FOXO3a and Posttranslational Modifications Mediate Glucocorticoid Sensitivity in B-ALL

Francesca Consolaro1,2, Sadaf Ghaem-Maghami1, Roberta Bortolozzi2, Stefania Zona1, Mattaka Khongkow1, Giuseppe Basso2, Giampietro Viola2, and Eric W.-F. Lam1

Abstract

Glucocorticoids are widely used to treat B acute lymphoblastic leukemia (B-ALL); however, the molecular mechanism underlying glucocorticoid response and resistance is unclear. In this study, the role and regulation of FOXO3a in mediating the dexamethasone response in B-ALL were investigated. The results show that FOXO3a mediates the cytotoxic function of dexamethasone. In response to dexamethasone, it was found that FOXO3a translocates into the nucleus, where it induces the expression of downstream targets, including p27Kip1 and Bim, important for proliferative arrest and cell death in the sensitive RS4;11 and SUP-B15 B-ALL cells. FOXO3a activation by dexamethasone is mediated partially through the suppression of the PI3K/Akt signaling cascade. Furthermore, two posttranslational modifications were uncovered, phosphorylation on Ser-7 and acetylation on Lys-242/245. These posttranslational events can be exploited as biomarkers for B-ALL diagnosis and as drug targets for B-ALL treatment, particularly for overcoming the glucocorticoid resistance.

Implications: FOXO3a and its posttranslational regulation are essential for dexamethasone response, and targeting FOXO3a and sirtuins may enhance the dexamethasone-induced cytotoxicity in B-ALL cells. Mol Cancer Res; 13(12); 1578–90. ©2015 AACR.

Introduction

B acute lymphoblastic leukemia (B-ALL) is one of the most common clonal malignant diseases in children, and it stems from unchecked proliferation of lymphoid progenitor cells. Glucocorticoids are the most effective and commonly used agents for treatment of B-ALL; however, their efficacy is often hampered by the development of resistance (1). In fact, glucocorticoid sensitivity at diagnosis has a major bearing on the eventual clinical outcome for patients with childhood B-ALL (1). In consequence, uncovering the mechanisms that underlie dexamethasone responsiveness will not only help identify reliable biomarkers for early diagnosis and for predicting disease relapse but also aid the design of targeted therapies to overcome glucocorticoid resistance in B-ALL. Despite this, the molecular mechanisms underlying glucocorticoid response and resistance remain poorly understood (1).

FOXO3a (previously known as FKHR-L1) is a member of the Forkhead family of transcription factors, which share a distinct forkhead DNA-binding domain (2). FOXO3a plays an important role in proliferation, apoptosis, autophagy, metabolism, inflammation, differentiation, and stress resistance (3, 4). The stability, subcellular localization, the DNA-binding affinity, and the transcriptional activity of FOXO3a are primarily regulated by a complex array of posttranslational modifications (5). FOXO3a is primarily regulated by the PI3K/Akt (PKB) signaling pathway (6–8). In the presence of growth factors, the PI3K/Akt axis is activated, and Akt phosphorylates the FOXO3a at three sites, Thr-32, Ser-253, and Ser-315, triggering the 14-3-3 protein binding, nuclear export, and subsequent degradation via the ubiquitin-mediated proteasome pathway (6–8). The Ser-315 residue locates within the nuclear export domain, and its phosphorylation has been shown to be important for FOXO3a nuclear export (9). The MAP kinase ERK has also been shown to phosphorylate FOXO3a on Ser-294, Ser-344, and Ser-425, driving its proteasomal degradation via ubiquitin E3 ligase, MDM2 (10). Conversely, the phosphorylation mediated by the other two MAPKs, p38 and JNK (c-jun-NH2-kinase), promotes FOXO3a nuclear localization and transcriptional activity. The stress-activated protein kinase p38 phosphorylates FOXO3a on Ser-7 promoting its nuclear localization, whereas JNK phosphorylates the FOXO3a-related FOXO4 at Thr-447 and Thr-451 (11, 12). Furthermore, JNK can also activate FOXO3a indirectly by repressing the PI3K/Akt activity (13). Resembling phosphorylation, acetylation can both promote and decrease the transcriptional activity of FOXO3a. FOXO
acetylation is co-ordinately controlled by the histone/lysine acetyltransferase and deacetylases. Co-precipitation analysis revealed that the acetyltransferase CBP/p300 binds the first 52 amino acids of the N-terminal region of FOXO3a (14). Interestingly, p300 also directly acetylates FOXO transcription factors at several conserved lysine residues, Lys-242, Lys-245, and Lys-262 of FOXO3a (15–17). However, p300-dependent acetylation has been shown to have a dual function in FOXO-mediated transcription; either it can attenuate FOXO transcriptional activity or it can promote the recruitment and assembly of the transcriptional machinery, increasing their DNA-binding ability and transcriptional activity (18, 19). FOXO3a acetylation status is further modulated by class III histone/lysine deacetylases (sirtuins), including SIRT1, SIRT2, SIRT3, and SIRT6 (20). For example, it has been demonstrated that SIRT1 can antagonize the p300-mediated acetylation and activation of FOXO3a (21). In agreement, studies conducted in breast cancer have also reported that SIRT6 overexpression correlates with FOXO3a inactivation and that SIRT6 depletion sensitizes breast cancer cells to both paclitaxel and epirubicin treatment (22).

FOXO3a functions primarily as a tumor suppressor in a number of hematologic malignancies, playing a crucial role in controlling cell-cycle arrest, apoptosis, and self-renewal of hematopoietic progenitor cells (23, 24). For example, hyperphosphorylation of FOXO3a has been shown to be correlated with adverse prognosis in acute myelogenous leukemia (AML; ref. 25). FOXO3a activation can induce apoptotic cell death in therapy-resistant T-cell acute lymphoblastic leukemia (T-ALL) cells (26). Furthermore, deletion of FOXO1/3a/4 in mice has been found to lead to the development of T-cell lymphoma (27). Hitherto, the involvement of FOXO3a in B-ALL and its role in treatment response has remained undefined. Nevertheless, it has been shown that in glucocorticoid-resistant patients with B-ALL, Bim, a downstream FOXO3a target (28), is downregulated compared with their sensitive counterparts (29). Moreover, FOXO3a expression has also been demonstrated to predict bortezomib sensitivity and patient remission in B-ALL (30). Together these findings led us to hypothesize that FOXO3a has a key role in glucocorticoid sensitivity in B-ALL. In this study, we investigated the role and regulation of FOXO3a in mediating the dexamethasone response in B-ALL. More specifically, we intended to determine how phosphorylation and acetylation, two major FOXO3a posttranslational modifications, influence FOXO3a subcellular localization and function.

**Materials and Methods**

**Cells, patient samples, and cell cultures**

B-ALL patient samples were obtained after informed consent according to the tenets of the Declaration of Helsinki. The study was approved by the Italian Association of Pediatric Onco-Hematology (AIEOP). All analyzed BCP-ALL samples were collected at the time of diagnosis before treatment, after Ficol-Hypaque (Pharmacia) separation of mononuclear cells as described previously (31). Patient samples were classified into different groups by using AIEOP criteria [poor responder patients (PPR), patients with at least 1,000 blast cells/μl peripheral blood after 7 days of prednisone monotherapy]. Human leukemia cell lines, REH (resistant) and RS4;11, SUP-B15 (sensitive), were grown in RPMI-1640 medium (Gibco) all supplemented with 115 units/ml penicillin G (Gibco), 115 μg/ml streptomycin (Invitrogen), 10% FBS (Invitrogen), and maintained at 37°C in a humidified atmosphere with 5% of CO2.

**Drug treatment**

Cells were grown to 60% confluence and then treated with dexamethasone (D4902; Sigma UK), SP600125 (S7979, SelleckChem), SB202190 (S1077, SelleckChem), PD98059 (S1177, SelleckChem), PDF-170, EX-527 (S1541, SelleckChem), and sirtinol (S7942, Sigma UK) at a stock concentration of 10 mmol/L and then used at different concentrations.

**MTT proliferative assay**

Cell proliferation was assessed by MTT (Sigma-Aldrich) assay after treatment. Equal numbers of cells were plated in triplicate in a 96-well plate and incubated with MTT (Sigma-Aldrich) for 4 hours. Absorbance was measured at 562 nm using Victor™ 1420 Multilabel Counter (PerkinElmer).

**Flow cytometric analysis of cell-cycle distribution**

For flow cytometric analysis of DNA content, 5 × 10⁵ of REH, RS4;11, and SUP-B15 cells in exponential growth were treated with dexamethasone at 1 μmol/L concentration for 24 hours. After the incubation period, cells were collected, centrifuged, and fixed with ice-cold ethanol (70%). Cells were then treated with lysis buffer containing RNase A and 0.1% Triton X-100 and then stained with propidium iodide (PI). Samples were analyzed on a Cytomix FC500 (Beckman Coulter) flow cytometer. DNA histograms were analyzed using FlowJo software (Millenyi Biotec Ltd.).

**Apoptosis assay**

Cell viability assay was performed by flow cytometric analysis of cells double stained with Annexin V/APC and PI using the Annexin V–FLUOS Staining Kit (Roche), following the manufacturer’s instructions. The FACSCalibur Flow Cytometer (BD Biosciences) with FACS Flow Supply System was used to measure the surface exposure of phosphatidylserine (PS) on apoptotic cells according to the manufacture Annexin-V Fluos (Roche Diagnostics). Cell populations were analyzed using FlowJo software.

**Subcellular fractionation, immunoprecipitation, immunoblotting, and immunofluorescent staining**

These procedures were performed as previously described (32). For details, see Supplementary Materials and Methods.

**Real-time quantitative PCR (RT-qPCR)**

Total RNA was isolated from frozen cell pellets using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Also, see Supplementary Materials and Methods.

**Statistical analysis**

Results are presented as the mean ± SD. The differences between different conditions were analyzed using the two-sided Student’s t test. \( P < 0.05 \) was considered significant. *, \( P \leq 0.05; **, P \leq 0.01; ***, P \leq 0.001.

**Results**

Dexamethasone treatment induces FOXO3a activation in B-ALL–sensitive cells

Deregulation of the PI3K/Akt/FOXO3a pathway has been shown to be involved in cancer development and contribute to
therapy resistance in different hematologic malignancies (33–36). To explore the potential role played by FOXO3a in dexamethasone response, we first examined the expression of both total and phosphorylated forms of FOXO3a in one dexamethasone-resistant B-ALL cell line (REH) and two dexamethasone-sensitive cell lines (RS4;11 and SUP-B15) following treatment with 1 μmol/L dexamethasone for 24 hours (Fig. 1A). Dose–response curves were previously obtained by treating cells for 72 hours with a range of dexamethasone concentrations (0–100 μmol/L), and the results confirmed the dexamethasone sensitivity of the B-ALL cells (Fig. 1B; Supplementary Fig. S1 and Supplementary Data). Western blot analysis showed that baseline FOXO3a is more hyperphosphorylated at Akt-targeted sites, inducing Thr-32 and Ser-315, in the dexamethasone-resistant REH cells compared with the sensitive counterparts, RS4;11 and SUP-B15 (Fig. 1A). The resistant REH cells expressed comparable levels of FOXO3a, P-FOXO3a (S315), P-FOXO3a (T32), P-FOXO3a (S253) before and after dexamethasone treatment. In contrast, in the sensitive cells, RS4;11 and SUP-B15, dexamethasone caused the downregulation of FOXO3a phosphorylation at Thr-32, Ser-253, and Ser-315, indicative of FOXO3a nuclear relocation and activation (4, 36). In response to dexamethasone, FOXO3a activation in the sensitive cells was further confirmed by the increased expression of the FOXO3a target Bim and the consequent activation of apoptosis, as evidenced by caspase-3, -7, and -9 cleavage and activation. In concordance, the expression of another FOXO3a downstream target p27Kip1 was also increased in sensitive cells following dexamethasone treatment. Notably, the p27Kip1 and Bim mRNA levels were also induced by dexamethasone in the sensitive and not the resistant cells, further supporting their transcriptional induction by FOXO3a (Fig. 1C). Interestingly, unlike FOXO3a, the expression of the other FOXO family members, FOXO1 and FOXO4, was expressed at low levels in the sensitive cells before and after dexamethasone, suggesting that FOXO1 and FOXO4 are unlikely to have a crucial part to play in dexamethasone response (Fig. 1A). Together, these results suggest that after treatment, FOXO3a becomes hypophosphorylated at Akt-dependent sites and consequently activated in the sensitive B-ALL cells. Conversely, in the resistant cells, FOXO3a remained phosphorylated and inactive. We next analyzed the expression of the components of PI3K/Akt signaling pathway after dexamethasone treatment (Fig. 1D). In sensitive cells, the Akt activator mTOR became dephosphorylated (at S2448), thus less active, after dexamethasone treatment. Accordingly, these data suggest that dexamethasone treatment leads to P38/Akt inactivation in sensitive cells, and as a consequence, FOXO3a becomes hypophosphorylated and activated. In contrast, FOXO3a remains phosphorylated and inactive in the resistant REH cells.

FOXO3a is hyperphosphorylated at Ser-315 in PPR

To confirm the physiologic relevance of our findings from the B-ALL cell lines, bone marrow cells from 5 B-ALL pediatric patients responsive to prednisone (Prednisone good responder, PGR) and 5 B-ALL pediatric patients not responsive to prednisone (Prednisone poor responder, PPR) were studied by Western blotting. In agreement with the data obtained from the cell culture models (Fig. 1E), Western blotting results showed that while there was little difference in FOXO3a levels between the two groups, FOXO3a was generally more phosphorylated on the Akt-targeted Ser-315 residue in PPR individuals (1–5) compared with patients with PGR (refs. 6–10; Fig. 1E). Collectively, these data indicated that FOXO3a at baseline conditions is more phosphorylated and therefore, less active in PPR, providing further evidence that FOXO3a has a role in modulating dexamethasone sensitivity.

Dexamethasone treatment leads to cell-cycle arrest and cell death in drug-sensitive B-ALL cells

To explore further the potential role of FOXO3a in dexamethasone response and resistance, we next studied the effects of dexamethasone on the B-ALLs by PI staining and flow cytometry. Consistent with the proliferation assays, the cell-cycle analysis showed that whereas there were no significant shifts in cell-cycle distribution of REH cells in response to dexamethasone, considerable cell-cycle phase changes indicative of cell proliferative arrest and cell death were observed for the sensitive RS4;11 and SLIP-B15 cells (Fig. 2A–C). Accordingly, in response to dexamethasone, there were also increases in sub-G1 cell population for the sensitive and not the resistant cells (Fig. 2B). We also detected a significant increase in G2–M population in SUP-B15 cells following treatment (Fig. 2C). Upon dexamethasone, we also observed a significant decrease in RS4;11 cells in S-phase with a corresponding increase in G2–M phase cells (Fig. 2C). The G2–M arrest observed can be because of the fact that FOXO3a negatively regulates the expression of genes, including cyclin B and FOXM1 (Fig. 1A) important for G2–M progression (2, 36). Collectively, these data suggest that dexamethasone arrests cell-cycle progression, particularly in G2–M phase, and induces cell death in the sensitive but not resistant B-ALL cells. It is also notable that this cell-cycle arrest and cell death induced by dexamethasone in the sensitive cells correlated with FOXO3a activation (Fig. 1), providing further evidence of a role of FOXO3a in the cytostatic and cytotoxic functions of dexamethasone in B-ALLs.

FOXO3a translocates to the nucleus after dexamethasone treatment in sensitive B-ALL cells

As Akt phosphorylation of FOXO3a promotes its relocation to the cytoplasm, we next analyzed whether dexamethasone treatment also influences FOXO3a subcellular localization. To this end, B-ALL cells were either untreated or treated with dexamethasone for 24 hours, fixed and stained with a specific FOXO3a fluorescent–conjugated antibody. The results showed that upon dexamethasone treatment, FOXO3a translocated from cytoplasm into nucleus in the sensitive cell lines RS4;11 and SUP-B15, but not in the resistant REH cells (Fig. 2D). To confirm this further, we examined the expression of FOXO3a in the cytoplasmic and nuclear fractions of the sensitive RS4;11 and resistant REH B-ALL cells in response to dexamethasone treatment. In agreement, Western blotting results showed that dexamethasone treatment increased the nuclear FOXO3a and p27Kip1 expression, the cytoplasmic Bim expression but reduced the nuclear P-FOXO3a, Akt, FOXM1, and Aurora B expression substantially in the sensitive and not the resistant cells (Fig. 2E). Together these results reinforce the idea that FOXO3a is activated in the dexamethasone-sensitive and not in the -resistant B-ALL cells.

FOXO3a is a critical mediator of dexamethasone-induced apoptosis in B-ALL

To test whether FOXO3a is essential for the cytotoxic function of dexamethasone in B-ALL, we depleted its expression using a smart pool of FOXO3a siRNA and assayed for the ability of dexamethasone to induce cell death in the sensitive RS4;11 cells. After 48 hours of transfection with FOXO3a siRNA or nonsilencing control
Figure 1.
Dexamethasone induces FOXO3a activation and proliferative arrest in sensitive but not resistant B-ALL cells. The REH, RS4;11, and SUP-B15 B-ALL cell lines were treated with 1 μmol/L dexamethasone for 0 and 24 hours. A, cell lysates were collected for Western blot analysis for the indicated proteins. B, MTT assay was performed on REH, RS4;11, and SUP-B15 cells. Results are the average ± SD of 3 independent experiments in triplicate. Statistical analysis performed using the Student t tests showed dexamethasone **, very significantly reduced RS4;11 and SUP-B15 at all concentrations studied, whereas dexamethasone only *significantly inhibited REH proliferation at >10 μmol/L dexamethasone (t test: 0.0001–100 μmol/L vs. 0 μmol/L dexamethasone of the same cell line; *, significant P < 0.05; **, very significant P < 0.01, and ns, not significant). C, Bim and p27Kip1 mRNA levels were analyzed by RT-qPCR analysis and results were normalized to L19 mRNA levels. Error bars show SD. Results are the average ± SD of 3 independent experiments in triplicate. Statistical analysis was performed using the Student t tests (t test: 0- vs. 24-hour treatment; *, significant P < 0.05, and ns, not significant). D, protein expression of p-mTOR (S2448), mTOR, Akt (S473), and β-tubulin was analyzed in the dexamethasone-treated B-ALL cells. E, protein expression of FOXO3a, FOXM1, Aurora B, and β-tubulin was analyzed at baseline conditions in five PPR and five PGR by Western blot analysis.
NSC siRNA, cells were treated for another 24 hours with dexamethasone before they were collected for subsequent cell death analysis. The knockdown of FOXO3a in RS4;11 was confirmed at mRNA and protein levels using real-time quantitative (RTq)-PCR (Fig. 3A) and Western blot analysis (Fig. 3B), respectively. Importantly, the expression of two FOXO3a targets, Bim and p27Kip1, also decreased substantially in the FOXO3a-silenced cells, confirming a depletion of FOXO3a activity. As shown in Fig. 3C and D, dexamethasone failed to induce apoptosis in RS4;11 cells with FOXO3a knockdown, indicating that FOXO3a depletion conferred dexamethasone resistance to the RS4;11 cells and therefore suggesting that FOXO3a plays a central role in mediating the cytotoxic function of dexamethasone in B-ALL.

Dexamethasone promotes FOXO3a phosphorylation on Ser-7

In addition to Akt, MAP kinases also phosphorylate and modulate FOXO3a activity (4). In particular, it has been reported that p38 and JNK regulates FOXO3a nuclear localization and that p38

Figure 2.
Dexamethasone treatment perturbs cell proliferation and induces FOXO3a nuclear translocation in sensitive B-ALL cells. A, REH, RS4;11, and SUP-B15 cells treated with dexamethasone (1 μmol/L) for 0 and 24 hours were subjects to flow cytometric analysis after PI staining. Representative cell-cycle profiles with and without dexamethasone treatment are shown. B and C, cell-cycle analysis of sub-G1 and G1-S-G2/M populations was performed on these dexamethasone-treated REH, RS4;11, and SUP-B15 cells. Error bars show SD. Results are the average ± SD of 3 independent experiments in triplicate. Statistical analysis was performed using the Student t tests (t test: 0- vs. 24-hour treatment; *, significant P < 0.05; **, very significant P < 0.01, and ns, not significant). D, after 0 and 24 hours of dexamethasone treatment (1 μmol/L), immunofluorescent staining was performed on the REH, RS4;11, and SUP-B15 cells using the mouse FOXO3a antibody and DAPI. All of the images shown are typical results obtained from at least 10 different fields. FOXO3a localization was detected using a secondary Alexa-conjugated antibody. Nuclei were stained with DAPI. Images were obtained using a video confocal microscope (Vico, Eclipse T80, Nikon), equipped with a digital camera. E, after treatment, cells were collected, and cytosolic (Cyto)/nuclear fractionation (Nuclear) procedures were performed. The resultant fractions were standardized according to protein content, followed by Western blot analyses using the indicated antibodies.
and JNK also phosphorylate FOXO3a on Ser-7 (11). To explore the molecular mechanisms by which dexamethasone modulates FOXO3a function, we next analyzed the expression patterns of FOXO3a and MAPK, including ERK, p38, and JNK, in REH, RS4;11, and SUP-B15 B-ALL cells in response to dexamethasone treatment. The results showed that the FOXO3a Ser-7 phosphorylation level increased in sensitive but not resistant cells following dexamethasone treatment (Fig. 4A). Furthermore, while there was an induction in activity of the two canonical MAPKs, p38 and JNK, as revealed by the phosphorylation-specific antibodies, ERK expression and activity remained relatively constant in RS4;11 and SUP-B15 cells after dexamethasone (Fig. 4A). Conversely, in REH cells, the JNK activity decreased whereas ERK activity increased marginally (Fig. 4A). These results indicate that p38 and JNK may have a role in mediating dexamethasone function in B-ALL. To test this conjecture, we next assessed whether inhibition of JNK, p38, or ERK kinases using small-molecule inhibitors can influence dexamethasone sensitivity. More specifically, REH and RS4;11 cells were treated for 48 and 72 hours with 1 μmol/L of dexamethasone combined with a range of concentrations (0–100 μmol/L) of ERK, p38, and ERK inhibitors, SP600125, SB202190, and PD98059, respectively, and cell viability analyzed by MTT assay (Fig. 4B and Supplementary Figs. S1 and S2). Interestingly, despite a strong induction of JNK activity by dexamethasone in the RS4;11 cells as revealed by the increase in JNK phosphorylation (Fig. 4A), we did not observe a significant decrease in the cytotoxicity of dexamethasone when administered with the JNK inhibitor, SP600125, in both the RS4;11 (Fig. 4B) and REH cells (Supplementary Figs. S1 and S2). Similarly, the dexamethasone cytotoxicity did not decrease substantially when combined with either the p38 or ERK inhibitors (Supplementary Figs. S1 and S2 and Supplementary Data). We have previously shown that JNK inhibition can cause a compensatory increase in the activity of p38, which can also phosphorylate FOXO3a on Ser-7 (11, 37). As a consequence, it is possible that the lack of a significant reduction in dexamethasone cytotoxicity upon JNK inhibition can be due to a compensatory increase in p38 activity. To test this conjecture, we depleted JNK using siRNA and tested for cell survival and p38 activity upon dexamethasone treatment in the RS4;11 cells after 48 hours. Western blot analysis showed an increase in p38 phosphorylation and activation upon JNK depletion (Fig. 4C, left). Viability assays also revealed that there was no significant difference in survival rates with or without JNK knockdown following dexamethasone treatment (Fig. 4C, right). To investigate this further, the RS4;11 cells were treated with dexamethasone in the absence or presence of 25 μmol/L SP600125 and
Figure 4.
Dexamethasone enhances FOXO3a Ser-7, JNK, and p38 phosphorylation in sensitive and not resistant B-ALL cell lines. A, REH, RS4;11, and SUP-B15 cells were treated with dexamethasone (1 μmol/L) for 0 and 24 hours. Cells were collected after treatment and were subjects to Western blot analysis with antibodies shown. B, RS4;11 cells were treated with the a range of concentrations of (0–100 μmol/L) SP600125 and/or dexamethasone. Cell viability analysis was performed after 48 hours using MTT assay (t test: 2-tailed: SP600125 with 1 μmol/L dexamethasone vs. dexamethasone at 1 μmol/L). *: significant P < 0.05; ns, not significant. C, RS4;11 cells were transiently transfected with either the nonsilencing control (NSC) siRNA or the JNK siRNA smart pool. Forty-eight hours after transfection, protein lysates were prepared from these cells and then analyzed for the expression of JNK, p-p38, total p38, and β-tubulin (left). The transfected dexamethasone-treated RS4;11 cells were assayed for cell viability by Annexin V (AV) and PI staining after 48 hours. Representative flow cytometric analysis was shown. Statistical significance was determined by the Student t test (two-sided; *, P < 0.05; significant, ns, nonsignificant). D, protein lysates prepared from RS4;11 cells with or without 25 μmol/L SP600125 pretreatment (1 hour) at 0, 6, and 24 hours following treatment with 1 μmol/L dexamethasone were subject to immunoprecipitation with a FOXO3a (αFOXO3a) or a control (IgG) antibody. The Input (1 of 10) and immunoprecipitates were then analyzed by Western blot analysis using antibodies against p-FOXO3a (Ser-7), Ac-FOXO3a (Lys-242/245), and FOXO3a. Representative co-immunoprecipitation results are shown.
subjected to immunoprecipitation with a polyclonal FOXO3a antibody (Fig. 4D). The immunoprecipitates were then probed for the expression of FOXO3a, (Ser-7) FOXO3a phosphorylation, and (Lys-242/5) FOXO3a acetylation. The result showed that JNK inhibition caused a prominent increase in FOXO3a levels as well as an increase in (Ser-7) FOXO3a phosphorylation and (Lys-242/5) FOXO3a acetylation, which have been shown to be associated with FOXO3a nuclear relocation, stabilization, and activation (11, 20, 38, 39). These results are consistent with the notion that dexamethasone activates FOXO3a through inducing JNK and p38 MAPKs coordinately in B-ALL.

FOXO3a nuclear localization is associated with phosphorylation on Ser-7 and acetylation on Lys-242/245 in B-ALL cells

Acetylation has been described to modulate the transcriptional activity of FOXO3a (22, 40). In addition, a recent study has also demonstrated that JNK phosphorylation can promote FOXO1 acetylation and activation (41). These findings have led us to propose that dexamethasone activates JNK to mediate FOXO3a phosphorylation and consequently, acetylation. To examine whether dexamethasone induces FOXO3a acetylation, immunoprecipitation experiments were performed on REH, RS4;11, and SUP-B15 cells using anti-FOXO3a antibodies. Western blotting results using pan-acetylation antibodies showed that in response to dexamethasone, there was a net increase in FOXO3a acetylation levels in the drug-sensitive RS4;11 and SUP-B15 but not in the resistant REH cells (Fig. 5A). In contrast, when the Western blot analyses were reprobed with a monoclonal anti-FOXO3a, these results indicated that the FOXO3a acetylation observed was not a result of the expression patterns of FOXO3a following dexamethasone treatment (Fig. 5A). Similar results were obtained, when the reverse immunoprecipitation and Western blotting were performed using a pan-acetylation and a FOXO3a antibody, demonstrating that dexamethasone induces FOXO3a acetylation in sensitive and not resistant B-ALL cells (Fig. 5B). We next used the FOXO3a (S7) antibody to immunoprecipitate the FOXO3a complex and probed with a pan-FOXO3a antibody. The Western blot analysis results showed that FOXO3a Ser-7 phosphorylation demonstrated similar expression patterns, as FOXO3a acetylation in both the sensitive and resistant B-ALL cells in response to dexamethasone (Fig. 5C), implicating that dexamethasone mediates both FOXO3a Ser-7 phosphorylation and acetylation in B-ALL.

FOXO3a Lys-242 and Lys-245 acetylation is associated with nuclear localization, Ser-7 phosphorylation, and JNK activation

The residues Lys-242 and Lys-245 (K242/5) located within the overlapping DNA-binding and nuclear localization signal (NLS) domains of FOXO3a have been demonstrated to be CBP acetylation sites (2, 39). It is possible that these posttranslational modifications could alter FOXO3a DNA-binding and/or nuclear localization ability and thereby impact its transcriptional activity. To investigate FOXO3a acetylation further, we generated an acetylation-specific antibody that recognizes the Lys-242 and Lys-245 acetylated FOXO3a. Using this Ac-FOXO3a (K242/5)-specific antibody in Western blot analysis, we found that FOXO3a increased in Lys-242/5 acetylation upon dexamethasone treatment in the sensitive but not resistant cells in a short time course (Fig. 6A). The Western blot analysis results also revealed that the expression patterns of FOXO3a Lys-242/5 acetylation were similar to that of Ser-7 phosphorylation and JNK phosphorylation, providing further evidence that JNK phosphorylation is associated FOXO3a Ser-7 phosphorylation and Lys-242/5 acetylation on Lys-242/5 (Fig. 6A). To test this idea further, we next examined the expression of total-acetylated, Lys-242/5 acetylated, and Ser-7 phosphorylated FOXO3a in the cytoplasmic and nuclear fractions of the sensitive and resistant B-ALL cells in response to treatment with dexamethasone following immunoprecipitation with the anti-FOXO3a (Fig. 6B). The results showed that the total acetylated, the Lys-242/5 acetylated, and the Ser-7 phosphorylated FOXO3a resided predominantly in the nuclei. In addition, Western blot analysis results clearly showed that in the sensitive cells, FOXO3a became more acetylated and phosphorylated after dexamethasone treatment. On the contrary, FOXO3a acetylation (total and Lys-242/245) and phosphorylation (Ser-7) decreased marginally following dexamethasone in the resistant REH cells. Collectively, these data suggest that Lys-242/245 acetylation and Ser-7 phosphorylation are associated with dexamethasone-mediated nuclear relocalization and activation of FOXO3a (Fig. 6B). In agreement, previous studies have shown that Ser-7 phosphorylation and Lys-242/245 acetylation promote nuclear relocalization and activation of FOXO3a and FOXO1, respectively (11, 17).

We also performed Western blot analysis with the FOXO3a Ser-7 phosphorylation antibody on the primary patient samples (Supplementary Fig. S5). As predicted, we only observed low basal expression levels of FOXO3a Ser-7 phosphorylation, consistent with our finding that FOXO3a Ser-7 phosphorylation is low at the baseline level and is induced by dexamethasone treatment. Nevertheless, we also observed generally higher levels of FOXO3a Ser-7 phosphorylation in good dexamethasone responders (GDR) compared with poor responders (PPR), further corroborating a role of FOXO3a Ser-7 phosphorylation in dexamethasone response.

FOXO3a acetylation is associated with p300 activation and SIRT1/2/6 downregulation

To investigate the mechanisms underlying FOXO3a acetylation, we studied the expression patterns of known mediators of FOXO3a acetylation, including the acetyltransferase CBP/p300 and the histone/lysine deacetylases SIRT1, SIRT2, and SIRT6, in response to dexamethasone in the B-ALL cell lines. Specifically, p300/CBP has been shown to mediate the FOXO3a acetylation on Lys-242, Lys-245, and Lys-262 residues, whereas SIRT1, 2, and 6 have been shown to target FOXO3a for deacetylation (22, 42–45). Western blot analysis showed that dexamethasone treatment upregulated CBP/p300 and downregulated SIRT1, 2, and 6 expression in the drug-sensitive RS4;11 and SUP-B15 cells (Fig. 6C). In contrast, dexamethasone treatment did not affect CBP/p300 expression as well as SIRT1, 2, and 6 levels after dexamethasone treatment in the resistant REH cells. Taken together, these data suggest that the dexamethasone-induced FOXO3a acetylation in B-ALL is mediated by mechanisms involving the downregulation of SIRT1/2 and upregulation of CBP/p300 and that these control mechanisms are deregulated in resistant cells. As we had already shown that FOXO3a has a role in B-ALL dexamethasone sensitivity, we next tested whether SIRT1, SIRT2, or general SIRT inhibition can resensitize the drug-resistant B-ALL cells to dexamethasone. To this end, we treated both the drug-sensitive RS4;11 and -resistant REH cells with 1 μmol/L of dexamethasone and a range of concentrations (0–100 μmol/L) of EX-527 (a SIRT1 inhibitor; ref. 44),
PDF-170 (a SIRT2 inhibitor; ref. 46), and Sirtinol (a pan-SIRT1/2/6 inhibitor; ref. 44; Fig. 6D and E). MTT proliferation assay revealed that only Sirtinol, but not EX-527 or PDF-170, enhanced the antiproliferative effects of dexamethasone in both the sensitive (RS4;11) and the resistant (REH) cell lines (Fig. 6E). These data suggest that sirtuins have a key role in modulating dexamethasone sensitivity and that the inhibition of at least SIRT1 and SIRT2 simultaneously is required for overcoming dexamethasone resistance. We next tested whether these SIRT inhibitors can combine with dexamethasone to restore FOXO3a acetylation (K242/5) in the resistant cells. Consistent with the proliferation assay results, Western blot analysis results showed that only Sirtinol and neither EX-527 nor PDF-170 could further enhance FOXO3a acetylation induced by dexamethasone in the resistant cells. Notably, FOXO3a acetylation (K242/5) was not enhanced by the SIRT inhibitors in the sensitive RS4;11 cells (Fig. 6D), and it is likely to be due to the fact that FOXO3a acetylation was already strongly induced by dexamethasone in these cells.

**Discussion**

It is well-established that Akt (PKB)-mediated phosphorylation and inactivation of FOXO3a culminate in cytoplasmic localization and cell proliferation (2, 3). Herein, we showed that in the sensitive B-ALL cell lines, RS4,11 and SUP-B15, FOXO3a became dephosphorylated on Akt-targeted sites, Ser253, Thr315, and Thr32 upon dexamethasone treatment, but its phosphorylation was unaffected by dexamethasone in the resistant REH cells. In concordance, recent studies show that mTOR/Akt pathway inhibition and REDD1 status may influence glucocorticoid action in leukemia (47). These findings suggest that the Akt/FOXO3a axis has a central role in mediating dexamethasone response. In agreement with this, FOXO3a activation by dexamethasone is confirmed by the increased expression of two FOXO3a targets, p27kip1 and Bim, important for cell-cycle arrest and cell death. Dexamethasone treatment also caused cleavage of caspase-3, -7, and -9, indicative of apoptosis in the sensitive B-ALL. FOXO3a can also suppress the expression of targets important for the G2–M
Figure 6. Dexamethasone enhances FOXO3a acetylation on Lys 242/245 in sensitive and not resistant B-ALL cell lines via Sirtuins. A, REH, RS4;11, and SUP-B15 cells were treated with dexamethasone (1 μmol/L) for 0, 2, and 4 hours. Cells were collected after treatment and were subjected to Western blot analysis with antibodies shown. B, REH and RS4;11 cells were treated with dexamethasone (1 μmol/L) for 0 and 24 hours. Co-immunoprecipitation (co-IP) was performed on the respective nuclear and cytoplasmic lysates with a FOXO3a antibody (rabbit) and probed for P-FOXO3a (S7), Ac-Lysine, Ac-FOXO3a (K242/K245), and FOXO3a (mouse). Inputs [(1 of 10 of immunoprecipitation (IP))] and IP products with IgG and specific antibodies were resolved on Western blot analysis and probed for proteins as indicated. C, REH, RS4;11, and SUP-B15 cells were treated with dexamethasone (1 μmol/L) for 0 and 24 hours. The treated cells were collected and subjected to Western blot analysis for the proteins indicated. D, REH and RS4;11 cells were either untreated or treated with the SIRT inhibitors indicated in the presence of 1 μmol/L dexamethasone for 24 hours and subjected to immunoblotting with antibodies indicated. E, REH and RS4;11 cells were treated with the a range of concentrations of (0–100 μmol/L) Sirtinol and/or dexamethasone. Cell viability analysis was performed after 24 hours using MTT assay (t test: Sirtinol with 1 μmol/L dexamethasone vs. dexamethasone at 1 μmol/L). *, significant $P < 0.05$; **, very significant $P < 0.01$; no marker, not significant.
phase transition, including cyclin B, Aurora B, and FOXM1 (2), and this may explain the G2 M arrest observed after FOXO3a induction by dexamethasone. Besides protein-coding genes, FOXO3a might also regulate the expression of non–protein-coding RNAs to modulate dexamethasone response. Indeed, long, noncoding RNAs (lncRNAs), including BALR-2, have been shown to be involved in the glucocorticoid response pathway in B-lymphoblastic leukemia (48). The dexamethasone-induced FOXO3a dephosphorylation at the Akt sites was accomplished by its translocation to the nucleus in the sensitive cells, whereas FOXO3a remained phosphorylated and retained in the cytoplasm in resistant cells, as revealed by immunofluorescent staining. The physiologic relevance of this finding is confirmed in B-ALL patient samples where we found that FOXO3a is predominantly more phosphorylated on the Ser-315 residue in patients of good response (PGR) compared with poor responders (PPR) to prednisone therapy. Crucially, depletion of FOXO3a in the sensitive RS4:11 cells rendered these B-ALL cells significantly less responsive to dexamethasone treatment, confirming further the central role played by this transcription factor in dexamethasone response.

Our findings also suggest that besides Akt-mediated phosphorylation, FOXO3a is differentially regulated by other posttranslational mechanisms in dexamethasone-sensitive and -resistant B-ALL cells. Intriguingly, we have also obtained preliminary data that the P38/Akt/mTOR signaling cascade might not have a central role in mediating dexamethasone resistance as, inhibitors of P38 and mTOR failed to enhance the cytotoxicity of dexamethasone in the resistant B-ALL cells (Supplementary Fig. S4).

Moreover, when we silenced FOXO3a using siRNA in the sensitive RS4:11 line, we showed that FOXO3a has a critical role in dexamethasone sensitivity, as depletion of FOXO3a rendered the sensitive RS4:11 cells refractory to dexamethasone. Taken together, these data suggest that FOXO3a has a key role in modulating dexamethasone sensitivity, but its regulation by the P38/Akt signaling cascade may not have a central role in dexamethasone resistance.

More specifically, our data indicate that dexamethasone induces activation of JNK and p38 MAPKs and the phosphorylation of FOXO3a on Ser-7 in the sensitive but not the resistant B-ALL cells. Consistent with this, we have previously demonstrated that DNA-damaging agents, such as doxorubicin, also activate the p38 MAPK, which in turn will phosphorylate FOXO3a on Ser-7 to promote its nuclear localization and activation to mediate cell-cycle arrest (11). Surprisingly, our proliferative analysis shows that JNK, but not ERK or p38, inhibitor can combine effectively with dexamethasone to impair sensitive and resistant B-ALL proliferation. Consistently, our previous work has shown that JNK can promote FOXO3a activity and expression by repressing Akt activity and also by direct phosphorylation in breast cancer cells (13, 49). In this context, JNK induces FOXO3a nuclear relocalization and the activation of its targets, including p27Kip1 and Bim, for cell-cycle arrest and cell death (13, 49). Consistent with this, we also observed upon dexamethasone treatment, the activation of JNK and p38 MAPKs, which are associated with FOXO3a nuclear translocation, dephosphorylation at Akt sites, induction of p27Kip1 and Bim expression, and cell proliferative arrest in the sensitive and not the resistant B-ALL cells. Our observations, in conjunction with the results of previous studies, suggest that dexamethasone targets FOXO3a via JNK and p38 to mediate its cytotoxic and cytostatic functions in B-ALL.

We also found that FOXO3a is acetylated in the sensitive and not the resistant B-ALL cells and that FOXO3a acetylation can be further enhanced upon dexamethasone treatment. In these cases, our acetylation-specific FOXO3a antibody revealed that dexamethasone also causes FOXO3a to be acetylated at Lys-242/5 in the sensitive and not the resistant cells. In attempting to explore the mechanism involved, we found that the expression of CREB-binding protein (CBP) is increased, whereas SIRT1 and SIRT2 levels are suppressed by dexamethasone treatment. In agreement, previous research has shown that FOXO1/3a acetylation at Lys-242 and Lys-245 is mediated through the antagonistic action of CBP and CBP-related protein (CBR) and the NAD-dependent histone lysine deacetylase sirtuins (17, 39). However, the authors of these studies also predict that acetylation at Lys-242/5 is associated with nuclear localization, induction of downstream antiproliferative targets, cell proliferative arrest, and sensitivity to dexamethasone. Hence, the analogy between this and our study is not perfect. However, in support of our findings, SIRT6 has recently been shown to regulate gluconeogenesis by promoting FOXO1 nuclear exclusion (50). Moreover, another recent study on pancreatic cancer also demonstrates that JNK phosphorylation can promote FOXO1 acetylation and activation as well as Bim expression (51). In this case, FOXO1 acetylation is also accompanied by increased expression of CREB-binding protein (CBP) and reduced SIRT1 expression (51). Furthermore, deacetylation of FOXO3a by SIRT1 or SIRT2 leads to Skp2-mediated FOXO3 ubiquitination and degradation (43). Critically, we showed that the pan-SIRT inhibitor Sirinol, but not the specific SIRT1 (EX527) or SIRT2 (PDF-170) inhibitor, can combine with dexamethasone to restrict both sensitive and resistant B-ALL cell proliferation, suggesting that at least both SIRT1 and SIRT2 have to be suppressed simultaneously to exert the antiproliferative effects of dexamethasone. This observation is in concordance with our earlier results showing that both SIRT1 and SIRT2 were both down-regulated in response to dexamethasone in the sensitive but not the resistant B-ALL cells. Together these data suggest a role for both SIRT1 and SIRT2 in restricting the cytotoxic and cytostatic functions of dexamethasone and that dexamethasone mediates it action through downregulating SIRT1 and SIRT2 expression in B-ALL. Aply, the pan-SIRT inhibitor Sirinol, but not the SIRT1 or SIRT2 inhibitor, can function in combination with dexamethasone to induce FOXO3a acetylation at Lys-242/5 in B-ALL cells. Collectively, ours and others’ results have led us to propose a signaling mechanism whereby dexamethasone can induce JNK to inhibit FOXO3a phosphorylation at Akt sites and induce phosphorylation at Ser-7 and acetylation at Lys-242/5 to promote nuclear localization and thereby transcription activity. As we have shown that FOXO3a has an essential role in dexamethasone responsiveness, it is therefore tempting to speculate that the sensitivity to dexamethasone is, at least in part, determined by acetylation and phosphorylation status of FOXO3a. However, to draw informed definitive conclusions on this model, additional in-depth experimental work is required to identify all the phosphorylation and acetylation sites involved and to define the regulation and function of these posttranslational modifications.

Nevertheless, our findings are of significance because they reveal a potential novel therapeutic strategy to treat B-ALL and to overcome dexamethasone resistance by concurrent upregulation of JNK and/or antagonism of sirtuins with dexamethasone.
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E.W.-F. Lam

References

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Consolaro, S. Ghaem-Maghami, G. Viola, E.W.-F. Lam

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Authors’ Contributions
Conception and design: S. Ghaem-Maghami, G. Viola, E.W.-F. Lam
Development of methodology: F. Consolaro, S. Ghaem-Maghami, R. Bortolozzi, E.W.-F. Lam
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Consolaro, S. Ghaem-Maghami, R. Bortolozzi

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Francesca Consolaro, Sadaf Ghaem-Maghami, Roberta Bortolozzi, et al.


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