FOXO3a Is a Major Target of Inactivation by PI3K/AKT Signaling in Aggressive Neuroblastoma

Evan E. Santo, Peter Stroeken, Peter V. Sluis, Jan Koster, Rogier Versteeg, and Ellen M. Westerhout

Abstract

Neuroblastoma is a pediatric tumor of the peripheral sympathetic nervous system with a highly variable prognosis. Activation of the phosphoinositide 3-kinase (PI3K)/AKT pathway in neuroblastoma is correlated with poor patient prognosis, but the precise downstream effectors mediating this effect have not been determined. Here we identify the forkhead transcription factor FOXO3a as a key target of the PI3K/AKT pathway in neuroblastoma. FOXO3a expression was elevated in low-stage neuroblastoma tumors and normal embryonal neuroblasts, but reduced in late-stage neuroblastoma. Inactivation of FOXO3a by AKT was essential for neuroblastoma cell survival. Treatment of neuroblastoma cells with the dual PI3K/mTOR inhibitor PI-103 activated FOXO3a and triggered apoptosis. This effect was rescued by FOXO3a silencing. Conversely, apoptosis induced by PI-103 or the AKT inhibitor MK-2206 was potentiated by FOXO3a overexpression. Furthermore, levels of total or phosphorylated FOXO3a correlated closely with apoptotic sensitivity to MK-2206. In clinical specimens, there was an inverse relationship between gene expression signatures regulated by PI3K signaling and FOXO3a transcriptional activity. Moreover, high PI3K activity and low FOXO3a activity were each associated with an extremely poor prognosis. Our work indicates that expression of FOXO3a and its targets offer useful prognostic markers as well as biomarkers for PI3K/AKT inhibitor efficacy in neuroblastoma. Cancer Res; 73(7); 1–10. ©2013 AACR.

Introduction

Neuroblastoma is a pediatric tumor derived from the peripheral sympathetic neural lineage with a highly variable prognosis. Low-stage tumors have an excellent prognosis, but high-stage tumors often are refractory to treatment. The genetic basis underlying the development of this tumor is poorly understood; the most common single gene events being MYCN amplification (~20% of cases) and ALK mutation (~8% of cases), but whole genome sequencing efforts recently identified many more mutated genes (1). Activation of the PI3K/AKT pathway has been implicated in neuroblastoma pathogenesis. Immunohistochemical analysis of neuroblastoma revealed an association between high-stage tumors and activation of AKT; in addition, PI3K/AKT pathway activation could confer chemotherapeutic resistance to neuroblastoma cell lines (2, 3). AKT activity via GSK3β inhibition has also been directly implicated in the postranslational stability of MYCN, which is supportive of cell proliferation in vitro and tumor growth in vivo (4, 5). Other than MYCN stabilization, few mechanisms downstream of PI3K/AKT that are essential for the proliferation and survival of neuroblastoma cells have been identified. The forkhead box-type O (FOXO) subfamily of forkhead domain-containing transcription factors are important downstream effectors of the PI3K/AKT pathway (6). This family consists of 4 members in humans, FOXO1, 3, and 4 and FOXO1/3/4 have been identified as bona fide tumor suppressors when simultaneously knocked out in mice (7). Recently, FOXO3a knockout has been found to expand the tumor spectrum of p33 knockout mice (8). Human alveolar rhabdomyosarcoma have frequent translocations of FOXO1 to PAX3 or PAX7, whereas FOXO3a and FOXO4 are commonly fused to MLL in mixed lineage leukemia, creating oncogenes in both cases (9–12). Recently, heterozygous FOXO3a deletions were described in natural killer (NK) cell neoplasms (13). Functional studies suggest a tumor suppressive role for FOXOs, as FOXO proteins were found to be inactivated by constitutively active PI3K/AKT or RAS/MAPK/ERK signaling (6, 14, 15). Activation of these pathways can directly result in phosphorylation of FOXOs and their subsequent cytoplasmic sequestration and/or degradation via the ubiquitin–proteasome pathway. In neuroblastoma, AKT-mediated phosphorylation of FOXO has previously been correlated with neuroblastoma cell survival in response to growth factor stimulation (16, 17). When FOXO is activated by inhibition of the PI3K/AKT pathway, FOXOs can promote a wide range of effects including cell-cycle arrest, cell differentiation, autophagy, and apoptosis via various mechanisms (18, 19). However, the case for FOXOs as tumor suppressors has become more complicated because of their alternative roles as stress response factors (20). In breast cancer, prolonged FOXO3a activity can cause resistance to the chemotherapeutic
doxorubicin (21). Similar effects were also found in chronic myelogenous leukemia cells (22). All of these findings emphasize that the context within which FOXO3a are activated is crucial to the cellular response attained.

Here, we use an experimental and computational approach that identified FOXO3a as a key downstream player in the PI3K/AKT signaling pathway in neuroblastoma. FOXO3a has an exceptionally high expression in normal embryonal neuroblasts and in neuroblastoma tumors. However, in aggressive neuroblastoma, FOXO3a expression is significantly reduced, suggesting a tumor suppressor role. On the protein level, FOXO3a is phosphorylated and inhibited by AKT signaling in neuroblastoma cell lines. We show that reactivation of FOXO3a after AKT inhibition triggers apoptosis, which is further increased by combined FOXO3a overexpression and AKT inhibition. From microarray profiling of FOXO3a manipulation, we identified a gene expression signature for FOXO3a activation, which predicts good prognosis in neuroblastoma.

Materials and Methods

Cell lines and compounds

All cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS, 20 mmol/L l-glutamine, 10 U/mL penicillin, and 10 μg/mL streptomycin and maintained at 37°C under 5% CO2. Cell line identities were confirmed by short tandem repeat profiling using the PowerPlex16 system and GeneMapper software (Promega). During the month of November 2012, the short tandem repeat profiles were validated by comparison with those from 3 public databases storing the cell lines (Leibnitz-Institut DSMZ, American Type Culture Collection, and the Children’s Oncology Group Cell Culture and Xenograft Repository). PI-103 was purchased from Tocris Bioscience and MK-2206 from Selleckchem. Doxycycline, isopropyl-β-D-thiogalactopyranoside (IPTG), and puromycin were from Sigma. Blasticidin was from Invitrogen.

FACS analysis

Cells were seeded in 6-well plates and each condition was carried out in triplicate. After harvesting and washing cells in PBS + 4 mmol/L EDTA, the cells were fixed in 70% ice-cold ethanol. The cells were then collected and incubated in a hypotonic solution of PBS, dH2O, propidium iodide (PI), and ethanol. The cells were then collected and incubated in a hypotonic solution of PBS, dH2O, propidium iodide (PI), and ethanol. The cells were then collected and incubated in a hypotonic solution of PBS, dH2O, propidium iodide (PI), and ethanol. The cells were then collected and incubated in a hypotonic solution of PBS, dH2O, propidium iodide (PI), and ethanol. The cells were then collected and incubated in a hypotonic solution of PBS, dH2O, propidium iodide (PI), and ethanol. The cells were then collected and incubated in a hypotonic solution of PBS, dH2O, propidium iodide (PI), and ethanol. The cells were then collected and incubated in a hypotonic solution of PBS, dH2O, propidium iodide (PI), and ethanol. The cells were then collected and incubated in a hypotonic solution of PBS, dH2O, propidium iodide (PI), and ethanol. The cells were then collected and incubated in a hypotonic solution of PBS, dH2O, propidium iodide (PI), and ethanol. The cells were then collected and incubated in a hypotonic solution of PBS, dH2O, propidium iodide (PI), and ethanol. The cells were then collected and incubated in a hypotonic solution of PBS, dH2O, propidium iodide (PI), and ethanol. The cells were then collected and incubated in a hypotonic solution of PBS, dH2O, propidium iodide (PI), and ethanol.

Microarray data

The 12 microarray profiles of PI-103 treatment and FOXO3a overexpression can be found at the Gene Expression Omnibus (GEO) database under accession GSE42762.

See Supplementary Data for additional Materials and Methods.

Results

FOXO3a expression is high in normal neuroblasts and low-stage neuroblastoma and reduced in high-stage tumors

As PI3K/AKT signaling is associated with a poor prognosis in neuroblastoma, we investigated downstream targets of this pleiotropic pathway for a role in this tumor. FOXO3a transcription factors are inactivated by AKT signaling. We therefore investigated expression of FOXO family genes (FOXO1, 3, 4, and 6) in a large collection of microarray data, including our series of 88 neuroblastoma of all stages (NB88) with the R2 MegaSample application in the bioinformatic program R2 (http://r2.amc.nl). Most strikingly, FOXO3a mRNA expression was found to be very high in neuroblastoma relative to all other tumor types and to normal tissues and this was not the case for FOXO1, 4, and 6 (Fig. 1A; data not shown). We also analyzed FOXO3a expression in a panel of 24 neuroblastoma cell lines and a series of cell lines from other tumors. FOXO3a expression was highest in the neuroblastoma cell lines, confirming the neuroblastoma-specific expression pattern of FOXO3a (Fig. 1B). We then analyzed whether FOXO3a is also highly expressed in the normal tissue from which neuroblastoma can arise. We therefore checked a dataset with 3 microdissected samples of normal human fetal adrenal neuroblasts that were profiled with neuroblastoma tumors of different stages (23). FOXO3a expression levels were nearly 2 times higher in normal human fetal adrenal neuroblasts and stage I tumors than in high-stage neuroblastoma tumors (Fig. 1C). The high expression in normal neuroblasts and relatively reduced expression in high-stage tumors indicates that FOXO3a is lineage-specific expressed in neuroblasts of the sympathetic nervous system and downregulated in high-stage tumors. We therefore inquired whether FOXO3a expression carries a prognostic value among stage III and IV tumors. Indeed, Kaplan-Meier survival analysis of the 53 stage III and IV tumors in our NB88 series showed that low FOXO3a expression was significantly associated with a poor prognosis (P = 6.8e-03; Fig. 1D).

PI3K/AKT signaling regulates FOXO3a phosphorylation, nuclear localization, and transcriptional activation in neuroblastoma

We first asked whether the extremely high FOXO3a expression in neuroblastoma was functionally related to the PI3K/AKT signaling route. We investigated regulation of FOXO3a by AKT in 2 neuroblastoma cell lines, one with and one without MYCN amplification [SKNBe(2c) and SY5Y, respectively]. Both cell lines were treated with the potent dual-specificity inhibitor of phosphoinositide 3-kinase (PI3K) and mTOR kinases PI-103 (24). PI-103 induced G1 cell-cycle arrest and apoptosis in both lines within 24 hours as shown by PI staining and fluorescence-activated cell sorting (FACS) analysis (Fig. 2A). Western blot analysis showed robust steady state phosphorylation of AKT and FOXO3a in both cell lines and complete dephosphorylation of these proteins within 2 hours following PI-103 treatment (Fig. 2B). This dephosphorylation coincided with a depletion of the cytoplasmic FOXO3a pool and its accumulation in the nucleus (Fig. 2C). To investigate whether FOXO3a was transcriptionally active following PI-103 treatment, we cloned synthetic FOXO reporters (FireFOX reporters) containing either 6 copies of a wild-type FOXO DNA-binding element (DBR; TTGTTTAC) or a mutated element (TCGTGTAC). Using these vectors, we observed a 2- to 3-fold increase in endogenous FOXO transcriptional activity at 24 hours post-PI-103 treatment in SY5Y and SKNBe(2c) (Fig. 2D). Therefore, the
apoptosis induced by PI-103 treatment in these 2 cell lines coincided with FOXO3a re-activation.

Inducible silencing of endogenous FOXO3a expression rescues SKNBE(2c) cells from PI-103–induced apoptosis

We next asked whether the apoptosis induced by PI103 was mediated by the reactivation of FOXO3a. We constructed a stable SKNBE(2c) line containing an IPTG-inducible FOXO3a-specific short hairpin RNA (shRNA). Addition of 0.5 mmol/L IPTG to this line induces efficient FOXO3a knockdown within 48 hours and no change in AKT activity as indicated by phosphorylation of the AKT substrate PRAS40 (ref. 25; Fig. 3A). After 48 hours of IPTG treatment, 2.5 and 5 μmol/L PI-103 was added to the cells for an additional 24 hours. The IPTG-induced silencing of FOXO3a by shRNA rescued the cells from PI-103–induced apoptosis, as evident from a reduction of the sub-G1 fraction by 76% to 82% (Fig. 3B and Supplementary Fig. S1A). In addition, the IPTG-treated cells showed an increase in G1 arrest after PI-103 treatment of 6.2% to 6.9% of the total population when compared with the −IPTG PI-103–treated cells. This suggests that the FOXO3a knockdown cells arrested in G1 instead of dying after PI3K/AKT inhibition; indicating that FOXO3a primarily drives apoptosis at the expense of G1 arrest. These data show that FOXO3a is a key mediator of apoptosis following PI3K/AKT pathway inhibition.

Inducible overexpression of FOXO3a increases PI-103–induced apoptosis in SY5Y

To further corroborate that FOXO3a activity drives apoptosis in neuroblastoma cells, we also tested whether increased FOXO3a expression enhances apoptosis. To probe this, we constructed a polyclonal SY5Y cell line containing an inducible hemagglutinin (HA)-tagged FOXO3a expression construct. Doxycycline addition induced FOXO3a overexpression, which became maximal between 24 and 48 hours (Fig. 4A). Western blot analysis showed a strong increase of total FOXO3a protein after 24 hours of doxycycline treatment, reflecting the contribution of the transgene to the endogenous pool (Fig. 4B,
compare lanes 1 and 3). A subsequent 6-hour treatment with 1 μmol/L PI-103 blocked phosphorylation of AKT and FOXO3a in these cells, regardless of doxycycline treatment. PI staining and FACS analysis of this experiment revealed an increase in the sub-G1 from 15.8% in the −dox condition to 34.3% in the +dox, indicating strongly increased apoptosis (Fig. 4C). Notably, a strong decrease in G1 arrest was observed in the +dox/+PI-103 condition, again showing FOXO3a drives apoptosis and not cell-cycle arrest in neuroblastoma. To quantify the overall decrease in viability, MTT assays were conducted at 24- and 48-hour time points. Increasing amounts of PI-103 or the highly specific pan-Akt inhibitor MK-2206 (Akti) were used in conjunction with FOXO3a overexpression by doxycycline addition (26, 27). Induction of FOXO3a expression resulted in a consistent decrease in cell viability relative to the −dox condition with all IC50 differences highly significant (P < 0.01; Fig. 4D). We conclude that high FOXO3a expression strongly potentiates the apoptosis induced in neuroblastoma cells by PI3K/AKT inhibitors.

**Induction of apoptosis by AKT inhibition positively correlates with total and phosphorylated FOXO3a protein levels across cell lines**

As FOXO3a expression was identified as a major determinant of neuroblastoma apoptosis in response to PI3K/AKT inhibition, we evaluated this relationship across a neuroblastoma cell line panel. From our Affymetrix array data, we chose a panel of 11 cell lines that exhibited a range of FOXO3a mRNA expression levels and we tested their response to a 48-hour MK-2206 (Akti) treatment. Because PI-103 is a dual inhibitor of PI3Ks and mTOR, we used MK-2206 (Akti) to more specifically probe the contribution of the AKT/FOXO3a axis and eliminate the influence of direct PI3K/mTOR inhibition. The 8-μmol/L MK-2206 treatments resulted in effective pathway inhibition in all cell lines as shown by Western blot analysis for phosphorylated FOXO3a and PRAS40 as well as AKT serine 473 phosphorylation (Fig. 5). Apoptosis was quantified by cell-cycle FACS analysis and was found to correlate with total FOXO3a expression induced in neuroblastoma cells by PI3K/AKT inhibitors.
PI3K and FOXO3a gene expression signatures are highly prognostic in neuroblastoma

The cell line experiments showed that FOXO3a is a major mediator of the apoptotic response of neuroblastoma cells upon PI3K/akt inhibition. This finding led us to analyze whether PI3K/akt signaling and FOXO3a transcriptional activity are identifiable in neuroblastoma tumors. We therefore used the mRNA profiles of the NB88 neuroblastoma series and analyzed which samples showed PI3K/akt activity and FOXO3a transcriptional activation. To that end, we generated signatures of the downstream genes regulated by PI3K/akt or FOXO3a activity in SY5Y cells. For consistency with subsequent experiments (see later), we used the SY5Y-TetR-FOXO3a cells without inducing the FOXO3a transgene. We first identified the genes regulated by PI3K/akt signaling in this line by treating the cells with 1 μmol/L PI-103 or vehicle for 6 hours and profiling mRNA expression on Affymetrix U133 plus2.0 arrays. This experiment was carried out in triplicate across different days. The array data identified 464 significantly regulated genes (P < 0.01), which represented a signature for the PI3K/akt pathway (Supplementary Table S1A).

To probe the activity of the PI3K/akt pathway in the series of 88 neuroblastoma tumors, we conducted a 2-group k-means clustering of the 464 genes. This cluster analysis yielded a clear separation in the NB88 dataset into 2 groups, one with 49 tumors and the other with 39 tumors (Fig. 6A). Kaplan–Meier survival analysis of these 2 groups revealed that PI3K/akt regulated genes have a very strong prognostic significance in neuroblastoma. High levels of PI3K/akt signaling are correlated with poor outcome (Fig. 6B; P = 1.3e-13). Further support for this interpretation was uncovered by checking the expression of PTEN within these 2 patient groups. PTEN was more highly expressed within the PI3K inactive group, which is consistent with its well-established role as a suppressor of the pathway (Fig. 6C; P = 6.9e-06). Within stage III and IV tumors,
the PI3K/AKT signature is also highly prognostic (Supplementary Fig. S3A; \( P = 1.0 \times 10^{-6} \)). We subsequently aimed to identify a signature for FOXO3a-regulated genes. We used the same SY5Y-TetR-FOXO3a cells, but now we extended the analyses with doxycycline-induced FOXO3a expression, with or without PI-103 treatment. Comparison of expression profiles of cells with and without doxycycline-induced FOXO3a expression, but without PI3K/AKT inhibition by PI-103, yielded only 33 very weakly regulated genes (data not shown). In contrast, the comparison of cells with or without PI3K/AKT inhibition by PI-103, but with FOXO3a transgene expression, identified 1,060 regulated genes. These genes include genes regulated by FOXO3a, but also genes regulated by PI3K/AKT signaling. The genes regulated by FOXO3a can be found by removing those also identified in the 464 gene PI3K/AKT signature, leaving a signature of 809 FOXO3a-regulated genes (Supplementary Table S1B).

When a 2-group \( k \)-means clustering was conducted with these 809 genes in the NB88 dataset, groups of 52 and 36 tumors were generated (Supplementary Fig. S3B). As with the PI3K/AKT signature, this division was also highly prognostic (\( P = 9.7 \times 10^{-15} \); Supplementary Fig. S3C).

Transcription factor–binding site analysis identifies putative direct FOXO3a target genes

To more stringently model FOXO3a regulatory activity and identify likely direct target genes of FOXO3a, we conducted a search for the FOXO DBE within the promoter regions of the 809 FOXO3a-regulated genes. As a prerequisite to this, we first confirmed that FOXO3a was indeed activating the DBE element following our brief 6-hour treatment of the cells with PI-103. For this, a FireFOX reporter assay was done in this line under conditions identical to those of the mRNA profiling (Supplementary Fig. S4A). We observed negligible transcriptional activity of FOXO3a with PI-103 treatment alone and a 3-fold increase in activity in the + dox/+ PI-103 condition relative to

Figure 4. FOXO3a overexpression sensitizes SY5Y cells to PI3K/AKT pathway inhibition. A, Western blot analysis of exogenous FOXO3a after addition of 0.1 \( \mu \)g/mL doxycycline (Dox). The Western blot analysis was probed with an anti-HA tag antibody; \( \alpha \)-tubulin was the loading control. B, Western blot analysis of doxycycline and PI-103–treated SY5Y-TetR-FOXO3a cells with the indicated antibodies. A total of 0.1 \( \mu \)g/mL doxycycline was applied for 24 hours before the incubation with 1 \( \mu \)L/P1-103 for 6 hours. C, cell nuclei from B were harvested and stained with PI for cell-cycle analysis by FACS. All combinations were carried out in triplicate. D, SY5Y-TetR-FOXO3a cells were seeded at a density of 10,000 cells per well in 96-well plates. MTT assays conducted at 24 and 48 hours to measure viability under ±0.1 \( \mu \)g/mL doxycycline when treated with a gradient of either PI-103 or MK-2206. Each concentration was carried out in triplicate.
to the –dox/–PI-103 control. Unexpectedly, we also observed a 1.5-fold increase in reporter activity with FOXO3a overexpression alone (+ dox/–PI-103), which did not fit with the lack of FOXO3a target regulation under this condition (33 weakly regulated genes when FOXO3a was overexpressed without PI-103 treatment; data not shown). Cell fractionation revealed that overexpressed FOXO3a was indeed entering the nucleus without PI-103 treatment (data not shown). Despite this, FOXO3a was apparently unable to activate its targets but still able to activate a high-copy reporter. This implies that the larger context of PI3K/AKT pathway inhibition may be essential for FOXO3a to regulate target genes in this cell line.

Having confirmed that FOXO3a is transcriptionally activating DBE elements under these conditions, we classified the FOXO3a-regulated genes into activated (n = 419) and repressed (n = 284) groups. Of the 419 promoters activated by FOXO3a, 167 contained the DBE element resulting in a highly significant FOXO motif enrichment (P = 8.7e-06; Fig. 6D). Strikingly, no DBE element enrichment was found within 284 repressed promoters (P = 0.99). This analysis is in agreement with a role for FOXO3a as an activator of gene transcription and suggests that repressed genes are indirectly regulated by FOXO3a. Of the 464 genes regulated by PI3K/AKT, the promoters of 407 upregulated genes only showed a marginally significant enrichment for the FOXO motif (P = 0.05), whereas promoters of downregulated genes were not enriched (P = 0.69).

As a further validation of our FOXO3a activation system, we investigated the regulation of 6 established FOXO target genes; p27, BIM, MXI1, PIK3CA, RICTOR, and INSR (refs. 28–33; Supplementary Fig. S4B). These genes were all strongly upregulated with the combination of FOXO3a overexpression and PI-103 treatment. Taken together with the reporter assay and FOXO-binding site analysis, these results show that this system is appropriate for the discovery of FOXO3a target genes.

PI3K/AKT and FOXO3a signatures give overlapping classifications of neuroblastoma tumors

Using the 167 genes that were activated by FOXO3a and contained the FOXO motif in their promoters, we conducted a k-means clustering of the NB88 dataset (Supplementary Table S1C and Fig. 6E). This identified a group of 49 tumors with higher expression of most FOXO3a target genes; p27, BIM, MXI1, PIK3CA, RICTOR, and INSR (refs. 28–33; Supplementary Fig. S4B). These genes were all strongly upregulated with the combination of FOXO3a overexpression and PI-103 treatment. Taken together with the reporter assay and FOXO-binding site analysis, these results show that this system is appropriate for the discovery of FOXO3a target genes.
within stage III and IV (Fig. 6G; \( P = 4.2e-05 \)). The interpretation of this clustering was further confirmed by the individual Kaplan–Meier survival curves for the known FOXO target genes p27, BIM, MXI1, and RICTOR (Supplementary Fig. S5A). High expression of these genes was associated with a good prognosis. Being that 3 of these genes are well-known tumor suppressors, this is an indication that FOXO3a may have a bias toward the activation of other such genes in neuroblastoma.

**Discussion**

In this work, we have shown that FOXO3a is a highly expressed and important mediator of cell death in neuroblastoma within the context of PI3K/AKT pathway inactivation. Silencing of FOXO3a expression was sufficient to rescue SkNBE(2c) cells from the apoptosis induced by the PI3K inhibitor PI-103 and overexpression potentiated this apoptotic effect in SY5Y cells. Across a panel of neuroblastoma cell lines, FOXO3a protein levels positively correlated with the induction of apoptosis by the AKT inhibitor MK-2206. Microarray profiling of the SY5Y experiment elucidated gene expression signatures for both the PI3K/AKT pathway and FOXO3a transcriptional activity, which were both powerfully prognostic and inversely correlated within our NB88 tumor dataset.

It has been reported in other tumors that PI3K/AKT pathway inhibition can lead to a wide spectrum of direct effects including cell-cycle arrest, induction of autophagy, sensitization to chemotherapeutics, inhibition of metastasis as well as cell differentiation and death (18, 34). As a result, activation of PI3K/AKT signaling in tumors is largely regarded as an oncogenic event with the implied corollary that FOXO inactivation would be a poor prognosis for these tumors. This is even more so in tumors that maintain the highest levels of PI3K/AKT signaling and are considered to be least responsive to therapies that target this pathway. As a consequence, the deregulation of FOXO3a expression and function may be an important determinant of patient outcomes and the success of therapy. The FOXO3a–PI3K/AKT pathway signature was found to have improved clinical outcome in patients treated with chemotherapy (Fig. 6G; \( P = 4.2e-05 \)).

*Figure 6. FOXO3a activation predicts good prognosis in neuroblastoma. A, a 2 group k-means clustering of genes regulated by PI-103 in the SY5Y-TetR-FOXO3a line in the absence of doxycycline (464 genes). Highest → lowest expression is colored red → blue. B, the Kaplan–Meier survival curves for the 2 PI3K k-means groups. C, the expression of PTEN within the 2 PI3K k-means groups. D, analysis of the promoter regions of genes regulated by PI-103 treatment alone and PI-103 treatment in combination with FOXO3a overexpression. The FOXO DBE motif was searched within the promoters of the regulated genes and in the promoters of random sets of genes that were not regulated. Promoters for some genes were not identifiable due to poor annotation of transcriptional start sites. E, a 2 group k-means clustering of FOXO3a activated genes that contain the FOXO motif within their promoter regions (167 genes). Green boxes denote tumors classified as PI3K inactive and red boxes PI3K active from the PI3K clustering. G, the Kaplan–Meier survival curves for the 2 FOXO3a k-means groups. G, the Kaplan–Meier survival curves for the 2 FOXO3a k-means groups in stage III and IV tumors.*
connection with the pathway. The notion that FOXO is a tumor suppressor finds robust support in mouse models where FOXO genes have been deleted (7). Moreover, numerous overexpression experiments of constitutively nuclear-localized FOXO constructs resulted in the apoptosis and cell-cycle arrest of various human tumor cell lines or blockade of transformation (30, 35, 36). However, these seemingly straightforward results are complicated by the increasing number of pathways and posttranslational modifications that regulate FOXO activity and in turn the myriad of outcomes FOXO activity can influence (19, 20). Recently, it has even been described that FOXO3a can promote metastasis downstream of PI3K/AKT inhibition in collusion with the WNT/β-catenin pathway in colon cancer (37). All of these findings highlight that the ultimate outcomes of FOXO transcriptional activation or inactivation are greatly dependent on the context within which they occur. Here, we have established that within the context of PI3K/AKT signaling in neuroblastoma FOXO3a is indeed a tumor suppressor.

Our findings may have clinical implications for neuroblastoma therapy and potentially many other tumor models where PI3K/AKT inhibitors are being considered for use. From our gene expression signature for FOXO3a activation and functional work, we conclude that the administration of PI3K/AKT inhibitors may induce cell death via re-activation of FOXO3a. In addition, these drugs may cause the restoration of a gene expression program within neuroblastomas associated with very good prognosis and response to current therapy. This makes a case for the use of these drugs in combination with current therapies as they are predicted to synergize with them. Support for this hypothesis has come from in vitro and in vivo studies using PI-103 and MK-2206 in neuroblastoma (38, 39). More directly, overexpression of constitutively active FOXO3a has also yielded chemosensitization effects in neuroblastoma cells (40). PI3K/AKT inhibitors may even be effective as single agents in neuroblastoma due to the sensitivity of these cells to FOXO3a-induced apoptosis, unlike glioma and acute or chronic myelogenous leukemia where these drugs are mainly cytostatic and the primary effect is a G1/2 block (41–43). Another possibility is FOXO-induced terminal neuronal differentiation as recently shown in SY5Y cells (44). Interestingly, the rescue of SKNBE(2c) from PI-103 showed an increased G1 arrest in the FOXO3a knockout background. This suggests that G1 arrest may be the default outcome of PI3K/AKT inhibition if FOXO is not abundantly present to drive apoptosis. This is further supported by the FOXO3a overexpression in SY5Y where despite increased p27 transcript levels following PI-103 treatment there was a decrease in G1 arrest as apoptosis increased. It is also notable that the SKNBE(2c) line used in this study is from a relapse bone marrow metastasis and is MYCN-amplified, radiation and multi-drug resistant and contains inactive p53 (ref. 45). Despite these properties, this line is still responsive to PI3K/AKT inhibition and FOXO3a activation. To our knowledge, this is also the first report to suggest that FOXO3a expression levels may be a biomarker for the efficacy of PI3K/AKT inhibitors. Further research will be needed to see if this finding can be extended to other systems and if the other FOXO family members may also have this correlation. Finally, the finding that FOXO3a may function as an important tumor suppressor in neuroblastoma will lead us to examine many other pathways that are known to regulate it. Previously, we identified oncogenic activation of FOXR1 in neuroblastoma as another potential inhibitor of FOXO function (46). In conjunction with the current work, this finding suggests a broader role for forkhead biology in the pathogenesis of neuroblastoma. This perspective may uncover additional prospects for targeted pharmacologic intervention.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: E.E. Santo, R. Versteeg
Development of methodology: E.E. Santo
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