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Article

PI3K/mTOR mediate mitogen-dependent HDAC1 phosphorylation in breast cancer: a novel regulation of estrogen receptor expression

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Histone deacetylase 1 (HDAC1) is an important epigenetic controller involved in transcriptional regulation through modification of chromatin structure. Genetic and epigenetic changes and deregulation of signal transduction pathways have been implicated in the development of breast cancer. Downregulation of estrogen receptor α (ERα) expression is one of the mechanisms behind the acquisition of endocrine resistance. Sustained and increased hormone and growth factor receptor signaling in breast cancer cells contribute to resistance to endocrine therapy. Both HDACs and the PI3K/mTOR signaling pathway are becoming promising targets in breast cancer, reversing also acquired hormone resistance. Here we show how mitogens, activating the PI3K/mTOR pathway, trigger the phosphorylation of HDAC1 in breast cancer cells, which is completely dependent on the activity of the p70 S6 kinase (S6K1). Our findings show that S6K1, overexpressed in many breast cancers, controls HDAC1-dependent transcriptional regulation of ERα levels upon mitogenic stimuli, controlling HDAC1 recruitment to the *ERα* promoter. Furthermore, cell treatment with both mTOR and HDACs inhibitors shows an additive effect in inhibiting breast cancer proliferation. This confirms the novel cross-talk between the HDAC1 and PI3K pathways with clinical implications towards the treatment of this malignant disease.

Keywords: breast cancer, estrogen receptor, HDAC1/mTOR/PI3K/S6K1

Introduction

Many genetic and epigenetic changes have been implicated in the development of breast cancer. Histone deacetylases (HDACs) are important epigenetic regulators and are overexpressed in multiple cancers, including breast cancer (Spiegel et al., 2012). Together with histone acetyltransferases (HATs), HDACs regulate the acetylation status of histone and non-histone proteins. In humans, HDACs are divided into four classes according to their sequence similarity and mechanism of catalysis. HDAC-class I includes four enzymes: HDAC1 and HDAC2, HDAC3 and HDAC8. HDAC1 is a phosphoprotein and is constitutively phosphorylated through the cell cycle (Cai et al., 2001; Pflum et al., 2001). Although HDACs are widely acknowledged to repress gene expression as part of corepressor complexes, recent findings have established a link between HDACs inhibition and repression of gene expression, suggesting that they might function also as coactivators (Smith, 2008). In some cases, as for the regulation of estrogen receptor α (ERα), HDACs inhibitors (HDACi) can have both a positive or negative impact on transcription, depending on cell context. It has been shown that the use of different HDACi such as trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) decreases the expression of ERα in ER-positive breast cancer cells (Margueron et al., 2004; Duong et al., 2006), whereas it reactivates the expression of the receptor in ER-negative cells (Yang et al., 2000; Margueron et al., 2004; Sharma et al., 2005).

ERα is expressed in ∼75% of human breast cancer and the ER pathway plays a critical role in breast cancer development and progression. Although endocrine therapy targeting estrogen action is the most important systemic therapy for ER-positive breast cancer, its efficacy is limited by intrinsic and acquired resistance. One mechanism of endocrine acquired resistance includes the loss of ERα expression, which occurs in 15%–20% of resistant breast carcinomas. The molecular mechanisms that regulate ERα gene expression and its transcriptional repression are not fully understood. Many studies have described different classes of chromatin-modifiers that may be recruited to the *ERα* promoter as multi-enzyme corepressor complexes containing component of the HDACs family and, in particular, HDAC1 in breast cancer cells (Macaluso et al., 2003; Vesuna et al., 2012; Kang et al., 2014).

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Together with epigenetic and genetic changes, deregulation of signal transduction pathways plays an important role in the development of breast cancer. Accumulating evidence suggests that the phosphoinositide 3-kinase (PI3K)/ mammalian target of rapamycin (mTOR) may be a promising target in breast cancer. More specifically, the PI3K/mTOR pathway plays a critical role in multiple cellular functions including metabolism, proliferation, growth, and survival (Dillon et al., 2007). The p70 S6 kinase (S6K1) is one of the best characterized downstream targets of mTOR and plays important roles in cell size control, protein translation, and cell proliferation (Fingar and Blenis, 2004). The mTOR inhibitor rapamycin, tested as an anticancer drug, rapidly dephosphorylates and inactivates S6K1. S6K1 is encoded by *RPS6KB1*, which is located at 17q23 and is amplified in 10%–30% of breast cancer cell lines and primary breast cancers and S6K1 is overexpressed in the majority of cell lines and primary tumors with this amplification (Monni et al., 2001). Moreover, *RPS6KB1* amplification and S6K1 overexpression are associated with poor prognosis in breast cancer patients (Barlund et al., 2000; van der Hage et al., 2004).

Both HDACs and mTOR inhibitors have been shown to reverse acquired hormone resistance in ER-positive cells (deGraffenried et al., 2004; Fedele et al., 2012).

In this study we have uncovered a mechanism by which the mTOR/S6K1 pathway regulates HDAC1 in breast cancer cell lines, promoting mitogen-mediated phosphorylation of HDAC1, which regulates the expression of ERα. We also show how the combination of treatment with HDACs and mTOR inhibitors additively inhibits breast cell line proliferation.

Results

Mitogens induce HDAC1 hyper-phosphorylation in breast cancer cells

HDAC1 is known to undergo to many posttranslational modifications (Segre and Chiocca, 2011). Comparing cell lysates from the breast cancer cell line MCF7 and the non-tumorigenic mammary cell line MCF10A we detected a slower migrating band of HDAC1 in MCF7 but not in MCF10A cells (Figure 1A). To assess whether this modification was modulated by the presence of mitogenic agents, serum-starved MCF7 cells were treated with different stimuli such as fetal bovine serum (FBS), epidermal growth factor (EGF), or insulin. Upon all treatments, we could detect the appearance of the slower migrating band of HDAC1 (Figure 1B). We then confirmed the specificity of this modification by immunoprecipitating HDAC1 in MCF7 cells treated with FBS and immunoblotting with a different anti-HDAC1 antibody (Figure 1C). Since phosphorylation is the canonical modification triggered by mitogenic stimuli, we incubated MCF7 cell lysates treated or not with FBS with calf intestinal phosphatase (CIP), demonstrating that the FBS-mediated HDAC1 modification was a phosphorylation (Figure 1D, compare lane 2 with lane 4). Thus, we found that mitogens are able to phosphorylate HDAC1 in breast cancer cells.

Mitogen-mediated HDAC1 phosphorylation is triggered by the activation of the PI3K/mTOR pathway

Insulin and growth factors can activate many cellular mitogenic cascades, such as mitogen-activated protein kinases (MAPKs), which include p44/p42 MAPK, JNK (c-Jun N-terminal kinase) and p38 or the PI3K/mTOR pathway. To identify which of these

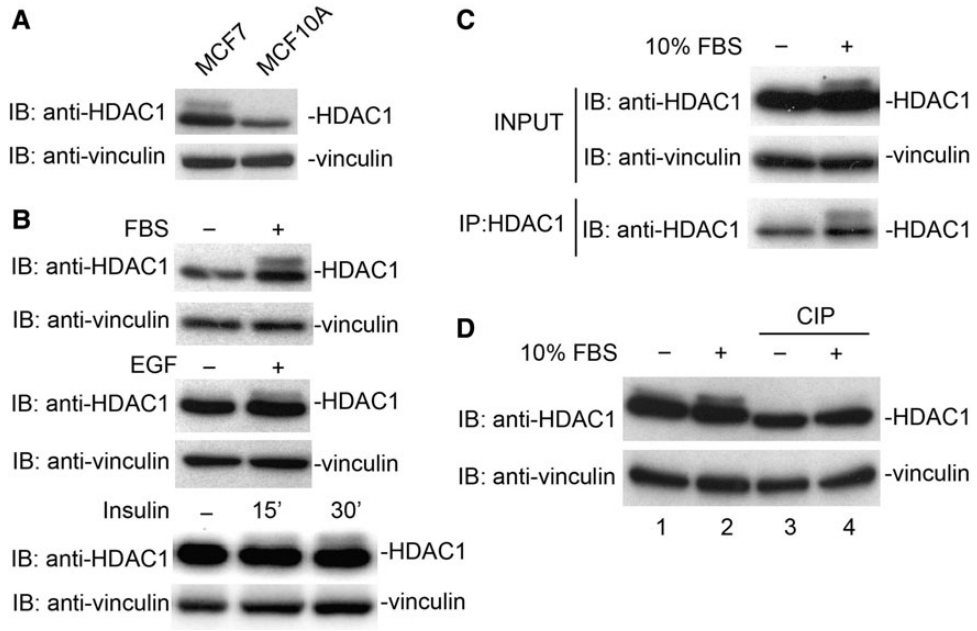

Figure 1 HDAC1 is phosphorylated upon mitogenic stimuli in MCF7 cells. (**A**) MCF7 and MCF10A cells were lysed and analyzed by immunoblotting (IB). (**B**) IB of lysates from MCF7 cells that were serum-starved for 24 h and treated with 10% FBS for 1 h, 10 ng/ml EGF for 30 min or 100 nM insulin for 15 and 30 min. (**C**) Serum-starved MCF7 cells were treated with 10% FBS, lysed, and whole-cell extracts were subjected to anti-HDAC1 immunoprecipitation (IP). IB with the indicated antibodies was then performed and whole-cell extracts were used as input controls. (**D**) Lysates from serum-starved MCF7 cells treated with 10% FBS were incubated or not for 30 min at 37°C with calf intestinal phosphatase (CIP) and analyzed by IB.

pathways was involved in mitogen-mediated HDAC1 phosphorylation we treated MCF7 cells with specific inhibitors of these kinases. Figure 2A shows that only pretreatment of MCF7 cells with the inhibitor of the PI3K (LY294002) and not with inhibitors of the other kinases prevented HDAC1 phosphorylation upon FBS treatment. Since mTOR is one of the main kinases downstream PI3K we tested whether it was involved in HDAC1 phosphorylation. Upon treatment of MCF7 cells with rapamycin, both FBS and EGF-mediated phosphorylation of HDAC1 was abolished (Figure 2B and C, respectively). We then treated cells with anisomycin, which is both a well-known activator of p38 and also an activator of S6K1. As shown in Figure 2D, anisomycin was able to increase the activity of both p38 and S6K1 and to induce HDAC1 phosphorylation. However, only rapamycin and not SB203580, an inhibitor of p38, was able to abolish FBS-mediated HDAC1 phosphorylation, suggesting that S6K1 and not p38 activation is involved in HDAC1 modification. Taken together, these data suggest that the PI3K/mTOR pathway mediates the mitogen-dependent phosphorylation of HDAC1.

S6K1 is required for mitogen-mediated HDAC1 phosphorylation, which occurs on serine 421 or 423

As shown in Figure 2D, S6K1 activation seems to be involved in mitogen-dependent HDAC1 phosphorylation. We confirmed that the presence of this kinase was necessary for HDAC1 phosphorylation upon mitogenic stimuli. As shown in Figure 3A and B, FBS treatment was able to induce HDAC1 phosphorylation only in MCF7 cells, which have *RPS6KB1* amplification and high expression of S6K1, and not in MCF10A cells (Figure 3A) nor in MDAMB453, another breast cancer cell line where S6K1 is low expressed and active (Figure 3B). To corroborate these data, we knocked-down S6K1 using specific siRNA in MCF7 cells. Figure 3C shows that FBS was no longer able to induce HDAC1 phosphorylation in the absence (compare lane 2 with lane 1), as well as upon inactivation of S6K1 (compare lane 4 with lane 3). All these data suggest that S6K1 activity is required for mitogen-mediated HDAC1 phosphorylation, which seems to occur only in cells overexpressing this kinase.

To assess which amino acid on HDAC1 was modified upon mitogenic treatment, we screened different HDAC1 mutants. However, as shown in Figure 3D only mutations of serine 421 or serine 423 were able to abolish FBS-mediated HDAC1 phosphorylation, suggesting that these sites are required for this modification. We confirmed these data also using an antibody specific for phospho421/423 HDAC1 (Supplementary Figure S1A), which recognized both the basal form of HDAC1, constitutively modified at least on one of these serines, and also the FBS-mediated HDAC1 phosphorylation (Figure 3E).

S6K1 is required to drive rapamycin-mediated transcriptional control of estrogen receptor α expression in breast cancer cells

HDACi are known to decrease the expression of ERα in MCF7 cells

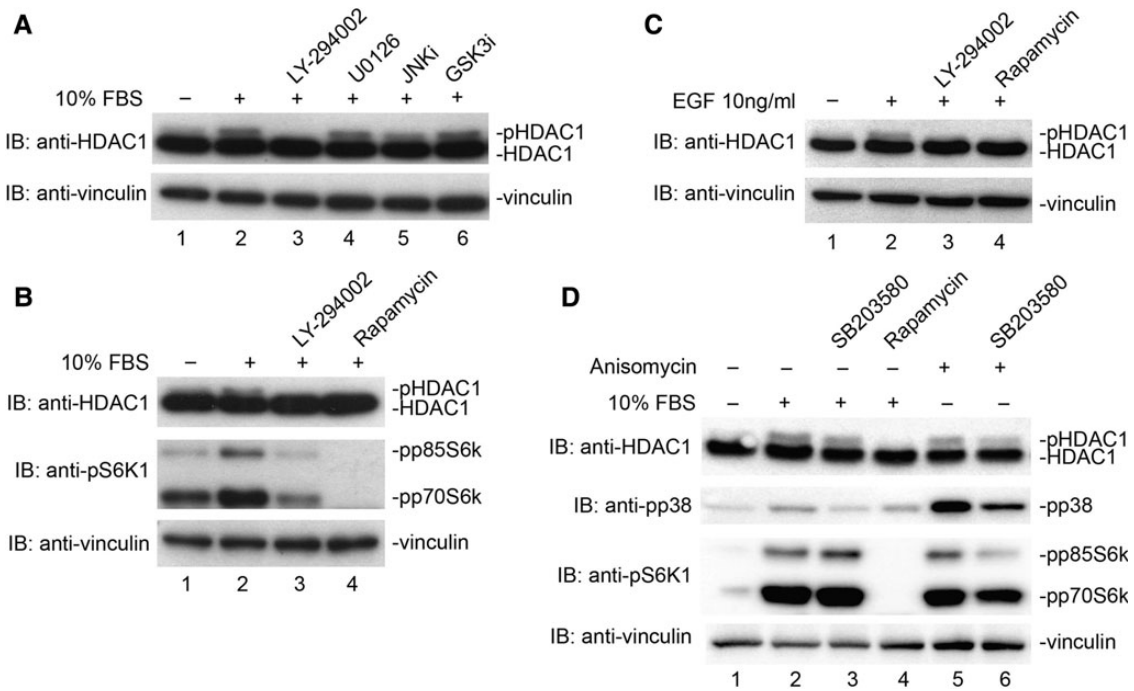

Figure 2 Activation of the PI3K/mTOR pathway induces HDAC1 phosphorylation in MCF7 cells. (**A**) MCF7 cells were serum-starved for 24 h and treated with 10% FBS for 1 h after preincubation for 30 min with the following inhibitors: 20 μM LY294002 (PI3K), 10 μM U0126 (ERK1/2), 10 μM JNK inhibitor V (JNK), or 5 μM GSK3 inhibitor (GSK3). Cell lysates were analyzed by western blotting. (**B** and **C**) Serum-starved MCF7 cells were preincubated for 30 min with 20 μM LY294002 or 100 nM rapamycin before treatment with 10% FBS for 1 h (**B**) or 10 ng/ml EGF for 30 min (**C**). Cell lysates were immunoblotted (IB) with the indicated antibodies. (**D**) MCF7 cells were serum-starved for 24 h and preincubated for 30 min with 100 nM rapamycin or 10 μM SB203580 before stimulation with 10% FBS or 1 μg/ml anisomycin for 1 h. Cell lysates were IB with the indicated antibodies.

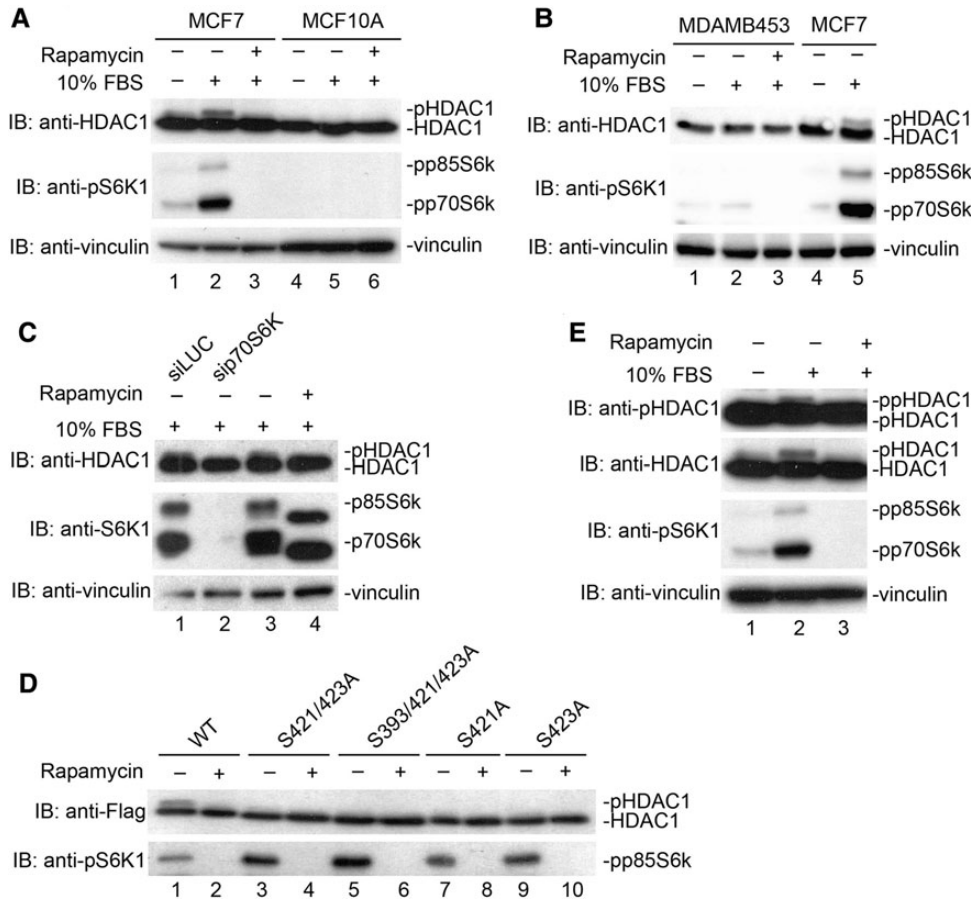

Figure 3 Mitogen-mediated HDAC1 phosphorylation requires S6K1 activity and occurs on serine 421 or 423. (**A**) MCF10A and MCF7 cells were serum-starved for 24 h and treated with 10% FBS for 1 h after pretreatment with 100 nM rapamycin. Cell lysates were immunoblotted (IB) with the indicated antibodies. (**B**) Serum-starved MDAMB453 and MCF7 cells were treated with 10% FBS for 1 h after pretreatment with 100 nM rapamycin, lysed, and IB. (**C**) MCF7 cells were transfected with siRNA targeting S6K1 or control siRNA targeting luciferase (siLUC). Forty-eight hours after transfection, cells were lysed and IB. (**D**) MCF7 cells infected with pBabe Flag-HDAC1, Flag-HDAC1 S421A, Flag-HDAC1 S423A, Flag-HDAC1 S421/423A, or Flag-HDAC1 S421/423/393A were serum-starved for 24 h and then incubated with 100 nM rapamycin for 30 min before treatment with 10% FBS for 1 h. Cells were lysed and IB. (**E**) MCF7 cells were serum-starved for 24 h and treated with 10% FBS for 1 h. Cell lysates were IB with the indicated antibodies.

(Margueron et al., 2004; Duong et al., 2006). We also confirmed these data showing that TSA treatment was able to almost completely abolish both ERα mRNA and protein expression in these cells (Figure 4A and B respectively), suggesting that deacetylase activity is necessary for proper ERα expression. PI3K inhibitors regulate ERα increasing its expression (Creighton et al., 2010) (Supplementary Figure S1B), prompting us to test whether mTOR was involved in this process. We used a more physiological concentration of rapamycin (1 nM), which was still inhibiting S6K1 phosphorylation (Supplementary Figure S1C) and, as shown in Figure 4C, rapamycin treatment both at 24 h and 48 h was able to significantly increase ERα mRNA expression in MCF7 cells. We then asked whether S6K1 had a role in this regulation by comparing breast cell lines with different expression of S6K1 and/or ERα. Figure 4D shows that among the breast cancer cell lines screened, only three have high expression of S6K1, namely MCF7, BT474, and MDAMB361, cell lines known to have 17q23 amplified and all ER-positive. Among these cell lines we chose to compare MCF7, which is ER-positive and has amplified S6K1, with T47D, which is ER-positive but with no amplification of S6K1 and MDAMB231, which has lower amount of S6K1 and is ER-negative. Treatment of these three cell lines with rapamycin increased the expression of ERα mRNA only in MCF7 cells and not in T47D or MDAMB231 (Figure 4E and also shown as fold change in Supplementary Figure S1D). Figure 4F shows how rapamycin affects also ERα protein levels only in MCF7 and not in T47D cells, suggesting that the presence of S6K1 is necessary for the effect of rapamycin on ERα expression. These data were corroborated knocking down S6K1 with specific siRNA in MCF7 cells. The absence of the kinase increased by itself ERα protein levels (Figure 4G, compare lane 3 with lane 1), similarly to the effect of rapamycin in control cells (Figure 4G, compare lane 2 with lane 1); moreover in siS6K cells the presence of rapamycin was not able to further increase ERα protein levels as it did in siLUC control cells (Figure 4G, compare lane 4 with lane 3, and lane 2 with lane 1). Taken together, these data confirmed that the rapamycin-dependent regulation of

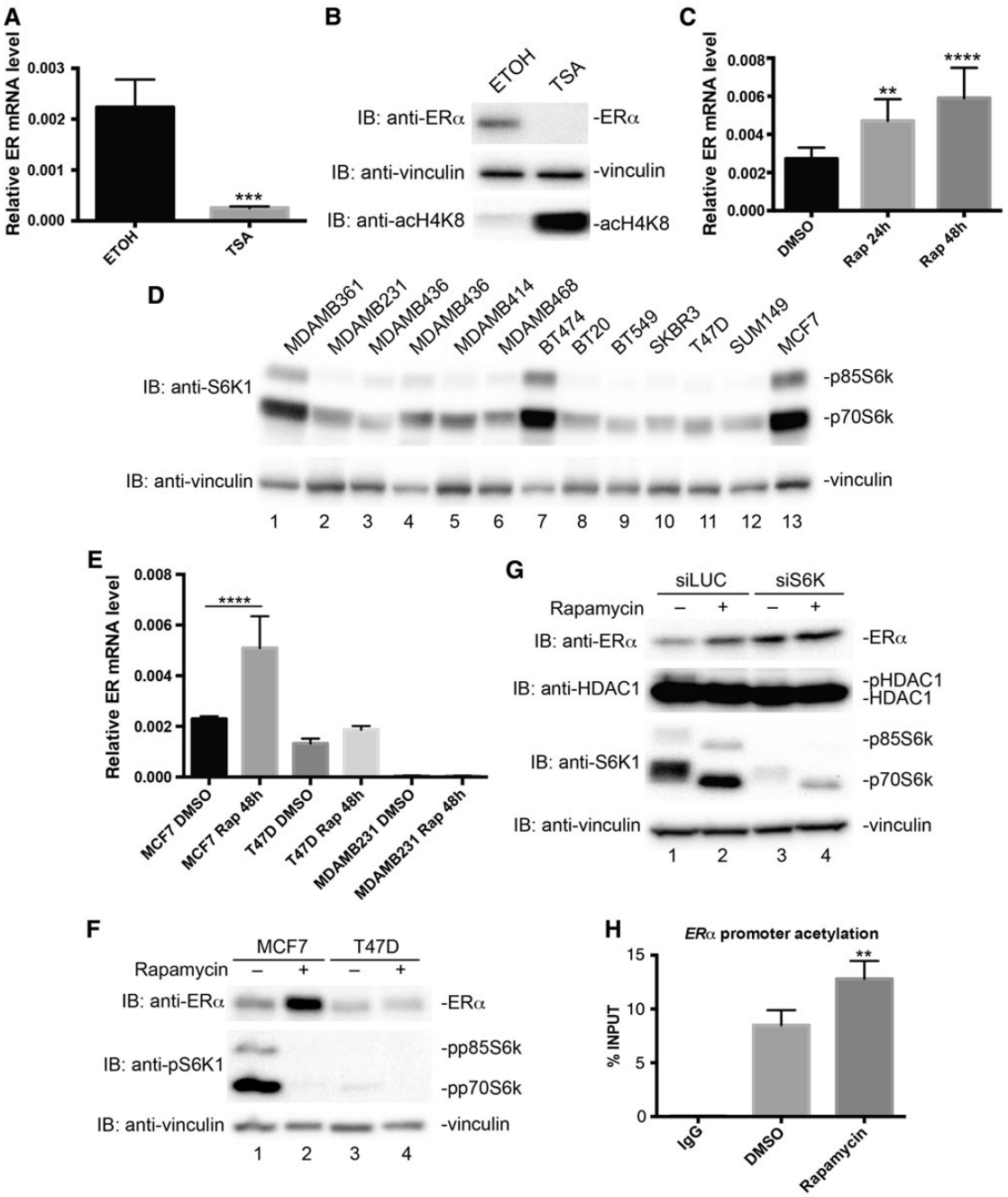

Figure 4 The PI3K/mTOR pathway regulates ERα expression through S6K1 in MCF7 cells. (**A**) Total RNAs from MCF7 cells treated with 330 nM TSA or vehicle for 18 h were isolated for RT–PCR. ERα expression was normalized to RpPO and expressed as mean ± SD. ***$P < 0.0005$. (**B**) MCF7 cells treated with 330 nM TSA or vehicle control for 18 h were lysed and immunoblotted (IB) with the indicated antibodies. (**C**) Total RNAs from MCF7 cells treated with 1 nM rapamycin or vehicle for 24 and 48 h were isolated for RT–PCR. ERα expression was normalized to RpPO and expressed as mean ± SD. **$P < 0.001$, ****$P < 0.0001$. (**D**) Breast cancer cell lines were lysed and IB. (**E**) Total RNAs from MCF7, T47D, and MDAMB231 cells treated with 1 nM rapamycin or vehicle for 48 h were isolated for RT–PCR. ERα expression was normalized to RpPO and expressed as mean ± SD. ****$P < 0.0001$. (**F**) MCF7 and T47D cells treated with 1 nM rapamycin or vehicle for 48 h were lysed and IB with the indicated antibodies. (**G**) MCF7 cells were transfected with siRNA against S6K1, and 48 h later were treated with 1 nM rapamycin for 24 h, lysed, and analyzed by IB. (**H**) MCF7 cells treated with 1 nM rapamycin or vehicle for 48 h were formaldehyde cross-linked and chromatin immunoprecipitated with the antibody specific for acetyl-H3K9 or non-immune IgG as control. QPCR was performed using primers spanning a specific region of the *ERα* promoter, and data were expressed as percent of input ± SD. **$P < 0.001$.

ERα expression requires S6K1 and interestingly, only inhibition of the PI3K/mTOR pathway and not of the p42/p44 MAPK pathway was able to increase ERα level in MCF7 cells (Supplementary Figure S2A). We then asked whether this rapamycin-mediated upregulation of ERα was due to transcriptional or posttranscriptional mechanisms. Since acetylation of the *ERα* promoter is an epigenetic determinant of ERα expression and epigenetic repressor complexes containing HDAC/HAT are known to regulate *ERα* promoter transcription, we performed a ChIP assay in MCF7 cells treated or untreated with rapamycin by using an anti-acetylated H3 antibody (specifically anti-H3K9ac). We detected an increased acetylation of the *ERα* promoter in the presence of the inhibitor (Figure 4H), suggesting that rapamycin, through inhibition of S6K1, regulates ERα expression at the transcriptional level, changing the *ERα* promoter acetylation status.

Rapamycin-mediated transcriptional control of estrogen receptor α expression in breast cancer cells requires HDAC1 activity

We have shown that S6K1 inhibition upregulates ERα expression whereas HDACs inhibition decreases ERα levels in MCF7 cells. We know that serum treatment promotes S6K1 activity and subsequently HDAC1 phosphorylation. By comparing normal growing MCF7 cells with starved MCF7 cells, we noticed that cell starvation, which leads to decreased S6K1 activity and absent HDAC1 phosphorylation, was able by itself to increase the expression of ERα (Figure 5A) as much as rapamycin treatment in both starved or normal growing cells (Figure 5A). Using specific siRNA against HDAC1 (Supplementary Figure S2B) we then showed that ERα expression in HDAC1 knocked-down cells grown under normal condition was similar to ERα levels in siLUC control cells (Figure 5B, left panel), whereas, in starved cells, where ERα expression is higher, the effect of HDAC1 knock-down on ERα level was notable (Figure 5B, right panel). Moreover, in the absence of the deacetylase, rapamycin was no longer able to increase ERα expression compared with siLUC control cells in both starved and not starved cells (Figure 5B). This was also confirmed at the protein level as shown in Figure 5C, where in the absence of HDAC1 rapamycin was unable to increase ERα protein expression.

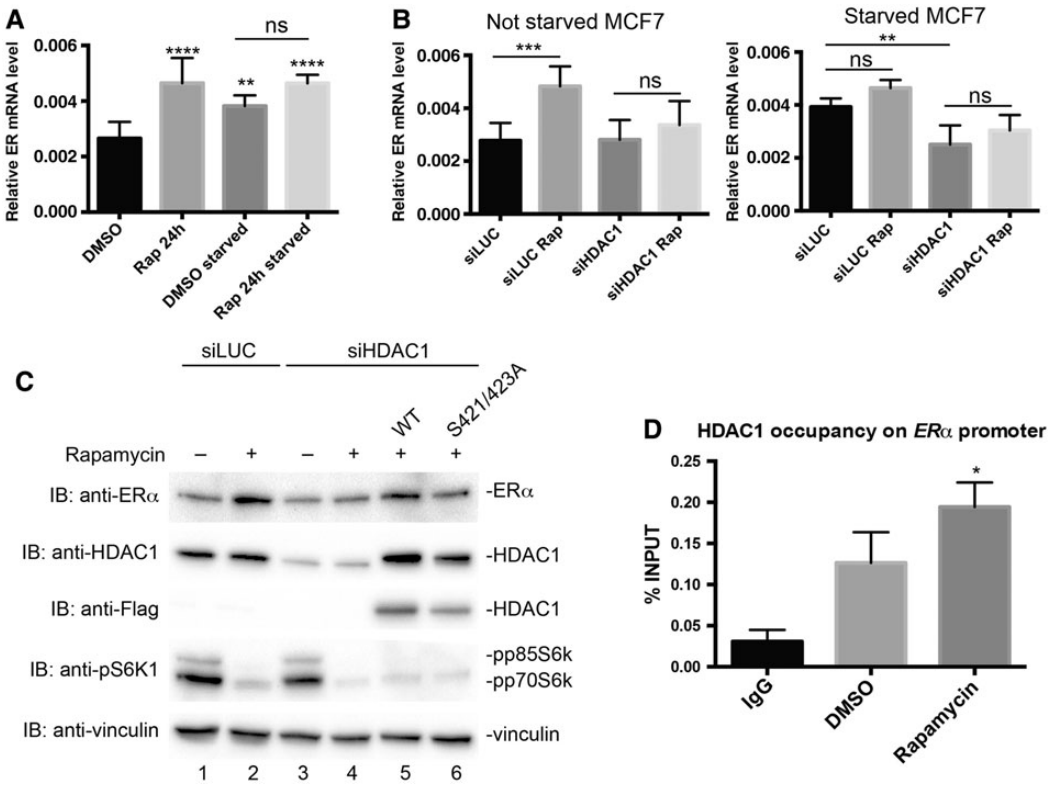

Figure 5 HDAC1 activity is required for rapamycin-dependent regulation of ERα expression. (**A**) Total RNAs from normal growing or serum-starved MCF7 cells were isolated for RT–PCR. ERα expression was normalized to RpPO and expressed as mean ± SD. $^{**}P < 0.001$, $^{****}P < 0.0001$. ns, not statistically significant. (**B**) MCF7 cells were transfected with siRNA against HDAC1 for 24 h. Cells were serum-starved (right panel) or not (left panel) for 24 h and then incubated with 1 nM rapamycin or vehicle for 24 h. Total RNAs were isolated for RT–PCR. ERα expression was normalized to RpPO and expressed as mean ± SD. $^{***}P < 0.0005$, $^{**}P < 0.001$. ns, not statistically significant. (**C**) MCF7 cells were transfected with siRNA against HDAC1 together with wt Flag-HDAC1 or the phosphorylation-deficient mutant Flag-HDAC1 S421A/S423A. Forty-eight hours after transfection, cells were treated with 1 nM rapamycin or vehicle for 24 h, lysed, and immunoblotted (IB) with the indicated antibodies. (**D**) MCF7 cells treated with 1 nM rapamycin or vehicle for 48 h were formaldehyde cross-linked and chromatin immunoprecipitated with the antibody specific for HDAC1 or non-immune IgG as control. QPCR was performed using primers spanning a specific region of the *ERα* promoter, and data were expressed as percent of input ± SD. $^{*}P < 0.005$.

Interestingly, this effect was only rescued by the re-expression of wt HDAC1 but not the phospho-deficient mutant (Figure 5C, compare lane 5 with lane 4 and lane 6 with lane 4), probably explained by the defects in enzymatic activity and binding properties of the S421/423A mutant. These data suggest that S6K1 drives ERα transcriptional regulation through the activity of HDAC1. Since HDAC1 is known to be recruited to the *ERα* promoter regulating its expression, we then checked whether rapamycin was affecting HDAC1 recruitment to the *ERα* promoter. As Figure 5D shows the binding of HDAC1 to the *ERα* promoter was increased in the presence of rapamycin, supporting the hypothesis of a positive role of unphosphorylated HDAC1 in the transcriptional regulation of ERα expression.

Mitogens activate S6K1 and regulate ERα expression through the activity of HDAC1; the combination of mTOR and HDAC inhibitors additively decreases the viability of breast cell lines

As shown in Figure 1C, not only FBS but also other mitogens were able to induce rapamycin-dependent HDAC1 phosphorylation and are also known to decrease ERα expression in breast cancer cells (Stoica et al., 2000a, b). We confirmed these data treating MCF7 cells at different times with insulin (Figure 6A) and EGF (Supplementary Figure S2C) and showing that both stimuli were able to significantly reduce ERα mRNA levels. Moreover, as shown in Figure 6B, after 24 h treatment with insulin the rapamycin-dependent HDAC1 phosphorylation still occurred. The effect of insulin on ERα mRNA level was evident only in MCF7 cells and not in T47D (Figure 6C, compare right and left panels) and both rapamycin (Figure 6C, right panel) and LY294002 (Supplementary Figure S2D) were able to completely rescue this effect. Finally, using siRNA against HDAC1 we showed that, in the absence of the deacetylase, insulin was not able to further decrease ERα mRNA level and rapamycin did not cause any effect (Figure 6D), suggesting that mitogens activate S6K1, which decreases ERα levels through the action of HDAC1. We then checked the acetylation status of the *ERα* promoter upon insulin treatment by ChIP experiments. As shown in Figure 6E, insulin treatment decreased *ERα* promoter acetylation compared with starved cells and this effect was completely reverted by rapamycin. This fully correlated with ERα expression levels and with rapamycin-dependent HDAC1 phosphorylation (Figure 6F).

Both HDACs and mTOR inhibitors show activity against some breast cancers (Margueron et al., 2004; Chang et al., 2007) and, from the data showed in this study, mitogens, responsible for breast cancer cell proliferation, regulate HDAC1 through the PI3K/mTOR pathway. Thus, we tested the effect of SAHA and rapamycin, alone or in combination, on the viability of different breast cancer cell lines. As shown in Figure 5G, as expected cell lines with higher expression of S6K1 (MCF7, MDAMB361, ZR75-1 and BT474) were more sensitive to mTOR inhibition compared with cells with S6K1 not amplified (T47D and MDAMB231) (Supplementary Figure S3A). Nevertheless, in all cases, regardless of the expression of S6K1 or ERα, the combination of treatment with SAHA and rapamycin showed an additive effect on the inhibition of cell proliferation (Figure 6G).

Discussion

HDAC1 activity is regulated by its binding to corepressor complex partners and depends on its posttranslational modifications. In particular, acetylation and phosphorylation of HDAC1 can modulate its enzymatic activity and complex formation (Pflum et al., 2001; Qiu et al., 2006), whereas sumoylation and ubiquitination regulate its stability (Li et al., 2010; Citro et al., 2013). In this current study we found that mitogens such as FBS, EGF and insulin induce HDAC1 hyper-phosphorylation in the breast cancer cell line MCF7 but not in the non-tumorigenic breast cell line MCF10A. MAPKs and GSK3 inhibitors are not able to inhibit this mitogen-dependent HDAC1 modification, whereas both the PI3K inhibitor LY294002 and the mTOR inhibitor rapamycin completely abolish this phosphorylation. The presence and activity of S6K1 is necessary to drive HDAC1 hyper-phosphorylation by mitogens. Thus, only in breast cell lines with *RPS6KB1* amplification and S6K1 overexpression this modification occurs. HDAC1 activity requires its phosphorylation on Ser421 and Ser423; alanine mutations on these two residues reduces HDAC1 enzymatic activity and its binding to interacting proteins (Pflum et al., 2001) (Supplementary Figure S3B and C, respectively). We found that alanine mutations on both Ser421 and Ser423 are able to completely abolish the mitogen-dependent phosphorylation of HDAC1, which is recognized by a specific phospho-S421/S423 antibody. We may speculate that mitogens driven HDAC1 hyper-phosphorylation occurs on one of these two serines while the other residue is constitutively phosphorylated and that mutation of only one of the two residues causes the deactivation also of the other residue probably due to a change in the enzyme conformation.

Although HDAC1 is associated with transcriptional repression, in recent years accumulating studies suggest that it may also positively regulate transcription. HDAC1 knock-out studies in yeast and mouse reveal a significant number of down-regulating genes (Zupkovitz et al., 2006; Yamaguchi et al., 2010). Moreover, genome wide ChIP of HDAC1 shows its enrichment at transcriptionally active loci in human (Wang et al., 2009), mouse (Kidder and Palmer, 2012), and yeast cells (Kurdistani et al., 2002). The recruitment of HDAC1 to active genes can be explained by the requirement of both activating and repressing epigenetic processes to facilitate the sequential and combinatorial assembly and disassembly of transcriptionally productive complex on a promoter (Metivier et al., 2003). Several specific mammalian genes have been reported to be repressed by treatment with HDACi (Polack et al., 1987; Bresnick et al., 1990; Lallemand et al., 1996; Kostyniuk et al., 2002) and some studies suggest that HDACi-mediated changes in gene expression, in addition to histone modification, require also phosphorylation events (Cuisset et al., 1997; Han et al., 2001). In particular, as we also show in this study, ERα expression is completely abolished upon TSA treatment in MCF7 cells, suggesting that HDACs facilitate ERα transcription. PI3K inhibitors are also able to regulate ERα increasing its expression (Creighton et al., 2010) and we show that also rapamycin modulates ERα expression through the activity of S6K1: only in cells with S6K1 overexpressed rapamycin can increase both mRNA and protein ERα levels, promoting the acetylation of its promoter.

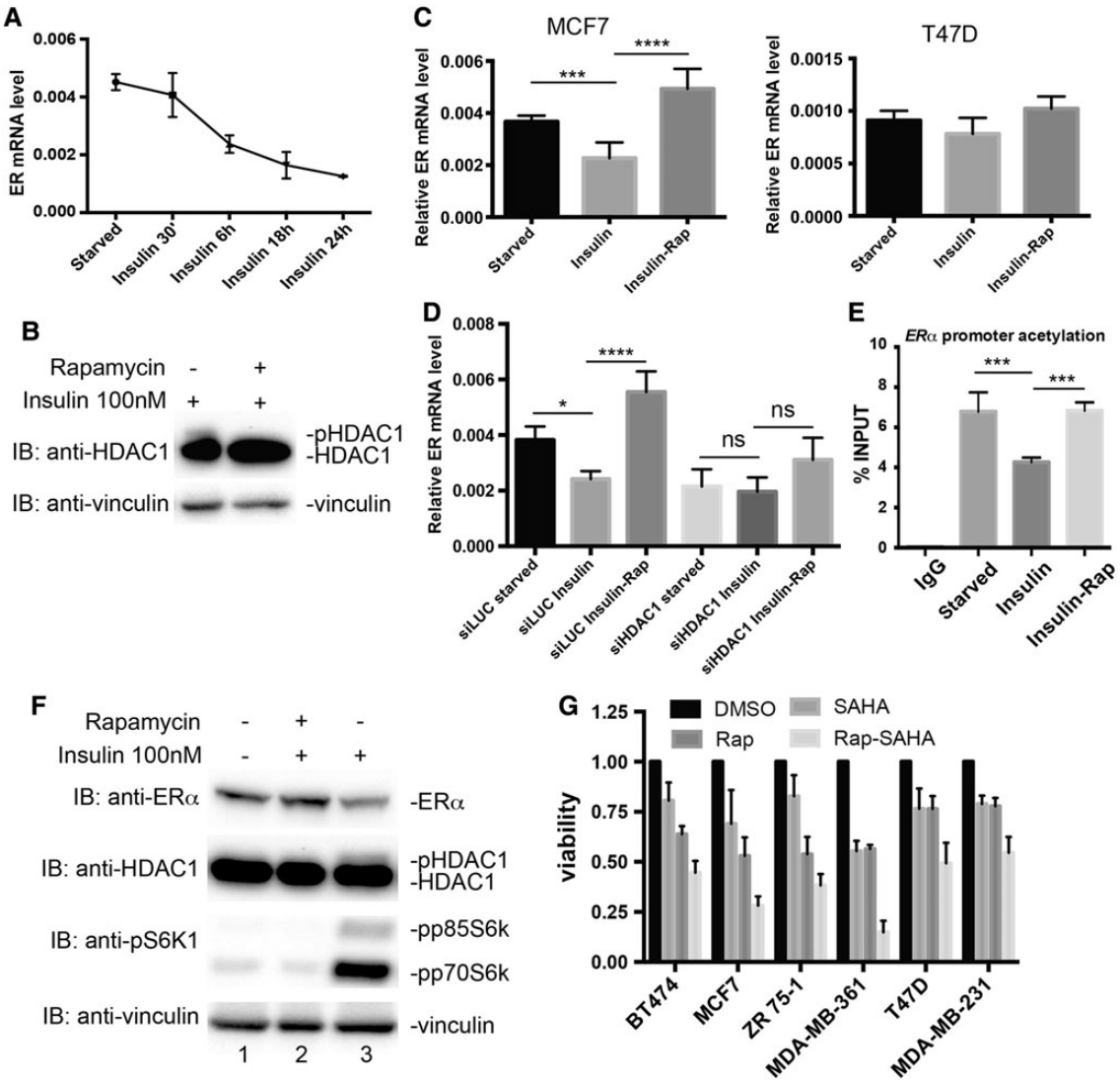

Figure 6 Mitogens regulate ERα expression through the PI3K/mTOR pathway, which requires the activity of HDAC1; mTOR and HDAC inhibitors reduce the viability of breast cell lines additively. (**A**) Total RNAs from starved MCF7 cells treated with 100 nM insulin at different time points were isolated for RT–PCR. ERα expression was normalized to RpPO. (**B**) Serum-starved MCF7 cells were preincubated for 30 min with 1 nM rapamycin before treatment with 100 nM insulin for 24 h, lysed, and immunoblotted (IB). (**C**) Serum-starved MCF7 and T47D cells were preincubated for 30 min with 1 nM rapamycin before treatment with 100 nM insulin for 24 h. Total RNAs were isolated for RT–PCR and ERα expression was normalized to RpPO and expressed as mean ± SD. ***$P < 0.0005$, ****$P < 0.0001$. (**D**) MCF7 cells were transfected with siRNA against HDAC1 for 24 h. Cells were serum-starved for 24 h and then incubated with 1 nM rapamycin for 30 min before treatment with 100 nM insulin for 24 h. Total RNAs were isolated for RT–PCR and ERα expression was normalized to RpPO and expressed as mean ± SD. *$P < 0.005$, ****$P < 0.0001$. ns, not statistically significant. (**E**) Serum-starved MCF7 cells were preincubated for 30 min with 1 nM rapamycin before treatment with 100 nM insulin for 18 h. Cells were formaldehyde cross-linked and chromatin immunoprecipitated with the antibody specific for acetyl-H3K9 or non-immune IgG as control. QPCR was performed using primers spanning a specific region of the *ERα* promoter, and data were expressed as percent of input ± SD. ***$P <$ 0.0005. (**F**) Serum-starved MCF7 cells were preincubated for 30 min with 1 nM rapamycin before treatment with 100 nM insulin for 18 h, lysed, and IB. (**G**) Breast cancer cell lines were treated with 20 nM rapamycin or 1 μM SAHA alone or in combination for 72 h. Cell viability was assessed using CellTiter-Glo® Luminescent Cell Viability Assay and expressed as viability relative to control cells (DMSO).

Moreover, HDAC1 activity and its binding to the *ERα* promoter are required for the rapamycin-dependent upregulation of ERα expression. Thus, when S6K1 is active and HDAC1 is hyperphosphorylated ERα expression decreases, whereas in the presence of rapamycin or cell starvation, S6K1 activation and mitogen-dependent HDAC1 phosphorylation are abrogated, increasing ERα levels in MCF7 cells. All these data suggest that mitogen-dependent phosphorylation of HDAC1 inhibits the positive transcriptional regulation of the deacetylase on ERα expression (Figure 7).

Hyper-activation of hormone and growth factor receptor signaling through multiple intracellular signaling pathways may

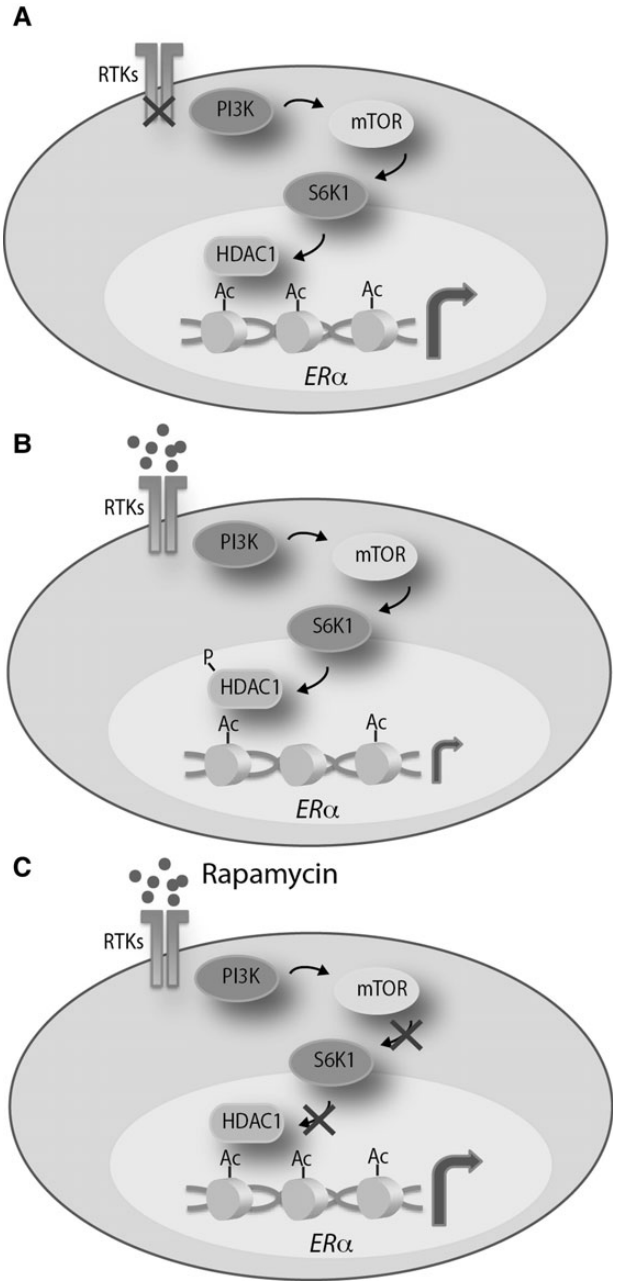

Figure 7 Schematic representation of mitogen-mediated HDAC1 phosphorylation and ERα transcriptional regulation. (**A**) During starvation, receptor tyrosine kinases (RTKs) are not stimulated and are not able to activate the PI3K/mTOR pathway. Under these conditions, HDAC1 is not phosphorylated, *ERα* promoter is more acetylated, and *ERα* is more expressed. (**B**) Upon RTKs activation by hormones and growth factors, PI3K/mTOR are activated. The subsequent activation of S6K1 controls HDAC1 phosphorylation, affecting its positive role in ERα regulation, reducing *ERα* promoter acetylation and gene expression. (**C**) In the presence of rapamycin, S6K1 is not active and is not able to phosphorylate HDAC1, thus promoting *ERα* promoter acetylation and gene expression.

contribute to the appearance of endocrine resistance, affecting ERα expression. Thus, manipulating these signaling networks has become an attractive strategy to potentially overcome resistance to endocrine therapy in breast cancer. We observed that, insulin and EGF treatment, while hyper-phosphorylating HDAC1, significantly decreases ERα expression in MCF7 cells reducing the acetylation of its promoter in a rapamycin and HDAC1-dependent way, uncovering a new mechanism by which hormones and growth factors regulate ERα expression in breast cancer cells. This mechanism is completely dependent of the activity of S6K1, corroborating a previous study on breast cancer patients that suggests how expression of phosphorylated-S6K1 might be a useful marker to assess the development of endocrine resistance in ER-positive breast cancer (Kim et al., 2011).

Growth factors are well known regulators of breast cancer cells proliferation and here we found a growth factors-dependent regulation of HDAC1 through the PI3K/mTOR pathway. Both HDACs and mTOR inhibitors are known to have anti-proliferative effect in human breast cancer cells (Margueron et al., 2004; Chang et al., 2007). It has been shown that ER-positive breast cancer cells show higher sensitivity to low concentrations of HDACi (Margueron et al., 2004), whereas rapamycin sensitivity seems to be determined by S6K1 overexpression (Noh et al., 2004). Treating different breast cancer cell lines with SAHA, we could not determine a great correlation between ERα expression and inhibition of cell viability, probably due to the concentration of the inhibitor used. Rapamycin instead showed a greater efficacy in the breast cell lines with S6K1 overexpressed compared with cells with normal expression of the kinase. We noticed that the effect of the combination of SAHA and rapamycin was equal to the summed effects produced by each individual drug, thus causing an additive inhibitory effect that may result from the action of the two drugs on different targets of the same pathway. Our results suggest that HDACs and mTOR/S6K inhibitors can be used in combination in the clinic for the treatment of breast cancer.

Materials and methods

Cell culture, plasmids, and reagents

T47D, MDAMB231, MDAMB453, BT474, MDAMB361, and MCF7 cell lines were grown in Dulbecco's modified Eagle's medium supplemented with antibiotics, 2 mM L-glutamine, and 10% FBS (Lonza). MCF10A cell line was maintained in F-12 Dulbecco's modified Eagle's medium (1:1) supplemented with horse serum, 20 ng/ml EGF, 0.5 μM hydrocortisone, 50 ng/ml cholera toxin, 10 μg/ml insulin, and antibiotics. ZR75-1 were grown in RPMI supplemented with antibiotics, 1% NaP, 10% fetal calf serum, and 10 mM HEPES. All cell lines were cultured in a humidified 37°C incubator with 5% CO_2. Plasmids for mammalian expression of human Flag-HDAC1, Flag-HDAC1S421/423A were constructed in pBJ5 or pBabe expression vector. Rapamycin and TSA were from Sigma and SAHA from Alexis Biochemicals.

Cell treatment, transfection, and lysis

Before stimulation with insulin, EGF, or FBS cells were starved for 24 h and pretreated with rapamycin or other inhibitors for 30 min. Cells were then lysed in either a sodium dodecyl sulfate (SDS) lysis buffer: a 1:3 mixture of buffer I (5% SDS, 0.15 M Tris–HCl [pH 6.8],

30% glycerol) and buffer II (25 mM Tris–HCl [pH 8.3], 50 mM NaCl, 0.5% NP-40, 0.1% SDS, 1 mM EDTA, and protease inhibitors) containing 0.5 mM N-ethylmaleimide (NEM), 0.5 mM NaF and 2 mM sodium Na_3VO_4 or in E1A buffer (50 mM HEPES pH7, 250 mM NaCl, 0.1% NP-40, proteases inhibitors, 0.5 mM NEM, 0.5 mM NaF, and 2 mM Na_3VO_4). Lipofectamine 2000 was used to transfect siRNA against HDAC1 (5′-UCUAUUUCUCUGUGUAUUUT T-3′) and S6K1 (5′-CCAAGGUCAUGUGAAACUATT-3′) following manufacturer's instructions.

Immunoblotting

After lysis, an equal amount of protein for each sample was resuspended in denaturing sample loading buffer, separated on SDS–PAGE gel and immunoblotted with the indicated antibodies. The following antibodies were used: monoclonal anti-HDAC1 and anti-AcH3K9 (UPSTATE), anti-phosphoS6K1, anti-S6K1, and anti-phosphop42/p44 (Cell Signaling), polyclonal anti-HDAC1, anti-HDAC2, anti-AcH4K8 and anti-H3 (Abcam), anti-FLAG M2 (Sigma) and anti-SIN3A (Santa Cruz).

RT-QPCR

RNA was extracted from cells with the QIAGEN RNEasy Mini Kit. cDNA was generated by reverse transcription-PCR with the M-MLV reverse transcriptase (Invitrogen). Relative levels of specific mRNAs were determined with the Fast SYBR Green detection chemistry system (Applied Biosystem). All PCR reactions were performed with a 7500 Fast Real-Time PCR system (Applied Biosystem). Ribosomal Phosphoprotein (RpPO) was used as a house-keeper gene for normalization.

Cell proliferation assay

To test the effect of rapamycin and SAHA alone or in combination on cell proliferation, cells were plated into 96-well plates with the density determined on the basis of the growth characteristic of each cell line (2000 cells/well T47D, 1800 cells/well MCF7, 1500 cells/well MDAMB231, 6000 cells/well BT474, 3500 cells/well MDAMB361, 5000 cells/well ZR75-1). Twenty-four hours later triplicate wells were treated with 20 nM rapamycin, 1 μM SAHA, or the combination of the two for 72 h. Cell proliferation was assayed after cell washing using CellTiter-Glo® Luminescent Cell Viability Assay and following manufacturer's instructions.

ChIP assay

ChIP analyses were performed using published procedure (Frank et al., 2001) with the following modifications. Chromatin samples were sonicated on ice three times for 10 sec each yielding a genomic DNA fragments with a bulk size of 300–600 bp. Chromatin fragments were pre-cleared with A-sepharose beads blocked with tRNA (Sigma) and immunoprecipitated DNA was purified with Qiaquick columns (Qiagen). The ER promoter was analyzed using the forward primer 5′-TGAACCGTCCGCAGCTCAAGATC-3′ and reverse primer 5′-GTCTGACCGTAGACCTGCGCGTTG-3′.

Histone deacetylation assay

Immunoprecipitates from transfected cells were incubated with [^{3}H]labeled acetylated histones for 1 h at 30°C. The reaction was terminated by adding a stop solution (1 M HCl and 0.4 M NaAc) and deacetylase activity was determined by scintillation counting of the ethyl acetate-soluble [^{3}H]acetic acid.

Statistical analysis

Statistical differences were evaluated using Tukey's multicomparison analysis after one-way analysis ANOVA to compare multiple samples or unpaired *t* test to compare only two samples (Graphpad software).

Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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References

Barlund, M., Forozan, F., Kononen, J., et al. (2000). Detecting activation of ribosomal protein S6 kinase by complementary DNA and tissue microarray analysis. J. Natl Cancer Inst. *92*, 1252–1259.

Bresnick, E.H., John, S., Berard, D.S., et al. (1990). Glucocorticoid receptor-dependent disruption of a specific nucleosome on the mouse mammary tumor virus promoter is prevented by sodium butyrate. Proc. Natl Acad. Sci. USA *87*, 3977–3981.

Cai, R., Kwon, P., Yan-Neale, Y., et al. (2001). Mammalian histone deacetylase 1 protein is posttranslationally modified by phosphorylation. Biochem. Biophys. Res. Commun. *283*, 445–453.

Chang, S.B., Miron, P., Miron, A., et al. (2007). Rapamycin inhibits proliferation of estrogen-receptor-positive breast cancer cells. J. Surg. Res. *138*, 37–44.

Citro, S., Jaffray, E., Hay, R.T., et al. (2013). A role for paralog-specific sumoylation in histone deacetylase 1 stability. J. Mol. Cell Biol. *5*, 416–427.

Creighton, C.J., Fu, X., Hennessy, B.T., et al. (2010). Proteomic and transcriptomic profiling reveals a link between the PI3K pathway and lower estrogen-receptor (ER) levels and activity in ER$^+$ breast cancer. Breast Cancer Res. *12*, R40.

Cuisset, L., Tichonicky, L., Jaffray, P., et al. (1997). The effects of sodium butyrate on transcription are mediated through activation of a protein phosphatase. J. Biol. Chem. *272*, 24148–24153.

deGraffenried, L.A., Friedrichs, W.E., Russell, D.H., et al. (2004). Inhibition of mTOR activity restores tamoxifen response in breast cancer cells with aberrant Akt Activity. Clin. Cancer Res. *10*, 8059–8067.

Dillon, R.L., White, D.E., and Muller, W.J. (2007). The phosphatidyl inositol 3-kinase signaling network: implications for human breast cancer. Oncogene *26*, 1338–1345.

Duong, V., Licznar, A., Margueron, R., et al. (2006). ERα and ERβ expression and transcriptional activity are differentially regulated by HDAC inhibitors. Oncogene *25*, 1799–1806.

Fedele, P., Calvani, N., Marino, A., et al. (2012). Targeted agents to reverse resistance to endocrine therapy in metastatic breast cancer: where are we now and where are we going? Crit. Rev. Oncol. Hematol. *84*, 243–251.

Fingar, D.C., and Blenis, J. (2004). Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. Oncogene *23*, 3151–3171.

Frank, S.R., Schroeder, M., Fernandez, P., et al. (2001). Binding of c-Myc to chromatin mediates mitogen-induced acetylation of histone H4 and gene activation. Genes Dev. *15*, 2069–2082.

Han, J.W., Ahn, S.H., Kim, Y.K., et al. (2001). Activation of p21(WAF1/Cip1) transcription through Sp1 sites by histone deacetylase inhibitor apicidin: involvement of protein kinase C. J. Biol. Chem. *276*, 42084–42090.

Kang, H.J., Lee, M.H., Kang, H.L., et al. (2014). Differential regulation of estrogen receptor α expression in breast cancer cells by metastasis-associated protein 1. Cancer Res. *74*, 1484–1494.

Kidder, B.L., and Palmer, S. (2012). HDAC1 regulates pluripotency and lineage specific transcriptional networks in embryonic and trophoblast stem cells. Nucleic Acids Res. *40*, 2925–2939.

Kim, E.K., Kim, H.A., Koh, J.S., et al. (2011). Phosphorylated S6K1 is a possible marker for endocrine therapy resistance in hormone receptor-positive breast cancer. Breast Cancer Res. Treat. *126*, 93–99.

Kostyniuk, C.L., Dehm, S.M., Batten, D., et al. (2002). The ubiquitous and tissue specific promoters of the human SRC gene are repressed by inhibitors of histone deacetylases. Oncogene *21*, 6340–6347.

Kurdistani, S.K., Robyr, D., Tavazoie, S., et al. (2002). Genome-wide binding map of the histone deacetylase Rpd3 in yeast. Nat. Genet. *31*, 248–254.

Lallemand, F., Courilleau, D., Sabbah, M., et al. (1996). Direct inhibition of the expression of cyclin D1 gene by sodium butyrate. Biochem. Biophys. Res. Commun. *229*, 163–169.

Li, Y., Li, X., and Guo, B. (2010). Chemopreventive agent 3,3′-diindolylmethane selectively induces proteasomal degradation of class I histone deacetylases. Cancer Res. *70*, 646–654.

Macaluso, M., Cinti, C., Russo, G., et al. (2003). pRb2/p130-E2F4/5-HDAC1-SUV39H1-p300 and pRb2/p130-E2F4/5-HDAC1-SUV39H1-DNMT1 multimolecular complexes mediate the transcription of estrogen receptor-α in breast cancer. Oncogene *22*, 3511–3517.

Margueron, R., Duong, V., Castet, A., et al. (2004). Histone deacetylase inhibition and estrogen signalling in human breast cancer cells. Biochem. Pharmacol. *68*, 1239–1246.

Metivier, R., Penot, G., Hubner, M.R., et al. (2003). Estrogen receptor-α directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. Cell *115*, 751–763.

Monni, O., Barlund, M., Mousses, S., et al. (2001). Comprehensive copy number and gene expression profiling of the 17q23 amplicon in human breast cancer. Proc. Natl Acad. Sci. USA *98*, 5711–5716.

Noh, W.C., Mondesire, W.H., Peng, J., et al. (2004). Determinants of rapamycin sensitivity in breast cancer cells. Clin. Cancer Res. *10*, 1013–1023.

Pflum, M.K., Tong, J.K., Lane, W.S., et al. (2001). Histone deacetylase 1 phosphorylation promotes enzymatic activity and complex formation. J. Biol. Chem. *276*, 47733–47741.

Polack, A., Eick, D., Koch, E., et al. (1987). Truncation does not abrogate transcriptional downregulation of the c-myc gene by sodium butyrate in Burkitt's lymphoma cells. EMBO J. *6*, 2959–2964.

Qiu, Y., Zhao, Y., Becker, M., et al. (2006). HDAC1 acetylation is linked to progressive modulation of steroid receptor-induced gene transcription. Mol. Cell *22*, 669–679.

Segre, C.V., and Chiocca, S. (2011). Regulating the regulators: the post-translational code of class I HDAC1 and HDAC2. J. Biomed. Biotechnol. *2011*, 690848.

Sharma, D., Blum, J., Yang, X., et al. (2005). Release of methyl CpG binding proteins and histone deacetylase 1 from the Estrogen receptor α (ER) promoter upon reactivation in ER-negative human breast cancer cells. Mol. Endocrinol. *19*, 1740–1751.

Smith, C.L. (2008). A shifting paradigm: histone deacetylases and transcriptional activation. Bioessays *30*, 15–24.

Spiegel, S., Milstien, S., and Grant, S. (2012). Endogenous modulators and pharmacological inhibitors of histone deacetylases in cancer therapy. Oncogene *31*, 537–551.

Stoica, A., Saceda, M., Doraiswamy, V.L., et al. (2000a). Regulation of estrogen receptor-α gene expression by epidermal growth factor. J. Endocrinol. *165*, 371–378.

Stoica, A., Saceda, M., Fakhro, A., et al. (2000b). Role of insulin-like growth factor-I in regulating estrogen receptor-α gene expression. J. Cell. Biochem. *76*, 605–614.

van der Hage, J.A., van den Broek, L.J., Legrand, C., et al. (2004). Overexpression of P70 S6 kinase protein is associated with increased risk of locoregional recurrence in node-negative premenopausal early breast cancer patients. Br. J. Cancer *90*, 1543–1550.

Vesuna, F., Lisok, A., Kimble, B., et al. (2012). Twist contributes to hormone resistance in breast cancer by downregulating estrogen receptor-α. Oncogene *31*, 3223–3234.

Wang, Z., Zang, C., Cui, K., et al. (2009). Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes. Cell *138*, 1019–1031.

Yamaguchi, T., Cubizolles, F., Zhang, Y., et al. (2010). Histone deacetylases 1 and 2 act in concert to promote the G1-to-S progression. Genes Dev. *24*, 455–469.

Yang, X., Ferguson, A.T., Nass, S.J., et al. (2000). Transcriptional activation of estrogen receptor α in human breast cancer cells by histone deacetylase inhibition. Cancer Res. *60*, 6890–6894.

Zupkovitz, G., Tischler, J., Posch, M., et al. (2006). Negative and positive regulation of gene expression by mouse histone deacetylase 1. Mol. Cell. Biol. *26*, 7913–7928.