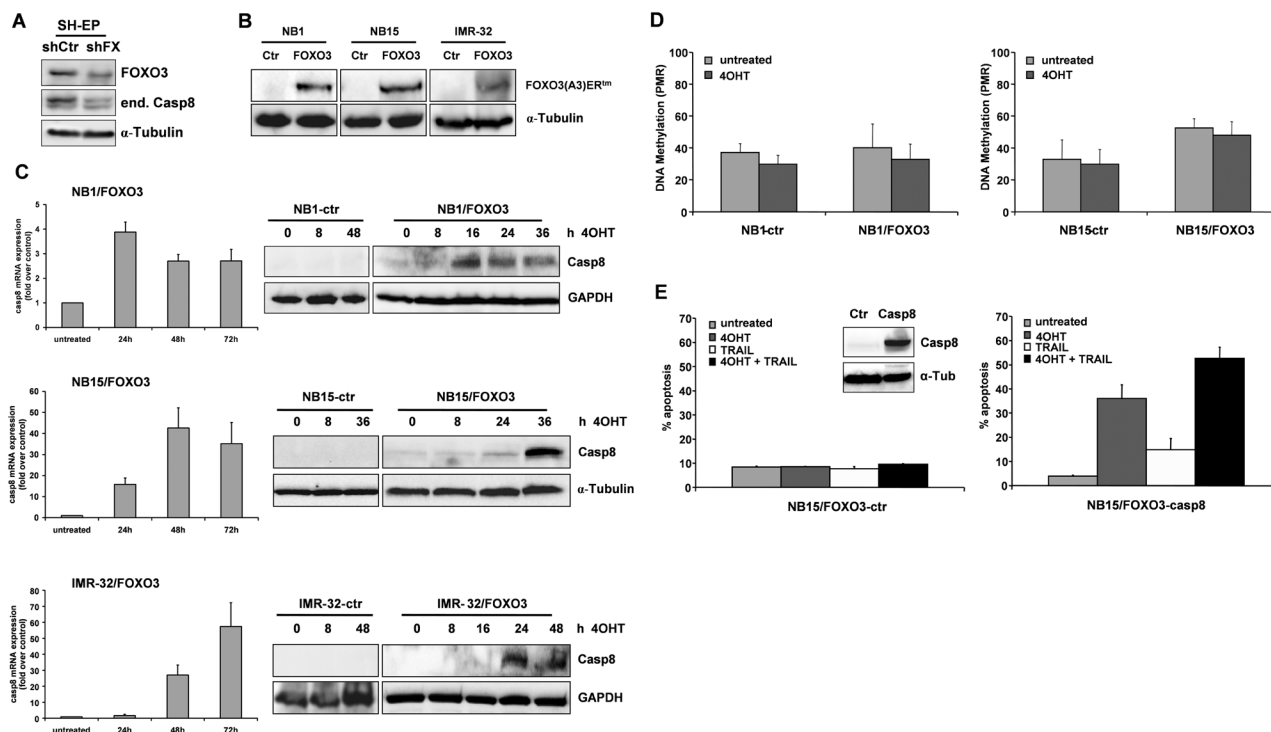
**FIGURE 2:** FOXO3 induces silenced caspase-8 independent of DNA methylation and increases sensitivity to TRAIL-induced apoptosis. (A) Caspase-8–positive SH-EP cells infected with lentiviral vectors. Scrambled shRNA control or FOXO3-specific shRNA (shFX) were subjected to immunoblot analyses for the expression of FOXO3 (75 kDa), caspase-8 (55 kDa), and α-tubulin (60 kDa). (B) A FOXO3(A3)ER$^{tm}$ (110 kDa) allele that can be activated by 4OHT was retrovirally transduced into caspase-8–deficient NB1, NB15, and IMR-32 cells. Mock-infected or FOXO3(A3)ER$^{tm}$-infected cells were subjected to immunoblot analysis. (C) NB1/FOXO3, NB15/FOXO3, and IMR-32/FOXO3 cells and mock-infected controls were treated with 4OHT (100 nM) for the times indicated to activate transgenic FOXO3 and then subjected to mRNA quantification (left) and immunoblot (right) of caspase-8 (55 kDa). For quantitative RT-PCR the mean ± SEM of three independent experiments is shown. One representative immunoblot out of four is shown, respectively. (D) NB1/FOXO3 and NB15/FOXO3 cells and mock-infected controls were treated with 100 nM 4OHT for 24 h and subjected to methylation-specific PCR of putative gene-regulatory sequences in the caspase-8 gene. (E) NB15/FOXO3 cells were infected with a retrovirus coding for human procaspase-8. Bulk-selected cells were subjected to immunoblot analysis (left). NB15/FOXO3-casp8 cells (right) and mock-infected controls (left) were treated with low-dose (10 nM) 4OHT, recombinant TRAIL (100 ng/ml), or a combination of both. Apoptosis was determined by propidium iodide–fluorescence-activated cell sorting analyses. The mean of three independent experiments is shown.

FOXO3 in biopsies was significantly associated with increased caspase-8 expression (p = 0.04). The differences between caspase-8 expression in biopsies with cytoplasmic or nuclear FOXO3 were assessed by the Wilcoxon signed rank test. To investigate a possible mechanistic relationship between FOXO3 and caspase-8 expression, we next studied the effects of active FOXO3 in caspase-8–negative neuroblastoma cells.

## FOXO3 regulates caspase-8 expression

To study whether steady-state levels of FOXO3 influence caspase-8 expression, we expressed short hairpin RNA (shRNA) directed against FOXO3 in caspase-8–positive SH-EP neuroblastoma cells. As shown in Figure 2A, knockdown of endogenous FOXO3 led to reduced endogenous caspase-8 expression, suggesting a connection between FOXO3 activity and caspase-8 expression. In a next step caspase-8–negative NB1, NB15, and IMR-32 cells were infected with a retrovirus coding for a 4-hydroxytamoxifen (4OHT)–inducible, PKB phosphorylation–independent FOXO3–estrogen receptor fusion protein (FOXO3(A3)ER$^{tm}$; Dijkers et al., 2000; Obexer et al., 2007). Transgene expression was verified by immunoblot (Figure 2B). The FOXO3(A3)ER$^{tm}$ allele consists of the FOXO3 coding sequence with mutations at all known PKB phosphorylation sites fused to a mutated estrogen receptor ligand–binding domain that has low affinity for estrogen and preferentially binds 4OHT. This fusion protein is constitutively expressed in the infected neuroblastoma cell lines but inactive in absence of the ligand 4OHT. In presence of 4OHT it undergoes a conformational change that activates it and causes translocation to the nucleus. Activation of ectopic FOXO3(A3)ER$^{tm}$ by 4OHT induced both mRNA and protein expression of caspase-8 in all investigated cell lines. Caspase-8 mRNA steady-state levels were increased fourfold in NB1/FOXO3 cells after 24 h. In NB15/FOXO3 cells we observed a 45-fold induction of caspase-8 mRNA after 48 h, and in IMR-32/FOXO3 cells caspase-8 mRNA was increased 60-fold within 72 h of FOXO3 activation (Figure 2C, left). Consistent with the quantitative real-time RT-PCR results, caspase-8 was reexpressed in NB1/FOXO3 cells already after 16 h and induced in IMR-32/FOXO3 and NB15/FOXO3 cells after 24–36 h in the presence of 4OHT (Figure 2C, right). 4OHT did not affect caspase-8 expression in mock-infected

control cell lines. These experiments indicate that FOXO3 regulates caspase-8 expression levels in neuroblastoma cells.

## Caspase-8 induction by FOXO3 does not correlate with changes in methylation of caspase-8 regulatory gene sequences

Because lack of caspase-8 in neuroblastoma has been ascribed to methylation of distinct regulatory sequences in the second intron of the caspase-8 gene (Teitz et al., 2000; Fulda et al., 2001), we next investigated whether FOXO3 affects DNA methylation of caspase-8 alleles. To determine the level of methylation at this critical regulatory region in cells with or without active FOXO3, we designed a methylation-specific methyLight PCR. However, the methylation status was not changed by FOXO3 activation within 24 h in NB1/FOXO3 and NB15/FOXO3 cells or in mock-infected controls (Figure 2D). This suggests that FOXO3 induces caspase-8 independent of the DNA-methylation status of caspase-8–regulatory sequences in the intron between exons 2 and 3 (Teitz et al., 2000).

## Increasing caspase-8 expression above a specific threshold does not induce spontaneous apoptosis but sensitizes neuroblastoma cells to tumor necrosis factor–related apoptosis-inducing ligand–induced cell death

To study whether caspase-8 sensitizes neuroblastoma cells in a dose-dependent manner to death receptor–induced apoptosis, we infected NB15/FOXO3 cells with a retrovirus coding for human pro–caspase-8. The expression of high levels of caspase-8 did not induce spontaneous cell death. This excludes the possibility that raising caspase-8 above a specific threshold leads to spontaneous caspase-8 cleavage and apoptosis of neuroblastoma cells. When NB15/FOXO3 cells were treated with low-dose 4OHT (10 nM), which is not sufficient to induce apoptotic cell death, NB15 cells remain refractory to tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) treatment (Figure 2E, left). However, ectopic expression of caspase-8 increased the sensitivity to TRAIL, and additional induction of endogenous caspase-8 via low-dose activation of FOXO3 synergized with ectopically expressed caspase-8 (Figure 2E, right). This suggests that a certain threshold of caspase-8 has to be reached before caspase-8 is efficiently activated by TRAIL to trigger cell death in neuroblastoma cells.

## Activation of FOXO3 induces phosphorylation of CREB in NB1/FOXO3 and NB15/FOXO3 cells

Caspase-8 expression can be induced by retinoic acid via activation of the transcription factor CREB (Jiang et al., 2008). Similar results have been achieved in NB15 cells (Supplemental Figure S1). Because FOXO3 was shown to functionally interact with ATM and to promote ATM downstream signaling (Tsai et al., 2008; Yalcin et al., 2008) and furthermore ATM activates CREB via phosphorylation at serine 133, we hypothesized that FOXO3 may induce caspase-8 expression via triggering ATM. To determine whether FOXO3 leads to phosphorylation of ATM at serine 1981 and thereby its activation, we performed immunofluorescence and immunoblot analyses in NB15/FOXO3 cells. ATM phosphorylation was detected already 4 h after FOXO3 activation, as analyzed by immunoblot and by fluorescence microscopy after 8 h of 4OHT treatment (Figure 3C). An increased phosphorylation of CREB on serine 133 was observed within 8 h of FOXO3 activation in NB1/FOXO3 and NB15/FOXO3 cells (Figure 3, A and B). Inhibition of ATM by the ATM inhibitor or via ATM-specific shRNA during FOXO3 activation prevented the phosphorylation of CREB at serine 133 and the reexpression of caspase-8 (Figure 3, A and B, and Supplemental Figure S6). To investi-

gate whether CREB is critical for FOXO3-induced caspase-8 reexpression, we transfected NB15/FOXO3-BclxL cells (Hagenbuchner et al., 2010), which are protected from FOXO3-induced cell death with a cytomegalovirus (CMV) promoter–containing control vector (CMV-ctr) or with plasmids coding for wild-type (wt) CREB or a dominant-negative CREB mutant that lacks the CREB-DNA–binding and dimerization domain (CREBΔLZ; Riccio et al., 2006). After transfection, cells were cultured in the presence or absence of 100 nM 4OHT for 40 h. The transfection of the dimerization-deficient CREB mutant (CREBΔLZ) but not of wt CREB clearly attenuated caspase-8 induction by FOXO3 in NB15/FOXO3 cells (Figure 3D). The combined results demonstrate that FOXO3 induces caspase-8 via ATM-CREB signaling in neuroblastoma cells.

## 5-azadC activates FOXO3 and triggers ATM-CREB signaling in caspase-8–negative neuroblastoma cells

5-azadC prevents DNA methylation in proliferating cells and is therefore widely used as a DNA-demethylating agent. In addition to inhibiting de novo methylation, 5-azadC may also induce a DNA-damage response via ATM (Palii et al., 2008). We therefore examined whether 5-azadC activates FOXO3 and, as a consequence, ATM-CREB signaling and caspase-8 expression. To address this question, we treated SH-EP neuroblastoma cells expressing an enhanced cyan fluorescent protein (ECFP)–FOXO3 fusion protein (Obexer et al., 2009) with 20 μM 5-azadC and analyzed them by live-cell fluorescence imaging. In untreated cells ECFP-tagged FOXO3 is localized exclusively in the cytoplasm, whereas addition of 5-azadC induces nuclear accumulation within 20–60 min (Figure 4A). After FOXO3 activation, 5-azadC induced caspase-8 mRNA and protein expression in three caspase-8–negative neuroblastoma cell lines (NB1, NB3, and NB15) as measured by quantitative RT-PCR (Figure 4B) and immunoblot (Figure 4C). Similar to 5-azadC, the DNA-damaging agents doxorubicin and etoposide induced the nuclear accumulation of FOXO3 (Supplemental Figure S3) and the expression of silenced caspase-8 (Supplemental Figure S4). This indicates that FOXO3 is activated by DNA-damaging drugs in neuronal cells and that caspase-8 induction is downstream of a common DNA-damage response pathway. To verify DNA damage and ATM activation by 5-azadC, we performed immunofluorescence staining for pH2Ax and pATM in NB15/BclxL cells, which are protected from cell death by overexpression of BclxL. 5-azadC treatment induced focal, nuclear staining of pH2Ax and pATM, which is characteristic for the initiation of DNA repair after damage (Supplemental Figure S5). To investigate whether 5-azadC treatment also triggers ATM-CREB signaling, which in turn leads to caspase-8 expression (Figure 3, A and B), we assessed serine 133 phosphorylation and steady-state expression of CREB in 5-azadC–treated NB1 and NB15 cells. As shown in Figure 5, A and B (left), the levels of CREB–serine 133 phosphorylation increased after 4–8 h in both cell lines, whereas CREB protein expression remained unaffected. This is consistent with Figure 3, A and B, in which elevated CREB phosphorylation is observed at 4 and 8 h after FOXO3 activation. Inhibition of ATM prevented the serine 133 phosphorylation of CREB in response to 5-azadC treatment. The proapoptotic BH3-only protein Bim, which is a transcriptional target of FOXO3 (Obexer et al., 2007), was upregulated by 5-azadC. Bim was induced already after 2 h in the presence of 5-azadC in both tested cell lines. Inhibition of ATM prevented the 5-azadC–mediated induction of Bim in NB1 and NB15 cells. To assess whether the induction of caspase-8 depends on FOXO3, we knocked down the endogenous FOXO3 by lentiviral expression of FOXO3-specific shRNA (Supplemental Figure S2). Lowering endogenous FOXO3 attenuated caspase-8 induction by 5-azadC up to 72 h compared with
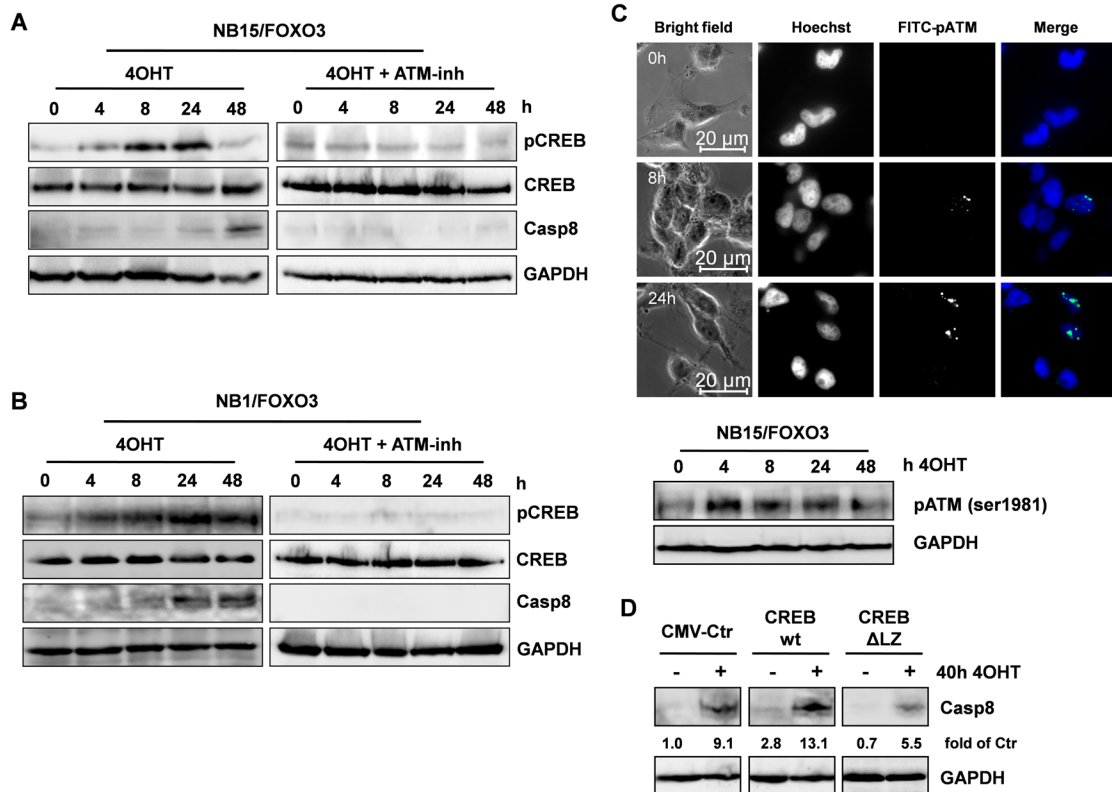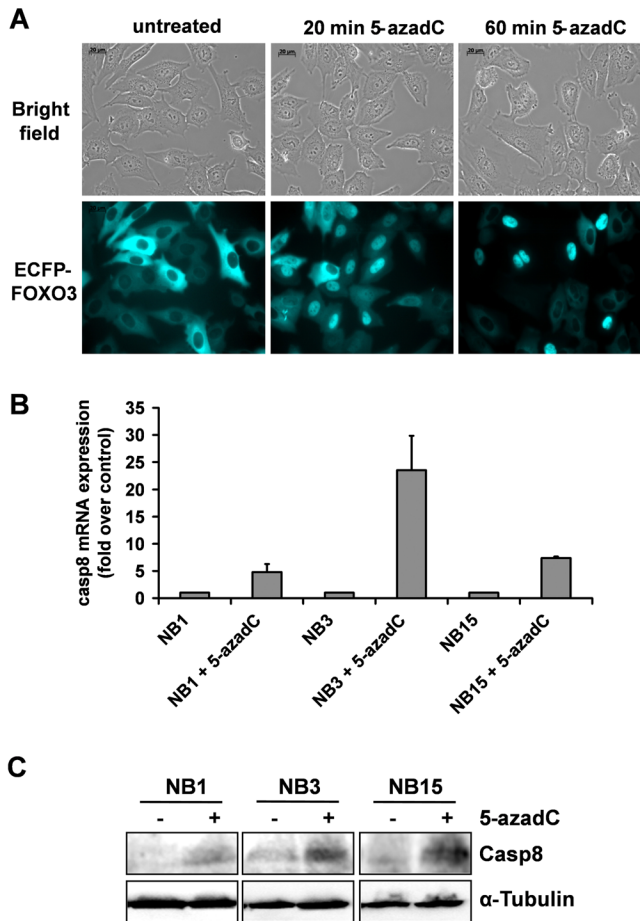
**FIGURE 3:** ATM-dependent phosphorylation of CREB is critical for caspase-8 induction by FOXO3. NB15/FOXO3 (A) and NB1/FOXO3 (B) cells were exposed to 100 nM 4OHT alone or to 4OHT (100 nM) and ATM kinase inhibitor (2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one, 10 µM) to switch on conditional FOXO3 and then harvested at the times indicated. Cell extracts were analyzed by immunoblotting using antibodies directed against CREB (43 kDa), the serine 133 phosphorylation site of CREB (46 kDa), caspase-8 (55 kDa), and GAPDH (38 kDa). One representative immunoblot out of six is shown, respectively. (C) NB15/FOXO3 cells were treated for 8 and 24 h with 4OHT and stained with a phospho-S1981–specific ATM antibody. Nuclei were visualized with Hoechst 33342 (100 ng/ml). NB15/FOXO3cells were treated for the times indicated with 100 nM 4OHT, and the serine 1981 phosphorylation site of ATM (370 kDa) and GAPDH (38 kDa) was detected by immunoblot. (D) NB15/FOXO3-BclxL cells were transiently transfected with a CMV promoter containing control vector (CMV-ctr) or with plasmids coding for wt CREB or a dominant-negative CREB mutant that lacks the CREB-DNA–binding and dimerization domain (ΔLZ). Transfection efficiency as assessed by cotransfection of a green fluorescent protein–expressing plasmid was ~40% with all three vectors. After transfection, cells were cultured in presence or absence of 100 nM 4OHT for 40 h. Equal amounts of protein were loaded. Caspase-8 (55 kDa) and GAPDH (38 kDa) expression levels were assessed by immunoblot. Basal and induced expression of caspase-8 is expressed in fold induction of untreated control.

controls (Figure 5C). The combined data demonstrate that 5-azadC induces caspase-8 in caspase-8–negative neuroblastoma via a DNA damage–response pathway involving FOXO3 activation.

## DISCUSSION

Hyperactive PKB is observed in cultured neuroblastoma cells (Obexer *et al.*, 2007) and correlates with adverse clinical outcome in neuroblastoma patients (Opel *et al.*, 2007). Active PKB also contributes to functional inactivation of FOXO3 and may thereby significantly hamper the apoptotic machinery downstream of FOXO3. In neuroblastoma cell lines we showed that FOXO3 triggers apoptotic cell death by inducing the BH3-only proteins Noxa and Bim (Obexer *et al.*, 2007). More recently we provided evidence that FOXO3 sensitizes neuroblastoma cells to chemotherapeutic agents by the repression of survivin (Obexer *et al.*, 2009) and triggers cell death via a biphasic ROS accumulation (Hagenbuchner *et al.*, 2012). Because caspase-8 deficiency is frequent in neuroblastoma (Teitz *et al.*, 2000; Fulda *et al.*, 2001) and FOXO3 activates components of the death receptor signaling pathway,

we investigated expression and localization of FOXO3 and caspase-8 in biopsies of neuroblastoma patients postchemotherapy. Nuclear (active) FOXO3 correlated with positive caspase-8 expression (Figure 1), suggesting a possible link between FOXO3 and caspase-8 in the analyzed postchemotherapy patient material. DNA-damaging agents such as doxorubicin and etoposide rapidly induce nuclear accumulation of FOXO3 (Hagenbuchner *et al.*, 2012) and caspase-8 expression (Supplemental Figures S3 and S4). Nuclear localization and the induction of caspase-8 in patient material might therefore result from chemotherapy and may not represent the status at diagnosis. To directly assess whether caspase-8 is regulated by FOXO3, we expressed a FOXO3(A3)ERtm allele that carries mutations at all three major PKB phosphorylation sites, which allows activation of FOXO3 independent of PKB activity in mammalian cells. Indeed, the activation of this PKB-insensitive FOXO3 allele (Figure 2B) induced caspase-8 expression on mRNA and protein levels in all investigated caspase-8–negative cell lines (Figure 2C), indicating that silenced caspase-8 can be reinduced via FOXO3. The converse

**A**
untreated     20 min 5-azadC     60 min 5-azadC

Bright field

ECFP-FOXO3

**B**

**C**

NB1    NB3    NB15
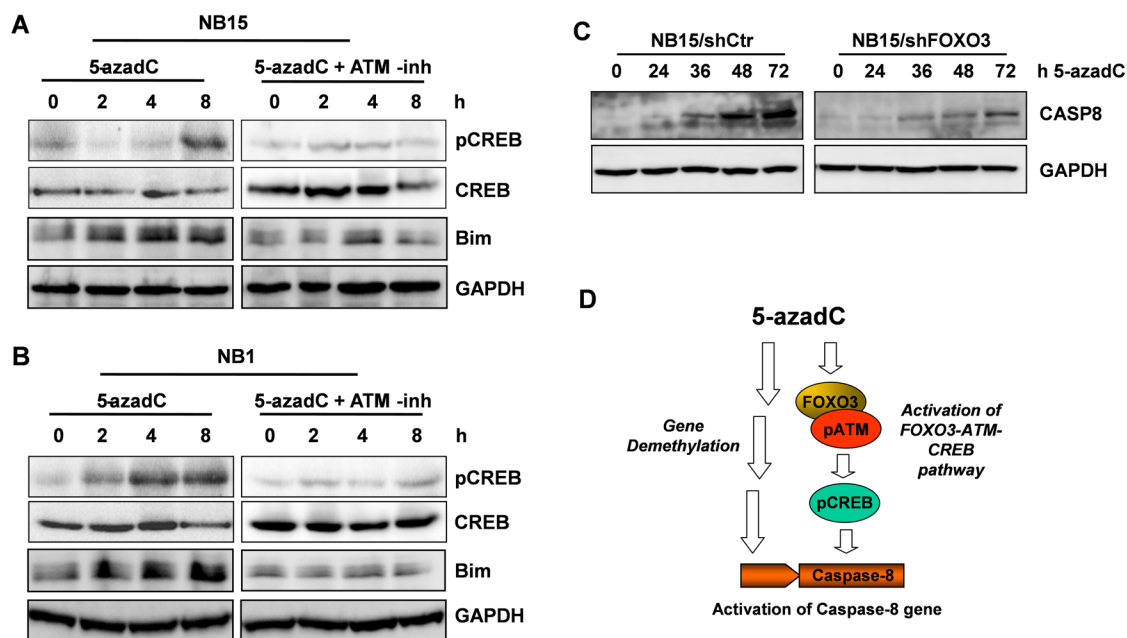
- +   - +   - +   5-azadC

Casp8

α-Tubulin

**FIGURE 4:** 5-azadC induces nuclear accumulation of FOXO3 and caspase-8 expression. (A) SH-EP/ECFP-FOXO3 cells were cultured on collagen-coated LabTek chamber glass slides and treated with 20 µM 5-azadC for the times indicated. Live-cell fluorescence imaging was performed by an Axiovert 200M microscope using AxioVision 4.7 software. Caspase-8–deficient NB1, NB3, and NB15 cells were treated for 96 h with 5-azadC (1 µM) and analyzed for their caspase-8 mRNA expression by quantitative RT-PCR (B) and for caspase-8 and α-tubulin protein expression by immunoblot (C). For quantitative RT-PCR the mean ± SEM of three independent experiments, and one representative immunoblot out of three is shown.

experiment, that is, lowering endogenous FOXO3 steady-state levels by shRNA technology in caspase-8–expressing SH-EP cells, resulted in reduced caspase-8 protein levels, supporting the hypothesis of a functional connection between FOXO3 and caspase-8 (Figure 2A). Caspase-8 induction by FOXO3 was not sufficient to sensitize the tumor cells to death ligand–induced apoptosis, although caspase-8 cleavage increased (Supplemental Figure S7). However, when raising caspase-8 expression above a certain threshold, FOXO3-induced endogenous caspase-8 further increased apoptosis by TRAIL (Figure 2E). This suggests that in neuroblastoma cells a specific threshold for caspase-8 activation exists that determines sensitivity to TRAIL-induced apoptosis.

Caspase-8 deficiency has been ascribed to DNA hypermethylation since the caspase-8 gene contains methylated regulatory sequences and 5-azadC treatment leads to reexpression of caspase-8 (Teitz et al., 2000; Fulda et al., 2001). FOXO3 activation did not change the methylation–status of these regulatory sequences as measured by methyLight PCR (Figure 2D). From this we concluded

that FOXO3 does not affect DNA-methylation patterns to induce caspase-8 in caspase-8–negative neuroblastoma, which is in line with several other studies: Banelli et al. (2002) provided evidence that DNA methylation of this region does not correlate with caspase-8 expression, and the induction of caspase-8 in response to γ-interferon (Fulda and Debatin, 2002; Tekautz et al., 2006) or retinoic acid (Fernandes et al., 2007) is also independent of changes in the methylation status of these intronic region. Retinoic acid–induced differentiation of neuroblastoma cells was shown to be associated with ATM-dependent phosphorylation of CREB (Fernandes et al., 2007). Another report demonstrated that caspase-8-induction during retinoic acid treatment can be triggered by binding of phosphorylated CREB to an intronic region in the caspase-8 gene (Jiang et al., 2008). ATM is a serine/threonine protein kinase that coordinates DNA damage repair but is also a sensor for cellular ROS (reviewed in Ditch and Paull, 2012). Of importance, in both cases ATM cooperates with FOXO3. In mouse embryonic fibroblasts and the tumor cell lines MCF7 and LNCaP, FOXO3 promotes phosphorylation of ATM at serine 1981 during DNA damage (Tsai et al., 2008). In hematopoietic stem cells FOXO3 regulates ROS via ATM, which is essential for stem cell maintenance (Yalcin et al., 2008). On the basis of these reports, we hypothesized that the ATM-CREB signaling pathway might connect FOXO3 to caspase-8 expression. As shown in Figure 3C, FOXO3 induced the phosphorylation of ATM on serine 1981 already within 4 h, and marked CREB phosphorylation at serine 133 was detected 8 h after activation of ectopic FOXO3(A3)ER. Of importance, both phosphorylation of CREB and induction of caspase-8 were blocked by inhibition of ATM (Figure 3, A and B, right). Because a dominant-negative CREB mutant that lacks the CREB-DNA–binding and dimerization domain also attenuated caspase-8 induction (Figure 3D), the combined data clearly demonstrate that FOXO3 activates caspase-8 expression via the ATM-CREB signaling pathway. In earlier studies, the DNA-methylation inhibitor 5-azadC was used as a proof for methylation-dependent caspase-8 silencing (Teitz et al., 2000; Fulda et al., 2001). However, besides its DNA-demethylating activity in proliferating cells, 5-azadC may also induce DNA damage response associated with ATM activation in some cell types (Palii et al., 2008). In cells with hyperactive PKB, FOXO3 can still be activated by stress-induced kinases during oxidative or genotoxic stress. Mammalian-sterile 20-like kinase-1 phosphorylates FOXO3 at serine 209, thereby promoting the dissociation of FOXO3 from 14-3-3 proteins, which leads to its nuclear accumulation and activation (Lehtinen et al., 2006). Jun-N-terminal kinase phosphorylates FOXO3 within its C-terminal part (Essers et al., 2004) and also 14-3-3 proteins at serine 184, which causes dissociation of FOXO3 from 14-3-3 proteins and nuclear accumulation of FOXO3 (Sunayama et al., 2005). We therefore tested the hypothesis that 5-azadC activates caspase-8 expression via FOXO3-ATM–mediated DNA damage response. Like other DNA-damaging drugs (Supplemental Figures S3 and S4), 5-azadC induced rapid nuclear accumulation of ECFP-tagged FOXO3 (Figure 4A) and led to reexpression of caspase-8 (Figure 4, B and C). DNA demethylation does not seem to be the reason for the primary caspase-8 induction, since knockdown of FOXO3 efficiently prevented caspase-8 induction in 5-azadC–treated cells at 36 h (Figure 5C). Consistent with our model, 5-azadC led to the expression of the proapoptotic, FOXO3-regulated target protein Bim and, of importance, to ATM-dependent CREB-phosphorylation at serine 133 (Figure 5, A and B). 5-azadC treatment therefore triggers an immediate response via the FOXO3-ATM-CREB pathway, which causes caspase-8 expression in caspase-8–negative neuroblastoma (Figure 5D). This

**FIGURE 5:** The 5-azadC–mediated induction of caspase-8 depends on FOXO3 and ATM activation. NB15 (A) and NB1 (B) cells were treated with 20 µM 5-azadC alone or in combination with 10 µM ATM inhibitor, respectively, for the indicated times and analyzed by immunoblot for protein levels of phospho-CREB (46 kDa), CREB (43 kDa), Bim (25 kDa), and GAPDH (38 kDa). One representative immunoblot out of six is shown. (C) NB15-shCtr and NB15-shFOXO3 cells were treated with 20 µM 5-azadC for the times indicated, and caspase-8 expression was assessed by immunoblot. (D) Model for caspase-8 induction by 5-azadC via an immediate stress response that triggers the FOXO3-ATM-CREB signaling pathway.

does not exclude the possibility that shutdown of caspase-8 expression in high-grade neuroblastoma results at least in part from methylation of regulatory sequences. Gradual DNA demethylation by long-term 5-azadC exposure may further increase caspase-8 levels, but our data clearly demonstrate that DNA demethylation is not critical for initial caspase-8 reactivation.

In this study we demonstrate for the first time a functional relationship between FOXO3 and caspase-8 expression in situ and in neuroblastoma cell lines. We provide evidence that FOXO3 activates caspase-8 via the ATM-CREB signaling pathway independent of caspase-8 gene methylation status. The DNA-methylation inhibitor 5-azadC provokes a DNA damage response and induces caspase-8 via FOXO3, suggesting that genotoxic stress increases death-ligand sensitivity via activation of the FOXO3-ATM-CREB–caspase-8 pathway, which might be highly important for combination therapy.

## MATERIALS AND METHODS
### Cell lines and culture conditions
The MYCN-amplified neuroblastoma cell lines STA-NB1, STA-NB3, and STA-NB15 were isolated at the St. Anna Children's Hospital (Vienna, Austria) and are termed NB1, NB3, and NB15, respectively (Narath et al., 2005). SH-EP (kindly provided by N. Gross, Lausanne, Switzerland), IMR-32 (ATCC, Rockville, MD), and Phoenix packaging cells for helper-free production of amphotropic retroviruses were cultured as previously described (Obexer et al., 2007). HEK293T packaging cells for production of lentiviral particles were cultured in DMEM medium (PAA, Pasching, Austria) containing 10% fetal calf serum (Life Technologies, Paisley, United Kingdom), 100 U/ml penicillin, 100 µg/ml streptomycin (PAA), and 2 µM L-glutamine (Lonza, Basel, Switzerland). 5-azadC was pur-

chased from Sigma (Steinheim, Germany) and the ATM kinase inhibitor 2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one (118500) from Calbiochem (La Jolla, CA).

### Plasmids
The retroviral vectors pLIB-MCS2-iresPuro, pLIB-MCS2-iresNeo, pLIB-FOXO3(A3)ERtm–iresNeo, and pLIB-ECFP-FOXO3wt-iresPuro were constructed as previously described (Obexer et al., 2007, 2009). For construction of pLIB-casp8-iresPuro the coding region of caspase-8 was amplified by PCR using the primers GGATCCATG-GACTTCAGCAGAAATCTTTATG and GTCGACTCAATCAGAAGG-GAAGACAAG and PCR product inserted the into the Sall-BamHI sites of the pLIB-MCS2-iresPuro plasmid. The plasmids coding for CREBwt, CREBΔLZ, and the appropriate control were as published (Riccio et al., 2006).

### Retroviral and lentiviral infection
For retrovirus production 6 × 10^{5} Phoenix packaging cells were transfected with 2 µg of retroviral vector and 1 µg of a plasmid coding for vesicular stomatitis virus G protein using 8 µl of Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Lentiviruses were produced by transfecting pLKO.1 vectors expressing FOXO3-specific shRNA or scrambled shRNA (Open Biosystems, Thermo Scientific, Huntsville, AL) and the packaging plasmid pCMV 8.91 into HEK293T cells. After 48 h, the virus-containing supernatants were filtered through 0.2-µm syringe filters (Sarstedt, Nuembrecht, Germany). The pLIB-FOXO3(A3)ERtm–iresNeo or control vector retroviral supernatants were used to infect NB1, NB15, and IMR-32 cells (NB1/FOXO3, NB15/FOXO3, and IMR-32/FOXO3). NB15/FOXO3 cells were infected with pLIB-casp8-iresPuro (NB15/FOXO3-casp8).

## DNA isolation and methylation analysis

Genomic DNA from cell lines was isolated using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). Bisulfite modification was performed using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA) according to the manufacturer's instructions. MethyLight analysis was done as described previously (Eads et al., 2000). Primers and probes for caspase-8 were determined with the assistance of the computer program Primer Express (Applied Biosystems, Foster City, CA) to produce a 116–base pair PCR amplicon (nucleotide positions 834–949 as defined by GenBank accession number AF210257). The primer specificity was verified on SssI-treated, bisulfite-modified DNA (100% hypermethylated) or on whole-genome amplified, bisulfite-modified DNA, respectively (100% hypomethylated), and on unmodified genomic DNA (unpublished data). The preparation of the hypermethylated and hypomethylated control DNA was described (Weisenberger et al., 2005). Primer sequences were 5′-ACTTAACCGTCGAC-CCCAAA-3′ (forward), 5′-TCGTTTCGAATGTAGATATACGGTTTT-3′ (reverse), and 5′FAM-TCTAAACTCTCCAAACGAATCCACA-3′BHQ1 (probe). The amplicon is located in the 5′-flanking CpG-rich region, which was analyzed by Teitz et al. (2000). This region is not a classic CpG island (C + G, 42%; CpG:GpC ratio, 0.26; Banelli et al., 2002). Three of the five CG sites analyzed by Teitz et al. are included in the amplicon of our MethyLight reaction, which covers six CG sites.

## Immunohistochemistry

Paraffin-embedded tissue sections of 27 neuroblastoma patients treated at the Department of Pediatrics, University Hospital Innsbruck (Innsbruck, Austria), from 1989 to 2008 were used for in situ analysis of caspase-8 and FOXO3 expression. The study was performed in compliance with and approval by the institutional review board, and written informed consent for the scientific use of paraffin-embedded material was obtained. All samples were made anonymous before analysis. Sections were stained using the streptavidin-biotin-peroxidase method (Vectastain, Vector Laboratories, Burlingame, CA) with antibodies specific for FOXO3 and caspase-8. Briefly, 3-μm-thick sections were deparaffinated and rehydrated. Slides were then boiled in a microwave oven in a plastic jar filled with citrate acid buffer solution, pH 6, for 15 min. The endogenous peroxidase activity was blocked using a hydrogen peroxide solution. Tissue sections were then incubated with normal bovine serum (10%) for 20 min to reduce nonspecific background staining. Primary antibodies specific for FOXO3 (Upstate Biotechnology, Temecula CA) and caspase-8 (BD-Pharmingen, Heidelberg, Germany) were applied to tissue sections for 60 min at room temperature, followed by incubation with a polyvalent antibody for 30 min and then an avidin-biotin-complex reagent (Vectastain) for 30 min at room temperature. In negative controls, sections were not incubated with primary antibody. Diaminobenzidine tetrahydrochloride was used for staining. All slides were counterstained with hematoxylin, dehydrated, and mounted. Immunostaining results were evaluated, graded, and scored by extent (0, none; 1, 1–25%; 2, 26–50%; 3, 51–75%; and 4, 76–100% of the ductal cells) and intensity (0, negative; 1, weak; 2, moderate; and 3, intense). An immunohistochemical score was calculated for each case in which the percentage of positive rating was multiplied by the intensity rating. All specimens were centrally evaluated, and their diagnosis was confirmed by the National Registry of Neuroblastoma (Vienna, Austria). To avoid artifacts, only well-Formalin-fixed tissue specimens with expression of neuroblastoma markers were studied.

## Immunoblotting

Immunoblot analysis was performed as described previously (Obexer et al., 2007) with the exception of the used lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/NaOH, 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 10% glycerol). For the detection of phospho-ATM S1981 a 4–20% gradient gel (Invitrogen) was used. The membranes were incubated with primary antibodies directed against human FOXO3 (Cell Signaling Technology, Beverly, MA), caspase-8, Bim (BD-Pharmingen), CREB-pSer133 (Upstate Biotechnology), CREB, phospho- ATM S1981 (Cell Signaling Technology), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Novus Biologicals, Littleton, CO), and α-tubulin (Calbiochem). Afterward the membranes were washed and incubated with horseradish peroxidase–conjugated anti-mouse or anti-rabbit secondary antibodies (GE Healthcare, Little Chalfont, United Kingdom). The blots were developed as described previously (Obexer et al., 2007). Densitometric analysis was done using LabWorks software (UVP BioImaging Systems, Upland, CA).

## Live-cell fluorescence microscopy

For live-cell analyses $3 \times 10^5$ SH-EP/ECFP-FOXO3 cells were grown on LabTek Chamber Slides (Nalge Nunc International, Rochester, NY) coated with 0.1 mg/ml collagen and treated with 20 μM 5-azadC, 0.25 μg/ml doxorubicin, or 20 μg/ml etoposide. Images were collected with an Axiovert 200M microscope with a 63× oil objective (Carl Zeiss, Jena, Germany).

## Transient transfection

For transient transfection experiments 2 μg of pLP-CMV-Myc-tag, pLP-CMV-Myc-CREBwt, and pLP-CMV-Myc-CREBΔLZ were transfected into NB15/FOXO3-BclxL cells using 6 μl of Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen). Cells from each transfection were split into two six-well plates and cultured with or without 100 nM 4OHT for 40 h.

## Immunofluorescence

Approximately $1 \times 10^4$ NB15 cells were seeded onto LabTek Chamber Slides, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. The cells were incubated with monoclonal phospho-ATM S1981 (Cell Signaling), antibody diluted in phosphate-buffered saline/2% bovine serum albumin/1% fetal calf serum for 60 min, washed, and detected with an Alexa 488–conjugated secondary antibody (Invitrogen). Analysis was performed in an Axiovert 200M microscope using AxioVision software (Zeiss).

## Statistical analysis

The nonparametric Wilcoxon signed rank test was used to assess association between FOXO3 subcellular localization and caspase-8 expression in patient biopsies. Statistical significance of differences between controls and treated cells in neuroblastoma cell culture experiments were calculated using unpaired t test. All statistical analyses were performed using Prism 4.0 software (GraphPad Software, La Jolla, CA).

## Quantitative RT-PCR

Total RNA was isolated from cells by using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 2 μg of RNA using SuperScriptII (Invitrogen) and oligo dT primer (Fermentas, St. Leon-Rot, Germany). To quantify caspase-8 mRNA levels, we designed RT-PCR assays, using the following primers: for caspase-8, forward, 5′-CTGGATGATGACATGAACCT-GCTG-3′, and reverse, 5′-GCTCTTGTTGATTTGGGCACAGAC-3′; and for GAPDH, forward 5′-TGTTCGTCATGGGTGTGAACC-3′, and reverse, 5′-GCAGTGATGGCATGGACTGTG-3′ (MWG Biotech, Ebersberg, Germany). Amplification efficiency was determined by

serial log$_2$ dilutions. All reactions were performed with iQ SYBR Green Supermix (BioRad Laboratories, Munich, Germany). RT-PCR was performed as described previously (Obexer *et al.*, 2007), using the annealing temperature 65°C for caspase-8.

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