LATS2 Is a Modulator of Estrogen Receptor Alpha

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Abstract. *Background: Estrogen Receptor α (ERα), a member of the nuclear receptor superfamily of transcription factors, plays a central role in breast cancer development. More than two-thirds of patients with breast cancer are ERα-positive; however, a proportion becomes resistant. Phosphorylation of ERα is one of the mechanisms associated with resistance to endocrine therapy. In a kinome screen, we have identified the large tumor suppressor homolog-2 (LATS2) as a potential kinase, acting on ERα. Materials and Methods: The role of LATS2 on activation of ERα transcription and its functional consequences was examined by various molecular and cellular biology techniques. Results: LATS2 co-localises with ERα in the nucleus. LATS2-silencing increases expression of ERα-regulated genes and inhibits proliferation. At the protein level, inhibition of LATS2 reduces the expression of cyclin-D1 and Nuclear Receptor Co-Repressor (NCoR) while increasing the expression of p27. Conclusion: Identifying novel kinases which modulate ERα activity is relevant to therapeutics. LATS2 modulates ERα-regulated gene transcription, through direct and/or indirect interactions with ERα.*

Over two-thirds of breast tumours express estrogen receptor-alpha (ERα) and patients with $ER\alpha^+$ disease respond to ERα antagonists (tamoxifen), compounds that inhibit the synthesis of 17-β estradiol (E_2) (aromatase inhibitors) or those that induce ERα down-regulation (fulvestrant) (1, 2); however, resistance to such therapy frequently occurs and patients experience relapse.

ERα activation can either be ligand-dependent, involving binding of E_2 to the receptor, or ligand-independent involving phosphorylation of ERα by a second messenger signalling pathway (3). There are a number of currently identified phosphorylation sites (4-8) and several kinases that are able to phosphorylate ERα (4, 9-20). Phosphorylation plays a significant role in transcriptional regulation of ERα and regulates a variety of important processes, including DNA and estrogen response element (ERE) binding (4, 10, 21), ligand binding (22), ER dimerization (11, 21, 22), co-activator function and recruitment (12) and transcriptional activation (11, 23, 24).

Upon activation, ERα undergoes conformational changes allowing for interaction with a variety of co-activators (*e.g.* steroid receptor co-activator 1 (SRC1), amplified in breast cancer 1 (AIB1), p300/CREB-binding protein (CBP) (16, 25-28) or co-repressors (*e.g.* nuclear receptor co-repressor (NCoR) and silencing mediator for retinoid/thyroid hormone receptors (SMRT) (7, 26, 27, 29, 30). The ERα co-activator complex binds to EREs within the promoters of target genes (23) and mediates the basal transcriptional machinery causing ERα-driven transcription (26, 31).

We previously performed an siRNA screening (20) and identified kinases whose silencing alters the genomic E_2 response. Amongst the most potent regulators was large tumor suppressor homolog-2 (LATS2), whose silencing significantly increased the transcriptional activity of ERα, indicated by elevated mRNA levels of trefoil factor 1 (*TFF1*).

LATS2 is a putative serine/threonine kinase, located on chromosome 13q11-12 (32), close to the breast cancer type-2 susceptibility protein (*BRCA2*) gene, commonly associated with breast cancer (33). LATS2 is a centrosomal protein (34) involved in regulating spindle organisation through recruitment of γ-tubulin to the centrosome (35). Overexpression of LATS2 leads to down-regulation of cyclin E/cyclin-dependent kinase-2 (CDK2) kinase activity, resulting in G_1/S cell phase arrest (36). LATS2 is also involved in G_2/M arrest (37). LATS2 over-expression induces apoptosis *via* down-regulation of BCL-2/ BCL-xl, which results in apoptosis *via* the caspase-9 pathway (38). LATS2 also regulates p53 *via* interactions with murine double minute-2 (MDM2) (39) and has been reported to be a negative regulator of the androgen receptor (40). Finally, LATS2 has also been characterized as a key mediator in the

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salvador/warts/hippo (SWH) tumor suppressor pathway which is characterized by a series of kinases associated with the control of organ size and homeostasis (32, 41).

Interestingly, LATS2 is down-regulated in many types of cancers including leukaemia (42), astrocytoma (43), prostate cancer (40) and breast cancer (44). Down-regulation of LATS2 in breast cancer is most likely mediated by hypermethylation of CpG islands in the promoter of the *LATS2* gene (44). Loss of heterozygosity is also frequently seen in this area. However, other studies have demonstrated the involvement of micro-RNA-373 (45) and tristetraprolin (TTP) (32) in the regulation of LATS2 levels. The silencing seen in many types of cancers may therefore be important in their resistance to apoptosis or cell-cycle arrest.

Alternative phosphorylation pathways activating ERα may be pivotal to the development of resistance to commonly used endocrine therapies (46) and in the pathophysiology of breast cancer progression (3, 6, 7, 9, 15). Identifying kinases able to phosphorylate and/or modulate ERα is of considerable biomarker and therapeutic interest (15, 17, 46). Since LATS2 is down-regulated in breast cancer we investigated its role in various breast cancer cell lines and in transcriptional activity of ERα.

Materials and Methods

Cell lines and reagents. MCF-7 (ERα-positive) and MDA-231 (ERα-negative) human breast cancer cell lines (47) were obtained from the European Collection of Cell Cultures (ECACC) (Salisbury, UK) and maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin/L-glutamine at 37˚C and with 5% CO_2. E_2 was obtained from Sigma (Dorset, UK) and was dissolved in ethanol. Charcoal dextran-stripped serum (DSS) was obtained from Gemini (Bolnet, UK). Antibodies to β-actin (mouse), ERα (mouse), LATS2 (rabbit), mSIN3A (rabbit) and NCOA2 (rabbit) were purchased from Abcam (Cambridge, UK). Mouse monoclonal antibody against ERα-Ser118 was purchased from Cell Signalling (Danvers, MA, USA). Antibodies to NCoR (goat), p27 (rabbit) p300 (rabbit), cyclin-D1 (mouse), BCL-2 (mouse) and cyclin A (mouse) were purchased from Santa Cruz (Heidelberg, Germany). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, goat anti-mouse IgG antibodies and goat anti-goat IgG antibodies were purchased from GE Healthcare (Slough, UK).

LATS2 siRNA transfection. MCF-7 cells were maintained in phenol red-free medium with 10% DSS and 1% penicillin/streptomycin/L-glutamine for 72 h prior to transfection. MCF-7 cells were transfected using the Hiperfect reagent according to the manufacturer's instructions (Qiagen, Crawley, UK). *LATS2* was silenced using two individual siRNAs (30 nM), targeting the sequences 5'-CACACTCACCTCGCCCAATAA-3' (*LATS2* number 1) and 5'-AAGGATGTCCTGAACC GGAAT-3' (*LATS2* number 2). Both siRNAs were used in western blotting (WB) and Real Time quantitative PCR (RT-qPCR). Seventy-two hours post-transfection cells were treated with either vehicle (ethanol) or E_2 (10 nM) and harvested (as indicated) for RNA or protein analyses.

RNA isolation and RT-qPCR. Total RNA was isolated using the RNeasy kit (Qiagen). RNA concentrations were quantified using Nanodrop (Thermo Scientific, Essex, UK) and 1 μg was reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Warrington, UK), according to the manufacturer's instructions. RT-qPCR was carried out using the Taqman Mastermix (Applied Biosystems) on a 7900HT Thermocycler (Applied Biosystems), using primers for Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), Progesterone Receptor (*PGR*), *TFF1*, *cyclin-D1*, *ERα* and growth regulation by estrogen in breast cancer-1 (*GREB1*) cDNAs (Applied Biosystems).

Western blotting. Seventy-two hours after E_2 or vehicle treatment, cells were harvested and whole-cell lysates were prepared in NP-40 lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% (v/v) glycerol, 1% NP40, 5 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM EGTA, 50 μM leupeptin and 30 μg/ml aprotinin]. Protein quantification was performed using the Bradford method (Bio-Rad, Hertfordshire, UK). Lysates were then boiled at 95˚C for 5 min in 5× sodium dodecyl sulfate (SDS) sample buffer. Total protein (20 μg) was size-fractionated using SDS-Polyacrylamide Gel Electrophoresis (PAGE). Proteins were transferred to a Hybond C super nitrocellulose membrane (GE Healthcare) and blocked using 5% non-fat milk in tris buffered saline (TBS) with 0.1% Tween20 for 1 h at room temperature (RT). Membranes were then probed overnight using various antibodies. Membranes were extensively washed using TBS/Tween20 (0.1%), and immunocomplexes were detected by incubating for 1 h with HRP-conjugated goat anti-rabbit IgG, goat anti-mouse IgG or goat anti-goat IgG (1:1000 dilution). Bands were visualised using enhanced chemiluminescense detection (ECL) (GE Healthcare). The band intensity was quantified using the Image J software (NIH, Bethesda, MD, USA).

Immunofluorescence. Cells were grown on poly-D-lysine-coated glass coverslips for 24 h prior to transfection with *LATS2* (number 2) siRNA. Forty-eight hours after transfection cells were treated with vehicle or E_2 for 24 h. Cells were then fixed in 4% formaldehyde for 15 min. Fixed cells were washed in PBS and incubated with 0.1% Triton X-100 for 10 min at RT. Fixed cells were washed in PBS and blocked with 10% AB serum in PBS for 10 min. Coverslips were then incubated with LATS2 anti-rabbit antibody (1:100 in AB serum) and ERα anti-mouse antibody (1:50 in AB serum) for 45 min at RT. Coverslips were then washed with PBS and incubated with Alexa Fluor®-488 secondary anti-rabbit antibody and Alexa Fluor®-555 anti-mouse secondary antibody (Invitrogen, Paisley, UK) at RT for 45 min. DNA was visualised by 4',6-diamidino-2-phenylindole (DAPI) staining. Coverslips were examined on an Axiovert-200 laser canning inverted microscope (Zeiss, Welweyn Garden City, UK), equipped with a confocal imaging system.

Sulphorhodamine B (SRB) assay. Cells were cultured in 96-well plates for 24 h prior to transfection with *LATS2* siRNAs. Cells were fixed on different days after transfection by adding 100 μl/well ice-cold 40% trichloroacetic acid and incubating for 1 h at 4˚C. Plates

were then washed five times in running cold tap water. Cells were stained using 100 μl of 0.4% SRB (Sigma) in 1% acetic acid for 30 min and then washed 5 times in 1% acetic acid. The bound dye was solubilised by adding 100 μl of 10 mM Tris base and the plates were then placed on a rotating platform for 10 min prior to reading absorbance using a spectrophotometer at 488 nm as described elsewhere (48).

Results

LATS2 silencing increases ERα-regulated gene expression. Since *LATS2* down-regulation is frequently seen in breast cancer (44), we examined the effects of *LATS2* silencing on the expression levels of various ERα-regulated genes (*TFF1*, *PGR* and *GREB1*) using RT-qPCR. E_2-deprived MCF-7 cells were transfected with control (CT) or *LATS2* siRNA and were then treated with vehicle (ethanol) or E_2 or for 24 h; subsequently, RT-qPCR was performed. Our results showed that the expressions of *TFF1* and *GREB1* were significantly increased in *LATS2*-silenced cells compared to CT siRNA-treated cells upon E_2 treatment (Figure 1A and C). The expression of *PGR* was also increased in E_2-treated cells but to a lesser extent (Figure 1B). Silencing of the *LATS2* gene was also confirmed by RT-qPCR (Figure 1D). These results indicate a negative regulation of ERα transcriptional activity by *LATS2*.

LATS2 is not an ERα-regulated gene. To investigate whether LATS2 is regulated by ERα, we examined *LATS2* gene expression at different time points after E_2 treatment. MCF-7 cells were E_2-deprived for 48 h and then treated with E_2 for different time periods (0, 3, 6, 12, 24 and 48 hours). The relative *LATS2* mRNA levels were measured using RT-qPCR. *GREB1*, an E_2-induced gene in breast cancer cells (49), was used as a positive control. As expected, upon E_2 treatment, *GREB1* mRNA increased with a maximum peak at 12 h (Figure 1E). In contrast, *LATS2* expression remained relatively constant after E_2 treatment (Figure 1F), suggesting that the *LATS2* gene is not a target of activated ERα.

Effects of LATS2 silencing on mRNA and protein levels of ERα. Based on our observations regarding an increase in *ERα*-regulated gene transcription, we examined whether these results were due to altered mRNA and/or protein levels of ERα. MCF-7 cells transfected with *CT* or *LATS2* siRNA were treated with E_2 or vehicle for 24 h. RT-qPCR analysis revealed an E_2-dependent increase in *ERα* mRNA levels when *LATS2* was silenced compared to *CT* siRNA-treated cells (Figure 2A). Conversely, ERα protein levels were slightly reduced in *LATS2*-silenced cells (Figure 2B).

LATS2 does not modulate ERα activity via phosphorylation of Ser118. We then examined phosphorylation of ERα-Ser118 phosphorylation site, which is already linked to the activity of ERα, by western blot analysis. In all E_2-treated cells an increase of ERα-Ser118 levels was seen (Figure 2B). This suggests that *LATS2* silencing does not affect phosphorylation of this site but could influence other phosphorylation sites. *LATS2* silencing was also confirmed by western blot analysis (Figure 2B).

LATS2 silencing modulates N-CoR protein levels. ERα transcriptional activity is regulated by co-activators and co-repressors as part of multi-protein complexes. To establish if *LATS2* silencing altered the protein levels of any of these co-factors, MCF-7 cells were transfected for 48 h with either CT or *LATS2* siRNA and then treated with ethanol (vehicle) or E_2 for an additional 24-h period. Equal amounts of protein extracts were used for western blot analyses. Membranes were probed with antibodies against known ERα co-activators and repressors. Our results indicate that N-CoR protein levels were significantly reduced in *LATS2*-silenced cells, irrespective of treatment (Figure 2C). NCOA2 levels were higher in E_2-treated cells than vehicle-treated cells (Figure 2C); however, there was no further alteration in *LATS2*-silenced cells. Finally, p300 and mSIN3A were unchanged by *LATS2*-silencing and E_2-treatment (Figure 2C). These findings suggest that LATS2 may selectively affect, directly or indirectly, certain co-factors of ERα.

LATS2 is a nuclear protein which co-localises with ERα. In order to investigate if the effects of LATS2-silencing on ERα transcription could be due to a direct interaction, we performed immunofluorescence microscopy in order to examine the localisation of ERα and LATS2 proteins in MCF-7 cells. It is already known that ERα is a nuclear protein (9). Although LATS2 has been described as a centrosomal protein (34), immunofluorescence analysis revealed that LATS2 is mainly localised in the nucleus of MCF-7 cells. Moreover, based on our results, LATS2 appears to co-localise with ERα in the nucleus (Figure 3), supporting the hypothesis of a direct interaction between these two proteins.

Effects of LATS2 silencing on growth of breast cancer cell lines. LATS2 is involved in both apoptosis and cell-cycle arrest, as previously reported (36-39). Therefore to investigate the effect of *LATS2* silencing on cell proliferation, MCF-7 cells were incubated in complete media, followed by transfection with CT or *LATS2* siRNA for different time periods (1, 3, 5 and 7 days), prior to fixing, SRB staining and detection of cell density. As presented in Figure 4A, silencing of *LATS2* resulted in a reduction in the growth of MCF-7 cells, compared to untreated and to CT siRNA-treated wells. Interestingly, *LATS2* knockdown did not significantly affect the cell proliferation rate of MDA-231, an ERα-negative cell line (Figure 4B).

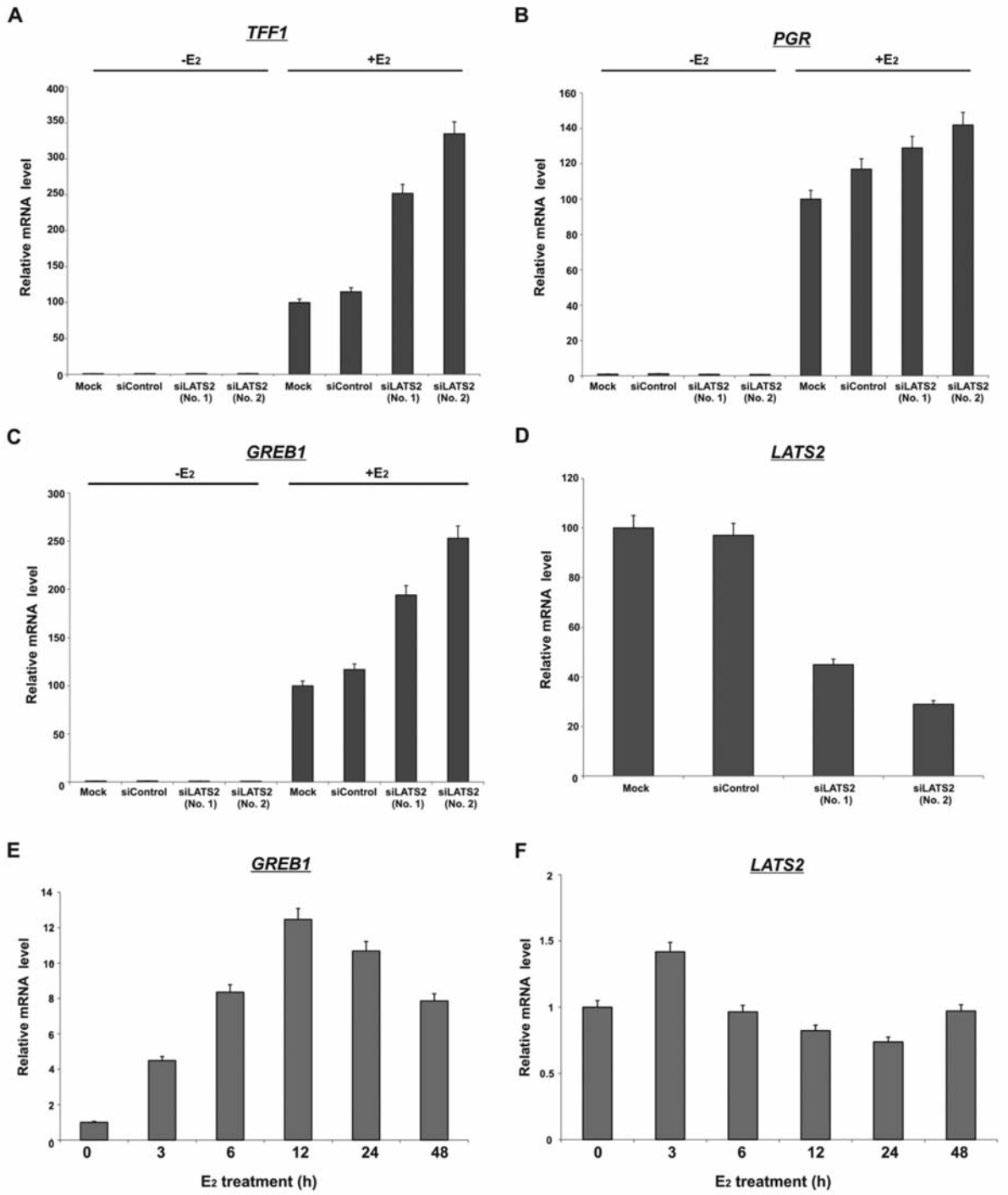

Figure 1. *Real time quantitative-PCR (RT-qPCR) analysis of estrogen receptor alpha (ERα)-regulated genes and large tumour suppressor homolog-2 (LATS2). Expression of ERα-regulated genes after LATS2 silencing. MCF-7 cells (5×10^4) were starved for 48 h and plated in 24-well plates with phenol-red-free dulbecco's modified eagle's medium (DMEM) with 10% double charcoal-stripped serum (DSS). Twenty-four hours later cells were transfected with either 30 nM siControl or siLATS2 and 72 hours later treated with 10 nM E_2 or vehicle (ethanol) for 24 h prior to RNA extraction. RT-qPCR was used to measure trefoil factor 1 (TFF1) (A), progesterone receptor (PGR) (B) and growth regulation by estrogen in breast cancer 1 (GREB1) (C) gene expression. Each gene was assessed in triplicate (100%=mRNA expression after E_2 treatment-alone). D: RT-qPCR validation of down-regulation of LATS2 mRNA levels using two different LATS2 siRNAs. GAPDH was used for normalisation. Error bars represent SD of two experiments, each in triplicate. LATS2 is not an ERα-regulated gene. MCF-7 cells (3×10^5) were plated in 6-well plates with phenol red-free DMEM, containing 10% DSS for 48 h. Cells were treated with 10 nM E_2 for the indicated time periods before total RNA extraction. RT-qPCR analysis was used to measure LATS2 (E) and GREB1 (F) gene expression. GAPDH was used for normalisation. Error bars represent SD of two experiments, each in triplicate.*

LATS2 silencing modulates cell-cycle-related proteins. To investigate the possible causes of the growth arrest observed in *LATS2*-silenced cells, MCF-7 cells were transfected with *CT* or *LATS2* siRNA, treated with E_2 or vehicle and total protein extracted for use in western blot analysis. Our results showed that in *LATS2*-silenced cells there was an increase in p27 and a decreased in cyclin-D1 protein levels compared to untreated and *CT* siRNA-treated cells (Figure 4C). The expression pattern was not dependent on ligand treatment.

Discussion

Phosphorylation of ERα is relevant for regulation of its transcriptional activity (11, 21, 23, 24). In this study, we identified LATS2 as a protein kinase able to modulate E_2-dependent ERα transcriptional activity in breast cancer cells. In E_2-treated MCF-7 cells, *LATS2*-silencing resulted in an increase of certain ERα-regulated genes (*TFF1*, *GREB1*, *PGR*). These data suggest that LATS2 may inhibit the binding of ERα to specific promoter elements of certain ERα-regulated genes. Powzaniuk *et al*. have shown that LATS2 overexpression in prostate cancer cells caused a promoter-specific reduction in androgen receptor (AR)-regulated gene expression. Further investigations using chromatin immunoprecipitation (CHIP) assays revealed that LATS2 was recruited to the regulatory domain of AR-regulated genes and thus acted as an AR co-repressor (40). Therefore, as our results suggested ERα-regulated genes were affected by LATS2-silencing in a specific manner, such additional investigation may provide further evidence regarding the possibility that LATS2 acts as an ERα co-repressor.

After *LATS2*-silencing, ERα mRNA levels slightly increased while protein levels slightly decreased. The observed difference could be explained by post-translational modifications of ERα and/or interactions of ERα with proteins affecting its stability. This may act as an internal control to prevent accumulation of large amounts of ERα protein within cells. In addition, the pattern we observed showed increased ERα transcriptional activation but a decline in the ERα protein level and is well-documented in E_2-dependent ERα activation (50). As upon E_2-binding, ERα degradation is accelerated (51).

As phosphorylation of ERα enhances its transcription, we next examined effects of *LATS2* silencing on the phosphorylation state of ERα. Western blot analysis of ERα -Ser118, the most commonly identified phosphorylation site of ERα (3, 7, 12, 16), did not change after *LATS2* inhibition compared to E_2-treatment-alone. This suggests that *LATS2* silencing does not affect phosphorylation of this particular site.

Taken together, these data suggest that LATS2 may block ERα-regulated gene transcription indirectly by modulating various co-activators or co-repressors associated with the ERα-induced transcriptional machinery (27). Our results indicate that *LATS2* silencing causes no changes in the protein levels of the co-activators NCOA2 or p300. NCOA2 is an ERα-regulated gene (52), and thus, as expected, after E_2 treatment NCOA2 protein levels increased in all cells; however, there was no additional change after *LATS2* silencing. ERα transcription can be repressed at the level of the transcriptional machinery by binding of co-repressors (30). In MCF-7 cells, when *LATS2* was silenced, the protein levels of the co-repressor mSIN3A remained unchanged. However, analysis of NCoR, another co-repressor, revealed a decrease in its protein levels after *LATS2* silencing which was independent of E_2 treatment. This observed reduction could partially explain the increase in ERα-regulated gene expression.

Immunofluorescence microscopy revealed that LATS2 co-localises with ERα in the nucleus of MCF-7 cells. When combined with the above observations, this may suggest that LATS2 interacts with the ERα complex either by modulating co-activators or co-repressors, which play a crucial role in ERα transcriptional competence, or by itself acting as a co-repressor. Alternatively, LATS2 may also be able to affect (directly or indirectly) phosphorylation of ERα at specific sites other than Ser 118, explaining our observed findings.

There has been much research recently regarding the regulation of *LATS2*, including the effects of TTP, microRNA and epigenetic modification (32, 44, 45). Our results indicate that *LATS2* is not an ERα-regulated gene, as the time course experiments with E_2 treatment did not result in any changes in *LATS2* gene expression levels. Therefore the effects of *LATS2* on ERα-regulated gene transcription do not appear to form a feedback loop in MCF-7 cells. Confirmation of these data in other cell lines, using ERα-negative controls, *in vivo* data and clinical correlates, would be required to confirm these findings.

Our results show that silencing of *LATS2* causes a reduction of MCF-7 cell proliferation but does not affect the growth of MDA-231 (ERα-negative cells) to the same degree, suggesting that LATS2 could have proliferative effects in ERα-positive breast cancer cells. A decrease in cell growth reflects changes in one of two process: cell-cycle arrest or apoptosis. LATS2 over expression has been associated with apoptosis and cell-cycle arrest. Li *et al*. describe that LATS2 overexpression causes down-regulation of the cyclin E/CDK2 kinase function (36), which leads to cell-cycle arrest at the G_1/S checkpoint. Furthermore, Aylon *et al*. have identified a novel tetraploidy G_1/S checkpoint in which ectopic LATS2 expression promotes apoptosis in nocodazole-treated cells by directly interacting with MDM2 and preventing it from ubiquitinising p53. This leads to an accumulation of p53 and apoptosis (39). LATS2 overexpression also down-regulated BCL-2 and BCL-xl leading to apoptosis (38). Therefore to elucidate the mechanism of its action on the growth of MCF-7 cells, we investigated several proteins involved in both cell cycle-arrest and apoptosis.

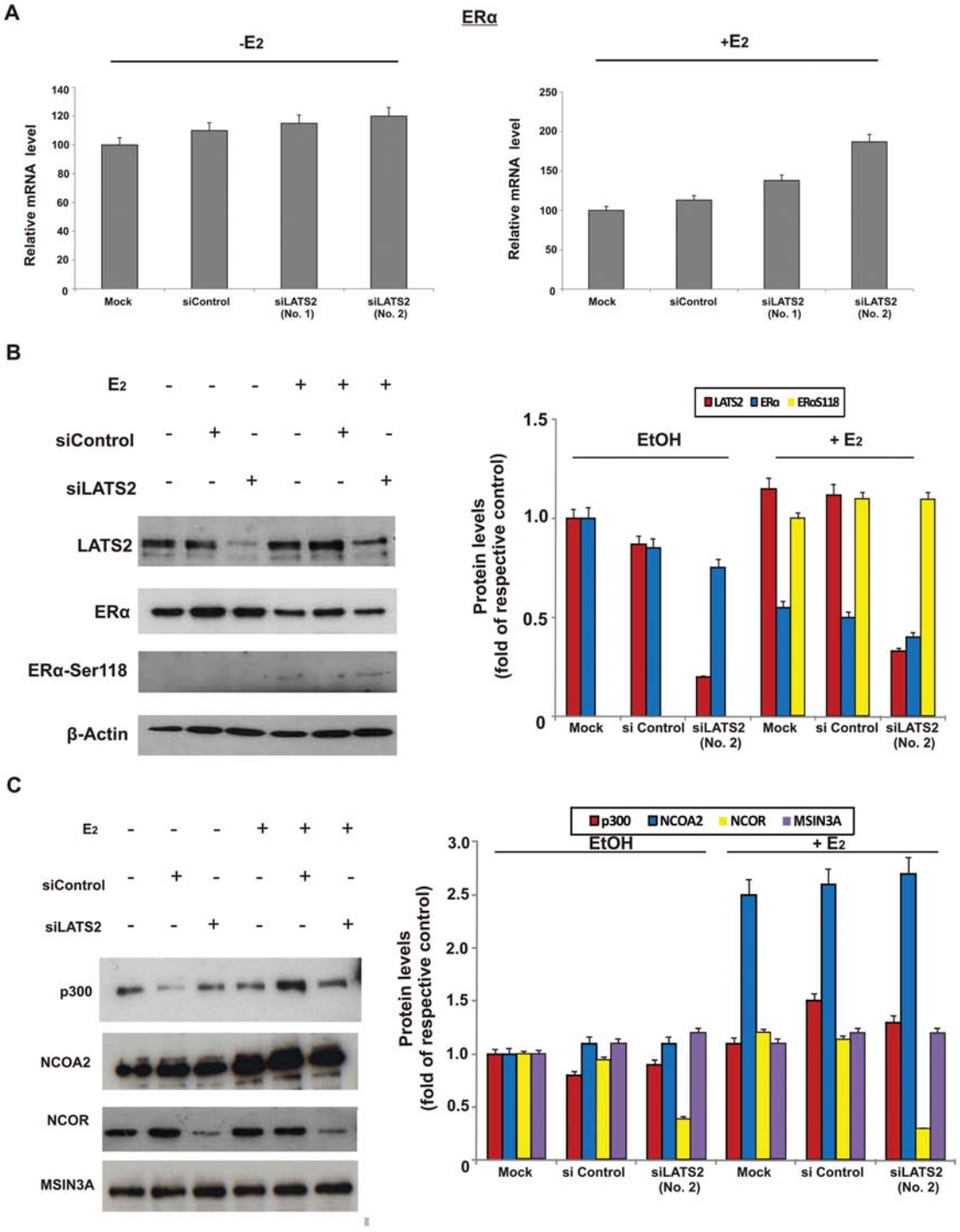

Figure 2. *Effects of large tumour suppressor homolog-2 (LATS2) silencing on mRNA and protein levels of various genes. MCF-7 cells (5×10^4) were plated in 24-well plates in phenol red-free dulbecco's modified eagle's medium (DMEM) with 10% double charcoal-stripped serum (DSS). Cells were transfected with either 30 nM LATS2 siRNAs or siControl siRNA for 72 h and then treated with 10 nM E_2 or vehicle. A: After 24 hours, RNA was extracted and RT-qPCR was performed to measure the ERα mRNA levels. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalisation. Error bars represent the SD of two experiments, each in triplicate. B: MCF-7 cells treated as described above were harvested at 24 h, lysed and equal amounts of protein were used for western blotting analysis using an anti-LATS2, anti-ERα or anti-ERα-Ser118 antibody. C: Western blot analysis was carried out after treatments as described above, using anti-p300, anti-nuclear receptor coactivator-2 (NCOA2), anti-nuclear receptor corepressor (NCoR) and anti-transcriptional corepressor Sin 3A (MSIN3A) antibodies. β-Actin was used as a loading control. Quantitative analysis of protein levels is given as fold of the loading control. Error bars represent SD of two experiments.*

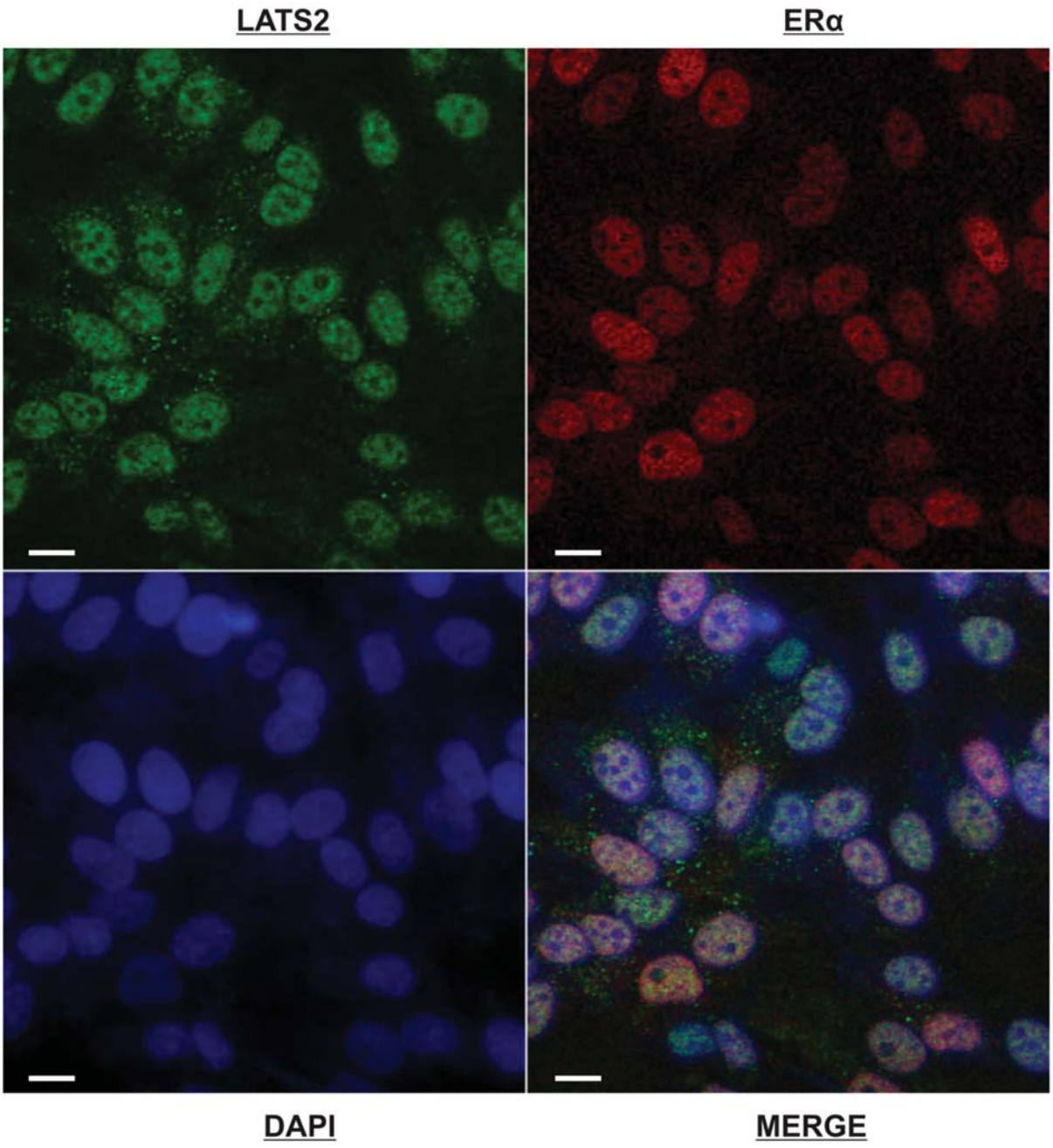

Figure 3. *Examination of large tumour suppressor homolog-2 (LATS2) localisation. MCF-7 cells (1×10^6) were plated in 6-well plates on poly-D-lysine-coated glass coverslips in phenol red-free dulbecco's modified eagle's medium (DMEM) with 10% double charcoal-stripped serum (DSS). Cells were treated with 10 nM E_2 for 24 h and fixed. Immunofluorescence microscopy was performed on MCF-7 cells stained with antibodies against estrogen receptor alpha (ERα) or large tumour suppressor homolog-2 (LATS2) followed by Alexa Fluor®-488 or -555 secondary antibodies. 4',6-diamidino-2-phenylindole (DAPI) staining was used to visualise the nuclei. The scale bar represents 10 μm.*

We showed that both cyclin-D1 and cyclin-A (data not shown) expressions decreased when *LATS2* is silenced. Cyclin-D1 is involved in a complex with CDK4/6 at the G_1/S checkpoint and cyclin-A/CDK2 at the start of the S phase. Therefore a decrease in the levels of these proteins would cause cell-cycle arrest and explain the reduction in proliferation. Additionally, p27, a member of the key intermediary protein (KIP) family of CDK inhibitors, was also increased in *LATS2*-silenced cells. p27 inhibits cyclin-D1 CDK4/6 and cyclin-A CDK2 kinase activity (53),

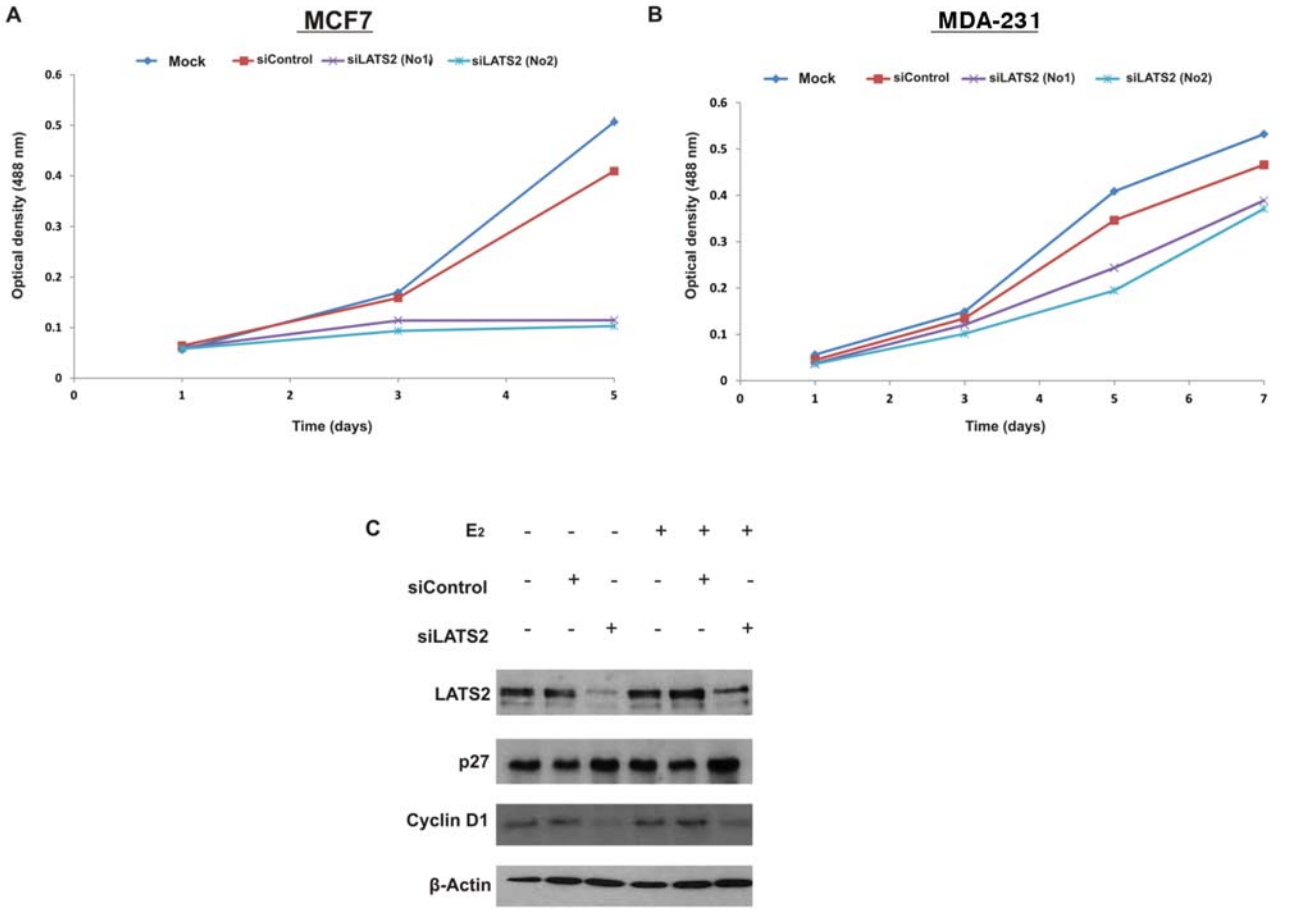

Figure 4. *Effects of large tumour suppressor homolog-2 (LATS2) silencing on cell proliferation and cell-cycle proteins. MCF-7 (3×10^3) (A) and MDA-231 (2×10^3) (B) cells were plated into 96-well plates in dulbecco's modified eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Cells were transfected with 30 nM siControl or siLATS2 for the indicated time periods before being fixed and stained with Sulphorhodamine B (SRB). Optical densities of the cells were read by spectrophotometry at 488 nm. The optical density represents the mean of 5 replicates for each condition minus the background reading. C: MCF-7 cells were starved of E_2 for 48 h prior to transfection with either 30 nM siControl or siLATS2 for 72 h. Cells were then treated with 10 nM E_2 for 24 h, prior to harvesting cells, lysing and protein quantification. Equal amounts of proteins were used for western blot analysis using antibodies against p27 and cyclin-D1.*

providing a further mechanism by which *LATS2* silencing reduces cyclin-D1 and thus reduces proliferation. These checkpoints are relevant because they prevent DNA-damaged cells from undergoing uncontrolled proliferation (39). Therefore, LATS2 may play a role in the prevention of uncontrolled cell growth, which is characteristic of cancer.

Many chemotherapeutic drugs are cell-cycle phase-specific. G_1/S arrest causes cells to be held in this specific phase. As *LATS2* silencing caused G_1/S arrest and *LATS2* is frequently down-regulated in breast cancer (44), this information could assist in selecting chemotherapy which is most appropriate to the *LATS2* status of an individual (54). This could improve efficacy as well as reducing unnecessary treatment. BCL-2 is an antiapoptotic protein that prevents apoptosis *via* the intrinsic pathway (55). BCL-2 is normally expressed in breast tissue (56), resulting in a tendency towards apoptosis in cells. Our results also showed that BCL-2 was reduced in E_2-treated *LATS2*-silenced cells (data not shown). This reduction would increase apoptosis of MCF-7 cells and thus provide an alternative pathway by which *LATS2* may exert its effects on MCF-7 cell proliferation. It has also been suggested that BCL-2 is an E_2-regulated gene (54). This adds further support to the hypothesis that *LATS2* differentially regulates certain ERα-regulated genes. Furthermore, cyclin-D1 is also an ERα-regulated gene (57), again supporting this hypothesis. However, cyclin-D1 is also involved in ERα transcriptional activation (58), acting as a bridge between the activation function-2 (AF-2) and SRC-1, a co-activator (17). Therefore, given the complexity of the multiple

pathways potentially involved, there is strong evidence that further research into the interactions between LATS2 and cyclin-D1 is required.

In conclusion, in MCF-7 cells *LATS2* silencing modulates ERα-regulated gene expression. It is likely that these effects are controlled through interactions with co-repressors such as NCoR, or through direct interaction with the ERα. In addition, *LATS2* silencing reduces proliferation of MCF-7 cells. *LATS2* silencing causes a decrease in cyclin D1 and cyclin A expression, as well as increases in p27, which results in reduced cell proliferation due to cell-cycle arrest at the G_1/S phase. These changes may be important due to the fact that this kind of inhibition would prove useful in arresting ERα-positive cancer cells within tumors while not affecting normal surrounding cells, where this abrogated growth due to *LATS2* silencing is unlikely to be seen. However, further research into the kinase function of LATS2 is vital for a complete understanding of its actions on ERα and its co-factors.

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