SIRT1 represses estrogen-signaling, ligand-independent ERα-mediated transcription, and cell proliferation in estrogen-responsive breast cells

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Abstract

In prostate and breast cancer, the androgen receptor and estrogen receptor (ER) mediate induction of androgen- and estrogen-responsive genes respectively and stimulate cell proliferation in response to the binding of their cognate steroid hormones. Sirtuin 1 (SIRT1) is a NAD+-dependent class III histone deacetylase that has been linked to gene silencing, control of the cell cycle, apoptosis, and energy homeostasis. In prostate cancer, SIRT1 is required for androgen antagonist-mediated transcriptional repression and growth suppression of prostate cancer cells. Whether SIRT1 plays a similar role in the actions of estrogen or antagonists had not been determined. We report here that SIRT1 represses the transcriptional and proliferative response of breast cancer cells to estrogens, and this repression is ERα dependent. Inhibition of SIRT1 activity results in the phosphorylation of ERα in an AKT-dependent manner, and this activation requires phosphoinositide 3-kinase activity. Phosphorylated ERα subsequently accumulates in the nucleus, where ERα binds DNA ER-responsive elements and activates transcription of estrogen-responsive genes. This ER-dependent transcriptional activation augments estrogen-induced signaling, but also activates ER signaling in the absence of estrogen, thus defining a novel and unexpected mechanism of ligand-independent ERα-mediated activation and target gene transcription. Like ligand-dependent activation of ERα, SIRT1 inhibition-mediated ERα activation in the absence of estrogen also results in breast cancer cell proliferation. Together, these data demonstrate that SIRT1 regulates the most important cell signaling pathway for the growth of breast cancer cells, both in the presence and the absence of estrogen.

Key Words
- sirtuin
- estrogen receptor
- SIRT1
- ligand-independent
- breast cancer

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Introduction

The sirtuins are a family of enzymes with increasingly recognized relevance to cancer (Moore 2011), but the full extent of their involvement in breast cancer genesis and evolution remains to be elucidated. Sirtuin 1 (SIRT1) is a NAD+-dependent histone deacetylase (HDAC) that has been linked to longevity, gene silencing, control of the cell cycle, apoptosis, and energy homeostasis (Landry *et al.* 2000, Blander & Guarente 2004, Borra *et al.* 2005, Haigis & Guarente 2006, Dai *et al.* 2007, Yamamoto *et al.* 2007). SIRT1 also has functions relating to inflammation and

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neurodegeneration (Yamamoto *et al*. 2007). In addition, SIRT1 interacts with PPARγ and PGC-1α in the differentiation of muscle cells, adipogenesis, fat storage, and metabolism in the liver (Fulco *et al*. 2003, Picard *et al*. 2004, Puigserver *et al*. 2005, Rodgers *et al*. 2005). SIRT1 is primarily a nuclear protein that targets the PGC-1α, FOXO, and NF-κB families of transcription factors, among others (Haigis & Guarente 2006). SIRT1 also associates with the tumor suppressor protein p53 (Haigis & Guarente 2006, Dai *et al*. 2007, Yamamoto *et al*. 2007) and has been suggested to be a tumor suppressor protein itself (Motta *et al*. 2004, Pruitt *et al*. 2006, Wang *et al*. 2006, Dai *et al*. 2007, Jin *et al*. 2007).

SIRT1 appears to serve multiple functions in human prostate cancer cells. The enzyme is overexpressed in prostate cancer cells and is present in both the nucleus and the cytoplasm, promoting cell survival (Dai *et al*. 2007, Byles *et al*. 2010). However, enforced cytoplasmic localization of SIRT1 enhanced sensitivity to apoptosis in one report (Jin *et al*. 2007). SIRT1 also suppresses specific tumor suppressor genes in hormone-refractory prostate cancer cells (Fu *et al*. 2006). Deacetylation of the androgen receptor (AR) by SIRT1 inactivates its ability to transform prostate cells. SIRT1 binds to, and deacetylates, the AR at a conserved lysine motif, downregulating its levels in the cell and repressing androgen-induced AR transcription (Dai *et al*. 2007, 2008, Fu *et al*. 2006).

SIRT1 is required for the actions of endocrine therapy in prostate cancer as well. Androgen antagonist-mediated transcriptional repression and growth suppression of prostate cancer cells require SIRT1 (Dai *et al*. 2007). It had not yet been determined whether SIRT1 plays a similar role in the action of estrogen antagonists. We here show that SIRT1 is not required for estrogen antagonist activity. Rather, SIRT1 functions to repress the estrogen response in the absence and in the presence of estrogen, limiting ligand-independent signaling through the estrogen receptor α (ERα).

Materials and methods

Cell culture

The MCF-7 breast cancer cell line (ATC HTB-22) is an epithelial cell line derived from an adenocarcinoma of the mammary gland. T47D cells (ATCC HTB-133) are a PTEN-negative breast cancer cell line that expresses the WNT7B oncogene. MDA-MB 231 (ATCC HTB-26) is an ERα-negative, estrogen-independent breast cancer cell line. All cells were cultured in phenol red-free DMEM (Invitrogen) plus 10% FBS or charcoal-treated FBS (Hyclone, Erie, PA, USA). Cells were treated with β-estradiol (R187933), sirtinol, splitomicin, 4-hydroxytamoxifen (4HT), faslodex (all from Sigma), LY294002, Wortmannin (both from CalBiochem, Gibbstown, NJ, USA), or vehicle (EtOH or DMSO).

Transfections

Two micrograms of plasmid (ERE-luciferase reporter construct (Addgene, Cambridge, MA, USA) (Hall & McDonnell 1999); SV40-β-gal reporter construct; D/N SIRT1 (H363Y); or ERα expression vector) were added to 100 μl OPTI-MEM (Invitrogen) and incubated for 15 min. Transfections were performed using Lipofectamine (Invitrogen), following the manufacturer's instructions.

Luciferase assays

The Promega Dual luciferase reporter assay system was used. Cellular harvest and assay were carried out according to the manufacturer's instructions. Luminescence was quantitated in a Turner Designs 20/20 luminometer. Results were normalized with a β-galactosidase assay (Promega).

Immunoblot and chromatin immunoprecipitation assays

Cellular extracts were obtained by harvesting cells using lysis buffer (20 mM HEPES (pH 7.4), 10% glycerol, 2 mM EDTA, 2 mM EGTA, 50 mM β-glycerophosphate, 1% Triton X, 1 mM dithiothreitol, 1 mM sodium vanadate, and 1× Protease Inhibitor Cocktail (Roche Scientific #04693132001)). Nuclear and cytoplasmic fractions were prepared using the Nu-Per kit (Thermo Scientific, LaFayette, CO, USA #78833), according to the manufacturer's instruction. Immunoblots were performed using the following antibodies. Primary antibodies used were as follows: α-SIRT1 (Upstate Biotechnology, Waltham, MA, USA) 1:1000; α-ERα H-184 1:500; β-actin 1:10 000; α-β-tubulin 1:1000 (all from Santa Cruz Biotechnologies); α-phospho-ERα [Ser118] 1:500; α-lamin A/C 1:1000; α-AKT 1:2000; α-phospho-AKT [Ser473] 1:2000; α-phospho-AKT [Thr308] 1:2000; FOXO3a 1:1000 (all from Cell Signaling Technology, Danvers, MA, USA); secondary antibodies were as follows: α-mouse IgG-HRP and α-rabbit IgG-HRP (both from GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK). All ChIP experiments were carried out using a Fisher Scientific 550 Sonic Dismembrator for chromatin shearing, and a ChampionChIP One-Day Kit

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(SABiosciences, Valencia, CA, USA #GA101) according to the manufacturer's instructions.

siRNA

siRNA transfections were carried out according to the manufacturer's instructions (Thermo Scientific Acell Smart Pool). Cells were incubated with Smart Pool for 18 h and then placed in media containing 10% charcoal-stripped FBS for 72 h. Cells were then treated for 24 or 48 h and assayed for mRNA expression via quantitative RT-PCR or for protein expression via immunoblotting. Smart Pool sequences were as follows: siSIRT1: GUCUUAUCCUCUAGUUCUU, GCAUCUUGCCUGAUUUGUA, CUGUGAUGUCAUAAUUAAU, and GUUCGGUGAUGAAAUUAUC; siERα: GAUCAAACGCUCUAAGAAG, GAAUGUGCCUGGCUAGAGA, GAUGAAAGGUGGGAUACGA, and GCCAGCAGGUGCCCUACUA; and siAKT: CAUCACACCACCUGACCAA, ACAAGGACGGGCACAUUAA, CAAGGGCACUUUCGGCAAG, and UCACAGCCCUGAAGUACUC.

Real-time PCR

RNA was purified using the PureLink RNA Mini Kit (Invitrogen) according to the manufacturer's instructions. cDNA was made using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. RT-PCR was carried out using the SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. RT-PCR was carried out using an Applied Biosystems 7500 Fast RT-PCR machine: 50 °C for 2 min, one cycle; 95 °C for 10 min, one cycle; and 95 °C for 15 min, 55 °C for 20 min, and 60 °C for 30 min 45 cycles. All primers were acquired from Invitrogen and sequences were as follows: pS2 F/R: TTGGAGCAGAGAGGAGGCAATGG, TGGTATTAGGATAGAAGCACCAGGG; SIRT1 F/R: GGAATTGTTCCACCAGCATT, AACATTCCGATGGCTTTTTG; ERα: F/R: CCAGGGAAGCTACTGTTTGC, GATGTGGGAGAGGATGAGGA; and β-actin F/R: 5′-GCTCGTCGTCGACAACGGCTC-3′, 5′-CAAACATGATCTGGGTCATCTTCTC-3′.

Cell proliferation assays

Cells (2.0×10^5) were plated in a six-well plate and incubated in media containing 10% charcoal-treated FBS for 48 h. Cells were then treated as indicated and incubated for 72 h. Viable cells were enumerated via a Trypan Blue (Invitrogen) exclusion assay on a Countess Automated Cell Counter (Invitrogen).

Results

SIRT1 represses basal and inducible expression of estrogen-responsive genes

In a reporter assay using a transiently transfected estrogen response element (ERE)-luciferase reporter construct (wherein the promoter consists of three repeats of an ERα binding motif), exposure to estrogen induced an approximately fourfold increase in reporter activity. This induction was ameliorated by 4HT, an estrogen antagonist, as expected, indicating that the host MCF-7 cells are estrogen responsive (data not shown). Estrogen treatment also induced mRNA expression of *pS2* (*PSEN2*), an endogenous estrogen-regulated gene, by approximately fivefold, in these cells, and this induction was blocked by co-exposure to 4HT, indicating that endogenous estrogen-regulated genes are responsive to estrogen and 4HT in these cells, in a pattern similar to the reporter gene (e.g. see below).

MCF-7 cells were exposed to sirtinol (Ota *et al*. 2006), a small-molecule inhibitor of SIRT1 enzymatic activity, to examine the effect of SIRT1 inhibition on estrogen-regulated gene activity (data not shown). Exposure to sirtinol consistently induced the activity of a transfected estrogen-responsive reporter gene (Fig. 1A) and the mRNA levels of the endogenous *pS2* gene (Fig. 1B) in the absence of estrogen. Furthermore, combining estrogen and sirtinol exposure produced a more-than-additive effect on estrogen-regulated gene activity, both for transfected and for endogenous genes (Fig. 1A and B). These results indicate that SIRT1 activity is required for basal repression of estrogen-regulated gene activity in the absence of estrogen.

Conversely, the effects of SIRT1 activation on basal expression levels of estrogen-regulated genes were investigated via luciferase reporter assays and by measuring endogenous ER-regulated mRNA gene expression (data not shown) by treating MCF-7 cells with resveratrol, a phytoestrogen, and an activator of SIRT1 (albeit not a specific one). SIRT1 activation via resveratrol, in conjunction with exposure to estrogen or SIRT1 inhibitors, did not significantly reduce ERα transcriptional activity when compared with estrogen- or SIRT1 inhibitor-treated controls (data not shown).

To confirm that this effect was not restricted to sirtinol, cells were exposed in parallel studies to splitomicin (Neugebauer *et al*. 2008), a chemically distinct SIRT1 inhibitor (data not shown). SIRT1 inhibition by splitomicin induced *pS2* mRNA expression levels comparably to estrogen treatment (Fig. 1C). This indicates that the induction of estrogen-responsive genes in response to

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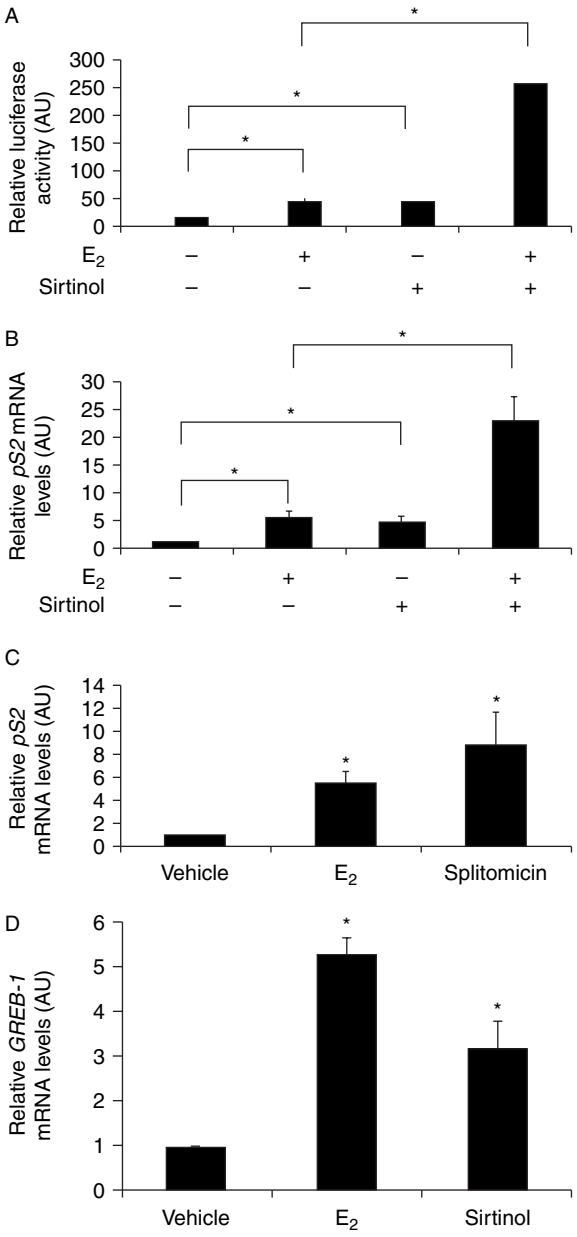

Figure 1

SIRT1 inhibition stimulates estrogen-responsive gene activity. MCF-7 cells were plated to 60% confluence in a 10 cm dish (6×10^5 cells per dish) in charcoal-treated (C/T) media, incubated for 24 h, and treated as follows: (A) transfection with an ERE-luciferase reporter plasmid and RSV-β-gal; then exposed to either vehicle, or E_2 (100 nM), or sirtinol (50 μM) or both; and assayed for reporter gene activity via luciferase assay. Results are normalized to β-galactosidase activity as a transfection control. In panels B, C, and D, cells were exposed to either vehicle, or E_2 (100 nM), or sirtinol (50 μM), or splitomicin (200 μM), or combinations thereof, and mRNA levels of the endogenous estrogen-regulated genes *pS2* (B and C) and *GREB1* (D) were assayed via quantitative RT-PCR and normalized to β-actin transcript levels. The data presented are average (±S.E.M.) of four independent experiments. *$P<0.002$ (A and B) and <0.01 (C and D) compared with vehicle controls. AU, arbitrary units.

SIRT1 inhibition is not an SIRT1 inhibitor-specific effect. The effects of SIRT1 inhibition on an independent, endogenous estrogen-responsive gene, *GREB1* (Carroll *et al*. 2006), were assessed. Upon exposure of MCF-7 cells to estrogen or sirtinol, *GREB1* mRNA expression levels increased five- and fourfold respectively (Fig. 1D), indicating that repression of estrogen-responsive genes by SIRT1 is a generalizable effect. These changes in endogenous estrogen-responsive gene mRNA levels correlated with changes in estrogen-responsive luciferase reporter gene expression in the same cells, indicating that they were the result of changes in transcriptional activity.

To determine whether SIRT1 inhibition of the estrogen response is generalizable, T47D cells, an estrogen-dependent breast cancer cell line known to express SIRT1 at high levels (Aoyagi & Archer 2008), were exposed to estrogen and/or sirtinol at varying concentrations. Estrogen induced *pS2* mRNA expression levels by approximately sixfold, whereas sirtinol induced *pS2* mRNA expression levels by threefold. Treatment with both estrogen and sirtinol increased *pS2* mRNA expression more than exposure to either drug alone (e.g. see below). This indicates that SIRT1 inhibition of the estrogen response is not cell line specific. Together, these data indicate that SIRT1 activity represses basal and estrogen-inducible expression of estrogen-regulated genes.

As chemical inhibitors are never completely specific with respect to their enzymatic target, more specific, genetically based methods of inhibiting SIRT1 activity were also used. Cells were transfected with a dominant-negative (D/N), deacetylase-defective SIRT1 mutant, together with the ERE-luciferase reporter plasmid, to determine whether the repression of estrogen-regulated gene activity is SIRT1 specific. In the absence of estrogen, expression of D/N SIRT1 induced estrogen-regulated gene activity by approximately threefold. When cells expressing D/N SIRT1 were exposed to estrogen, estrogen-regulated gene activity was induced in an additive manner (Fig. 2A). These results are consistent with the findings obtained using SIRT1 inhibitors.

As another independent and specific approach, SIRT1 levels in MCF-7 cells were knocked down with *SIRT1* siRNA. SIRT1 knockdown was verified by RT-PCR (data not shown) and immunoblotting (Fig. 2B). Knockdown of SIRT1 in the absence of estrogen resulted in an induction of *pS2* mRNA by approximately sixfold. *pS2* induction by SIRT1 knockdown was additive in the presence of estrogen, confirming that SIRT1 represses the estrogen response in the absence and in the presence of estrogen and that this effect is SIRT1 specific.

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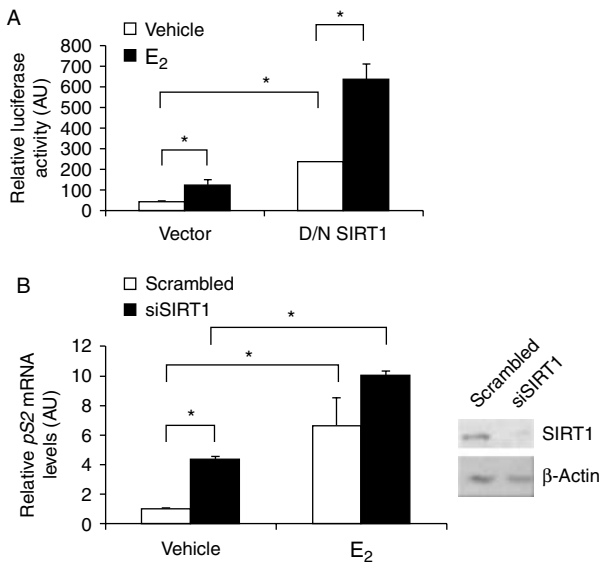

Figure 2

Repression of estrogen-regulated gene activity is SIRT1 specific. MCF-7 cells were plated to 60% confluence in a 10 cm dish (6×10^5 cells per dish) in C/T media, incubated for 24 h, then transfected with ERE-luciferase reporter plasmid plus either empty vector or H343Y D/N SIRT and RSV-β-gal (A) or with 500 ng scrambled siRNA or SIRT1-siRNA (B) and incubated for 18 h, then incubated for a further 24 h (A) or 72 h (B) in fresh C/T media. The cells were then exposed to vehicle or E_2 (100 nM), as indicated, for 24 h. Estrogen-regulated gene activity was determined via luciferase assay normalized to β-galactosidase activity (A), or mRNA expression, as determined via quantitative RT-PCR and normalized to β-actin transcript levels (B). The efficiency of SIRT1 knockdown was determined by analyzing protein expression via immunoblot (B, inset). The data presented are the average (±S.E.M.) of three independent experiments. *$P<0.005$.

SIRT1 is not required for repression of estrogen-responsive genes by estrogen antagonists

To determine whether SIRT1 is required for the function of estrogen antagonists, MCF-7 and T47D cells were exposed to estrogen in combination with sirtinol and 4HT (Fig. 3A) or faslodex (Fig. 3B), a pure estrogen antagonist. SIRT1 inhibition did not affect the ability of 4HT or faslodex to repress the estrogen response, indicating that SIRT1 is not required for estrogen antagonist function (Fig. 3A and B), in contrast to its essential role in mediating the effects of androgen antagonists (Dai *et al*. 2007, 2008).

ERα is required for regulation of estrogen-responsive genes by SIRT1 inhibition

Interestingly, 4HT and other estrogen antagonists repressed the induction of estrogen-responsive gene activity by SIRT1 inhibition as well as their induction by estrogen. One possible explanation would be that the transcriptional activation of estrogen-regulated genes observed upon SIRT1 inhibition is also dependent on the ER, through which 4HT and faslodex inhibit the estrogen response.

As one test of the potential dependency of SIRT1 repression of the estrogen response on ERα, MDA231 cells, which have silenced ERα expression grow in an estrogen-independent manner, and are known to express high levels of SIRT1 (Alvala *et al*. 2012), were exposed to estrogen or sirtinol. *pS2* mRNA expression was not induced by estrogen, as expected, nor by sirtinol, compared with untreated control (data not shown, but see the empty vector controls in Fig. 4A for a comparable experiment). ERα was then ectopically expressed in the MDA231 cells by transfection, and the effects of exposure to estrogen or sirtinol on a co-transfected ERE-luciferase reporter gene was determined. In the absence of ERα (empty vector), neither estrogen nor sirtinol induced estrogen-regulated gene activity (Fig. 4A). However, in the presence of ERα, estrogen-regulated gene activity increased two- to three-fold when exposed to sirtinol or estrogen respectively. ERα expression was verified by immunoblotting.

To demonstrate that the observed estrogen-regulated gene activity in MDA231 cells is due to the inhibition of SIRT1, rather than a nonspecific action of sirtinol, the cells were transfected with the ERE-luciferase reporter, plus or minus ERα and D/N SIRT1 expression vectors. In the absence of ERα, neither estrogen treatment nor expression of D/N SIRT1 induced estrogen-regulated gene activity. When ERα and D/N SIRT1 were expressed together, estrogen-regulated gene activity increased 15- and 20-fold, in the absence and presence of estrogen respectively (data not shown).

As an alternative way of testing the requirement of ERα for SIRT1 activity, T47D cells were pre-treated with faslodex, a pure anti-estrogen that targets the ERα for degradation, and then exposed to estrogen, sirtinol, or both, in combination with faslodex. Pre-treatment with faslodex reduced ERα protein levels, as verified by immunoblotting (Fig. 3B). *pS2* mRNA expression increased after estrogen or sirtinol exposure in vehicle-treated cells, but this induction was abrogated in cells where the ERα had been degraded by faslodex treatment (Fig. 3B).

As a further test of the dependency of SIRT1 signaling on the ERα, knockdown of ERα by siRNA was carried out in MCF-7 cells and the cells were then exposed to estrogen, sirtinol, or both and assayed for estrogen-regulated gene induction. Knockdown of ERα was confirmed by immunoblotting (Fig. 4B) and RT-PCR (not shown). In the presence

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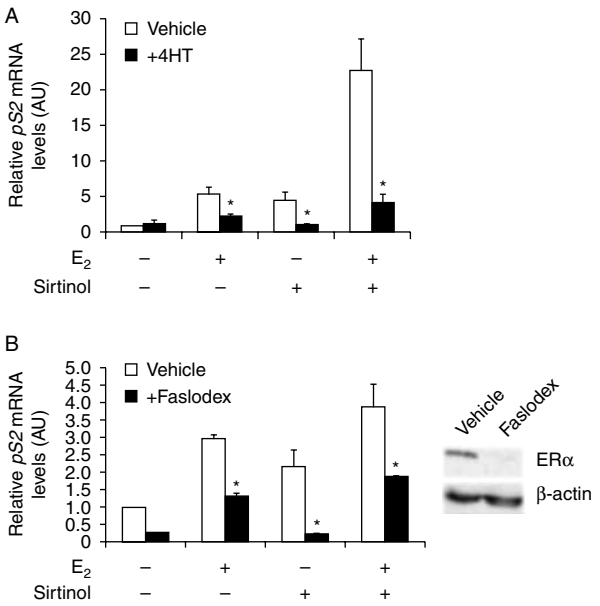

Figure 3
SIRT1 is not required for estrogen antagonist function. MCF-7 (A) or T47D (B) cells were plated to 60% confluence in a 10 cm dish (6×10^5 cells per dish) in C/T media and incubated for 24 h; then exposed to vehicle, E_2 (100 nM), and/or sirtinol (50 μM), 4HT (1 μM) (A) and/or faslodex (100 nM) (B), as indicated; incubated for an additional 24 h; and mRNA was harvested. *pS2* mRNA expression was determined via quantitative RT-PCR and normalized to β-actin transcript levels. ERα protein expression was determined via immunoblot (B, inset). The data presented are average (±S.E.M.) of six independent experiments (A) and three independent experiments (B). The increases in mRNA levels induced by the treatments were significant compared with controls ($P<0.001$ (A) and <0.01 (B)). Inhibition of mRNA levels by the estrogen antagonists was significant (*$P<0.001$ (A) and <0.01 (B)).

of ERα, exposure to estrogen or sirtinol caused significant induction of *pS2* mRNA expression, as expected. ERα knockdown effectively blunted *pS2* mRNA induction by both estrogen and sirtinol. These changes in endogenous estrogen-responsive gene mRNA levels correlated with changes in estrogen-responsive luciferase reporter gene expression in the same cells, indicating that they were the result of changes in transcriptional activity. Collectively, these findings indicate that SIRT1 repression of the estrogen-regulated gene activity in the absence and presence of estrogen is both SIRT1 specific and ERα dependent.

Effect of SIRT1 inhibition on ERα localization

ERα is a steroid receptor that is present in both the nucleus and the cytoplasm, depending upon ligand availability and the metabolic state of the cell. The nuclear fate and protein turnover of the receptor is dependent upon the chemical structure of the agonist (or antagonist). Upon estrogen ligation, ERα accumulates in the nucleus. In the cell lines used throughout this study, ERα has been shown to be expressed predominately in the nucleus in cells growing in the presence of estrogen (Welsh *et al.* 2012). However, ERα expressed on the cell surface as well as in the cytoplasm can be readily detected by flow cytometry as well as other analytical methods (Ford *et al.* 2011). In order to determine whether SIRT1 represses ligand-independent accumulation of ERα in the nucleus, MCF-7 cells were exposed to estrogen or sirtinol, and cytoplasmic and nuclear fractions were prepared. The purity of the subcellular fractions was determined by immunoblotting with β-tubulin (a cytoplasmic protein) and lamin A/C (a nuclear protein). In cells grown in media containing estrogen, we found that ERα was predominately expressed in the nucleus (data not shown), which is in agreement with the literature. However, after 24 h of incubation in media containing charcoal-treated serum, which is devoid of all steroid hormones including estrogen, ERα was expressed predominately in the cytoplasm (Fig. 5A, untreated lanes). In response to estrogen or sirtinol exposure, ERα accumulated in the nucleus (Fig. 5A), indicating that SIRT1 regulates ligand-independent ERα nuclear accumulation.

SIRT1 represses ligand-independent ERα activation and DNA binding

Once in the nucleus, ERα binds to cognate sequences on the genome and activates the transcription of estrogen-regulated genes. To determine whether SIRT1 regulates ligand-independent binding of ERα to estrogen-regulated promoters, MCF-7 cells were exposed to estrogen or sirtinol, and ERα or RNA polymerase II binding to endogenous estrogen-regulated promoters was assessed via ChIP assays. In response to estrogen, ERα association with the estrogen-regulated gene pS2 promoter increased by ~15-fold, as expected. Exposure to sirtinol alone also increased ERα binding to the endogenous pS2 promoter, by ~20-fold (Fig. 5B). To determine the generalizability of this effect, ERα binding to the promoter of another estrogen-responsive gene, *GREB1*, was investigated in parallel. Estrogen or sirtinol exposure increased ERα binding by approximately five- and sixfold respectively. In response to estrogen or sirtinol exposure, the association of RNA polymerase II with these estrogen-responsive promoters increased by approximately nine- and sevenfold respectively (data not shown). ERα binding to the

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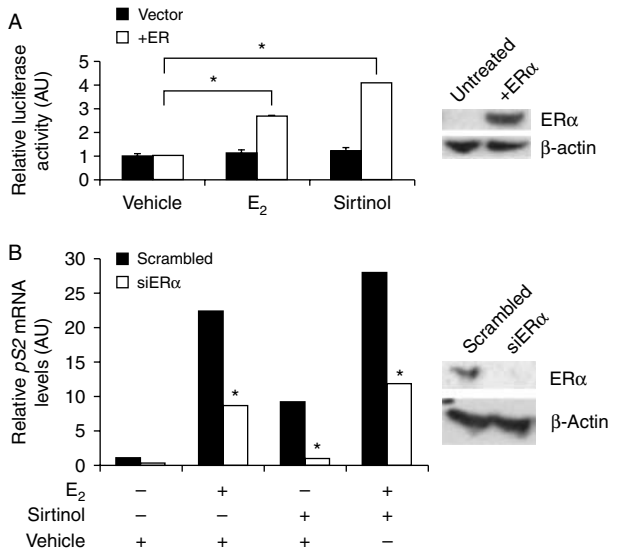

Figure 4
SIRT1 repression of estrogen-regulated gene activity is ERα dependent. MDA231 (A) or MCF-7 (B) cells were plated to 60% confluence in a 10 cm dish (6×10^5 cells per dish) in C/T media, incubated for 24 h, then transfected with an ERE-luciferase reporter plasmid plus either empty vector or an ERα expression vector (A) or 500 ng scrambled siRNA or ERα-specific siRNA (B), and incubated for 18 h, then incubated for 24 h (A) or 72 h (B) in fresh C/T media. The cells were then exposed to either vehicle, or E_2 (100 nM), or sirtinol (50 μM), or both, as indicated, for 24 h. Cells were then harvested and estrogen-regulated gene activity was determined via luciferase assay normalized to β-galactosidase activity (A), or mRNA expression via RT-PCR normalized to β-actin transcript levels (B). In panel A, ectopic ERα expression was verified by immunoblot (inset). In panel B, the efficiency of ERα knockdown was determined by analyzing ERα protein expression via immunoblot (inset). For panel A, the data presented are average (±S.E.M.) of three independent experiments. *$P<0.001$. For panel B, the experiment was repeated five times with comparable relative ERα knockdown. In each experiment, *pS2* mRNA expression levels increased in response to E_2 or sirtinol treatment or the combination and were significantly reduced in cells in which ERα had been knocked down. A representative experiment is presented (B). *$P<0.01$ compared to paired black bar from scrambled siRNA-transfected cells.

promoter of GAPDH, a non-estrogen-regulated gene, was not increased by estrogen or sirtinol.

SIRT1 represses ligand-independent ERα activation

ERα is activated by a phosphorylation event at serine[118]. To determine whether this activating phosphorylation of ERα occurs after SIRT1 inhibition, MCF-7 cells were exposed to estrogen or sirtinol, and cell lysates were immunoblotted for phospho-ser[118]-ERα (pERα). In vehicle-treated cells, pERα was undetectable. Upon exposure to estrogen, pERα levels accumulated in the cell, as expected (Fig. 5C). Exposure to sirtinol alone produced a comparable rise in pERα levels. Collectively, these data indicate that SIRT1 represses ligand-independent ERα activation, nuclear localization, and binding to estrogen-regulated promoters.

SIRT1 represses estrogen-independent activation of AKT

ERα is phosphorylated at ser[118] by the serine/threonine kinase AKT1 in response to estrogen. To determine whether AKT is activated in response to SIRT1 inhibition, lysates of MCF-7 cells exposed to estrogen or sirtinol were blotted for phospho-ser[473]-AKT1 (pAKT), the activated form of the kinase. pAKT1 was undetectable in the vehicle-treated cells. Upon exposure to estrogen, pAKT1 levels accumulated in the cell, as expected (Fig. 5C). Exposure to sirtinol alone also produced a comparable rise in activated AKT1.

To determine whether SIRT1 repression of estrogen-regulated genes is AKT1 dependent, MCF-7 cells were treated with siRNA directed against AKT1 or a scrambled siRNA, and the treated cells were then exposed to estrogen or sirtinol. In the presence of AKT1, estrogen and sirtinol increased *pS2* mRNA expression by ~12-fold (Fig. 5D). In cells where AKT1 was knocked down, neither estrogen nor sirtinol exposure increased *pS2* mRNA expression. AKT1 knockdown was confirmed by immunoblotting (Fig. 5D). Activation of estrogen-regulated genes by SIRT1 inhibition, like activation by estrogen, is therefore AKT1 dependent.

Phosphoinositide 3-kinase activity is required for activation of ERα signaling following SIRT1 inhibition or estrogen exposure

Activation of AKT1 occurs through the phosphatidylinositol 3-kinase (PI3K) pathway. To determine whether the ligand-independent activation of ERα following SIRT1 repression requires PI3K activity, as does ligand-dependent activation, MCF-7 cells were exposed to estrogen or sirtinol, in combination with wortmannin, a PI3K pathway inhibitor. In cells exposed to estrogen or sirtinol, pERα accumulated in the cell. When cells were co-treated with wortmannin, pERα was undetectable (Fig. 5E). Together, these findings suggest that SIRT1 activity may represses estrogen-independent activation AKT1 by the PI3K pathway.

SIRT1 activity is required for FOXO3a expression in the cytoplasm of breast cancer cells

The FOXO family of proteins play a role in ER signaling, as well as the regulation of PI3K activity, and are in turn

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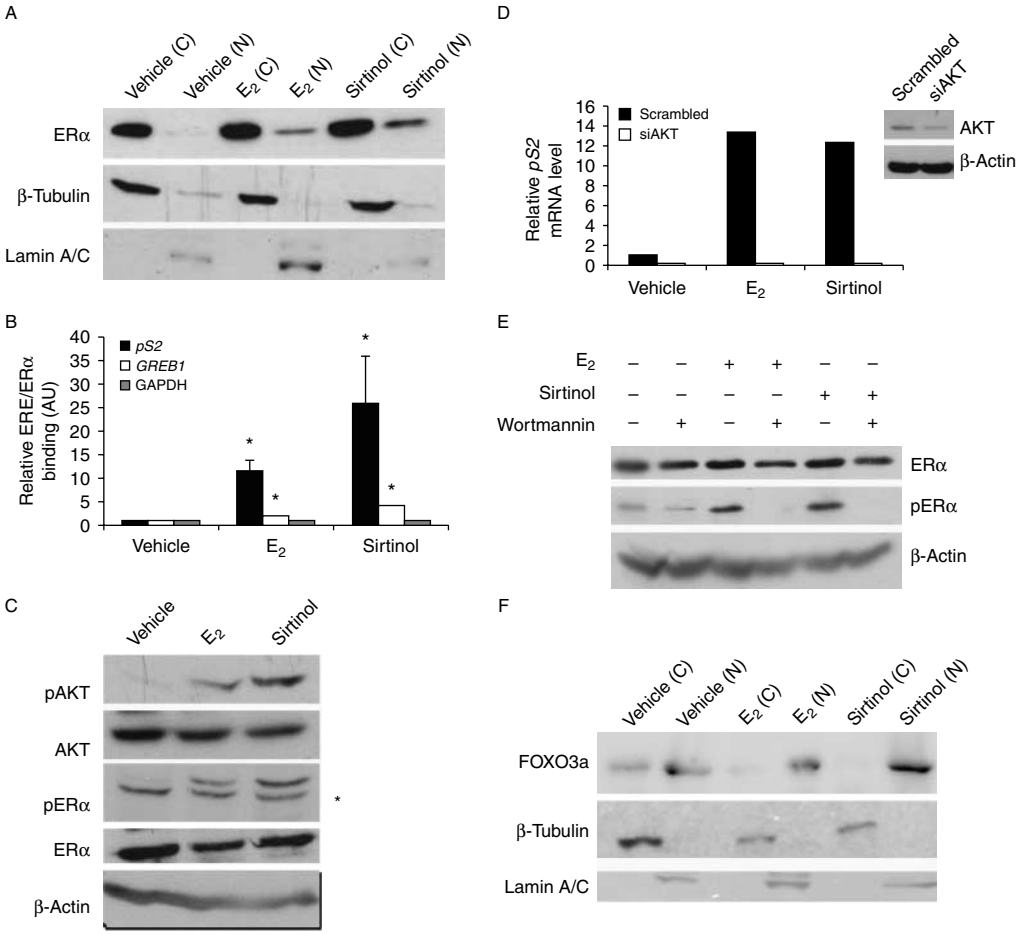

Figure 5

SIRT1 represses estrogen-independent ERα nuclear accumulation, promoter binding, and activation, in an AKT-dependent manner. (A) MCF-7 cells were plated to 60% confluence in a 10 cm dish (6×10^5 cells per dish) in C/T media and incubated for 24 h; then exposed to vehicle, E_2, or sirtinol as indicated; and incubated for an additional 24 h. The cells were then harvested and levels of ERα protein were analyzed by immunoblotting. β-Tubulin and lamin A/C were used as markers to determine the purity of the cytoplasmic and nuclear extracts respectively. The figure presented is representative of three independent experiments. (B) MCF-7 cells were plated to 40% confluence in a 10 cm dish (4×10^5 cells per dish) in C/T media; incubated for 48 h; then exposed to vehicle, E_2, or sirtinol for 24 h; harvested; and ChIP analysis was performed. Immunoprecipitation was carried out using α-ERα or –Pol II antibodies (data not shown). The promoter regions of the *pS2* and *GREB1* genes were amplified via quantitative PCR. The GAPDH promoter regions were also amplified, as a specificity control. The data are mean (±S.E.M.) of three independent experiments. Immunoprecipitations carried out using nonspecific antibodies or pooled antisera did not yield products up to 45 cycles of amplification (not shown). *$P<0.01$ compared with corresponding vehicle controls. (C) MCF-7 cells were plated to 60% confluence in a 10 cm dish (6×10^5 cells per dish) in C/T media and incubated for 24 h; then treated with vehicle, E_2, or sirtinol as indicated; incubated for an additional 24 h; and then harvested. Protein expression levels of pERα, ERα, pAKT, AKT, and β-actin were analyzed via immunoblotting. A blot representative of three independent experiments is shown. (*nonspecific band seen even in cell lacking ERα.) (D) MCF-7 cells were plated at 60% confluence in a 10 cm dish (6×10^5 cells per dish) in C/T media, incubated for 24 h, then transfected with 500 ng scrambled siRNA or AKT1-specific siRNA, and incubated for 18 h and then incubated for 72 h in fresh C/T media. The cells were then exposed to vehicle, E_2, or sirtinol for 24 h; harvested; and mRNA was collected. *pS2* mRNA expression levels were determined via quantitative RT-PCR and normalized to β-actin transcript levels. The efficacy of AKT knockdown was determined by immunoblot (insert). The experiment was repeated four times with similar relative AKT knockdown and consistent effects on *pS2* mRNA induction. Shown is a representative experiment. (E) MCF-7 cells were plated to 60% confluence in a 10 cm dish (6×10^5 cells per dish) in C/T media; incubated for 24 h; then exposed to vehicle, E_2, or sirtinol, and/or wortmannin (100 nM) (a PI3K inhibitor), as indicated; and incubated for an additional 12 h. The cells were then harvested and protein levels of pERα, ERα, and β-actin were analyzed via immunoblotting. The experiment was repeated three times with comparable results. A representative blot is shown. (F) MCF-7 cells were plated to 60% confluence in a 10 cm dish (6×10^5 cells per dish) in C/T media and incubated for 24 h; then exposed to vehicle, E_2, or sirtinol as indicated; and incubated for an additional 24 h. The cells were then harvested and levels of FOXO3a protein were analyzed by immunoblotting. β-Tubulin and lamin A/C were used as markers to determine the purity of the cytoplasmic and nuclear extracts respectively. The figure presented is representative of three independent experiments.

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regulated by SIRT1 (Zou *et al.* 2008). In order to investigate the link between deregulation of the FOXO family of proteins by inhibition of SIRT1 activity and ligand-independent activation of ERα, AKT, and the PI3K pathway, MCF-7 cells were exposed to estrogen or sirtinol, and cytoplasmic and nuclear fractions were prepared. The purity of the subcellular fractions was determined by immunoblotting with β-tubulin (a cytoplasmic protein) and lamin A/C (a nuclear protein). In untreated cells, FOXO3a was relatively evenly distributed between the cytoplasm and the nucleus. In response to estrogen or sirtinol exposure, FOXO3a disappeared from the cytoplasm (Fig. 5F), indicating that SIRT1 regulates the cytoplasmic expression of FOXO3a. Nuclear expression of FOXO3a remained relatively unchanged.

SIRT1 represses estrogen-independent breast cancer cell growth

Activation of ERα by estrogen results in cellular proliferation, as well as induction of ERα-responsive genes. To determine whether estrogen-independent activation of the ERα via repression of SIRT1 activity was sufficient to induce estrogen-independent proliferation, estrogen-dependent MCF-7 cells were exposed to estrogen or sirtinol for 72 h and viable cells were enumerated. Compared to cells incubated in charcoal-stripped media, estrogen-treated cells proliferated by ~2.5-fold over that interval. Cells exposed to sirtinol alone showed comparable proliferation. Cells exposed to estrogen plus sirtinol proliferated by approximately fivefold (Fig. 6). Cell viability remained at 96–99% throughout the study.

To determine whether this ligand-independent cell proliferation in response to SIRT1 inhibition is ERα dependent, cells were exposed to estrogen or sirtinol in combination with 4HT, an estrogen antagonist. 4HT inhibited cell growth induced by either estrogen, or sirtinol, or the combination (Fig. 6). Collectively, these findings indicate that SIRT1 normally functions to repress estrogen-independent, ERα-dependent cell proliferation.

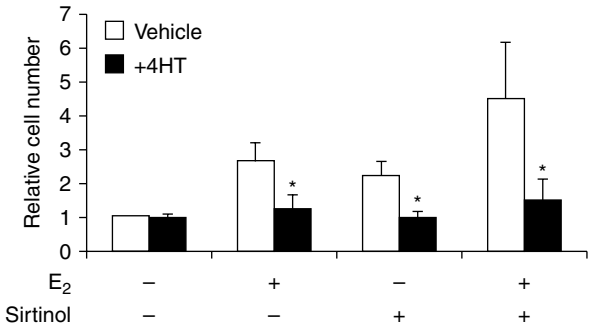

Figure 6
SIRT1 inhibition induces estrogen-independent, ERα-dependent breast cancer cell proliferation. MCF-7 cells (2×10^5) were incubated in phenol red-free medium containing C/T serum for 24 h and then exposed to either vehicle, estrogen (100 nM), or sirtinol (50 μM), and/or 4HT (1 μM), as indicated, for 72 h. Viable cells were then enumerated. The data presented are the mean (±S.E.M.) of four independent experiments. Increases in cell number in response to estrogen and/or sirtinol are significant ($P<0.01$). *$P<0.03$ compared to paired open bars (vehicle-treated cells).

Discussion

These studies demonstrate that the type III HDAC SIRT1 serves an important role in regulating ER signaling. In MCF-7 cells, an estrogen-dependent breast cancer cell line, we found that SIRT1 repressed the basal expression levels of estrogen-regulated genes, as well as their response to estrogen. In the absence of estrogen, SIRT1 repression by small-molecule inhibitors or a D/N SIRT1, or siRNA knockdown, induced estrogen-regulated gene activity to a level comparable to the induction seen when the cells are exposed to estrogen. When SIRT1 was inhibited by various independent means in the presence of estrogen, estrogen-regulated gene activity was induced in an additive manner. These results are generalizable, and the effects are specific for SIRT1, as they are recapitulated by the specific genetic techniques of siRNA knockdown of SIRT1 or expression of a D/N SIRT1. Interestingly, and in contrast to its function in androgen signaling (Dai *et al.* 2007), SIRT1 was not required for estrogen antagonist activity.

The repression of estrogen-regulated genes by SIRT1 is dependent on the ERα as demonstrated by a number of independent approaches. 4HT and faslodex, which serve as ER ligand antagonists, were able to suppress the induction of estrogen-responsive genes produced by inhibition of SIRT1, both in the presence and in the absence of estrogen. Furthermore, MDA231 cells, which lack ERα, did not show induction of ER-regulated genes upon exposure to SIRT1 inhibitors (or to estrogen). When ERα was ectopically expressed in MDA231 cells, however, SIRT1 inhibition stimulated the induction of estrogen-responsive genes.

Ligand-independent activation of estrogen-responsive genes by SIRT1 inhibitors shares a number of common elements with ligand (estrogen)-dependent activation signaling. SIRT1 inhibition resulted in ligand-independent phosphorylation and activation of ERα, accumulation of phospho-ERα in the nucleus, and subsequent association

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of ERα with the promoters of estrogen-responsive genes, in a pattern similar to that induced by estrogen.

ERα is known to be acetylated at multiple sites *in vivo* (Wang *et al*. 2001). Mutational modification of some of these acetylation sites may modestly influence the activity of the receptor, raising the possibility that the deacetylase SIRT1 might act on ERα directly, through modulation of ERα acetylation status. Furthermore, the deacetylase activity of SITR1 is required for its effects on ERα signaling, as the D/N SIRT1 mutant we used is deacetylase deficient. We found, however, that the PI3K/AKT pathway was required for ligand-independent activation of ERα following SIRT1 inhibition just as it is for activation by estrogen, indicating that the actions of SIRT1 on ERα are likely indirect, rather than direct. Thus, SIRT1 inhibitors subvert the same pathway for ligand-independent activation of the ERα that estrogen uses for ligand-dependent activation.

Under normal conditions, SIRT1 appears to repress ERα-mediated cell growth when estrogen is absent, blocking estrogen-independent breast cancer cell proliferation. Inhibition of SIRT1 activity allows proliferation in the absence of estrogen. This proliferation can be blocked by estrogen antagonists, indicating that this proliferation, like the induction of estrogen-regulated genes, is dependent on ERα.

A link between SIRT1 activity and the repression of estrogen-independent ER signaling has not been previously established. However, a recent report indicated that within the nucleus, SIRT1 co-localizes and binds with ERα in several breast and breast cancer cell lines (Elangovan *et al*. 2011). One possible model is that SIRT1 may be inhibiting ERα, PI3K, or AKT activation directly. However, any direct link between SIRT1 and PI3K regulation, with subsequent downstream effects on AKT activity, or functionally related acetylation of p85, p110, and AKT has not been identified to date. Furthermore, while ERα-SIRT1 binding requires SIRT1 catalytic activity, SIRT1 does not affect the acetylation status of ERα (Elangovan *et al*. 2011). We speculate instead that SIRT1 regulation of ERα and PI3K/AKT activity is more likely carried out in an indirect manner, through co-regulatory proteins.

One potential co-regulatory protein is PTEN (Maehama & Dixon 1998, Myers *et al*. 1998, Stambolic *et al*. 1998, Ikenoue *et al*. 2008). SIRT1 deacetylates PTEN at L^{402}, thereby inactivating PTEN and relieving repression of PI3K signaling (Ikenoue *et al*. 2008). However, our studies show an increase in PI3K signaling (AKT phosphorylation) in response to SIRT1 inhibition, so it is unlikely that PTEN plays a role in SIRT1-mediated repression of estrogen-independent PI3K activity.

The FOXO family of proteins plays a role in ERα signaling, as well as the regulation of PI3K activity, and is in turn regulated by SIRT1. Much of the activity seen in MCF-7 cells when FOXO family members are inhibited mirrors the findings of this report. For example, the silencing of endogenous FOXO3a increases expression of estrogen-regulated genes and can convert non-tumorigenic, estrogen-dependent breast cancer cells into tumorigenic, estrogen-independent cells (Zou *et al*. 2008). Herein, we report that SIRT1 catalytic activity is required to maintain FOXO3a expression in the cytoplasm. We hypothesize that it is this maintenance of FOXO3a expression and activity in the cytoplasm that prevents FOXO3a from becoming disassociated with ERα in the absence of ligand, thereby preventing the ligand-independent activation of ERα, transcription of estrogen-regulated genes, and breast cancer cell proliferation (Fig. 7). We are currently further investigating a link between deregulation of the FOXO family of proteins by inhibition of SIRT1 activity and ligand-independent activation of ERα, AKT, and the PI3K pathway.

The findings presented in this report differ from those of several previous reports. Other studies have found that sirtuin inhibition results in cell death and p53 acetylation (Peck *et al*. 2010), or cell senescence (Ota *et al*. 2006), or that it represses expression levels of ERα (Yao *et al*. 2010). Elangovan *et al*. suggested that SIRT1 is required for oncogenic signaling in breast cancer cells. Furthermore, Yao *et al*. indicated that SIRT1 inhibition decreases levels of ERα protein expression. Those studies differ from this report in one major way, in that the previously mentioned studies did not starve the breast cancer cells of steroid hormones before SIRT1 inhibition. Rather, SIRT1 was inhibited while the breast cancer cells were growing logarithmically in the presence of estrogen with ERα engaged by estrogen ligand. In this study, however, cells were incubated in charcoal-treated media, thereby depriving the cells of any steroidal hormone signaling, disengaging the Ras-MAPK pathway (Ota *et al*. 2006), halting cell growth without effects on cell viability (as shown in Fig. 6), and allowing study of the effects of SIRT1 depletion on quiescent cells with unengaged ERα. The Ras-MAPK pathway (Ota *et al*. 2006), as well as p53 (Peck *et al*. 2010), may contribute to differential regulation of estrogen signaling by SIRT1 in the presence or absence of hormonal signaling. Another methodological difference between our approach and some of the papers cited above is our use of reagents highly specific for SIRT1 (shRNA and D/N

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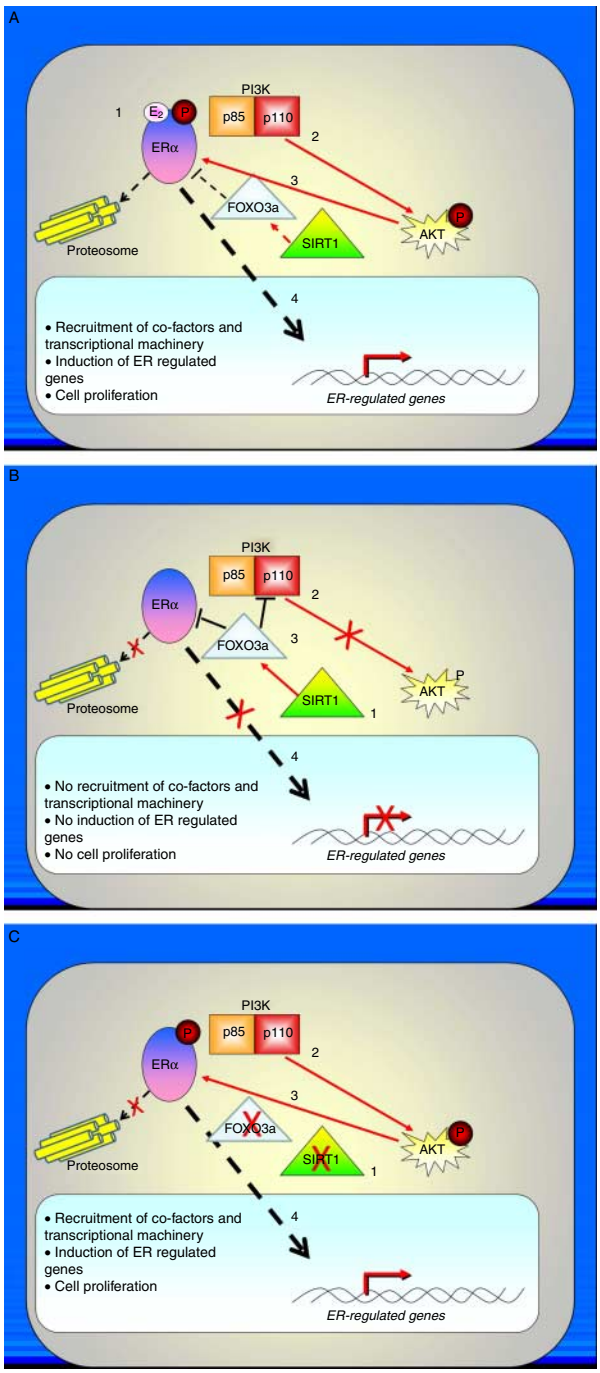
proteins) to confirm the role of SIRT1 rather than relying only on chemical inhibitors, which target SIRT2 as well as having other off-target effects. Peck *et al*. (2010) showed that the effects of SIRT inhibitors on p53 and cell death they observed required inhibition of both SIRT1 and SIRT2. Lastly, as shown in Fig. 5A, C and E, we did not detect any significant change in ERα protein expression in response to the experimental conditions presented herein.



It is important to note that we used two- to threefold less of the chemical SIRT1 inhibitors in these studies than those used in the study of Yao *et al*. It is possible that the differences in ERα expression in response to treatment may be the result of cell toxicity or unintended off-target effects of using inhibitors of higher concentrations. Cell viability remained between 96 and 99% as measured by trypan blue exclusion assay throughout the experiments presented herein. Significantly, ERα expression levels did not change in same cells when SIRT1 was depleted by shRNA, again indicating that any reduction of ERα expression levels may have been an unintended side effect due to nonspecific actions of chemical inhibitors at higher concentrations. The data presented here therefore highlight a potentially significant difference between the regulation of ER signaling in the presence of estrogen compared with ligand-independent signaling in the absence of estrogen. The results presented here, therefore, describe an important mechanism by which breast cancer cells might transition from an estrogen-dependent to an estrogen-refractory state, particularly in the setting of estrogen depletion or estrogen antagonists.

The findings presented in this report support the concept that SIRT1 serves as a tumor suppressor gene in breast cancer cells (Jin *et al*. 2007, Moore *et al*. 2012). These findings also highlight the potential role of SIRT1 in regulating breast cancer cell dependency upon estrogen.

Figure 7
Model of SIRT1 repression of ligand-independent ERα activation. (A) Normal estrogen signaling. Estrogen (E_2) enters the cell and binds to ERα in the cytoplasm (1). This activates PI3K, which phosphorylates AKT (long red arrow) (2). This in turn phosphorylates ERα (3), which then translocates to the nucleus (large black arrow) (4), where it recruits cofactors and other transcriptional machinery, induces ER-regulated genes, and leads to cancer cell proliferation. SIRT1 and FOXO3a act as tonic inhibitors of ERα and PI3K in the presence of E_2, as demonstrated by the fact that SIRT1 inhibition in addition to E_2 treatment yields additive induction of ER-regulated genes (dashed red arrow and black line). (B) SIRT1 inhibition in the absence of E_2. SIRT1 stabilizes FOXO3a in the cytoplasm through SIRT1 deacetylase activity (small red arrow) (1), which in turn represses ligand-independent ERα, PI3K, and AKT activation (solid black line) (2 and 3). The ERα does not translocate to the nucleus (4) and is not degraded in the cytoplasm via the proteasome. ER-regulated genes are not induced nor does the cell proliferate. (C) Effect of SIRT1 inhibition on ligand-independent ERα activation. SIRT1 inhibition (1) destabilizes FOXO3a cytoplasmic localization, which relieves ligand-independent repression of PI3K and ERα (2). This leads to PI3K-mediated activation of AKT, which in turn phosphorylates and activates ERα (3). ERα translocates to the nucleus where it recruits cofactors and transcriptional machinery and initiates transcription of ER-regulated genes, leading to cancer cell proliferation (4). Due to a lack of E_2 ligation, ERα is not degraded via the proteosome. Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-12-0102.

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When coupled with the findings that SIRT1 increases the expression of drug-resistant genes (Chu *et al*. 2005), SIRT1 may have the potential to be an important biomarker for breast cancer prognosis or future patient-specific tailored chemotherapeutic strategies (Chu *et al*. 2005, Bhat-Nakshatri *et al*. 2008). Similarly, modulation of SIRT1 activity may prove useful for next-generation breast cancer therapeutics.

In view of the major regulatory effects of SIRT1 on ERα signaling in breast cancer, further study of the transcriptional and post-translational regulation of SIRT1 in breast cancer cells will likely prove relevant to our understanding of the genesis of breast cancer and its evolution to a hormone-independent state.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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