Targeting the BRD4/FOXO3a/CDK6 axis sensitizes AKT inhibition in luminal breast cancer

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BRD4 assembles transcriptional machinery at gene super-enhancer regions and governs the expression of genes that are critical for cancer progression. However, it remains unclear whether BRD4-mediated gene transcription is required for tumor cells to develop drug resistance. Our data show that prolonged treatment of luminal breast cancer cells with AKT inhibitors induces FOXO3a dephosphorylation, nuclear translocation, and disrupts its association with SirT6, eventually leading to FOXO3a acetylation as well as BRD4 recognition. Acetylated FOXO3a recognizes the BD2 domain of BRD4, recruits the BRD4/RNAPII complex to the CDK6 gene promoter, and induces its transcription. Pharmacological inhibition of either BRD4/FOXO3a association or CDK6 significantly overcomes the resistance of luminal breast cancer cells to AKT inhibitors in vitro and in vivo. Our study reports the involvement of BRD4/FOXO3a/CDK6 axis in AKTi resistance and provides potential therapeutic strategies for treating resistant breast cancer.
Breast cancer is a heterogeneous disease\textsuperscript{1,2}, characterized into at least four different subtypes: luminal A, luminal B, ERBB2 overexpression, and basal-like\textsuperscript{3,4}. Mutations of PIK3CA gene, which encodes the catalytic subunit (p110α) of PI3Ka, occur in almost 40% of ER\textsuperscript{+}/luminal subtype. In addition, mutations of PTEN and INPP4B contribute to activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway in this subtype\textsuperscript{5}. The PI3K/AKT pathway has key roles in regulating growth, survival, and metabolism in both normal and malignant cells. For example, AKT inhibits Forkhead box O (FOXO)-induced expression of growth inhibition and apoptosis induction genes by phosphorylating FOXOs and blocking their nuclear translocation\textsuperscript{6,7}. These findings indicate that activation of the PI3K/AKT pathway is likely a causal genetic event in the luminal subtype of breast cancer; thereby, inhibition of this pathway represents a top priority for therapeutic intervention. Indeed, numerous clinical trials have evaluated the efficacy of over 30 drugs targeting different steps of the PI3K/AKT pathway in breast and other cancers, including several AKT inhibitors (AKTis) such as MK2206, AZD5363, and GSK690693. Although these inhibitors have shown evidence of suppressing growth and inducing apoptosis of luminal breast cancer cells, responses of solid tumors to monotherapy have been modest and accompanied by rapid emergence of drug resistance. For example, AKT inhibition induces the expression and phosphorylation of HER3, IGF-1R, and insulin receptor through FOXO-dependent transcriptional activation, suggesting that targeting different nodes of feedback regulation of PI3K/AKT inhibition may improve the killing effects of these inhibitors. Intriguingly, FOXOs proteins are usually deemed as tumor suppressors because of their growth-inhibitory and cell death-inducing ability; the functional roles and downstream target genes of FOXOs involved in drug resistance remain obscure.

The Cancer Genome Atlas (TCGA) data also indicate frequent amplification of cyclin D1 (40\%) and low levels of RB1 mutations in luminal-type breast cancer\textsuperscript{8}. The cyclin D1/CDK4/6 complex phosphorylates the retinoblastoma (Rb) protein, which leads to cell cycle activation\textsuperscript{9}. Results from several studies indicate that CDK4 and CDK6 have an important role in estrogen-stimulated proliferation of breast cancer cells in early to mid G\textsubscript{1} phase\textsuperscript{9,10}. Thus, CDK4 and CDK6 represent valuable therapeutic targets of ER\textsuperscript{+} advanced breast cancer. Consistent with this idea, combination of a CDK4/6 inhibitor with an aromatase inhibitor achieves significant effect on suppressing advanced ER\textsuperscript{+}/luminal subtype of breast cancer\textsuperscript{11}. In addition, a combinatorial drug screen on multiple PIK3CA mutant cancers with decreased sensitivity to PI3K inhibitors revealed that combined CDK4/6-P13K inhibition synergistically reduces cell viability\textsuperscript{12}. Although the combination of PI3K and CDK4/6 inhibitors overcomes intrinsic and adaptive resistance leading to tumor regressions in PIK3CA mutant xenografts, the molecular mechanism underlying the resistance of AKTi and the synergy seen on the PI3K inhibitors and CDK4/6 inhibitors remain elusive.

Recently, inhibitors of BRD4, a BET (bromodomain and extra-terminal domain) family member, have shown significant effects in hindering tumor growth by suppressing the expression of oncogenes\textsuperscript{13,14}. BRD4 has the capacity to assemble diverse transcriptional complexes on gene super-enhancers and activate RNA polymerase II-dependent transcriptional elongation. In the later, BRD4 was found to preferentially occupy on oncogene super-enhancers and maintain their high expression levels in tumor cells\textsuperscript{15}, explaining why BET inhibitors could specifically suppress tumor cell growth and induce apoptosis. Our recent study also demonstrates that BET inhibitors disrupt the Twist/BRD4 interaction and effectively inhibit invasion and cancer stem cell-like property of basal-like breast cancer (BLBC) cells\textsuperscript{16}. Although it is well accepted that BRD4-guided gene expression mediates diverse processes during tumor development and progression, whether and how BRD4 assembles transcriptional machinery on chromatin to activate feedback survival genes expression is totally unclear. Here our study discovered the novel role of FOXO3a/BRD4/CDK6 axis in AKTi resistance of luminal breast cancer cells.

Results

Bromodomain inhibitor enhances growth suppressive effects of AKTi. As luminal subtype of breast cancer has activation of the PI3K/AKT pathway and the effect of monotherapy of PI3K/AKTis is moderate\textsuperscript{17}, we sought to identify additional strategies that may increase the efficacy of AKTi by rational combination therapies that will increase synergy and overcome emergence of resistance. We treated four luminal breast cancer cell lines (BT474, T47D, MDA-MB-453, and MDA-MB-361) with three different AKTi inhibitors (MK2206, AZD5363, and GSK690693). Consistent with previous studies that monotherapy of AKTi did not induce substantial growth suppression, about 50–80\% of these tumor cells remain alive after treatment with these inhibitors (1 \mu M) for 4 days detected by both cell count and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays (Fig. 1a, b). Treatment with two individual BET inhibitors (JQ1 and MS417, 1 \mu M) also did not result in substantial suppression of growth and proliferation. However, the combination of JQ1 or MS417 with AKTi produces significant more growth suppressive effect than either single agent alone (Fig. 1a, b). These three AKTi have different mechanistic action on AKT inhibition. MK2206 suppresses the allosteric activation of AKT\textsuperscript{18}, AZD5363 binds to the catalytic pocket of AKT\textsuperscript{19}, and GSK690693 is an ATP-competitive pan-AKT kinase inhibitor, which exhibits efficacy irrespective of the mechanism of AKT activation involved\textsuperscript{20}. Therefore, the synergistic effect seen in the combination of three different AKTi with BET inhibitors is not likely due to off-target effect. To further confirm this observation, we knocked down the expression BET family members BRD4, BRD3, BRD2, and BRDT individually and treated these cells with AKTi for 3 days. We found that only BRD4 knockdown greatly enhanced the suppressive effect of AKTi (Fig. 1c). These results indicate that BRD4 inhibition improves the growth suppressive effect mediated by AKTi, suggesting BRD4 and its related transcription machinery mediated oncogene expression is involved in AKTi resistance.

Prolonged treatment of AKTi induces FOXO3a acetylation and its association with BRD4. FOXO3a, a member of Forkhead family of transcription factors that regulates diverse gene expression in controlling various cellular processes, is a key target of AKT. AKT phosphorylates FOXO3 at three conserved serine/threonine residues (T32, S253, and S315), resulting in the binding of FOXO3 with 14-3-3 and thus the cytoplasmic retention as well as inactivation of this transcription factor\textsuperscript{21,22}. Consistent with AKT inhibition, treatment with MK2206 (1 \mu M) in BT474 and T47D cells resulted in the nuclear translocation of FOXO3a in these cells (Fig. 2a). Our recent study identified that a histone H4 mimic GK-X-GK motif in Twist is responsible for the interaction of Twist with BRD4\textsuperscript{16}. Intriguingly, we noticed that FOXO transcription factor family members also contain this GK-X-GK motif (Fig. 2b); notably, lysine 242 and 245, which are near the nuclear localization signal (NLS) of FOXO family proteins, have been identified as two major acetylation modification residues in FOXO3a protein previously and are involved in FOXO3a-mediated transcriptional activation\textsuperscript{23–25}, although the underlying mechanism remains unclear. We found that treatment with three different AKTi (1 \mu M) for 4 days in BT474 and T47D cells...
significantly induced FOXO3a acetylation and BRD4 association, concomitant with the suppression of FOXO3a phosphorylation (Fig. 2c). The level of endogenous FOXO3a acetylation and its interaction with BRD4 induced by AKTi appeared at day 1 and gradually reached to maximum at day 4 after MK2206 treatment (Fig. 2d). This finding suggests that the FOXO3a-BRD4 interaction became eminent after prolonged exposure to AKTi, and that this interaction may be involved in drug resistance against AKTi. The FOXO3a-BRD4 interaction is sensitive to bromodomain blockade, because the addition of JQ1 or MS417 (2 μM) in the
immunoprecipitation (IP) buffer completely disrupts the FOXO3a-BRD4 interaction in BT474 cells (Fig. 2e and Supplementary Figure 1). To further extend this observation, we treated cells with de-acetylase inhibitors Trichostatin A (TSA) (2 μM) and nicotinamide (4 mM) overnight. Endogenous FOXO3a became robustly acetylated and its interaction with BRD4 was disrupted by adding JQ1 or MS417 in the IP buffer (Fig. 2f and Supplementary Figure 1). These data indicate that AKTì treatment induces endogenous FOXO3a nuclear translocation, acetylation, and its interaction with BRD4, which can be disrupted by BET inhibitors.
BD2 of BRD4 recognizes K242/245 di-acetylated FOXO3a. BRD4 contains two tandem bromodomains (BD1 and BD2) that are responsible for interaction with di-acetylated substrates. To determine which bromodomain of BRD4 is responsible for the FOXO3a-BRD4 interaction, we co-expressed FOXO3a with either BD1 or BD2 of BRD4 in HEK293 cells, and found that FOXO3a interacted with BD2 but not BD1 of BRD4, and the interaction could be disrupted by the addition of JQ1 or MS417 in the IP buffer (Fig. 3a). As lysine 242 and 245 (K242/245) of FOXO3a are located in GK-X-GK motif similar to Twist K73/76 (Fig. 2b), we co-expressed wild-type (WT) or KR mutant (in which K242/245 were changed to arginine) of FOXO3a with BRD4 in HEK293 cells. We found that MK2206 treatment induced the acetylation of WT-FOXO3a and its interaction with BRD4; however, the acetylation of KR mutant and its interaction with BRD4 were completely abolished (Fig. 3b). In addition, treatment of TSA and nicotinamide, which inhibits the deacetylation event in cells, induced robust acetylation of WT-FOXO3 and its interaction with BD2 of BRD4 (Fig. 3c). This effect was completely abolished in KR-FOXO3a, indicating that K242/245 di-acetylation of FOXO3a is critical for the interaction with BD2 of BRD4.

We also performed a two-dimensional $^1$H-$^1$N heteronuclear single quantum coherence nuclear magnetic resonance (NMR) titration study and showed that BRD4-BD2 exhibited substantially more extended chemical shift perturbations upon binding to the di-acetylated FOXYO peptides than those produced by BRD4-BD1, including K242/245 of FOXO3a (Fig. 3d). Our analyses indicated that the three-dimensional (3D) NMR structure of the BRD4-BD2 bound to a FOXO3a-K242ac/K245ac peptide and confirmed that indeed the BRD4-BD2 recognition of the di-acetylated site of FOXO3a is highly similar to that of Twist. The key residues of His437 and Asn433 at the acetyl-lysine binding site that are responsible for the BRD4-BD2’s specificity for this molecular recognition are colored in red and green, respectively (Fig. 3e and Supplementary Figure 2). To further confirm these observations, we generated a His437 and Asn433 double-mutant of full-length BRD4 (HN), in which both His437 and Asn433 were changed to Alanine. Compared with WT-BRD4, HN-BRD4 completely lost the interaction with FOXO3a in a co-IP experiment (Fig. 3f). These results indicate that the FOXO3a-BRD4 interaction is dependent on BD2 of BRD4-mediated recognition of di-acetylated K242/245 of FOXO3a. A recent study applied motif analyses and predicted the involvement of FOXO transcription factors in BRD4-enriched regions on chromatin. Our study elucidates the exact molecular basis of combined action of FOXO3a and BRD4.

**AKTi induces FOXO3a acetylation by disrupting the FOXO3a-SirT6 interaction.** To define the underlying mechanism responsible for AKTi-induced FOXO3a acetylation, we co-expressed FOXO3a with seven SirT de-acetylase family members in HEK293 cells, we found only SirT6 and SirT7 (to a less extent) associate with FOXO3a (Fig. 4a). With IP endogenous FOXO3a from BT474 and T47D cells, only endogenous SirT6 but not SirT7 was detected (Fig. 4b and data not shown). Interestingly, all three AKTi (1 μM) blocked the endogenous interaction of FOXO3a with SirT6. To examine whether SirT6 is a deacetylase of FOXO3a, we knocked down endogenous SirT6 in BT474 cells and found MK2206-induced FOXO3a acetylation was greatly elevated (Fig. 4c). In an in vitro deacetylation assay, the addition of purified active SirT6 protein removed the acetylation of endogenous FOXO3a protein immunoprecipitated from BT474 cells induced by TSA and nicotinamide treatment (Fig. 4d). These data indicate that SirT6 interacts with and deacetylates FOXO3a, which inhibited by AKTi.

When AKT is inhibited, FOXO3a phosphorylation can be quickly removed by PP2A, resulting in the dissociation of 14-3-3, the exposure of NLS of FOXO3a and the consequent nuclear translocation of this molecule. To further investigate the FOXO3a-SirT6 interaction, we expressed either WT or TM mutant of FOXO3a (T32/S253/S315 were mutated to alanine, which detains FOXO3a in the nucleus) in cells. WT-FOXO3a is mainly localized in the cytoplasm, whereas TM-FOXO3a is exclusively resided in the nuclear (Supplementary Figure 3). AKT inhibition induces nuclear translocation of WT-FOXO3a. However, endogenous SirT6 exclusively resides in the nucleus even in the presence of AKTi (Fig. 2a and Supplementary Figure 3). Interestingly, when WT- and FOXO3a are immunoprecipitated, TM mutant binds to SirT6 much stronger than WT-FOXO3a (Fig. 4e). MK2206 treatment disrupted the interaction of SirT6 with both WT and TM mutant of FOXO3a, suggesting that the disruption of FOXO3a-SirT6 interaction by MK2206 is independent of FOXO3a phosphorylation and cellular localization. A recent study indicated that S338 of SirT6 is phosphorylated by AKT, and that this phosphorylation is critical for SirT6 to interact with its binding partners. In line with this finding, we found that IGF-1 treatment in BT474 and T47D cells induces SirT6-S338 phosphorylation detected by antibodies against S338 phosphorylation and AKT phospho-substrates; this phosphorylation was abolished by pre-treatment with MK2206 (1 μM) (Fig. 4f). In addition, the endogenous interaction of AKT with SirT6 was completely disrupted by MK2206 in BT474 and T47D cells (Fig. 4g). To further extend this observation, we co-expressed WT, S338A (cannot be phosphorylated), and S338E (mimic phosphorylation) SirT6 with FOXO3a in HEK293 cells. WT-SirT6 interacted with FOXO3a and this interaction was reduced by MK2206. However, S338A lost this interaction whereas S338E remained bound to FOXO3a partially even in the presence of MK2206 (Fig. 4h). Together, these data suggest an interesting model, in which under normal condition, FOXO3a diffuses in the whole cells, part of them associates with SirT6. Upon extracellular stimuli, hyper-activated AKT phosphorlates and detains FOXO3a in the cytosol; meanwhile, it maintains the phosphorylation status and substrate-binding activation of SirT6. On the
contrary, AKT inhibition enhances FOXO3a nuclear translocation, but it also reduces the ability of SirT6 targeting to its substrate, resulting in the separation of SirT6 from FOXO3a, although they both reside in the nucleus, ultimately leading to the increased FOXO3a acetylation and its interaction with BRD4 (left panel, Fig. 5a). These observations elucidate the exquisite interplay among AKT, SirT6, and FOXO3a, renovate the understanding on regulation of activity of FOXOs protein, implicating the potential mechanism of drug resistance to AKT inhibition.
AKTi induces CDK6 expression. To test whether the interaction of SIRT6 with FOXO3a is involved in the drug resistance of AKTi, we expressed vector, WT-SirT6, S338A-SirT6, or S338E-SirT6 in BT474 and T47D cells treated with MK2206 (1 μM) for 4 days. Expression of WT-SirT6, S338A-SirT6, or S338E-SirT6 did not affect the overall cell growth in the absence of MK2206 compared with vector control (middle and right panel, Fig. 5a). Although WT-SirT6 expression conferred partial sensitivity to MK2206-mediated growth inhibition, expression of S338E-SirT6 endowed cells with more sensitivity to MK2206, whereas S338A-SirT6 expressing cells became completely insensitive to MK2206. These results support the notion that S338 phosphorylation of SirT6 increases its interaction with and deacetylation of FOXO3a, which prevents BRD4 interaction and suppresses the transcriptional activity of FOXO3a.

To further investigate the mechanism of FOXO3a-BRD4 interaction in mediating AKTi resistance, we sought to identify the target genes of the FOXO3a-BRD4 complex by performing RNA-sequencing analyses on BT474 and T47D cells treated with MK2206 for 3 days (GSE118148) (Fig. 5b and Supplementary Figure 4). We reasoned that survival or growth-promoting genes that are transcriptionally induced by FOXO3a could have a role in AKTi resistance. Among 78 overlapping genes that differen-

CDK6 is a targeted gene of the FOXO3a-BRD4 complex. We noticed two typical FOXO3a-binding sites in the CDK6 gene promoter (Fig. 6a). When CDK6 promoter luciferase reporter was co-expressed with BRD4 and WT or KR mutant of FOXO3a in HEK293 cells, FOXO3a and BRD4 barely enhanced CDK6 reporter activity in the absence of MK2206. However, MK2206 significantly induced CDK6 promoter luciferase activity in the presence of FOXO3a; the addition of BRD4 further increased the luciferase activity (Fig. 6b). KR-FOXO3a, which cannot interact with BRD4, lost its ability to induce CDK6 promoter luciferase activity. We also constructed three mutant CDK6 promoter luciferase reporters (M1, M2, and M3), in which the first and the second FOXO3a-binding element were mutated, respectively, or both (Fig. 6a). Luciferase assay indicated that M1 and M3 totally lost reporter activity, whereas M2 retained large percent of activity, suggesting that the FOXO3a-binding element distal to the transcription starting site is the major binding site for the FOXO3a-BRD4 complex at the CDK6 promoter (Fig. 6c). Indeed, chromatin IP from BT474 and T47D cells displayed that FOXO3a, BRD4, and RNA polymerase II are targeted to this region of CDK6 promoter upon MK2206 treatment, and that these associations were abolished by JQ1 (Fig. 6d). We also performed the sequential chromatin immunoprecipitation (ChIP) analyses to further validate the association of the FOXO3-BRD4 complex at the CDK6 promoter (Fig. 6e). Using a control IgG in the first cycle of ChIP, we did not detect the association of FOXO3a or BRD4 at the CDK6 promoter in the second cycle of ChIP. However, using either FOXO3a or BRD4 antibody in the first cycle of ChIP, we found the interaction of BRD4 or FOXO3a, respectively, at the CDK6 promoter in the sequential ChIP. In addition, we knocked down FOXO3 in BT474 cells and found that FOXO3 knockdown significantly reduced BRD4 association at the CDK6 promoter (Fig. 6f). Together, these data further strengthen our findings that the interaction of FOXO3a-BRD4 complex is critical for the transcription of CDK6.

To further validate the transcriptional activation of CDK6 by the FOXO3a-BRD4 complex, we knocked down FOXO3a in BT474 cells, and then followed up with a rescue expression of WT or KR-FOXO3a in these cells. FOXO3a knockdown inhibited MK2206-induced CDK6 expression (Fig. 6g); rescue
expression of WT- but not KR-FOXO3a, completely restored CDK6 expression. In addition, FOXO3a knockdown enhanced the sensitivity of cells to MK2206-induced growth inhibition, re-expression of KR mutant lost the resistance ability as WT-FOXO3a did (bottom panel, Fig. 6g). We also knocked down BRD4 expression in BT474 and T47D cells (Supplementary Figure 5b and Fig. 6h). BRD4 knockdown completely abolished MK2206-induced CDK6 expression. Rescue expression of WT-BRD4 but not HN-BRD4, which cannot interact with FOXO3a, restored CDK6 expression in BT474 cells (Fig. 6h). Together, these results indicate that AKTi-induced CDK6 expression is mediated through the association of the FOXO3a-BRD4 complex at the CDK6 promoter and disruption of the FOXO3a-BRD4 complex by JQ1 or MS417 abolishes CDK6 induction.

Inhibition of CDK6 confers drug sensitivity to AKTi. To further determine the biological role of CDK6 induction in
AKTi-mediated drug resistance, we generated MK2206-resistant cells in T47D andZR75 cell lines by exposing them with gradually increasing concentration of MK2206 (from 1 μM to 20 μM) over a 4-month period (left panel, Fig. 7a). Both resistant cell lines can grow in the presence of 2 μM MK2206 with no apparent growth inhibition, because with 2 μM MK2206 treatment, significantly fewer of these cells have decreased S-phase of cell cycle compared with that of the parental cells (middle panel, Fig. 7a). This is likely due to the induction of CDK6, which phosphorylates Rb (serine 780) and disrupts cell cycle arrest (left panel, Fig. 7a). Intriguingly, JQ1 or Palbociclib conferred sensitivity of these MK2206-resistant cells (right panel, Fig. 7a). To validate this finding, we generated CDK6-expression stable clones in BT474 and T47D cells (left panel, Fig. 7b). Again, these CDK6-expressing cell lines induced Rb phosphorylation and have a lower population of cells with decreased S-phase when exposed to 2 μM MK2206 (middle panel, Fig. 7b). As expected, CDK6-expressing cell lines were less sensitive to MK2206 and they were also less sensitive to growth inhibition mediated by the combination of MK2206 and JQ1 (right panel, Fig. 7b). This is because exogenous CDK6 expression was not under the control of endogenous CDK6 promoter, which can be activated by the FOXO3a-BRD4 complex upon AKTi. However, these CDK6-expressing cell lines remained highly sensitive to the growth suppression mediated by the combination of MK2206 and Palbociclib. These results clearly indicate that AKTi-induced CDK6 expression, which is mediated by the FOXO3a-BRD4 complex, is critical for drug resistance to AKT inhibition.

To further extend our finding that the FOXO3a-BRD4-CDK6 axis promotes resistance of P13K/AKT inhibition, we treated BT474 cells with Lapatinib (1 μM), which is an EGFR/HER2 dual tyrosine kinase inhibitor that is commonly used in breast cancer treatment. Similar to AKTi, prolonged treatment of Lapatinib for 4 days induced FOXO3a acetylation and its interaction with BRD4, as well as the consequent CDK6 induction (Fig. 7c). Combination of JQ1 or Palbociclib greatly enhanced the growth suppressive effect mediated by Lapatinib. Together, our data not only confirm that CDK6 is a major feedback survival factor against AKTi, but also suggest that the FOXO3a-BRD4-CDK6 axis is a common drug resistance mechanism of P13K/AKT targeting.

Pharmacological inhibition of BRD4/FOXO3a or CDK6 sensitizes luminal breast cancer cells to AKTi in vivo. To extend our finding in vivo, we inoculated BT474 cells into the 6-week-old female athymic mice subcutaneously. When tumors reached 250 mm³, the mice were equally divided into six groups (six mice/group) and received the following treatments: (1) control vehicle; (2) JQ1 (50 mg/kg); (3) Palbociclib (100 mg/kg); (4) MK2206 (90 mg/kg); (5) MK2206 plus JQ1; and (6) MK2206 plus Palbociclib for 2 weeks. Consistent with the cell culture studies in vitro, monotherapy with JQ1, Palbociclib, or MK2206 slightly reduced tumor growth; however, the combinatory treatments of, either MK2206 plus JQ1 or MK2206 plus Palbociclib, significantly inhibited tumor growth (Fig. 8a, c). To further explore the clinical relevance of CDK6 expression, we performed immunohistochemistry (IHC) analysis of a tissue microarray featuring 343 cases of breast tumor samples that contains 56 cases of recurrence disease from Kentucky. We scored different levels of CDK6 expression in these tumor samples and found that high CDK6 expression correlated with recurrent disease in patients with ER+/PR+/HER− luminal subtype of breast cancer (Fig. 8d, e). In vivo data support our findings in cell culture experiments and further strengthen the hypotheses that the FOXO3a-BRD4-CDK6 axis is critical for conferring drug resistance to AKTi and inhibitors of FOXO3a-BRD4 and CDK6 can sensitize the growth suppressive effects mediated by AKTi in luminal breast cancer.

Discussion

Our study reveals a novel mechanistic insight for drug resistance mediated by the FOXO3a-BRD4-CDK6 signaling axis. In proliferating tumor cells, AKT not only phosphorylates FOXO3a to retain it in the cytoplasm, but also phosphorylates SirT6 at serine 338 to maintain its substrate-binding ability. When cells are treated with AKTi, FOXO3a undergoes de-phosphorylation and translocates into the nucleus; at the same time, de-phosphorylated SirT6 loses its capacity to associate with FOXO3a, resulting in the increased FOXO3a acetylation. Acetylated FOXO3a interacts with BD2 of BRD4, forming a transcription activation complex with pTEFb and RNA polymerase II on the CDK6 gene promoter and activates CDK6 transcription (Fig. 8f).

Our study provides several potential therapeutic strategies for overcoming drug resistance in luminal breast cancer. Luminal breast tumors have been shown to be dependent on the P13K-AKT-mTOR signaling for survival and proliferation; therefore, they are highly sensitive to inhibition of pathway components. Although corresponding small inhibitors have been shown to possess a robust efficiency in killing tumor cells, intrinsic drug resistance, a hallmark associated with poor clinical outcomes, inevitably arises. We found that individual treatment with BET or CDK6 inhibitors have only a mild suppressive effect on proliferation and survival of luminal breast cancer. However, either...
A compound exhibits a synergistic effect with AKTi in the inhibition of cell growth in vitro and in vivo, indicating that the induction of CDK6 by the FOXO3a-BRD4 complex has a critical role in the development of intrinsic AKTi resistance. Targeting either the FOXO3a-BRD4 complex or CDK6 greatly potentiates the anti-proliferative and pro-apoptotic effects of AKTi.

Our study also provides new insight into the pro-survival role of FOXO3a for tumor cells. FOXO proteins have long been considered tumor suppressors, because they inhibit tumor cell proliferation and induce apoptosis, which are negatively regulated by AKT, a central regulator of cell proliferation and survival. Specifically, FOXOs can inhibit proliferation by inducing expression of cell cycle inhibitors p15, p19, p21, and p27, while repressing cyclin D. FOXOs also induce apoptosis by upregulating expression of several pro-apoptotic molecules, including FasL, TRAIL, Puma, and Bim. Unexpectedly, we identified a pro-survival function of FOXO3a in tumor cells via an upregulation of CDK6 gene expression. CDK4/6 function as the major oncogenic drivers in cancer cells by promoting G1-S cell cycle transition and by preventing cancer cell-intrinsic tumor suppression. For example, CDK4/6 prevent senescence and apoptosis by activating downstream signaling such as Rb/E2F and...
FOXO3a regulates CDK6 expression and functions as a transcriptional activator. FOXO3a-mediated gene transcription is critical for its association with DNA sequence and transcriptional activity. Our data demonstrated that K242/245 di-acetylation of FOXO3a gradually increases after prolonged AKTi treatment. Similar to Twist, this di-acetylation modification is located in a GC-X-GK motif, which can be recognized and bound by the second bromodomain of BRD4, as revealed by NMR structural and binding analysis. The K242/245 R mutant of FOXO3a loses its ability to interact with BRD4 and to induce CDK6 expression. Our study demonstrated that FOXO3a uses its di-acetylation motif to recruit BRD4 and RNA polymerase II for activating gene transcription.

Methods

Cell culture. All cell lines were purchased from ATCC. Breast cancer cell line BT474, MDA-MB-453, and MDA-MB-361 were grown in Dulbecco’s modified Eagle’s medium/F-12 medium plus 10% fetal bovine serum (FBS). T47D and ZR75 were grown in RPMI-1640 plus 10% FBS. MK2206-resistant cancer cells were obtained by stepwise increased concentrations of MK2206. BT474 and ZR75 cells were incubated with 1 μM MK2206 for 2 days in the beginning. Then the medium was changed to fresh one without MK2206 and cells were cultured until they grow well. Whenever we subcultured, the cells were incubated with gradual increasing concentration of MK2206 for 2 days. Some aliquots of the cells were stored whenever we subcultured it. Cells that grew at the maximum concentration (20 μM) of MK2206 were stored for further analyses. For selection of stable clone cells, puromycin (2 μg/ml) or blasticidin (5 μg/ml) was used.

siRNA and antibodies. Palbociclib, MK2206, AZD5363, and GSK690693 were obtained from Selleckchem (Houston, TX). Smart pool siRNA (small interfering RNA) against human BRD4, BRD2, BRD3, and BRD7 were obtained from Dharmacon (Chicago, IL). Short hairpin RNA against human FOXO3a and antibodies against Flag tag and actin were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies for phospho-SirT6 (Ser338), SirT6, phospho-AKT substrate, AKT, BRD4, phospho-FOXO3a (Thr32), FOXO3a, and Pan acetyl-lysine were purchased from Cell Signaling (Danvers, MA). Details regarding the dilutions of antibodies used were presented in Supplementary Table 1.

Protein structure analysis by NMR. NMR samples contained a protein/peptide complex of 0.5 mM in a 100 mM sodium phosphate buffer (pH 6.5) that contains 5 mM perdeuterated diethanol and 0.5 mM EDTA in H2O/D2O (9:1) or H2O. All NMR spectra were collected at 30 °C on NMR spectrometers of 800, 600, or 500 MHz. The 1H, 13C, and 15N resonances of the protein in the complex were assigned by stepwise increased NMR spectra collected with a 13C/15N-labeled and 75% deuterated BRD4-BD2 bound to an unlabeled FOXO3a peptide. The distance restraints were obtained from 3D 1H-15N-Cα Overhauser spectroscopy (NOESY) spectra, 15N-NOE spectra, and 13Cα-1Hα CCSS spectra. Protein structures were calculated with distance geometry-simulated annealing protocol using X-PLOR that was aided with iterative automated NOE assignment by ARIA for refinement. Structure quality was assessed by PROCHECK-NMR. The structure of the protein ligand complex was determined using intermolecular NOE-derived distance restraints that were obtained from 13Cα-edited (F2) 13Cα/15N-filtered (F3) 3D NOESY spectra. Statistics of NMR structures of the BRD4-BD2/FOXO3a peptide complex are presented in Supplementary Table 2.
**Cell proliferation and MTT assay.** The cell proliferation was analyzed by counting the number of viable cells via Vi-CELL XR (Beckman Coulter). Breast cancer cells with the same number were seeded in culture dishes followed by specific treatment with different compounds for the indicated time, then trypsinized and counted the viable cells again. The cell proliferation rate was calculated by the difference of number of viable cells from two-times count. For MTT assay, seed cells in a 96-well plate at a density of $10^5$–$10^6$ cells/well in 100 µL of culture medium with compounds to be tested, culture the cells in a CO2 incubator for indicated time. Then wash them by removing the medium and replacing it with 100 µL of fresh medium. Add 10 µL of the MTT stock solution to each well and
Fig. 6 CDK6 is a targeted gene of FOXO3a/BRD4. a Schematic depiction of the CDK6 promoter and CDK6 reporter luciferase constructs used. Two consensus FOXO3a-binding motifs are indicated. b Effects of WT and KR mutant of FOXO3a, BRD4 on the activity of CDK6 promoter luciferase in the presence or absence of MK2206. c Effects of WT-FOXO3a, BRD4 on the activation of WT or mutant CDK6 promoter luciferase in the presence of MK2206. For b and c, \( p < 0.01 \). d FOXO3a, BRD4, H4ac4, and RNA-Pol II association at the CDK6 promoter as assessed by ChIP. \( p < 0.01 \) when FOXO3a-ChIP or H4ac-ChIP in either MK2206-treated group or MK2206 plus JQ1-treated group is compared with control group. \( p < 0.01 \) when BRD4-ChIP or RNA-Pol II-ChIP in MK2206-treated group is compared with control group. e Cells treated with MK2206 were analyzed by sequencing ChIP. The first-round of immunoprecipitation used anti-IgG, anti-FOXO3a or anti-BRD4 antibodies, followed by a second-round of immunoprecipitation using anti-IgG, anti-FOXO3a, or BRD4 antibodies. Pulldown DNA was analyzed by RT-PCR using specific CDK6 promoter primers and nonspecific primers. \( p < 0.01 \) when individual group with specific antibodies (FOXO3a and BRD4) used in the first-round of ChIP is compared with the corresponding group using IgG in the first-round of ChIP. f Effects of FOXO3a-knockdown, MK2206 treatment, or both on the association of FOXO3a, BRD4, RNA-Pol II, and H4ac at the CDK6 promoter as assessed by ChIP. \( p < 0.01 \) when specific ChIP in MK2206-treated group is compared with the corresponding ChIP in control, or FOXO3a-knockdown or combination groups. g WT or KR mutant of FOXO3a was expressed in BT474 cells with endogenous FOXO3a knockdown. Rescue CDK6 expression was analyzed by western blotting; growth inhibition was assessed by cell count. \( p < 0.01 \) when WT-FOXO3a group is compared with either vector or KR-FOXO3a group. H WT or HN mutant of BRD4 was expressed in BT474 cells with endogenous BRD4 knockdown. Rescue CDK6 expression and growth inhibition were assessed as in g. \( p < 0.01 \) when WT-BRD4 group is compared with either vector or HN-BRD4 group. From b to h, data are representative of three experiments performed in triplicate as the mean ± SD.

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Luciferase reporter assay. HEK293 cells were seeded in 60 mm dishes and transfected with mentioned plasmids by FuGene 6 transfection reagent (Roche) for 24 h, cell lysates were extracted with passive lysis buffer (Promega, Madison, WI) and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). All experiments were performed for three times in triplicate. Representative experiment was shown.

Flow cytometry. Cell cycle was measured via flow cytometry analysis. Briefly, after specified treatment, cells were trypsinized and fixed with 70% ethanol for over an hour. Cells were then pelleted and washed with phosphate-buffered saline plus 20 mM EDTA. RNA was removed by incubating samples with RNase (1 mg/ml) at 37 °C for 2 h. Cells were then stained with propidium iodine (final concentration: 30 μg/ml). DNA content was analyzed by an LSR II flow cytometer (BD Biosciences). All experiments were performed for three times, statistical analysis (mean ± SD) from three separate experiments is shown.

Mice model. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky College of Medicine and conform to the legal mandates and federal guidelines for the care and maintenance of laboratory animals. Animals were maintained and treated under pathogen-free conditions. Female athymic nude mice (6 weeks old, Taconic) were injected with breast cancer BT474 (5 × 10⁶ cells/mouse) cells subcutaneously. When tumors reached 250 mm³, the mice were divided into six groups (six mice/group) and received the following treatments: (1) control vehicle; (2) IQ1 (50 mg/kg); (3) Palbociclib (100 mg/kg); (4) MK2206 (90 mg/kg); (5) MK2206 plus IQ1; and (6) MK2206 plus Palbociclib every 2 days. Tumor growth was monitored with caliper measurements. When tumors were approximately 1.0 cm in size, mice were killed and tumors excised.

Statistical analysis. Data are presented as mean ± SD. Student’s t-test (two-tailed) was used to compare two groups \( p < 0.05 \) was considered significant unless otherwise indicated.

Data availability. RNA-sequencing data are available at the GEO data repository with the accession code GSE118148. The solution structure of the BRD4 BD2 in complex with Foxo3α-K242ac/K245ac peptide and the NMR spectral data are deposited in Protein Data Bank (PDB) ID 6MNL and BioMagResBank (BMRB) ID 30373,
Fig. 7 Inhibition of CDK6 confers drug sensitivity to AKTi. **a** Left panel: MK2206-resistant cell lines were established by growing T47D and ZR75 cells in increasing concentrations of MK2206 for 4 months. Inductions of CDK6 expression was examined by western blot. Middle panel: the growth suppressive effect of MK2206 was presented by the percentage of S-phase decrease in parental or resistant cells after 2 μM MK2206 treatment for 4 days. Data are representative of three experiments performed in triplicate as the mean ± SD.* p < 0.01 when resistant cells were compared with parental cells. Right panel: the growth inhibition of these cells responded to the combination of MK2206 with JQ1 or Palbociclib was measured by cell count assays. Data are representative of three experiments performed in triplicate as the mean ± SD.* p < 0.01 when MK2206-treated group was compared with untreated group in parental cells;# p > 0.05 when MK2206-treated group was compared with untreated group in resistant cells. **b** Left panel: induction of CDK6 expression in parental and CDK6-overexpressing cells were examined by western blotting. Middle panel: the growth suppressive effect of MK2206 was presented by the percentage of S-phase decreased in parental or CDK6-overexpressing cells after 2 μM MK2206 treatment for 4 days. Data are representative of three experiments performed in triplicate as the mean ± SD.* p < 0.01 when CDK6-expressing cells were compared with vector control cells. Right panel: cell count assay was used to detect the growth inhibition effects of the combination of MK2206 with JQ1 or Palbociclib. Data are representative of three experiments performed in triplicate as the mean ± SD.** p < 0.01 when MK2206 plus JQ1 treatment group in CDK6-expressing cells was compared with corresponding group in parental cells. **c** Lapatinib (1 μM) also induced FOXO3a acetylation, its association with BRD4 and CDK6 expression in BT474 cells as analyzed by IP-western blotting. The growth inhibition effects of the combination of Lapatinib with JQ1 or Palbociclib on BT474 cells were reflected by cell count assays. Data are representative of three experiments performed in triplicate as the mean ± SD. * p < 0.01 when combinatory treatment group was compared with Lapatinib-treatment group.
The FOXO3a-BRD4-CDK6 axis is critical for AKTi resistance in vivo. a, b BT474 cells were injected into athymic mice subcutaneously. When tumors from mice reached 250 mm³, mice were divided into six groups (six mice/group) and treated with solvent control, MK2206 (90 mg/kg), JQ1 (50 mg/kg), Palbociclib (100 mg/kg), or in combinations, respectively. The size of tumor was recorded every 3 days. Data are represented as a mean ± SEM from six mice. */# p < 0.01 when combinatory treatment group is compared with either control or single-agent treatment group. c Tumor weight was also measured from above. Data are represented as a mean ± SEM from six mice. */ p < 0.01 when combinatory treatment group is compared with either control or single-agent treatment group. ‡ p > 0.05 when both combinatory treatment groups were compared. d, e The 343 surgical specimens of breast cancer were immuno-stained using antibody against CDK6 and the control serum (data not shown). Images with consecutive IHC staining of CDK6 and H&E staining in four cases of breast tumors are shown (Scale bar = 100 μm). Recurrence rate was calculated according to CDK6 scores and clinical information of these patients. f A proposed model illustrating the formation of FOXO3a-BRD4 complex at the enhancer/promoter of CDK6, which leads to the transcriptional activation of CDK6 gene in response to AKTi. Either targeting FOXO3a-BRD4 complex by BET inhibitors or inhibition of CDK6 by Palbociclib reverses the AKTi resistance.
respectively. All uncropped western blot images were presented in Supplementary Figure 6. All tumor images and source data of this study are available upon request.

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Author contributions

L.J., S.J., and Z.B.P. conceived and designed the study. L.I., S.J., D.Z., G.W., W.Y., C.Y., T. F., W.Y., L.Y., and H.Y. performed most of the study. Z.L., Z.Q., G.S., and Z.M.M. performed the NMR analyses and data interpretation. S.R.L. and L.S. performed the IHC and tumor sample analyses. W.C. performed statistical analyses. D.J., L.P.C., and E.B.M. discussed the results, conceived some experiments, and provided critical reagents and comments. L.J., S.J., Z.M.M., and Z.B.P. wrote the manuscript.
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