

Doxorubicin activates FOXO3a to induce the expression of multidrug resistance gene *ABCB1* (*MDR1*) in K562 leukemic cells

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Abstract

Using the doxorubicin-sensitive K562 cell line and the resistant derivative lines KD30 and KD225 as models, we found that acquisition of multidrug resistance (MDR) is associated with enhanced FOXO3a activity and expression of *ABCB1* (*MDR1*), a plasma membrane P-glycoprotein that functions as an efflux pump for various anticancer agents. Furthermore, induction of *ABCB1* mRNA expression on doxorubicin treatment of naive K562 cells was also accompanied by increased FOXO3a activity. Analysis of transfected K562, KD30, and KD225 cells in which FOXO3a activity can be induced by 4-hydroxytamoxifen showed that FOXO3a up-regulates *ABCB1* expression at protein, mRNA, and gene promoter levels. Conversely, silencing of endogenous FOXO3a expression in KD225 cells inhibited the expression of this transport protein. Promoter analysis and chromatin immunoprecipitation assays showed that FOXO3a regulation of *ABCB1* expression involves binding of this transcription factor to the proximal promoter region. Moreover, activation of FOXO3a increased *ABCB1* drug efflux potential in KD30 cells, whereas silencing of FOXO3a by siRNA significantly reduced *ABCB1* drug efflux ability. Together, these findings suggest a novel mechanism that can

contribute towards MDR, involving FOXO3a as sensor for the cytotoxic stress induced by anticancer drugs. Although FOXO3a may initially trigger a program of cell cycle arrest and cell death in response to doxorubicin, sustained FOXO3a activation promotes drug resistance and survival of cells by activating *ABCB1* expression. [Mol Cancer Ther 2008;7(3):670–8]

Introduction

Acquired resistance to chemotherapeutic agents remains a major obstacle for the effective treatment of many advanced and metastatic cancers. Several mechanisms are thought to be involved in the development of multidrug resistance (MDR), defined by simultaneous cross-resistance to anticancer drugs that differ in their chemical structures, modes of action, and molecular targets (1, 2). Cancer cells can decrease expression or activity of transporter molecules that regulate the uptake of hydrophilic chemotherapeutic drugs. An additional mechanism entails up-regulation of transporters involved in energy-dependent efflux of a wide variety of hydrophobic chemotherapeutic agents that enter cells freely by diffusion through the plasma membrane. One such transporter molecule is *ABCB1*, also called *MDR1* or P-glycoprotein (P-gp), a well-characterized member of the ATP-binding cassette transporter superfamily. In certain cancers, such as chronic or acute myeloid leukemia and breast cancer, overexpression of *ABCB1* is a prognostic indicator for clinical outcome and correlates with a poor response to chemotherapy (3–6). Malignant cells may also counteract the cytotoxic and/or cytostatic effects of therapeutic agents via amplification of proliferation and survival signals, increased DNA damage repair, and altered drug metabolism (2).

The phosphoinositide 3-kinase (PI3K)/Akt pathway is frequently implicated in tumorigenesis and chemotherapeutic resistance (7, 8). PI3Ks are a family of lipid kinases that serve as mediators of signals generated by many different activated growth factor receptors and adhesion molecules. When activated by growth factors, PI3K generates PtdIns(3,4,5)P₃, which leads to the recruitment and activation of 3'-phosphoinositide-dependent kinase, Akt/protein kinase B serine/threonine kinase, and G-proteins (e.g., Rac-GTPase). The FOXO subfamily of forkhead transcription factors are the most recently identified downstream targets of the PI3K/Akt pathway. Phosphorylation of FOXO proteins by Akt prevents their nuclear translocation, thereby inhibiting transactivation of target genes [e.g., *p27^{Kip1}*, *p130(RB2)*, *cyclin D*, *Bim*, and *Bcl-x_L*] important for cell proliferation, apoptosis, and differentiation (9).

Chronic myeloid leukemia is a malignant clonal hematopoietic stem cell disorder characterized by the expression

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of the Philadelphia chromosome, generated by a reciprocal translocation between the long arms of chromosomes 9 and 22 (10). The Philadelphia translocation culminates in the fusion of a truncated *Bcr* gene to 5' sequences of the *c-Abl* gene (11), which encodes for chimeric Bcr-Abl proteins with constitutively active tyrosine kinase activity (10). In this study, we used drug resistant K562 chronic myeloid leukemia cell lines (12) to study the molecular mechanisms that regulate ABCB1 expression. Our results show that doxorubicin-mediated activation of FOXO3a enhances *ABCB1* gene expression, which may represent an important step in the genesis of MDR.

Materials and Methods

Cell Lines, Culture, and Treatments

The naive K562 and derivatives KD30 and KD225 have been described previously (12). KD30 cells were generated following one-step exposure of K562 cells to 30 nmol/L doxorubicin for 2 weeks and KD225 by multistep exposures with final doxorubicin tolerance of 225 nmol/L. All cells were cultured in RPMI (Sigma UK) with 10 % fetal bovine serum supplemented with 2 mmol/L L-glutamine, penicillin, and streptomycin in 5% CO₂. KD30 and KD225 were maintained in 30 and 225 nmol/L doxorubicin, respectively. One week before experiments were done, doxorubicin was removed from the resistant cell lines. For drug treatment, exponentially growing cells seeded at 5×10^5 /mL were incubated with 1 μ mol/L doxorubicin (Sigma UK) for the indicated times. These drug concentrations used were determined previously to cause morphologic changes indicative of cytotoxic stress.

Plasmids and Transfections

The FOXO3a expression vector pLPC-FOXO3a(wt) and pLPC-FOXO3a(A3) have been described previously (13, 14). The 4-hydroxytamoxifen (4-OHT)-inducible pBabe-puro-FOXO3a(A3):ER and pBabe-puro-ER plasmids have been mentioned previously (13, 14). The psiRNA-FOXO3a expression vector and the control scramble vector were generated by cloning small synthetic oligonucleotides encoding two complementary sequences of 19 nucleotides separated with a loop sequence into the Invivogen psiRNA-h7SKzeo vector (Autogen Bioclear). The target sequence for FOXO3a was 5'-TCACTGCATAGTCGATTCA-3' and the scramble sequence was 5'-CATATGTGCGTACCTAGCA-3' (Invivogen).

For generating stable transfectants, 10 μ g of respective vector DNA were electroporated into 1×10^7 cells (Bio-Rad GenePulser II) in 500 μ L conditioned medium at 0.35 V and 950 μ F. The transfected cells were selected with 1 μ g/mL puromycin (Invivogen) or 100 μ g/mL zeocin (Invivogen) for 14 days and maintained in 0.5 μ g/mL puromycin or 50 μ g/mL zeocin (Invitrogen). Single-cell clones were obtained with serial dilution and tested by Western blotting. Inducibility was checked with the addition of 200 nmol/L 4-OHT (Sigma) at 5×10^5 cells/mL for 24 h. For transient transfection, cells were collected at 72 h unless specified after transfection and puromycin selection for

either Western blot, real-time quantitative PCR (RTQ-PCR), or drug efflux analysis.

Western Blotting and Antibodies

Western blotting was done on whole-cell extracts as described previously (15). Supernatant was collected as cytosolic extract and the pellet was resuspended in high salt buffer with 400 mmol/L NaCl for nuclear extract. Protein concentration was determined by BCA protein assay (Pierce, Perbio Science). Protein (50 μ g) was size fractionated using SDS-PAGE and electrotransferred onto Protran nitrocellulose membranes (Schliecher & Schuell). Antibodies recognizing total FOXO3a (06-951) was purchased from Upstate. Antibodies against p27^{Kip1} (C-19), Bcl-6, actin, β -tubulin, and total Akt were purchased from Santa Cruz Biotechnology (Autogen Bioclear). Antibodies against phospho-Akt (Ser⁴⁷³), phospho-FOXO3a (Thr³²), I κ B α , phospho-I κ B α (Ser³²/Ser³⁶), and Bim were from Cell Signaling Technologies. Lamin B1 was from Abcam. For detection of ABCB1 and ABCC1, anti-P-gp monoclonal C219 (Calbiochem) and anti-MRP1 (Caltag-MedSystems) were used at 1:500. Primary antibodies were detected using horseradish peroxidase-linked anti-mouse, anti-goat, or anti-rabbit conjugates as appropriate (DAKO) and visualized using the enhanced chemiluminescence detection system (Amersham Biosciences).

Flow Cytometric Analysis of P-gp Activity

P-gp activity was examined by determination of intracellular accumulation of the fluorescent anionic dye, calcein-AM (Sigma, M2566; ref. 16) using the Multi-Drug Resistance Quantitation Kit (Sigma) according to the manufacturer's protocol. Briefly, 4×10^6 cells/mL were loaded with calcein-AM (10 nmol/L) with or without MDR/MRP or MRP-specific inhibitors at 37°C for 10 min. The cells were subsequently analyzed for their cellular drug fluorescence using a flow cytometer (FACSCanto, BD Biosciences). Propidium iodide-stained cells were gated out as dead cells before flow cytometric analysis using BD FACSDiva Software (BD Biosciences). F_{\max} is the maximum mean fluorescence intensity (MFI) obtained after treatment with the MDR/MRP inhibitor (Sigma, M2066), whereas F_{MRP} is that obtained after treatment with the MRP-specific inhibitor (Sigma, 2316) and F_0 from without inhibitors. The total MDR activity factor, MAF_T , equals to $100 \times (F_{\max} - F_T) / F_{\max}$, whereas MRP1-related $\text{MAF}_{\text{MRP}} = 100 \times (F_{\text{MRP}} - F_0) / F_{\max}$. The mean MDR1-specific MDR activity was calculated after subtraction of the MRP1-related activity ($\text{MAF}_{\text{MDR}} = \text{MAF}_T - \text{MAF}_{\text{MRP}}$) according to the manufacturer's instruction. Notably, the MAF of <20 indicates negligible MDR and the range of the assay is normally between 0 and 50 units.

Luciferase Reporter Assay

The ABCB1 promoter (-1,202 DSP in pGL2-basic) is a kind gift from Kathleen Scotto (17–19). Cells (10^7) were transfected with 10 μ g ABCB1 promoter and 1 μ g *Renilla* (pRL-TK; Promega) as internal transfection control. For promoter analysis, 24 h after transfection, the cells were collected, washed twice in PBS, and harvested for firefly/*Renilla* luciferase assays using the Dual-Glo Luciferase

reporter assay system (Promega) according to manufacturer's instruction.

Real-time Quantitative PCR

Total RNA was isolated using the RNeasy Mini kit (Qiagen). Total RNA (2 µg) was reverse transcribed using the SuperScript III reverse transcriptase and random primers (Invitrogen), and the resulting first-strand cDNA was used as template in the real-time PCR. All samples were done in triplicates. The following gene-specific primer pairs were designed using the ABI Primer Express software: *FOXO3a*-sense 5'-TCTACGAGTGGATGGTGCCTT-3' and *FOXO3a*-antisense 5'-CGACTATGCAGTGACAGGTTGTG-3', *ABCB1*-sense: 5'-TGGTTCAGGTGGCTCTGGAT-3' and *ABCB1*-antisense: 5'-CTGTAGACAAACGATGAGCTATCACAA-3', and *L19*-sense 5'-GCGGAAGGGTACAGCCAAT-3' and *L19*-antisense 5'-GCAGCCGGCGCAAA-3'. Specificity of each primer was determined using National Center for Biotechnology Information BLAST module. Real-time PCR was done with ABI PRISM 7700 Sequence Detection System using SYBR Green MasterMix (Applied Biosystems). *FOXO3a*, *ABCB1*, and *L19* transcript levels were quantified using the standard curve method. *L19*, a nonregulated ribosomal housekeeping gene, was used as an internal control to normalize input cDNA.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated by using the RNeasy kit (Qiagen), and the concentration and purity of each sample were assessed by absorbance at 260 nm and by the 260/280 nm ratio, respectively. The integrity of the RNA was verified by observing the rRNA bands in ethidium bromide-stained gel under UV irradiation. RNA (20 µg), prepared as described above, was resolved on 1.4% formaldehyde-agarose gels. Following electrophoresis, RNAs were transferred to Hybond-N⁺ membrane (Amersham Biosciences) and subjected to Northern blotting as described previously (20). *ABCB1* and *GAPDH* were detected by hybridization with their respective full-length ³²P-labeled human cDNA probes described previously (12, 20). The probes were labeled with Ready-to-Go DNA labeling beads (Amersham Biosciences) and purified with ProbeQuant G-50 Microcolumns (Amersham Biosciences).

Generation of Human ABCB1 Promoter Deletion Constructs

The full-length promoter construct (-1,202/+118) was digested with *Tth*III and the resultant promoter fragments were blunt-ended and cloned into the *Sma*I site of pGL2 basic (Promega) vector to give construct (-278/+118). The other deletion constructs were generated by PCR, restriction digested, and then cloned into the *Mlu*I and *Bgl*II sites of the pGL2-basic (Promega) reporter vector. The deletion construct inserts (-988/+118), (-624/+118), and (-181/+118), (-142/+118), and (-41/+118) were amplified with the common reverse PCR primer 5'-TCAGATCTACTTAGATCTCGAGCTTGGAAACGG-3' and the forward PCR primers 5'-TCACGCGTGTGGAAACATCCTCAGACTATGCAG-3', 5'-TCACGCGTAAACACGGCATTGATCTGACG-3', and 5'-TCACGCGTAAGCCAGAACATTCCTCCTG-3', 5'-TCACGCGTAACCTGTTTCGCAGTTTC-3', 5'-TCACGCG-

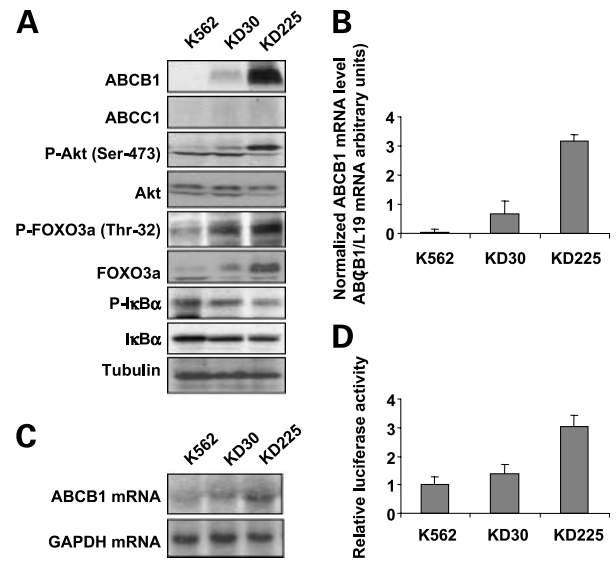


Figure 1. ABCB1 expression correlates with FOXO3a expression and doxorubicin resistance in K562 cells. **A**, the naive and drug-resistant K562 cell lines, as indicated, were analyzed by Western blot using specific antibodies against ABCB1, ABCC1, phospho-Akt (Ser⁴⁷³), Akt, phospho-FOXO3a (Thr³²), FOXO3a, phospho-IκBα, IκBα, and tubulin. **B**, ABCB1 mRNA levels of the K562, KD30, and KD225 cells were analyzed by RT-PCR using ABI PRISM 7700 Sequence Detection System and normalized with L19 mRNA levels. **C**, Northern blotting analysis was done by running 20 µg K562, KD30, and KD225 mRNA on a formaldehyde/formaldehyde agarose gel, transferring onto a Hybond-N⁺ membrane (Amersham Biosciences), and incubating with ³²P-radiolabeled ABCB1 and GAPDH cDNA probes. **D**, K562, KD30, and KD225 cells were transiently transfected with 1 µg human full-length (-1,202/+118) ABCB1 promoter/reporter construct. The transfected cells were harvested 24 h later and assayed for luciferase activity. The relative luciferase activity values are corrected for cotransfected *Renilla* activity. Average of three independent experiments; bars, SD.

TAGTTTCTCGAGGAATCAG-3', and 5'-TCACGCGCAGCCGCTTCGC-3', respectively. Putative forkhead site mutagenesis was done using the Stratagene QuikChange site-directed mutagenesis kit and the oligonucleotide 5'-TCAGATCTTTAGATCTCGAGCTTGGAAACG-3'.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) assay were done as described (14) using 2.5 × 10⁶ inducible FOXO3a(A3):ER K562 cells treated with or without 200 nmol/L 4-OHT for 24 h before harvesting. DNA fragments were purified using the QIAquick Spin Kit (Qiagen). For PCR, 1/25th of the extracted DNA was used and amplified in 25 PCR cycles using specific primers.

PCRs were then done on the purified DNA using the following primers: S1 (-1,190/-964) 5'-TTGCAGTGAGCCGAGATCCT-3' and 5'-CTGCATAGTCTGAGGATGTTTCAC-3', S2 (-989/-747) 5'-AGTGGAACATCCTCAGACTATGC-3' and 5'-CCTGTCCACTATTACTCAAACCTGAGG-3', S3 (-793/-608) 5'-GCAGATTGCAGTACTTTTCC-3' and 5'-GATCAATGCCCCGTGTTTTTC-3', S4 (-619/-363) CGGGCATTGATCTGACGTCTGAAGTT-3' and 5'-CTCCGACCTCTCCAATTCTGTATCACCT-3', S5 (-376/-159) 5'-TGGAGAGGTCGGAGTTTTTG-3' and 5'-TTCCAGGAGGAATGTTTCTGG-3', and S6 (-181/+68)

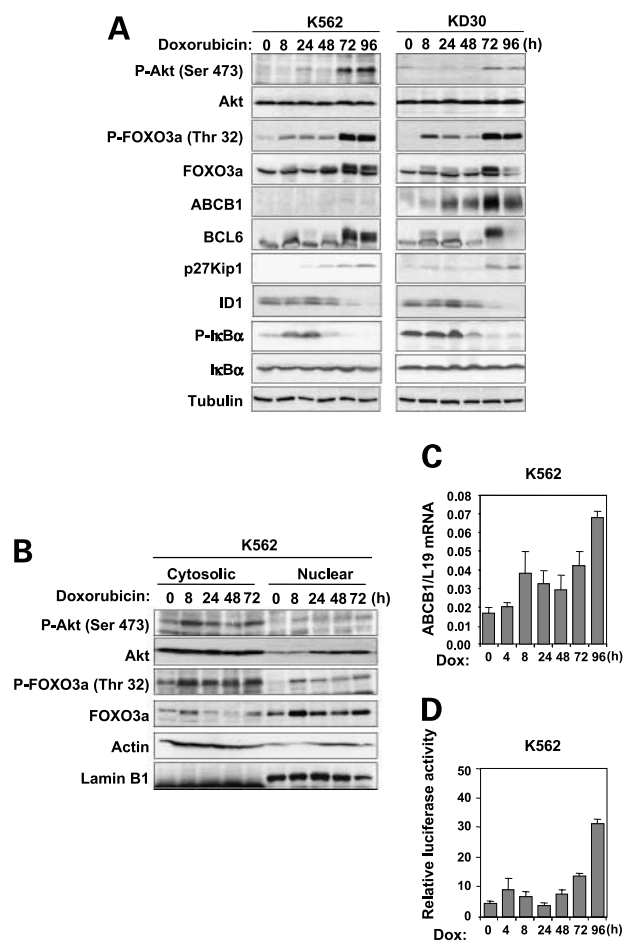


Figure 2. Doxorubicin treatment causes an activation of ABCB1 expression accompanied by an induction of FOXO expression and activity and Akt phosphorylation. K562 and KD30 cells were treated with 1 $\mu\text{mol/L}$ doxorubicin. **A**, protein lysates of K562 were prepared at the times indicated and protein expression levels were analyzed by Western blotting using antibodies against specific antibodies against ABCB1, phospho-Akt (Ser⁴⁷³), Akt, phospho-FOXO3a (Thr³²), FOXO3a, BCL6 p27^{Kip1}, Id1, phospho-I κ B α , I κ B α , and tubulin. **B**, cytoplasmic and nuclear extracts isolated from K562 cells treated with 1 $\mu\text{mol/L}$ doxorubicin were subjected to Western blotting with antibodies against phospho-Akt (Ser⁴⁷³), Akt, phospho-FOXO3a (Thr³²), FOXO3a, actin, and lamin B1. **C**, ABCB1 mRNA levels of the K562 cells were analyzed by RTQ-PCR as described. **D**, the K562 cells were transiently transfected with 1 μg human full-length (-1,202/+118) ABCB1 promoter/reporter construct. The transfected cells were stimulated with doxorubicin 24 h later, harvested at times indicated, and assayed for luciferase activity.

5'-AAGCCAGAACATTCCTCCTG-3' and 5'-TGGAAA-GACCTAAAGGAAACGA-3'. Analysis of the PCR products was done on a standard 2% (w/v) agarose gel by electrophoresis in Tris-acetate EDTA buffers.

Results

Increased Expression and Phosphorylation of FOXO3a Transcription Factor in Doxorubicin-Resistant K562 Cells

To investigate the mechanism responsible for the development of MDR, we examined the expression

patterns of ATP-binding cassette transporters (that is, ABCB1/MDR1/P-gp and ABCC1/MRP1) and components of the PI3K/Akt signaling pathway (that is, Akt and FOXO3a) in the doxorubicin-sensitive naive K562 cell line and the resistant derivative lines KD30 and KD225 (Fig. 1). Western blot analysis showed that ABCB1 expression was undetectable in naive K562 cells but expressed in resistant KD30 and KD225 cells (Fig. 1A). Furthermore, the level of ABCB1 expression correlated with the increase in doxorubicin resistance in these cells. In contrast, ABCC1 levels appear low in both naive and derivative lines. RTQ-PCR analysis showed that ABCB1 transcripts were almost undetectable in the naive K562 cells, more abundant in KD30 cells, and even higher in KD225 cells, consistent with the ABCB1 protein levels in these cells (Fig. 1B). The increased ABCB1 mRNA levels in the resistant cell lines were confirmed by Northern blotting (Fig. 1C). Transient transfection studies showed higher basal ABCB1 promoter activity in drug-resistant KD225 cells when compared with K562 cells (Fig. 1D), suggesting that increased expression of this transport molecule in drug-resistant cells could be due in part to enhanced promoter activity (12). Total FOXO3a protein levels were also higher in KD225 cells when compared with KD30 and naive K562 cells, although a significant proportion was phosphorylated, probably reflecting the higher phospho-Akt levels in the doxorubicin-resistant cell lines (Fig. 1A). Together, these results suggested that the development of drug resistance in K562 clones is associated with increased ABCB1 expression, enhanced PI3K/Akt activity, and higher FOXO3a levels. Previous reports have shown that Akt can induce ABCB1 expression through the nuclear factor- κ B (NF- κ B) pathway (21). However, in the resistant KD30 and KD225 cell lines, there was an inverse correlation between ABCB1 and phospho-I κ B α expression, suggesting that the I κ B α /NF- κ B pathway is unlikely to be responsible for the augmented ABCB1 expression in this cell model (Fig. 1A).

Doxorubicin Induces Akt Phosphorylation as well as FOXO3a and ABCB1 Expression in K562 Cells

To explore further the potential role of PI3K/Akt/FOXO3a pathway in drug resistance, we treated naive K562 chronic myeloid leukemia cells with 1 mol/L doxorubicin and studied the expression and activity of Akt and FOXO3a over a 96-h time course. Western blot analysis showed that doxorubicin caused a net increase in FOXO3a expression, detectable as early as 8 h after treatment (Fig. 2A). Akt activity, as determined by its phosphorylation status and that of FOXO3a, also increased in response to doxorubicin treatment, especially at later time points. However, two lines of evidence indicated that activation of the PI3K/Akt pathway did not inactivate or sequester FOXO3a into the cytoplasm of doxorubicin-treated cells. First, expression of BCL-6 and p27^{Kip1}, FOXO3a targets, increased in doxorubicin-treated cells (13, 22) Moreover, Id1 levels, which recently has been shown to be repressed by FOXO3a (23), declined on doxorubicin treatment. Second, analysis of cytosolic and nuclear protein fractions showed increased nuclear

FOXO3a levels after 8 h of doxorubicin treatment, implying increased FOXO3a transcriptional activity (Fig. 2B). Consistent with previous data, ABCB1 protein was barely detectable in K562 cells treated with doxorubicin (12). ABCB1 is highly regulated at the translational level and the lack of increased ABCB1 protein expression in doxorubicin-treated K562 cells is due to a strong translational block (23), which is only overcome in resistant cells following long-term drug exposure (12). Consistent with this, doxorubicin treatment induced expression of ABCB1 in the resistant cell line KD30 (Fig. 2A). Notably, in the K562 and KD30 cells treated with doxorubicin, there was a decrease in phospho-I κ B α , further suggesting that the I κ B α /NF- κ B pathway is unlikely to be involved in doxorubicin-induced ABCB1 expression in this cell system. Together, the results showed that, despite increased PI3K/Akt signaling, doxorubicin induces nuclear accumulation of FOXO3a in K562 cells and increased transcriptional activity. The data also suggested the possibility that FOXO3a regulates ABCB1 expression in response to doxorubicin.

FOXO3a Activates ABCB1 Transcription in K562 Cells

We next examined if doxorubicin regulates ABCB1 expression at a transcriptional level. RTQ-PCR analysis showed that exposure of K562 cells to doxorubicin caused an induction of ABCB1 mRNA expression, and consistent with the changes in protein levels, this induction was characterized by a discrete rise in transcript levels at 8 h and a more pronounced induction after 96 h (Fig. 2C). Transient transfection experiments showed that the full-length (-1,202/+118) ABCB1 promoter was also inducible on doxorubicin treatment; again, the response appeared biphasic with a more pronounced induction after prolonged exposure to doxorubicin (that is, ≥ 96 h; Fig. 2D). These observations show that doxorubicin-induced ABCB1 expression is mediated at promoter level in addition to regulation at the level of mRNA turnover and translation, as reported previously (12). Furthermore, the biphasic transcriptional response on doxorubicin treatment of K562 cells correlated with the pattern of nuclear FOXO3a accumulation (Fig. 2B). To investigate if FOXO3a regulates ABCB1 expression, we generated a clonal K562 cell line (K562-FOXO3a:ER) stably expressing the transcriptionally active FOXO3a(A3) mutant, which contains alanine substitutions at the Akt phosphorylation sites, fused to the ligand-binding domain of the estrogen receptor (FOXO3a:ER). In untreated cells, FOXO3a:ER resides inactively in the cytoplasm but, on 4-OHT exposure, it translocates to the nucleus where it activates FOXO3a target genes (13). Figure 3A shows that treatment with 4-OHT induces expression of p27^{Kip1} and Bim, well-characterized FOXO target genes (14, 24), in K562-FOXO3a:ER cells but not in K562 cells stably transfected with ER alone. It is notable that the induction of Bim by FOXO3a was modest, consistent with the fact that FOXO3a activation primarily causes cell cycle arrest and differentiation but not apoptosis in K562 cells (25). Not unexpectedly, 4-OHT did not induce ABCB1 expression at protein level in K562-FOXO3a:ER cells, in keeping with the presence of the

strong translational block in the parental cells (12). This conjecture was supported by Northern blotting analysis showing increased ABCB1 transcript levels in 4-OHT-treated K562-FOXO3a:ER cells but not control cells (Fig. 3A, bottom). This finding was verified by RTQ-PCR analysis, where an induction of ABCB1 mRNA was observed after 8 h (Fig. 3B). Similarly, 4-OHT also triggered ABCB1 promoter activity in K562-FOXO3a:ER cells but not in the parental K562 cells or K562 cells harboring ER only (Fig. 3C). Furthermore, ABCB1 promoter activity was also enhanced, albeit discretely, in K562 cells transiently transfected with increasing amounts of wild-type FOXO3a and much more so in transient expression of the constitutively active FOXO3a(A3) mutant (Fig. 3D).

FOXO3a-Stimulated ABCB1 mRNA Expression Is Independent of *De novo* Protein Synthesis and Akt Activity

To determine if *de novo* protein synthesis was required for the FOXO3a-induced ABCB1 transcription, K562-FOXO3a:ER cells were pretreated with or without cycloheximide for 30 min and then stimulated with or without 4-OHT. RTQ-PCR analysis revealed that cycloheximide treatment alone enhances ABCB1 mRNA levels (Fig. 4A) as reported previously (26). However, cycloheximide failed to

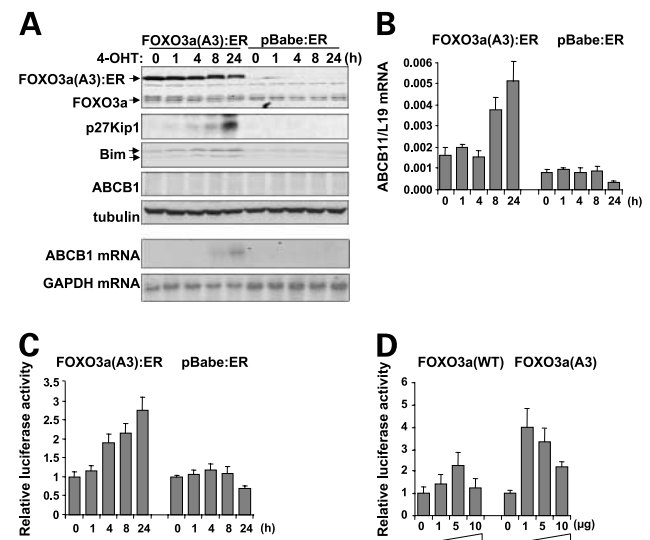


Figure 3. Activation of FOXO3a induces ABCB1 gene promoter activity and mRNA expression in K562 cells. K562-FOXO3a(A3):ER and K562-ER cells were treated with 4-OHT for the indicated times. **A**, cell lysates were prepared at the times indicated. The expression of FOXO3a, p27^{Kip1}, ABCB1, Bim, and tubulin was analyzed by Western blotting. Total RNA was extracted and analyzed for ABCB1 mRNA expression by Northern blotting as described previously. **B**, RNA from **A** was analyzed to quantify ABCB1 mRNA levels using RTQ-PCR. **C**, K562-FOXO3a(A3):ER and K562-ER cells as well as the parental K562 cells were transiently transfected with 1 μ g of the human full-length (-1,202/+118) ABCB1 promoter/reporter construct. The transfected cells were stimulated with 4-OHT 24 h later, harvested at times indicated, and assayed for luciferase activity. **D**, K562 cells were transiently transfected with 1 μ g of the human full-length ABCB1 promoter/reporter construct together with increasing amounts (0, 1, 5, and 10 μ g) of pLPC FOXO3a-WT or -A3. Cells were harvested 24 h after transfection and assayed for luciferase activity.

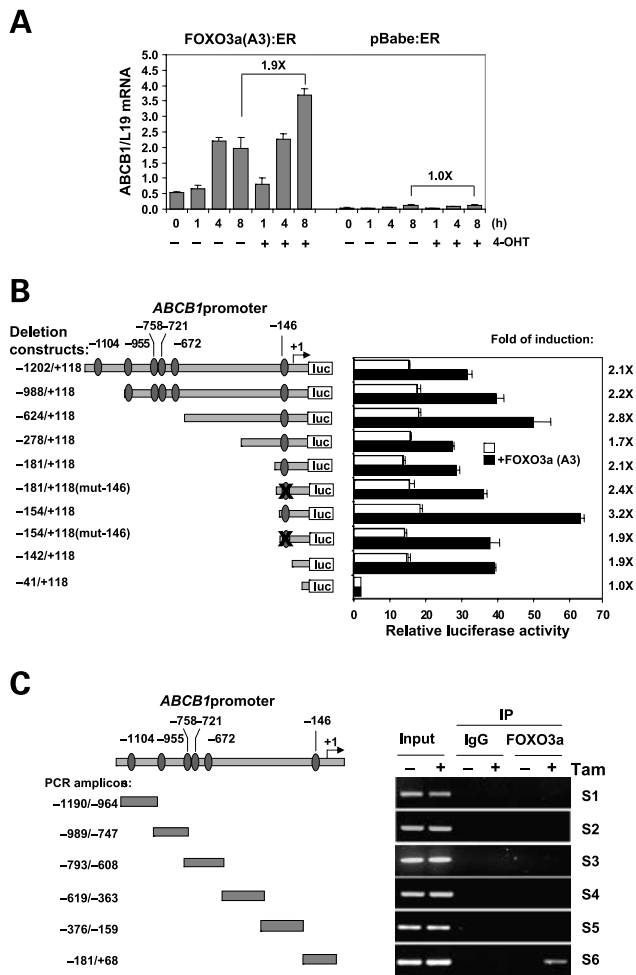


Figure 4. FOXO3a induces *ABCB1* mRNA expression independent of *de novo* protein synthesis and through its promoter. **A**, K562-FOXO3a(A3):ER and K562-ER cells were pretreated with 100 μ mol/L cycloheximide for 30 min before stimulation with 4-OHT for the indicated times. Total RNA was extracted and analyzed for *ABCB1* mRNA expression of *ABCB1* using RTQ-PCR as described. Average of three experiments; bars, SD. **B**, comparison of the ability of FOXO3a to transactivate different deletion constructs of the human *ABCB1* promoter. Top, a schematic representation of the human *ABCB1* promoter showing the locations of putative forkhead response elements. K562 cells were transiently transfected with different human *ABCB1* promoter-luciferase reporter constructs (molar equivalence of 1 μ g of the full-length human *ABCB1* promoter) together with 1 μ g pLPCFOXO3a(A3). Cells were harvested 24 h after transfection and assayed for luciferase activity. Right, fold induction of the *ABCB1* promoter by FOXO3a. **C**, ChIP analysis of the human *ABCB1* promoter. Protein-DNA complexes from untreated and 4-OHT-treated K562 FOXO3a(A3):ER cells were subjected to immunoprecipitation with antibodies against IgG (nonspecific) FOXO3a as indicated. After cross-link reversal, the coimmunoprecipitated DNA was amplified by PCR using the indicated primers and resolved in 2% agarose gels.

abrogate the additional induction of *ABCB1* transcripts in K562-FOXO3a:ER cells treated with 4-OHT for 8 h. The data therefore suggested that *ABCB1* expression is, at least partially, under FOXO3a control as the response to 4-OHT did not require *de novo* synthesis of other transcription factors. Using a K562 cell line harboring the 4-OHT-inducible Akt expression vector Akt(Myr):ER (Supplemen-

tary Fig. S1),⁴ we further confirmed that enhanced *ABCB1* expression is mediated directly by FOXO3a rather than indirectly through increased Akt activity.

FOXO3a Binds to the Proximal Region of the *ABCB1* Promoter to Activate Transcription

To identify the transcriptional elements in the *ABCB1* promoter responsive to FOXO3a, a series of deletion/mutation reporter constructs were generated and cotransfected into K562 cells together with either the constitutively active FOXO3a(A3) mutant or an empty control vector. As shown in Fig. 4B, deletion of the 5' distal region (-1,202 to -142 bp) had little effect on either basal or FOXO3a(A3)-dependent *ABCB1* promoter activity. A potential FOXO3a-responsive element was identified at position -146; however, when mutated or deleted, *ABCB1* promoter activity and its induction by FOXO3a(A3) was maintained. These results suggested that the proximal region of the *ABCB1* promoter (-142/+118) is required for transactivation by FOXO3a. Consistent with this idea, the basal promoter activity was significantly lower and induction by FOXO3a(A3) lost when cells were transfected with reporter constructs (-41/+118) that lack this proximal promoter region (Fig. 4B). We next investigated the endogenous promoter occupancy by FOXO3a using ChIP. To this end, K562-FOXO3a:ER cells were cultured in the presence or absence of 4-OHT for 4 h, the chromatin precipitated with a specific FOXO3a antibody, and analyzed by PCR using primer sets that covered the entire length (-1,190/+68) of the *ABCB1* promoter. Figure 4C is a representative result of three independent ChIP experiments. Consistent with the cotransfection studies, ChIP analysis showed that FOXO3a binding was confined to the proximal region of the *ABCB1* promoter (-181/+68). Together, the results establish that FOXO3a regulates *ABCB1* expression through binding and transactivation of its proximal promoter region.

FOXO3a Regulates *ABCB1* Expression in the Drug-Resistant K562 Cell Lines

It was not possible to show that FOXO3a regulates *ABCB1* expression at protein level in K562-FOXO3a:ER cells because of the strong translational block (12). On the other hand, drug-resistant KD225 and KD30 cells express substantial levels of *ABCB1* protein but are resistant to establishment of permanent transfectants. To show definitively that FOXO3a enhances *ABCB1* expression, KD225 cells were transfected with expression vectors that encode either for the FOXO3a:ER fusion protein or ER, cultured for 10 days in the presence of 1 μ g/mL puromycin, and then stimulated with or without 4-OHT for 24 h. Western blot, RTQ-PCR, and promoter analyses showed that 4-OHT induced *ABCB1* expression at protein (Fig. 5A), mRNA (Fig. 5B), and gene promoter (Fig. 5C) levels, respectively, in FOXO3a:ER but not ER-expressing cells. Dose-dependent induction of *ABCB1* by FOXO3a:ER was also confirmed in the KD30 cell line (Fig. 5D). To investigate if FOXO3a activation induces

⁴ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org>).

functional ABCB1 protein, we next examined ABCB1 (P-gp) expression and activity in KD30 cells following FOXO3a induction. To this end, KD30 cells were transfected with expression vectors that encode either for the FOXO3a:ER fusion protein or ER, selected with puromycin for 10 days, and then stimulated with or without 4-OHT for 24 h before analysis of ABCB1 expression and transporter efflux activity using Western blotting and flow cytometry (Supplementary Fig. S2),⁴ respectively. Despite the endogenous FOXO3a expression, both ABCB1 expression and transporter activity were increased on FOXO3a:ER expression in KD30 cells (Fig. 6A). This induction (MAF = 30; 50% versus the control transfected with pBabe-puro-ER) was observed even in the absence of 4-OHT treatment and can be accounted for by the forced nuclear localization of the FOXO3a:ER fusion protein when highly overexpressed. Nevertheless, treatment with 4-OHT further enhanced ABCB1 expression and increased its activity by 76% (MAF = 35) in FOXO3a:ER-transfected cells but not in the ER control cells (Fig. 6A and B). To extend these findings, we next examined the effects of FOXO3a silencing on ABCB1 expression. To this end, KD225 cells were transfected with a short hairpin RNA vector targeting FOXO3a or a scramble control vector and harvested 72 h later. FOXO3a knockdown was confirmed by RTQ-PCR and Western blot analysis (Fig. 6C). As expected, silencing of FOXO3a expression in KD225 cells resulted in a corresponding down-regulation of ABCB1 expression at mRNA and protein levels (Fig. 6C). Furthermore, we also found that silencing of FOXO3a also significantly reduced the ABCB1-related transporter efflux activity (Fig. 6D; Supplementary Fig. S3),⁴ thereby unequivocally showing that *ABCB1* is a transcriptional target gene of FOXO3a.

Discussion

Chemotherapy is the preferred treatment for hematopoietic cancers, metastatic diseases, and most solid tumors. The majority of cancers are initially sensitive to chemotherapy; however, in most cases, acquired resistance invariably occurs, leading to disease relapse through expansion of MDR cancer cells. Thus, defining the mechanisms responsible for development of MDR is a priority in research into anticancer chemotherapy. Using the naive K562 chronic myeloid leukemia cell line and its doxorubicin-resistant derivatives KD30 and KD225 as models, we found that drug resistance is associated with an induction in FOXO3a and ABCB1 expression. Consistent with previous data, we found that doxorubicin treatment induces ABCB1 expression in K562 cells and that doxorubicin resistance is not related to ABCC1 (MRP1) expression in K562 cells (27–29). In addition to increasing the levels of phosphorylated Akt and FOXO3a, doxorubicin treatment also increased the levels of transcriptionally active nuclear FOXO3a. This is not necessarily paradoxical as phosphospecific Akt and FOXO3a antibodies indicate the levels of inactive phosphorylated FOXO3a but do not provide information on the abundance of transcriptionally active unphosphory-

lated FOXO3a. Additionally, doxorubicin treatment and cellular stress increase phosphorylation and subsequent activation of JNK (30), which in turn has been shown to activate FOXO proteins by phosphorylating multiple sites. Importantly, JNK activation of FOXO3a is not only opposite to but also dominant over Akt-mediated inactivation (31).

Treatment of FOXO3a:ER K562 cells with 4-OHT was sufficient to induce ABCB1 expression, suggesting that the increase in FOXO3a activity is causative for the induction of ABCB1 on doxorubicin exposure. ABCB1 confers drug resistance by increasing the ability of cells to pump out hydrophobic cytotoxic drugs, such as doxorubicin (28). Thus, although induction of FOXO3a in response to anticancer drug treatment may cause cell cycle arrest and apoptosis (32), prolonged FOXO3a activation and increased ABCB1 expression may be critical to the development of acquired MDR. However, the molecular mechanism by which high levels of FOXO3a expression and activity are maintained in resistant cells remains to be elucidated. Recently, the ABCB1 expression and anticancer drug

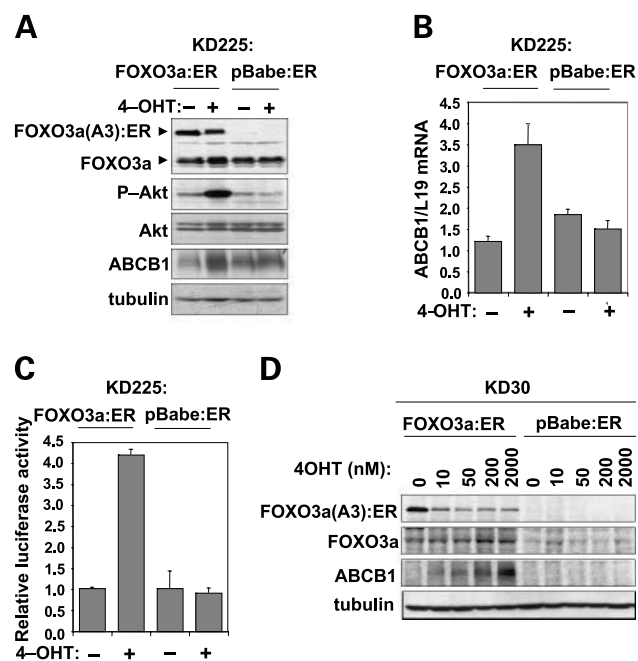


Figure 5. Effect of FOXO3a induction on ABCB1 expression and gene promoter activity in KD225 cells. KD225 or KD30 cells were transiently transfected with either pBabe-puro-FOXO3a(A3):ER or pBabe-puro-ER constructs and selected with puromycin for 10 days. The transfected cells were then treated with 4-OHT for 24 h. **A**, KD225 cell lysates were prepared from these transfectants. The expression of FOXO3a, ABCB1, and tubulin was analyzed by Western blotting. **B**, total RNA was extracted from KD225 cells and analyzed for *ABCB1* mRNA expression of *ABCB1* using RTQ-PCR as described. **C**, transfected KD225 cells were transiently transfected with 1 μ g of the human full-length (-1,202/+118) *ABCB1* promoter/reporter construct before induction with 4-OHT for 24 h. The transfected cells were then harvested and assayed for luciferase activity. **D**, KD30 cell lysates were prepared from the transfectants and the expression of FOXO3a, ABCB1, and tubulin was analyzed by Western blotting.

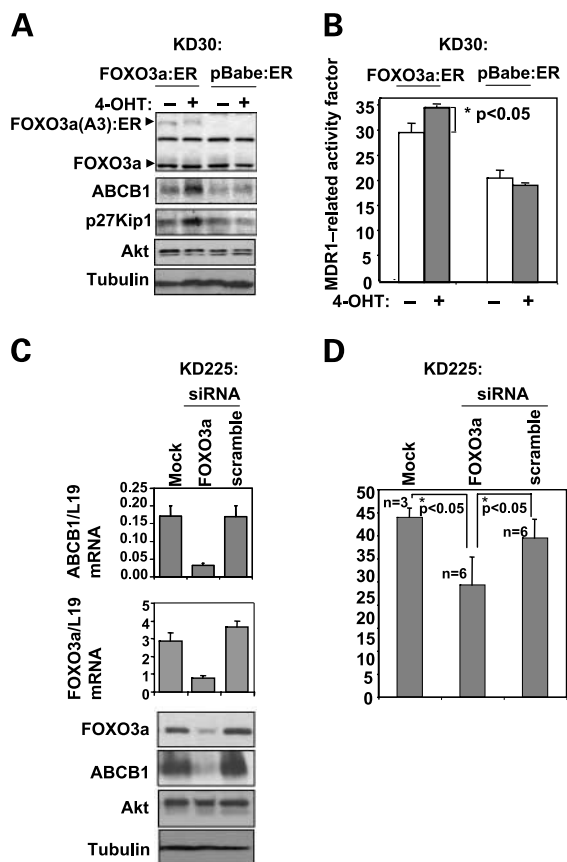


Figure 6. Effects of FOXO3a induction and silencing of FOXO3a expression on ABCB1 activity and expression in KD30 and KD225 cells. KD30 cells were transfected with FOXO3a(A3):ER or pBabe-puro-ER were treated with or without 200 nmol/L 4-OHT for 24 h. **A**, cell lysates were prepared from the transfectants and the expression of FOXO3a, ABCB1, p27^{Kip1}, and tubulin was analyzed by Western blotting. **B**, MDR activity analysis was done using the Multi-Drug Resistance Quantitation Kit (Sigma UK). Fluorescence of intracellular calcein-AM was analyzed by flow cytometry. Each study was done in triplicate and one set of experiments is shown in Supplementary Fig. S2.⁴ MDR1-related resistance activity factors calculated for KD30 transfected with FOXO3a(A3):ER or pBabe-puro-ER with or without 4-OHT are shown (mean \pm SD). Compared with the pBabe-puro-ER-transfected controls, transfection with FOXO3a(A3):ER with and without 4-OHT resulted in 50% and 76% increases in the mean, respectively. KD225 cells were transiently transfected with the psiRNA-FOXO3a plasmid or a control scramble plasmid. Cells were harvested 72 h after transfection. **C**, total RNA was extracted from KD225 cells and analyzed for FOXO3a and ABCB1 mRNA expression using RTQ-PCR as described in Fig. 1 and normalized to the level of L19 RNA. Transfected cells were also analyzed by Western blot using specific antibodies as indicated. **D**, MDR1-related drug efflux activity analysis was done and fluorescence of intracellular calcein-AM was analyzed by flow cytometry. Each study was done at least in triplicate and one set of experiments is shown in Supplementary Fig. S3.⁴ MDR1-related efflux activity factors calculated for KD225 mock-transfected and transfected with FOXO3a short hairpin RNA or control short hairpin RNA are shown (mean \pm SD). *t* test was done on the results, and the decrease in MDR1-related efflux activity is significant compared with the controls.

resistance have been linked to the expression of the NAD-dependent deacetylase Sirt1 (33). This observation is consistent with the notion that FOXO3a regulates ABCB1 expression as the longevity regulatory protein Sirt1 is well

known to modulate the transcriptional activity of FOXO proteins (34–36).

The regulation of *ABCB1* expression is complex and has been shown previously to be mediated at least at two distinct levels, mRNA turnover and translational regulation (12). We now show that *ABCB1* expression is also regulated at promoter level in response to drug challenges. However, this mechanism does not cause increased *ABCB1* protein expression in K562 due to a strong translational block, which is overcome in the resistant KD30 and KD225 cells as a result of long-term exposure to chemotherapeutic agents (12). PI3K/Akt activity has been shown previously to induce the expression of the rat *ABCB1* gene via NF- κ B activation (21). However, our results show Akt activation does not induce *ABCB1* mRNA expression. Moreover, induction of *ABCB1* expression by doxorubicin does not appear to involve NF- κ B. Therefore, it is unlikely that the PI3K/Akt/NF- κ B pathway directly regulates *ABCB1* in this system. Instead, our promoter and ChIP analyses defined a promoter region proximal to the transcription start site to be responsive to FOXO3a induction. This region overlaps with binding sites for the basal transcriptional factors and has been identified previously as the “*MDR1* enhancosome,” where various environmental signals, including those from hormones, histone deacetylase inhibitors, chemotherapeutic agents, and phorbol esters, etc., converge to modulate *ABCB1* transcription (37). The promoter region consists of an inverted CCAAT box (-79/-75, also known as Y-box) and a GC-rich element (-56/-43), which interact with trimeric transcription factor NF-Y and Sp family members (that is, Sp1 and Sp3), respectively (18). Consistent with our results, previous reports have shown that mutation or removal of one or both of the elements leads to a complete loss of *ABCB1* transcription (37). Interestingly, these two promoter elements have been shown to cooperate to recruit histone acetyltransferases, which have been shown to interact with FOXO proteins (38, 39). Together, our data suggest that FOXO3a is recruited to the *ABCB1* promoter indirectly through these and other basal transcription factors, which bind to the “*MDR1* enhancosome” region. Furthermore, it is also possible that FOXO3a can also bind to elements distant to the transcription start site and our promoter sequence.

In summary, this study leads us to propose a novel mechanism that underlies MDR, which involves FOXO3a as both sensor and regulator of cytotoxic stress caused by chemotherapeutic agents. We show that FOXO3a activity is induced in response to doxorubicin and that sustained activation of this transcription factor promotes cell survival by enhancing expression of *ABCB1*, which functions as an effluent pump for hydrophobic cytotoxic drugs. Our findings suggest that targeting FOXO3a could be a novel strategy for the prevention of MDR in cancer.

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