

# Estrogen receptor alpha (ER $\alpha$ /ESR1) mediates the p53-independent overexpression of MDM4/MDMX and MDM2 in human breast cancer

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## ABSTRACT

**MDM2 and MDM4 are heterodimeric, non-redundant oncoproteins that potently inhibit the p53 tumor suppressor protein. MDM2 and MDM4 also enhance the tumorigenicity of breast cancer cells in *in vitro* and *in vivo* models and are overexpressed in primary human breast cancers. Prior studies have characterized Estrogen Receptor Alpha (ER $\alpha$ /ESR1) as a regulator of MDM2 expression and an MDM2- and p53-interacting protein. However, similar crosstalk between ER $\alpha$  and MDM4 has not been investigated. Moreover, signaling pathways that mediate the overexpression of MDM4 in human breast cancer remain to be elucidated. Using the Cancer Genome Atlas (TCGA) breast invasive carcinoma patient cohort, we have analyzed correlations between ER $\alpha$  status and MDM4 and MDM2 expression in primary, treatment-naïve, invasive breast carcinoma samples. We report that the expression of MDM4 and MDM2 is elevated in primary human breast cancers of luminal A/B subtypes and associates with ER $\alpha$ -positive disease, independently of p53 mutation status. Furthermore, in cell culture models, ER $\alpha$  positively regulates MDM4 and MDM2 expression via p53-independent mechanisms, and these effects can be blocked by the clinically-relevant endocrine therapies fulvestrant and tamoxifen. Additionally, ER $\alpha$  also positively regulates p53 expression. Lastly, we report that endogenous MDM4 negatively regulates ER $\alpha$  expression and forms a protein complex with ER $\alpha$  in breast cancer cell lines and primary human breast tumor tissue. This suggests direct signaling crosstalk and negative feedback loops between ER $\alpha$  and MDM4 expression in breast cancer cells. Collectively, these novel findings implicate ER $\alpha$  as a central component of the p53-MDM2-MDM4 signaling axis in human breast cancer.**

## INTRODUCTION

MDM2/HDM2 and MDM4/MDMX/HDMX are homologous, heterodimeric, E3-ubiquitin protein ligases that are classically known for their non-redundant abilities to inhibit the p53 tumor suppressor protein (reviewed in [1–4]) p53 regulates a variety of cellular processes, including DNA repair, apoptosis, cell cycle arrest, senescence, autophagy and metabolism, amongst others (reviewed in [5–9]). MDM2 and MDM4 constrain p53 function by binding to and inhibiting the transactivation domain of p53, by cooperating to poly-ubiquitinate p53, and by facilitating

the translocation of p53 to the cytosol [10–30]. p53 is also known for its ability to upregulate *MDM2* and *MDM4* gene expression through an auto-inhibitory negative feedback loop [31–37]. However, p53-independent mechanisms by which *MDM2* and *MDM4* gene expression are regulated remain poorly understood.

*MDM2* is an established oncogene in breast cancer. *In vitro*, MDM2 enhances the proliferation of human breast cancer cells and antagonizes apoptosis [38–43]; *in vivo*, *Mdm2* transgene expression initiates mammary gland tumorigenesis in murine models [44]; and in breast cancer patients, MDM2 protein overexpression and *MDM2* gene

amplification are associated with decreased overall and/or disease-free survival [45–48]. Consistent with its oncogenic role, the *MDM2* gene is overexpressed at the mRNA and protein levels in 26–73% of primary human breast cancers [47, 49–54]. Since *MDM2* gene amplifications are relatively infrequent [45, 52, 53, 55–58], the overexpression of MDM2 in breast cancer is likely mediated by aberrant gene regulation.

Estrogen receptor alpha/estrogen receptor 1 (*ERα/ESR1*) is a nuclear hormone receptor and oncoprotein that is expressed in approximately 70% of breast cancers [59, 60]. Interestingly, MDM2 expression positively correlates with *ERα* expression in primary human breast tumors and human breast cancer cell lines, and *ERα* has been proposed to upregulate MDM2 expression [38, 50, 51, 56, 61–68]. In addition, MDM2 also forms a protein complex with *ERα* and facilitates the ubiquitination and degradation of *ERα* [41, 43, 66, 69]. This establishes a negative feedback loop between MDM2 and *ERα*. However, the ability of *ERα* and MDM4 to similarly interact with one another and to regulate one another's expression remains to be elucidated.

Like MDM2, MDM4 also plays a protumorigenic role in human breast cancer cells that are cultured *in vitro* or *in vivo* as murine xenografts [39, 40, 55, 70–72]. Knockdown of MDM4 inhibits the proliferation of breast cancer cells, induces the expression of the cyclin dependent kinase inhibitor CDKN1A/p21<sup>waf1/cip1</sup>, and causes G<sub>1</sub>-phase cell cycle arrest and senescence [39, 40, 55, 70]. Additionally, loss of MDM4 reduces cell viability, sensitizes cells to agent-induced apoptosis and upregulates p53 in breast cancer cell culture models [30, 39, 40, 71]. MDM4 also cooperates with MDM2 to facilitate the ubiquitination of p53 in breast cancer cells [30]. In the clinic, these protumorigenic functions of MDM4 are likely facilitated by the overexpression of the *MDM4* gene, which occurs in approximately 20–55% of primary human breast tumors [49, 53, 55, 70]. However, mechanisms that mediate the overexpression of MDM4 in breast cancer have not been identified, and factors capable of regulating *MDM4* gene expression in human cells remain largely unknown, with only two main pathways having been identified to date: p53 and mitogen-activated protein kinase (MAPK) [35, 73]. Since MDM4 expression is frequently elevated in luminal breast cancers [55], and the majority of luminal tumors are *ERα*-positive [74], we propose that *ERα* and MDM4 may be coexpressed with one another in human breast cancer and may regulate each other's expression.

The objective of the present study is to examine signaling crosstalk between *ERα*, MDM4, MDM2 and p53 in human breast cancer at the levels of gene expression and protein-protein interactions. We have used treatment-naive primary human breast carcinomas with corresponding gene expression data from the Cancer Genome Atlas invasive breast carcinoma (TCGA BRCA) patient cohort, as well as complementary cell culture models, to demonstrate that *ERα* mediates the overexpression of *MDM4* and *MDM2* genes in human breast cancer. We also provide evidence

that, like MDM2, MDM4 forms a protein complex with *ERα* and negatively regulates *ERα* expression, thereby establishing a negative feedback loop between *ERα* and MDM4/MDM2 at the expression level.

## RESULTS

### ***MDM4* and *MDM2* mRNA expression is elevated in *ERα*-positive primary breast invasive carcinoma samples**

It is well-established that MDM2 protein is coexpressed with *ERα* in human breast cancer cell lines and primary human breast tumors [50, 51, 56, 62, 63, 68]. However, it is not known if *MDM2* mRNA expression also correlates with *ERα* expression in primary breast tumors. Moreover, analyses of correlations between the MDM2 homolog, MDM4, and *ERα* expression have not been performed. In the present study, we utilized The Cancer Genome Atlas invasive breast carcinoma (TCGA BRCA) RNA-Seq gene expression dataset to analyze the expression of *MDM4* and *MDM2* mRNA in treatment-naive primary human breast invasive carcinoma samples. The samples had been previously categorized according to *ERα* status and intrinsic molecular subtype using the PAM50 50-gene subtype predictor model [75, 76]. To study *MDM4* and *MDM2* mRNA expression patterns that were independent of *MDM4* and *MDM2* gene amplification, samples that were known to have *MDM4* or *MDM2* gene amplifications (as described in [75]) were excluded from our *MDM4* and *MDM2* gene expression analyses, respectively. We observed that *MDM4* and *MDM2* mRNA expression was elevated in primary breast invasive carcinomas of luminal A and luminal B molecular subtypes, as compared to HER2-enriched, basal, and normal-like subtypes (Figure 1, panels A and B). Notably, luminal A and luminal B subtypes are enriched for *ERα*-positive tumors, as compared to HER2-enriched, basal, or normal-like subtypes [74], indicating that *MDM4* and *MDM2* gene expression may correlate with *ERα* expression. Indeed, Pearson product-moment correlation coefficient tests identified significant positive correlations between *ERα/ESR1* and *MDM4* mRNA expression (Figure 1C) and between *ERα/ESR1* and *MDM2* mRNA expression (Figure 1D). In addition, *MDM4* and *MDM2* mRNA expression was significantly higher in *ERα*-positive tumors, as compared to *ERα*-negative tumors (Figure 1, panels E and F). These findings establish a co-expression pattern for *ERα* and MDM4, as well as for *ERα* and MDM2, in primary human invasive breast carcinomas. Furthermore, this correlation is independent of *MDM4* and *MDM2* gene amplifications, suggesting that mechanisms other than gene amplification govern the upregulation of *MDM4* and *MDM2* mRNA in *ERα*-positive tumors.

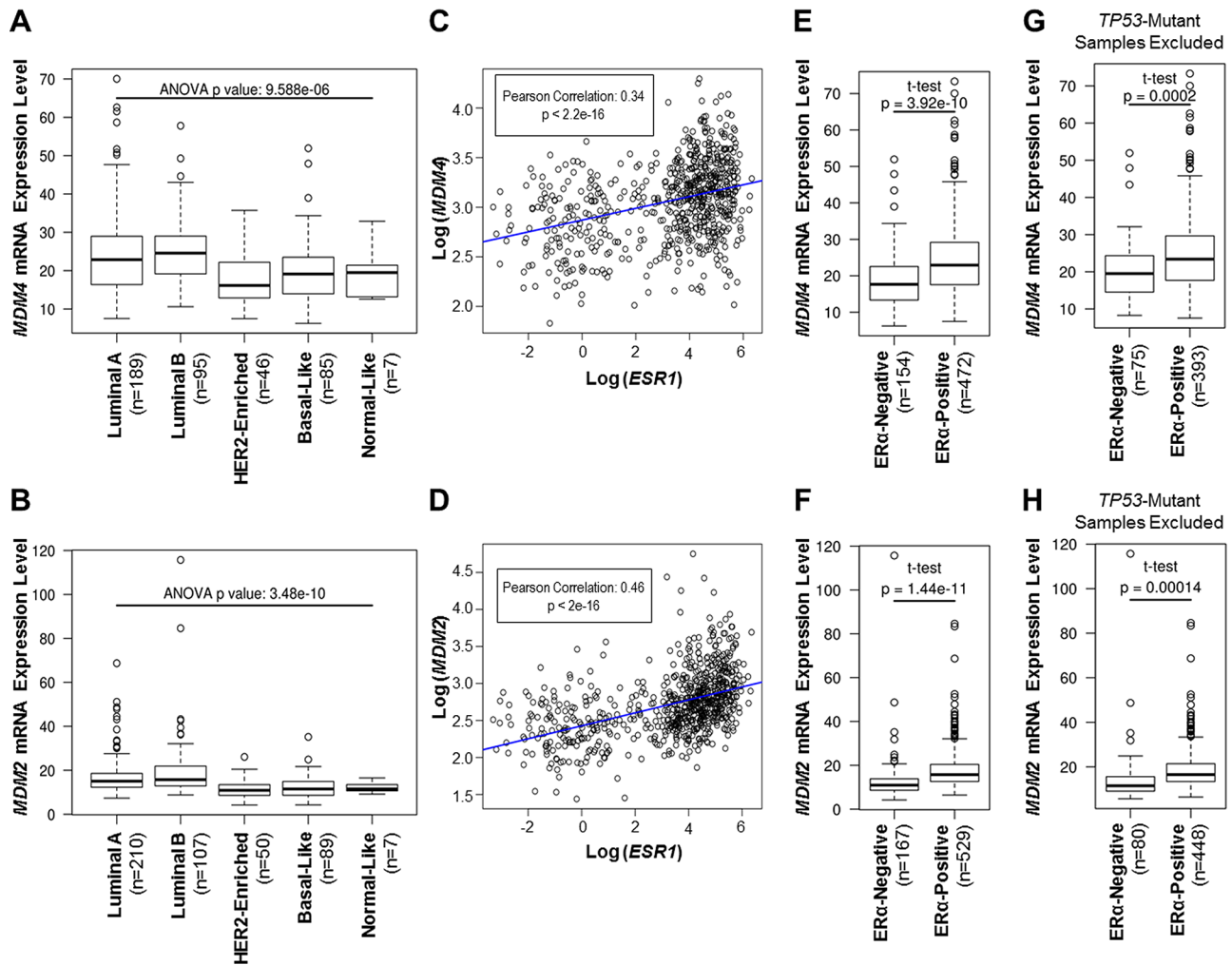
Due to the well-characterized roles of MDM4 and MDM2 in the p53 pathway, we also measured *MDM4* and *MDM2* gene expression in terms of *TP53* mutation status. We observed that *MDM4* and *MDM2* mRNA expression

was elevated in *TP53* wild type tumors, as compared to *TP53* mutant tumors (Supplementary Figure S1), consistent with wild type p53 being a positive transcriptional regulator of *MDM4* and *MDM2* genes [31–37]. It is known that the majority of *TP53*-mutant breast tumors are ER $\alpha$ -negative [77], and our analyses have revealed that ER $\alpha$ -negative tumors have reduced expression of *MDM4* and *MDM2* mRNA (Figure 1, panels E and F). Therefore, we tested if our observed positive correlations between ER $\alpha$  and either *MDM4* or *MDM2* gene expression were dependent upon *TP53* mutation status. To do this, we excluded samples with *TP53* mutations and analyzed *MDM4* and *MDM2* gene expression in the *TP53* wild type group. This analysis revealed that *MDM4* and *MDM2* mRNA expression was still higher in the ER $\alpha$ -positive tumors than in the ER $\alpha$ -

negative tumors, even after exclusion of the *TP53* mutant tumors (Figure 1, panels G and H). This finding indicates that the associations between ER $\alpha$  and either *MDM4* or *MDM2* gene expression are independent of *TP53* mutations. As such, we postulated that ER $\alpha$  may regulate *MDM4* and *MDM2* expression via p53-independent mechanisms.

### Loss of ER $\alpha$ and/or inhibition of ER $\alpha$ downregulates *MDM4* and *MDM2* in breast cancer cells

Since ER $\alpha$ -positive primary invasive breast carcinomas have elevated expression of *MDM4* and *MDM2* genes, we hypothesized that ER $\alpha$  mediates the upregulation of *MDM4* and *MDM2* in human breast cancer.



**Figure 1: *MDM4* and *MDM2* mRNA expression is elevated in ER $\alpha$ -positive primary breast invasive carcinoma samples.** (A–H) Analyses of *MDM4* or *MDM2* mRNA expression in TCGA breast invasive carcinoma cohort. (A, B) Tumor types are classified according to the PAM50 model of intrinsic subtype classification. (C, D) Pearson product-moment correlation coefficient test analysis of correlations between *ESR1/ER $\alpha$*  mRNA and either *MDM4* mRNA (panel C) or *MDM2* mRNA (panel D). (E, F) Tumor types are classified according to ER $\alpha$  status. (G, H) As in panels E and F, but *TP53*-mutant tumors were excluded from the analysis to study gene expression patterns that were independent of *TP53* mutations. Note that for panels A, C, E and G, samples with *MDM4* gene amplifications were excluded from the analysis to study patterns in *MDM4* mRNA expression that were independent of *MDM4* gene amplification. For panels B, D, F and H, samples with *MDM2* gene amplifications were excluded from the analysis to study patterns in *MDM2* mRNA expression that were independent of *MDM2* gene amplification.

Previous reports have proposed that ER $\alpha$  modulates MDM2 expression [38, 50, 61, 62, 64–68]. However, it is not known if ER $\alpha$  also regulates MDM4 expression. We used the luminal human breast adenocarcinoma cell lines ZR-75-1 and MCF7 as *in vitro* model systems of ER $\alpha$ -positive breast cancer, and we investigated how loss of ER $\alpha$  expression affected *MDM4* gene expression at the protein and mRNA levels, as compared to its effects on *MDM2* gene expression. As shown in Figure 2, panel A, we used ER $\alpha$  siRNA to efficiently knock down ER $\alpha$  expression by approximately 90% in ZR-75-1 cells, which resulted in the decreased expression of MYC, a well-characterized ER $\alpha$ -target gene [78, 79] shown here as a positive control. Notably, when ER $\alpha$  was knocked down, we also observed significant reductions in MDM4 and MDM2 protein expression, by approximately 40% and 60%, respectively, demonstrating that ER $\alpha$  positively regulates the expression of both MDM4 and MDM2 (Figure 2A). Substantial decreases in MDM4 and MDM2 protein expression were also observed using a second ER $\alpha$  siRNA and a second cell line, MCF7, demonstrating the specificity of the effect (Supplementary Figure S2, panel A). qPCR analysis of *MDM4* and *MDM2* mRNA expression in MCF7 and ZR-75-1 cells that had been transfected with ER $\alpha$  siRNA or non-silencing control siRNA revealed that loss of ER $\alpha$  also resulted in a significant downregulation of *MDM4* and *MDM2* mRNA expression (Figure 2B). This finding is in agreement with our data from Figure 1, where we observed that ER $\alpha$  expression positively correlated with *MDM4* and *MDM2* mRNA expression in primary breast carcinomas. Therefore, we conclude that ER $\alpha$  is a positive regulator of not only *MDM2* gene expression, but also of *MDM4* gene expression.

Having demonstrated that ER $\alpha$  regulates *MDM4* and *MDM2* mRNA expression (Figure 2B), and additionally, that *MDM4* and *MDM2* mRNA expression is specifically elevated in ER $\alpha$ -positive primary human breast tumors (Figure 1), we postulated that ER $\alpha$  transcriptionally regulates *MDM4* and *MDM2* genes. We mined 8 different publicly-available ER $\alpha$  ChIP-Seq and ChIP-Chip datasets from the Nuclear Receptor Cistrome database [80] and the ENCODE Project database [81], where the ER $\alpha$  ChIP was performed in p53 wild type MCF7 human breast cancer cells or p53 mutant T-47D human breast cancer cells. We used Integrated Genomics Viewer (IGV) software to visualize the binding of ER $\alpha$  to the *MDM4* and *MDM2* genes in these ChIP datasets. Consensus estrogen receptor binding sites (ERBS) within the *MDM4* and *MDM2* genes were identified as regions that were bound by ER $\alpha$  in at least 2 or more of the 8 ChIP datasets. We observed 4 consensus ERBS within a downstream regulatory element of the *MDM4* gene (Supplementary Figure S3); likewise, we similarly observed 4 consensus ERBS within an upstream regulatory element of the *MDM2* gene (Supplementary Figure S4). To determine if these consensus ERBS were directly bound by ER $\alpha$ , we analyzed their DNA sequences for the presence of putative estrogen response element (ERE) sequences and/or ERE half sites using Dragon ERE Finder software [82]. We

found that each of the consensus ERBS that we identified in both the *MDM4* and *MDM2* genes contained one or more ERE or half site (Supplementary Tables 1 and 2), indicating that ER $\alpha$  has the capability to directly bind to these regions.

Additionally, as further supportive evidence to demonstrate that ER $\alpha$  directly regulates *MDM4* and *MDM2* gene expression, we assessed the binding of the nuclear hormone receptor-associated pioneer transcription factor, forkhead box protein A1 (FOXA1). FOXA1 binding has previously been demonstrated to occur within close proximity to EREs, to predict the genomic locations of functional EREs, and to determine ER $\alpha$ -binding specificity [83–86]. We found that FOXA1 ChIP-Seq peaks were clustered near the consensus ERBS that we identified within the *MDM4* and *MDM2* genes (Supplementary Figure S5). Therefore, we conclude that ER $\alpha$  and FOXA1 directly access *MDM4* and *MDM2* chromatin, supporting a role for these factors in regulating *MDM4* and *MDM2* transcription.

Thus far, we have provided evidence that ER $\alpha$  positively regulates the expression of the *MDM4* and *MDM2* genes at the mRNA level (Figure 2B), which translates to the protein level (Figure 2A). We next used immunofluorescent microscopy to visualize the effect of loss of ER $\alpha$  on the subcellular localization and expression of MDM4 and MDM2 protein. Immunofluorescent staining of ER $\alpha$  in MCF7 cells revealed that the receptor was predominantly nuclear (Figure 2C, red fluorescence). Colabeling of MDM4 (green fluorescence) and ER $\alpha$  (red fluorescence) in the same cells demonstrated that MDM4 and ER $\alpha$  were both localized in the nuclear compartment (Figure 2C). Similar results were observed for MDM2 and ER $\alpha$  (Figure 2D). We next used ER $\alpha$  siRNA to transiently deplete ER $\alpha$ . Under this condition, nuclear MDM4 immunofluorescent signal was significantly reduced by approximately 50% (Figure 2C; green fluorescence), as was MDM2 signal (Figure 2D; green fluorescence). Due to the accessibility of many commonly-used, commercially-available MDM2 antibodies, we went on to validate our MDM2 immunofluorescence data with two additional MDM2 antibodies, both of which showed similar results (Supplementary Figure S2, panel B). We also verified that the immunofluorescent signals of our MDM4 and MDM2 antibodies were specific by demonstrating that siRNA-mediated knockdown of MDM4 and MDM2 could markedly reduce the immunofluorescent staining of each protein, respectively (Supplementary Figure S2, panels C and D).

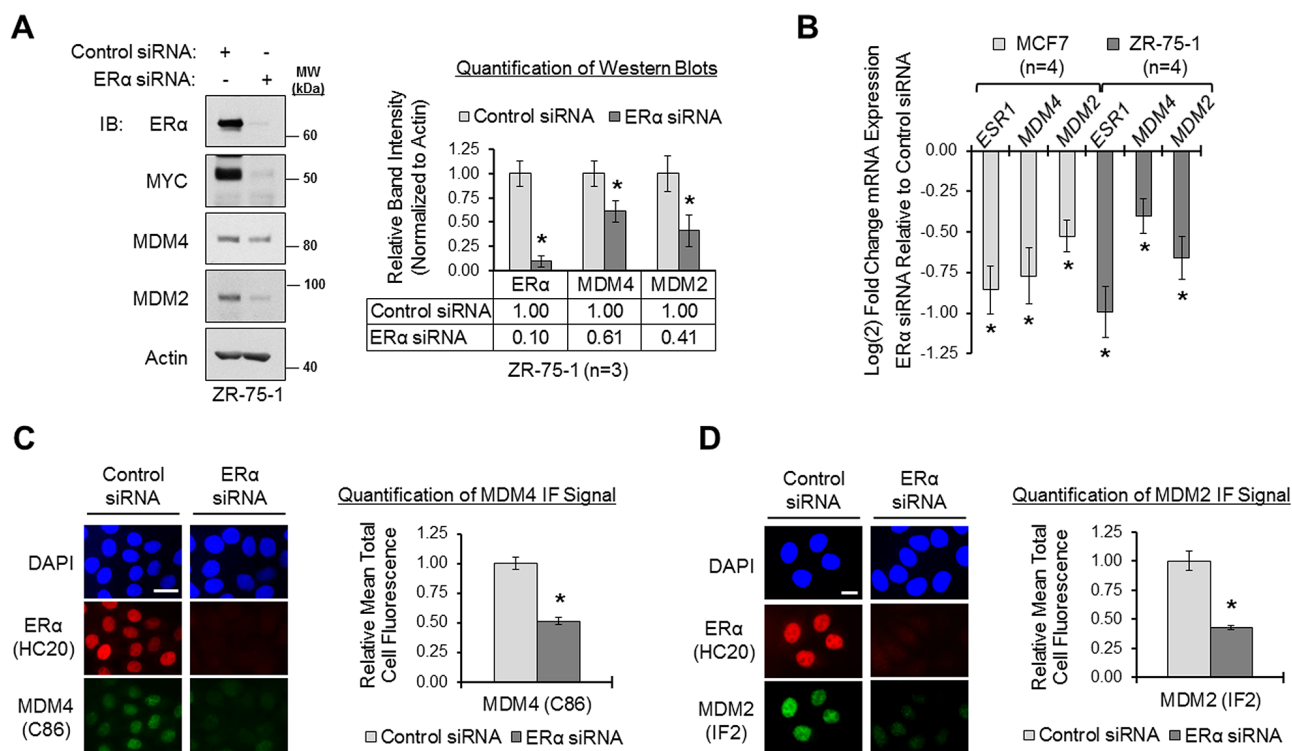
To complement our ER $\alpha$  siRNA experiments, we next tested if MDM4 and MDM2 expression could be modulated pharmacologically with the clinically-relevant ER $\alpha$  antagonists, fulvestrant and 4-hydroxytamoxifen. Fulvestrant is a pure anti-estrogen that inhibits ER $\alpha$  function and downregulates the receptor [87–89]. 4-hydroxytamoxifen is a selective estrogen receptor modulator that inhibits ER $\alpha$  function but upregulates the receptor [90–92]. We observed that fulvestrant downregulated MDM4 and MDM2 expression in MCF7 cells by 64% and 41%, respectively, and in ZR-75-1

cells by 42% and 54%, respectively (Figure 3A). Similar to fulvestrant, 4-hydroxytamoxifen also downregulated both MDM4 and MDM2 expression by approximately 40% (Figure 3B). Collectively, the data presented thus far establish ER $\alpha$  as a positive regulator of MDM4 and MDM2 expression. Furthermore, we conclude that two ER $\alpha$  antagonists with different mechanisms of action, fulvestrant and 4-hydroxytamoxifen, can be used to inhibit MDM4 and MDM2 expression.

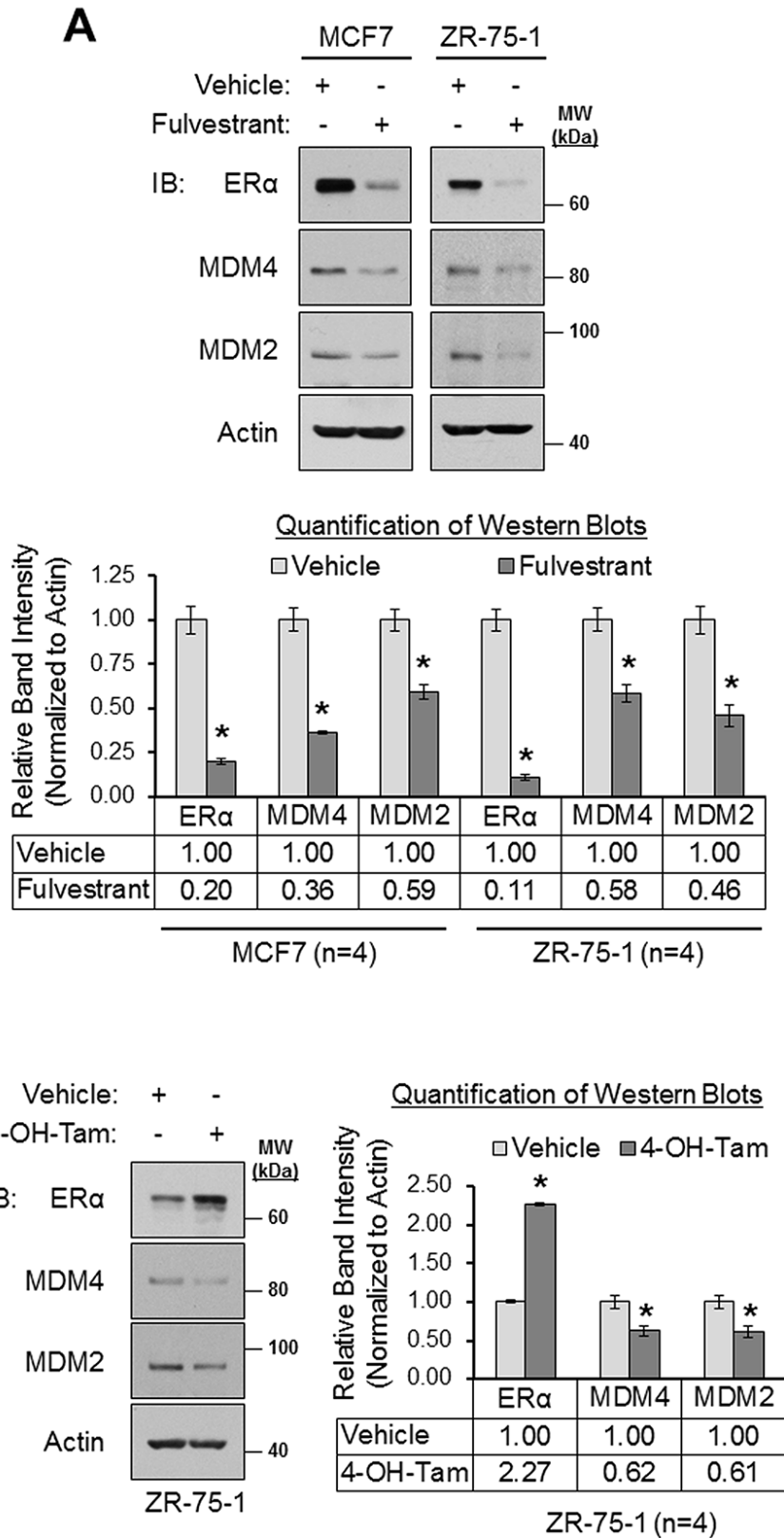
### ER $\alpha$ regulates p53 expression, but p53 is dispensable for ER $\alpha$ -mediated regulation of MDM4 and MDM2

Our results from Figures 2 and 3 revealed that loss of ER $\alpha$  signaling resulted in the downregulation of MDM4 and MDM2. Since MDM4 and MDM2 cooperate to negatively regulate p53 expression by promoting the polyubiquitination

and proteasomal degradation of p53 [16, 20, 30], we tested if the downregulation of MDM4 and MDM2 that we observed in response to loss of ER $\alpha$  signaling would result in a subsequent increase in p53 expression. Surprisingly, we observed that conditions which downregulated MDM2 and MDM4 (i.e. ER $\alpha$  siRNA, fulvestrant and 4-hydroxytamoxifen; Figures 2 and 3) did not upregulate p53. Rather, they downregulated p53. As shown in Figure 4, ER $\alpha$  siRNA decreased p53 expression by approximately 40% in MCF7 cells and by approximately 50% in ZR-75-1 cells (panel A); fulvestrant downregulated p53 by approximately 40% in MCF7 and ZR-75-1 cells (panel B); and 4-hydroxytamoxifen reduced p53 expression by approximately 40% in ZR-75-1 cells (panel C). Therefore, we conclude that inhibition of ER $\alpha$  downregulates p53 expression along with MDM4 and MDM2. This is consistent with previous reports that demonstrated that ER $\alpha$  upregulates p53 expression [93-97] by binding directly to



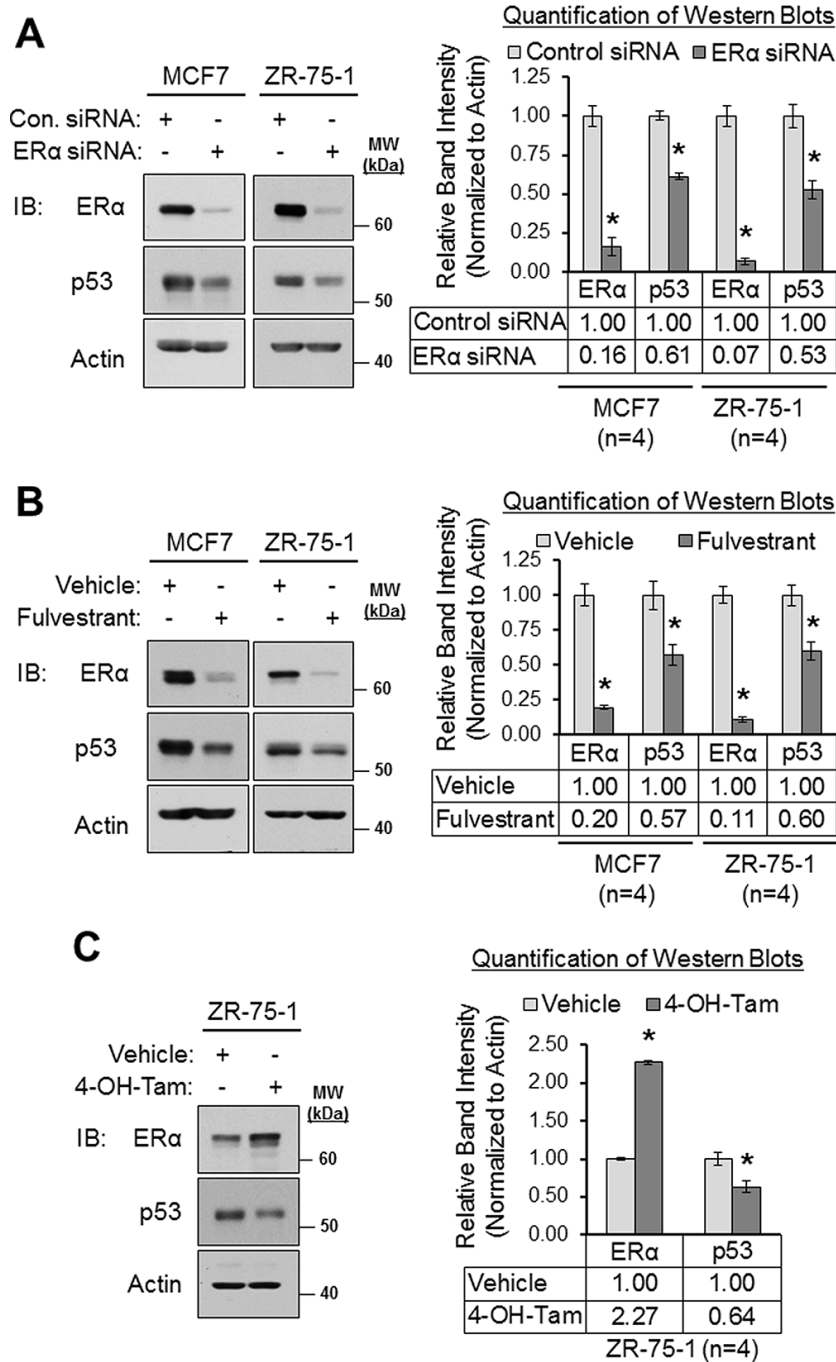
**Figure 2: Loss of ER $\alpha$  downregulates MDM4 and MDM2 in breast cancer cells.** (A) Western blot analysis of ZR-75-1 cells transfected with control or ER $\alpha$  siRNA (sequence #1) for 22 h. Left panel: representative blot. Right panel: quantification of western blots. Error bars = SEM of biological replicates ( $n = 3$ ). (B) qPCR analysis of MCF7 and ZR-75-1 cells transfected with control or ER $\alpha$  siRNA for 22 h. *ESR1/ER $\alpha$* , *MDM4* and *MDM2* mRNA expression values were normalized to *ACTB*, and ER $\alpha$  siRNA-transfected samples were relatively compared to control samples according to the  $\Delta\Delta C_q$  method. Control samples are set at 0 on the y-axis. Fold change values are  $\log_2$  transformed to ensure an accurate representation of values less than 1; therefore, a change of 1 unit on the  $\log_2$  scale would be equivalent to a 2-fold change in expression on a linear scale. Error bars = SEM of biological replicates ( $n = 4$ ). (C) Immunofluorescent (IF) microscopy of ER $\alpha$  and MDM4 in MCF7 cells that were transfected with control or ER $\alpha$  siRNA for 22 h. Nuclei were labeled with DAPI. Exposure times were equal for control and ER $\alpha$  siRNA-transfected cells. Left panel: representative image. Right panel: quantification of MDM4 IF signal. Error bars = SEM of total cell fluorescence ( $n = 15$  cells from 3 independent coverslips). (D) IF microscopy of ER $\alpha$  and MDM2 in MCF7 cells that were transfected with control or ER $\alpha$  siRNA for 22 h. Nuclei were labeled with DAPI. Exposure times were equal for control and ER $\alpha$  siRNA-transfected cells. Left panel: representative image. Right panel: quantification of MDM2 IF signal. Error bars = SEM of total cell fluorescence ( $n = 15$  cells from 3 independent coverslips). \* $p < 0.05$ , unpaired  $t$ -test. Microscopy scale bar = 20  $\mu$ m. DAPI = 4',6-diamidino-2-phenylindole. IB = immunoblot. kDa = kilodaltons. MW = molecular weight. SEM = standard error of the means.



**Figure 3: Pharmacologic inhibition of ER $\alpha$  results in the downregulation MDM4 and MDM2 in breast cancer cells.** (A) Western blot analysis of MCF7 and ZR-75-1 cells that were treated with vehicle (ethanol) or fulvestrant (1  $\mu$ M) for 24 h. Upper panel: representative western blots. Lower panel: quantification of western blots ( $n = 4$ ). (B) Western blot analysis of ZR-75-1 cells that were treated with vehicle (ethanol) or 4-hydroxytamoxifen (1  $\mu$ M) for 24 h. Left panel: representative western blot. Right panel: quantification of western blots ( $n = 4$ ). Error bars = SEM of biological replicates. \* $p < 0.05$ , unpaired  $t$ -test. 4-OH-Tam = 4-hydroxytamoxifen. IB = immunoblot. kDa = kilodaltons. MW = molecular weight.

the *TP53/p53* promoter and transcriptionally activating the gene [93]. In addition, this finding also indicates that the downregulation of MDM4 and MDM2 in response to inhibition of ER $\alpha$  signaling is unlikely to have resulted in a significant loss of ubiquitination of p53, as an accumulation of p53 protein was not observed under these conditions.

We next tested if ER $\alpha$ 's ability to positively regulate MDM4 and MDM2 expression was p53-dependent because we had observed that ER $\alpha$  positively regulates p53 expression (Figure 4), and it is known that p53 transcriptionally upregulates the *MDM4* and *MDM2* genes [31–37]. We generated two different pools of ZR-75-1 cells

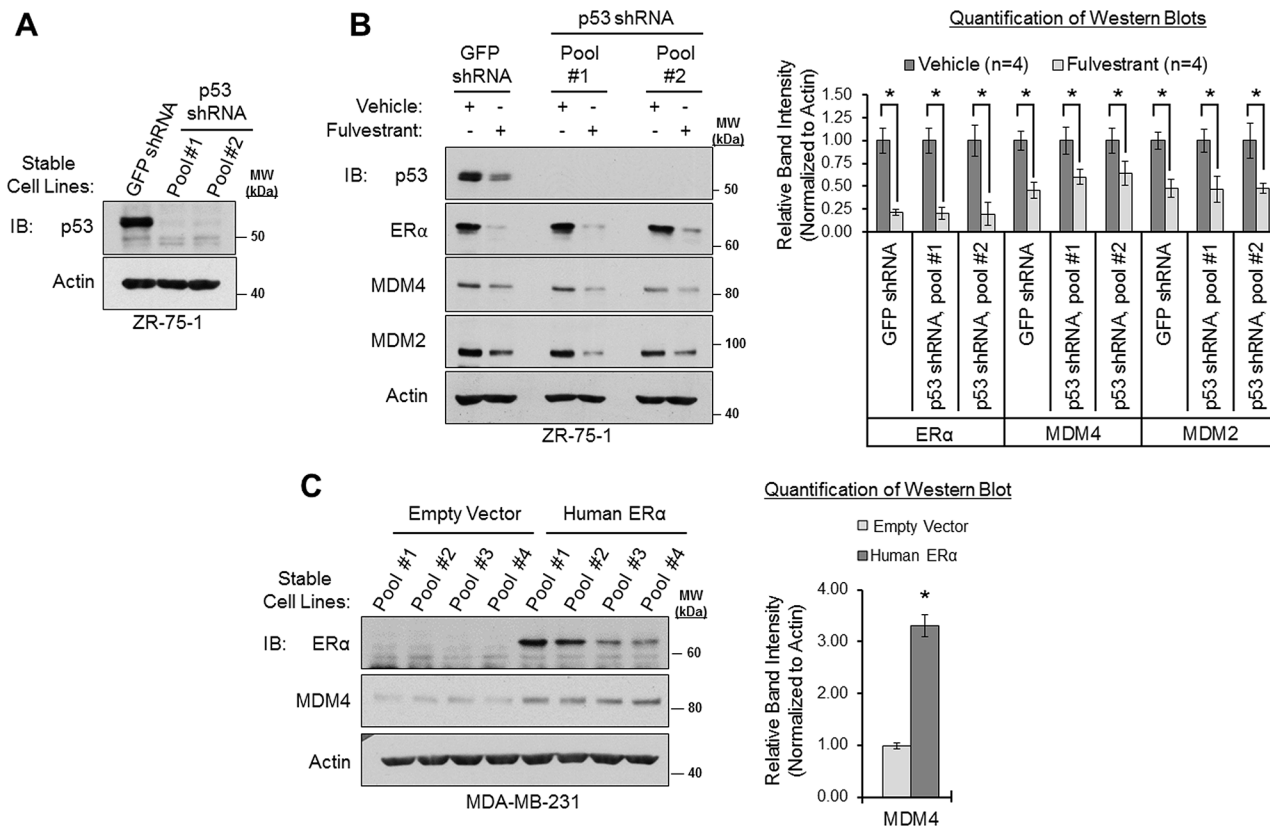


**Figure 4: Loss of and/or inhibition of ER $\alpha$  downregulates p53 expression.** (A) Western blot analysis of MCF7 and ZR-75-1 cells that were transfected with control siRNA or ER $\alpha$  siRNA for 22 h. Left panel: representative western blot. Right panel: quantification of western blots ( $n = 4$ ). (B) Western blot analysis of MCF7 and ZR-75-1 cells that were treated with vehicle (ethanol) or fulvestrant (1  $\mu$ M) for 24 h. Left panel: representative western blot. Right panel: quantification of western blots ( $n = 4$ ). (C) Western blot analysis of ZR-75-1 cells that were treated with vehicle (ethanol) or 4-hydroxytamoxifen (1  $\mu$ M) for 24 h. Left panel: representative western blot. Right panel: quantification of western blots ( $n = 4$ ). Error bars = SEM of biological replicates.  $*p < 0.05$ , unpaired  $t$ -test. 4-OH-Tam = 4-hydroxytamoxifen. Con. = control. IB = immunoblot. kDa = kilodaltons. MW = molecular weight.

that stably expressed a p53-silencing shRNA construct. As shown in Figure 5A, the p53 shRNA efficiently knocked down p53 to nearly undetectable levels, when compared to control cells that expressed GFP shRNA. We then treated these shRNA-engineered cell lines with the ER $\alpha$  antagonist fulvestrant to downregulate and inhibit ER $\alpha$ , and we measured the expression of MDM4 and MDM2 by western blotting. We observed that fulvestrant downregulated MDM4 and MDM2 in both the p53-shRNA-expressing and the GFP-shRNA-expressing ZR-75-1 cells (Figure 5B). Notably, two different p53 shRNA-infected ZR-75-1 cell pools yielded similar findings, demonstrating the specificity of the effect (Figure 5B). In addition, comparable results were also observed in MCF7 cells, where we found that fulvestrant retained the ability to downregulate MDM4 and MDM2 in the absence of p53 in two different p53 shRNA-expressing MCF7 cell pools (Supplementary Figure S6). From these findings, we conclude that the ER $\alpha$  antagonist fulvestrant downregulates MDM4 and MDM2 via p53-independent mechanisms.

As a complementary approach, we also stably overexpressed ER $\alpha$  in the ER $\alpha$ -negative breast adenocarcinoma cell line, MDA-MB-231. This cell line

expresses a hot spot point mutation (R280K) within the DNA-binding domain of p53, which significantly hinders p53's ability to bind to cognate DNA response elements and, therefore, renders p53 transcriptionally inactive [98, 99]. A previous study has demonstrated that overexpression of ER $\alpha$  in this cell line results in the upregulation of MDM2 [68]. Therefore, we hypothesized that overexpression of ER $\alpha$  would also upregulate MDM4. Consistent with our hypothesis, we found that stable overexpression of human ER $\alpha$ /ESR1 cDNA in four different pools of MDA-MB-231 cells led to a more than 3-fold upregulation of MDM4, as compared to four cell pools that stably expressed an empty vector (Figure 5C). This indicates that ER $\alpha$  can upregulate MDM4 in the absence of functional, transactivation-competent p53 protein. To summarize, the collective findings of Figure 5 and Supplementary Figure S6 indicate that ER $\alpha$  positively regulates MDM4 and MDM2 expression via p53-independent mechanisms. This is in agreement with our findings from Figure 1, where we observed that ER $\alpha$  expression correlated with MDM4 and MDM2 gene expression in primary breast carcinomas, independently of TP53 mutational status.



**Figure 5: ER $\alpha$  modulates MDM4 expression via p53-independent mechanisms.** (A) Western blot analysis of ZR-75-1 cells that stably overexpress GFP shRNA or p53 shRNA (two different cell pools). (B) Western blot analysis of cells from panel A that were treated with vehicle (ethanol) or fulvestrant (1  $\mu$ M) for 22 h. Left: representative western blot. Right: Quantification of western blots (n = 4). Error bars = SEM of biological replicates. (C) Western blot analysis of p53-mutant MDA-MB-231 cells that stably overexpress human ER $\alpha$  cDNA or an empty vector. Left: western blot. Right: quantification of western blot. Error bars = SEM of 4 different cell pools for each condition. \*p < 0.05, unpaired t-test. IB = immunoblot. kDa = kilodaltons. MW = molecular weight.



## MDM4 and MDM2 negatively regulate ER $\alpha$ expression and form a protein complex with ER $\alpha$

We have presented evidence that ER $\alpha$  positively regulates MDM4 and MDM2 expression. However, a previous study has also demonstrated that MDM2 negatively regulates ER $\alpha$  expression [69]. Together, these findings suggest that a negative feedback loop exists between ER $\alpha$  and MDM2 at the expression level. We further hypothesized that a similar negative feedback loop may exist to opposingly regulate ER $\alpha$  and MDM4 expression. To test this hypothesis, we first confirmed that MDM2 negatively regulates ER $\alpha$  expression. Consistent with a previous report [69], western blot analysis of MCF7 cells that had been transiently transfected with MDM2 siRNA or non-silencing control siRNA revealed that loss of MDM2 resulted in an approximately 50% increase in ER $\alpha$  expression (Supplementary Figure S7, panel A). Similarly, siRNA-mediated knockdown of MDM4 also increased ER $\alpha$  expression by approximately 60% in MCF7 cells (Figure 6A). Therefore, not only does ER $\alpha$  positively regulate MDM4 and MDM2 expression (Figures 2 and 3), but MDM4 and MDM2 also negatively regulate ER $\alpha$  expression (Figure 6A and Supplementary Figure S7, panel A). This finding is indicative of the existence of negative feedback loops that regulate ER $\alpha$  and MDM4 expression and that similarly regulate ER $\alpha$  and MDM2 expression.

It has been proposed that MDM2 negatively regulates ER $\alpha$  expression by forming a protein complex with ER $\alpha$ , which either directly or indirectly facilitates the ubiquitination and proteasomal degradation of the receptor [69]. However, studies have demonstrated that MDM2 and MDM4 preferentially exist as a heterodimer [16, 100], and the MDM2/MDM4 heterodimeric complex is a more potent E3 ubiquitin ligase than the MDM2 homodimer [10, 16, 20, 30, 101]. Based on these lines of evidence, we hypothesized that MDM4, like MDM2, would be capable of forming a protein complex with ER $\alpha$ . Although protein complexes consisting of MDM2-ER $\alpha$ , MDM2-MDM4, MDM2-p53, MDM4-p53 and ER $\alpha$ -p53 have been identified [18, 21, 23, 27, 28, 41, 43, 66, 69, 102–108], complexes consisting of MDM4-ER $\alpha$  have never before been described. In addition, protein complexes comprised of endogenously-expressed MDM2 and ER $\alpha$  proteins have not been characterized in breast cancer cells.

Having already established that ER $\alpha$ , MDM4 and MDM2 are all localized in the nuclear compartment of breast cancer cells (Figure 2), we next used size exclusion column chromatography and co-immunoprecipitation (co-IP) assays to assess the ability of ER $\alpha$  to form a protein complex with MDM4, as compared to MDM2 and p53, which served as positive controls. Size exclusion column chromatographic analyses of MCF7 lysates revealed that a portion of ER $\alpha$  co-fractionated with MDM4, MDM2 and p53 in high molecular weight fractions (Figure 6B), supporting the potential for these proteins to form a complex with one another. We

then analyzed endogenous protein complexes comprised of ER $\alpha$  and MDM4 by co-IP in ZR-75-1 cells (Figure 6C) and MCF7 cells (Supplementary Figure S7, panel B). Using MDM4 antibody (lane 5) or ER $\alpha$  antibody (lane 6) in reciprocal co-IP assays, we found that MDM4 was indeed in complex with ER $\alpha$  in both cell lines. As positive controls for previously identified protein complexes, we observed that p53 antibody (lane 3) co-immunoprecipitated MDM2, MDM4, and ER $\alpha$ . Also, MDM2, MDM4, and ER $\alpha$  antibodies (lanes 4, 5, and 6, respectively) co-immunoprecipitated p53 in reciprocal co-IP experiments. Likewise, interactions between endogenous MDM2 and MDM4 were also detected in co-IP assays when we used either MDM2 antibody (lane 4) or MDM4 antibody (lane 5) for immunoprecipitation, as were interactions between ER $\alpha$  and MDM2 when we used either ER $\alpha$  antibody (lane 6) or MDM2 antibody (lane 4) for immunoprecipitation. As a negative control, normal IgG (lane 2) failed to immunoprecipitate any of our proteins of interest.

To determine if protein complexes consisting of ER $\alpha$  and MDM4 were relevant *in vivo*, we conducted a co-IP experiment using lysate from an ER $\alpha$ -positive, treatment-naive, primary breast carcinoma patient tissue sample. Again, we observed that MDM4 co-immunoprecipitated with ER $\alpha$  (Figure 6D). The interaction between ER $\alpha$  and p53 is shown as a positive control. As a negative control, normal IgG failed to immunoprecipitate ER $\alpha$ , MDM4, and p53. Therefore, we conclude that ER $\alpha$  is found in complex with MDM4 in human breast cancer cell lines and in patient breast tumors. This is the first report to demonstrate that ER $\alpha$  and MDM4 exist in a protein complex with one another, and it is also the first report to demonstrate that endogenous ER $\alpha$  and MDM2 similarly complex with one another in breast cancer cells.

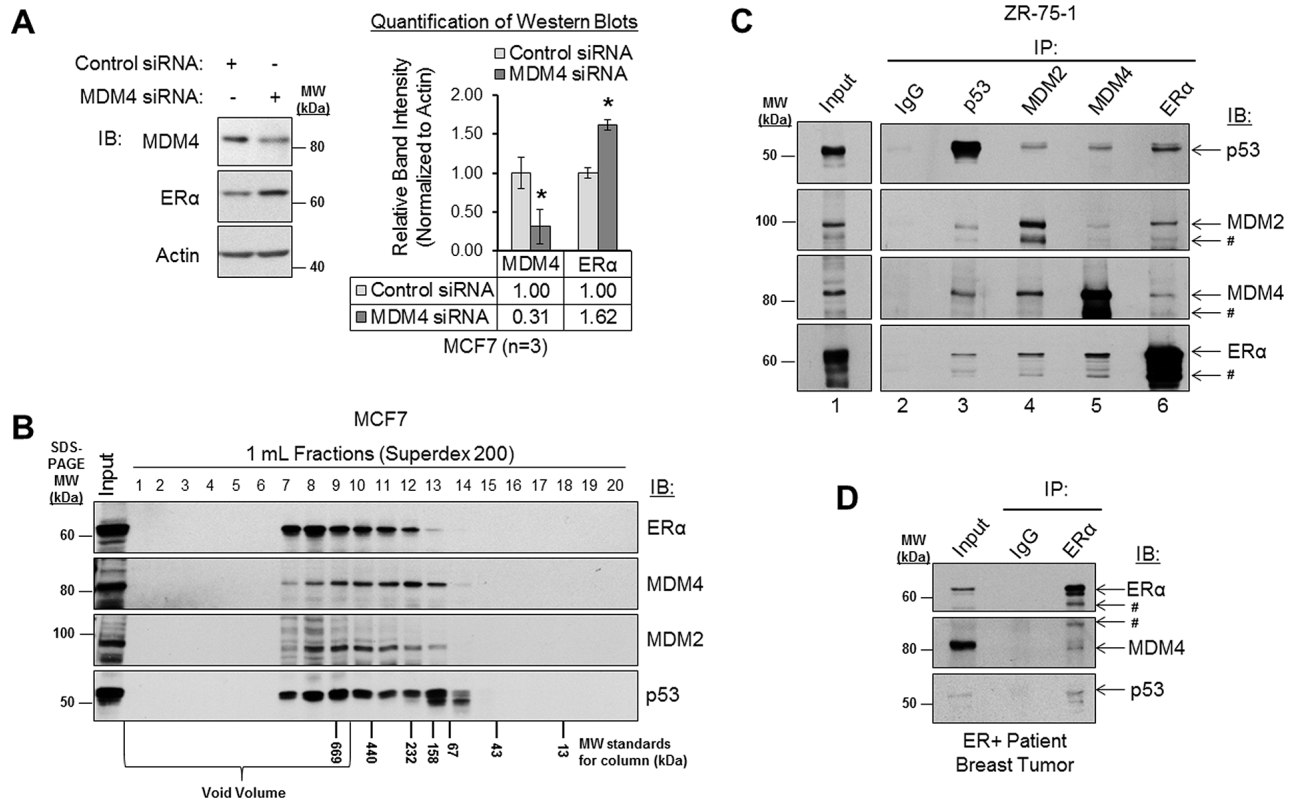
To summarize, our collective findings describe a negative feedback loop, wherein ER $\alpha$  upregulates MDM4 and MDM2 expression, and in turn, MDM4 and MDM2 downregulate ER $\alpha$  expression. This builds upon the complex regulatory loops that have previously been described for p53, ER $\alpha$ , MDM4, and MDM2 (summarized in Figure 7) and link ER $\alpha$  to MDM4 for the first time. In addition, we have identified MDM4 as a novel ER $\alpha$ -interacting protein, and we confirm prior reports that MDM2 and p53 also form a protein complex with ER $\alpha$ . These findings point to the existence of important signaling crosstalk amongst ER $\alpha$ , MDM4, MDM2 and p53 in human breast cancer.

## DISCUSSION

Previous studies have demonstrated that MDM2 is overexpressed in human breast cancer and that ER $\alpha$  is coexpressed with MDM2 and regulates *MDM2* gene expression [38, 47, 49–54, 56, 61–68]. The MDM2 homolog, MDM4, is also overexpressed in human breast cancer [49, 53, 55, 70]. However, the mechanisms underlying its overexpression remained to be elucidated. In

the present work, we report that ER $\alpha$  expression correlates not only with *MDM2* gene expression in primary breast carcinoma samples but also with *MDM4* expression. In addition, we demonstrate that tumors of luminal A and luminal B molecular subtypes have the highest expression of *MDM2* and *MDM4* mRNA, in agreement with a prior study that observed that MDM4 protein expression is similarly elevated in luminal A/B breast tumors [55]. We also provide evidence that ER $\alpha$  mediates the p53-independent upregulation of MDM4 in human breast cancer cell lines, thereby providing an explanation for why MDM4 is overexpressed in ER $\alpha$ -positive luminal breast cancer and also identifying a novel p53-independent mechanism of regulating MDM4 expression. Other groups have also reported additional factors that similarly regulate either MDM2 or MDM4 expression independently of p53, such as MAPK, which regulates MDM4 expression, and NF-KB, TGF $\beta$ /SMAD and PTEN, which regulate MDM2

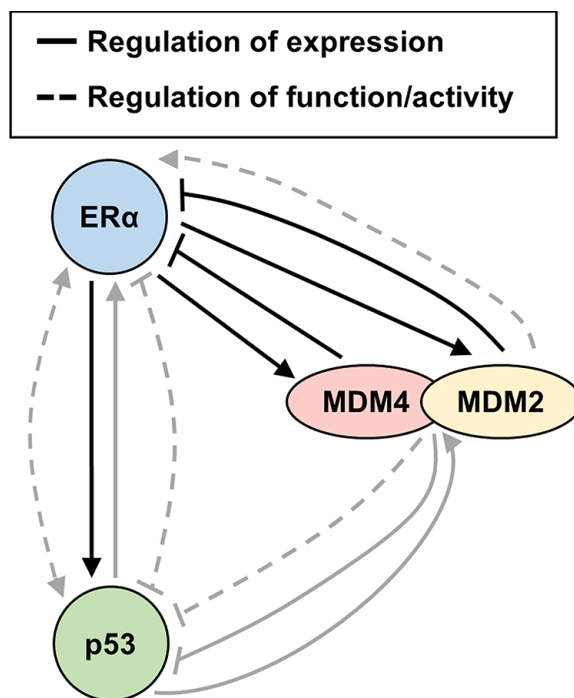
expression [73, 109–112]. Based on our observations from ChIP datamining studies, we postulate that ER $\alpha$ -dependent overexpression of MDM4 and MDM2 in human breast cancer is mediated, at least in part, by the ability of ER $\alpha$  to directly bind to and transcriptionally upregulate the *MDM4* and *MDM2* genes. We also observed that ER $\alpha$  positively regulates p53 expression; a finding that is supported by a recent study that reported that ER $\alpha$  directly regulates *TP53/p53* gene transcription [93]. Together, these findings indicate that ER $\alpha$  supports the expression of the entire MDM4-MDM2-p53 signaling axis. We have herein provided evidence that ER $\alpha$ -targeting therapies block the ability of ER $\alpha$  to modulate MDM4 and MDM2 expression. Therefore, as enthusiasm for the preclinical development of novel MDM4/MDM2 inhibitors builds (reviewed in [3]), we propose that these future agents may show utility in combination with hormonal therapies for the treatment of MDM4/MDM2-driven breast cancers.



**Figure 6: MDM4 and MDM2 negatively regulate ER $\alpha$  expression and form a protein complex with ER $\alpha$ .** (A) Western blot analysis of MCF7 cells that had been transfected with control siRNA or MDM4 siRNA for 48 h. Left panel: representative western blot. Right panel: quantification of western blots ( $n = 3$ ). Error bars = SEM of biological replicates.  $*p < 0.05$ , unpaired  $t$ -test. (B) Western blot analysis of MCF7 lysates that were subjected to size exclusion column chromatography gel filtration. The fraction numbers are labeled above the blot. The molecular weight markers for column fractionation are labeled below the blot. The molecular weight markers for SDS-PAGE are labeled to the right of the blot. (C) Co-immunoprecipitation (co-IP) analysis of protein complexes in ZR-75-1 lysates. Immunoprecipitation (IP) was performed with normal IgG, p53 antibody, MDM2 antibody, MDM4 antibody or ER $\alpha$  antibody, as labeled above the blot. Immunoblot (IB) detection was performed using p53, MDM2, MDM4, or ER $\alpha$  antibodies, as labeled to the right of the blot. IgG is shown as a negative control. Interactions between p53-MDM2, p53-MDM4 and p53-ER $\alpha$  are shown as positive controls. (D) Co-IP analysis of protein complexes in lysate from a primary ER $\alpha$ -positive patient breast tumor sample. IP was performed with normal IgG or ER $\alpha$  antibody, as labeled above the blot. IB detection was performed using ER $\alpha$ , MDM4 or p53 antibodies, as labeled to the right of the blot. IgG is shown as a negative control. Interaction between p53-ER $\alpha$  is shown as a positive control. # = related isoform, protein fragment, or non-specific band. IB = immunoblot. IgG = immunoglobulin G. IP = immunoprecipitation. kDa = kilodaltons. MW = molecular weight.

Why does ER $\alpha$  upregulate MDM4 and MDM2 expression, and what is the physiological relevance of such regulation? Since MDM4 and MDM2 play well-characterized proliferative and prosurvival roles in breast cancer models [38–44, 55, 70–72], ER $\alpha$ -dependent upregulation of MDM4 and MDM2 surely enhances these processes. In addition, ER $\alpha$  may modulate MDM4 and MDM2 expression for the purpose of inhibiting p53 function, despite upregulating p53 expression. Surprisingly, we found that the downregulation of MDM4 and MDM2 in response to loss of ER $\alpha$  signaling did not result in an increase in p53 expression, indicating that the p53 degradation pathway was unlikely to have been compromised. This points to the existence of MDM4/MDM2-independent p53 degradation pathways in breast cancer cells. Indeed, many other E3 and E4 ubiquitin ligases have been implicated in p53 ubiquitination (reviewed in [113]). Aside from ubiquitinating p53, MDM4 and MDM2 also inhibit p53's transactivation domain. We propose that ER $\alpha$ -dependent modulation of MDM4 and

MDM2 expression might not affect p53 ubiquitination but may negatively impact the ability of p53 to regulate gene transcription. In support of this hypothesis, in our previous studies, we have demonstrated that loss of ER $\alpha$  results in the upregulation of p53-target genes and that ER $\alpha$  antagonizes p53's transactivation function in breast cancer cells [102, 103]. In these reports, we discovered that ER $\alpha$ -dependent inhibition of p53-target gene expression was mediated, at least in part, by the ability of ER $\alpha$  to form a protein complex with p53 and to recruit a co-repressor complex to p53 response elements [102, 103]. We now demonstrate in the present study that ER $\alpha$  not only forms a protein complex with p53, but also with MDM4 and MDM2; and furthermore, ER $\alpha$  upregulates the expression of MDM4 and MDM2—two proteins that can potentially repress p53's transactivation domain [18, 23, 26–28]. Therefore, future studies should determine if ER $\alpha$  partly antagonizes the ability of p53 to regulate target gene transcription via MDM4/MDM2-dependent mechanisms. Such a finding would likely have a global impact on p53



**Figure 7: Summary diagram.** Schematic diagram of feedback loops and signaling crosstalk amongst MDM4, MDM2, ER $\alpha$  and p53 that govern their expression and function. Findings from the present report are indicated with black arrows. Findings from prior studies are indicated with grey arrows. The present study demonstrates that ER $\alpha$  positively regulates MDM2 and MDM4 expression, while MDM2 and MDM4 negatively regulate ER $\alpha$  expression, in agreement with a previous report that also demonstrated that MDM2 negatively regulates ER $\alpha$  expression [69]. In addition, it is known from prior reports that MDM2 enhances the transactivation function of ER $\alpha$  [41, 43]. In prior studies, p53 and ER $\alpha$  have been demonstrated to mutually support one another's expression, in agreement with our finding from the present study where we observed that ER $\alpha$  positively regulates p53 expression; however, it is known that p53 and ER $\alpha$  inhibit one another's ability to regulate the expression of some genes and cooperate with one another to mutually upregulate other genes [93–97, 102, 105, 108, 114, 141–145]. MDM2 and MDM4 negatively regulate p53 expression via ubiquitination and also repress the transactivation function of p53; yet, p53 transcriptionally induces MDM2 and MDM4 expression [1–4, 31–37]. Additional levels of regulation that are not included in the diagram include the ability of MDM4 to promote p53-mediated transcriptional upregulation of MDM2 [130], the ability of the MDM2/MDM4 heterodimer to negatively regulate its own expression via auto-ubiquitination [10, 20, 30, 101, 146], and the ability of MDM2 and MDM4 to enhance p53 translation under conditions of cell stress [147, 148]. Furthermore, we have herein demonstrated that all four of the illustrated proteins form endogenous protein complexes with one another in breast cancer cells.

gene expression signatures in ER $\alpha$ -positive cells, as we have herein demonstrated that MDM4 and MDM2 are highly expressed in ER $\alpha$ -positive tumors, and Bailey et al. have demonstrated in a genome-wide breast cancer study that ER $\alpha$  antagonizes the expression of approximately 150 putative p53-regulated transcripts [114].

We also studied crosstalk between ER $\alpha$ , MDM2, and MDM4 in terms of protein-protein interactions. Despite protein-protein interactions having been described previously for MDM2-ER $\alpha$ , MDM2-MDM4, MDM2-p53, MDM4-p53 and ER $\alpha$ -p53 [18, 21, 23, 27, 28, 41, 43, 66, 69, 102–108], our study is the first to describe an interaction between ER $\alpha$  and MDM4 and to identify protein complexes comprised of endogenously-expressed ER $\alpha$  and MDM2 in breast cancer cells. Whether or not ER $\alpha$  binds directly to MDM4, or if MDM2 and/or p53 facilitate their interaction remains to be elucidated in future studies. However, it is conceivable that the four proteins exist in a single complex with one another and may function together to regulate several biological processes. At present, few MDM4-interacting proteins have been discovered, highlighting the novelty of our work. However, MDM2 has been reported to interact with a great number of proteins, including ATF3, AR, DYRK2, GR, HIPK2, hnRNP-K, IGF-1R, JMY, Nbs1, Notch4, Numb, TAB1, and various ribosomal proteins, to name only a few [2, 115–117]. It is likely that the protein interaction between ER $\alpha$  and MDM2/MDM4 and the ability of ER $\alpha$  to upregulate MDM2/MDM4 expression either directly or indirectly influences the abilities of MDM2/MDM4 to bind to and to regulate the function or expression of other proteins; therefore, ER $\alpha$  may contribute to breast oncogenesis by impinging upon MDM2/MDM4-dependent cellular processes that had not been speculated previously to be associated with ER $\alpha$  activity.

Additionally, the ER $\alpha$ -MDM4-MDM2 protein complex might also play important roles in positively regulating the already characterized protumorigenic functions of ER $\alpha$ , such as the ability of ER $\alpha$  to transactivate target genes. Two groups of investigators have shown that MDM2 enhances ER $\alpha$ 's transactivation function [41, 43] and that MDM2 may play a general role in mediating transcriptional crosstalk between ER $\alpha$  and steroid receptor coactivator (SRC) proteins [41]. Furthermore, MDM2 is recruited with ER $\alpha$  to ER $\alpha$ -target genes in chromatin immunoprecipitation assays [89, 118, 119], and loss of MDM2 blocks the ability of ER $\alpha$  to transcriptionally upregulate its targets [41]. It has long been known that transcriptional activation of ER $\alpha$ -target genes by 17 $\beta$ -estradiol is linked to the subsequent proteasomal degradation of the receptor [87, 120–124]; therefore, it is possible that an additional function of the ER $\alpha$ -MDM4-MDM2 protein complex might be to regulate the ubiquitination of activated-ER $\alpha$  in concert with gene transcription. Since MDM4 and MDM2 preferentially exist as a heterodimer, and since heterodimerization is required for their potent E3 ligase activity [10, 16, 20,

21, 30, 100], we speculate that both MDM4 and MDM2 would be required to ubiquitinate ER $\alpha$ . In support of this hypothesis, we have demonstrated that both MDM4 and MDM2 form a protein complex with ER $\alpha$  and both proteins negatively regulate ER $\alpha$  expression. While MDM2 has been implicated in either the direct or indirect ubiquitination of many proteins—including the nuclear receptors ER $\alpha$ , ER $\beta$ , and androgen receptor [69, 125–127]—few studies have assessed the ability of MDM4 to ubiquitinate substrate proteins, other than its MDM2-dependent roles in auto-ubiquitination and the ubiquitination of p53 and MDM2 [10, 16, 20, 30, 101]. Additional investigation is presently underway to assess the role of MDM4 in regulating both the activity and proteasomal degradation of nuclear receptors via MDM2-dependent processes.

In conclusion, we have herein demonstrated that ER $\alpha$  expression associates with *MDM4* and *MDM2* gene expression in primary breast invasive carcinoma samples. Our *in vitro* analyses of human breast cancer cells reveal that ER $\alpha$  positively regulates MDM4 and MDM2 expression via p53-independent mechanisms, while MDM4 and MDM2 negatively regulate ER $\alpha$  expression. In addition, ER $\alpha$  forms a protein complex with MDM4, MDM2 and p53, suggesting direct signaling crosstalk amongst these four proteins. Future studies are needed to ascertain the intricate mechanisms which govern signaling crosstalk between ER $\alpha$ , MDM4 and MDM2, and to delineate the physiological relevance of such crosstalk in terms of p53 and ER $\alpha$  biology, breast tumorigenesis, and therapeutic response.

## MATERIALS AND METHODS

### Ethics statement

For the co-IP assay, de-identified patient breast tumor tissue was obtained from the Roswell Park Cancer Institute (RPCI) Pathology Resource Network. Investigation has been conducted in accordance with ethical standards of, the Declaration of Helsinki and has been approved by the authors' institutional review board. For the analysis of the Cancer Genome Atlas (TCGA) breast invasive carcinoma (BRCA) cohort, de-identified patient data from the public domain were used in accordance with TCGA data portal user guidelines.

### Gene expression analysis of the cancer genome atlas invasive breast carcinoma cohort

The Cancer Genome Atlas invasive breast carcinoma (TCGA BRCA) dataset is available from TCGA data portal (<https://tcga-data.nci.nih.gov/tcga/>). We downloaded the following data (as described by TCGA Network [75]) for TCGA BRCA cohort: de-identified clinical data of 949 patients, somatic mutation data of 825 patients, intrinsic molecular subtype assignment of

522 patients, and normalized RNA-Seq gene expression data of 778 patients using RNA-Seq by Expectation Maximization (RSEM) [128].

Patients with available RNA expression data, available clinical ER $\alpha$  status, available *TP53*, *MDM2*, and *MDM4* somatic mutation/amplification data, and available intrinsic molecular subtype assignment data were used to study *MDM2* and *MDM4* gene expression. 38 patients with *MDM2* amplification and 109 patients with *MDM4* amplification (according to [75]) were excluded from the analyses of *MDM2* and *MDM4* gene expression, respectively, to study effects on gene expression that were independent of gene amplification. The patient cohort was classified into five different intrinsic molecular subtypes (luminal A, luminal B, HER-enriched, basal-like and normal-like) using RNA expression of the PAM50 50-gene subtype predictor [76], according to [75]. For gene expression analysis between two groups, the *t*-test was applied. For gene expression analysis amongst more than two groups, an analysis of variance (ANOVA) was applied. The Pearson correlation test was applied to assess correlations between *MDM2/MDM4* and *ESR1/ER $\alpha$*  mRNA expression. All statistical tests were carried out using R statistical software and *p*-values of less than 0.05 were considered to be statistically significant.

### Cell culture and reagents

The human breast cancer cell lines MCF7, ZR-75-1 and MDA-MB-231 (ATCC) were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Mediatech) supplemented with 10% fetal bovine serum (FBS; Life Technologies) at 37°C, under a humidified atmosphere of 5% carbon dioxide. 4-hydroxytamoxifen (Sigma) and fulvestrant (Tocris Bioscience) were solubilized in ethanol, and final ethanol concentrations in the media of treated cells were less than 0.01%. The ER $\alpha$  expression plasmid was kindly provided by Dr. Carolyn Smith (Baylor College of Medicine, Houston, TX).

### Small interfering RNA (siRNA)

siRNA transfections were performed as previously described [129]. In brief, siRNA at a final concentration of 50 nM was transfected into cells using Lipofectamine 2000 (Life Technologies), according to the manufacturer's instructions. Cells were harvested post-transfection at the times indicated in the figure legends. The ER $\alpha$  siRNA sequences were as follows: sequence #1 (Ambion, siRNA ID # s4824) and sequence #2 (Dharmacon, catalog # M-003401-04-0020). The *MDM2* siRNA was a 1:1 mix of two different sequences: sequence #1 (Qiagen, catalog # SI02653392) and sequence #2 (Ambion, siRNA ID # HSS142909). The *MDM4* siRNA was a 1:1 mix of two different sequences: sequence #1 (Qiagen, catalog # SI00037163) and sequence #2 (Ambion, custom sequence; sense 5'-GGA UAU UCC AAG UCA AGA CUU-3';

antisense 5'-GUC UUG ACU UGG AAU AUC CAU-3'; as described in [130]). The non-silencing control siRNA was from Ambion (catalog # 4390843).

### Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

MCF7, ZR-75-1 and MDA-MB-231 cells were harvested in phosphate buffered saline (PBS; Mediatech) and pelleted by centrifugation at 1,000 rpm. Cell pellets were then lysed in ice cold lysis buffer [20 mM Tris, pH 8.0; 150 mM NaCl; 5 mM MgCl<sub>2</sub>; 0.5% NP40; 2 mM PMSF; 1X EDTA-free Complete protease inhibitor cocktail (Roche)] for 30 minutes with intermittent gentle vortexing. Lysates were centrifuged at 10,000 rpm for 10 min at 4°C to remove debris. Protein concentrations were assessed by the Bradford method using Protein Assay Dye Reagent Concentrate (Bio-Rad), a BSA standard curve, and a BioTek Synergy 2 plate reader with Gen5 v1.11.5 software. Protein lysates were mixed with 4X Laemmli buffer [200 mM Tris, pH 6.8; 8% SDS; 40% glycerol; 20% 2-mercaptoethanol; 0.4% bromophenol blue] at ratios that yielded a 1X final concentration of Laemmli buffer, boiled at 100°C for 10 min, and subjected to 8% SDS-PAGE. Proteins were transferred to PVDF membranes, blocked for 45 min with 5% non-fat milk dissolved in Tris-buffered saline-tween (TBST) [10 mM Tris, pH 8.0; 150 mM NaCl; 0.05% Tween-20], and incubated with primary antibody diluted in 0.5% non-fat milk-TBST overnight at 4°C with gentle rocking. The following day, membranes were washed 3 times with TBST, incubated with horseradish peroxidase-conjugated secondary antibody diluted in 0.5% non-fat milk-TBST for 3 h at room temperature with gentle rocking, and then washed 3 times with TBST. Bands were visualized using the chemiluminescent method with ECL Western Blotting Substrate (Pierce), and films were developed using a Kodak X-Omat 200A Processor. Films were scanned using a Bio-Rad SG-800 calibrated densitometer and Quantity One v4.6.7 software. Bands were quantified using Image J software, and relative band intensity was calculated as relative to control, after having been normalized to  $\beta$ -Actin. Error bars are representative of standard error of the means of either triplicate or quadruplicate biological replicates, as indicated in the figure legends. Statistically significant differences in the means were calculated using unpaired *t*-tests.

Primary antibodies for western blotting were as follows: p53 (DO-1, Santa Cruz, catalog # sc-126) used at 1:1,000; ER $\alpha$  (HC-20, Santa Cruz, catalog # sc-543) used at 1:10,000; *MDM2* (IF2, EMD Millipore, catalog # MABE340) used at 3:1,000; *MDM4* (Bethyl Labs, catalog # A300-287A) used at 1:10,000; c-Myc (Y69, Abcam, catalog # ab32072) used at 1:2,000; and  $\beta$ -Actin (AC-15, Sigma, catalog # A5441) used at 1:10,000.

Secondary antibodies for traditional western blotting were as follows: goat anti-rabbit IgG HRP-conjugate (EMD Millipore, catalog #12-348) used at 1:10,000 and goat anti-mouse IgG HRP-conjugate (EMD Millipore, catalog # 12-349) used at 1:10,000. For western blotting following co-immunoprecipitation, light chain-specific secondary antibodies were used to prevent the detection of the antibody heavy chain. Goat anti-mouse light chain specific IgG-HRP conjugate was used at 1:10,000, and mouse anti-rabbit light chain specific IgG-HRP conjugate was used at 1:20,000 (Jackson Immuno Research, catalog # 211-032-171 and # 115-035-174, respectively).

### Quantitative real time polymerase chain reaction (qPCR)

Total RNA was isolated using Trizol Reagent (Life Technologies) and then purified by sodium acetate ethanol precipitation. RNA was quantified using a Nanodrop 8000 spectrophotometer. 1 µg of total RNA was subjected to DNase I (Life Technologies; amplification grade) digestion in a 10 µL reaction, according to the manufacturer's instructions. DNase I-digested RNA was then reverse transcribed in a 20 µL reaction using the iScript cDNA Synthesis Kit (Bio-Rad) and a Bio-Rad C1000 Touch thermocycler, according to the cycling parameters described in the manufacturer's instructions. cDNA was diluted 4-fold with molecular grade water prior to qPCR. qPCR was carried out in an ABI 7300 Real Time PCR System with 7300 System SDS Software (Applied Biosystems) in a 10 µL reaction containing iTaq Universal SYBR Green Supermix (Bio-Rad), 1.5 µL of diluted cDNA, and 500 nM of specific primers. The cycling conditions were as follows: 50°C for 15s (1 cycle); 95°C for 10 min (1 cycle); 95°C for 15 s, followed by 60°C for 45 s (35 cycles). The detection of a single amplicon was verified using a dissociation curve. Normalized, relative mRNA levels were calculated according to the  $\Delta\Delta C_q$  method, using endogenous *ACTB* as a reference gene for normalization. Error bars are representative of standard error of the means of quadruplicate biological replicates, as indicated in the figure legends. Statistically significant differences in the means were calculated using unpaired *t*-tests.

Primer efficiencies were calculated from a dilution curve and determined to be within the acceptable range of 90–110% efficiency [131]. Primers were designed using Primer 3 software [132, 133] or taken from published sources, as indicated below. Primers were verified to amplify a single PCR product by traditional agarose gel electrophoresis. The following primer sets were used for qPCR: *ACTB* (RefSeq NM\_001101.3), 5'-ATG GGT CAG AAG GAT TCC TAT-3' and 5'-AAG GTC TCA AAC ATG ATC TGG G-3', as described in [102]; *ESR1* (RefSeq NM\_000125.3), 5'-GCA GTG TGC AAT GAC TAT G-3' and 5'-CGT TAT GTC CTT GAA TAC TTC-3'; *MDM4* (RefSeq NM\_002393.4), 5'-ATC TGA CAG TGC TTG CAG GA-3' and 5'-GCT GCA TGC AAA ATC TTC AA-3'; and *MDM2* (RefSeq NM\_002392.5), 5'-GGT CGA

CCT AAA AAT GGT TGC A-3' and 5'-GGG CAG GGC TTA TTC CTT TTC-3', as described in [134].

### Chromatin Immunoprecipitation-Sequencing (ChIP-Seq) and ChIP-on-Chip (ChIP-Chip) cistromic data mining

For ER $\alpha$  ChIP-Seq and ChIP-Chip datasets, normalized ER $\alpha$  ChIP bed files and wig files were downloaded from the Nuclear Receptor Cistrome Project database ([http://cistrome.org/NR\\_Cistrome/](http://cistrome.org/NR_Cistrome/) Cistrome.html; [80]) and the ENCODE Project database (<https://www.encodeproject.org/>; [81]). The following datasets were from Nuclear Receptor Cistrome: Antoni Hurtado dataset, unpublished data; Edison Liu dataset, (Gene Expression Omnibus (GEO) Accession: GSE23701) [85]; Hendrik Stunnenberg dataset, (GEO Accession: GSE14664) [135]; Duncan Odom dataset, (Array Express Accession: E-TABM-828) [136]; Arul Chinnaiyan dataset, (GEO Accession: GSE19013) [137]. Myles Brown dataset ([http://research.dfci.harvard.edu/brownlab/datasets/index.php?dir=ER\\_whole\\_human\\_genome](http://research.dfci.harvard.edu/brownlab/datasets/index.php?dir=ER_whole_human_genome)) [84]; and Kevin White dataset, (GEO Accession: GSE15244) [138]. The following dataset was from ENCODE: Richard Meyers/ENCODE Consortium dataset, (GEO accession: GSE32465) [81]. Bed and wig files were viewed using Integrated Genomics Viewer (IGV) software [139]. Data are displayed in hg19 alignment format.

Normalized FOXA1 ChIP bed files were downloaded from the from the Jason Carroll lab website (<http://www.carroll-lab.org.uk/data>) and are described in [86]. Bed files were converted from hg18 genomic coordinates to hg19 genomic coordinates using the UCSC Batch Coordinate Conversion Lift Over Tool software (<https://genome.ucsc.edu/cgi-bin/hgLiftOver>), described in [140]. Bed files were viewed using IGV.

### Immunofluorescent (IF) microscopy

MCF7 cells were seeded onto sterile glass coverslips in 6-well plates at a density of 0.2 million cells per well. The following day, cells were transfected with siRNA. After the times indicated in the figure legends, cells were rinsed twice with PBS (Mediatech) and then fixed with 2 mL of fixation buffer [4% paraformaldehyde; PBS, pH 7.4] for 15 min at room temperature with gentle rocking. Coverslips were then rinsed 4 times with PBS prior to incubation with 2 mL of permeabilization buffer [0.5% Triton X-100; PBS, pH 7.4] for 10 minutes at room temperature with gentle rocking. Coverslips were rinsed 3 times with PBS and then blocked with blocking buffer [3% non-fat milk; PBS, pH 7.4] for 1 h at room temperature with gentle rocking. Coverslips were rinsed once with PBS and then incubated with primary antibodies diluted in 1% non-fat milk-PBS for 3.5 h at room temperature. Coverslips were rinsed 3 times with PBS and then incubated with secondary antibodies diluted in 0.5% non-fat milk-PBS for 1.5 h at room

temperature. Coverslips were rinsed once with PBS and then incubated with a 1 mg/mL solution of 4',6-diamidino-2-phenylindole (DAPI) diluted in PBS for 15 min at room temperature. Coverslips were rinsed 2 times with PBS and then 1 time with water, prior to being mounted onto slides with ProLong Gold antifade medium (Life Technologies) and CoverGrip Coverslip Sealant (Biotium).

Cells were visualized with a Zeiss epifluorescence microscope and 100X Plan-Neofluar, 63X Plan-Apochromat, and 40X Plan-Neofluar objective lenses. Images were obtained with a digital camera and Image-Pro Plus v4.5.1.29 or Spot Advanced v5.2 software.

Primary antibodies for IF were as follows: ER $\alpha$  HC-20 antibody (Santa Cruz, catalog # sc-543) at 1:200; MDM2 SMP14 antibody (Santa Cruz, catalog # sc-965) at 1:100; MDM2 4B11 antibody (EMD Millipore, catalog # OP143) at 1:25; MDM2 IF2 antibody (EMD Millipore, catalog # MABE340) at 1:50; and MDM4 C86 antibody (Millipore catalog # 04-1555) at 1:25.

Secondary antibodies for IF were as follows: Alexa Fluor 488 Goat anti-Mouse IgG (H+L) (Invitrogen Molecular Probes, catalog # A11001) and Alexa Fluor 595 Goat anti-Rabbit IgG (H+L) (Invitrogen Molecular Probes, catalog # A11012), both used at 1:500.

### Generation of stable cell lines

To generate p53 knockdown cells and corresponding control cells, MCF7 and ZR-75-1 cells were infected with lentiviral particles expressing pLKO.1-p53 shRNA or pLKO.1-GFP shRNA (kind gifts from Dr. Xinjiang Wang, Roswell Park Cancer Institute, described in [134]), followed by selection with puromycin for one week at 2  $\mu$ g/mL. The appropriate puromycin concentration was determined from a kill curve. Two different p53 shRNA pools were generated for each cell line.

To generate ER $\alpha$ -overexpressing cells, MDA-MB-231 cells were transfected with 6  $\mu$ g of PCR3.1 plasmid (Invitrogen), encoding either human ER $\alpha$  cDNA (described in [103]) or no insert. Cells were selected with 800  $\mu$ g/mL of G418 sulfate (Mediatech) for 3 weeks and were maintained in 400  $\mu$ g/mL of G418 sulfate. The appropriate G418 sulfate concentration was determined from a kill curve. Four different ER $\alpha$ -overexpressing pools and four different empty vector pools were generated.

### Size exclusion column chromatography

MCF7 cells were lysed in ice cold gel filtration lysis buffer [50 mM HEPES, pH 7.4; 150 mM NaCl; 0.5% NP-40; 1X EDTA-free Complete Protease Inhibitor Cocktail (Roche)] at 4°C with gentle rotation for 30 min. Lysates were then centrifuged twice at 15,000 rpm for 15 min to remove debris. 2.5 mg of lysate was then fractionated by size-exclusion chromatography at 4°C, using a Superdex 200 10/300 GL column, an AKTA Purifier system and Unicorn 5.1 software (GE Healthcare). Protein complexes

were eluted using column buffer [25 mM HEPES, pH 7.4; 150 mM NaCl], and 24 fractions of 1 mL each were collected. Equal volumes of each of the different fractions were then analyzed by western blotting. The column was calibrated with the following molecular weight standards: Blue Dextran 2000, thyroglobulin, ferritin, catalase, aldolase, bovine serum albumin, ovalbumin, and ribonuclease A (GE Healthcare). Based on the Blue Dextran 2000 elution profile, the void volume was determined to be 9 mL, in agreement with the manufacturer's predicted void volume.

### Co-immunoprecipitation (Co-IP)

ZR-75-1 and MCF7 cells were harvested by scraping in PBS (Mediatech) and pelleted by centrifugation at 1,000 rpm. Cell pellets were lysed in ice cold lysis buffer [20 mM Tris, pH 8.0; 150 mM NaCl; 5 mM MgCl<sub>2</sub>; 0.5% NP40; 1X EDTA-free Complete protease inhibitor cocktail (Roche)] for 30 minutes with intermittent gentle vortexing. Lysates were centrifuged at 15,000 rpm for 10 min at 4°C and then pre-cleared with protein G agarose (Life Technologies) for 1 h at 4°C with gentle rotation. 2.5 mg of precleared lysate was then incubated overnight with 6  $\mu$ g of antibody at 4°C with gentle rotation. The following day, immunogen-antibody complexes were captured by incubation with protein G agarose for 3 h at 4°C with gentle rotation. The protein G agarose was then washed 4 times with wash buffer [20 mM Tris, pH 8.0; 150 mM NaCl; 5 mM MgCl<sub>2</sub>; 1X EDTA-free Complete protease inhibitor cocktail (Roche)], the agarose pellets were collected, and residual wash buffer was removed. Protein complexes were eluted in 2X Laemmli buffer [100 mM Tris, pH 6.8; 4% SDS; 20% glycerol; 10% 2-mercaptoethanol; 0.2% bromophenol blue], boiled at 100°C for 10 min and then analyzed by 7% SDS-PAGE, followed by western blotting, as described above.

Co-IP of human breast tumor lysate was conducted with the following modification; 3 mg of fresh, frozen breast carcinoma tissue from a treatment-naive patient was pulverized using a dounce homogenizer prior to lysis.

IP antibodies were as follows: Normal mouse and normal rabbit IgG (EMD Millipore, catalog # 12-371 and # 12-370); p53 antibodies as a 1:1:1 mix of DO-1 (Santa Cruz, catalog # sc-126), FL-393 (Santa Cruz, catalog # sc-6243) and PAb421 (EMD Millipore, catalog # OP03L); ER $\alpha$  antibodies as a 1:1 mix of HC-20 (Santa Cruz, catalog # sc-543) and D-12 (Santa Cruz, catalog # sc-8005); MDM2 antibodies as a 1:1:1 ratio of SMP14 (Santa Cruz, catalog # sc-965), N-20 (Santa Cruz, catalog # sc-913) and 4B11 (EMD Millipore, catalog # OP143); and MDM4 antibody (Bethyl Labs, catalog # A300-287A).

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## CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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