Published in final edited form as:

Oncogene. 2015 January 8; 34(2): 154–164. doi:10.1038/onc.2013.550.

Systems level-based RNAi screening by high content analysis identifies UBR5 as a regulator of estrogen receptor- α protein levels and activity

MJ Bolt, F Stossi, AM Callison, MG Mancini, R Dandekar, and MA Mancini

Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA

Abstract

Estrogen receptor- α (ER α) is a central transcription factor that regulates mammary gland physiology and a key driver in breast cancer. In the present study, we aimed to identify novel modulators of ER α -mediated transcriptional regulation via a custom-built siRNA library screen. This screen was directed against a variety of coregulators, transcription modifiers, signaling molecules and DNA damage response proteins. By utilizing a microscopy-based, multi-end point, estrogen responsive biosensor cell line platform, the primary screen identified a wide range of factors that altered ER α protein levels, chromatin remodeling and mRNA output. We then focused on UBR5, a ubiquitin ligase and known oncogene that modulates ER α protein levels and transcriptional output. Finally, we demonstrated that UBR5 also affects endogenous ER α target genes and E2-mediated cell proliferation in breast cancer cells. In conclusion, our multi-end point RNAi screen identified novel modulators of ER α levels and activity, and provided a robust systems level view of factors involved in mechanisms of nuclear receptor action and pathophysiology. Utilizing a high throughput RNAi screening approach we identified UBR5, a protein commonly amplified in breast cancer, as a novel regulator of ER α protein levels and transcriptional activity.

Keywords

estrogen receptor; UBR5; high content screening; high throughput microscopy; ubiquitin ligase

INTRODUCTION

Estrogen receptor- α (ER α) is expressed in 70% of breast cancers,^{1,2} where it modulates several tumorigenic processes through its transcription factor activity.³ Upon ligand binding, ER α dimerizes and binds to estrogen response elements throughout the genome recruiting histone modifying enzymes and coregulators that change chromatin structure allowing for transcriptional regulation. ER α induction of oncogenes, such as c-Myc, drives cell proliferation and tumorigenesis.⁴ Therapeutics that target ER α activation, either through

Correspondence: Dr MA Mancini, Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, DeBakey Building, Room 132C, Houston, TX 77030, USA. mancini@bcm.edu.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

Supplementary Information accompanies this paper on the *Oncogene* website (<http://www.nature.com/onc>)

antagonism or degradation,⁵ are the main defense against breast cancer. However, these therapies are only effective in ~70% of cases (ER+), of which ~40% develop resistance over time.^{5,6} One additional way to target ER α would be to decrease the ER α protein level through inhibition of upstream factors that control mRNA production and/or protein stability; or to restore ER α protein in ER- tumors, which could then be targeted with anti-ER α therapies.

While ER α function has many important links to pathophysiological conditions, the proteins that regulate ER α levels remain understudied. ER α mRNA is differentially regulated in various tissues/cell types: by FOXA1 in breast tissue,⁷ the leptin pathway in chondrocytes,⁸ ER β in uterine stromal cells and ER α autoregulation in MCF-7 breast cancer cells.⁹ At the protein level, ER α turnover through proteasomal degradation via E6-AP ubiquitylation,¹⁰ or the Skp, Cullin, F-box complex,¹¹ has been shown to be necessary for transactivation, while turnover through STUB1 controls its steady-state levels.¹² High throughput screening to define modulators of ER α levels and other mechanistic steps in disparate biochemical or cell-based assays can be useful for identifying novel therapeutic targets and diagnostic/prognostic avenues. Previous RNAi screens focused upon defining signaling pathways that affect ER-mediated transcription,¹³ estrogen-independent growth¹⁴ or tamoxifen resistance,¹⁵ but have not yet carefully examined proteins that regulate ER α levels, especially in single cell-based assays. Determining which factors modulate ER α protein levels, without changing its mRNA, could have direct implications in a subset of breast tumors that have been shown to express ER α mRNA, but not protein, possibly due to a hyperactivation of the c-Src pathway.

We have previously described an estrogen receptor biosensor cell line (GFP-ER α : PRL-HeLa, hereafter referred to as PRL array cells)¹⁶ that has been utilized to study a variety of mechanistic aspects of ER α -mediated transcription: ligands that cause ER α activation,^{17,18} functions of ER α domains,¹⁹ and ER α interplay with other transcription factors on chromatin.²⁰ It is important to note that virtually all RNAi screens, including those referenced above, have been based upon single end point assays. In contrast, the PRL array system allows us to further our analysis of ER α -mediated transcriptional regulation since we can measure, simultaneously, several mechanistically relevant end points (ER α protein levels and nuclear translocation, DNA binding, chromatin remodeling and reporter gene transcriptional output). This approach is possible due to our ability to perform high throughput microscopy coupled with automated image analysis (for example, high content analysis/high content screening). Utilizing a custom designed RNAi library containing 281 selected transcriptional modulators and coregulators identified by the Nuclear Receptor Signaling Atlas (www.nursa.org; R Lanz), we interrogated the effects of their knockdown on 17- β estradiol (E2)-activated ER α nuclear levels, ER α SRC-3 and RNA polymerase II recruitment to the PRL array, chromatin remodeling and transcriptional output.

The success of the primary screen was confirmed by the identification of a series of known ER α coregulators, including PPARGC1A (PGC1 α)²¹ and MED1.²² We further chose to validate two hits that were not previously known to interplay with ER α , UBR5 and Supt5H, which we found to oppositely modulate ER α protein levels, chromatin remodeling and transcriptional activation. We then further focused upon UBR5 and showed that knockdown

and overexpression oppositely modulate ER α levels and activity in MCF-7 breast cancer cells, in terms of cell proliferation and target gene regulation. The comprehensive and mechanistic data presented in this novel high content analysis-based study advances the knowledge of ER α protein level regulation and, additionally, provides a systems biology analysis of ER α transcription activation that includes identification of a new, prognostically relevant, target protein important in ER α -positive cancers.

RESULTS

A custom RNAi screen of selected coregulators identifies modulators of ER α protein level, chromatin remodeling and transcriptional output

To define novel factors that affect various stages of ER α -mediated transcription regulation, we utilized the previously characterized PRL-HeLa array cell line stably expressing GFP-ER α (PRL array, described thoroughly in Sharp *et al.*¹⁶). Briefly, we engineered HeLa cells with a stably integrated $\sim 100 \times$ repeat of the estrogen responsive prolactin enhancer/promoter regulatory unit controlling a dsRED2 reporter gene, and also stably expressing GFP-ER α . This model cell line allows visualization and quantitation of multiple mechanisms linked to ER α transcription regulation. For example, the array area measurement is used as an indicator of chromatin remodeling, as agonists induce larger arrays than antagonists. The ER α nuclear and array intensities are a measure of total ER α protein in the nucleus and loaded on the array, respectively, providing a measure of how knockdowns will affect total levels of ER α and the ability of ER α to bind the response elements within the array. We also utilized the RNA FISH array intensity measure to quantify reporter gene mRNA output from the transcriptional array.

To perform the screen, PRL array cells were reverse transfected for 72 h with a custom library of siRNA pools against 281 putative transcription modulators (for full list of targeted proteins, see Supplementary Table 1). Each siRNA pool (consisting of three separate siRNAs to each target) transfection was performed in quadruplicate wells of a 384 multiwell plate, for 72 h, and the cells were then treated with 10 nM E2 for 30 min (Figure 1a), then processed for dsRED2 mRNA FISH (Stellaris, Biosearch Technologies, Novato, CA, USA). Array measurements were replicated three times, while the mRNA FISH experiment was performed once, but was then repeated for the identified hits. The cells were imaged on either an IC-200 (Vala Sciences, San Diego, CA, USA) or an In Cell 6000 (GE Healthcare, Issaquah, WA, USA) high throughput microscope; subsequently, data were analyzed utilizing customized workflows developed on a PipelinePilot (Accelrys, San Diego, CA, USA) image analysis software platform.¹⁷ Standard measurements included ER α nuclear fluorescence intensity, PRL array fluorescence intensity and area, total dsRED2 mRNA FISH signal at the array (total FISH Array intensity) and cell number (Figure 1b, see Materials and methods). As expected, utilizing POLR2A siRNA as a control for knockdown efficiency and toxicity (for example, loss of POLR2A leads to cell death²³), we saw a large decrease in cell number when compared with the non-targeting control siRNA (Figure 1c). The effect of POLR2A siRNA was confirmed across all the experimental plates, with an average Z -score of 0.615, suggesting excellent assay quality in terms of siRNA knockdown efficiency and plate-to-plate variation (Figure 1d). To remove the possibility of cell toxicity

interfering with *bona fide* coregulator action, we excluded from further analysis all siRNAs that caused an >30% decrease (Z -score of -6 compared with the mean of the control siRNA) in cell number compared with control siRNA in at least two of the assays (Figure 1e, dotted line). The full list of toxic siRNAs is presented in Supplementary Table 2.

A positive 'hit' for each measured output was defined as an siRNA that caused Z -score ± 2 away from the mean of the non-targeting siRNA control (Figures 2a–c). In this primary screen, we simultaneously evaluated ER α nuclear intensity, ER α array intensity, array area and dsRED2 mRNA FISH intensity at the array as mechanistic end points, with cell number serving for a measure of toxicity. Several general observations were readily apparent. First, utilizing Pearson's correlation coefficient, we observed a significant correlation ($r = 0.88$) between the siRNA hits affecting ER α nuclear intensity and ER α array intensity (Supplementary Figure 1A), suggesting that none of the siRNAs in this library have the ability to specifically block ER α DNA binding to the PRL array. Figure 2a shows all siRNAs and their effects on ER α nuclear intensity with the purple box signifying a Z -score of ± 1 from the mean of the control and the pink box being up to Z -score ± 2 away. Indicated in the figure are the names of siRNAs having the largest effect on each of the selected features. There were six siRNAs affecting ER α nuclear intensity (three reduced and three increased). The ER α array intensity (Figure 2b) was influenced by 21 siRNAs (16 reduced and 5 increased). The array area measurement, which is linked to chromatin remodeling (Figure 2c), was modulated by 13 factors (9 reduced and 4 increased); while total mRNA FISH intensity (Figure 2d) was the most affected by knockdowns (24 repressed and 5 induced). We then compared all of the hits that caused a greater than two standard deviation reduction (Figure 2e) or increase (Figure 2f) in each feature. Although we observed siRNAs that affect all three categories simultaneously (for example, Supt5H), there are some siRNAs that affect only select features. For example, MED1 siRNA affected array area and mRNA FISH whereas UBR5 siRNA induced ER α and reporter gene mRNA levels, without significantly altering array area.

Several well-established ER α coregulators were present in the screen and caused changes to RNA output in the PRL system. For example, PPARGC1A (PGC1 α) is a well-known coactivator of ER α in ovarian and cervical cell lines and its knockdown led to significant decreases in array area and mRNA FISH (Figure 3). However, individual knockdown of other known ER α coactivators, including SRC-1, SRC-2 and SRC-3, did not pass the filter of Z -score ± 2 applied to the data set. Individual SRC knockdowns did have quantifiable effects (~10–20% reduction) on ER nuclear intensity, array area and RNA FISH, with SRC-1 causing the greatest loss of reporter gene mRNA levels (35% reduction). The lack of maximal effects of the p160 coactivators in this system is not surprising, as our previous manuscript has shown compensation between at least SRC-2 and SRC-3 in regards to recruiting other TFs and serine-5 phosphorylation of RNA polymerase II at the PRL array locus.²⁰ Compensation has also been reported for gene-specific regulation of ER α targets by SRC-1, SRC-2 and SRC-3.²⁴ This is also not surprising data as it has been suggested previously that the regulation of prolactin by ER α is mainly mediated by SRC-1 in pituitary cells.^{25,26}

An additional interesting observation from the screen was the extensive and factor-specific effect of knockdown of single components of the mediator complex. Mediator has been shown to have central roles in nuclear receptor-mediated transcriptional regulation especially through direct association between nuclear receptor and specific subunits, including MED1,^{22,27} THRAP1 (MED13), THRAP4 (MED24), THRAP5 (MED16) and THRAP6 (MED30). The mediator complex is viewed as a four module scaffold composed of head, middle, tail and CDK substructures.^{28,29} The results of mediator knockdowns are displayed in Supplementary Figure 2A, with MED1 (middle), THRAP1 (kinase), THRAP5 (tail) and THRAP6 (middle) all having varying effects on all the features measured while THRAP4 (middle) had no effect. MED1, THRAP1 and THRAP6 knockdown all caused a decrease in ER α nuclear intensity, array area and RNA FISH; whereas THRAP5 knockdown caused an increase in all three features, indicating differential regulation by members of the mediator complex. Further, rather than the usual approach of running disparate single point assays in parallel (or sequentially), the simultaneous multiplexing performed here provides a more integrated data set that offers a systems level view of ER α functions.

Analysis of factors that affect RNA FISH, RNA Polymerase II phosphorylation and/or SRC-3 recruitment

To delve deeper into the mechanism of action of hits from the primary screen that affected E2-stimulated mRNA FISH, we performed additional knockdown experiments in the PRL array cells measuring effects upon array recruitment of RNA Polymerase II (Pol II) that was phosphorylated at either serine-5 (S5P) or serine-2 (S2P), or SRC-3 (Figure 3). The activating post-translational modifications on the CTD of Pol II are known for representing transcription initiation (S5P³⁰) and transcription elongation (S2P³¹), while SRC-3 is a well-studied ER α coactivator that is recruited to many ER α enhancers including the PRL array.^{20,32} For a more complete picture of the mechanism of action of the hits from the primary screen, we combined results from these additional experiments with the features already described in Figure 2. We employed unsupervised clustering using Z-score from the mean for each feature as a measure. The majority of hits affecting mRNA FISH output also showed an effect on S5P or S2P, with only select siRNAs (transferrin, MED1 and ST13) affecting SRC-3 intensity at the array. This analysis allowed us to see different actions of the primary hits with regard to how they affected transcription. For example, UBR5 siRNA increased ER α levels, array area, mRNA FISH intensity, SRC-3 recruitment and transcription elongation (S2P), whereas transferrin affected ER α levels, array area, mRNA FISH intensity, but specifically increased transcription initiation (S5P), and not elongation (S2P) or SRC-3 recruitment. We also observed a similar difference in the repressive hits where Supt5H reduces all features except S2P, with the knockdown of THRAP1 reducing all features except S5P and SRC-3. A few siRNAs reduced all features including ST13 (an HSP70/HSP90 adapter protein³³) and C17ORF31 (a telomere-associated protein³⁴) suggesting that while the overall end point result is similar, the mechanism leading to the change is different (for example, decreased ER α levels vs reduced chromatin remodeling vs change to Pol II phosphorylation).

Validation of UBR5 and Supt5H as modifiers of ER α protein levels We chose to further validate and explore two hits from the primary screen, UBR5 and Supt5H, which showed

opposite effects on ER α levels and mRNA FISH output. Supt5H is a transcription initiation factor component of the DRB sensitivity-inducing factor complex and has a role in transcription initiation.³⁵ UBR5, on the other hand, is an E3 ubiquitin ligase³⁶ that has roles in cell cycle and DNA damage response. It has been shown to suppress ubiquitinylation of histones near damaged DNA³⁷ and is a known coactivator of the progesterone receptor.³⁸ As a first validation step we used different sets of siRNAs in the PRL array cells, which also led to a loss of ER α nuclear intensity (images in Supplementary Figure 1C), array area and mRNA FISH intensity for Supt5H (Figure 4a), and an increase in ER α nuclear intensity, array area and mRNA FISH intensity for UBR5 (Figure 4b). We also found these changes (ER α intensity increases with UBR5 knockdown and ER α intensity decreases with Supt5H knockdown) to occur regardless of ligand (Supplementary Figures 2A and B; E2, 4-hydroxytamoxifen (Tam), fulvestrant (Ful)).

We next wanted to determine whether the observed effects were specific to ER α . We performed siRNA knockdown of Supt5H (Figure 4c) or UBR5 (Figure 4d) in the PRL array cells and then immunolabeled for glucocorticoid receptor (GR) and SRC-3. Following knockdown of Supt5H or UBR5, we saw no significant gain or loss of either of these proteins, suggesting a specific effect of these proteins on ER α . The knockdown efficiency, as measured by IF, approximates 65% for Supt5H (Figure 4e) and 45% for UBR5 (Figure 4f). By quantifying these changes on a single cell basis, we can record a continuum of analysis based on the knockdown efficiency; and by doing so we observed a marked shift in the population between control siRNA vs Supt5H (Supplementary Figure 2C) or UBR5 (Supplementary Figure 2D) that is followed by changes in the ER α protein levels. We also confirmed knockdown of UBR5 and Supt5H in PRL array cells via western blotting (Figure 4g). Collectively, this type of analysis and resultant data suggest a specific role for both Supt5H and UBR5 in the regulation of ER α protein levels.

Next, we also wanted to determine whether these proteins were components of the ER α transcription complex in the PRL array cell line. To do this, we treated PRL array cells with 10 nM E2 for 30 min and then immunolabeled with antibodies against Supt5H (Supplementary Figure 2E) or UBR5 (Supplementary Figure 2F). Interestingly, neither Supt5H nor UBR5 localized to the PRL array with ER α suggesting that their control over ER α levels does not occur at the PRL reporter gene locus.

UBR5 knockdown modulates ER α , but not GR or SRC-3 levels in MCF-7 cells

Due to the importance of ubiquitin ligases in ER α -mediated transcription and protein level modulation, we performed additional follow-up studies with UBR5. As shown in Figure 4g, immunoblotting showed that a loss of UBR5 led to an increase in ER α levels. By design, ER α expression in PRL-HeLa cells is driven by a CMV containing promoter; further, the protein is GFP tagged. As such, we sought to determine whether UBR5 knockdown would also affect endogenously expressed ER α . We therefore performed UBR5 siRNA knockdown in MCF-7 breast cancer cells, and then immunolabeled them with antibodies to ER α , GR or SRC-3. Similar to results from the PRL array cell line, MCF-7 cells also showed an increase in ER α intensity with UBR5 knockdown, but no changes in GR or SRC-3 intensity (Figure 5a). We then validated this result by western blotting (Figure 5b). We further validated the

effect of UBR5 knockdown on ER α in T47D cells (Supplementary Figure 2G). Collectively, these data confirmed our RNAi screening results in the PRL-HeLa cells and suggest that the changes in ER α level exerted by UBR5 are not dependent upon cell type, CMV promoter or GFP tag.

Next, in order to determine whether this new ER α modulator has translational relevance, we utilized the cBio Cancer Genomics Portal,³⁹ which allows a user to query a specific gene, or set of genes, and determine their alterations (that is, amplifications, deletions and mutations) across a variety of cancer genomics data sets. Interestingly, we observed that alterations in the UBR5 locus occur in many breast tumors (~30%) and are a prognostic factor for a poor outlook (Supplementary Figure 3A), especially in the luminal A subtype (Figure 5c) compared with the other subtypes (Supplementary Figure 3B–D). These data were also confirmed by querying the TCGA database.⁴⁰

UBR5 modulates ER α -induced gene expression and proliferation through its ubiquitin ligase activity

We next sought to determine whether UBR5 ubiquitin ligase activity was required for modulation of ER α levels. As such, we overexpressed Flag-tagged UBR5 or Flag-tagged UBR5 containing a C2768A point mutation that disrupts its ubiquitin ligase activity³⁸ in MCF-7 cells. Using immunofluorescence combined with single cell analysis, overexpression of Flag-UBR5 was shown to greatly reduce ER α protein levels (Figure 5d), and overexpression of the inactive UBR5 mutant caused no change when compared with non-transfected cells. These results confirmed the effect of UBR5 as a negative regulator of ER α protein levels and indicated a mechanistic dependence for ubiquitin ligase activity.

After exploring the effects of UBR5 modulation of ER α protein level, we wanted to determine whether it also affected ESR1 mRNA in MCF-7. Upon UBR5 knockdown, we observed an ~50% decrease in ESR1 mRNA (Figure 6a) which is consistent with the previously reported⁹ observation that ER α auto-regulates its own expression by binding to its gene locus and recruiting a Sin3A containing repressor complex. To determine whether this mechanism could occur also in this scenario, we performed chromatin immunoprecipitation of ER α after control or UBR5 siRNA transfection. As shown in Figure 6b, upon UBR5 depletion, ER α occupancy at the ESR1 locus does increase in a ligand-independent manner.

We then wanted to determine whether the increase in ER α protein upon UBR5 knockdown lead to changes in transcriptional activation of an endogenous ER target gene. We quantified the E2-induced expression of GREB1 mRNA, a well-known ER α target gene, by qPCR. Knockdown of UBR5 (Figure 6c) resulted in an increased induction of GREB1 in cells treated with 10 nM E2 without changes in the basal mRNA level, thus suggesting a possible role for UBR5 functioning as a rheostat that controls the magnitude of ER α responses.

To test whether UBR5 also impacted E2-induced cell proliferation, we performed EdU pulse labeling (~10 min), which captures cells in S phase of the cell cycle. Here, MCF-7 cells were treated with 10 nM E2 for 24 h following knockdown of UBR5. Figure 6d shows that, in control siRNA samples, ~23% of cells were EdU positive; however, UBR5 knockdown

resulted in an >50% increase in EdU-positive cells (~35%). These data were confirmed by monitoring the mRNA expression of Cyclin D1, another ER α target gene, which showed a marked increase in E2 stimulation with UBR5 knockdown compared with control (Figure 6e). This increase in proliferation upon loss of UBR5 was not recapitulated in the ER α -negative MDA-MB-231 cell line (Figure 6f). These data provide further evidence that regulation of ER α levels by UBR5 lead to downstream effects on ER α signaling, thus providing us with new possible prognostic and therapeutic targets for ER α -mediated disease.

DISCUSSION

ER α is a key transcription factor in many physiological processes including breast and bone development.^{41,42} Alterations in ER α signaling are observed in many pathophysiological conditions including breast cancer and osteoporosis. While ER α is expressed in 70% of breast cancers,^{1,2} it is a protein coregulator that aids in the modulation of transcriptional regulation and can be amplified in disease.^{43,44} It is for this reason that we set out to utilize our PRL array system, which can be used to simultaneously observe many mechanistic steps of ER α -mediated transcriptional activation,^{17,19,45} and identify those factors that mediate ER levels and activity.

In our primary screen using a custom RNAi library containing 281 putative transcription regulators (see Supplementary Table 1) we confirmed the role for a variety of known ER α coregulators in our model system, including PPARGC1A (PGC1 α) and MED1. PGC1 α has been shown to coactivate ER α through the ER α hinge region.^{21,46–48} Our screen identified five other subunits of the Mediator complex, four of which showed effects in the PRL array line when knocked down. The only mediator subunit not to affect ER activity was THRAP4 (MED24), whereas MED1, THRAP1 (MED13), THRAP5 (MED16) and THRAP6 (MED30) all showed robust effects. Although mediator components are more known for their role in RNA Polymerase II recruitment, we observed that two of the mediators (MED1 and MED16) have larger effects upon array area. These data agree with a recent report suggesting that these proteins can interact with long, non-coding RNAs to affect chromatin remodeling.⁴⁹ Interestingly, while the loss of the majority of the mediator proteins caused a decrease in transcription, MED16 knockdown increased reporter activity, suggesting that its presence may actually have an overall repressive effect on transcription. Of note, MED16 repression on transcription has been reported previously on ribosomal protein mRNAs.⁵⁰ We also found a potentially interesting modulator of ER α -mediated transcription in FTH1, the cellular iron storage protein, whose knockdown led to decreased array area and dsRED2 mRNA production. These findings help confirm that our screen can identify known coregulators and, simultaneously, can also efficiently ascribe certain mechanistic details about their *modus operandi*.

To validate and extend the results from our RNAi screen, we chose two proteins, Supt5H and UBR5, whose reduction showed opposite effects on all ER α features; further, we then focused deeper on UBR5, an E3 ubiquitin ligase reported to play roles in cell cycle,⁵¹ DNA damage repair pathways,³⁷ and known to ubiquitylate PEPCK³⁶ and CDK9,⁵² which we identify as a novel negative prognostic factor in breast cancer. Our results are the first linking UBR5 to ER α protein regulation, although it has been shown to regulate the activity

of the progesterone receptor.³⁸ It is known that other ubiquitin ligase family members (for example, E6AP⁵³) can ubiquitinyrate ER α after Src activation leading to an increase in both protein turnover and activity upon E2 treatment.¹⁰ It is commonly thought that this activation is through mono-ubiquitinylation of K302/303,⁵⁴ whereas polyubiquitinylation of unliganded ER α leads to degradation.^{55,56} This ubiquitinylation event is different upon fulvestrant treatment that causes ER α localization to nuclear intermediate filaments followed by ubiquitinylation and subsequent degradation.⁵⁷ Interestingly, UBR5 knockdown showed increases in ER α levels independent of ligand, providing further evidence that it can regulate steady-state levels of the protein.

The identification of UBR5 as a potential novel ubiquitin ligase for ER α is of clinical relevance since *UBR5* gene is located in a region of the genome (8q22) amplified in ~30% of breast cancers,^{58,59} providing a possible mechanism for ER α protein-negative, ER α -mRNA-positive tumors. Furthering the results in the PRL array model, we showed a loss of UBR5 in MCF-7 resulted in an increase in ER α transcriptional activity and proliferative capabilities. We also demonstrate that increased UBR5 levels lead to a decrease in ER α protein and that this is dependent upon an active ubiquitin ligase domain in UBR5. These findings open the potential for new drug screening and therapeutic options for breast cancer subtypes linked to UBR5 expression.

In conclusion, our highly multiplex and mechanism-oriented RNAi screen has provided an expanded view of known and novel factors that impinge upon ER α -mediated transcriptional regulation. Furthermore, the robustness and the translatability of the results obtained from the PRL array platform will permit us to explore additional RNAi libraries or specific small molecule inhibitors to identify novel mediators and pathways affecting ER actions. For example, ER α is well known to be under control of a variety of kinase signaling pathways but interpreting the effects of these pathways upon the ER 'complexosome' is complicated by disparate experimental approaches. Utilization of a highly multiplexed siRNA screen to the human kinome will help elucidate some of these mechanisms, while expanding the approach to a genome-wide siRNA will provide a global systems level view of proteins that can influence ER α -mediated transcription. These and other approaches are poised to define further prognostic and therapeutic targets for ER α + diseases.

MATERIALS AND METHODS

RNAi screen, cell culture and transfections

GFP-ER α :PRL-HeLa and MCF-7 cells were maintained as previously described.²⁰ For the RNAi screen, GFP-ER α :PRL-HeLa cells were plated in 5% stripped-dialyzed FBS, phenol red-free DMEM onto Aurora 384-well plastic bottom plates which had been seeded with Lipofectamine RNAiMax reagent (1:50 dilution) and 40 nM siRNA duplex. Cells were incubated for 72 h followed by treatment with 10 nM E2 for 30 min. The cells were then processed for antibody or mRNA FISH using a Beckman Coulter Biomek FX robot (Beckman-Coulter, Brea, CA, USA).

siRNA transfection in MCF-7 cells was done as mentioned above using a custom Stealth siRNA library (a kind gift from Invitrogen, Carlsbad, CA, USA).

For immunolabeling, we followed the same protocol as in Bolt *et al.*²⁰ using anti-GR (GeneTex, Irvine, CA, USA, GTX101120), ER α (Millipore, Billerica, MA, USA, 04-820), Ser5-phospho RNA Polymerase II (ABCAM (Cambridge, MA, USA, ab5401), Ser2-phospho RNA Polymerase II (ABCAM, ab5095), Supt5H (ABCAM, ab126592), UBR5 (ABCAM, ab70311), SRC-3 (BD Transduction Labs, San Jose, CA, USA, # 611105), Alexa Fluor 647 goat anti-rabbit IgG or Alexa Fluor 647 goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA).

For mRNA FISH, cells were fixed in 4% formaldehyde in RNase-free PBS for 15 min and then permeabilized with 70% ethanol in RNase-free water at 4 °C for 1 h. Cells were washed in 1 ml wash buffer (2 $\times$ SSC, 10% formamide) followed by hybridization in hybridization buffer (comprises 1 g dextran sulfate, 1 ml 20 $\times$ SCC buffer, 1 ml formamide, 8 ml nuclease-free water) with RNA probes (dsRED2 Stellaris probes; Biosearch Technologies Inc) for 4 h at 37 °C followed by one wash in wash buffer for 30 min at 37 °C and then DAPI staining for 10 min at 37 °C. Cells were held and imaged in 2 $\times$ SCC buffer. GFP-UBR5, pCMV-Tag2B-UBR5 and pCMV-Tag2B-UBR5 C2768A have been characterized previously.³⁸

Imaging and quantification

Automated imaging was carried out using an IC-200 image cytometer (Vala Sciences) or an In Cell 6000 (GE Healthcare). In each instance, image acquisition was performed with a Nikon S Fluor $\times$ 40/0.90NA objective. Z-stacks were imaged at 0.3 μ m intervals at 1 $\times$ 1 binning. Nuclear, array segmentation and automated image analyses were performed using PipelinePilot image analysis software as previously described.¹⁷

Western blotting

Cell lysates were obtained from scraping cells into ice-cold NP-40 lysis buffer. Lysates were then combined with LDS Sample Buffer (Novex, Carlsbad, CA, USA) and boiled for 5 min. Samples were then loaded onto 12-well 8–12% acrylamide gels and run at 200 V at 4 °C for 90 min. Gels were then transferred onto Immobilon transfer membranes at 4 °C for 2 h at 80 V. Non-specific antibody binding was blocked by treating membranes with 5% milk in TBS-T.

Quantitative RT-PCR

Total RNA was isolated using Trizol (Life Technologies, Carlsbad, CA, USA) as per the manufacturer's instructions. RT and qPCR were performed as previously described.²⁰ Reactions were carried out in an Applied Biosystems StepOne Plus (Carlsbad, CA, USA). The fold change in expression was calculated using the $\Delta\Delta$ Ct comparative threshold cycle method with the ribosomal protein 36B4 mRNA as an internal control.

Statistical analysis and software

Data presented were acquired from a minimum of three independent experiments performed on different days using similar cell passage numbers. *P*-value was determined using Student's *t*-test for single comparisons or one-way ANOVA for independent samples and a Tukey HSD *post hoc* test for comparisons across multiple samples.

The data for the heat map in Figure 3 were generated using Cluster 3.0 utilizing City Block Distance clustering with a centroid linkage method⁶⁰ and then visualized using Java Treeview.⁶¹ The Venn diagrams in Figure 2 were generated using the Venny software (Oliveros JC 2007, <http://bioinfogp.cnb.csic.es/tools/venny/index.html>).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge ZD Sharp for aid in the development of the PRL-HeLa cell line, FJ Ashcroft for creation of the GFP-ERα:PRL-HeLa cells, and I Mikic, TJ Moran and JY Newberg for help in developing the automated high-content analysis tools used in this study. We also acknowledge R Lanz and C Stephan for curation, handling and upkeep of the RNAi library. We acknowledge NIEHS funding from 7RC2ES018789 (MAM), the Keck Foundation pre-doctoral fellowship and imaging/automation resource support from the John S Dunn Gulf Coast for Chemical Genomics (PJ Davies and MAM), Dan L Duncan Baylor Cancer Center (K Osborne), Center for Reproductive Biology (FJ Demayo), Keck Center NLM Training Program in Biomedical Informatics of the Gulf Coast Consortia National Library of Medicine (T15LM007093 to MB), and the Diana Helis Henry Medical Research Foundation (MAM) through its direct engagement in the continuous active conduct of medical research in conjunction with Baylor College of Medicine and the Cancer Program. This project was supported by the Integrated Microscopy Core at Baylor College of Medicine with funding from the NIH (HD007495, DK56338 and CA125123), the Dan L Duncan Cancer Center, and the John S Dunn Gulf Coast Consortium for Chemical Genomics.

References

1. Dickson RB, Lippman ME. Control of human breast cancer by estrogen, growth factors, and oncogenes. *Cancer Treat Res.* 1988; 40:119–165. [PubMed: 2908648]
2. Jacquemier JD, Hassoun J, Torrente M, Martin PM. Distribution of estrogen and progesterone receptors in healthy tissue adjacent to breast lesions at various stages—immunohistochemical study of 107 cases. *Breast Cancer Res Treat.* 1990; 15:109–117. [PubMed: 2322649]
3. Frasar J, Danes JM, Komm B, Chang KC, Lyttle CR, Katzenellenbogen BS. Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. *Endocrinology.* 2003; 144:4562–4574. [PubMed: 12959972]
4. Dubik D, Shiu RP. Mechanism of estrogen activation of c-myc oncogene expression. *Oncogene.* 1992; 7:1587–1594. [PubMed: 1630819]
5. Ali S, Coombes RC. Endocrine-responsive breast cancer and strategies for combating resistance. *Nat Rev.* 2002; 2:101–112.
6. Esslimani-Sahla M, Simony-Lafontaine J, Kramar A, Lavaill R, Mollevi C, Warner M, et al. Estrogen receptor beta (ER beta) level but not its ER beta cx variant helps to predict tamoxifen resistance in breast cancer. *Clin Cancer Res.* 2004; 10:5769–5776. [PubMed: 15355905]
7. Bernardo GM, Lozada KL, Miedler JD, Harburg G, Hewitt SC, Mosley JD, et al. FOXA1 is an essential determinant of ERalpha expression and mammary ductal morphogenesis. *Development (Cambridge, England).* 2010; 137:2045–2054.
8. Li XF, Wang SJ, Jiang LS, Dai LY. Stage specific effect of leptin on the expressions of estrogen receptor and extracellular matrix in a model of chondrocyte differentiation. *Cytokine.* 2013; 61:876–884. [PubMed: 23357303]
9. Ellison-Zelski SJ, Solodin NM, Alarid ET. Repression of ESR1 through actions of estrogen receptor alpha and Sin3A at the proximal promoter. *Mol Cell Biol.* 2009; 29:4949–4958. [PubMed: 19620290]
10. Sun J, Zhou W, Kaliappan K, Nawaz Z, Slingerland JM. ERalpha phosphorylation at Y537 by Src triggers E6-AP-ERalpha binding, ERalpha ubiquitylation, promoter occupancy, and target gene expression. *Mol Endocrinol (Baltimore, Md).* 2012; 26:1567–1577.

11. Bhatt S, Xiao Z, Meng Z, Katzenellenbogen BS. Phosphorylation by p38 mitogen-activated protein kinase promotes estrogen receptor alpha turnover and functional activity via the SCF(Skp2) proteasomal complex. *Mol Cell Biol.* 2012; 32:1928–1943. [PubMed: 22431515]
12. Fan M, Park A, Nephew KP. CHIP (carboxyl terminus of Hsc70-interacting protein) promotes basal and geldanamycin-induced degradation of estrogen receptor-alpha. *Mol Endocrinol* (Baltimore, Md). 2005; 19:2901–2914.
13. Giamas G, Filipovic A, Jacob J, Messier W, Zhang H, Yang D, et al. Kinome screening for regulators of the estrogen receptor identifies LMTK3 as a new therapeutic target in breast cancer. *Nat Med.* 2011; 17:715–719. [PubMed: 21602804]
14. Fox EM, Miller TW, Balko JM, Kuba MG, Sanchez V, Smith RA, et al. A kinome-wide screen identifies the insulin/IGF-I receptor pathway as a mechanism of escape from hormone dependence in breast cancer. *Cancer Res.* 2011; 71:6773–6784. [PubMed: 21908557]
15. Mendes-Pereira AM, Sims D, Dexter T, Fenwick K, Assiotis I, Kozarewa I, et al. Genome-wide functional screen identifies a compendium of genes affecting sensitivity to tamoxifen. *Proc Natl Acad Sci USA.* 2012; 109:2730–2735. [PubMed: 21482774]
16. Sharp ZD, Mancini MG, Hinojos CA, Dai F, Berno V, Szafran AT, et al. Estrogen-receptor-alpha exchange and chromatin dynamics are ligand- and domain-dependent. *J Cell Sci.* 2006; 119(Pt 19):4101–4116. [PubMed: 16968748]
17. Ashcroft FJ, Newberg JY, Jones ED, Mikic I, Mancini MA. High content imaging-based assay to classify estrogen receptor-alpha ligands based on defined mechanistic outcomes. *Gene.* 2011; 477:42–52. [PubMed: 21256200]
18. Berno V, Amazit L, Hinojos C, Zhong J, Mancini MG, Sharp ZD, et al. Activation of estrogen receptor-alpha by E2 or EGF induces temporally distinct patterns of large-scale chromatin modification and mRNA transcription. *PLoS One.* 2008; 3:e2286. [PubMed: 18509470]
19. Zwart W, de Leeuw R, Rondaij M, Neefjes J, Mancini MA, Michalides R. The hinge region of the human estrogen receptor determines functional synergy between AF-1 and AF-2 in the quantitative response to estradiol and tamoxifen. *J Cell Sci.* 2010; 123(Pt 8):1253–1261. [PubMed: 20332105]
20. Bolt MJ, Stossi F, Newberg JY, Orjalo A, Johansson HE, Mancini MA. Coactivators enable glucocorticoid receptor recruitment to fine-tune estrogen receptor transcriptional responses. *Nucleic Acids Res.* 2013; 41:4036–4048. [PubMed: 23444138]
21. Tcherepanova I, Puigserver P, Norris JD, Spiegelman BM, McDonnell DP. Modulation of estrogen receptor-alpha transcriptional activity by the coactivator PGC-1. *J Biol Chem.* 2000; 275:16302–16308. [PubMed: 10748020]
22. Wu Q, Burghardt R, Safe S. Vitamin D-interacting protein 205 (DRIP205) coactivation of estrogen receptor alpha (ERalpha) involves multiple domains of both proteins. *J Biol Chem.* 2004; 279:53602–53612. [PubMed: 15471764]
23. Mook OR, Baas F, de Wissel MB, Fluiter K. Allele-specific cancer cell killing in vitro and in vivo targeting a single-nucleotide polymorphism in POLR2A. *Cancer Gene Ther.* 2009; 16:532–538. [PubMed: 19165236]
24. Karmakar S, Foster EA, Smith CL. Unique roles of p160 coactivators for regulation of breast cancer cell proliferation and estrogen receptor-alpha transcriptional activity. *Endocrinology.* 2009; 150:1588–1596. [PubMed: 19095746]
25. Dong J, Tsai-Morris CH, Dufau ML. A novel estradiol/estrogen receptor alpha-dependent transcriptional mechanism controls expression of the human prolactin receptor. *J Biol Chem.* 2006; 281:18825–18836. [PubMed: 16651265]
26. Schaufele F. Regulation of estrogen receptor activation of the prolactin enhancer/promoter by antagonistic activation function-2-interacting proteins. *Mol Endocrinol* (Baltimore, Md). 1999; 13:935–945.
27. Zhang X, Krutchinsky A, Fukuda A, Chen W, Yamamura S, Chait BT, et al. MED1/TRAP220 exists predominantly in a TRAP/Mediator subpopulation enriched in RNA polymerase II and is required for ER-mediated transcription. *Mol Cell.* 2005; 19:89–100. [PubMed: 15989967]
28. Ansari SA, Morse RH. Mechanisms of Mediator complex action in transcriptional activation. *Cell Mol Life Sci.* 2013; 70:2743–2756. [PubMed: 23361037]

29. Toth-Petroczy A, Oldfield CJ, Simon I, Takagi Y, Dunker AK, Uversky VN, et al. Malleable machines in transcription regulation: the mediator complex. *PLoS Comput Biol*. 2008; 4:e1000243. [PubMed: 19096501]
30. Guenther MG, Levine SS, Boyer LA, Jaenisch R, Young RA. A chromatin landmark and transcription initiation at most promoters in human cells. *Cell*. 2007; 130:77–88. [PubMed: 17632057]
31. Hirose Y, Ohkuma Y. Phosphorylation of the C-terminal domain of RNA polymerase II plays central roles in the integrated events of eukaryotic gene expression. *J Biochem*. 2007; 141:601–608. [PubMed: 17405796]
32. Gonzalez-Arenas A, Hansberg-Pastor V, Hernandez-Hernandez OT, Gonzalez-Garcia TK, Henderson-Villalpando J, Lemus-Hernandez D, et al. Estradiol increases cell growth in human astrocytoma cell lines through ERalpha activation and its interaction with SRC-1 and SRC-3 coactivators. *Biochim Biophys Acta*. 2012; 1823:379–386. [PubMed: 22142990]
33. Prapapanich V, Chen S, Nair SC, Rimerman RA, Smith DF. Molecular cloning of human p48, a transient component of progesterone receptor complexes and an Hsp70-binding protein. *Mol Endocrinol (Baltimore, Md)*. 1996; 10:420–431.
34. Snow BE, Erdmann N, Cruickshank J, Goldman H, Gill RM, Robinson MO, et al. Functional conservation of the telomerase protein Est1p in humans. *Curr Biol*. 2003; 13:698–704. [PubMed: 12699629]
35. Stanlie A, Begum NA, Akiyama H, Honjo T. The DSIF subunits Spt4 and Spt5 have distinct roles at various phases of immunoglobulin class switch recombination. *PLoS Genet*. 2012; 8:e1002675. [PubMed: 22570620]
36. Jiang W, Wang S, Xiao M, Lin Y, Zhou L, Lei Q, et al. Acetylation regulates gluconeogenesis by promoting PEPCK1 degradation via recruiting the UBR5 ubiquitin ligase. *Mol Cell*. 2011; 43:33–44. [PubMed: 21726808]
37. Gudjonsson T, Altmeyer M, Savic V, Toledo L, Dinant C, Grofte M, et al. TRIP12 and UBR5 suppress spreading of chromatin ubiquitylation at damaged chromosomes. *Cell*. 2012; 150:697–709. [PubMed: 22884692]
38. Henderson MJ, Russell AJ, Hird S, Munoz M, Clancy JL, Lehrbach GM, et al. EDD, the human hyperplastic discs protein, has a role in progesterone receptor coactivation and potential involvement in DNA damage response. *J Biol Chem*. 2002; 277:26468–26478. [PubMed: 12011095]
39. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov*. 2012; 2:401–404. [PubMed: 22588877]
40. Comprehensive molecular portraits of human breast tumours. *Nature*. 2012; 490:61–70. [PubMed: 23000897]
41. Horner-Glister E, Maleki-Dizaji M, Guerin CJ, Johnson SM, Styles J, White IN. Influence of oestradiol and tamoxifen on oestrogen receptors-alpha and -beta protein degradation and non-genomic signalling pathways in uterine and breast carcinoma cells. *J Mol Endocrinol*. 2005; 35:421–432. [PubMed: 16326830]
42. Rudnik V, Sanyal A, Syed FA, Monroe DG, Spelsberg TC, Oursler MJ, et al. Loss of ERE binding activity by estrogen receptor-alpha alters basal and estrogen-stimulated bone-related gene expression by osteoblastic cells. *J Cell Biochem*. 2008; 103:896–907. [PubMed: 17614350]
43. Burandt E, Jens G, Holst F, Janicke F, Muller V, Quaas A, et al. Prognostic relevance of AIB1 (NCoA3) amplification and overexpression in breast cancer. *Breast Cancer Res Treat*. 2013; 137:745–753. [PubMed: 23322234]
44. Gururaj AE, Peng S, Vadlamudi RK, Kumar R. Estrogen induces expression of BCAS3, a novel estrogen receptor-alpha coactivator, through proline-, glutamic acid-, and leucine-rich protein-1 (PELP1). *Mol Endocrinol (Baltimore, Md)*. 2007; 21:1847–1860.
45. Garcia-Becerra R, Berno V, Ordaz-Rosado D, Sharp ZD, Cooney AJ, Mancini MA, et al. Ligand-induced large-scale chromatin dynamics as a biosensor for the detection of estrogen receptor subtype selective ligands. *Gene*. 2010; 458:37–44. [PubMed: 20347019]

46. Bourdoncle A, Labesse G, Margueron R, Castet A, Cavailles V, Royer CA. The nuclear receptor coactivator PGC-1 α exhibits modes of interaction with the estrogen receptor distinct from those of SRC-1. *J Mol Biol.* 2005; 347:921–934. [PubMed: 15784253]
47. Chen YJ, Lee MT, Yao HC, Hsiao PW, Ke FC, Hwang JJ. Crucial role of estrogen receptor- α interaction with transcription coregulators in follicle-stimulating hormone and transforming growth factor β 1 up-regulation of steroidogenesis in rat ovarian granulosa cells. *Endocrinology.* 2008; 149:4658–4668. [PubMed: 18511507]
48. Stokes K, Alston-Mills B, Teng C. Estrogen response element and the promoter context of the human and mouse lactoferrin genes influence estrogen receptor α -mediated transactivation activity in mammary gland cells. *J Mol Endocrinol.* 2004; 33:315–334. [PubMed: 15525592]
49. Lai F, Orom UA, Cesarini M, Beringer M, Taatjes DJ, Blobel GA, et al. Activating RNAs associate with Mediator to enhance chromatin architecture and transcription. *Nature.* 2013; 494:497–501. [PubMed: 23417068]
50. Willis IM, Chua G, Tong AH, Brost RL, Hughes TR, Boone C, et al. Genetic interactions of MAF1 identify a role for Med20 in transcriptional repression of ribosomal protein genes. *PLoS Genet.* 2008; 4:e1000112. [PubMed: 18604275]
51. Smits VA. EDD induces cell cycle arrest by increasing p53 levels. *Cell Cycle (Georgetown, Tex).* 2012; 11:715–720.
52. Cojocaru M, Bouchard A, Cloutier P, Cooper JJ, Varzavand K, Price DH, et al. Transcription factor IIS cooperates with the E3 ligase UBR5 to ubiquitinate the CDK9 subunit of the positive transcription elongation factor B. *J Biol Chem.* 2011; 286:5012–5022. [PubMed: 21127351]
53. Bernassola F, Karin M, Ciechanover A, Melino G. The HECT family of E3 ubiquitin ligases: multiple players in cancer development. *Cancer Cell.* 2008; 14:10–21. [PubMed: 18598940]
54. La Rosa P, Pesiri V, Marino M, Acconcia F. 17 β -Estradiol-induced cell proliferation requires estrogen receptor (ER) α monoubiquitination. *Cell Signal.* 2011; 23:1128–1135. [PubMed: 21356307]
55. Metivier R, Penot G, Hubner MR, Reid G, Brand H, Kos M, et al. Estrogen receptor- α directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell.* 2003; 115:751–763. [PubMed: 14675539]
56. Reid G, Hubner MR, Metivier R, Brand H, Denger S, Manu D, et al. Cyclic, proteasome-mediated turnover of unliganded and liganded ER α on responsive promoters is an integral feature of estrogen signaling. *Mol Cell.* 2003; 11:695–707. [PubMed: 12667452]
57. Long X, Nephew KP. Fulvestrant (ICI 182,780)-dependent interacting proteins mediate immobilization and degradation of estrogen receptor- α . *J Biol Chem.* 2006; 281:9607–9615. [PubMed: 16459337]
58. Chin SF, Teschendorff AE, Marioni JC, Wang Y, Barbosa-Morais NL, Thorne NP, et al. High-resolution aCGH and expression profiling identifies a novel genomic subtype of ER negative breast cancer. *Genome Biol.* 2007; 8:R215. [PubMed: 17925008]
59. Clancy JL, Henderson MJ, Russell AJ, Anderson DW, Bova RJ, Campbell IG, et al. EDD, the human orthologue of the hyperplastic discs tumour suppressor gene, is amplified and overexpressed in cancer. *Oncogene.* 2003; 22:5070–5081. [PubMed: 12902990]
60. de Hoon MJ, Imoto S, Nolan J, Miyano S. Open source clustering software. *Bioinformatics (Oxford, England).* 2004; 20:1453–1454.
61. Saldanha AJ. Java Treeview—extensible visualization of microarray data. *Bioinformatics (Oxford, England).* 2004; 20:3246–3248.

Figure 1.

Experimental workflow and quality control for high throughput RNAi screening. **(a)** Flow chart depicting experimental steps of the custom RNAi screen being performed in the PRL array cell line. The 30-min time point has previously been determined to be maximal for the transcriptional activity of the reporter gene. **(b)** Representative images of features measured in the primary screen, which include ER α nuclear levels and ER α at array, array area and mRNA FISH intensity. **(c)** Representative images (DAPI stained) from non-targeting or POLR2A RNAi, which was used as a positive control for toxicity and knockdown efficiency. **(d)** Z' -score of the primary screen calculated based on the number of cells across plate replicates comparing non-targeting and POLR2A siRNAs. **(e)** Primary screen results in terms of cell toxicity as evaluated by cell number. Dotted line represents the 30% cell toxicity cutoff. POLR2A and RAN, two lethal siRNAs, are circled in red.

Figure 2.

Identification of hits from the primary screen. All siRNAs are charted against their changes in ERα nuclear intensity (a), ERα array intensity (b), array area (c) and total RNA FISH intensity (d), and normalized to non-targeting siRNA, with the largest inducer and repressor being labeled. The purple box represents all siRNAs with at most a Z-score of ± 1 from the control, the pink box represents all hits between 1 and 2 Z-scores from the control. Any siRNA falling outside both boxes has a Z-score of ± 2 or greater from the control. (e, f) Venn diagrams representing the overlap between all primary hits affecting negatively (e) or positively (f) ERα nuclear intensity, ERα array intensity, array area or total RNA FISH.

Figure 3. Hierarchical clustering of the hits from the primary RNAi screen visualized as the *Z*-scores when compared with controls. Eight features are compared: ERα nuclear intensity, ERα array intensity, array area, FISH array intensity, SRC-3 array intensity, Serine 5-phospho Pol II (S5P) array intensity, Serine 2-phospho Pol II (S2P) array intensity and total RNA FISH intensity (mRNA FISH). Yellow represents values above control siRNA and blue represents values below control siRNA.

Figure 4.

Validation of Supt5H and UBR5 knockdown in PRL array cells. **(a, b)** ER α nucleoplasm intensity, array area and total RNA FISH array intensity measured in PRL array cells treated with 10 nM E2 after 72 h knockdown $\pm$ Supt5H **(a)** or UBR5 **(b)** siRNA. **(c, d)** ER α , GR and SRC-3 nuclear intensity measured after 72 h control or Supt5H **(c)**, or UBR5 **(d)** knockdown in PRL array cells. **(e, f)** Supt5H **(e)** or UBR5 **(f)** nuclear intensity measured after 72 h knockdown of control or their respective siRNAs. **(g)** Western blotting of UBR5, ER α and beta actin following 72 h knockdown with control or UBR5 siRNA in PRL array cells. *Denotes P -value <0.05 compared with siControl of same feature.

Figure 5.

UBR5 knockdown affects ERα levels in MCF-7 cells. **(a)** ERα, GR and SRC-3 nuclear intensity measured after 72 h control or UBR5 knockdown in MCF-7 cells. **(b)** Western blot of UBR5, ERα and beta actin following 72 h knockdown with control or UBR5 siRNA in MCF-7 cells. **(c)** Kaplan–Meier plot of PAM50 Luminal A breast invasive carcinoma patients when UBR5 has (red) or has not (blue) been altered. Kaplan–Meier plots were created from Cbioportal for Cancer Genomics (<http://www.cbioportal.org/public-portal/>). **(d)** MCF-7 cells transfected with Flag-tagged UBR5 or UBR5 C2768A were immunolabeled for ERα and Flag. Images were quantified in ImageJ for nuclear ERα and Flag intensity ($N > 50$ for each group). *Denotes P -value < 0.05 compared to UBR5 (–) control.

Figure 6.

Loss of UBR5 alters ER α -mediated target gene expression and proliferation. (a) ESR1 mRNA levels were measured by qPCR in MCF-7 cells after 48 h of knockdown using UBR5 siRNA. (b) ChIP assay showing ER α or IgG recruitment to ESR1 Promoter A genomic locus after 48 h of knockdown $\pm$ UBR5 siRNA. (c) GREB1 mRNA levels were measured by qPCR in MCF-7 cells treated with 10 nM E2, or 5% EtOH, for 24 h after 48 h of knockdown using UBR5 siRNA. (d) EdU labeling was quantified in MCF-7 cells treated with 10 nM E2 for 24 h after 48 h of control of UBR5 siRNA transfection. (e) Cyclin D1 mRNA levels were measured by qPCR in MCF-7 cells treated with 10 nM E2, or 5% EtOH, for 24 h that had been transfected with siRNA to UBR5 for 48 h. (f) EdU labeling was quantified in MDA-MB-231 cells after 48 h of control of UBR5 siRNA transfection. *Denotes *P*-value < 0.05 compared with siControl of same treatment.