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14. ABSTRACT

Breast cancer is the most common cancer in women. Tamoxifen has been a front-line treatment for estrogen receptor alpha (ERα)-positive breast tumors in premenopausal women. However resistance to tamoxifen occurs in many patients. ERα still plays a critical role in the growth of breast cancer cells with acquired tamoxifen resistance, suggesting that ERα remains a valid target for treatment of tamoxifen-resistant breast cancer. In an effort to identify novel regulators of ERα signaling, through a small-scale siRNA screen against histone methyl modifiers, we found WHSC1, a histone H3K36 methyltransferase, as a positive regulator for ERα signaling in breast cancer cells. We demonstrated that WHSC1 is recruited to the ERα gene by interacting with the BET protein BRD3/4, and facilitates ERα gene expression. The small-molecule BET protein inhibitor JQ1 potently suppressed the classic ERα signaling pathway and the growth of tamoxifen-resistant breast cancer cells in culture. By performing a comprehensive mechanistic study, we found that JQ1 targets both ERα and MYC pathways on cell cycle-related genes in tamoxifen-resistant breast cancer cells. In addition, using a tamoxifen-resistant breast cancer xenograft mouse model, we are the first to show the *in vivo* anti-breast cancer activity by JQ1 and a strong long-lasting effect of combination therapy with JQ1 and the ER degrader fulvestrant.

15. SUBJECT TERMS
Endocrine, tamoxifen, bromodomain, resistance

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Final report for DoD Award W81XWH-13-1-0159
TREATMENT OF ENDOCRINE-RESISTANT BREAST CANCER WITH A SMALL MOLECULE C-MYC INHIBITOR

1. Introduction

Breast cancer is the most common noncutaneous cancer in U.S. women. Tamoxifen has been a front-line treatment for estrogen receptor alpha (ERα)-positive breast tumors in premenopausal women. However resistance to tamoxifen occurs in many patients. ERα still plays a critical role in the growth of breast cancer cells with acquired tamoxifen resistance, suggesting that ERα remains a valid target for the treatment of tamoxifen-resistant breast cancer. With the support by this DoD award, we have demonstrated using preclinical mouse model that JQ1 is a promising drug in treatment of tamoxifen-resistant breast cancer by targeting the expression of ERα and MYC at the transcriptional level.

2. Keywords

Breast cancer, Endocrine-resistant, tamoxifen, MYC, JQ1

3. Accomplishments

A. Major goals of the projects:

(1) Dissect the molecular mechanisms of JQ1 effect on tamoxifen-resistant MCF7 breast cancer cells by microarray analysis;
(2) determine the therapeutic activity of JQ1 on a panel of well-characterized preclinical endocrine resistant breast cancer cell models;
(3) Investigate in vivo JQ1 anti-tumor activity using the tamoxifen-resistant breast cancer xenograft model in nude mouse.

B. Accomplishments under these goals (Figures and legends are shown in Appendices Section)

(1) Dissect the molecular mechanisms of JQ1 effect on tamoxifen-resistant MCF7 breast cancer cells by microarray analysis;

Under this goal, we have found:

- Bromodomain inhibitor JQ1 targets both ERα and MYC pathways in Tamoxifen-resistant cells;
- JQ1 exerts tumor suppression effect mainly through regulating cell cycle genes;
- JQ1 gene signature correlates with better clinical outcomes;
- JQ1 delays the development of tamoxifen-resistance in breast cancer cells.

Specifically, the following are the results we have obtained:

a. Microarray analysis identifies cell cycle genes as major targets of JQ1

My preliminary results showed that JQ1 represses ERα gene expression. To determine the global signaling pathways that are altered by JQ1 in addition to ER, microarray analysis was performed using Tam-R MCF7 cells treated with vehicle or 0.2 μM of JQ1 (in triplicates). The Affymetrix GeneChip Human Gene 1.0 ST array containing 764,885 distinct probes covering 28,869 well-annotated genes on a single array has been used. When applying a threshold of log2 < -0.2 or log2 > 0.2, we identified 652 down-regulated genes and 219 up-regulated genes in JQ1-treated cells (Fig. 1a). Fig. 1b showed the biological pathways negatively affected by JQ1, and Table 1 lists all the genes up-regulated or down-regulated in major biological pathways by KEGG pathway analysis. Among them, the cell cycle is the primary pathway being affected since cell cycle-related gene expression was significantly altered by JQ1 treatment (Fig. 2). Consistent with this observation, by flow cytometry analysis, we found that Tam-R MCF7 cells were arrested at G1 phase after JQ1 treatment for 24 hrs, while parental cells were arrested at G1 phase after > 48 hrs of JQ1 treatment (Fig. 3).

b. JQ1 targets both ERα and MYC pathways in tamoxifen-resistant breast cancer cells

JQ1 was reported to inhibit MYC signaling in previous studies. In our microarray analysis, many altered cell cycle-regulated genes were MYC target genes, such as E2f1, MCM5, Cdc25A, Cdc25C, CKD6, and Cdc6 (Fig. 2). Thus we determined the effect of JQ1 treatment on the mRNA levels of MYC in parental and Tam-R MCF7 cells. Shown in Fig. 4, MYC was dramatically reduced in JQ1-treated Tam-R MCF7 cells, but only slightly reduced in parental MCF7 cells. This result indicates that MYC is indeed a JQ1 target gene in Tam-R MCF-7 cells, and this partially explain why JQ1 has more potent inhibitory effect on the growth of Tam-R cells than on the parental cells. Our result suggests that JQ1 targets both ERα and MYC pathways in tamoxifen-resistant breast cancer cells.

c. JQ1 gene signature correlates with better clinical outcomes

Next we want to find out how JQ1-regulated gene pathways correlate with clinical outcomes. Therefore, in collaboration with Dr. Chad Creighton at Duncan Cancer Center, we generated a JQ1-regulated gene signature with these JQ1 target genes[1]. Using a compendium of nine separate breast tumor expression array datasets, we scored human breast tumors based on the manifestation of the JQ1 gene signature. In ER-positive tumors (N=682), high manifestation of the JQ1 signature was associated with better patient outcome (Fig. 5, Log-rank P=0.001), while in ER-negative tumors (N=309), no survival association was found. This data further support the functional significance of JQ1 on ER signaling in breast cancer.

d. JQ1 delays the development of tamoxifen-resistance in breast cancer cells

In our preliminary results we have shown that JQ1 could overcome the tamoxifen resistance in MCF7 cells after the resistance is established. However, it is not clear if JQ1 could prevent the development of tamoxifen resistance. In order to address this question, we treat MCF7 cells with 4-hydroxytamoxifen (4-HT), in the absence or presence of JQ1, and evaluate the effect of JQ1 in development of tamoxifen-resistance by monitoring the cell growth. As shown in Fig. 6, although breast cancer cell growth was initially inhibited by single treatment of 4-HT, cancer cells eventually started to grow even in the presence of tamoxifen. Importantly, when these two compounds were combined, the cell growth was completely abolished. This result suggests that JQ1 not only represses the growth of breast cancer cells after tamoxifen resistance is established, but also prevent the development of tamoxifen resistance if cancer cells were treated by JQ1 at the same time when treated with tamoxifen.

(2) determine the therapeutic activity of JQ1 on a panel of well-characterized preclinical endocrine resistant breast cancer cell models;

Under this goal, we have found:

- We have shown that JQ1 has potent anti-tumor activity against tamoxifen-resistant (Tam-R) and estrogen-deprivation-resistant (EDR) breast cancer cells in multiple resistant models;
- We have successfully dissected the molecular mechanisms of how the histone methyltransferase WHSC1 complexes with acetylated histone binding protein BRD3/4, and how this complex regulate ERα gene expression at the chromatin level;
- We observed that tamoxifen-resistant breast cancer cells are more sensitive to JQ1 treatment; and GATA3 appear to be one of determinants for JQ1 sensitivity.

Specifically, the following are the results we have obtained:

a. JQ1 has anti-tumor activity in multiple tamoxifen-resistant (Tam-R) and estrogen-deprivation-resistant (EDR) breast cancer cells.

Previously we have investigated if the BRD3/4 inhibitor JQ1 inhibits growth of breast cancer cells. We treated parental MCF7 cells and a tamoxifen-resistant derivative with different doses of JQ1 or vehicle control. JQ1 has shown potent growth inhibition in Tam-R MCF7 cells, whereas it only moderately

inhibited the growth of parental MCF7 cells at a concentration of 0.2 uM. Here we further tested three more ER+ breast cancer cell lines including T47D, MCF7RN, and ZR75-1 cells. We found that JQ1 inhibited the growth of all of these breast cancer cell lines, with higher efficacy towards the tamoxifen resistant cells. Interestingly, JQ1 also inhibited four estrogen-deprivation-resistant (EDR; mimics aromatase inhibitor resistance) lines (Fig. 7a). Similar to MCF7 cells, JQ1 downregulated ERα mRNA levels in MCF7RN and ZR75-1 cells. We also measured the mRNA level of ERα dominant negative splicing variant ERΔ7, the major alternatively spliced form in most human breast tumors and cancer cell lines [2]. Similarly to wild-type ERα, the mRNA level of ERΔ7 is also downregulated by JQ1 treatment (Fig. 7b and 7c).

b. Further dissecting the molecular mechanisms of how JQ1 affect ERα gene expression.

BRD4 interacts with WHSC1 through its N-terminal 470 amino acids. Previously we identified WHSC1 as a regulator for ERα gene expression, and BRD3 and BRD4 have been identified as interacting proteins of WHSC1. BRD3 and BRD4 belong to the BET (bromodomain and extraterminal domain) family of proteins. In humans, there are four BET proteins including BRD2, BRD3, BRD4, and BRDT [3]. JQ1 potently inhibits all four BET proteins. BET family members contain two bromodomain and one extraterminal domain. The bromodomain specifically recognizes acetylated lysine residues on the histone tails. BRD3 and BRD4 are implicated in the transcription elongation process by association with the PAF1 complex and the pTEFb complex, respectively [4]. BRD4 also regulates the expression of G1 cell cycle genes. We next determined the domain responsible for the interaction between WHSC1 and BRD3/4 by deletion mapping. Shown in Fig. 8a and 8b, BRD4 interacts with WHSC1 through its NH2-terminal 470 amino acids, which contains two bromodomains. Interestingly, treatment of cells with JQ1 failed to disrupt the interaction between WHSC1 and BRD4, suggesting BRD4 can bind to WHSC1 and acetylated lysine simultaneously (Fig. 8C).

Loss of BRD3/4 abolished the recruitment of WHSC1 to ERα gene. To further test the hypothesis that WHSC1 is recruited to the ERα gene promoter through BRD3 and BRD4, these two genes were knocked down in MCF7 cells, and the recruitment of WHSC1 to the ERα gene and the level of histone H3 K36 methylation were determined by ChIP-qPCR assay. As shown in Fig. 9, knockdown of BRD3/4 dramatically reduced the recruitment of WHSC1 to the ERα gene. Consistent with the fact that WHSC1 is a methyltransferase for histone H3K36, the levels of H3K36me2 and H3K36me3 were both significantly reduced, suggesting that BRD3/4 functions as a scaffold to recruit WHSC1, which promotes the transcription elongation of the ERα gene.

c. GATA3 as one of determinants of JQ1 sensitivity

My preliminary results showed that Tam-R breast cancer cells are more sensitive to JQ1. Related to this observation, we noticed when cells were treated with JQ1 for up to three days, ERα mRNA was persistently suppressed in Tam-R MCF-7 cells. In contrast, in parental MCF-7 cells, ERα mRNA level was abolished initially, but recovered after prolonged treatment (Fig. 4). And MYC mRNA level responded to JQ1 treatment similarly to ERα in parental and Tam-R MCF7 cells. These results demonstrate that ERα and MYC are JQ1 target genes in Tam-R MCF-7 cells, and that sustained suppression of ERα and MYC by JQ1 probably contributes to its more potent anticancer activity on Tam-R breast cancer cells. To gain more mechanistic insight into this observation, we found that GATA3, a key regulator of ER gene expression, is highly expressed in parental MCF-7 cells, but not in Tam-R cells (Fig. 10a and 10b). And in parental MCF-7 cells, GATA3 expression is further increased by JQ1 treatment (Fig. 10a). When we knocked down GATA3 using siRNA, the parental MCF-7 cells became more sensitive to JQ1 treatment (Fig. 10d). Thus, our results suggest that other key transcription factors, such as GATA3 in parental MCF-7 cells, could have contributed to the JQ1 resistance with prolonged treatment. A decrease in such factors might contribute to epigenomic environmental changes on the ERα promoter, resulting greater JQ1 sensitivity in Tam-resistant lines.

(3) Investigate in vivo JQ1 anti-tumor activity using the tamoxifen-resistant breast cancer xenograft model in nude mouse.

Under this goal, we have found:

- JQ1 has anti-cancer activity against tamoxifen-resistant tumor *in vivo*;
- Most importantly, we have demonstrated that JQ1 potently inhibits growth of tamoxifen-resistant tumor *in vivo* when combined with fulvestrant therapy.

Specifically, the following are the results we have obtained:

a. JQ1 has *in vivo* anticancer activity against tamoxifen-resistant breast cancer

After we found that JQ1 has potent inhibitory effect on cancer cell growth, we next sought to determine the *in vivo* anti-tumor activity of JQ1. Ovariectomized nude mice were transplanted subcutaneously with Tam-R MCF7 tumors, and were randomized into two groups when tumor volumes reached 200 mm^3. JQ1 or control vehicle was given to these mice by intraperitoneal injection daily. A pilot experiment was performed to treat the mice for 7 days to test if JQ1 could downregulate ERα expression *in vivo*. As shown in Fig. 11a, the ERα mRNA level was indeed reduced about 30% in JQ1 treated tumors. MYC mRNA level was not significantly reduced, although there was a trend. Immunochemical staining confirmed a decreased level of ERα protein in JQ1-treated tumors, and a reduced proliferation rate was observed with Ki67 and histone H3 phospho-Ser10 staining (Fig. 11b). This result demonstrates that JQ1 has *in vivo* anticancer activity against tamoxifen-resistant breast cancer.

b. JQ1 potently inhibits growth of tamoxifen-resistant tumor *in vivo* when combined with fulvestrant therapy

In our pilot experiment, JQ1 exhibits mild *in vivo* anticancer activity against tamoxifen-resistant breast tumor when JQ1 was used alone. Gene expression and histochemical staining indicates that ERα mRNA level has mild reduction upon JQ1 treatment, but far from the potent inhibition effect as what we observed in cultured cells (Fig. 11). In order to further downregulate ERα and achieve an optimal drug response *in vivo*, we tested a combination of JQ1 and fulvestrant/ICI 182,780, an ERα protein degrader, in Tam-R MCF7 xenograft tumors. While single treatment of JQ1 or fulvestrant moderately inhibited tumor growth, the combination of JQ1 and fulvestrant showed a synergistic anti-tumor activity in the Tam-R tumors. In the vehicle-treated group, the volumes of all the tumors quickly tripled within 17 days, while in the group that received combination therapy, none of the tumors tripled their size after 40 days of treatment, and only about half of the tumors tripled after a prolonged treatment (90 days) (Fig. 12a). Time-to-tumor tripling from the four groups of treatment was compared using the generalized Wilcoxon test as shown in Fig. 13. Although JQ1-treated animals lose weight initially and then regain it, generally there is no difference among the four groups in the baseline weights, indicating that the JQ1 is well tolerated by mice (Fig. 12b). By Western blot analysis, the protein levels of ERα were dramatically downregulated in the tumors that received combination therapy of fulvestrant and JQ1, despite that single treatment of each drug only partially decreased ERα protein level (Fig. 12c). Immunochemical staining for proliferation markers Ki67 and histone H3 phospho-Ser10 confirmed that the combination therapy potently inhibited the tumor cell proliferation (Fig. 12d).

(4). Based on the results we obtained, we have extended this study to address several important questions related to this project. And we have further demonstrated:

- JQ1 derivatives with improved drug stability have been developed;
- JQ1 synergizes with SRC-3 inhibitor sangivamycin to inhibit proliferation of cancer cells.

Specifically, the following are the results we have obtained:

a. Development of amide derivatives of JQ1 with improved stability

Although JQ1 has been reported as a potent anti-cancer compound in multiple publications, it is very unstable *in vivo*. The half-life of JQ1 is about 30 mins in plasma. The ester bond in JQ1 is subjected to hydrolysis by esterases in plasma and leads to its turnover. Therefore, in order to achieve the sustained drug concentration *in vivo,* one way is to modify the ester bond and stabilize JQ1. In collaboration with Dr. Yongcheng Song in the Department of Pharmacology at the Baylor College of Medicine, we designed and synthesized two amide derivatives of JQ1, named as JQ1-685 and JQ1-686, respectively (Fig. 14a). We then tested their anti-cancer activities on the growth of tamoxifen-resistant MCF7 cells. In comparison to JQ1, JQ1-685 shows similar or even more potent anti-proliferation activity, whereas JQ1-686 appeared to be less effective (Fig. 14b).

Because JQ1 exerts its anti-tumor function through shutting down the expression of both ERα and MYC at the transcriptional level, we next examined the gene expression of ERα and Myc after treatment of JQ1 and its derivatives. Shown in Fig. 15a, both JQ1 derivatives reduced expression of ERα and Myc in MCF7 parental cells. However, in MCF7 tam-R cells, JQ1-686 did not repress ERα and Myc mRNA levels, although it repressed ERα protein level at relative high concentrations (Fig. 15b). These data suggest that JQ1-685, but not JQ1-686, might be a promising drug for *in vivo* use in the future.

b. JQ1 synergizes with SRC-3 inhibitor sangivamycin to inhibit cancer cell growth

SRC-3 is a transcription coactivator and amplified in up to 60% of breast tumors. Overexpression of SRC-3 is associated with tamoxifen resistance in patients, and activation of SRC-3 by phosphorylation through growth receptor signaling pathways further contributes to endocrine therapy resistance. When we performed the microarray analysis and gene expression validation, we noticed that SRC-3 expression is upregulated in JQ1-treated breast cancer cells. Importantly, EGFR and Her2, the two growth receptors that eventually phosphorylate and activate SRC-3 through the growth receptor signaling pathways, are especially highly expressed in tamoxifen-resistant cells (Fig. 16). Furthermore, in xenograft mouse models, JQ1 inhibits tumor growth but does not shrink the tumor, suggesting that other signal pathways sustain the tumor growth. Therefore, we tested whether combination therapy of JQ1 and SRC-3 inhibitors can eradiate the tumors.

High throughput screening of SRC inhibitors conducted in Dr. David Lonard and Dr. Bert O'Malley's lab in our Department has identified several potent SRC-3 inhibitors, including bufalin, sangivamycin, and cephaelin (Fig. 17a). All three compounds inhibits cell proliferation at nM scales. We treated the tamoxifen-resistant MCF7 cells with JQ1 and the three SRC-3 inhibitors at different concentrations, and found that sangivamycin showed synergistic anti-cancer activity when combined with JQ1 (Fig. 17b). Next we also examined the activity of sangivamycin and JQ1 on other tamoxifen-resistant cell models T47D and ZR75-1, and further confirmed their synergistic anti-cancer activities (Fig. 18). These results indicate that the SRC-3 inhibitor sangivamycin is a promising compound for a combinatory therapy with JQ1 for future *in vivo* studies.

<u>Opportunities for training and professional development</u>

With support of this award, the PI has further obtained two additional grants from NIH; and Dr. Zheng Zhang is the postdoc exclusively supported by this award. Baylor College of Medicine provides many opportunities to foster development of trainees. In addition to lab research work, Dr. Zhang has attended the Career Development Series which consists of approximately 25 hours of lecture/discussion. The series address six central core competencies (i.e., discipline-specific conceptual knowledge, research skill development, communication skills, professionalism, leadership and management skills, and responsible conduct of research). And she also attended several Career-building activities such as Resume-building and Mock Interview workshops that prepare trainees for job applications. As a result, after Dr. Zhang has

completed her training in my lab, she obtained a professional research position at the MD Anderson Cancer Center.

Results disseminated to communities of interest
Nothing to report

Plans to do during the next reporting period to accomplish the goals
Nothing to report as this is a final report

4. Impact
Impact on the development of the principle disciplines of the project
Using preclinical mouse models, our work has established a novel therapeutic strategy for the treatment of ER-positive breast cancer. BET protein inhibitors such as JQ1 targets both ERα and Myc at transcription level, suggesting that this group of compounds can also be used to treat recurrent ER+ breast cancers, particularly in breast cancers with ER fusion mutants, which cannot be targeted by traditional endocrine therapy (personal communication with Dr. Matthew Ellis, the director of Lester and Sue Smith Breast Center at Baylor College of Medicine).

Impact on other disciplines
During our studies, we have generated multiple endocrine-resistant breast cancer cell models, which are valuable tools for the study of endocrine-resistant breast cancer.

Impact on technology transfer
Nothing to report

Impact on society beyond science and technology
Nothing to report

5. Changes/problems
Nothing to report in this category

6. Products
Relevant publications, conference papers, and presentations
So far we have generated the following publications that are directly related to this grant during the funding period:

1. Feng Q, Zhang Z, Shea MJ, Creighton CJ, Coarfa C, Hilsenbeck SG, Lanz R, He B, Wang L, Fu X, Nardone A, Song Y, Bradner J, Mitsiades N, Mitsiades CS, Osborne CK, Schiff R, O'Malley BW. 2014. An epigenomic approach to therapy for tamoxifen-resistant breast cancer. *Cell Res.* 24(7): 809-19. PMID: 24874954
2. Feng Q, O'Malley BW. 2014. Nuclear Receptor Modulation – Role of coregulators in selective estrogen receptor modulator (SERM) actions. *Steroids*. 90: 39-43. PMID: 24945111

I have attended the 2014 Cold Spring Harbor Asia conference (Epigenetics, Chromatin & Transcription), and gave an oral presentation "An epigenomic approach to therapy for tamoxifen-resistant breast cancer".

Other products

We have generated multiple endocrine-resistant breast cancer cell models, which are now available to the scientists in the field upon request.

7. Participants and other collaborating organizations

Individuals have worked on the project

Name	Qin Feng
Project Role	PI
Nearest person month worked	4
Contribution to project	Dr. Feng has designed, performed cell-based experiments, routinely reviewed data, and prepared manuscripts

Name	Rachel Schiff
Project Role	collaborator
Nearest person month worked	1
Contribution to project	Dr. Schiff has provided the endocrine-resistant tumor cell lines and advised on how to performf the studies of the endocrine-resistant xenograft mouse models.

Name	Zheng Zhang
Project Role	Postdoc associate
Nearest person month worked	12
Contribution to project	Dr. Zhang has performed most of the experiments in this project, including cell culture work and xenograft mouse model studies

Name	Martin Shea
Project Role	Research assistant
Nearest person month worked	3
Contribution to project	Dr. Shea has worked with Dr. Zhang to perform the drug treatment of xenograft mice and measured the tumor growth

Changes in the active other support of the PD/PI or senor/key personnel since the last reporting period

Dr. Feng has obtained a grant from NIH/NIAID during the last funding period:
1R21AI122418 (FENG) 12/8/2015 - 11/30/2017, 50% effort
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES (NIAID) $135,000.00
Title: AN EPIGENOMIC APPROACH TO REACTIVATE LATENT HIV

Other organizations involved as partners

Nothing to report

8. Special reporting requirement

Nothing to report in this category

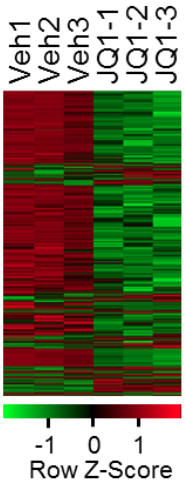
9. Appendices

Figures and legends (see following pages)

Supporting data:

Figures and Tables

a



b

KEGG Pathways	Gene Count	% of genes	P Value
Cell cycle	27	4.19906687	3.36E-14
DNA replication	10	1.55520995	2.22E-06
Pyrimidine metabolism	11	1.71073095	0.0013837
Systemic lupus erythematosus	11	1.71073095	0.00189615
Homologous recombination	6	0.93312597	0.00229482
Purine metabolism	13	2.02177294	0.00582703
Progesterone-mediated oocyte maturation	8	1.24416796	0.02703209
Mismatch repair	4	0.62208398	0.04219004

Fig. 1. (a) Heatmap of expression levels for the genes differentially expressed upon treatment with JQ1. Tam-R MCF7 cells were treated with 0.2 µM of JQ1 or vehicle (DMSO) for 24 hrs before harvest for microarray analysis. (b) Biological pathways were identified by microarray analysis. KEGG pathways were determined by the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov) based on the gene list that is down-regulated by JQ1.

Table 1a, pathways upregulated by JQ1

KEGG pathways	Genes up-regulated in JQ1 treated cells
Systemic lupus erythematous	HIST2H2AA, HIST2H4A/B, HIST1H2AC, HIST1H2BC, HIST1H3H, HIST1H2BD
Nitrogen metabolism	CPS1, CTH, GLS

Table 1b, pathways downregulated by JQ1

KEGG pathways	Genes down-regulated in JQ1 treated cells
Cell cycle	CHEK2, cdc23, cdc45, ESPL1, CDK1, TGFB2, BUB1B, TTK, ORC6L, MCM3, MCM6, MCM5, CCNA2, CHEK1, WEE1, SKP2, E2F3, MCM2, E2F2, ORC1L, CDK6, cdc25C, cdc25A, CDC6, RBL2, MAD2L1, MYC
DNA replication	POLE2, MCM3, MCM6, MCM5, DNA2, RFC4, RFC5, POLA1, PRIM1, MCM2
Pyrimidine metabolism	CAD, POLE2, PNPT1, CTPS, POLR1B, POLA1, PRIM1, POLR3G, DHODH, CANT1, NME6
Systemic lupus erythematous	HIST2H2AB, HIST1H2AL, HIST1H2BL, HIST1H2AD, HIST1H2AE, H2AFY2, HIST1H2BL, HIST1H3L, HIST1H2AB, HIST1H4A, HIST1H2BM, HIST1H2BN, HHIST1H2AJ, HIST1H3J
Homologous recombination	RAD54L, BRCA2, XRCC2, EME1, RAD51, BLM
Purine metabolism	POLE2, AK2, PDE3B, PRIM1, CANT1, PFAS, ADSL, NMD6, ADCY3, PNPT1, POLA1, POLR1B, POLR3G
Progesterone mediated oocyte maturation	PDE3B, CDC25C, CDC23, CCNA2, CDC25A, ADCY3, MAD2L1, CDK1
Mismatch repair	RFC4, RFC5, MSH2, EXO1

Table 1a and 1b. pathways upregulated or downregulated by JQ1. Upregulated and downregulated genes in different pathways are listed.

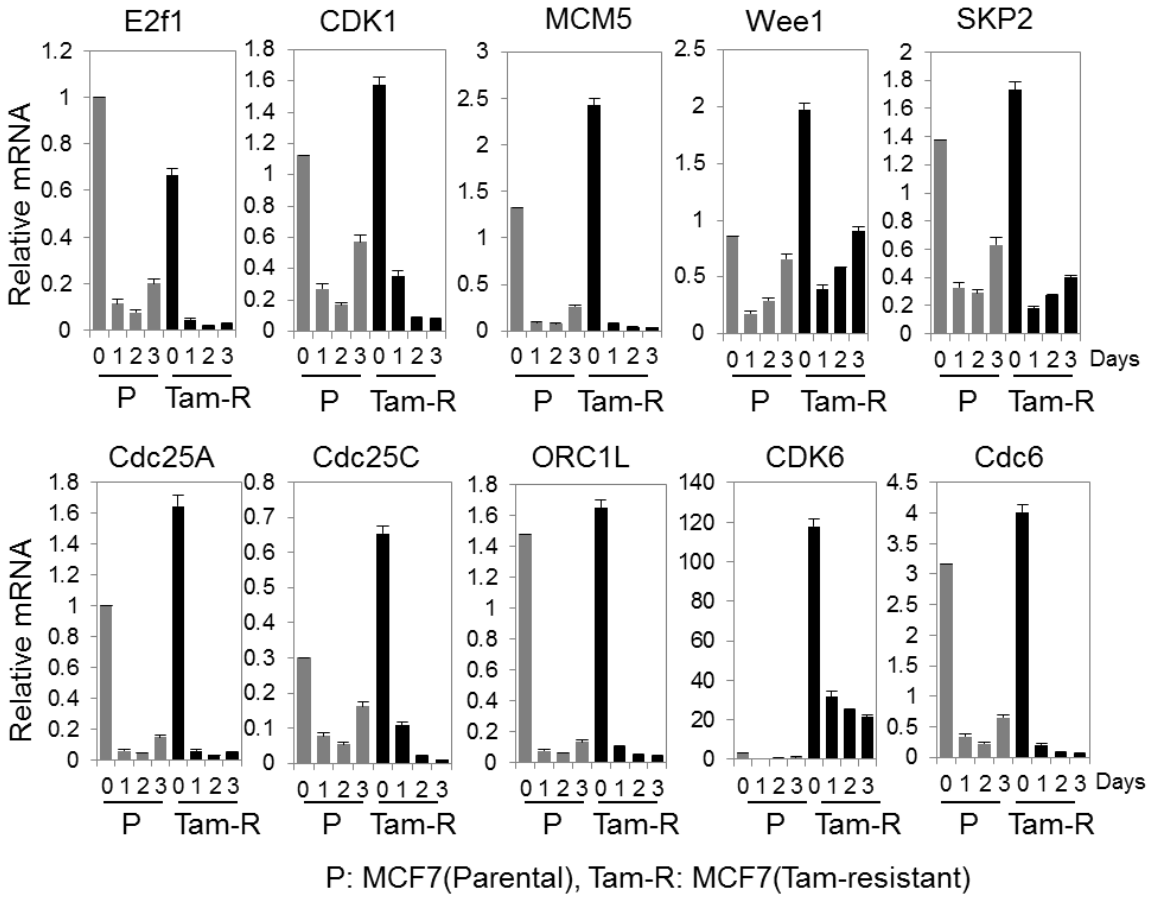


Fig. 2. Expression of cell cycle-related genes in JQ1-treated breast cancer cells. Parental and Tam-R MCF7 cells were treated with 0.2 μM of JQ1 for different days and gene expression level was determined by RT-qPCR. Error bars were shown as s.e.m.

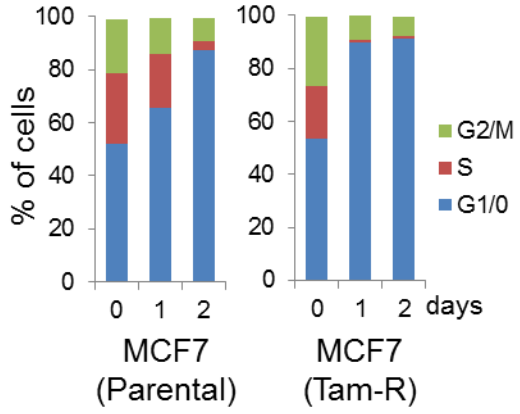


Fig. 3. Tam-R MCF7 cells are more sensitive to JQ1-induced G1 cell cycle arrest. Cells were fixed and stained with propidium iodide (PI) before being analyzed by flow cytometry.

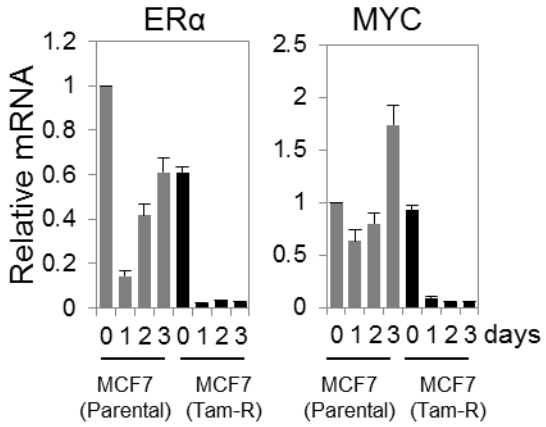


Fig. 4. JQ1 suppressed both ERα and MYC signaling pathways in Tam-R MCF7 cells. Parental and Tam-R MCF7 cells were treated with JQ1 for different days, and mRNA levels of ERα and MYC were analyzed by RT-qPCR.

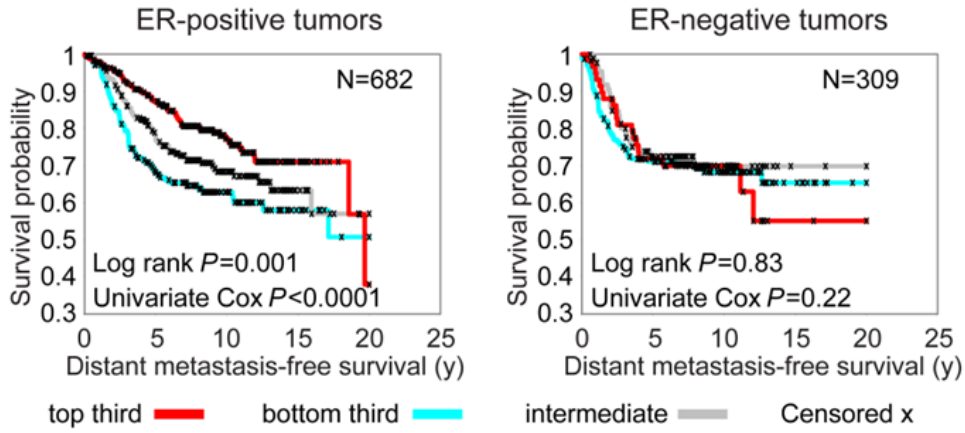


Fig. 5. Association of the gene expression signature of JQ1 treatment with breast cancer patient survival. For ER-positive and ER-negative subsets, the differences in risk between tumors, according to degree of manifestation of the JQ1 gene signature, is compared using Kaplan-Meier plots (top third, "strong manifestation"; bottom third, "weak manifestation"; middle third, "intermediate manifestation").

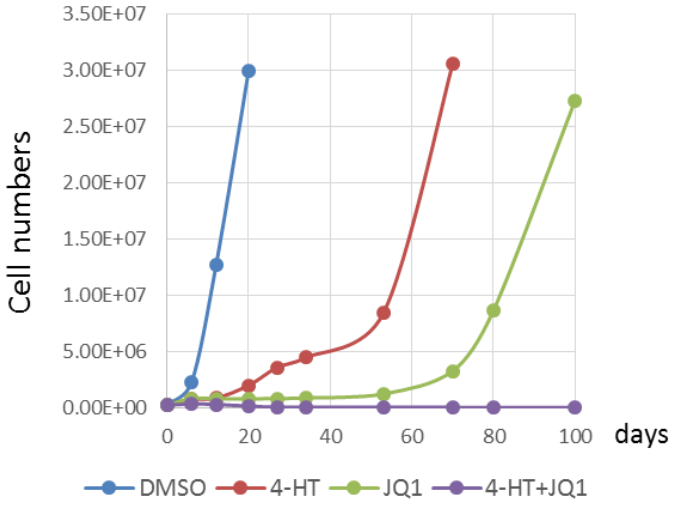


Fig. 6. JQ1 prevented the development of tamoxifen-resistance in MCF7 cells. 0.2 uM of JQ1 were used to treat MCF7 cells individually or in combination with 1 mM of 4-HT. Cells were supplemented with fresh media every three days.

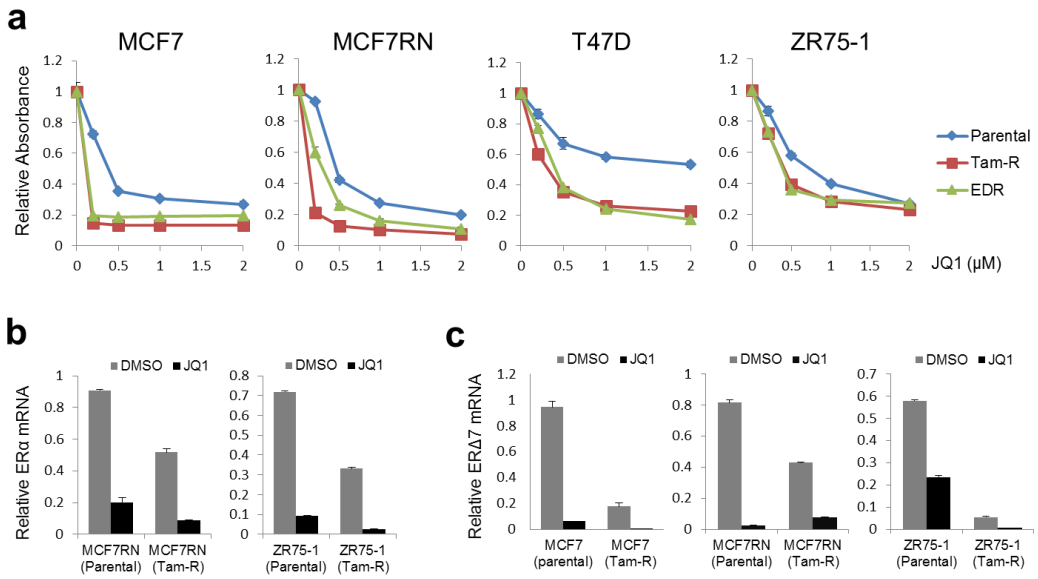


Fig. 7. (a) JQ1 inhibits the growth of multiple Tam-R and EDR (estrogen-deprivation-resistant) breast cancer cells. The anti-tumor effect of JQ1 was evaluated in several ER-positive, Tam-R, or EDR cell lines including MCF7, T47D, MCF7RN, and ZR75-1. Cell growth was determined after 5 days of JQ1 treatment. Error bars were shown as s.e.m. (b) JQ1 downregulates ERα gene expression in multiple ER-positive breast cancer cell lines. Here MCF7RN and ZR75-1 parental and Tam-R cells were tested. 0.5 μM of JQ1 was used to treat the cells 24 hours before harvest. (c) JQ1 also reduces expression of ERΔ7, a major ERα isoform, in multiple ER-positive cell lines. 0.5 μM of JQ1 was used to treat the cells 24 hours before harvest.

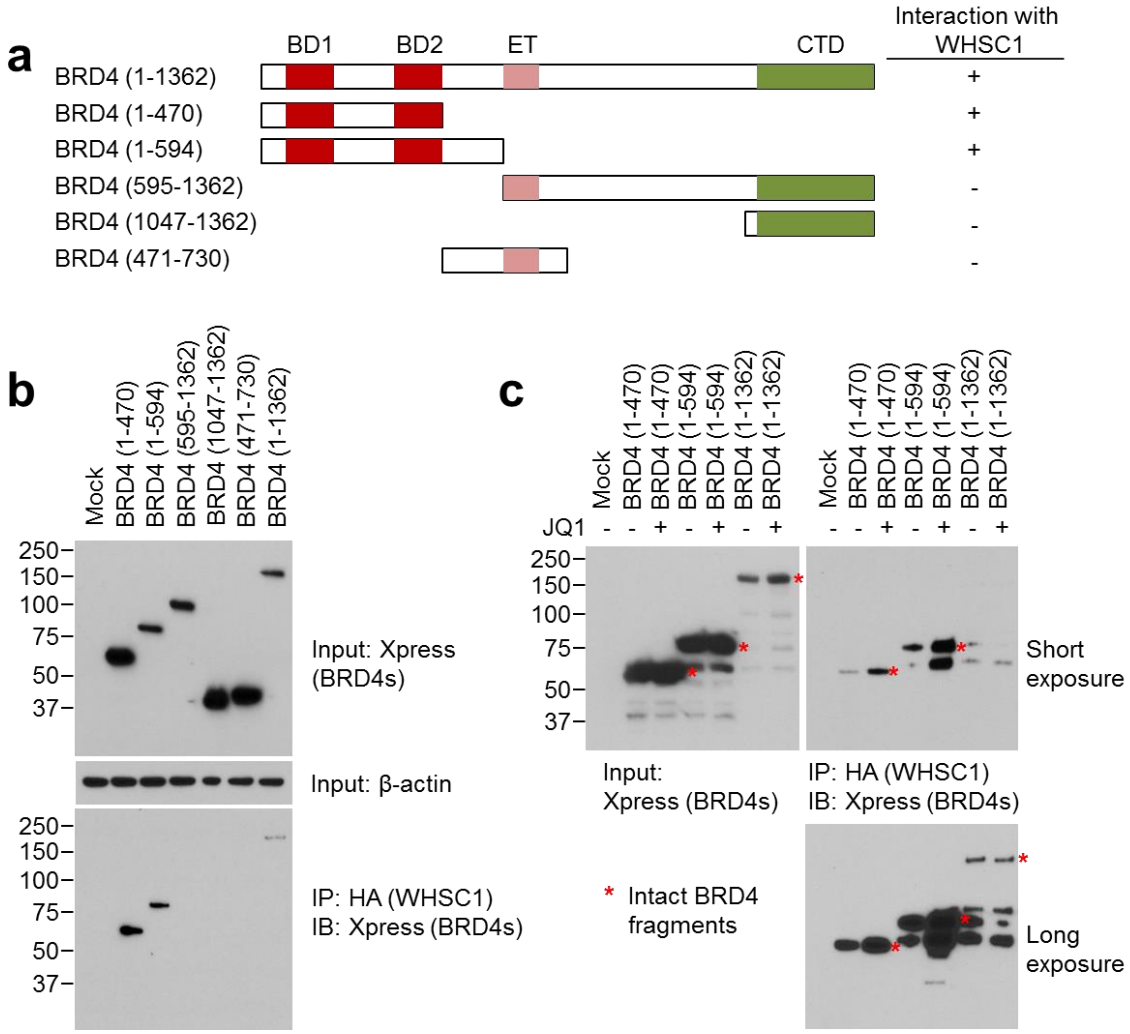


Fig. 8. Deletion mapping of the interacting region between BRD4 and WHSC1. (a) Diagram of BRD4 deletion mutants and the result of their interactions with WHSC1. BD, bromodomain; ET, extraterminal domain; CTD, C-terminal domain. (b) Interaction between WHSC1 and BRD4 deletion mutants by Co-IP experiment. 293T cells were transiently transfected with vectors expressing HA-tagged WHSC1 and Xpress-tagged BRD4 deletion mutants. IP antibody: anti-HA; blot antibody: anti-Xpress. (c) JQ1 does not interfere with the interaction between WHSC1 and BRD4. 0.5 μM of JQ1 was added to cell culture medium 1 hour before cell harvest. Same concentration of JQ1 was also supplemented in cell lysates with JQ1 treatment during immunoprecipitation. A long exposure image is included to show the immunoprecipated full-length BRD4 protein. The intact BRD4 fragments were marked by red asterisks.

Fig. 9

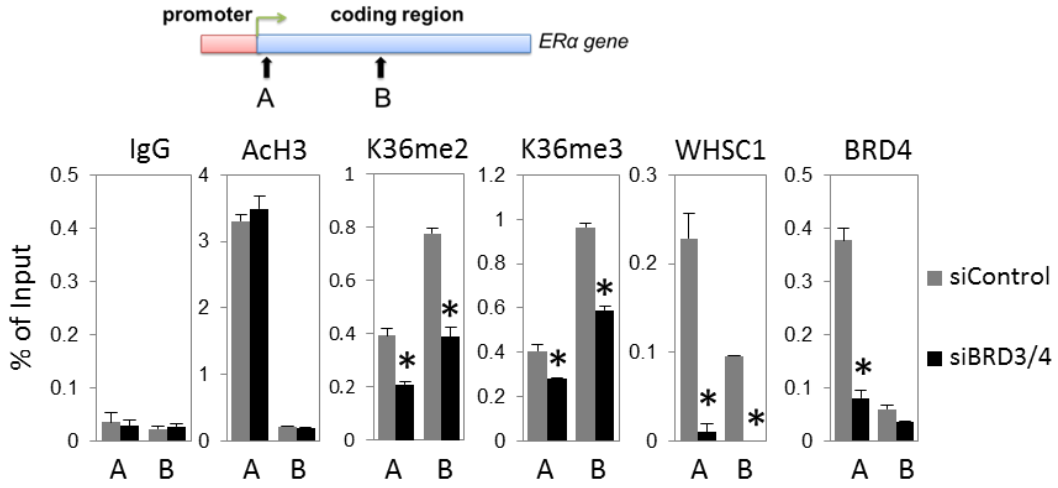


Fig. 9. Loss of BRD3/4 abolished the recruitment of WHSC1 to ERα gene. Chromatin-IP was performed in MCF7 cells treated with BRD3/4 siRNA or control siRNA for 2 days. Primer pair A locates next to promoter region and primer pair B locates in the gene body region of ERα. Each IP was duplicated and average values were shown. Error bars indicate s.e.m. *, p<0.05 by t-test.

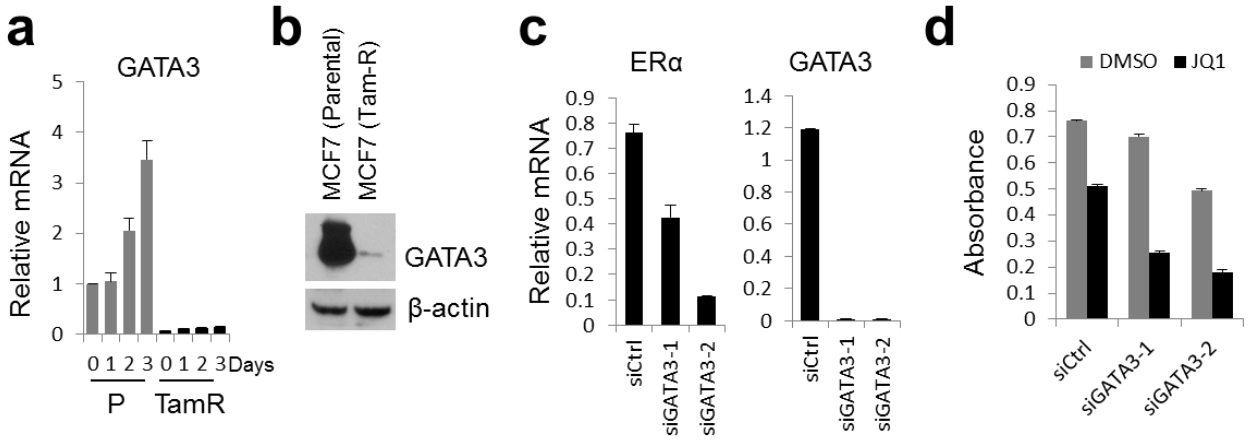


Fig. 10. (a) Expression of GATA3 in MCF7 parental and Tam-R cells after JQ1 treatment. Error bars were sown as s.e.m. (b) Comparison of GATA3 protein levels by Western blot analysis in MCF7 parental and Tam-R cells. (c) Knockdown of GATA3 by siRNA reduces ERα gene expression. Error bars were shown as s.e.m. (d) Knockdown of GATA3 by siRNA enhances JQ1 inhibition function in MCF7 parental cells. Error bars were shown as s.e.m.

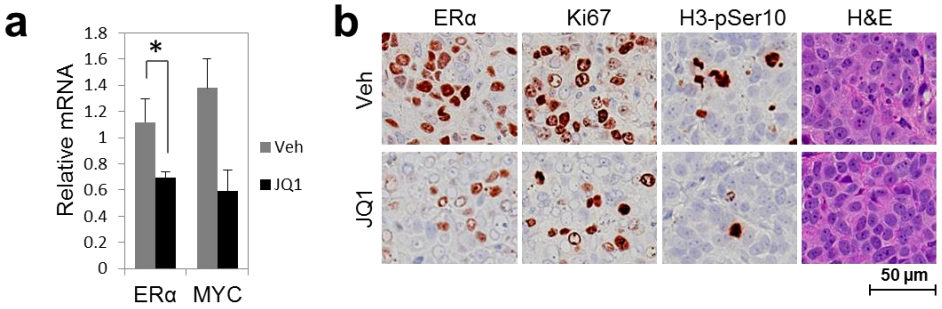


Fig. 11. (a) JQ1 downregulates ERα mRNA in JQ1-treated xenograft tumor tissue. JQ1 was administered by intraperitoneal injection at 50 mg/kg daily for 7 days. 6 mice per group. *, $p<0.05$ by t-test. Error bars were shown as s.e.m. (b) Immunohistochemistry staining of ERα, Ki67 and histone H3 phospho-serine 10 in xenograft Tam-R tumor from Vehicle or JQ1 treated group.

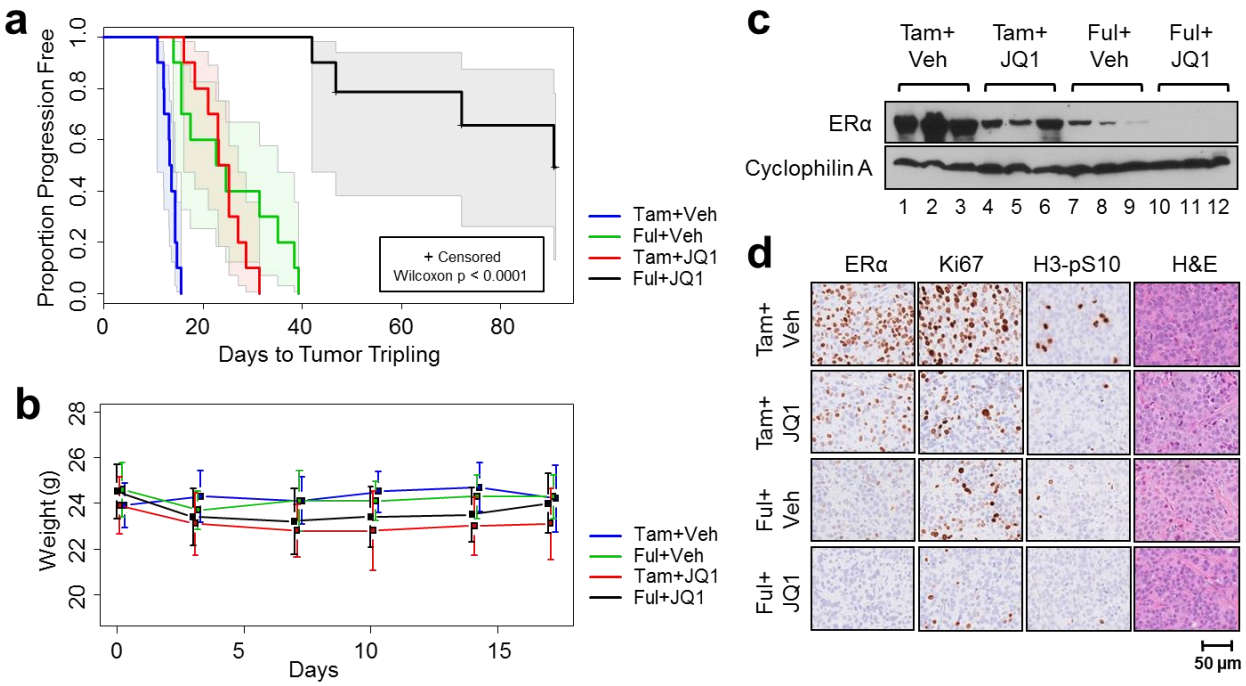


Fig. 12. A combination therapy of JQ1 and fulvestrant in Tam-R xenograft mouse model. (a) Ovariectomized mice bearing Tam-R established MCF7 tumors were randomized (on day 0) into 4 groups of treatment: Tam+Vehicle, Tam+JQ1, fulvestrant+Vehicle, and fulvestrant+JQ1, with 10 mice per group. JQ1 was administered daily at 50 mg/kg, while 5 mg fulvestrant was given by subcutaneous injection weekly. Tumors were harvested when they reached 1000 mm3 or three months after treatment. (b) Body weight measurement for xenograft experiment shown in (a). The error bars show means +/- s.e.m. (c) Tumors were harvested by the end of the treatment, and Western blot was performed using antibodies against ERα and cyclophilin A. (d) Immunohistochemical staining of ERα, Ki67 and histone H3 phospho-serine 10 in xenograft Tam-R tumors from four groups treated with Tam+Vehicle, Tam+JQ1, fulvestrant+Vehicle, or fulvestrant+JQ1.

The *LIFETEST* Procedure

Stratum 1: Fulvestrant + JQ1

Quartile Estimates				
		95% Confidence Interval		
Percent	Point Estimate	Transform	[Lower	Upper)
75	.	LOGLOG	90.7000	.
50	90.7000	LOGLOG	42.0000	.
25	72.0357	LOGLOG	42.0000	.

Stratum 3: Tam + Vehicle

Quartile Estimates				
		95% Confidence Interval		
Percent	Point Estimate	Transform	[Lower	Upper)
75	14.3865	LOGLOG	13.2714	15.5455
50	13.4754	LOGLOG	10.8636	14.3865
25	12.1205	LOGLOG	10.8636	13.6793

Stratum 2: Tam + JQ1

Quartile Estimates				
		95% Confidence Interval		
Percent	Point Estimate	Transform	[Lower	Upper)
75	35.0191	LOGLOG	22.5904	39.2530
50	23.5931	LOGLOG	14.0000	35.0191
25	15.5904	LOGLOG	14.0000	24.5957

Stratum 4: Fulvestrant + Vehicle

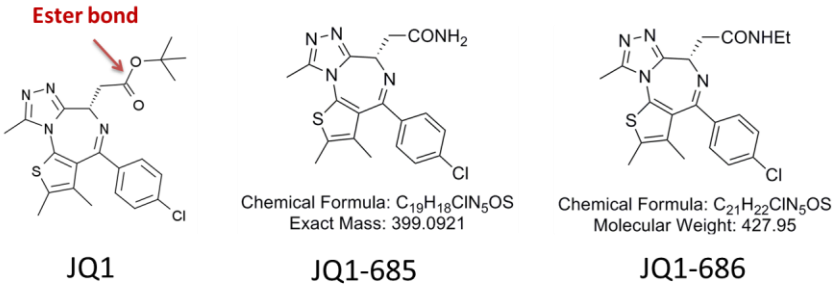
Quartile Estimates				
		95% Confidence Interval		
Percent	Point Estimate	Transform	[Lower	Upper)
75	27.1024	LOGLOG	23.0508	31.3444
50	24.1017	LOGLOG	16.1034	27.1024
25	21.0517	LOGLOG	16.1034	25.1527

Test of Equality over Strata			
Test	Chi-Square	DF	Pr > Chi-Square
Wilcoxon	56.6203	3	<.0001

Adjustment of Multiple Comparisons for the Wilcoxon Test				
Strata Comparison			p-Values	
treatment	treatment	Chi-Square	Raw	Sidak
Fulvestrant + JQ1	Tam + JQ1	7.5171	0.0061	0.0361
Fulvestrant + JQ1	Tam + Vehicle	45.7664	<.0001	<.0001
Fulvestrant + JQ1	Fulvestrant + Vehicle	6.8020	0.0091	0.0534
Tam + JQ1	Tam + Vehicle	10.8938	0.0010	0.0058
Tam + JQ1	Fulvestrant + Vehicle	0.0143	0.9049	1.000
Tam + Vehicle	Fulvestrant + Vehicle	11.7782	0.0006	0.0036

Fig. 13. Time to tumor tripling from four treatment groups (Tam+Vehicle, Tam+JQ1, fulvestrant+Vehicle, and fulvestrant+JQ1, with 10 mice per group) was compared using the generalized Wilcoxon test. Adjustments for multiple comparisons were also shown.

a



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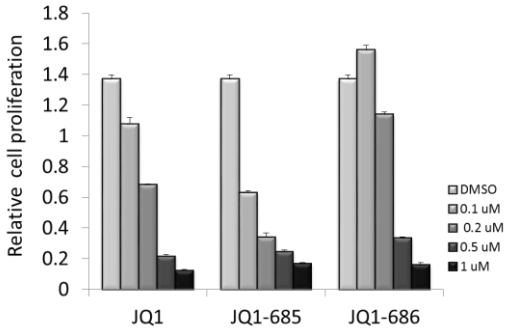

Fig. 14. Development of amide derivatives of JQ1. (a) Chemical structure of JQ1 and its two amide derivatives JQ-685 and JQ686. (b) The inhibitory effect of JQ1 derivatives on the growth of tamoxifen-resistant MCF7 cells. Cell proliferation were determined by MTS assay after three days of treatment.

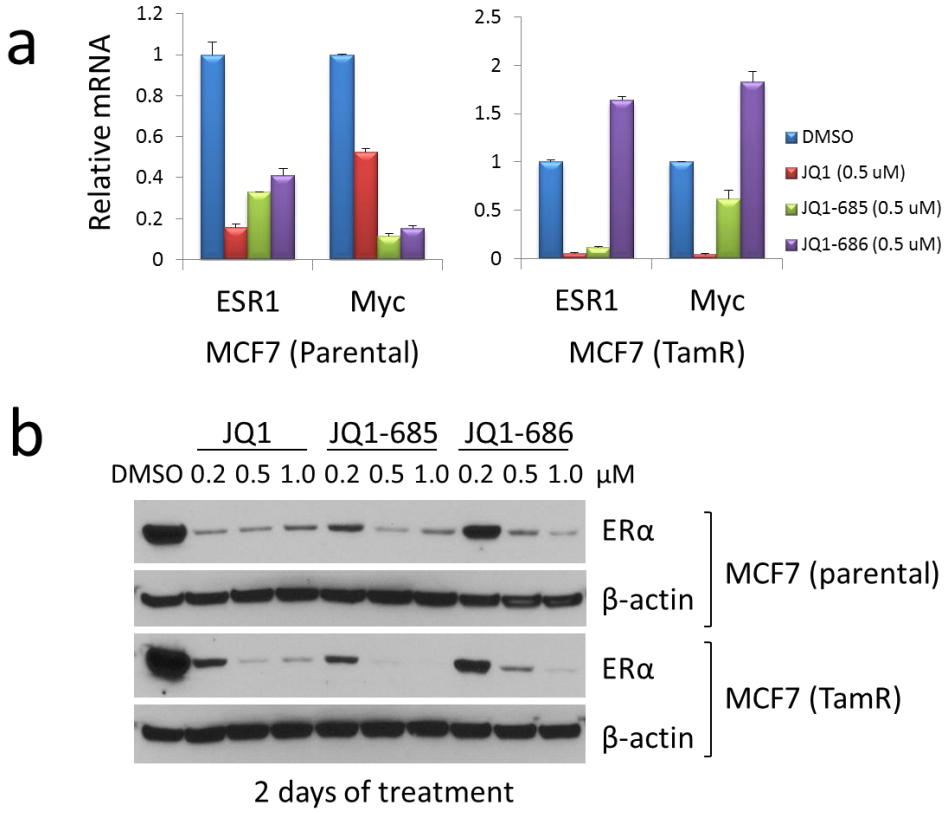

Fig. 15. Similar to JQ1, its amide derivatives also repressed gene expression of ESR1 and Myc. (a) MCF7 parental and tamoxifen-resistant cells were treated with JQ1 and its amide derivatives at 0.5 uM, and expression levels of ESR1 and Myc were measured by RT-qPCR. (b) JQ1 and its amide derivatives reduce ERα protein level in MCF7 cells. Cells were treated with listed compounds for 2 days before being harvested for Western blot analysis.

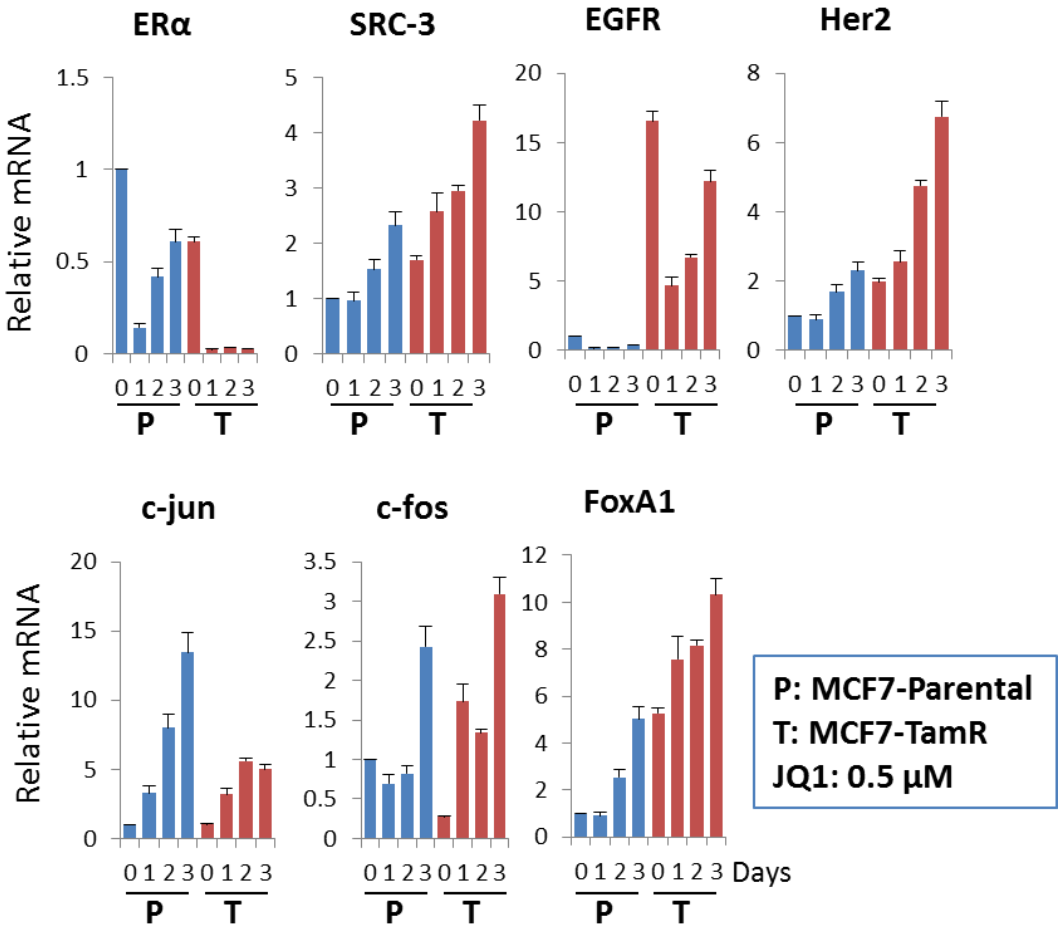

Fig. 16. The SRC-3 coactivator and growth receptor pathways were enhanced upon JQ1 treatment in tamoxifen resistant cells. Gene expression assay was performed after JQ1 treatment in parental and tamoxifen-resistant MCF7 cells.

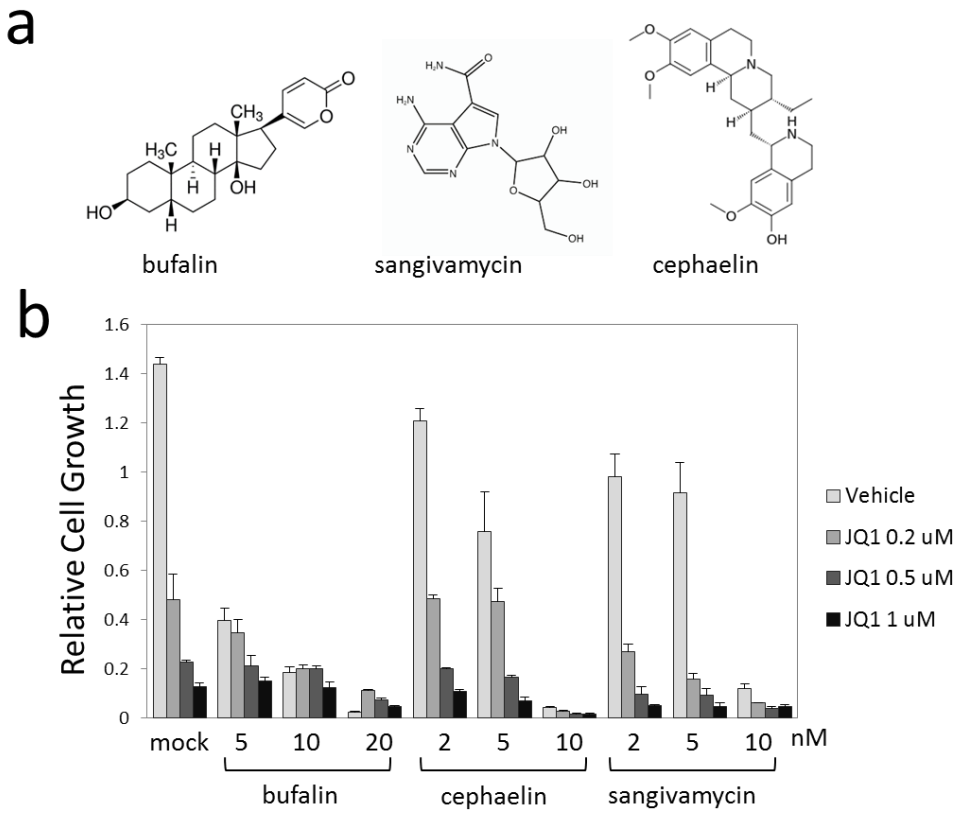

Fig. 17. Combinatory treatment of MCF7 TamR cells with JQ1 and SRC-3 inhibitors. (a) Chemical structure of three potent SRC-3 inhibitors identified from a small molecule screen. (b) MCF7 TamR cells were treated with listed compounds for 3 days, and relative cell proliferation was determined by MTS assay.

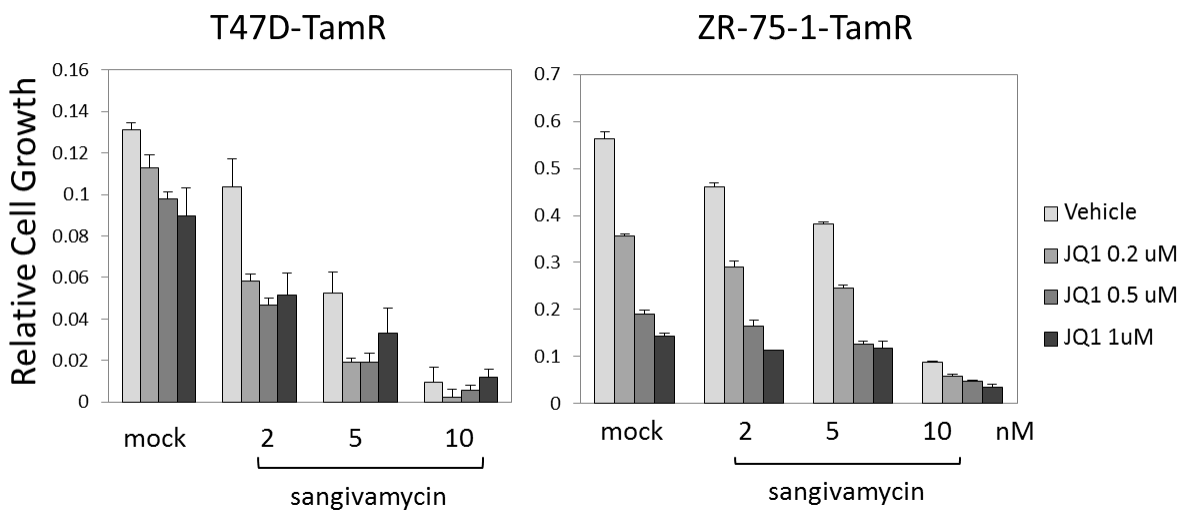

Fig. 18. Evaluation of anti-proliferation effect of sangivamycin in combination with JQ1 in additional tamoxifen-resistant cell models including T47D and ZR-75-1.

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ORIGINAL ARTICLE

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An epigenomic approach to therapy for tamoxifen-resistant breast cancer

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Tamoxifen has been a frontline treatment for estrogen receptor alpha (ERα)-positive breast tumors in premenopausal women. However, resistance to tamoxifen occurs in many patients. ER still plays a critical role in the growth of breast cancer cells with acquired tamoxifen resistance, suggesting that ERα remains a valid target for treatment of tamoxifen-resistant (Tam-R) breast cancer. In an effort to identify novel regulators of ERα signaling, through a small-scale siRNA screen against histone methyl modifiers, we found WHSC1, a histone H3K36 methyltransferase, as a positive regulator of ERα signaling in breast cancer cells. We demonstrated that WHSC1 is recruited to the *ERα* gene by the BET protein BRD3/4, and facilitates *ERα* gene expression. The small-molecule BET protein inhibitor JQ1 potently suppressed the classic ERα signaling pathway and the growth of Tam-R breast cancer cells in culture. Using a Tam-R breast cancer xenograft mouse model, we demonstrated *in vivo* anti-breast cancer activity by JQ1 and a strong long-lasting effect of combination therapy with JQ1 and the ER degrader fulvestrant. Taken together, we provide evidence that the epigenomic proteins BRD3/4 and WHSC1 are essential regulators of estrogen receptor signaling and are novel therapeutic targets for treatment of Tam-R breast cancer.

***Keywords*:** epigenomic; tamoxifen; breast cancer

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Introduction

Estrogen signaling is crucial for the development of normal mammary gland and breast cancer. Estrogen binds to and activates estrogen receptors (ERs), resulting in expression of genes involved in cell proliferation and survival. By blocking estrogen binding to ER alpha (ERα), the selective ER modulator, tamoxifen remains a frontline treatment for ERα-positive breast cancer [1]. However, eventually many tumors develop tamoxifen resistance. Interestingly, ERα is still important for the growth of breast cancer cells with acquired drug resistance. For instance, recent chromatin immunoprecipitation (ChIP)-seq analysis on primary breast tumors from patients clearly shows that ERα is still recruited to chromatin in drug-resistant breast cancer [2]. Moreover, in the absence of estrogen, epidermal growth factor could induce AP-1-dependent ERα genomic targets [3]. Therefore, there is an urgent need to develop novel treatments to further suppress ER signaling in Tam-R breast cancer. Recent data show that mTOR inhibition is effective in overcoming hormone resistance [4]. However, mTOR inhibition is associated with several side effects, limiting its use in patients who must take it for a long time. Combinations of HDAC inhibitor vorinostat and tamoxifen also showed some effect in reversing hormone resistance [5]. In this study, we are exploring a novel strategy to overcome endocrine resistance by shutting down expres-

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sion of the *ER* gene itself.

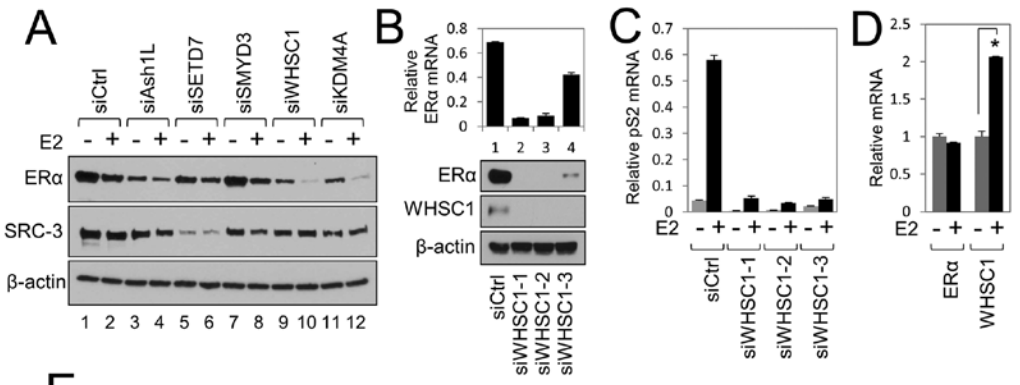
Results

WHSC1 regulates ERα gene expression

Recent progress has shown that histone-modifying enzymes and coregulators play important roles in the regulation of gene expression [6-8]. In this study, we were interested in understanding how histone modifications, particularly histone methylation, regulate ERα signaling in breast cancer cells. To this end, we performed a small-scale siRNA screen to identify epigenomic enzymes involved in estrogen signaling. We knocked down histone methyltransferases (HMTs) or demethylases (HDMTs) individually in MCF7 cells by siRNA, and determined the expression levels of ERα and its target genes *GREB1* and *pS2*. We focused on the HMTs and HDMTs that target lysine residues, because the diverse roles of histone lysine methylation in gene expression have been well established. The role of 29 HMTs and 18 HDMTs in estrogen signaling was tested by knockdown using individual siRNAs. Supplementary information, Figure S1 shows the mRNA levels of ERα and its target gene *GREB1*. Knockdown of SMYD3 led to an increased level of *GREB1* mRNA, while knockdown of three HMTs, ASH1L, SETD7 and WHSC1, and two HDMTs, KDM4A and KDM7C reduced *GREB1* mRNA level by > 60%. Similar effects were observed for *pS2* gene expression (data not shown). ERα mRNA levels were also significantly reduced in these samples, indicating that these epigenomic enzymes are critical for ERα and its target gene expression. We confirmed the reduction of ERα protein levels by western blot analysis as shown in Figure 1A.

WHSC1 encodes a HMTs that methylates histone H3K36 [9, 10]. Methylation of H3K36 is a key histone mark for transcription elongation. *WHSC1* was initially found to be deleted in Wolf-Hirschhorn syndrome (WHS), which is a malformation syndrome associated with a hemizygous deletion of the distal short arm of chromosome 4. Later studies revealed that *WHSC1* is significantly overexpressed in many cancers including breast cancer [11, 12]. Importantly, its expression is associated with tumor aggressiveness and bad prognosis in several breast cancer studies [13, 14]. However, the underlying molecular mechanism of its role in breast cancer development remains unknown. Therefore, we focused on WHSC1 for further study.

Using three individual *WHSC1* siRNAs, we confirmed the essential role of WHSC1 in expression of ERα and its target genes (Figure 1B and 1C). Interestingly, *WHSC1* mRNA levels were increased by two-fold in MCF7 cells treated with estradiol, indicating that *WHSC1* itself is an ERα-regulated gene (Figure 1D), forming a positive



E

Tumor Type	ER-positive	Her2	Luminal A	Luminal B
Pearson's r	0.245	0.037	0.285	0.125
P value	$8.72*10^{-6}$	0.4025	$3.48*10^{-5}$	0.095

Figure 1 WHSC1 regulates *ERα* gene expression. **(A)** Confirmation of siRNA screening results by western blot analysis. Five genes were selected from the siRNA screening. The protein levels of ERα and SRC-3 were measured by western blot analysis. **(B)** Knockdown of WHSC1 by three different siRNAs all decreased mRNA and protein levels of ERα in MCF7 cells. **(C)** Knockdown of *WHSC1* reduced the expression of ERα target gene *pS2* in MCF7 cells. 10 nM of estradiol (E2) was added 24 h before cell harvest. **(D)** Enhanced expression of WHSC1 by treatment of 10 nM E2 for 24 h in MCF7 cells. mRNA levels of ERα and WHSC1 were measured by RT-qPCR. **P* < 0.05 by a two-tailed *t*-test. **(E)** Correlation of ERα mRNA and WHSC1 mRNA levels in a subset of the TCGA breast cancer samples.

feedback regulatory loop. In support of this, we found a positive correlation in mRNA levels between WHSC1 and ERα in ER-positive patients in the TCGA database, particularly in luminal-A-type of breast tumors (Figure 1E).

WHSC1 and BRD3/4 coordinately regulate ERα expression and function

Because WHSC1 does not contain a DNA-binding domain, it cannot bind directly to the ERα gene promoter/enhancer to regulate its transcription. To investigate how WHSC1 activates ERα gene expression, we searched the Epicome database (http://epicome.org), a mass spectrometry-based proteomics database generated by the Nuclear Receptor Signaling Atlas (NURSA) [15, 16], to identify WHSC1-interacting proteins. BRD3 and BRD4, two bromodomain-containing proteins, are at the top of a list of potential WHSC1-interacting proteins (Supplementary information, Figure S2A). Their association with WHSC1 was confirmed by immunoprecipitation/western blot analysis using either exogenously expressed or endogenous proteins. As shown in Figure 2A-2C, BRD3 and BRD4 were co-immunoprecipitated with WHSC1 protein and vice versa, but another BET family protein, BRD2, did not interact significantly with WHSC1. Similar interaction was detected between endogenous BRDs and WHSC1 in MCF7 cells (Supplementary information, Figure S2), and this is in agreement with a recent report of an interaction between WHSC1 and BRD4 in MEF cells [17].

BRD3 and BRD4 belong to the BET (bromodomain and extraterminal domain) family of proteins. In humans, there are four BET proteins including BRD2, BRD3, BRD4, and BRDT [18]. BET family members contain two bromodomain and one extraterminal domain. The bromodomain specifically recognizes acetylated lysine residues on the histone tails. BRD3 and BRD4 are implicated in the transcription elongation process by association with the PAF1 complex and the pTEFb complex, respectively [19]. BRD4 also regulates the expression of G1 cell cycle genes [20]. We next determined the domain responsible for the interaction between WHSC1 and BRD3/4 by deletion mapping. As shown in Supplementary information, Figure S2D and S2E, BRD4 interacts with WHSC1 through its N-terminal 470 amino acids, which contains two bromodomains. Interestingly, treatment of cells with JQ1 failed to disrupt the interaction between WHSC1 and BRD4, suggesting that BRD4 can bind to WHSC1 and acetylated lysine simultaneously (Supplementary information, Figure S2F).

Given the key role of WHSC1 in ERα gene expression and the physical association between WHSC1 and BRD3/4, we hypothesized that WHSC1 is recruited to the ERα gene promoter by BRD3/4 which binds directly to acetylated histone tails and subsequent methylation of K36 on histone H3 by WHSC1, then could facilitate the transcription elongation of the *ERα* gene. To test this hypothesis, we asked whether BRD3/4 are required for the expression of ERα and its target genes. As shown in Figure 2D, simultaneous knockdown of BRD3 and BRD4 dramatically reduced the mRNA levels of ERα and *pS2*, indicating that BRD3 and BRD4 are crucial for the expression of ERα.

To further test the above hypothesis, BRD3 and BRD4 genes were knocked down in MCF7 cells, and the recruitment of WHSC1 to the *ERα* gene and the level of histone H3 K36 methylation were determined by ChIP-qPCR assay. As shown in Figure 2E, knockdown of BRD3/4 dramatically reduced the recruitment of WHSC1 to the *ERα* gene and the levels of H3K36me2 and H3K36me3 were both significantly reduced, suggesting that BRD3/4 functions as a scaffold to recruit WHSC1, which promotes the transcription elongation of the *ERα/ESR1* gene.

In our model shown in Figure 2F, BET proteins (BRD3/4) recognize acetylated lysine residues on histone tails in the *ERα/ESR1* gene promoter. After recruitment to the *ERα* gene by interacting with BRD3/4, WHSC1 methylates H3K36 and subsequently increases transcription elongation of ERα. On the other hand, estrogen stimulates the expression of WHSC1, forming a positive feedback regulatory loop. Interestingly, we also found that WHSC1 and BRD4 are potent coactivators for ER in an ERE-luciferase gene reporter assay (Supplementary information, Figure S3A). Taken together, our results indicate that WHSC1 is a key regulator of ER signaling, as it is not only a positive epigenomic regulator of ER gene expression, but also a coactivator for ER itself.

Small-molecule inhibitors have been recently developed and published for the BET family of proteins. For instance, JQ1, an acetylated lysine analog, has been reported in recent studies to be a potent BET inhibitor and can be used to treat a number of cancers including multiple myeloma and acute myeloid leukemia [21-23]. Based on our results, we tested whether JQ1 can suppress ERα expression. As shown in Figure 2G, treatment of MCF7 cells with JQ1 significantly reduced the mRNA levels of ERα and its target genes *pS2, GREB1*, and *Cyclin D1*, but not other breast cancer genes such as *FoxA1, SRC-3*, and *Her2* (Supplementary information, Figure S3B). Another bromodomain inhibitor I-BET had a similar effect, although at a slightly higher concentration (Figure 2H). Similar experiments were performed on prostate cancer LNCaP cells. Interestingly, androgen receptor mRNA

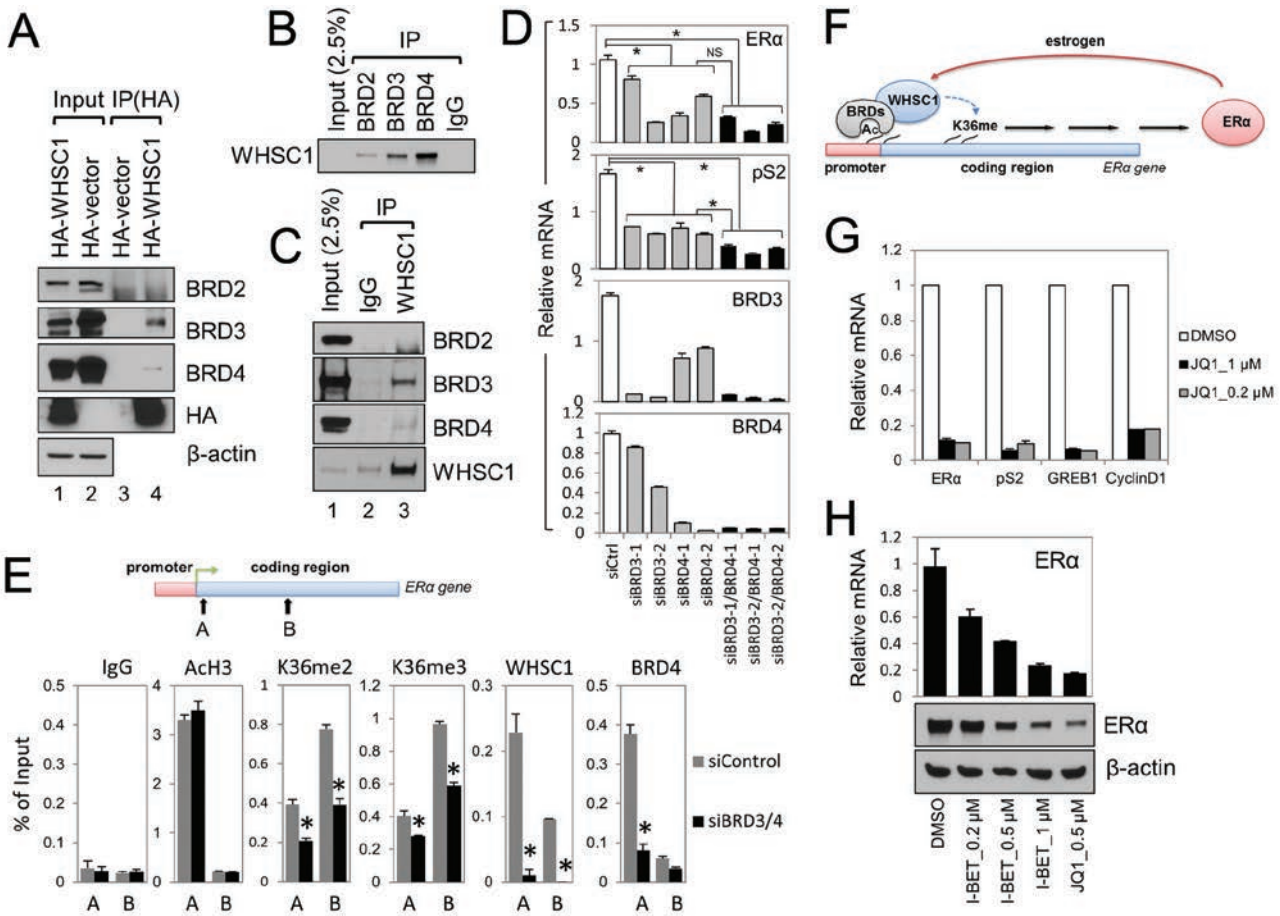


Figure 2 WHSC1 and BRD3/4 coordinately regulate ERα expression and function. **(A)** Co-immunoprecipitation (co-IP) of WHSC1 and BRD proteins from transiently transfected 293T cells. HA-tagged WHSC1 or empty vector was transiently transfected to 293T cells for 48 h. The whole-cell lysate was cleared and immunoprecipitated with anti-HA antibody. Input, 2.5%. **(B)** Co-IP of endogenous WHSC1 and BRD proteins from HeLa nuclear extract. Input, 2.5%. **(C)** Reciprocal co-IP of endogenous WHSC1 and BRD proteins from HeLa nuclear extract. Input, 2.5%. **(D)** Knockdown of BRD3 and BRD4 reduced expression of ERα and its target gene *pS2* in MCF7 cells. Error bars indicate SEM. **P* < 0.01 by *t*-test. **(E)** Loss of BRD3/4 abolished the recruitment of WHSC1 to *ERα* gene. ChIP was performed in MCF7 cells treated with BRD3/4 siRNA or control siRNA for 2 days. Primer pair A locates next to promoter region and primer pair B locates in the coding region of ERα. Each IP was duplicated and average values were shown. Error bars indicate SEM. **P* < 0.05 by *t*-test. **(F)** A hypothetical model of regulation of ERα gene expression by WHSC1 and BRD3/4. **(G)** Bromodomain inhibitor JQ1 efficiently reduced the expression of ERα and its target genes in MCF7 cells. Cells were treated with JQ1 for 24 h before harvest. **(H)** Another Bromodomain inhibitor I-BET had similar function in suppressing ERα expression. MCF7 cells were treated with I-BET for 24 h before harvest.

levels were not altered upon JQ1 treatment, indicating that the suppression of ERα expression by JQ1 is selective (Supplementary information, Figure S3C).

JQ1 inhibits growth of Tam-R breast cancer cells

Next, we investigated whether the BRD3/4 inhibitor JQ1 inhibits growth of breast cancer cells. We treated parental MCF7 cells and a Tam-R derivative with different doses of JQ1 or vehicle control. As shown in Figure 3A, JQ1 potently inhibited the growth of Tam-R MCF7 cells, while it only moderately inhibited the growth of parental MCF7 cells at a concentration of 0.2 μM (At a higher concentration of 0.5 μM, JQ1 also inhibited parental MCF7 cell growth.). We further tested three more ER-positive breast cancer cell lines including T47D, MCF7 RN, and ZR75-1 cells. We found that JQ1 inhibited the growth of all of these breast cancer cell lines, with higher efficacy toward the Tam-R cells. Interestingly, JQ1 also inhibited four estrogen-deprivation-resistant (mimics aromatase inhibitor resistance) lines (Supplementary information, Figure S4A). Simlar to MCF7 cells, JQ1 downregulated ERα mRNA levels in MCF7

RN and ZR75-1 cells. The ERα dominant-negative splicing variant ERΔ7 was similarly downregulated [24] (Supplementary information, Figure S4B and S4C).

Our ChIP-qPCR analysis provided further evidence that disruption of BRD3/4/WHSC1/ERα axis by JQ1 suppressed ERα gene expression. As shown in Figure 3B, overall, the levels of histone modifications marking active promoters, such as acetylated histone H3 and H3K4me3, were significantly lower on the ERα gene promoter in Tam-R MCF7 cells, likely due to lack of other key transcriptional factor(s). JQ1 treatment in Tam-R cells eliminated the recruitment of BRD3/4 and WHSC1 to the ERα gene promoter, and dramatically reduced the level of H3K4me3 and H3K36me3, two histone marks of transcription activation. In parental MCF7 cells, the recruitment of BRD3/4 and WHSC1 was still maintained at relatively high levels, although it was reduced by JQ1. BRD3 and BRD4 mRNA levels were not reduced (actually increased) by JQ1, while WHSC1 was reduced by JQ1 both at the mRNA and protein levels (Figure 3C and 3D).

We next wished to understand why Tam-R breast cancer cells are more sensitive to JQ1. Knockdown of WHSC1 alone reduced the ERα mRNA levels similarly in both parental and Tam-R breast cancer cells, and inhibited the growth of parental and Tam-R MCF7 cells similarly (Supplementary information, Figure S4D and S4E). This result suggests that WHSC1 is an important regulator of ERα gene expression and cell growth, but does not cause extra JQ1 sensitivity in Tam-R cells. However, we noticed that when cells were treated with JQ1 for up to 3 days, ERα mRNA was persistantly suppressed in Tam-R MCF7 cells (Figure 4A). In contrast, in parental MCF7 cells, ERα mRNA level was abolished initially, but recovered after prolonged treatment (Figure 4A). Moreover, JQ1 was reported to inhibit MYC signaling in previous studies [21, 25, 26]. Thus, we measured expression of MYC in JQ1-treated MCF7 cells. As shown in Figure 4A, MYC mRNA level responded to JQ1 treatment similarly to ER in parental and Tam-R MCF7 cells. These results demonstrate that ERα and MYC are JQ1 target genes in Tam-R MCF7 cells, and that sustained sup-

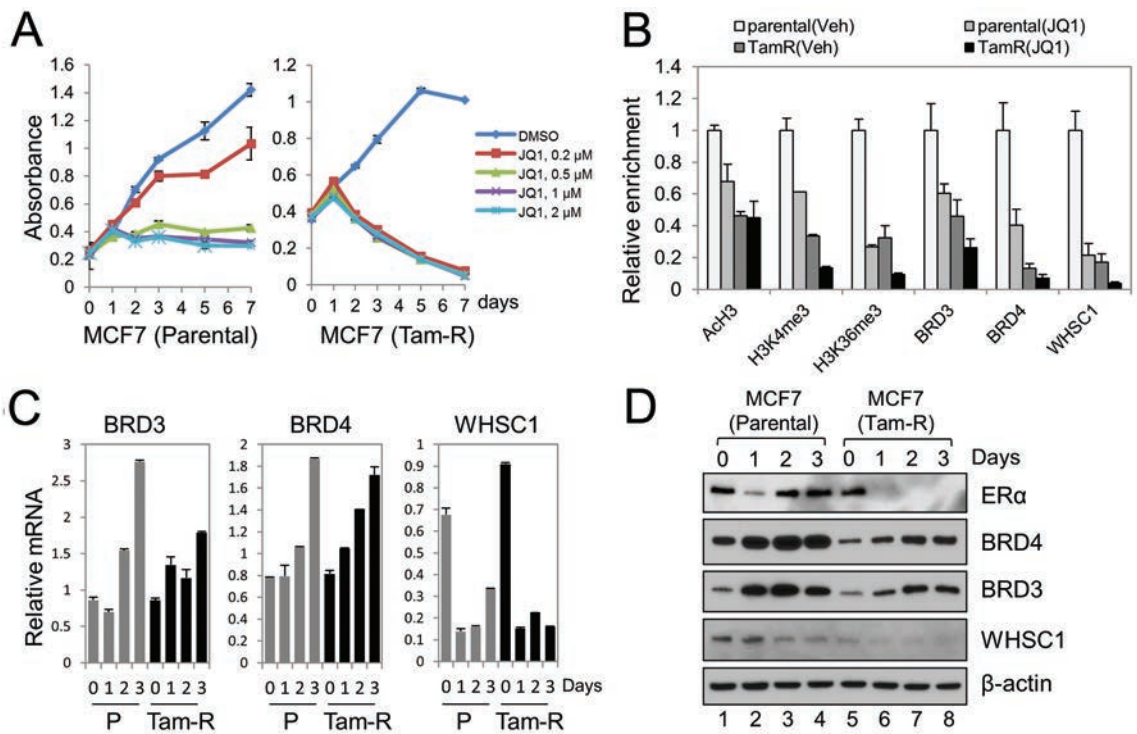


Figure 3 JQ1 inhibits growth of Tam-R cells. **(A)** JQ1 efficiently inhibited the growth of Tam-R MCF7 breast cancer cells as determined by MTS assay. Error bars indicate SEM. **(B)** ChIP assay to determine the level of histone modifications and recruitment of BRD3/4 and WHSC1 to ERα promoter. MCF7 parental and Tam-R cells were treated with DMSO (Veh) or 0.2 M of JQ1 for 24 h before cell harvest. Primer Pair A (shown in Figure 2E) was used for qPCR analysis. Error bars were shown as SEM. **(C)** Expression of BRDs and WHSC1 in 0.2 μM JQ1-treated MCF7 cells. **(D)** Western blot analysis of ERα, BRDs, and WHSC1 proteins from 0.2 μM JQ1-treated MCF7 cells.

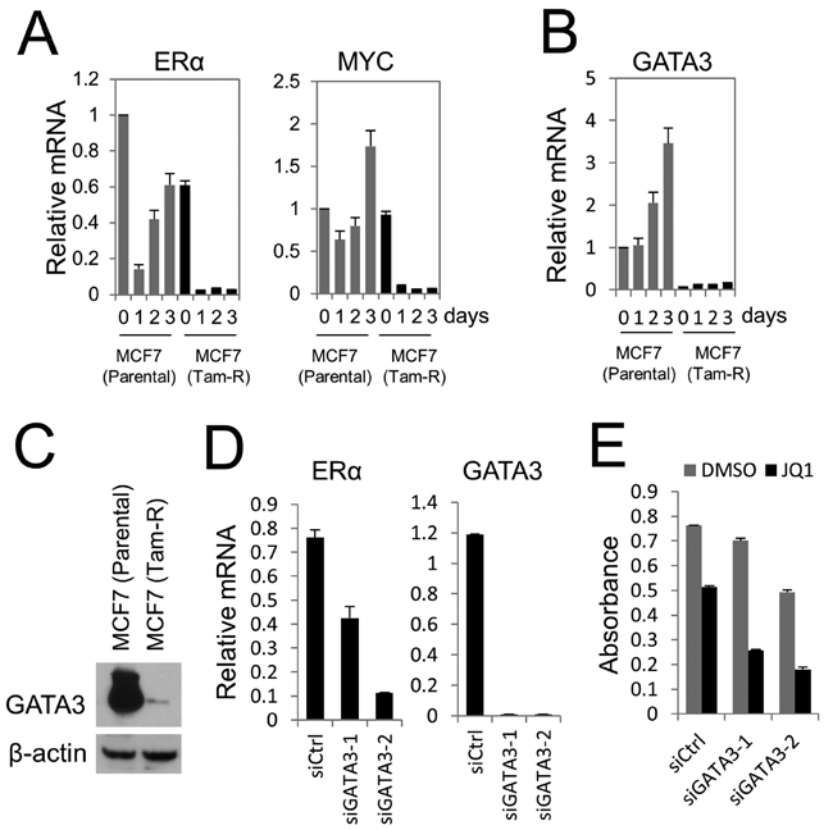
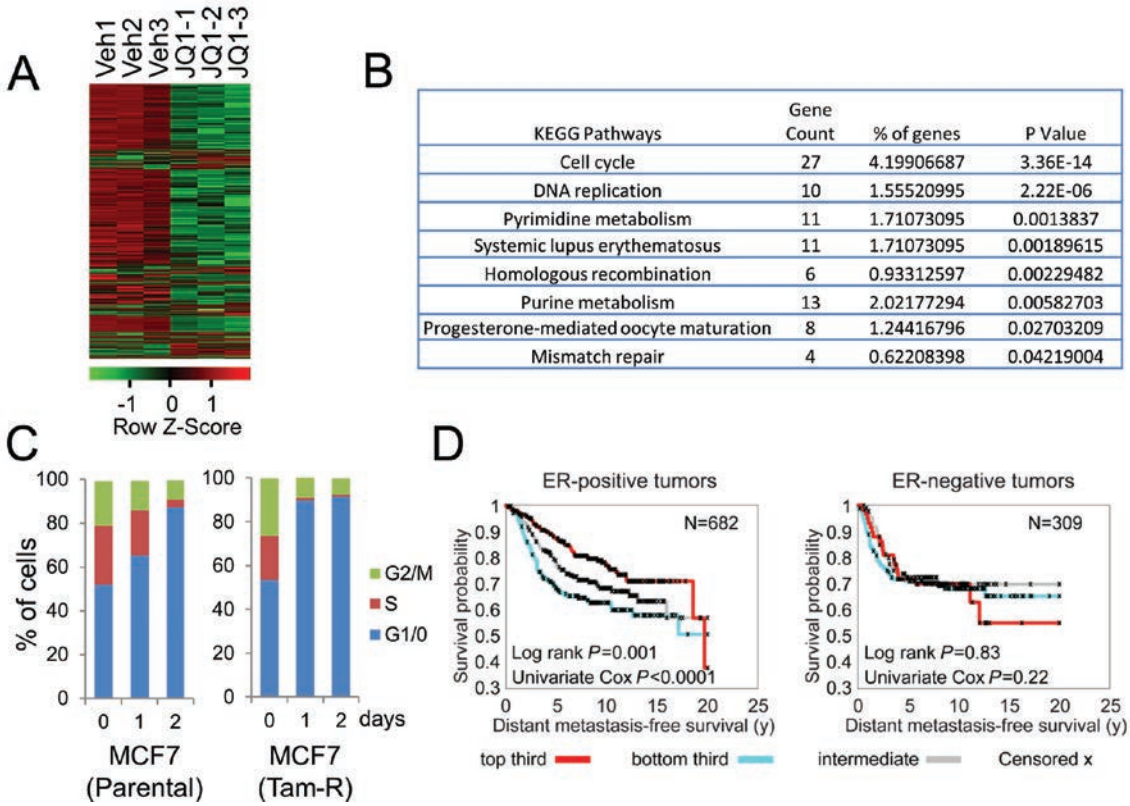


Figure 4 GATA3 is a potential factor to regulate JQ1 sensitivity in MCF7 cells. **(A)** JQ1 suppressed both ERα and MYC signaling pathways in Tam-R MCF7 cells. Parental and Tam-R MCF7 cells were treated with 0.2 μM of JQ1 for different days, and mRNA levels of ERα and MYC were analyzed by RT-qPCR. **(B)** Expression of GATA3 in MCF7 parental and Tam-R cells after JQ1 treatment. **(C)** Comparison of GATA3 protein levels by western blot analysis in MCF7 parental and Tam-R cells. **(D)** Knockdown of GATA3 by siRNA reduces ERα gene expression. **(E)** Knockdown of GATA3 by siRNA enhances JQ1 inhibition function in MCF7 parental cells. All error bars were shown as SEM.

pression of ERα and MYC by JQ1 probably contributes to its more potent anticancer activity on Tam-R breast cancer cells. To gain more mechanistic insight into this observation, we found that GATA3, a key regulator of ER gene expression [27], is highly expressed in parental MCF7 cells, but not in Tam-R cells (Figure 4B and 4C). In parental MCF7 cells, GATA3 expression is further increased by JQ1 treatment (Figure 4B). When we knocked down GATA3 using siRNA, the parental MCF7 cells became more sensitive to JQ1 treatment (Figure 4E). Thus, our results suggest that other key transcription factors, such as GATA3 in parental MCF7 cells, could have contributed to the JQ1 resistance with prolonged treatment. A decrease in such factors (Figure 4B) might contribute to epigenomic environmental changes on the ERα promoter, resulting in greater JQ1 sensitivity in Tam-R lines.

To determine the global signaling pathways that are altered by JQ1 in addition to ER and MYC, microarray analysis was performed on Tam-R MCF7 cells treated with vehicle or 0.2 μM of JQ1. When applying a threshold of $\log2 < -0.2$ or $\log2 > 0.2$, we identified 652 downregulated genes and 219 upregulated genes in JQ1-treated cells (Figure 5A). Supplementary information, Table S1A lists all the genes upregulated or downregulated in major biological pathways by KEGG pathway analysis. Figure 5B shows the biological pathways negatively affected by JQ1. Among them, the cell cycle is an important pathway being affected since cell cycle-related gene expression was significantly altered by JQ1 treatment (Supplementary information, Figure S5A). Consistent with this observation, we found by flow cytometry analysis that Tam-R MCF7 cells were arrested in G1 phase after JQ1 treatment for 24 h, while parental cells were arrested in G1 phase after > 48 h of JQ1 treatment (Figure 5C). Moreover, using these JQ1 target genes, we generated a JQ1-regulated gene signature using the same method as previously described [28]. Using a compendium of several expression array studies, we scored human breast tumors based on the manifestation of the JQ1 gene signature. As shown in Figure 5D, in ER-positive tumors (N = 682), high JQ1 signature activity was associated with better patient outcome (log-rank P = 0.001), while in ER-negative tumors (N = 309), no survival association was found. These data further support the functional significance of JQ1 in ER signaling in breast cancer.

After 2 days of JQ1 treatment, Tam-R cells began to die, suggesting that prolonged cell cycle arrest may induce apoptosis (Figure 3A). This was confirmed by the appearance of cleaved PARP-1 protein in Tam-R MCF7 cells (Supplementary information, Figure S5B). In con-



KEGG Pathways	Gene Count	% of genes	P Value
Cell cycle	27	4.19906687	3.36E-14
DNA replication	10	1.55520995	2.22E-06
Pyrimidine metabolism	11	1.71073095	0.0013837
Systemic lupus erythematosus	11	1.71073095	0.00189615
Homologous recombination	6	0.93312597	0.00229482
Purine metabolism	13	2.02177294	0.00582703
Progesterone-mediated oocyte maturation	8	1.24416796	0.02703209
Mismatch repair	4	0.62208398	0.04219004

Figure 5 Cellular pathways targeted by JQ1. **(A)** Heatmap of expression levels for the genes differentially expressed upon treatment with JQ1. Tam-R MCF7 cells were treated with 0.2 μM of JQ1 or vehicle (DMSO) for 24 h before harvest for microarray analysis. **(B)** Biological pathways were identified by microarray analysis. KEGG pathways were determined by the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov) based on the gene list that is downregulated by JQ1. **(C)** Tam-R MCF7 cells are more sensitive to JQ1-induced G1 cell cycle arrest. Cells were fixed and stained with propidium iodide (PI) before being analyzed by flow cytometry. **(D)** Association of the gene expression signature of JQ1 treatment with breast cancer patient survival. For ER-positive and ER-negative subsets, the differences in risk between tumors, according to degree of manifestation of the JQ1 gene signature, is compared using Kaplan-Meier plots (top third, "strong manifestation"; bottom third, "weak manifestation"; middle third, "intermediate manifestation").

trast, parental MCF7 cells did not undergo apoptosis (Supplementary information, Figure S5B).

JQ1 inhibits tumor growth in Tam-R xenograft mouse model when combined with fulvestrant therapy

Next, we sought to determine the *in vivo* antitumor activity of JQ1. Ovariectomized nude mice were transplanted subcutaneously with Tam-R MCF7 tumors, and were randomized into two groups when tumor volumes reached 200 mm^3. JQ1 or control vehicle was given to these mice by intraperitoneal injection daily. We first performed a pilot experiment to treat the mice for 7 days to test whether JQ1 could downregulate ERα expression *in vivo*. As shown in Supplementary information, Figure S6A, the ERα mRNA level was indeed reduced by about 30% in JQ1-treated tumors; MYC mRNA level was not significantly reduced, although there was a trend. Immunohistochemical staining confirmed a decreased level of ERα protein in JQ1-treated tumors, and a reduced proliferation rate was observed with Ki67 and histone H3 phospho-Ser10 staining (Supplementary information, Figure S6B). This result demonstrates that JQ1 has *in vivo* anticancer activity against Tam-R breast cancer. To achieve an optimal drug response *in vivo*, we tested a combination of JQ1 and fulvestrant/ICI 182,780, an ERα protein degrader, in Tam-R MCF7 xenograft tumors. While single treatment of JQ1 or fulvestrant moderately inhibited tumor growth, the combination of JQ1 and fulvestrant showed a synergistic antitumor activity in the Tam-R tumors. In the vehicle-treated group, the volumes of all the tumors quickly tripled within 17 days, while in the group that received combination therapy, none of the tumors tripled their size after 40 days of treatment, and only about half of the tumors tripled after a prolonged

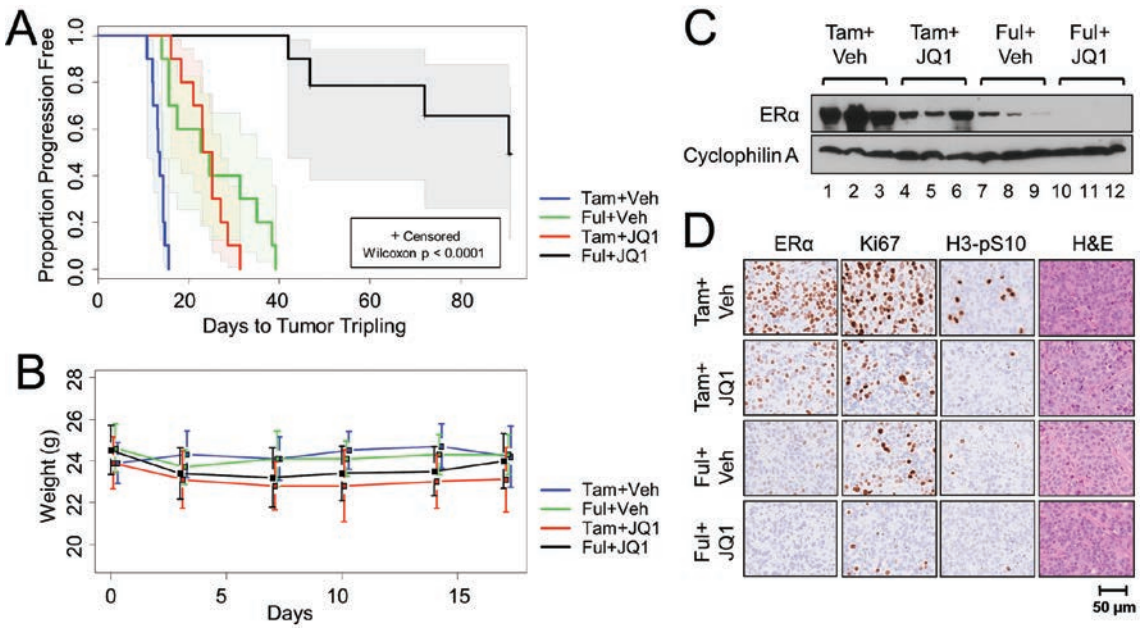


Figure 6 A combination therapy of JQ1 and fulvestrant in Tam-R xenograft mouse model. **(A)** Ovariectomized mice bearing Tam-R-established MCF7 tumors were randomized (on day 0) into four groups of treatment: Tam+vehicle, Tam+JQ1, fulvestrant+vehicle, and fulvestrant+JQ1, with 10 mice per group. JQ1 was administered daily at 50 mg/kg, while 5 mg fulvestrant was given by subcutaneous injection weekly. Tumors were harvested when they reached 1 000 mm^3 or 3 months after treatment. **(B)** Body weight measurement for xenograft experiment shown in **A**. The error bars show means ± SEM. **(C)** Tumors were harvested by the end of the treatment, and western blot was performed using antibodies against ERα and cyclophilin A. **(D)** Immunohistochemical staining of ERα, Ki67, and histone H3 phospho-Ser10 in xenograft Tam-R tumors from four groups treated with Tam+vehicle, Tam+JQ1, fulvestrant+vehicle, or fulvestrant+JQ1.

treatment (90 days) (Figure 6A). Time-to-tumor tripling from the four groups of treatment was compared using the generalized Wilcoxon test as shown in Supplementary information, Figure S6C. Although JQ1-treated animals lose weight initially and then regain it, generally there is no difference among the four groups in the baseline weights, indicating that JQ1 is well tolerated by mice (Figure 6B). By western blot analysis, the protein levels of ERα were dramatically downregulated in the tumors that received combination therapy of fulvestrant and JQ1 (Figure 6C). Immunohistochemical staining for Ki67 and histone H3 phospho-Ser10 confirmed that the combination therapy potently inhibited the tumor cell proliferation (Figure 6D).

Discussion

Epigenetics is defined as heritable changes caused by mechanisms other than changes in DNA sequence, including DNA methylation, histone modifications, and noncoding RNA. Epigenomic proteins such as chromatin regulators have emerged as novel therapeutic targets for cancer. For instance, DNA methylase inhibitors, such as Vidaza and Decitabine, and an HDAC inhibitor, such as Vorinostat, have been used clinically in treating hematological malignancies [29]. Specific inhibitors against H3K79 methylase hDOT1L and H3K27 methylase EZH2 also are being developed for treatment of a variety of cancers [30-32]. The small-molecule inhibitor of epigenomic reader bromodomain JQ1 has shown potent anticancer activity in hematological cancer by targeting the MYC pathway. JQ1 is also thought to be effective in a subset of human lung adenocarcinoma cell lines by inhibiting the expression of an oncogenic transcriptional factor FOSL1 [33]. In a previous report, breast cancer cell lines only show modest sensitivity to JQ1 [34]. In agreement with that observation, we found that the growth of the parental MCF7 and T47D cells was only partially inhibited by JQ1 at high concentrations. Strikingly, the Tam-R cells are more sensitive to JQ1 treatment. In our JQ1 treatment experiment, the ER mRNA level in MCF7 parental cells initially was suppressed significantly but returned to a normal level after prolonged treatment. MYC mRNA levels were only slightly changed by JQ1 in MCF7L parental cells. In contrast, in Tam-R cells, both ER and MYC mRNA levels were consistently sup-

pressed by JQ1 even after 3 days of treatment. Our result provides an explanation for the increased JQ1 sensitivity of Tam-R breast cancer cells compared to parental cells, and further indicates that JQ1 targets both ER ad MYC pathways in Tam-R cells. More importantly, JQ1 shows *in vivo* anticancer activity in suppressing the Tam-R breast cancer growth in the xenograft mouse model. A combination treatment of fulvestrant and JQ1 more effectively downregulated ERα and inhibited *in vitro* and *in vivo* tumor growth, providing a new potential approach for treating Tam-R and ERα-dependent breast cancer.

Materials and Methods

Small-scale siRNA screening

A customized small siRNA library contains individual Stealth RNAi siRNAs (Life Technologies) targeting 29 histone lysine methyltransferases and 18 histone lysine demethylases. All siRNAs used in this study were transfected to MCF7 cells at the final concentration of 20 nM using Lipofectamin RNAiMAX reagent.

Cell culture and transfection

MCF7, MCF7 RN, T47D, and ZR75-1 cells were maintained in RPMI 1640 medium supplemented with 10% FCS. MCF7 cells were initially obtained from Dr Marc Lippman in 1985 at the National Cancer Institute, Bethesda, MD. T47D cells were purchased from ATCC. MCF7 RN cells were initially obtained from Dr Robert Nicholson [35]. The Tam-R lines were established from a long-time treatment of 100 nM 4-hydroxy tamoxifen (4-HT) (Sigma) until cell growth was resumed. The parental cells were cultured in RPMI1640 supplemented with 10% of FCS, whereas their Tam-R derivative line was cultured in phenol red-free medium containing 10% charcoal-dextran-stripped FCS and 100 nM of 4-HT. All cell lines were authenticated once the resistance was established, and mycoplasma contamination is tested once every 6 months. For estradiol-induced experiments, MCF7 cells were maintained in phenol red-free medium containing 10% charcoal-dextran-stripped FCS until hormone addition. Fugene 6 transfection reagent (Roche) was used for transient overexpression experiments. All siRNAs used in this study were individual Stealth RNAi siRNAs from Life Technologies, and transfected at the final concentration of 20 nM. Lipofectamin RNAiMAX reagent was used for all the siRNA transfections. The mammalian expression vector of WHSC1/MMSET (pCEFL-MMSET-II) and its control vector plasmids were kindly provided by Dr Zhenkun Lou at Mayo Clinic [36]. The six different mammalian expression vectors of BRD4 used for deletion mapping in Supplementary information, Figure S2D were obtained from Addgene.

Co-IP and western blot analysis

Co-IP experiments were done to determine the interaction between WHSC1 and BRD proteins. Two days after transient transfection, 293T cells were harvested and washed with ice-cold phosphate-buffered saline before being disrupted with lysis buffer (50 mM Tris, 100 mM NaCl, 0.1% NP-40, 50 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of aprotinin, 0.5 μg/ml of leupeptin, and 0.7 μg/ml of pepstatin). 0.8 mg of protein lysates was incubated with 5 μl of anti-HA antibody (Roche) at 4 °C for 4 h, followed by the addition of 10 μl of protein G slurry (Santa Cruz Biotechnology) for 1 h. Endogenous IP and co-IP were performed using nuclear extract generated from HeLa cells as previously described [37]. After three washes with lysis buffer, the immunoprecipitated proteins were separated by SDS-PAGE and analyzed by western blot. The antibodies used in co-IP and western blot are anti-BRD2 (Bethyl Labs, A302-583A), anti-BRD3 (Bethyl Labs, A302-368A), anti-BRD4 (Bethyl Labs, A301-985A50), anti-WHSC1 (Abcam, ab75359), anti-β-actin (Sigma, A2228), anti-ERα (Santa Cruz Biotechnology, sc-543), and anti-cyclophilin A (Cell Signaling Technology, 2175S). The specificity of the BRD antibodies was confirmed by IP/western blot analysis (Supplementary information, Figure S2B). The validation profiles of all the antibodies are available from their company websites.

Chromatin IP

The ChIP-IT Express kit (Active Motif) was used for ChIP assay in this study following the manufacturer's protocol. The additional antibodies used in ChIP assay were rabbit IgG (Santa Cruz Biotechnology, sc-2027), anti-acetyl-histone H3 (Millipore, 06-599), anti-H3K36me2 (Active Motif, 39255), anti-H3K4me3 (Active Motif, 39159), and anti-H3K36me3 (Active Motif, 61101). These antibodies are all ChIP grade and have been validated by their companies. The ChIP PCR primers for amplification of *ERα/ESR1* gene next to the promoter region (A) are: Forward, 5′-CCCACTCAACAGCGTGTCT-3′; Reverse, 5′-CTGCAGGAAAGGCGACAG-3′. The ChIP primers for amplification of *ERα* gene in the coding region (B) are: Forward, 5′-GAAGAAGCATGGGTAAATGTCA-3′; Reverse, 5′-TCAGCCCTGAACCCAGTG-3′.

RNA isolation and reverse transcription-qPCR analysis

Total RNA was extracted with TriReagent (Molecular Research Center) (for siRNA screening) or RNeasy Mini Kit (Qiagen). To measure the relative mRNA levels, real-time reverse transcription-PCR (RT-PCR) was performed in an Applied Biosystems 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA). The primers for gene expression assays were designed using online Roche website: https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp. The primers for amplication of total *ERα*: Forward, 5′-ATCCACCTGATGGCCAAG-3′; Reverse, 5′-GCTCCATGCCTTTGTTACTCA-3′. The primers for amplification of specifically *ERΔ7*: Forward, 5′-TGCTGGCTACATCATCTCGGTT-3′; Reverse, 5′-CCATGCCTTTGTTACAGAATTAAGCA-3′ [24]. The SensiFast SYBR one-step Kit (Bioline) was used for RT-qPCR analysis. For all RT-qPCR experiments in this study, samples were duplicated and the error bars were shown as SEM.

Correlation analysis and microarray analysis

For the correlation analysis shown in Figure 1E, gene expression and clinical data were downloaded from the TCGA Breast Cancer [38]. Pearson correlation between WHSC1 and ESR1 was computed using the *R*-statistical system within the following subtypes of breast cancer: Luminal A, Luminal B, Her2, and ER positive. For microarray analysis, the RNA was extracted with RNeasy Mini Kit (Qiagen), and the array was performed on GeneChip Human Gene 1.0 ST Array (Affymetrix) at Asuragen Inc. (Austin,

TX) with triplicated samples. A heatmap was built using the gene expression for the genes differentially expressed upon treatment with JQ1 when a threshold of log2 < −0.2 or log2 > 0.2 was applied. Gene expression was transformed by subtracting the mean value and dividing by SD for each individual gene. A heatmap was generated using the *R*-statistical system. Raw microarray data can be accessed at Gene Expression Omnibus (GEO), with accession number GSE49124.

JQ1 signature analysis

For the "compendium" data set of nine separate breast tumor expression profiling data sets for survival analysis, gene transcription profiling data sets (all on Affymetrix U133 array, A set, and all with DMFS as an outcome measure) were previously obtained from previous studies and consolidated into one data set [39]. Genes within each data set were first normalized to SD from the median, where multiple human array probe sets referenced the same gene, the probe set with the highest variation was used to represent the gene. To score each human breast tumor profile, for similarity to the gene signature of JQ1-treated cells, we derived a "*t*-score" metric for each human tumor in relation to the experimental signature, similar to what we have done in previous analyses [28, 40]; briefly, the *t*-score was defined for each external profile as the two-sided *t*-statistic comparing, within the profile, the average of the genes high in the signature with the average of the genes low in the signature.

MTS assay

Breast cancer cells were seeded at a density of 2×10^3 cells per well in flat-bottomed 96-well plates (day 0) and their growth was measured on days 1, 2, 3, 5, and 7 after JQ1 treatment. Cell media were changed every 2 days. CellTiter 96 AQueous One Solution Reagent (Promega) was added to each well following the manufacturer's instructions. After 1 h of incubation, the cell viability was determined by measuring the absorbance at 490 nm using the Multiskan FC microplate photometer (Thermo Scientific). For all the MTS assays done in this study, samples were treated in quadruplicate and error bars were shown as SEM.

Apoptosis assay

Parental and Tam-R MCF7 cells were treated with various dosages (0.2, 0.5, 1 μM) of JQ1 for 2 days followed by being harvested for western blot. The cleaved (89 kDa, C terminus) and full-length (116 kDa) forms of PARP-1 protein were detected by PARP-1 antibody (Santa Cruz Biotechnology, sc-8007, dilution 1:200).

In vivo Tam-R breast cancer xenograft studies and immunohistochemistry

The antitumor effect of JQ1 and JQ1/fulvestrant was evaluated in Tam-R breast cancer xenograft mouse model. Briefly, tamoxifen citrate-treated 4-5-week-old ovariectomized athymic mice were subcutaneously implanted with Tam-R breast tumor fragments at the hypogastrium area. Three-five weeks later when the tumor size reaches 150-200 mm^3, mice were randomized into four treatment groups by simple randomization method, totally 10 mice per group. The allocation started from the first to the fourth group, and then from the fourth to the first group for the next round. Unless any animals die from an unknown reason, no animal has been excluded from the study. JQ1 or DMSO (vehicle) was administered daily at 50 mg/kg with 10% hydroxypropyl beta-cyclodextrin solution as a carrier, while 5 mg fulvestrant was given by subcutaneous injection weekly [41]. Tumors were monitored and tumor volumes and body weight were measured twice a week. Blind measurements were carried out to avoid unconscious biases. Tumors were harvested for molecular studies after 3 months of treatment or when they reached the size of 1 000 mm^3. Small pieces of the tumors were fixed and embedded in paraffin, and additional materials were kept at –80 °C. H&E staining was performed to examine the overall structure of the tumors, and the cell proliferation was determined by immunohistochemical staining of Ki67 and phosphorylated histone H3 at Ser10. The antibodies used were histone H3 phospho-Ser10 (Millipore 06-570, 1:300), Ki67 (Dako M7240, 1:200), and ERα (Vector Laboratories VP-E613, 1:200). Other tumor parts were used for RNA extraction and protein preparation. All *in vivo* animal studies were conducted under a protocol approved by Institutional Animal Care and Use Committees (IACUC) at the Baylor College of Medicine.

Acknowledgments

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Nuclear receptor modulation – Role of coregulators in selective estrogen receptor modulator (SERM) actions

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ABSTRACT

Selective estrogen receptor modulators (SERMs) are a class of small-molecule chemical compounds that bind to estrogen receptor (ER) ligand binding domain (LBD) with high affinity and selectively modulate ER transcriptional activity in a cell- and tissue-dependent manner. The prototype of SERMs is tamoxifen, which has agonist activity in bone, but has antagonist activity in breast. Tamoxifen can reduce the risk of breast cancer and, at same time, prevent osteoporosis in postmenopausal women. Tamoxifen is widely prescribed for treatment and prevention of breast cancer. Mechanistically the activity of SERMs is determined by the selective recruitment of coactivators and corepressors in different cell types and tissues. Therefore, understanding the coregulator function is the key to understanding the tissue selective activity of SERMs.

1. Introduction

The pleiotropic effects of estrogens are mediated by their two cognate nuclear receptors, estrogen receptor alpha and beta (ERα and ERβ), which are members of the nuclear hormone receptor superfamily [1]. Estrogens are essential for the maintenance of normal functions of the female reproductive tissues and non-reproductive tissues including metabolic homeostasis, skeletal homeostasis, the cardiovascular system, and central nervous system (CNS) [2,3]. Diminished estrogen levels in postmenopausal women are associated with dysfunctions in those tissues. Hormone replacement therapy (HRT; estrogen plus progestin) is effective in relieving the symptoms associated with menopause but is associated with an increased incidence of breast cancer. Replacement with pure estrogen is associated with increased risk of uterine cancers [4,5]. Selective estrogen receptor modulators (SERMs) can selectively activate or inhibit ER transcriptional activity in different tissues, and therefore retain beneficial effects while reducing harmful effects [6]. SERMs have been widely used for treatment of ER-positive breast cancer which occurs in 70% of breast cancer cases. The most commonly used SERMs in clinic is tamoxifen, and it has been used clinically for more than 30 years as a front-line treatment for the ERα-positive breast tumors at all stages [7]. Other second generation SERMs that have decreased stimulatory effects on the uterus are now available.

2. Structure and function of estrogen receptors

Estrogen receptors ERα and ERβ contain three major functional domains including an amino-terminal Activation Function-1 (AF-1) domain, a central DNA-binding domain (DBD), and a carboxyl-terminal ligand binding domain (LBD) (Fig. 1A). The variable NH_2-terminal AF-1 domains of ERs are not conserved among the nuclear receptor superfamily. The AF-1 domain is intrinsically unstructured in solution and forms secondary structure when engaged in interaction with coregulators. The transcriptional activity of AF-1 is controlled by phosphorylation through the MAPK pathway [8,9] and is hormone-independent. The centrally located DBD is the most conserved region and is a signature domain of nuclear receptors. The DBD is composed of two C4-type zinc fingers and recognizes specific DNA sequences in enhancer or promoter regions of target genes, known as hormone response elements (HREs). The carboxyl-terminal LBD contains 12 alpha helices (H1–H12) which form alpha helical sandwich conformation [10]. The hydrophobic ligand binding cavity is within the interior of the LBD and binds estrogen with high specificity and affinity. Hormone binding subsequently induces a conformational change and creates a novel interacting surface for coregulators. The primary sequence of LBDs is moderately conserved among nuclear receptors.

ERα mainly functions as a transcription factor in the nucleus. Hormone-bound ER protein binds to estrogen response elements (EREs) located on enhancer/promoter regions of target genes, and directly regulates gene expression in response to estrogens. ERα

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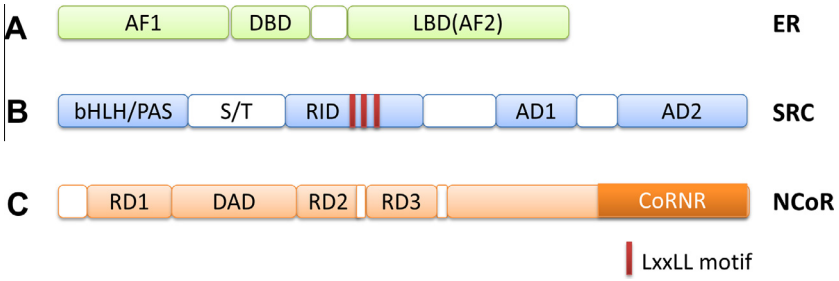

Fig. 1. Domain structure of ER, SRC coactivators and NCoR corepressors. (A) ER is composed of NH2-terminal AF1, DBD (DNA binding domain), and carboxyl-terminal LBD (Ligand binding domain). There is a hinge region between DBD and LBD. (B) SRC coactivators contain several major domains, including bHLH/PAS, RID that contains three LxxLL motifs, AD1 that interacts with p300/CBP, and AD2 that interacts with CARM1. (C) NCoR coreporessors, NCoR1 and NCoR2 (SMRT), contain several repression domains including RD1, RD2, RD3 and DAD (deacetylase activating domain) that interact with HDACs, and CoRNR (nuclear receptor interacting domain in corepressors) domain that interacts with nuclear receptor.

protein itself does not possess intrinsic enzymatic activity. Instead ERα recruits coregulators that contain diverse enzymatic activities, including histone acetyltransferase (HAT), histone methyltransferase (HMT), kinase, etc. These coregulators have no DBD and cannot bind directly to genomic DNA. They are targeted to specific genomic sites by interactions with DNA-bound ER protein. Once enriched at the specific genomic regions, the coregulators can covalently modify histones and DNA, and subsequently alter chromatin structure to facilitate or suppress the transcription of target genes. Thus, the transcriptional activity of ER is essentially carried out by coregulators. More than 450 coregulators have been reported for nuclear hormone receptors in the literature, and many of them contain a variety of different enzymatic activities.

3. The SRC family of transcriptional coactivators are "primary" ER coactivators

The SRC/p160 family of coactivators are the best characterized coactivators for steroid hormone receptors including ER. This family consists of three members. Steroid receptor coactivator 1 (SRC-1) is the first bona fide nuclear receptor coactivator, cloned in 1995 as an interacting partner for the progesterone receptor LBD through a yeast two-hybrid screening [11]. The subsequent identification and characterization of SRC-2 (GRIP1, TIF2) and SRC-3 (p/CIP, RAC3, ACTR, AIB1, and TRAM-1) has established the SRC/p160 family of coactivators [12]. Although SRCs are named as coactivators for steroid hormone receptors, it is clear that they also function as coactivators for non-steroid groups of nuclear receptors, such as thyroid hormone receptors, retinoid receptors, vitamin D3 receptor, and many non-nuclear receptor transcription factors as well, such as E2F1 [13], Ets-2 [14] and NF-κB [15].

The three SRC family members are highly homologous and share an overall similarity of 50–55% in their primary sequences [16]. SRC coactivators contain several functional domains, including a NH_2-terminal basic helix-loop-helix-Per/Ah receptor nuclear translocation/Sim (bHLH/PAS) domain, a receptor interacting domain (RID), and carboxyl-terminal Activation Domains 1 (AD1) and 2 (AD2) (Fig. 1B). The RID contains three amphipathic leu-x-x-leu-leu (LxxLL) motifs [17] and is responsible for direct interaction with agonist-bound receptor LBD [18]. Upon estrogen binding, the carboxyl-terminal alpha helix 12 of ER folds back toward the ER LBD and, together with helix 3 and 5, forms a hydrophobic cleft, which interacts with the hydrophobic surface of LxxLL motifs of SRCs. Because they are directly recruited by hormone-bound ER, SRC coactivators are considered as primary coactivators. Subsequently SRCs serve as bridging molecules to bring in secondary coactivators through AD1 and AD2, including p300/CBP [19] and CARM1 [20]. p300 and CBP are potent histone acetyltransferases, whereas CARM1 is a histone methyltransferase. The AD1 of SRCs physically associates with p300/CBP and shows potent transcriptional activity in reporter gene assay when tethered to a heterologous DNA binding domain such as GAL4 DBD [21]. AD2 binds to CARM1 and shows relatively weaker transcriptional activity when tethered to GAL4 DBD [21]. Interestingly SRC-1 and SRC-3 also possess intrinsic acetyltransferase activity toward histones [19,22]. However their HAT activity is weak, suggesting that their cognate substrates might not be histones.

In addition to conventional reporter gene assays, recruitment of SRC coactivators by ER to its target genes is strongly supported by state-of-the-art genome-wide chromatin immunoprecipitation followed by sequencing (ChIP-seq) analysis. In MCF7 breast cancer cells, mapping of the chromatin binding sites of the three members of SRCs revealed that the genomic recruitment of SRC proteins clearly followed ERα ligand stimulation, indicating that SRC recruitment by ER indeed occurs in real time in cells [23]. However the targeting genes regulated by SRC-3 are not shared with the other two SRC family members. This is in agreement with other studies showing that each SRC protein regulates a unique set of genes in MCF7 cells, suggesting that they play non-redundant roles in breast cancer development [24].

There also is ample biochemical evidence showing that SRC coactivators are bona fide coactivators for ER. In immunoprecipitation-mass spectrometry analyses, purification of E2-bound ER-associated protein complexes revealed that all there SRC members interact with ER in a hormone-dependent manner [25]. Similarly, purification of SRC-3-associated protein complexes confirms that CBP/p300 form a complex with SRC-3 [25]. In chromatin-based *in vitro* transcription assays, SRCs and CBP/p300 significantly enhance ER-mediated transcription [26]. Our recent Cryo-EM study reveals that two SRC-3 molecules simultaneously interact with an ERα dimer on DNA and form an asymmetric protein surface, providing a structural basis for further recruitment of a large number of secondary coactivators (unpublished).

The *in vivo* biological roles of SRC coactivators in female reproductive functions in animal have been well documented. For instance, SRC-1 null mice show partial resistance to steroid hormones [27]. SRC-2 is essential for progesterone-dependent uterine function and mammary gland morphogenesis in mouse models [28]. SRC-3 is required for female reproductive function and mammary gland development [29]. Finally it is worthy to note that SRC-3/AIB1 is a bona fide oncogene in breast cancer. The SRC-3 gene is amplified in ~10% of breast tumor samples [30] and SRC-3 mRNA is overexpressed in the majority of primary breast cancers. Forced overexpression of SRC-3 in mammary gland caused mammary tumor development [31]. Collectively, these molecular, cellular, biochemical, and animal data have unequivocally established that SRC proteins are bona fide coactivators for nuclear receptors and are key components of the estrogen/ER signaling pathway.

4. The NCoR1/SMRT corepressor family

In contrast to coactivators, there are a group of transcriptional factors, termed corepressors, which oppose the action of coactivators in nuclear receptor-mediated transcriptional regulation; the existence of coactivators and corepressors suggests a yin-yang relationship for regulation of gene transcription. NCoR1 (nuclear receptor corepressor (1) and SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) are prototypical nuclear receptor corepressors. NCoR1 and SMRT were initially cloned as hormone-independent TR or RXR interacting proteins in yeast two-hybrid screening [32,33]. NCoR1 and SMRT interact with nuclear receptors in the absence of hormone and their interaction is disrupted by agonist binding. NCoR1 and SMRT contain a receptor interacting domain (RID) and repression domains (RDs) (Fig. 1C). The RID contains a L/I-x-x-I/V-I motif (CORNR motif) to interact with nuclear receptors [34–36], reminiscent of the L-x-x-L-L motif (also known as NR box) of coactivators. Importantly NCoR1 and SMRT do not have intrinsic enzymatic activity. Instead NCoR1 and SMRT function as scaffold proteins, and they recruit histone deacetylases including HDAC3 through several repression domains (RD1, RD2 and RD3) [37,38]. In the absence of hormone, nuclear receptors TR, RAR and RXR bind to enhancers of target genes, and recruit NCoR1 and/or SMRT and associated HDACs to remove histone acetylation marks that suppress gene transcription. When receptors bind an agonistic ligand, the corepressors NCoR1/SMRT and their associated HDACs are dissociated, and SRC coactivators in turn are loaded to the agonist-bound receptors to stimulate gene transcription [39].

It is important to note that although NCoR1 and SMRT were initially identified as corepressors for non-steroid nuclear receptors, later studies have clearly established that NCoR1/SMRT can also function as corepressors for steroid receptors and suppress their target gene transcription [40–43]. For example, NCoR1 is recruited to the pS2 gene promoter by ER in the absence of hormone, and tamoxifen treatment enhances NCoR recruitment [44]. NCoR1 and SMRT can also function as corepressors for many non-nuclear receptor transcriptional factors [45].

5. Determination of SERM activity by coactivators and corepressors

The unique feature of a SERM is its cell- and tissue-selective activity. The first implication that relative expression of coactivator and corepressors in cells contribute to SERM function is based on transient transfection luciferase reporter assays. In this study, overexpression of SRC-1 enhanced 4-hydroxytamoxifen (4HT)-stimulated ER activity, whereas overexpression of SMRT strongly suppressed 4HT-stimulated ER activity [40]. When SRC-1 and SMRT were co-overexpressed, SMRT blocked the SRC-1 coactivation of 4HT-depependent ER activity [40]. For the first time, these results suggested that the relative expression of SRC-1 and SMRT can modulate the 4HT-dependent ER activity.

This observation is further supported by a study showing that tamoxifen is antiestrogenic in breast cells but estrogenic in endometrial cells. It was found that a high expression level of SRC-1 in endometrial carcinoma cell lines is responsible for induction of c-Myc and IGF-1 by tamoxifen-stimulated ER. Growth stimulatory effects of tamoxifen in uterine cells were abolished by depletion of SRC-1 by siRNA, indicating the SRC-1 levels are critical for agonist activity of tamoxifen in the endometrium [46].

In an *in vitro* model, up-regulated SRC-3 coactivator expression is associated with acquired tamoxifen resistance in MCF-7 cells cultured in tamoxifen-containing media [47]. Importantly in breast cancer patients, high SRC-3 level also correlates with tamoxifen resistance [48]. In cultured breast cancer cells, overexpression of SRC-3 and Her2 is able to convert tamoxifen from an antagonist into an ER agonist, resulting in de novo resistance [49]. Under this condition, tamoxifen treatment causes recruitment of coactivator complexes (SRC-3 and p300/CBP) to, and dissociation of corepressor complexes (NCoR/HDAC3) from, the ER-regulated pS2 gene promoter. In agreement with this, depletion of SRC-3 expression by siRNA restored the inhibitory effect of tamoxifen on cell proliferation in breast cancer BT474 cells [50]. PKA-induced tamoxifen resistance is associated with increased recruitment of SRC-1 by phosphorylation of S305 [51].

On the other hand, in breast cancer cells, corepressor NCoR1 and SMRT are required for the antagonistic effects of tamoxifen. They prevent tamoxifen from stimulating proliferation through suppression of a subset of ERα target genes involved in cell proliferation. Silencing of NCoR1 and SMRT promoted tamoxifen-induced cell cycle progression and proliferation [52]. In a breast cancer mouse model, reduced expression levels of NCoR1 correlate with the development of tamoxifen resistance [53]. In a cohort of ERα-positive unilateral invasive primary breast tumors from 99 postmenopausal patients who only received tamoxifen as adjuvant hormone therapy after primary surgery, low NCoR1 expression was associated with a significantly shorter relapse-free survival [54], substantiating a role of corepressor NCoR1 in tamoxifen resistance. In further support of this notion, based on ChIP analysis, over-expression of coactivator SRC-1 or corepressor SMRT is sufficient to alter the transcriptional action of tamoxifen on a number of target genes in breast and endometrial cancer cells [55].

Recent data reveal another mechanism of tamoxifen resistance caused by increased SRC-3 level. It is shown that the paired box 2 gene product (PAX2) is a tamoxifen-recruited transcriptional repressor of the ERBB2 gene. Increased SRC-3 can compete with PAX2 for ERRB2 gene binding and result in overexpression of ERRB2 and cause subsequent tamoxifen-resistant cell growth [56].

The importance of coregulators in determination of tamoxifen activity is also true for other SERMs. For instance, raloxifene promotes ERα interaction with NCoR1 both *in vivo* and *in vitro* [42]. In rat uterus, ormeloxifene antagonizes *in vivo* ERα activity by increasing the recruitment of NCoR1 corepressor and reducing the recruitment of SRC-1 coactivator [57]. In addition to SRCs, other coactivators can also play a role in SERM activity. For example, recruitment of peroxisome proliferator-activated receptor-γ coactivator (PGC)-1β with ER LBD induced by tamoxifen contributes to the agonistic activity of tamoxifen in cultured cells [58].

6. SERMs regulate coregulator expression level and activities

Coregulators determine the SERM activity. On the other hand, SERMs can regulate the expression levels of coregulators, forming a feedback regulatory loop. For instance, in cultured cells, 4-hydroxytamoxifen and raloxifene increased protein stability and function of SRC-1 and SRC-3 coactivators in an ERα-dependent manner [59]. The increased coactivators subsequently enhance the transcriptional activity of other nuclear receptors, suggesting that these SERMS have broad biological actions in the cells [59]. In human patients, tamoxifen therapy leads to significantly increased expression of coactivators, particularly SRC-3, in both normal and malignant breast tissues [60]. In human skeletal muscle cells, tamoxifen and raloxifene increase SRC-1 mRNA levels while decreasing SMRT mRNA levels [61].

SERMs not only impact coactivator protein stability, but also can regulate the coactivator activity though posttranslational modifications such as phosphorylation. In response to anti-estrogen treatment, multiple growth signaling pathways are up-regulated (HER2, PI3K, PKC, etc). Activated kinases phosphorylate SRC-3,

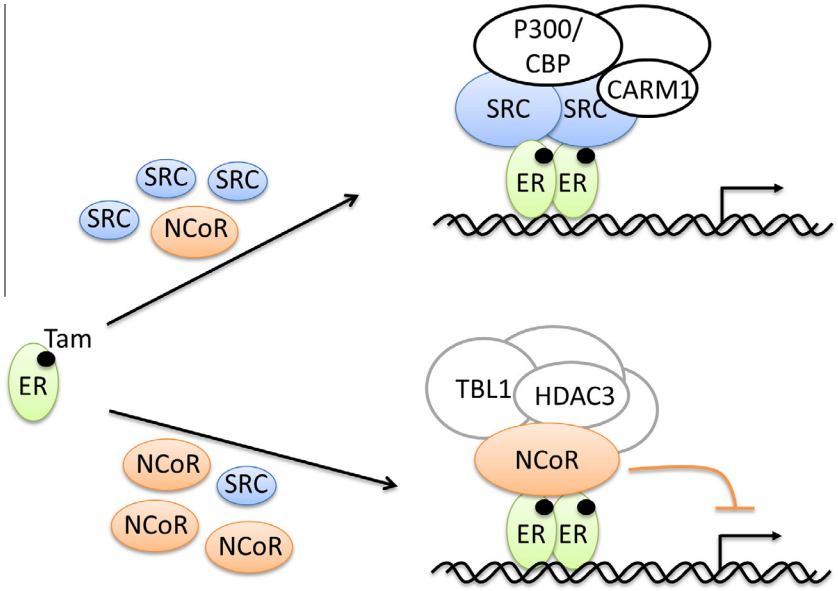

Fig. 2. The selective recruitment of coactivators and corepressors determines the SERM activity. Shown in the diagram is one representative mechanism in which the relative abundance of SRCs and NCoRs governs the recruitment. Other mechanisms could also influence recruitment, such as increased SRC recruitment by PKA-induced phosphorylation (See text for details).

enhance its coactivator activity, affect cell growth, and eventually contribute to resistance [62,63].

7. Summary

Since the cloning of the first nuclear receptor coactivator SRC-1 in 1995 [11], the last two decades have seen explosive growth of the coregulator field, with total numbers of about 450 coregulators characterized to date. Numerous studies have established that coregulators play pleiotropic roles in animal pathophysiology. In this review, we focused on the critical role of coregulators in SERM function since the literature reveals growing evidence to support that SERM activity is mainly determined by the selective recruitment of coactivators and corepressors to ER target gene in specific types of cells and tissues (Fig. 2). Better understanding of coactivator and corepressor functions should enhance future development of new generations of SERMs which have more beneficial and less harmful effects.

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