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The Forkhead Transcription Factor FOXO3a Increases Phosphoinositide-3 Kinase/Akt Activity in Drug-Resistant Leukemic Cells through Induction of PIK3CA Expression▽

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The phosphoinositide-3 kinase (PI3K)/Akt signal pathway plays a key role in the tumorigenesis of many cancers and in the subsequent development of drug resistance. Using the K562 chronic myelogenous leukemia (CML) cell line and the doxorubicin-resistant derivative lines KD30 and KD225 as models, we observed that enhanced PI3K/Akt activity and the acquisition of chemoresistance correlated unexpectedly with the increased expression and nuclear accumulation of FOXO3a. Moreover, we found that the induction of FOXO3a activity in naïve K562 cells was sufficient to enhance PI3K/Akt activity and to confer resistance to the cytotoxic effects of doxorubicin. Conversely, the knockdown of endogenous FOXO3a expression reduced PI3K/Akt activity and sensitized these cells to doxorubicin. Further chromatin immunoprecipitation and promoter mutation analyses demonstrated that FOXO3a regulates the expression of the PI3K catalytic subunit p110α through the activation of a promoter region proximal to a novel untranslated exon upstream from the reported transcription start site of the p110α gene *PIK3CA*. As was the case for FOXO3a, the expression or knockdown of p110α was sufficient to amplify or reduce PI3K/Akt activity, respectively. Thus, our results suggest that the chronic activation of FOXO3a by doxorubicin in CML cells can enhance survival through a feedback mechanism that involves enhanced p110α expression and hyperactivation of the PI3K/Akt pathway.

Chemotherapy is widely used for the treatment of leukemia and other advanced or metastatic cancers. However, its efficacy is often hampered by the development of intrinsic or acquired multidrug resistance (MDR), characterized by simultaneous cross-resistance to anticancer drugs that differ in their chemical structures, modes of action, and cellular targets (35). An understanding of the mechanisms of MDR is important for the development of more effective therapies. At the cellular level, three general mechanisms confer MDR in cancer cells, including decreased hydrophilic drug uptake, increased hydrophobic drug efflux, and enhanced cell survival signals or mechanisms. The first one involves a decrease in the expression or activity of transporters that regulate the uptake of hydrophilic chemotherapeutic drugs, such as folate antagonists, nucleoside analogues, and cisplatin. The second mechanism entails an upregulation of transporters, resulting in an increased energy-dependent efflux of a wide variety of hydrophobic chemotherapeutic agents. Finally, cancer cells often counteract the cytotoxic effects of therapeutic agents by amplifying the activity of proliferation and survival signal pathways, by increasing DNA damage repair, or by altering drug metabolism.

Phosphoinositide-3 kinases (PI3Ks) are a family of lipid kinases that serve as mediators of signals generated by many different activated growth factor receptors and adhesion molecules. The class IA PI3Ks are heterodimers composed of a p110 catalytic subunit and a p85 regulatory subunit (25). When activated by growth factors, the p85 subunit recruits and activates the p110 catalytic subunits at the plasma membrane, generating the phosphatidylinositol (3,4,5)-P_3, which leads to the recruitment and activation of 3′-phosphoinositide-dependent kinase (PDK), Akt (PKB) serine/threonine kinase, and G proteins (e.g., Rac-GTPases). These intermediate molecules in turn regulate the activity of downstream effectors including glycogen synthase kinase 3β (GSK-3β), mammalian target of rapamycin (mTOR), p70S6 kinase, endothelial nitric oxide synthase (eNOS), the FOXO (forkhead box O [forkhead members of the O subclass]) family of transcription factors, and several proapoptotic proteins such as Bad, Bim, and caspase 9 (45).

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The PI3K signal transduction pathway has emerged as a critical regulator of cell proliferation, differentiation, and apoptosis. Perturbation in the PI3K signaling pathway underpins many prevalent diseases ranging from cardiac and neurological disorders to autoimmune/inflammatory conditions, cancer, and chemoresistance (15, 30, 42–44). Hyperactivation of the PI3K pathway has been reported in a large proportion of human tumors of different tissue origins and is frequently a consequence of an enhanced expression of genes that encode class I PI3K subunits (e.g., p110α and p110β) or Akt or a result of somatic deletions or mutations of *PTEN* (phosphatase and tensin homologue deleted on chromosome 10), an antagonist of PI3K activity. The mammalian FOXO family of transcription factors, consisting of FOXO1, FOXO3a, and FOXO4, functions downstream of the PI3K signaling pathway and is a direct substrate of the protein kinase Akt (24). FOXO family members interact with a core consensus DNA sequence, GT AAA(C/T)A, to modulate target gene expression. The phosphorylation of FOXOs by Akt results in their nuclear exclusion and prevents the *trans*-activation of target genes [e.g., $p27^{Kip1}$, *p130*(*RB2*), *cyclin D*, *Bim*, *Fas ligand*, and *Bcl-*X_L, etc.] that are important for cell proliferation, apoptosis, and differentiation.

Chronic myelogenous leukemia (CML) is a malignant clonal hematopoietic stem cell disorder characterized by the expression of the Philadelphia chromosome, generated by a reciprocal translocation between the long arms of chromosomes 9 and 22 (33). Philadelphia translocation gives rise to the fusion of a truncated *Bcr* gene to 5′ sequences of the c-*Abl* gene (8), which encodes chimeric Bcr-Abl proteins with constitutively active tyrosine kinase activity (33). The PI3K/Akt signaling pathway is one of the downstream signaling cascades hyperactivated by the constitutively active Bcr-Abl kinase and has been demonstrated to be essential for the survival functions of Bcr-Abl in CML. The Bcr-Abl tyrosine kinase was originally shown to activate PI3K by a mechanism that requires the binding of Bcr-Abl to p85α and the recruitment of p110α to the regulatory subunit of PI3K (36). Subsequently, other potential routes of PI3K activation, including those through Grb2-Gab2, the adapter proteins Crkl and c-Cbl, and oncogenes such as Src, Ras, and insulin receptor substrate 1, have also been characterized (17, 18). Recently, it has also been shown that Bcr-Abl can also promote PI3K activity through inducing autocrine insulin-like growth factor 1 signaling (23).

Here, we used naïve and multidrug-resistant K562 CML cell lines to study the molecular mechanism responsible for the development of chemotherapy resistance (46). We found that FOXO3a plays an important role in sensing cellular stress induced by doxorubicin, an observation in agreement with other studies implicating this transcription factor in effecting cell cycle arrest and apoptosis in response to cytotoxic stress (6, 7, 9–12, 21, 24, 37, 38). However, we now report that the chronic induction and nuclear accumulation of FOXO3a can also lead to the hyperactivation of the PI3K/Akt pathway, which in turn provides a survival advantage and contributes to the development of multidrug resistance.

MATERIALS AND METHODS

Cell lines, culture, and treatments. The naïve K562 and the doxorubicin-resistant derivative KD30 lines were described previously (46), and KD225 was a kind gift from Selina Raguz and Ernesto Yagüe (Medical Research Council Clinical Sciences Centre, Hammersmith, United Kingdom). KD30 cells were generated following a one-step exposure of K562 cells to 30 nM doxorubicin for 2 weeks, and KD225 cells were generated by multistep exposures with a final doxorubicin tolerance of 225 nM. All cells were cultured in RPMI medium (Sigma, Poole, United Kingdom) with 10% fetal bovine serum supplemented with 2 mM L-glutamine, penicillin, and streptomycin in 5% CO_2. For drug treatment, exponentially growing cells seeded at 10^6 cells/ml were incubated with 1 mM doxorubicin (Sigma) for the indicated times. The drug concentrations used were previously shown to cause morphological changes indicative of cytotoxic stress (46).

Plasmids, siRNA, and transfections. The FOXO3a expression vectors pLPC-FOXO3a and pLPC-FOXO3a(A3), encoding wild-type and constitutively active FOXO3a, respectively, were previously described (9, 12). The conditional expression vectors pBabe-(myr)Akt:ER and pBabe-FOXO3a(A3):ER, containing the coding region of the myristoylated active form of Akt and the active FOXO3a(A3) mutant fused to the hormone binding domain of the estrogen receptor (ER), respectively, were also described previously (6, 9, 12, 22). The expressed fusion proteins are in an inactive form and become activated in the presence of 4-hydroxytamoxifen (4-OHT) (Sigma, Poole, United Kingdom). For generating stable transfectants, 10 μg of vector DNA was electroporated into 10×10^6 cells (Bio-Rad GenePulser II) in 500 μl conditioned medium at 0.35 V and 950 μF. The transfected cells were selected with 1 μg/ml puromycin (Invivogen) or 100 μg/ml zeocin (Invivogen) for 14 days and maintained in 0.5 μg/ml puromycin or 50 μg/ml zeocin (Invitrogen, Paisley, United Kingdom). Single-cell clones were obtained by serial dilution, and expression was tested by Western blotting. The inducibility of the conditional expression vectors was checked with the addition of 200 nM 4-OHT (Sigma) at 5×10^5 cells/ml for 24 h. Plasmids pSG5-p110α and pSG5-p110αCAAX (18) were kind gifts from Julian Downward, Cancer Research-UK London Research Institute, London, United Kingdom, and expression vector pSG5-p110αCAAX:ER (6) was provided by Paul Coffer, University Medical Centre, Utrecht, The Netherlands. In transient transfection studies, cells were collected at 72 h after transfection. For small interfering RNA (siRNA)-mediated silencing experiments, 1×10^6 K562 cells were collected and resuspended in 100 μl of Nucleofect solution V (Amaxa GmbH, Cologne, Germany) according to the manufacturer's protocol. A total of 50 nM *PIK3CA*, *FOXO3a*, or nonspecific Smartpool siRNA (Dharmacon, Lafayette, CO) was added to the cells and nucleofected with program T16 (Amaxa).

Preparation of nuclear and cytoplasmic extracts. Cells were lysed on ice for 20 min in cytosolic buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM dithiothreitol), after which NP-40 was added to a final concentration of 1%, and the cells were then vortexed for 10 s. Nuclei were sedimented by 30 s of centrifugation at 13,000 × *g*, and the supernatant containing the cytoplasmic fraction was snap-frozen. The pellet containing the nuclei was resuspended in cytosolic buffer with 400 mM NaCl–1% NP-40 and rotated at maximum speed on a wheel at 4°C for 15 min. The samples were then centrifuged at 4°C for 5 min at 13,000 × *g*. The supernatant containing the nuclear fraction was snap-frozen and kept at −70°C.

Western blotting and antibodies. Western blotting was performed on whole-cell extracts prepared by lysing cells with a two-times-packed cell volume of NP-40 lysis buffer (1% Nonidet P-40, 100 mM NaCl, 20 mM Tris-HCl [pH 7.4], 10 mM NaF, 1 mM sodium orthovanadate, and protease inhibitors [Complete protease inhibitor cocktail; Roche, Lewes, United Kingdom]). Protein concentration was determined by use of a Bio-Rad (Hemel Hempstead, United Kingdom) Dc protein assay. Total protein lysates (25 μg) were size fractionated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred onto Protran nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Antibodies recognizing FOXO3a phosphorylated at Thr32 and total FOXO3a (06-951) were purchased from Upstate (Dundee, United Kingdom). Mouse monoclonal antibody against FOXO3a (F-1304) was purchased from Sigma Aldrich (Poole, United Kingdom). Antibodies against $p27^{Kip1}$ (C-19), ID1 (C-20), β-actin (C-4), and β-tubulin (D-10) were purchased from Santa Cruz Biotechnology (Autogen Bioclear). Antibodies against phospho-Akt(Ser-473), phospho-Akt(Thr-308), phospho-FOXO3a(Thr-32), phospho-GSK3, total GSK3, phospho-p70S6K, total p70S6K, and total Akt were obtained from Cell Signaling Technologies (Hitchin, United Kingdom). Lamin B1 was obtained from Abcam (Cambridge, United Kingdom). The monoclonal p110α-specific antibody J1A (19) was a kind gift from Anke Kippel, Merck Research Laboratories, Boston, MA. Primary antibodies were detected using horseradish peroxidase-linked anti-mouse, anti-goat, or anti-rabbit conjugates as appropriate (Dako, Ely, United Kingdom) and visualized using the ECL detection system (Amersham Biosciences, United Kingdom).

Luciferase reporter assay. A total of 10×10^6 cells were transfected with 10 μg of reporter vector (in pGL4-basic; Promega, Southampton, United King-

dom), 1 μg of *Renilla* (pRL-TK), which served as an internal transfection control, and variable amounts of expression vector, as indicated. Cells were collected 24 h after transfection and washed twice in phosphate-buffered saline, and firefly and *Renilla* activities were measured using the Dual-Glo luciferase reporter assay system (Promega).

RTQ-PCR. Total RNA was isolated using the RNeasy kit (Qiagen, Crawley, United Kingdom). Total RNA (2 μg) was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) and random primers (Invitrogen), and the resulting first-strand cDNA was used as a template in the real-time quantitative PCR (RTQ-PCR) analysis. All measurements were performed in triplicate. The mRNAs analyzed encoded FOXO3a, PIK3CA (p110α), PIK3R1 (p85α), insulin-like growth receptor (IGFR1), PDPK1 (PDK1), and L19, which served to normalize for variances in input cDNA. The following gene-specific primer pairs were designed using ABI Primer Express software: 5′-TCTACGAGTGGATG GTGCGTT-3′ and 5′-CGACTATGCAGTGACAGGTTGTG-3′ for *FOXO3a*, 5′-AAATGAAAGCTCACTCTGGATTCC-3′ and 5′-TGTGCAATTCCTATG CAATC-3′ for*PIK3CA*, 5′-AGGATATTGGGCTTTACAACCT-3′ and 5′-GG CTTATTCCCCACAATGTAGT-3′ for *IGFR1*, 5′-GATTCTCAGCAGCCA GCTCTGAT-3′ and 5′-GCAGGCTGTCGTTCATTCCA-3′ for *PIK3R1*, 5′-AGGTTTTAGATGCCACAAA-3′ and 5′-CCAGCTTACATTGCCATA-3′ for *PDPK1*, and 5′-GCGGAAGGGTACAGCCAAT-3′ and 5′-GCAGCCGG CGCAAA-3′ for L19. The specificity of each primer was determined using the NCBI BLAST module. The detection of the transcripts was performed with Sybr green (Applied Biosystems, Brackley, United Kingdom) and an ABI Prism 7700 sequence detection system (Applied Biosystems) using the relative standard curve method.

RLM–5′-RACE. To identify the transcription start site of *PIK3CA*, 5′ rapid amplification of cDNA ends (5′-RACE) was performed with a FirstChoice RNA ligase-mediated (RLM) RACE kit (Ambion) according to the manufacturer's instructions. Briefly, 10 μg of total RNA from FOXO3a(A3):ER K562 cells exposed to 4-OHT for 24 h was treated with calf intestine alkaline phosphatase to remove free 5′-phosphates from rRNA, tRNA, and contaminating genomic DNA. Tobacco acid pyrophosphatase was then added to remove the cap structure from full-length mRNA, leaving a 5′-monophosphate. Afterwards, a 45-base RNA adaptor oligonucleotide was ligated into the full-length decapped mRNA at the 5′ end using T4 RNA ligase. A random-primed reverse transcription reaction was performed. The resulting cDNA was used as a substrate in two sequential PCRs using primers from the kit and two nested gene-specific PCR antisense primers, 5′-TTTCTTCACGGTTGCCTACTGGTTC-3′ and 5′-AGTT GATGGAGG GGGTATTTTCTTGC-3′, respectively. The nested PCR product was also cloned into the pCRII-TOPO vector using a TOPO TA cloning kit (Invitrogen) and then sequenced with primers M13 Forward and M13 Reverse. The resultant sequence was then aligned and analyzed using a Human Blat search (http://genome.ucsc.edu/cgi-bin/hgBlat?command=start).

Generation of human PIK3CA promoter constructs. The full-length *PIK3CA* promoter constructs (positions −1,888 to +51) were generated using PCR primers 5′-TTCGATATCTTATTGGTCTCTTTTTCCGCTCACATC-3′ and 5′-AG TCTCGAGCGGCTGAAGTCTGTAATCCCAACA-3′ from K562 genomic DNA and cloned into the XhoI and EcoRV sites of the pGL4.22 basic vector (Promega, Southampton, United Kingdom). Other deletion constructs (positions −1534 to +51, −637 to +51, −452 to +51, and −281 to +51) were generated after excising from the pGL4-(−1889/+51) 5′ upstream fragments using restriction enzymes XmaI, EcoRI, XcmI, and BsiWI, respectively. Putative forkhead site mutagenesis was performed using a Stratagene QuikChange site-directed mutagenesis kit and oligonucleotides Site1F (5′-GATTGATTTACTCTCAA GCGGGCAGACTTCTAAGGTACGCAGC-3′), Site1R (5′-GCTGCGTAC CTTAGAAGTCTGCCCGCTTGAGAGTAAATCAATC-3′), Site2F (5′-GC TCGCCTGCTGCTCGTAGGGGCGGGTATACTACACGTACGCTG-3′), Site2R (5′-CAGCGTACGTGTAGTATACCCGCCCCTACGAGCAGC AGGCGAGC-3′), Site3F (5′-CTGCACAAGACGGATCATTGGGCGGGC GTCAGAAGAGCAGCCCC-3′), and Site3R (5′-GGGGCTGCTCTTCTGA CGCCCGCCCAATGATCCGTCTTGTGCAG-3′) (restriction sites are underlined).

ChIP assay. Chromatin immunoprecipitation (ChIP) assays were performed as described previously (9) using 2.5×10^6 inducible FOXO3a(A3):ER K562 cells treated with or without 200 nM 4-OHT for 24 h before harvesting. DNA fragments were purified using the QIAquick Spin kit (Qiagen, United Kingdom). For PCR, 1/25 of the extracted DNA was used and amplified in 25 PCR cycles using specific primers. Analysis of the PCR products was performed on a standard 2% (wt/vol) agarose gel by electrophoresis in Tris-acetate-EDTA buffer.

MTT assay. Cell proliferation/survival was quantitated using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) tetrazolium salt assay according to the manufacturer's protocol (Sigma, United Kingdom).

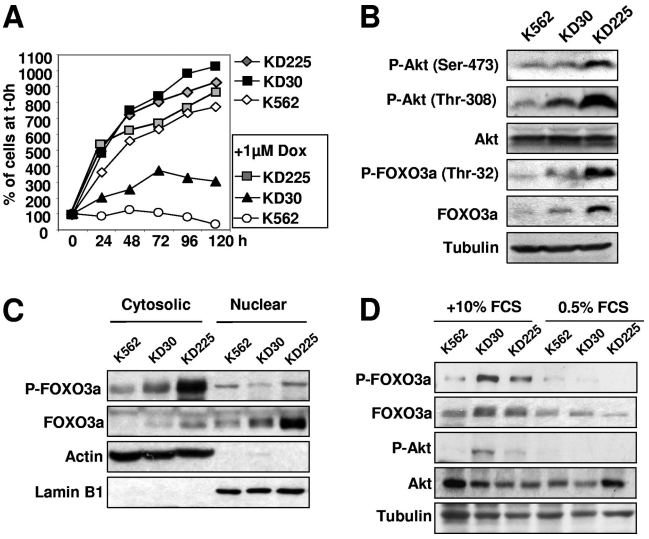

FIG. 1. Doxorubicin resistance correlates with FOXO3a expression and Akt phosphorylation in K562 cells. (A) Growth curves of K562, KD30, and KD225 cells in the presence or absence of 1 μM doxorubicin. (B) The naïve and drug-resistant K562 cell lines, as indicated, were analyzed by Western blotting using specific antibodies against P-Akt(Ser-473), P-Akt(Thr-308), Akt, P-FOXO3a(Thr-32), FOXO3a, and β-tubulin. (C) Cytoplasmic and nuclear extracts isolated from K562 cell lines were Western blotted with antibodies against FOXO3a(Thr-32), FOXO3a, actin, and lamin B1. (D) K562, KD30, and KD225 cells were cultured for 24 h in 0.5% FCS and then restimulated with 10% FCS for 24 h. Cell lysates from these K562 clones before and after serum stimulation were Western blotted for P-Akt-(Ser-473), Akt, P-FOXO3a(Thr-32), FOXO3a, and β-tubulin.

RESULTS

Doxorubicin resistance is associated with an increase in Akt phosphorylation and FOXO3a expression in K562 cells. To explore the potential role of the PI3K/Akt signaling pathway in the development of drug resistance in CML, we determined the expression and phosphorylation levels of Akt and its downstream target FOXO3a in the doxorubicin-sensitive naïve K562 cell line and the derivative cell lines KD30 and KD225, which exhibit intermediate and strong doxorubicin resistance, respectively (46). The cell proliferation assay showed the highest activity in KD30 cells, more so than KD225 cells, which in turn proliferated faster than K562 cells (Fig. 1A). Treatment with 1 μM doxorubicin significantly reduced cell proliferation in both K562 and KD30 cells but had relatively little effect on KD225 (Fig. 1A). Western blot analysis showed that the basal PI3K/Akt activity, as determined by the levels of phosphorylated Akt and FOXO3a in untreated cells maintained in the presence of serum, correlated with the relative drug resistances of these cell lines (Fig. 1B). Unexpectedly, total FOXO3a expression levels were also higher in drug-resistant KD30 and KD225 cells. This accumulation of FOXO3a could possibly be accounted for by increased PI3K/Akt-dependent phosphorylation and sequestration of this transcription factor in the cytoplasm (24, 39). We therefore examined the subcellular distribution of FOXO3a in the three cell lines by Western blot analysis of cytoplasmic and nuclear protein fractions (Fig. 1C). The results demonstrated higher levels of FOXO3a in both nuclear and cytoplasmic compartments of drug-resistant KD30

and KD225 cells than in naïve K562 cells. Lamin B1 and actin were also blotted to control for effective nuclear and cytoplasmic fractionation. The results indicate that despite enhanced PI3K/Akt activity, the development of doxorubicin-resistant K562 clones is associated with the increased expression and nuclear accumulation of FOXO3a.

We next examined if this enhanced Akt activity in drug-resistant K562 clones depends on growth factor stimulation. To this end, we studied the phosphorylation statuses of Akt and FOXO3a in K562 clones cultured under low-serum conditions (0.5% fetal calf serum [FCS]) for 24 h and upon restimulation with 10% FCS for 24 h. As shown in Fig. 1D, under serum-deprived conditions, PI3K activity, as reflected by Akt and FOXO3a phosphorylation, was equally decreased in both drug-sensitive and -resistant cells. Upon 24 h of serum stimulation following serum starvation, the pattern of Akt phosphorylation in the different lines was comparable to that of cells maintained in 10% serum. Phospho-Akt levels were higher in both K30 and KD225 cells than in the drug-sensitive K562 cells. The relatively lower levels of phospho-Akt in KD225 cells could be due to cell death, which in turn may reflect the abundance of FOXO3a in these cells. These results suggest that the elevated PI3K/Akt activity in the resistant cells requires upstream growth factor signaling.

Doxorubicin induces Akt phosphorylation as well as FOXO3a expression in K562 cells. To investigate the role of PI3K/Akt signaling in drug resistance further, we determined the effect of 1 μM doxorubicin on total and phosphorylated Akt and FOXO3a levels over a 96-h period in the three cell lines. Western blot analysis demonstrated that the FOXO3a expression level increases within 8 h of treatment of K562 and KD30 cells with doxorubicin (Fig. 2A). Although total Akt levels remained relatively constant throughout the time course, Akt and FOXO3a phosphorylation levels also increased upon doxorubicin treatment of K562 and KD30 cells albeit with delayed kinetics compared to those with the induction of total FOXO3a. Phosphorylated Akt levels were already high in KD225 cells (Fig. 1B), although a modest increase was still noted 24 h after doxorubicin exposure. The expression levels of *ID1*, a gene known to be repressed by FOXO3a (1), decreased at 24 h following doxorubicin treatment in all three K562 cell lines, indicating enhanced FOXO3a activity in response to doxorubicin (Fig. 2A). We further examined if doxorubicin treatment would increase the nuclear levels of transcriptionally competent FOXO3a in K562 cells (Fig. 2B). Consistent with the above-described data, FOXO3a accumulated in the nucleus within 8 h upon doxorubicin treatment, and nuclear levels peaked at 24 h, after which they appear to decline gradually. However, there was also a general decline in nuclear protein expression at 72 and 96 h, which was attributable to the considerable amounts of cell death induced by prolonged doxorubicin exposure. The results also show that the nuclear accumulation of FOXO3a in doxorubicin-treated cells precedes the increase in phosphorylated Akt levels. We further performed Western blot analysis on K562 cells before and after 24 h of doxorubicin treatment to study if other FOXO proteins are also induced by doxorubicin (Fig. 2C). HEC1B endometrial cells and NIH 3T3 cells were also included in the analysis as positive controls for FOXO1 and FOXO4 expression, respectively. The result shows that FOXO3a is the predominant FOXO family member expressed in K562 cells irrespective of doxorubicin treatment.

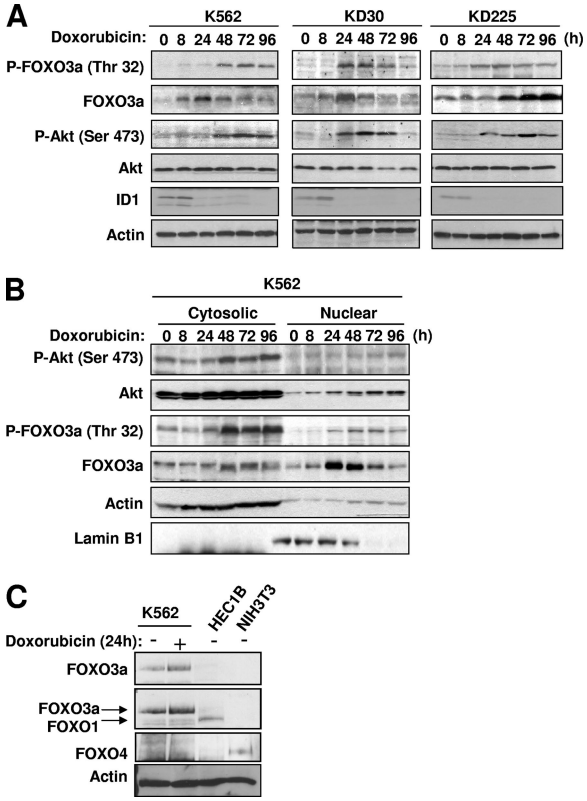

FIG. 2. Doxorubicin treatment causes an induction of FOXO3a expression and activity and Akt phosphorylation. Naïve and drug-resistant K562 cells were treated with 1 μM doxorubicin. (A) Protein lysates were prepared at the times indicated, and protein expression levels were analyzed by Western blotting using specific antibodies against P-Akt(Ser-473), Akt, P-FOXO3a(Thr-32), FOXO3a, ID1, and actin. The lower exposure blots for P-FOXO3a and P-Akt are shown in order to demonstrate the kinetics of induction upon doxorubicin treatment. (B) Cytoplasmic and nuclear extracts isolated from K562 cells treated with 1 μM doxorubicin were Western blotted with antibodies against P-Akt(Ser-473), Akt, P-FOXO3a(Thr-32), FOXO3a, actin, and lamin B1. (C) Lysates were prepared from K562 cells with or without 1 μM doxorubicin for 24 h and HEC1B and NIH 3T3 cells and immunoblotted for FOXO3a, FOXO1, and FOXO4 expression.

FOXO3a activation enhances PI3K/Akt activity. We next explored if the induction and activation of FOXO3a by doxorubicin could be causally linked to the subsequent increase in PI3K/Akt signaling. To this end, we stably transfected the K562 cell line with an expression vector that encodes the hormone binding domain of the ER fused to FOXO3a(A3), a constitutively active FOXO3a mutant (6, 9, 12). In these K562-ER:FOXO3a(A3) cells, FOXO3a activity can be conditionally induced upon the addition of 4-OHT. As shown in Fig. 3A, 4-OHT treatment of K562-ER:FOXO3a(A3) cells, but not of control cells stably transfected with ER, induced the expression of the FOXO3a target $p27^{Kip1}$. The result also revealed that FOXO3a activation enhances Akt phosphorylation without altering total Akt expression levels. This increase in Akt phosphorylation, and thus PI3K/Akt activity, was mediated specifically by FOXO3a, as no such response was observed in

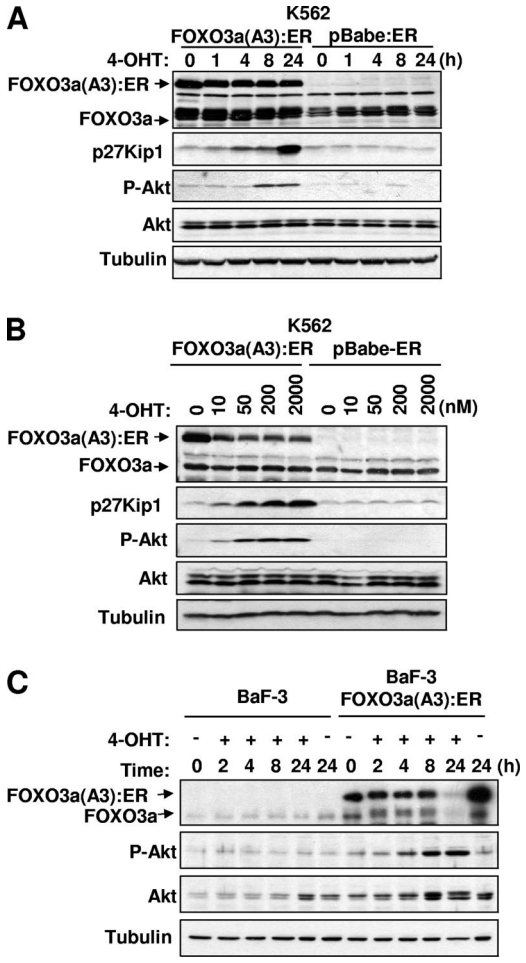

FIG. 3. Effect of FOXO3a induction on Akt phosphorylation and activity in K562 and BaF3 cells. K562-FOXO3a(A3):ER and K562-ER cells were treated with 4-OHT for the indicated times. (A) Cell lysates were prepared at the times indicated, separated on polyacrylamide gels, and subjected to immunoblotting with specific antibodies. The expression levels of FOXO3a, P-FOXO3a, $p27^{Kip1}$, P-Akt(473), Akt, and β-tubulin were analyzed by Western blotting. (B) Protein lysates were prepared from K562 cells treated with indicated doses of 4-OHT for 24 h, and protein expression levels were analyzed by Western blotting using specific antibodies against FOXO3a, P-FOXO3a, $p27^{Kip1}$, P-Akt(473), Akt, and β-tubulin. (C) BaF3-FOXO3a(A3):ER and BaF3-ER cells were treated with 4-OHT for the indicated times. Protein lysates were prepared from BaF3 cells treated with 4-OHT for the indicated times, and protein expression levels were analyzed by Western blotting using specific antibodies against FOXO3a, P-FOXO3a, P-Akt(473), Akt, and β-tubulin.

control K562 cells expressing ER alone. This conjecture was further supported by treating K562-ER:FOXO3a cells with increasing amounts of 4-OHT for 24 h, demonstrating a dose-dependent increase in Akt phosphorylation that paralleled the increased induction of $p27^{Kip1}$ (Fig. 3B). The ability of FOXO3a to enhance PI3K/Akt signaling was not confined to K562 cells, as the results could be recapitulated upon 4-OHT treatment of hematopoietic BaF-3 cells stably expressing ER:FOXO3a(A3) (Fig. 3C). It is noteworthy that the prolonged induction of FOXO3a activity appears to lead to a specific reduction in FOXO3a protein levels in BaF-3 cells. The reason for this is unclear. We previously reported that K562 cells differentiate into red blood cells rather than undergoing apoptosis in response to PI3K inhibition and FOXO3a induction (1). However, in contrast to K562 cells, the prolonged induction of FOXO3a activity in BaF-3 cells causes considerable cell death (6), resulting in the global degradation of proteins, which accounts for the apparent decline in Akt and tubulin levels at 24 h. FOXO3a expression appears to be even more sensitive for reasons not yet entirely clear. However, FOXO3a was previously shown to activate the Bim-caspase 3 pathway in these cells (6), and the induction of caspase 3, as well as related caspases, has been shown to specifically cleave FOXO3a (3). This mechanism could account for the reduction in FOXO3a protein levels upon the prolonged induction of FOXO3a activity.

Chronic FOXO3a induction and PI3K/Akt activity confer resistance to doxorubicin cytotoxicity in K562 cells. To determine if PI3K/Akt activity confers resistance to doxorubicin, we generated a K562 cell line that expresses a conditionally active form of Akt [(myr)Akt:ER] (7). The treatment of K562-(myr)Akt:ER cells with 4-OHT elicited the rapid hyperphosphorylation of the exogenous Akt fusion protein (Fig. 4A). Notably, there was a decrease in total FOXO3a levels over time in response to Akt activation. The phosphorylation of FOXO3a by Akt has been shown to lead to FOXO3a degradation (24), and this could account for the decrease in total FOXO3a levels over time in the presence of active (myr)Akt:ER. Growth curves and MTT proliferative assays performed in the presence of serum (10% FCS) showed no difference in growth rates between the K562-(myr)Akt:ER cells cultured in the presence of 4-OHT and those in the absence of 4-OHT (data not shown and Fig. 4C). As shown in Fig. 4B and C (right), doxorubicin-mediated cytotoxicity was attenuated when K562-(myr)Akt:ER cells were cultured in the presence of 4-OHT. However, 4-OHT did not protect K562 cells expressing ER alone against the cytotoxic effects of doxorubicin (Fig. 4B and C), demonstrating that resistance in K562-(myr)Akt:ER cells was a consequence of increased PI3K/Akt activity. To investigate the role of FOXO3a in drug resistance, we transfected siRNA targeting the endogenous expression of this transcription factor in K562 cells, and the knockdown of FOXO3a was confirmed at the protein level (Fig. 4D, left) as well as at the mRNA level (Fig. 4D, right). Consistent with the above-described observations, FOXO3a knockdown reduced the levels of phosphorylated Akt without affecting the total expression of this kinase (Fig. 4D). Furthermore, the silencing of endogenous FOXO3a expression had little or no effect on the proliferative activity of K562 cells (Fig. 4E, left) but sensitized these cells to doxorubicin-mediated cell loss (Fig. 4E, right).

FOXO3a directly activates class I PI3K subunit p110α expression. We hypothesized that FOXO3a may regulate the expression of components of the PI3K pathway upstream of Akt. Hence, we studied the expression levels of IGFR1, PDK1, p110α, and p85α in K562-ER:FOXO3a(A3) cells treated with 4-OHT. Western blot analysis showed that the induction of FOXO3a activity had no effect on the expression of the PI3K regulatory subunit p85, IGFR1, or PDK but selectively enhanced the expression of the class I PI3K catalytic subunit p110α (Fig. 5A). We next studied the expression levels of these

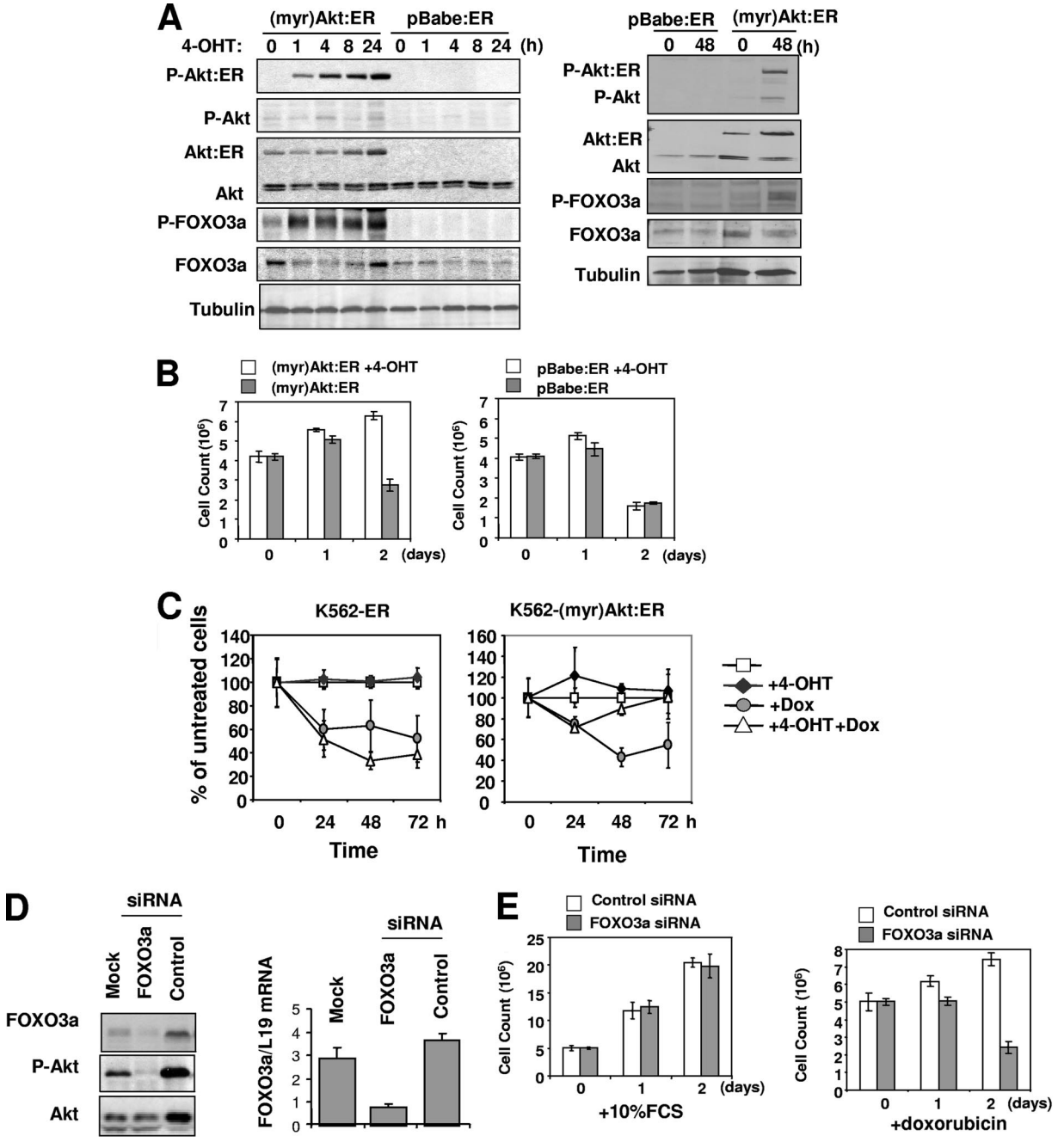

FIG. 4. Effects of Akt induction and FOXO3a silencing on K562 proliferation. (A) K562 cells harboring (myr)Akt:ER and K562-ER cells were treated with 4-OHT for the indicated times. Cell lysates were prepared at the times indicated, separated on polyacrylamide gels, and subjected to immunoblotting with specific antibodies. The expression levels of FOXO3a, P-FOXO3a, $p27^{Kip1}$, P-Akt(473), Akt, and β-tubulin were analyzed by Western blotting. (B) (myr)Akt:ER and K562-ER cells were treated with or without 4-OHT for 2 h and then treated with 1 mM doxorubicin. Cells were counts at the times indicated following doxorubicin treatment. (C) (myr)Akt:ER and K562-ER cells were treated with or without 4-OHT for 2 h and then treated with 1 mM doxorubicin. MTT assays were performed at the times indicated following doxorubicin treatment. (D) KD562 cells were transiently transfected using Nucleofect solution V (Amaxa) with either no siRNA, *FOXO3a* Smartpool siRNA, or control Smartpool siRNA (Dharmacon). Cell lysates and mRNA were prepared from these transfectants after 48 h and were subjected to Western blotting with FOXO3a, P-Akt(473), and Akt antibodies and RTQ-PCR analysis for FOXO3a and L19 RNA expression, respectively. (E) The siRNA-transfected K562 cells were then treated with 1 mM doxorubicin and counted at the times indicated following doxorubicin treatment. All data shown represent the averages of data from three independent experiments performed together, and the error bars show the standard deviations.

PI3K signaling components at the transcriptional level. Consistent with the analysis at the protein level, RTQ-PCR analysis demonstrated no change in p85α or PDK1 mRNA levels but a sixfold maximal increase in p110α mRNA levels in 4-OHT-treated K562-ER:FOXO3a(A3) cells (Fig. 5B). There was also a modest increase in IGFR1 mRNA expression levels, but this induction was much less pronounced than that of p110α transcripts. We then determined if de novo protein synthesis was

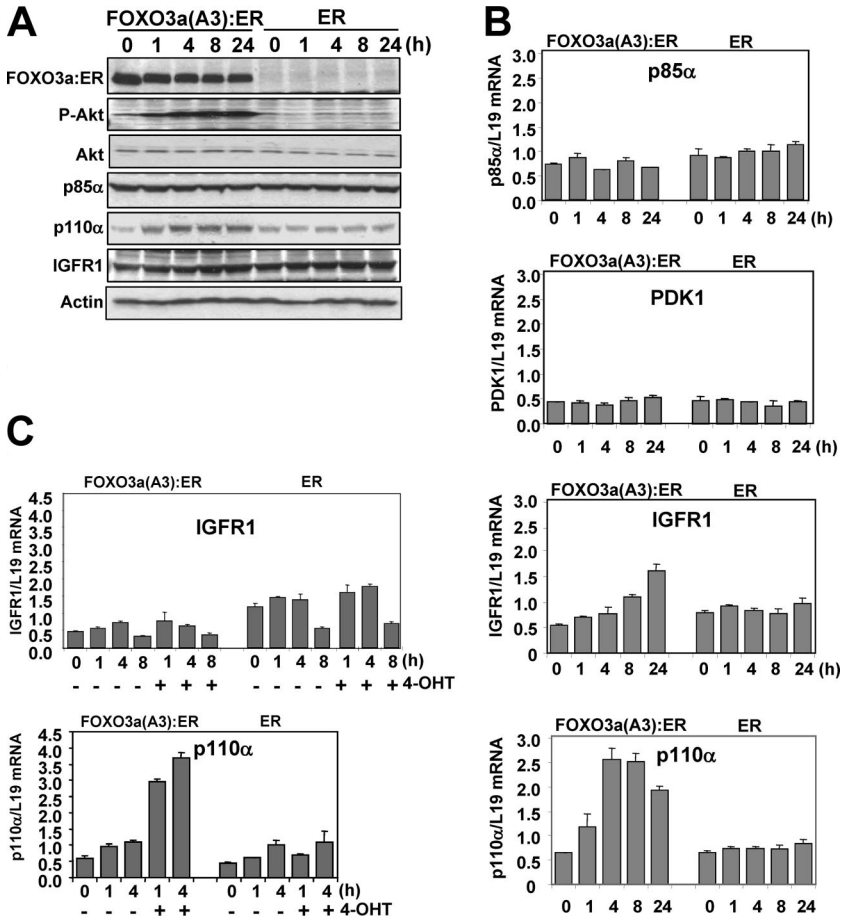

FIG. 5. FOXO3a induced *p110*α gene expression directly to mediate Akt phosphorylation. K562-FOXO3a(A3):ER and K562-ER cells were treated with 4-OHT for the indicated times. (A) Cell lysates were prepared at the times indicated, separated on polyacrylamide gels, and subjected to immunoblotting with specific antibodies. The expression levels of FOXO3a, P-FOXO3a, $p27^{Kip1}$, P-Akt(473), Akt, and β-tubulin were analyzed by Western blotting. (B) Total RNA was extracted and analyzed for p85α, p110α, PDK1, and IGFR1 mRNA expression using RTQ-PCR as described in the text and normalized to the level of L19 RNA. (C) K562-FOXO3a(A3):ER and K562-ER cells were pretreated with 100 μM cycloheximide for 30 min before stimulation with 4-OHT for the indicated times. Total RNA was extracted and analyzed for p110α and IGFR1 mRNA expression using RTQ-PCR as described in the text and normalized to the level of L19 RNA. All data shown represent the averages of data from three experiments, and the error bars show the standard deviations.

required for FOXO3a-induced p110α and IGFR1 mRNA expression. K562-ER:FOXO3a(A3) cells were pretreated with or without cycloheximide for 30 min and then stimulated with or without 4-OHT for either 1, 4, or 8 h (Fig. 5C). RTQ-PCR analysis revealed that cycloheximide treatment blocked the FOXO3a-mediated induction of IGFR1 mRNA levels. However, cycloheximide failed to abrogate the induction of p110α transcripts in K562-ER:FOXO3a(A3) cells treated with 4-OHT. Thus, the de novo synthesis of other transcription factors was not required for p110α mRNA induction, suggesting that FOXO3a directly regulates the expression of *PIK3CA* in K562 cells.

We also studied the effects of the pan-PI3K inhibitor LY294002 and the p110α-specific inhibitor PI-387 on the induction of Akt phosphorylation and activity by FOXO3a. The results showed that LY294002 blocks the induction of Akt phosphorylation by FOXO3a in K562-FOXO3a:ER cells (Fig. 6A). Moreover, LY294002 also abrogated the phosphorylation of FOXO3a, p70S6K, and GSK3β, which are direct downstream targets of Akt. More importantly, the results also showed that the induction of Akt phosphorylation by FOXO3a can be blocked by the p110α-specific inhibitor PI-387, indicating that the FOXO3a-induced PI3K activity and Akt phosphorylation are mediated through the p110α subunit, further supporting our hypothesis. Next, we examined if the expression of p110α would suffice to increase PI3K/Akt activity. K562 cells were transfected with expression vectors encoding either wild-type p110α, a membrane-targeted p110α (p110αCAAX), or a 4-OHT-inducible, membrane-targeted p110α-ER fusion protein (p110α-CAAX:ER). Transfected cells were then cultured for 7 days in the presence of 10 μg/ml puromycin (Fig. 6B). Western blot analysis demonstrated that the overexpression of membrane-targeted (i.e., p110α-CAAX and p110α-CAAX:ER) or wild-type p110α was sufficient to strongly enhance the levels of phosphorylated Akt and, thus, PI3K/Akt activity. Notably, it is not clear why the transfection of the inducible p110α-CAAX:ER could strongly activate Akt phosphorylation in the absence of 4-OHT. However, these experiments were

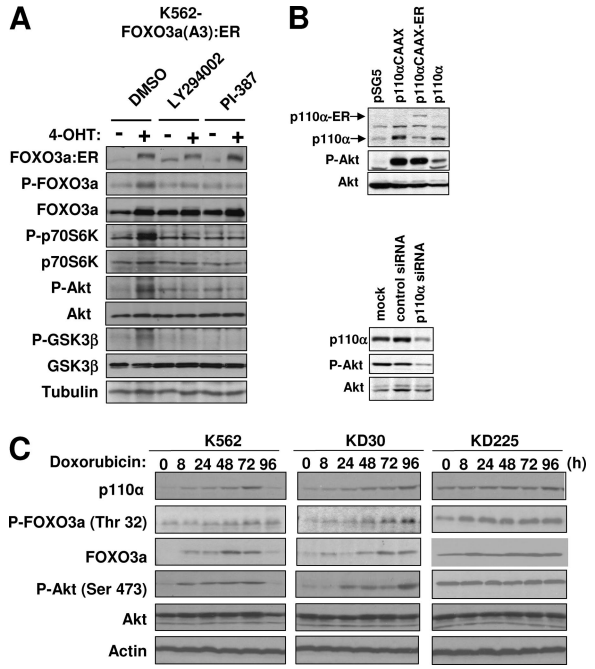

FIG. 6. FOXO3a induces Akt phosphorylation and activation via *p110α* in K562 cells. (A) K562-FOXO3a(A3):ER cells were cultured for 24 h with or without 4-OHT in the presence of vehicle (dimethyl sulfoxide [DMSO]), 30 μM LY294002, or 15 μM PI-387. Cell lysates prepared from these cells were separated on polyacrylamide gels and subjected to immunoblotting with specific antibodies. The expression of levels of Akt, P-Akt(473), FOXO3a, P-FOXO3a(Thr-32), p70S6K, P-p70S6K, GSK3β, and P-GSK3β were analyzed by Western blotting. (B) K562 cells were either mock transfected or transfected with p110α, control Smartpool siRNA (Dharmacon), pSG5, pSG5-p110α, pSG5-p110αCAAX-ER, or pSG5-p110α. Cell lysates were prepared from these transfectants after 48 h and Western blotted for p110α, Akt, and P-Akt(473). (C) The naïve K562 and drug-resistant KD30 and KD225 cell lines were treated with 1 μM doxorubicin. Protein lysates were prepared at the times indicated, and protein expression levels were analyzed by Western blotting using specific antibodies against p110α, P-Akt(Ser-473), P-Akt(Thr-308), Akt, P-FOXO3a(Thr-32), FOXO3a, and β-tubulin.

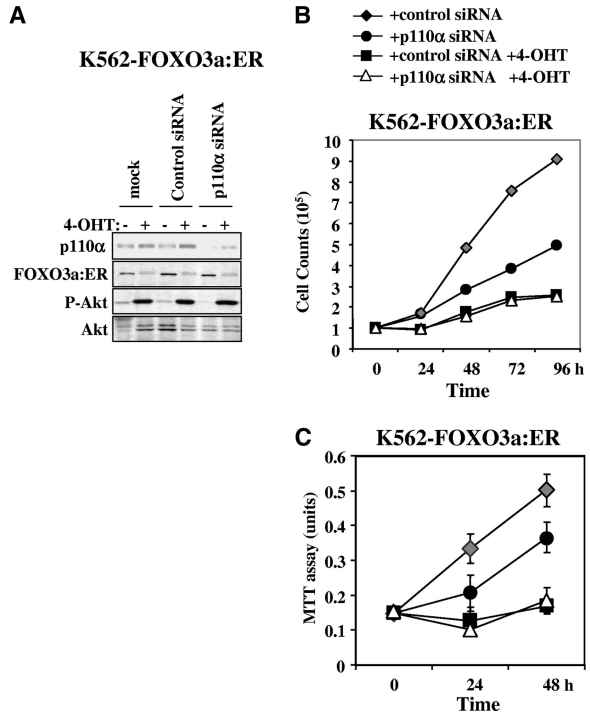

FIG. 7. Effects of p110α silencing on Akt activity and K562 proliferation. (A) K562 cells harboring FOXO3a:ER cells were transfected with control siRNA and siRNA and then treated with 4-OHT for 24 h. Cell lysates were prepared, separated on polyacrylamide gels, and subjected to immunoblotting with specific antibodies. The expression levels of FOXO3a:ER, P-Akt(473), Akt, and p110α were analyzed by Western blotting. These results indicate that p110α silencing significantly decreases P-Akt expression. (B) Cell counts were performed on these cells, showing that the knockdown of p110α decreases the cell proliferation rate but has no effect on cell proliferation in response to FOXO3a induction. (C) MTT assays were performed on these cells, again indicating that the knockdown of p110α decreases the cell proliferation rate but has no effect on cell proliferation in response to FOXO3a induction.

performed in medium containing phenol red, which can mimic ER-ligand. Moreover, p110CAAX-ER was overexpressed in this study, and this might cause a fraction of the transfected p110CAAX-ER not to be sequestered in the cytoplasm. To further elaborate on these findings, we examined the effects of p110α knockdown on Akt phosphorylation. To this end, K562 cells were transfected with either an siRNA pool targeting p110α or nontargeting siRNA and harvested 72 h later. As shown in Fig. 6B (bottom), p110α knockdown was confirmed by Western blot analysis and resulted in a corresponding downregulation in phosphorylated but not total Akt levels. Together, the results unequivocally demonstrate that the FOXO3a-dependent induction of p110α in K562 cells amplifies PI3K/Akt activity.

Akt phosphorylation is normally regulated by p110α in the K562 cells. We next investigated if the knockdown of p110α expression can prevent the induction of Akt phosphorylation by FOXO3a. To this end, K562 cells harboring FOXO3a:ER cells were transfected with control siRNA or siRNA targeting p110α and then treated with 4-OHT for 24 h. Cell lysates separated on polyacrylamide gels were then immunoblotted with specific antibodies for the detection of FOXO3a:ER, P-Akt(473), Akt, and p110α. The results show that p110α silencing decreased phospho-Akt levels in FOXO3a:ER K562 cell not treated with 4-OHT but had no effect on the induction of Akt phosphorylation upon 4-OHT stimulation (Fig. 7A). Cell counts and MTT assays were also performed, showing that the knockdown of p110α decreases cell proliferation rate, but again, no effect was observed in response to FOXO3a induction (Fig. 7B and C). These results indicate not only that Akt phosphorylation is normally regulated by p110α in the K562 cells but also that FOXO3a activity can phosphorylate Akt via alternative mechanisms. Indeed, insulin/insulin-like growth factor, IGFR, and other upstream PI3K signaling components were previously shown to be targets of FOXO proteins (5, 27–29).

FOXO3a induces p110α gene expression at the promoter level through a novel 5′ exon. We next sought to determine if FOXO3a induces *PIK3CA* activation at the promoter level. Genomic DNA from K562 cells was used to clone a 296-bp region and a 970-bp region (Fig. 8A), representing the untrans-

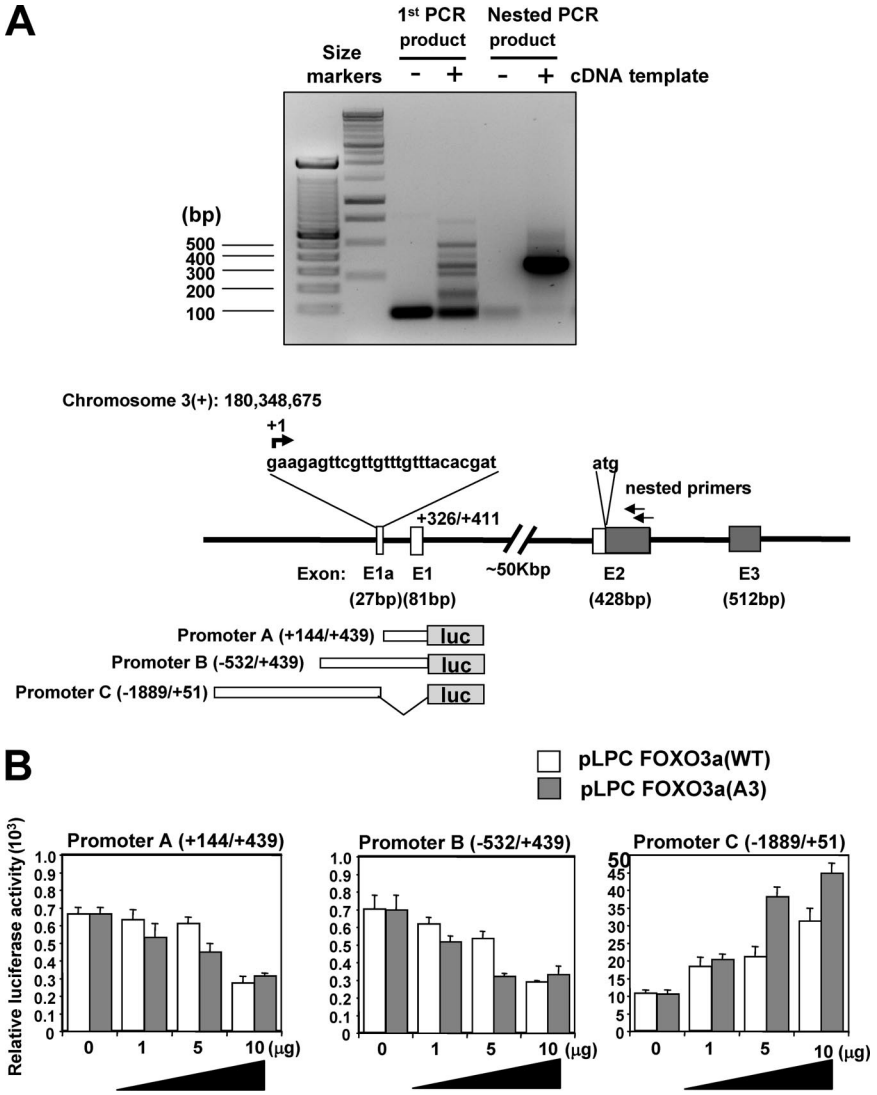

FIG. 8. FOXO3a induces the expression of the human *PIK3CA* gene through a promoter region upstream of a novel 5′ exon. (A) Mapping of the human p110α transcription start site using 5′-RACE. The total RNA prepared from K562-FOXO3a(A3):ER cells after 24 h of 4-OHT induction was reverse transcribed, and the cDNA was subjected to two sequential PCRs using primers from the Ambion FirstChoice RLM-RACE kit and nested PCR primers specific to exon 2 of the human *p110*α gene. The nested PCR products were analyzed by agarose gel electrophoresis, and the major PCR product was clearly visible as a band of about 300 bp. Molecular size markers (base pairs) are indicated on the left of the promoter sequence relative to the major transcription start site. (Bottom) Schematic representation of the 5′ region of the human *PIK3CA* genes. The exons (boxes), their sizes in base pairs, and their positions relative to the major transcription start site are indicated. Introns are represented by thick lines. The sequence and genome location of the novel exon 1A are shown. Boxes underneath show the relative positions of the three putative human *PIK3CA* promoter constructs A, B, and C. (B) K562 cells were transiently transfected with 1 μg of each of the three putative human *p110*α promoter/reporter constructs, together with increasing amounts (0, 1, 5, and 10 μg) of pLPCFOXO3a-WT or -A3. Cells were harvested 24 h after transfection and assayed for luciferase activity. All relative luciferase activity values are corrected for cotransfected *Renilla* activity. All data shown represent the averages of data from three independent experiments, and the error bars show the standard deviations. (C) K562 cells were transiently transfected with 1 μg of each of the human *PIK3CA* promoter/reporter constructs, together with 0 or 5 μg of pLPCFOXO3a-A3, and processed as described above. The induction of the *PIK3CA* promoter by FOXO3a is shown on the right. (D) ChIP analysis of the human *PIK3CA* promoter. Protein-DNA complexes from K562 FOXO3a(A3):ER cells cultured in the presence or absence of 4-OHT for 4 h were subjected to immunoprecipitation with antibodies against immunoglobulin G (IgG) (nonspecific) and FOXO3a, as indicated. After cross-link reversal, the coimmunoprecipitated DNA was amplified by PCR using the indicated primers and resolved in 2% agarose gels. The primer pairs used were 5′-GCTTTTTCTGCTATGACACACAACTTC-3′ and 5′-GCACCTGGCCTATTTGT GATTTTTA-3′ (positions −2000 to −1741), 5′-GTGAAACTACGACCACAAAGAGGA-3′ and 5′-AGCATCGGTTCCTCCTTGAA-3′ (positions −1127 to −946) 5′-CGCCTTCGGGATGGTATACAA-3′ and 5′-GAGGGTGTTGTGTCATCCTAGGAC-3′ (positions −548 to −251), and 5′-GA CACAACACCCTCACTACTGCA-3′ and 5′-GGCAGAGCCTACAATCCCC-3′ (positions −264 to −55).

lated exon 1 (E1) of *PIK3CA* and variable lengths of the putative upstream promoter region, into a luciferase reporter construct (Fig. 8B). Both of these promoter-reporter constructs, designated promoters A (positions +144 to +439) and B (positions −532 to +439), exhibited basal promoter activity when transiently expressed in K562 cells, which surprisingly was suppressed upon the cotransfection of increasing amounts of either wild-type or constitutively active FOXO3a (Fig. 8B).

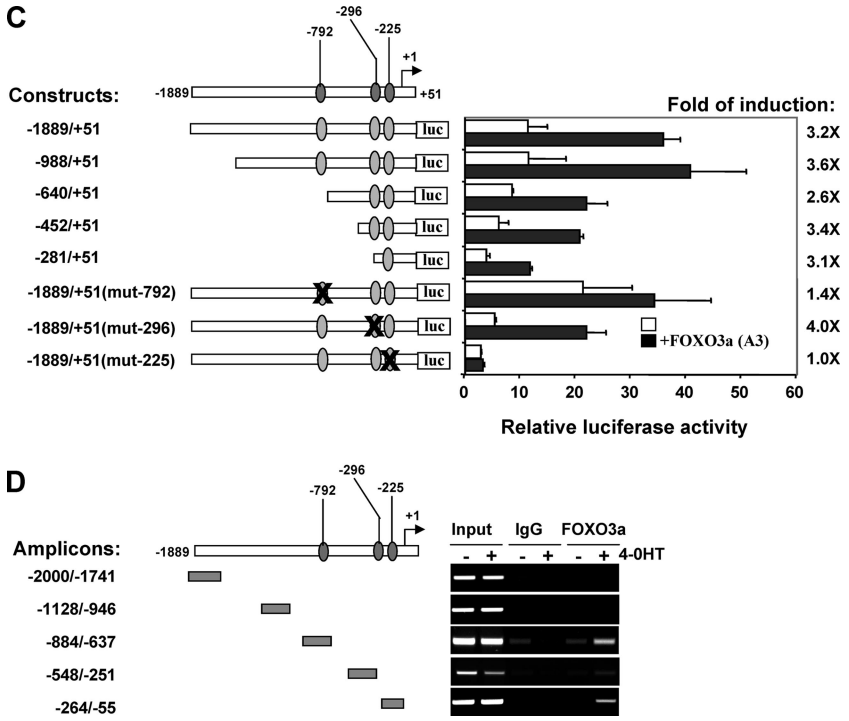

FIG. 8—*Continued.*

This paradoxical observation prompted us to examine *PIK3CA* promoter usage in 4-OHT-treated K562-ER:FOXO3a(A3) cells by mapping the transcriptional start site using 5′-RACE. Sequence analysis demonstrated that transcription is initiated at a novel 27-bp exon, termed exon 1a (E1a), located 326 bp upstream of E1 (Fig. 8A). We next cloned a 1.9-kb genomic fragment containing exon 1a and its upstream region, promoter C (positions −1889 to +51), into a luciferase reporter vector (Fig. 8A). Cotransfection studies demonstrated a dose-dependent induction of promoter C activity with increasing amounts of wild-type FOXO3a or the constitutively active FOXO3a(A3) mutant (Fig. 8B). In order to identify the FOXO3a-responsive elements, a series of deletion reporter constructs was first generated and cotransfected into K562 cells together with either the constitutively active FOXO3a(A3) mutant or an empty control vector. As shown in Fig. 8C, the sequential deletion of the 5′-distal region from −1889 to −281 bp decreased the basal *PIK3CA* promoter activity but had little effect on FOXO3a(A3)-dependent promoter activity. Next, we mutated each of the three putative FOXO3a-responsive elements located at positions −792, −296, and −225, respectively. As shown in Fig. 8C, the mutation of site 1 (position −792) resulted in a marginal increase in basal promoter activity that coincided with a blunted induction upon FOXO3a(A3) overexpression. When site 2 (position −296) was mutated (positions −1889 to +51 [mut-296]) or deleted (positions −281 to +51), basal promoter activity and induction by FOXO3a(A3) were unchanged. This contrasted to the consequences of mutating the proximal response element, site 3 (position −225), which not only dramatically reduced basal promoter activity but also abolished induction by FOXO3a(A3). Together, the data indicate that the putative FOXO-binding site in the proximal promoter region of *PIK3CA* is indispensable for *trans*-activation by FOXO3a. We next investigated the in vivo promoter occupancy by FOXO3a using ChIP assays. To this end, K562-ER:FOXO3a cells were cultured in the presence or absence of 4-OHT for 4 h, and the chromatin was precipitated with anti-FOXO3a antibody and analyzed by PCR using primer sets that covered the proximal as well as other distal promoter regions. Interestingly, ChIP analysis demonstrated in vivo FOXO3a binding to the *PIK3CA* promoter in regions that encompass site 1 (positions −884 to −637) as well as site 3 (positions −264 to −55) but not site 2 (positions −548 to −251). Notably, we occasionally found FOXO3a binding to the region that contained the putative site 2, which is possibly due to its proximity to sites 1 and 3. In control experiments, we performed ChIP analysis of two regions distal to the transcriptional start site (positions −2000 to −1741 and −1128 to −946), but FOXO3a binding was not detected. Together, these data suggest that FOXO3a increases PI3K/Akt activity in K562 cells, at least in part, by transcriptionally activating *PIK3CA* expression.

DISCUSSION

Many hematopoietic cancers are responsive to chemotherapy, but the emergence of multidrug-resistant cancer clones can lead to treatment failure and disease relapse. Despite the clinical imperative, the molecular mechanisms responsible for the development of MDR remain largely undefined. Nevertheless, there is abundant evidence that survival signals are required for the initial selection of cancer clones impervious to the cytotoxic effects of chemotherapeutic drugs. Furthermore, the observation that acquired resistance develops only after

drug treatment indicates that cytotoxicity and the emergence of drug-resistant cancer cell clones are intricately linked. By comparing naïve K562 and doxorubicin-resistant KD30 and KD225 CML cells, we found that the development of MDR is associated with increased FOXO3a expression levels and enhanced PI3K/Akt activity. Time course analysis demonstrated that the induction and nuclear accumulation of FOXO3a preceded the increase in phosphorylated Akt levels in doxorubicin-treated K562 CML cells. Furthermore, the induction of FOXO3a activity with 4-OHT was sufficient to enhance PI3K/Akt signaling and to confer doxorubicin resistance in K562 cells stably transfected with the conditional ER:FOXO3a(A3) expression vector. Conversely, the siRNA knockdown of endogenous FOXO3a attenuated PI3K/Akt activity in K562 cells and enhanced their sensitivity to doxorubicin.

Our data show that resistant cells have higher levels of active FOXO3a, which in turn feed back and increase PI3K activity and Akt and FOXO3a phosphorylation. However, this increase in Akt activity was insufficient to completely phosphorylate and inactivate the entire FOXO3a pool. Consistent with this notion, subcellular fractionation studies showed elevated nuclear FOXO3a levels in resistant cells despite considerable levels of phosphorylated FOXO3a in the cytoplasm. We proposed that the elevated levels of unphosphorylated nuclear FOXO3a drive PI3K activity, thereby promoting survival and resistance to doxorubicin and other drug treatments.

The observation that FOXO3a amplifies PI3K/Akt signaling appears paradoxical for several reasons. First, FOXO3a has been shown to induce apoptosis in various cell systems in response to cellular stress. For instance, we previously showed that the induction of FOXO3a in breast cancer cells exposed to paclitaxel, another chemotherapeutic drug, mediates cell death by activating *BIM* (*BCL2L11*) expression (37, 38). We have also demonstrated that FOXO3a induces cell death in primary endometrial cells and hematopoietic cell lines when exposed to oxidative stress (16, 40) and that its expression in CML cell lines leads to cell cycle arrest followed by apoptosis (9, 12). Second, FOXO transcription factors are inactivated by Akt, and thus, the FOXO3a-dependent hyperactivation of the PI3K/Akt pathway should be transient. However, this homeostatic mechanism is disrupted in doxorubicin-resistant KD30 and KD225 cells, resulting in constitutively nuclear FOXO3a expression concurrent with increased PI3K/Akt activity.

The mechanism that allows FOXO3a to evade PI3K/Akt-dependent cytoplasmic sequestration and inactivation in drug-resistant cells is yet unknown but may involve protein modifications. For instance, the targeted phosphorylation of FOXO factors by the stress-activated JNK pathway has been shown to induce their nuclear accumulation, to promote the recruitment of SIRT1, a NAD-dependent protein deacetylase, and to favor the activation of genes involved in cellular defenses against environmental or caloric stress (2, 4, 13, 14, 20, 47). Therefore, it is conceivable that acute exposure to cytotoxic drugs may lead to the FOXO3a-dependent activation of a proapoptotic gene program, whereas prolonged or chronic exposure promotes the selection of cells expressing modified, PI3K/Akt-insensitive FOXO3a that governs the expression of genes involved in cellular survival and drug resistance. Consistent with this idea, we found that the majority of doxorubicin-treated K562 cells underwent apoptosis and that only a very small population acquired resistance and survived (Fig. 1A). The resistant K562 line KD30, which was derived from one-step doxorubicin treatment for 2 weeks (46), is representative of these resistant cells and is characterized by elevated FOXO3a expression and activity.

We identified *PIK3CA*, the gene encoding p110α, as a FOXO3a target involved in survival responses and drug resistance in CML cells. Transfection studies demonstrated that the overexpression of the PI3K catalytic subunit p110α alone is enough to enhance PI3K/Akt activity in K562 cells. Although the underlying mechanism is not entirely understood, it could be due to a number of reasons. First, previous studies from p85 knockout mice showed that an imbalance in the levels of p85 and p110 subunits suffices to significantly modulate PI3K/Akt activity (41). In addition, gene deletion studies of mice have shown that p110α is essential not only for proper growth factor signaling but also for oncogenic transformation (48). Secondly, activating PIK3CA mutations are common in a whole spectrum of cancers, and an increased level of PIK3CA expression under these circumstances can further induce PI3K/Akt activity (34). Indeed, the amplification or mutations of *PIK3CA* are present in many other malignancies, including those of the breast, colon, lung, and ovary, and in chemoresistant cancers (15, 30, 42–44). Finally, the hyperactivation of growth factor receptors or the expression of oncogenes such as BCR-ABL can constitutively activate signaling upstream of PI3K. The induction of p110α expression, which is rate limiting under these circumstances, will consequently augment PI3K/Akt activity. Consistent with this, our serum deprivation and restimulation experiments (Fig. 1D) showed that growth factor signaling is required for the induction of PI3K/Akt activity by FOXO3a. Moreover, the finding that the overexpression of wild-type p110α alone is sufficient to activate Akt further confirms this notion (Fig. 6B). These results are also consistent with our hypothesis that FOXO3a induction activates the expression of p110α, the catalytic subunit of class 1a PI-3K. As p110α is a catalytic rather than a regulatory subunit, its upregulation will function only to alter the threshold of PI3K activation in resistant cells while having little effect on PI3K activity in the absence of growth factors or cytokines. In the case of CML, the constitutively active BCR-ABL tyrosine kinase will recruit the excess p110α subunits to the cellular membrane via p85α to further activate PI3K/Akt activity (36). Our data therefore provide more evidence that FOXO transcription factors can engage in a feedback mechanism that determines the activity of the upstream PI3K/Akt pathway. Recently, FOXO1 was shown to enhance Akt phosphorylation in hepatocytes by repressing the expression of tribble 3 (Trb3), a pseudokinase capable of binding Akt and inhibiting its phosphorylation (26). In *Drosophila melanogaster*, the dFOXO orthologue was shown to induce the expression of the insulin receptor (dInR) (31, 32), resulting in increased PI3K activity and cell growth under low-nutrient conditions. In addition to p110α, our reverse transcription-PCR data revealed the induction of IGFR1 transcripts upon FOXO3a activation in K562 cells, although based on the cycloheximide experiments, *IGFR1* is unlikely to be a direct target gene. In line with these observations, a recent DNA microarray study identified *IGFR1* and *PI3KCA* as being gene targets of FOXO3a in a colon carcinoma cell line (5). Together, these observations indicate

that FOXO3a can activate the PI3K/Akt signaling pathway through multiple direct and indirect mechanisms, including via the induction of IGFR1, InR, Tbr3, or p110α expression.

In summary, we propose that the peculiar ability of FOXO3a to regulate the expression of genes involved in opposing cell fate decisions underpins the development of MDR in CML cells. FOXO3a activity is rapidly enhanced in response to doxorubicin and thought to mediate the initial apoptotic response (9, 12). However, we now show that FOXO3a also *trans*-activates *PIK3CA*, which in turn augments PI3K/Akt activity, thereby contributing to increased cell survival and drug resistance. This unexpected dual role of FOXO3a may have important clinical ramifications. Specifically, our data suggest that a combination of conventional chemotherapy and PI3K/Akt-specific inhibitors could potentially prevent or overcome MDR by disabling the feedback mechanism that drives cell survival.

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