Molecular and Cellular Endocrinology 415 (2015) 76–86

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

The estrogen receptor alpha nuclear localization sequence is critical for fulvestrant-induced degradation of the receptor

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ARTICLE INFO

Article history:
Received 13 May 2015
Received in revised form
5 August 2015
Accepted 5 August 2015
Available online 10 August 2015

Keywords:
Breast cancer
Degradation
Endocrine therapy
Estrogen receptor
Fulvestrant
Nuclear localization sequence

ABSTRACT

Fulvestrant, a selective estrogen receptor down-regulator (SERD) is a pure competitive antagonist of estrogen receptor alpha (ERα). Fulvestrant binds ERα and reduces the receptor's half-life by increasing protein turnover, however, its mechanism of action is not fully understood. In this study, we show that removal of the ERα nuclear localization sequence (ERΔNLS) resulted in a predominantly cytoplasmic ERα that was degraded in response to 17-β-estradiol (E2) but was resistant to degradation by fulvestrant. ERΔNLS bound the ligands and exhibited receptor interaction similar to ERα, indicating that the lack of degradation was not due to disruption of these processes. Forcing ERΔNLS into the nucleus with a heterologous SV40-NLS did not restore degradation, suggesting that the NLS domain itself, and not merely receptor localization, is critical for fulvestrant-induced ERα degradation. Indeed, cloning of the endogenous ERα NLS onto the N-terminus of ERΔNLS significantly restored both its nuclear localization and turnover in response to fulvestrant. Moreover, mutation of the sumoylation targets K266 and K268 within the NLS impaired fulvestrant-induced ERα degradation. In conclusion, our study provides evidence for the unique role of the ERα NLS in fulvestrant-induced degradation of the receptor.

1. Introduction

The female sex hormone estradiol is implicated in breast cancer pathogenesis. The effects of 17-β-estradiol (E2), the most potent estrogen, are mediated via estrogen receptors (ERs) ERα and ERβ. Endocrine therapy targets ERα and approximately 70% of breast cancers are ERα-positive (McGuire, 1975). Moreover, the clinical value of ERα status in determining response to endocrine therapy has been established (Maynard et al., 1978).

ERα has a modular structure with several distinct domains, including an amino-terminally located ligand-independent transcriptional activation function (AF-1) domain (amino acids 1–184), a DNA binding domain (DBD; amino acids 185–250), a hinge region (amino acids 251–354), and a ligand-dependent AF-2 domain (amino acids 355–549). The hinge region of several nuclear receptors was originally thought of as a flexible linker between the DBD and the AF-2 domain (Khorasanizadeh and Rastinejad, 2001). However, for many nuclear receptors, including ERα, this region also serves important regulatory functions, serving as a site for a

Abbreviations: AF-1, activation function 1; AF-2, activation function 2; ChIP, Chromatin immunoprecipitation; DBD, DNA binding domain; DMSO, Dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; E2, 17-β-estradiol; ERΔNLS, ERα with deletion of nuclear localization sequence; IP, Immunoprecipitation; LBD, ligand binding domain; MEM, Minimum essential medium; NLS, nuclear localization sequence; PBS, phosphate buffered saline; q-RT-PCR, real-time quantitative reverse transcription polymerase chain reaction; SEM, standard error of mean; SERD, selective estrogen receptor down-regulator.

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http://dx.doi.org/10.1016/j.mce.2015.08.007
0303-7207/

number of post-translational modifications, including acetylation (Wang et al., 2001), phosphorylation (Cui et al., 2004), sumoylation (Sentis et al., 2005), methylation (Zhou et al., 2009), and ubiquitination (Berry et al., 2008). Additionally, it is important for both ERα DNA binding (Schultz et al., 2002; Melvin et al., 2002; Melvin et al., 2004) and receptor subcellular localization (Ylikomi et al., 1992). Its importance for localization stems from the fact that the ERα nuclear localization sequence (NLS) is located within the hinge region.

Deletion and fusion experiments have identified the amino acids within ERα that are critical for its nuclear accumulation. ERα amino acids 256–303 are sufficient to target a heterologous protein, β-galactosidase, to the nucleus (Picard et al., 1990). However, deletions within the context of the endogenous ERα protein showed that amino acids 274–298 do not possess any NLS function (Ylikomi et al., 1992), but that an ERα mutant with a deletion of amino acids 250–274 (identical to the deletion in our ERΔNLS) was completely cytoplasmic in the absence of ligand. Thus, the functional ERα NLS lies within these amino acids. An ERα mutant protein with every lysine and arginine between amino acid positions 253–271 (9 residues in total) mutated to alanine was completely cytoplasmic in the absence of hormone (Burns et al., 2011). Interestingly, a hormone-inducible NLS has also been identified within the ligand binding domain (LBD) of ERα (Ylikomi et al., 1992). While this NLS can cooperate with the hinge region NLS, on its own, it is insufficient to promote nuclear localization of the receptor.

Regulation of ERα target genes is critical for breast cancer progression. Fulvestrant, belongs to the class of anti-estrogens known as selective estrogen receptor down-regulators (SERDs). Fulvestrant (ICI 182,780) is currently approved by the United States Food and Drug Administration for the treatment of ERα-positive metastatic breast cancer in postmenopausal women with disease progression following prior anti-estrogen therapy (Bross et al., 2003; Bross et al., 2002). Fulvestrant is a competitive antagonist with a very similar structure to the endogenous ligand E2 and thus competes with E2 for binding to the LBD of ERα. However, a long hydrophobic side chain gives the drug its unique anti-estrogenic properties. The binding of both E2 and fulvestrant results in ERα degradation via the ubiquitin/proteasome pathway (Nawaz et al., 1999; Wijayaratne and McDonnell, 2001). However, while the turnover induced by E2 is associated with an actively functioning state of ERα (Nawaz and O'Malley, 2004), the degradation elicited by fulvestrant is associated with receptor inhibition (Osborne et al., 2004), suggesting different mechanisms of action. Despite this, the exact mechanism of fulvestrant action, including whether or not receptor degradation is actually required for its anti-estrogenic function, is currently unknown. In fact, several reports have attributed some of fulvestrant's properties to its ability to influence ERα subcellular localization. One report suggested that fulvestrant disrupts ERα nucleo-cytoplasmic shuttling, resulting in cytoplasmic accumulation of the receptor (Dauvois et al., 1993). On the other hand, other reports argue that fulvestrant treatment results in receptor immobilization and strong interaction of ERα with the nuclear matrix (Stenoien et al., 2000; Stenoien et al., 2001; Long and Nephew, 2006; Long et al., 2010; Kocanova et al., 2010).

Given the conflicting reports regarding the effect of fulvestrant on ERα localization, we decided to examine this relationship in more detail. We generated an ERα mutant with a deletion of the NLS domain (amino acids 250–274). Not only did the deletion promote receptor cytoplasmic localization, but it also influenced the degradation response elicited by fulvestrant. Mutation of two ERα sumoylation targets within the NLS does not affect nuclear localization, but significantly impaired fulvestrant induced degradation of the receptor suggesting that sumoylation of the NLS domain is required for fulvestrant-induced degradation of ERα.

2. Materials and methods

2.1. Cell culture

HEK293 and MCF-7 human breast cancer cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Cellgro) supplemented with 5% characterized fetal bovine serum (HyClone), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (antibiotics and antimycotic from Cellgro). C4-12 cells are ER-negative variants of MCF-7 cells that were derived by clonal selection from MCF-7 cells grown in the absence of estrogen for 9 months (Oesterreich et al., 2001). These cells were routinely maintained in minimum essential medium (MEM) alpha without phenol red (Invitrogen) supplemented with 5% charcoal/dextran-treated fetal bovine serum (HyClone), 100 IU/ml penicillin (Cellgro), 100 μg/ml streptomycin (Cellgro), 2 mM glutamine (Invitrogen), 10 mM HEPES (Invitrogen), and 10 μg/ml insulin (GIBCO). C4-12 cells stably expressing estrogen receptor constructs were maintained in phenol red-free MEM alpha (Invitrogen) supplemented with 5% charcoal/dextran-treated fetal bovine serum (HyClone), 100 IU/ml penicillin (Cellgro), 100 μg/ml streptomycin (Cellgro), 2 mM glutamine (Invitrogen), and 500 μg/ml geneticin (Invitrogen). Prior to ligand treatment, for all experiments, cells were starved in charcoal-stripped serum for at least one day. For ligand stimulation, cells were treated with various concentrations of 17-β-estradiol (E2; Sigma) and fulvestrant (Sigma). Vehicle control treated samples were stimulated with 0.01% ethanol. In some experiments, cells were pre-treated for 2 h with the proteasome inhibitor MG132 (2 μg/ml; Sigma) prior to ligand stimulation.

2.2. Transient and stable transfections

HEK293 cells were plated in 10 cm dishes and transiently transfected with ER-Flag (1 μg) and either GFP-ER or GFP-ERΔNLS (1 μg each). Transfections were carried out using Lipofectamine 2000 (Invitrogen) and maintained for 4 h. Following transfection, medium was aspirated, cells washed with PBS and replaced with fresh medium containing charcoal-stripped FBS. Stable transfections of GFP-ER and GFP-ERΔNLS were performed both in MCF-7 cells and in C4-12 cells. Cells were plated in 10 cm dishes and transfected with 1 μg DNA using Lipofectamine 2000 (Invitrogen). Transfection was maintained overnight (approximately 16 h) at which point cells were washed with PBS and switched to fresh medium. Selection with geneticin (1000 μg/ml) was begun approximately 48 h following transfection. Cells were maintained under high selection pressure until individual colonies began to grow. These individual colonies were selected and screened for ERα expression by Western blot analysis.

2.3. Immunoblotting

Proteins were resolved on 8% SDS-PAGE and transferred to nitrocellulose. The membrane was blocked in 5% milk dissolved in phosphate-buffered saline + 0.1% Tween 20 (PBST) for 1 h at room temperature. Primary antibodies used include anti-ERα (1:1000, Santa Cruz Biotechnology; sc-8002), anti-HA (1:1000, Covance; MMS-101P), and anti-β-actin (1:5000, Sigma; A5441). All the primary antibodies were diluted in PBST + 5% milk and the membranes were incubated at room temperature for 3 h or overnight at 4 °C. After washing three times for 5 min with PBST, the membranes were incubated with an anti-mouse secondary antibody conjugated to either IRDye700 or IRDye800 (Rockland) for 1 h at room temperature. Secondary antibodies were diluted 1:5000 in PBST + 5% milk. After incubation, membranes were washed three times for 5 min with PBST, and the signal was visualized using the Odyssey

imaging system (Li-Cor Biosciences).

2.4. RNA extraction and qRT-PCR

Total RNA was isolated using the RNeasy RNA isolation kit (Qiagen) as recommended by the supplier. Triplicate RNA samples were prepared for each treatment group. RNA was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen) in accordance with manufacturer's instructions. The PCR reaction was then carried out on an ABI 7500 fast real-time thermocycler (Applied Biosystems) using the SYBR green master mix (Applied Biosystems) and 150 nM each of both the forward and reverse primers. The cycling conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s. The sequences of the primers used are as follows: pS2, forward 5′–CCTCCCAGTGTGCAAATAAGG–3′ and reverse 5′–TCTTCTGGAGGGACGTCGAT–3'; β-actin, forward 5′–CCCTGGCACCCAGCAC–3′ and reverse 5′–GCCGATCCACACGGAGTAC–3'. The fold change for each gene was calculated using the cycle threshold ($\Delta\Delta C_T$) method as previously described (Livak and Schmittgen, 2001), and data are represented as E2-mediated fold change over vehicle-treated samples. For each sample, real-time quantitative reverse transcription-PCRs (qRT-PCRs) were done in triplicate for both the gene of interest (*pS2*) and the reference gene (*β-actin*) to normalize for input cDNA.

2.5. Immunoprecipitation

HEK293 cells were plated in 10 cm dishes and transfected with ER-Flag (1 μg) and either GFP-ER (1 μg) or GFP-ERΔNLS (1 μg). Transfection was performed using Lipofectamine 2000 (Invitrogen) and was maintained for 4 h. Following transfection, cells were starved overnight in charcoal-stripped serum and subsequently stimulated with E2 (100 nM) for 1 h. Cells were lysed in RIPA buffer and 1 mg of protein was used for immunoprecipitation (IP). IP samples were diluted out in TNESV buffer and immune-cleared with protein G/sepharose beads. IP was performed with either 2 μg anti-Flag antibody (Stratagene/Agilent; #200472-21) or 2 μg anti-IgG (Santa Cruz; sc-2025). Following IP, ERα protein on Western blot was detected using anti-ERα antibody (Santa Cruz; sc-7207).

2.6. ChIP assays

Chromatin immunoprecipitation (ChIP) assays were performed as previously described (Shang et al., 2002). Briefly, C4-12 cells stably expressing GFP-ER or GFP-ERΔNLS/SV40-NLS (5×10^6) were plated in MEMα. The next day, cells were washed twice with PBS and maintained under charcoal-stripped serum conditions. Following approximately 48 h starvation, cells were treated with vehicle or E2 (10 nM) for 45 min. After DNA purification (QIAquick PCR purification kit; Qiagen), the *pS2* ERE region was amplified by qRT-PCR using the following primer pair: forward 5′–GGCCATCTCTCACTATGAATCACTTCTGC–3′; reverse 5′–GGCAGGCTCTGTTTGCTTAAAGAGCG –3′. Data are represented as "% Input". The ChIP antibodies used were ERα (Santa Cruz Biotechnology; sc-7207) and IgG (Santa Cruz Biotechnology; sc-2027).

2.7. Immunofluorescence and confocal microscopy

Cells were grown on glass coverslips in 6-well plates and fixed in 4% paraformaldehyde. Following permeabilization with Triton X-100, cells were blocked with 5% horse serum. Primary antibodies were diluted in 1% horse serum solution and cells were incubated with antibody overnight at 4 °C. Primary antibodies used include ERα (Santa Cruz Biotechnology; sc-7207) and HA (Covance; MMS-101P). Secondary antibodies were conjugated to either AlexaFluor 488, 546, or Cy5 (Invitrogen). Nuclear counterstain was performed using DAPI (Vector Laboratories). Proteins were visualized by confocal microscopy (Leica Microsystems; CTR 6500).

2.8. Competitive radioligand binding assays

MCF-7, C4-12/GFP-ER, and C4-12/GFP-ERΔNLS cells were plated as biological duplicates in 6-well plates. Cells were starved overnight in 5% charcoal-stripped serum. Next day, cells were placed in 5% charcoal-stripped medium containing 1.5 pmol tritiated estradiol (estradiol [2, 4, 6, 7-3H (N)]; Perkin Elmer) and increasing concentrations of non-radiolabeled E2 or fulvestrant. Treatment was maintained for 2 h at 37 °C. After treatment, medium was aspirated, and cells were washed in 0.01% ethanol. A portion of the cell/ethanol mixture was combined with scintillation fluid, and counts of ionizing radiation per minute were determined using a scintillation counter. Similar experiments were also performed with HEK293 cells, which do not express ERα protein, as a negative control.

2.9. Plasmids and cloning

The cloning of N-terminal HA-tagged full-length ERα has been previously described (Oesterreich et al., 2000). To generate the GFP-tagged protein, full-length HA-ERα was subcloned into the *EcoRI* and *BamHI* restriction sites located within the multiple cloning site (MCS) of the pEGFP-C2 vector (Clontech). The 25 amino acid deletion of pEGFP/ERΔNLS (amino acids 250–274) was generated by two separate PCR reactions that amplified the sequence of the N-terminus and the sequence of the C-terminus, omitting the 75 nucleotides that encode the NLS. Following amplification of both ends, the fragments were fused together by blunt-end ligation. This ERΔNLS sequence was then inserted into pEGFP-C2 at the *EcoRI* and *BamHI* restriction sites of the MCS. To generate pEGFP-ERΔNLS/SV40-NLS, the pEGFP/ERΔNLS vector was first digested with *XhoI* and *EcoRI*. This vector contains each restriction site only once, and both are located within the MCS. Double-stranded oligos encoding the SV40-NLS and possessing *XhoI* and *EcoRI* sticky ends (SV40-NLS oligo sequences; sense strand 5′–TCGAGGCCAAAGAAGAAGCGTAAGGTTGGTG–3′ and antisense strand 5′–AATTCACCAACCTTACGCTTCT TCTTTGGCC–3′) were then ligated into the *XhoI* and *EcoRI* sites in the pEGFP/ERΔNLS vector. The same exact strategy (including identical restriction sites) was used to generate the pEGFP-ERΔNLS/endog-NLS vector, except that double-stranded oligos encoding the endogenous ERα NLS were used (endogenous ERα NLS oligo sequences; sense strand 5′-TCGAGGATGATGAAAGGTGGGATACGAAAAGAC CGAAGAGGAGGGAGAATGTTGAAACACAAGCGCCAGAGAGATGATGGGG–3′ and antisense strand 5′–AATTCCCCATCATCTCTCTGGCGCTTGTGTTTCAACATTCTCCCTCC T CTTCGGTCTTTTCGTATCCCACCTTTCATCATCC–3′). All vector sequences were confirmed by DNA sequencing. The plasmid pCR3.1-ERα-Flag was a kind gift from Dr. Carolyn Smith and has been previously described (Karmakar et al., 2010). To generate pCR3.1-ERα KR-Flag, QuikChange Lightning Multi Site-Directed Mutagenesis Kit was used (Agilent).

3. Results

3.1. Deletion of ERα NLS domain causes cytoplasmic localization but doesn't impair ligand binding

To understand the relationship between receptor localization and response to ligands, we generated a GFP-ERα mutant

containing a deletion of the 25 amino acids encoding the NLS domain (Fig. 1A). GFP was included at the N-terminus, as previous studies have shown that deletion of the NLS resulted in an ERα whose molecular weight (~55 kDa) is small enough to allow passive diffusion through the nuclear pore (Lang et al., 1986; Paine et al., 1975). GFP-ERΔNLS is of sufficient size to eliminate this possibility. We next performed stable transfections in C4-12 cells (an ERα-negative derivative of MCF-7 cells) with either N-terminal GFP-tagged wild-type ERα (GFP-ER) or GFP-tagged ERα with the NLS deleted (GFP-ERΔNLS). Immunofluorescence and confocal microscopy showed that while wild-type ERα was mainly nuclear, deletion of the NLS domain resulted in cytoplasmic localization of the receptor (Fig. 1B). As expected, the parental C4-12 cells did not express any ERα protein.

To ensure that the deletion of the NLS did not impair aspects of normal ERα function, we first tested the ability of GFP-ERΔNLS to bind both 17-β-estradiol (E2) and fulvestrant. In a whole cell competitive binding assay, C4-12 cells stably expressing either GFP-ER or GFP-ERΔNLS were incubated with a fixed amount of 1.5 pmol tritiated E2 ([^{3}H]-E2) and increasing concentrations of non-

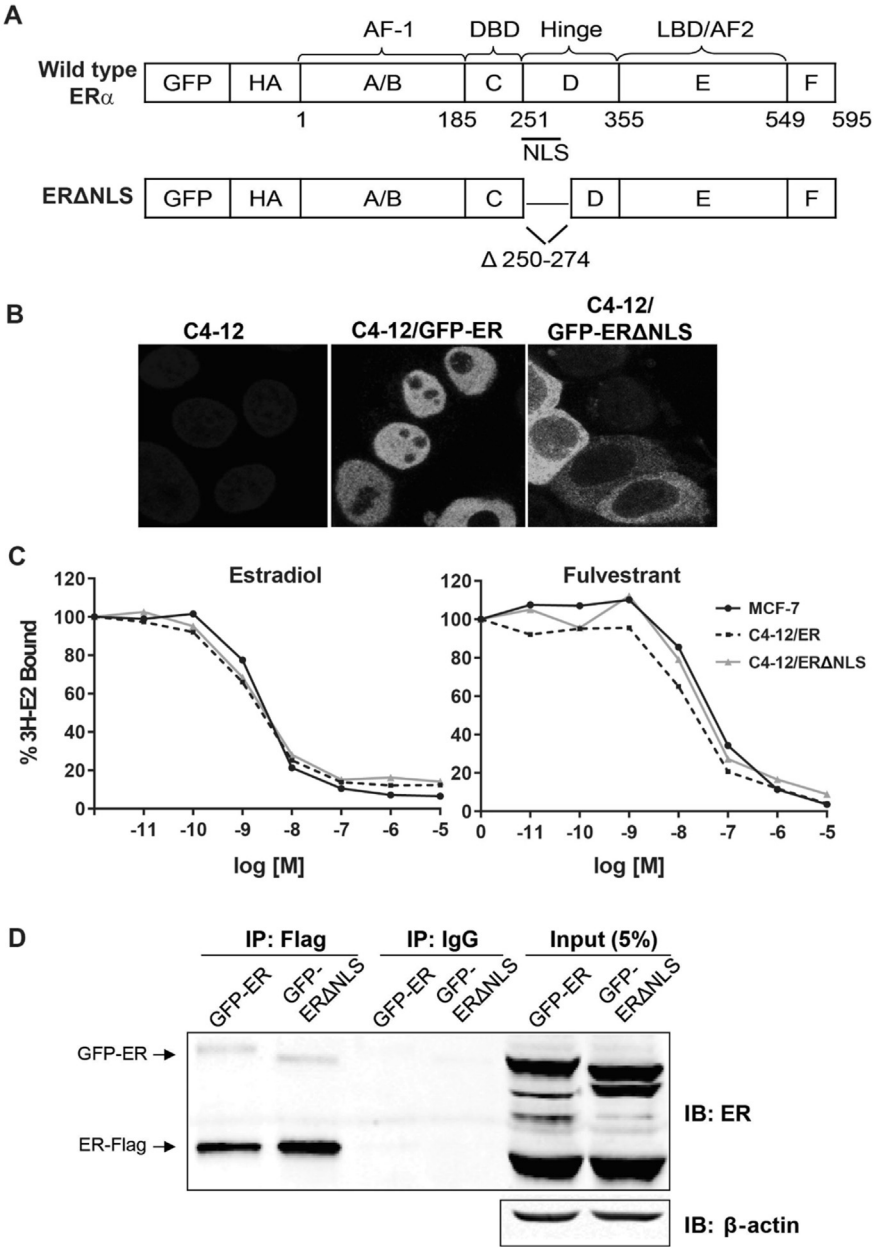

Fig. 1. (A) Schematic representation of the protein domain structures of GFP-tagged and N-terminal HA-tagged wild-type ERα (GFP-ER) and the deletion mutant (GFP-ERΔNLS). AF-1, AF-2 – activation function 1 and 2; DBD – DNA binding domain; LBD – ligand binding domain; NLS – nuclear localization sequence. (B) Localization of wild type ER and ERΔNLS. Confocal microscope images of immunofluorescence staining for ERα in parental C4-12 cells and C4-12 cells stably expressing GFP-ER or GFP-ERΔNLS (C) Competitive binding of E2 and Fulvestrant to wild type ER and ERΔNLS. Whole cell competitive binding studies were performed in MCF-7, C4-12/GFP-ER, and C4-12/GFP-ERΔNLS cells. The graph shows the displacement of 1.5 pM [^{3}H]-E2 by the ligands at various concentrations. IC50 for binding of E2 was found to be 2.5 nM, 1.7 nM and 1.7 nM and that of fulvestrant was 40.7 nM, 18.9 nM and 27.4 nM in MCF-7, C4-12/GFP-ER, and C4-12/GFP-ERΔNLS cells respectively. (D) Deletion of NLS does not impair interaction with ERα. HEK293 cells transfected with ER-Flag and either GFP-ER or GFP-ERΔNLS were stimulated with E2 (100 nM) for 1 h. Cells were lysed and 1 mg protein was immunoprecipitated with either anti-Flag antibody or anti-IgG. Following IP, samples were run on Western blot, and ERα protein was detected using anti-ERα antibody.

radiolabeled E2 (Fig. 1C, left panel) or fulvestrant (Fig. 1C, right panel). As a control, the same experiment was also performed with MCF-7 cells, which express endogenous ERα. For all three cell lines tested, we found that increasing concentrations of E2 or fulvestrant were able to compete with [^{3}H]-E2 for binding to both GFP-ER and GFP-ERΔNLS. Thus, deletion of the NLS had no major effect on the ability of the receptor to bind E2 or fulvestrant, indicating that there hasn't been a major disruption of folding of the AF-2 domain. Of note, the binding affinity of fulvestrant for ERα was approximately 10-times lower than that of E2, which is in accordance with earlier publications (Gibson et al., 1991). Taken together, the data show that the NLS deletion does not impair ligand binding, and GFP-ERΔNLS binds E2 and fulvestrant with the same affinity as GFP-ER.

We next examined whether or not the deletion hindered interaction with ERα. HEK 293 cells were transiently co-transfected with flag-tagged ERα and either GFP-ER or GFP-ERΔNLS. Cells were treated with E2 (100 nM) for 1 h and then subjected to immunoprecipitation with an anti-Flag antibody or anti-IgG antibody as control (Fig. 1D). Both GFP-tagged ERα and ERΔNLS co-immunoprecipitated with Flag-ER. Furthermore, we detected approximately equal levels of GFP-ER and GFP-ERΔNLS. Thus, the data show that the deletion of amino acids 250–274 did not affect ERα ligand binding or receptor–receptor interaction, suggesting that there have not been major changes in protein folding or conformation.

3.2. GFP-ERΔNLS is degraded in response to E2 treatment but is resistant to fulvestrant-induced degradation

Even though E2 is an ERα agonist and fulvestrant antagonizes receptor function, binding of either ligand to ERα results in receptor degradation. We sought to determine the degradation response of both GFP-ER and GFP-ERΔNLS to E2 and fulvestrant. C4-12 cells stably expressing either wild-type ERα or the NLS deletion mutant were treated with increasing concentrations of either ligand for 24 h (Fig. 2A). As expected, the wild-type receptor was degraded by increasing concentrations of either E2 or fulvestrant. However, in contrast to GFP-ER, GFP-ERΔNLS was degraded in response to increasing amounts of E2, but it was not degraded by fulvestrant. We next examined whether or not the length of exposure to ligand influenced the degradation response. The stable cell lines were treated with E2 (100 nM) or fulvestrant (100 nM) for 8 h, 48 h, or 72 h (Fig. 2B). Both GFP-ER and GFP-ERΔNLS were degraded by E2 at all the time points examined. However, GFP-ERΔNLS remained completely resistant to fulvestrant-induced degradation even after 72 h of treatment. To ensure that this effect was not unique to C4-12 cells, we examined the effect of E2 and fulvestrant on GFP-ERΔNLS stably expressed in MCF-7 cells (Fig. 2C). The endogenous ERα (~65 kDa) present in MCF-7 cells provides a useful internal control and can be easily distinguished from the GFP-tagged deletion mutant (which is approximately 90 kDa). In MCF-7 cells, just as we found in C4-12 cells, both wild-type ERα and GFP-ERΔNLS were degraded in response to increasing concentrations of E2. However, there was a striking differential response between wild-type receptor and the deletion mutant with regard to fulvestrant treatment. While endogenous ERα was degraded by increasing doses of fulvestrant, GFP-ERΔNLS was not.

Finally, we examined the role of the proteasome pathway in the degradation responses we observed. Previous publications have established that E2 and fulvestrant both degrade ERα via the ubiquitin/proteasome pathway (Nawaz et al., 1999; Wijayaratne and McDonnell, 2001). However, given that GFP-ERΔNLS contains a 25 amino acid deletion and has altered subcellular localization compared to the wild-type receptor, we wanted to ensure that E2-induced degradation of GFP-ERΔNLS still occurs via the proteasome. C4-12 cells stably expressing either GFP-ER or GFP-ERΔNLS were pre-treated with the proteasome inhibitor MG132 (2 μg/ml) or DMSO control for 2 h. This was followed by treatment with vehicle control (0.01% ethanol), E2 (100 nM), or fulvestrant (100 nM) for 24 h. As expected, degradation of GFP-ER elicited by either E2 or fulvestrant was abrogated in the presence of proteasomal inhibitor MG132 (Fig. 2D, left panel). E2-induced degradation of GFP-ERΔNLS also required a functional proteasome pathway as pre-treatment with MG132 blocked this turnover as well (Fig. 2D, right panel).

3.3. Cloning the SV40-NLS onto GFP-ERΔNLS forces it into the nucleus but does not restore sensitivity to fulvestrant-induced degradation

Fig. 2 shows that GFP-ERΔNLS is resistant to degradation by fulvestrant. To determine if the cytoplasmic localization of GFP-ERΔNLS was responsible for the loss of degradation in response to fulvestrant, we directed GFP-ERΔNLS back into the nucleus by cloning the heterologous SV40-NLS downstream of GFP at the amino-terminus (Fig. 3A). Expression of this construct in C4-12 cells followed by immunofluorescence and confocal microscopy showed that addition of SV40-NLS to GFP-ERΔNLS (GFP-ERΔNLS/SV40-NLS) was sufficient to promote nuclear localization of the deletion mutant (Fig. 3B). In fact, the localization of GFP-ERΔNLS/SV40-NLS was identical to the localization of wild-type receptor. We generated C4-12 cells stably expressing GFP-ERΔNLS/SV40-NLS, and the degradation response to both E2 and fulvestrant was assessed (Fig. 3C). While GFP-ERΔNLS/SV40-NLS was degraded by increasing concentrations of E2, it remained resistant to degradation by fulvestrant, thus mimicking the response of GFP-ERΔNLS (see Fig. 2). Thus, receptor localization in and of itself is not a critical determinant of fulvestrant-induced degradation.

3.4. The ability to bind DNA is not a prerequisite for fulvestrant-induced degradation of ERα

Restoring the nuclear localization of our deletion mutant with the SV40-NLS prompted us to examine the ability of GFP-ERΔNLS/SV40-NLS to stimulate estrogen target gene expression. C4-12 cells stably expressing GFP-ER, GFP-ERΔNLS, or GFP-ERΔNLS/SV40-NLS were treated with vehicle or E2 (10 nM) for 16 h (Fig. 4A). As expected, cells expressing wild-type receptor showed E2-mediated induction of the well-described target gene *pS2*, and cells expressing the cytoplasmic deletion mutant failed to induce pS2 mRNA. Interestingly, even though present in the nucleus, GFP-ERΔNLS/SV40-NLS was unable to increase pS2 transcript levels. We hypothesized that this may be due to a lack of ability of GFP-ERΔNLS/SV40-NLS to bind DNA which was then directly examined (Fig. 4B). C4-12 cells stably expressing either GFP-ER or GFP-ERΔNLS/SV40-NLS were treated with vehicle or E2 (10 nM) for 45 min. ChIP assay was performed using either an antibody directed against ERα or IgG antibody as control. Quantitative real-time RT-PCR was performed using primers that encompassed a well-characterized ERE within the *pS2* promoter. While GFP-ER was detected at the *pS2* ERE following E2 treatment, GFP-ERΔNLS/SV40-NLS was not found to occupy this promoter region. Thus, the data show that the NLS deletion abrogates the ability of the receptor to bind DNA.

This led us to examine whether or not DNA binding is a prerequisite for fulvestrant-induced degradation of ERα. To address this, we obtained C4-12 cells stably expressing HA-tagged wild-type ERα or one of two different DBD mutants of ERα. One ERα DNA binding mutant (DBM1) contains the two point mutations E203A and G204A, and the other (DBM2) contains the mutations C202H

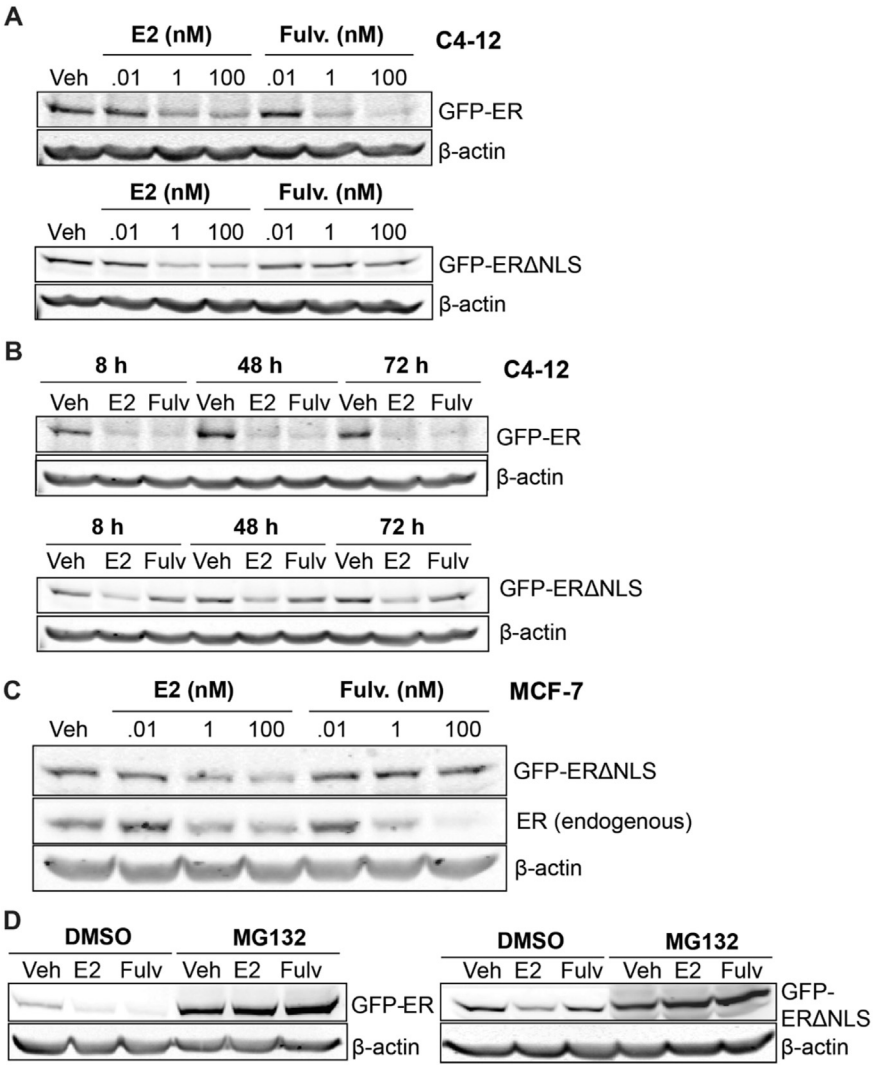

Fig. 2. GFP-ERΔNLS is degraded in response to E2 but is resistant to fulvestrant-induced degradation. (A) C4-12 cells stably expressing GFP-ER or GFP-ERΔNLS were treated with vehicle or increasing concentrations of E2 or fulvestrant (Fulv.) for 24 h. ERα protein detected by immunoblot. (B) C4-12 cells stably expressing GFP-ER (top panel) or GFP-ERΔNLS (bottom panel) were treated with E2 (100 nM) or fulvestrant (100 nM) for increasing lengths of time and ERα protein detected by immunoblot. (C) MCF-7 cells stably expressing GFP-ERΔNLS were treated with vehicle or increasing concentrations of E2 or fulvestrant for 24 h. ERα protein detected by immunoblot. (D) C4-12 cells stably expressing GFP-ER or GFP-ERΔNLS were pre-treated with either DMSO or MG132 (2 μg/mL) for 2 h. This was followed by 24 h treatment with vehicle, E2 (100 nM), or fulvestrant (100 nM) and ERα protein was detected by immunoblot.

and C205H. These ERα point mutants are present in the nucleus and their inability to bind DNA has been previously described (DeNardo et al., 2007). Each stable cell line was incubated in the presence of vehicle (0.01% ethanol) or increasing concentrations of E2 or fulvestrant for 24 h. Both of the DNA binding mutants, as well as wild-type ERα, were degraded by E2 and fulvestrant (Fig. 4C). Thus, the data show that DNA binding is not a prerequisite for degradation by fulvestrant. Furthermore, even though GFP-ERΔNLS/SV40-NLS is unable to bind DNA, this is likely not the reason why it is resistant to fulvestrant-induced degradation.

3.5. *The ERα NLS domain is sufficient for fulvestrant-induced degradation of ERα*

The data thus far have shown that ERα with the NLS deletion is resistant to degradation by fulvestrant. However, the cytoplasmic localization of the receptor and the inability of this deletion mutant to bind DNA are not critical determinants of the resistance phenotype. This led us to hypothesize that there is something unique about the ERα NLS domain itself (i.e. the specific amino acid sequence that comprises this domain) that is critical for the fulvestrant-induced degradation process. To address this hypothesis, we cloned the endogenous ERα NLS back onto GFP-ERΔNLS. However, to examine if the NLS can act in a heterologous manner, we cloned the sequence near the amino-terminus of the receptor (where we had previously inserted the SV40-NLS) instead of in its original location, which lies within the hinge region of the protein (Fig. 5A). Expression of this construct in C4-12 cells followed by immunofluorescence and confocal microscopy revealed that the presence of the endogenous NLS on GFP-ERΔNLS (GFP-endogNLS-ERΔNLS), even in a non-native position, was sufficient to restore most of the receptor's nuclear localization (Fig. 5B). However, despite its presence, some of the receptor did remain cytoplasmic, suggesting that some of its intrinsic ability to act as an NLS may be compromised by its non-native position. We generated C4-12 cells stably expressing GFP-endogNLS-ERΔNLS, and the degradation response to both E2 and fulvestrant was assessed (Fig. 5C). GFP-endogNLS-ERΔNLS was strongly degraded by increasing

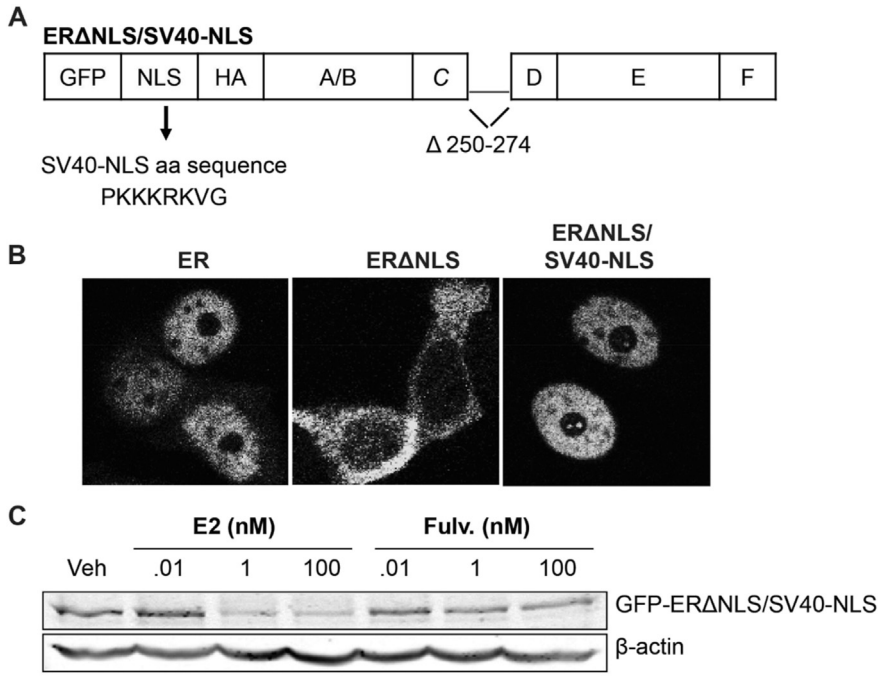

Fig. 3. Cloning the SV40-NLS onto GFP-ERΔNLS forces it into the nucleus but does not restore sensitivity to fulvestrant-induced degradation. (A) Schematic representation of GFP-ERΔNLS with the SV40-NLS (GFP-ERΔNLS/SV40-NLS) cloned near the protein's N-terminus. (B) Confocal microscope images of immunofluorescence staining for ERα using anti-HA antibody in parental C4-12 cells transiently transfected with GFP-ER, GFP-ERΔNLS, or GFP-ERΔNLS/SV40-NLS. (C) C4-12 cells stably expressing GFP-ERΔNLS/SV40-NLS were treated with vehicle, increasing concentrations of E2 or fulvestrant for 24 h. Cells were lysed and ERα protein detected by immunoblot.

concentrations of E2 and at least partially degraded by increasing concentrations of fulvestrant. While GFP-endogNLS-ERΔNLS was degraded in the presence of fulvestrant, the response was reduced compared to the degradation of wild-type ERα by the anti-estrogen. Similar to the situation of incomplete rescue of nuclear localization, the fact that the endogenous NLS domain is not in its native position within the protein may compromise some of its normal function with regard to ERα turnover by fulvestrant. ERα sumoylation is required for fulvestrant-mediated anti-estrogen activity and ERα transcriptional activity (Sentis et al., 2005; Hilmi et al., 2012). We asked whether fulvestrant-mediated sumoylation of the NLS is required for ER degradation. We generated a Flag-tagged ERα mutant where the two lysine residues are mutated (K266R/K268R). Although this mutant retains nuclear localization (Fig. 5D), fulvestrant-induced degradation is impaired, suggesting that sumoylation of these residues is required for fulvestrant-induced degradation (Fig. 5E). Taken together, the data show that the ERα NLS domain itself, and not merely receptor localization, is a critical determinant of the degradation response to fulvestrant.

4. Discussion

We report the critical role of the ERα NLS in fulvestrant-induced degradation of ERα. A number of earlier publications have reported the influence of fulvestrant treatment on ERα subcellular localization, often with conflicting results. For example, Dauvois et al. reported that fulvestrant treatment leads to the overall cytoplasmic accumulation of ERα (Dauvois et al., 1993). Other reports have argued that fulvestrant administration results in strong interaction of the receptor with the nuclear matrix (Stenoien et al., 2000, 2001). A subsequent study showed that ERα degradation by fulvestrant required keratins 8 and 18 (Long and Nephew, 2006). However, to the best of our knowledge, this is the first report describing the importance of the NLS domain itself in the degradation response elicited by fulvestrant.

We initially generated C4-12 stable cell lines expressing either GFP-tagged wild-type ERα (GFP-ER) or an ERα mutant with a 25 amino acid deletion of the NLS domain (GFP-ERΔNLS). When cells expressing GFP-ERΔNLS were cultured in medium containing charcoal-stripped serum, the deletion receptor was completely cytoplasmic. This is in accordance with earlier data from Pierre Chambon's laboratory (Ylikomi et al., 1992) and establishes amino acids 250–274 of ERα as critical for nuclear localization. In addition to the bipartite NLS located between amino acids 250–274, this same publication from Chambon's group described a hormone-inducible NLS in ERα located between amino acids 299–303. They reported that a small percentage (8%) of an ERα mutant with amino acids 250–274 deleted but 299–303 intact (very similar to our GFP-ERΔNLS construct) was found in the nucleus following E2 stimulation. However, we were unable to detect any nuclear GFP-ERΔNLS even after treatment with E2 (data not shown). One possibility is that this is due to cell line-dependent effects. Taken together, the data show that even if the hormone-inducible NLS possesses some type of nuclear localizing ability, it is negligible compared to the ability of amino acids 250–274 to promote nuclear localization of ERα.

Strikingly, in addition to its altered localization, GFP-ERΔNLS had a distinct degradation response to fulvestrant. As expected, GFP-ER was degraded by both E2 and fulvestrant. However, while E2 degraded GFP-ERΔNLS just as it did the wild-type receptor, fulvestrant failed to do so. Since the deletion mutant was degraded in response to E2, and since both E2 and fulvestrant bind the LBD of ERα, we hypothesized that the inability of fulvestrant to degrade GFP-ERΔNLS could be due to an inability of the deletion mutant to bind the anti-estrogen. We formally tested this by performing a competitive radioligand binding assay and found that the deletion did not impair ligand binding, and GFP-ERΔNLS bound fulvestrant with the same affinity as GFP-ER. We further concluded that the

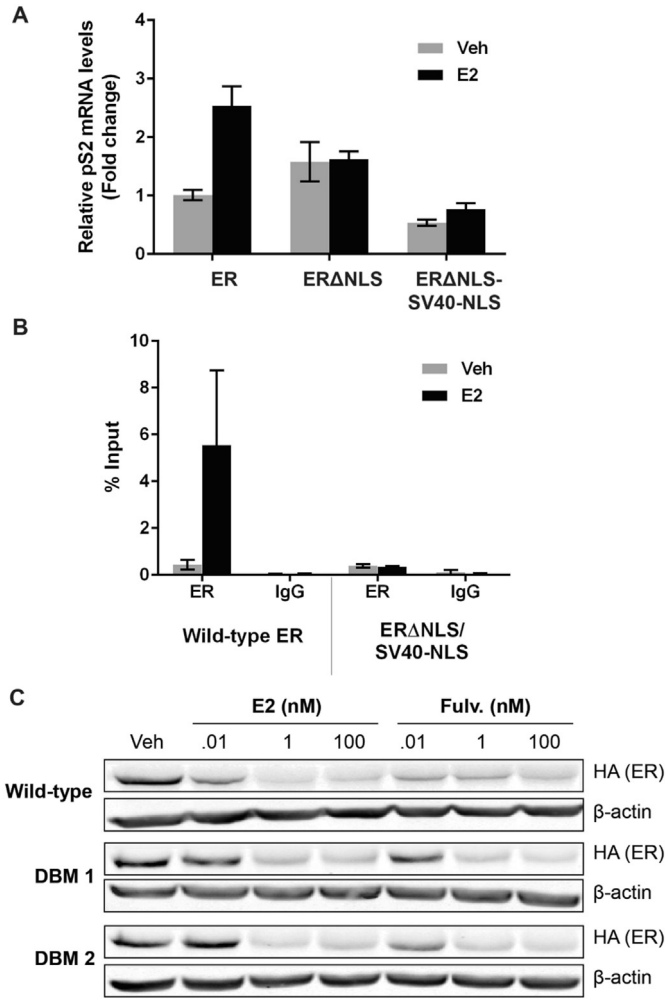

Fig. 4. DNA binding is not a prerequisite for fulvestrant-induced degradation of ERα. (A) C4-12 cells stably expressing GFP-ER, GFP-ERΔNLS, or GFP-ERΔNLS/SV40-NLS were treated with vehicle or E2 (10 nM) for 16 h and pS2 mRNA levels were detected by qRT-PCR. Data shown are an average of three independent experiments, and error bars represent standard error of the mean (SEM). (B) ChIP assays were performed in C4-12 cells stably expressing either GFP-ER or GFP-ERΔNLS/SV40-NLS. Cells were treated with vehicle or E2 (10 nM) for 45 min. ChIP was performed using an antibody directed against ERα or an IgG antibody as control. qRT-PCR was performed using primers that encompassed a well-characterized ERE within the *pS2* promoter. Data are represented as percent of input (% input) and are an average of three independent experiments. Error bars represent SEM. (C) C4-12 cells stably expressing HA-tagged wild-type ERα or one of two ERα DNA binding domain point mutants (DBM1 or DBM2) were treated with vehicle or increasing concentrations of E2 or fulvestrant for 24 h. Cells were lysed and HA (ERα) protein detected by immunoblot.

NLS deletion does not impair receptor–receptor interaction. Taken together, the data highlight some key points regarding both E2 and fulvestrant-induced degradation of ERα. First, the fact that GFP-ERΔNLS is able to bind E2 and fulvestrant with similar affinity as GFP-ER, and that E2 elicits degradation of GFP-ERΔNLS, suggests that the deletion of the 25 amino acids of the NLS domain has not severely disrupted the secondary protein structure of ERα. Second, the fact that E2 and fulvestrant show differential degradation potential on GFP-ERΔNLS suggests that they degrade ERα by different and distinct mechanisms—an observation that has been previously made by others (Wijayaratne and McDonnell, 2001; Marsaud et al., 2003). Third, ERα can be degraded by E2 even when it is present in the cytoplasm and is unable to alter transcription. Previous reports have argued that there is a link between ERα transcriptional activity and receptor turnover (Wijayaratne and McDonnell, 2001; Nawaz and O'Malley, 2004; Lonard et al., 2000). In fact, the degradation rate of several transcription factors seems to directly correlate with their transcriptional activity (Salghetti et al., 2000; Molinari et al., 1999). However, other reports have separated the transcription and degradation capabilities of ERα (Valley et al., 2005). The data we present in this report do not necessarily contradict these previous findings. Although ERα turnover may be critical for efficient transcriptional activity, a receptor that is not physically engaged in the process of transcription may still be degraded in response to E2 binding.

The fact that GFP-ERΔNLS is resistant to fulvestrant-induced degradation implicated either subcellular localization or the specific amino acid sequence of the NLS domain itself in the turnover process. The SV40-NLS has been shown to be both necessary and sufficient for the nuclear accumulation of a number of heterologous proteins (Roberts, 1989). Similarly, it promoted nuclear localization of the deletion mutant, but was still resistant to degradation by fulvestrant. Thus, the data implicates the role of the NLS domain itself, and not merely receptor localization, in the degradation process. This may be a unique feature of ERα, as the turnover of other proteins has been shown to rely predominately on their subcellular localization. For example, SRC-3 mutants with a deletion of the NLS are cytoplasmic and insensitive to proteasome-dependent turnover (Li et al., 2007). This same study showed that fusion of the SV40-NLS to the SRC-3 mutant restored both its nuclear localization and its sensitivity to proteasome degradation. Other examples include turnover of Smad2, which occurs in the nucleus (Lo and Massague, 1999) and cyclin D1, which requires nuclear export prior to degradation (Diehl et al., 1998).

To formally prove that it is indeed the ERα NLS domain itself that is critical for receptor turnover in the presence of fulvestrant, we cloned the endogenous ERα NLS back onto GFP-ERΔNLS. However, to examine if it could act in a heterologous manner, we positioned the NLS at the N-terminus (as replacing the NLS to its endogenous position would clearly restore function). Quite strikingly, GFP-endogNLS-ERΔNLS showed a mainly nuclear localization (albeit weaker than GFP-ERΔNLS/SV40-NLS). This is important since there have been surprisingly few studies of the ERα NLS. Even more surprising, we found that adding the ERα NLS to the N-terminus of GFP-ERΔNLS conferred fulvestrant-induced degradation. As no degradation was noted with SV40-NLS cloned onto the N-terminus, this specifically defines the ERα NLS as a protein domain that can confer fulvestrant-induced degradation. However, it should be noted that the degradation with fulvestrant was only partial and not as strong as that seen with GFP-ER. Whether this is due to the fact that not all ERα was present in the nucleus, or whether this is due to the fact that the NLS is not within its native context in the protein, is currently unclear. However, it is known that the efficiency of an NLS is sensitive to variations in protein context (Roberts et al., 1987). Despite this fact, our data argue that the NLS domain itself is a critical determinant of the degradation response to fulvestrant. The specific role of NLS in the degradation process is unclear. One possibility is that the NLS amino acid sequence is post-translationally modified, leading to subsequent receptor degradation. This may be the case given the frequent occurrence of such modifications within the hinge region of ERα. One post-translational modification that occurs within the NLS is sumoylation and this modification is essential for ERα transcriptional activity (Sentis et al., 2005). Also, sumoylation of multiple lysine residues is required for fulvestrant-mediated anti-estrogen activity (Hilmi et al., 2012). We show here that mutation of sumoylation sites within the NLS makes ERα more resistant to fulvestrant-induced degradation. Sumoylation of these lysine residues could lead to ubiquitination of other lysine residues and target the protein for degradation. A previous publication by Berry et al. reported that

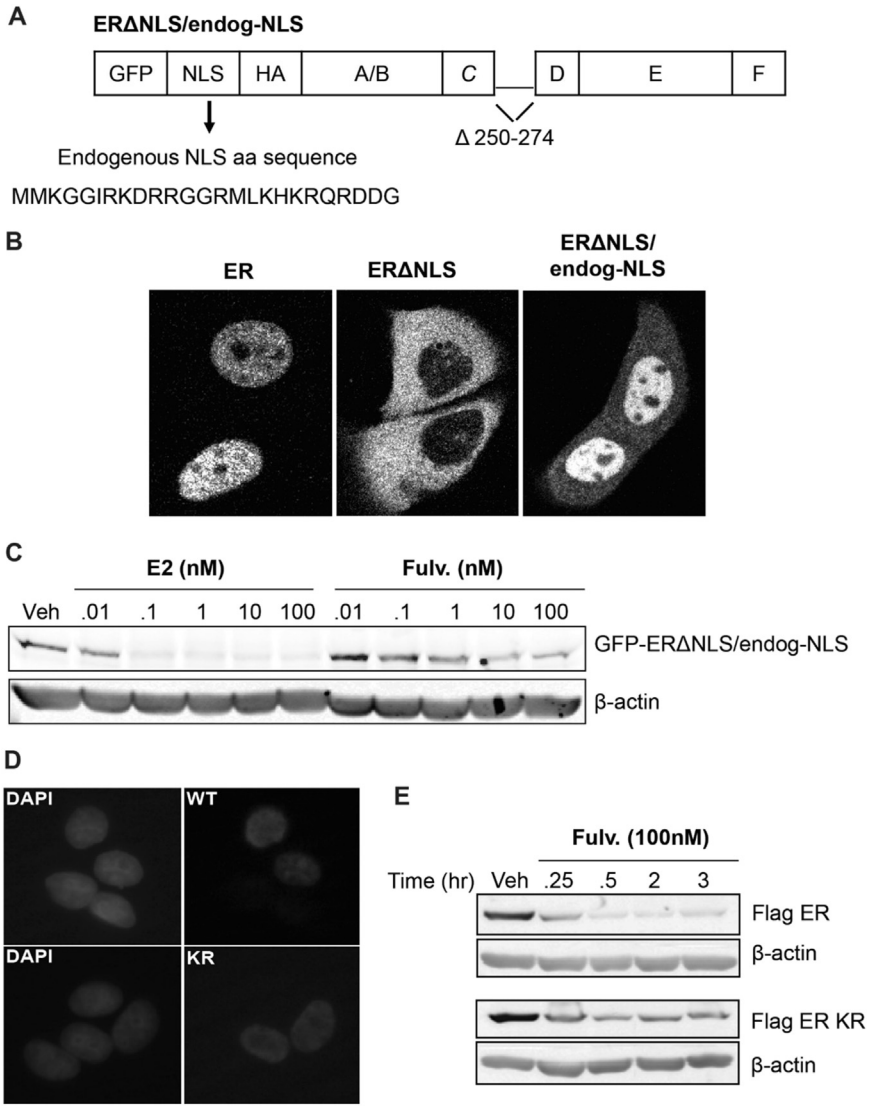

Fig. 5. The ERα NLS domain is sufficient for fulvestrant-induced degradation of ERα. (A) Schematic representation of GFP-ERΔNLS with the ERα endogenous NLS (GFP-endogNLS-ERΔNLS) cloned near the protein's N-terminus. (B) C4-12 cells were transiently transfected with GFP-ER, GFP-ERΔNLS, or GFP-endogNLS-ERΔNLS and ERα was detected by anti-HA antibody using confocal microscopy. (C) C4-12 cells stably expressing GFP-endogNLS-ERΔNLS were treated with Vehicle, or increasing concentrations of E2 or fulvestrant for 24 h. Cells were lysed and ERα protein was detected by immunoblot. (D) MCF-7 cells transiently expressing Flag-ERα or Flag-ERα-KR. Transfected ERα was detected using anti-Flag antibody. Representative cells are shown. (E) HEK293 cells transiently expressing Flag-ERα or Flag-ERα-KR were treated with fulvestrant for increasing lengths of time. ERα protein was detected by immunoblot.

two lysines, K302 and K303, within the hinge region of ERα are ubiquitinated in response to fulvestrant (Berry et al., 2008). These two amino acids are still present in our deletion mutant. However, there is the possibility that a modification within the NLS deleted region may serve as a priming modification for subsequent ubiquitination at K302 and K303. This has been previously described for other proteins. For example, degradation of cyclin E is triggered by prior phosphorylation by at least two kinases (Clurman et al., 1996; Won and Reed, 1996; Welcker et al., 2003). Another possibility of why the NLS domain is critical for the fulvestrant-induced degradation process is that the NLS may serve as a region of interaction with other proteins that are essential for the turnover process. Indeed, fulvestrant binding to ERα has been previously shown to result in receptor interaction with other proteins (Long and Nephew, 2006; Jaber et al., 2006). Of course, these two possibilities are not mutually exclusive. It is possible that post-translational modification within the ERα NLS promotes interaction with an essential protein, ultimately leading to receptor degradation.

In conclusion, our data provide evidence for a unique role of the ERα NLS in the fulvestrant-induced degradation process. It will be important to further evaluate the mechanism of this degradation. Not only may we better be able to identify patients who might not respond to this anti-estrogen, but we may also be able to develop better therapies to treat non-responders.

Funding

This work was supported by grants from the Department of Defense Breast Cancer Research Program DAMD17-02-1-0286 (AVL), W81XWH-06-1-0714 (AJC) and Susan G Komen PDF14301091 (SS). AVL is a recipient of a Scientific Advisory Council award from Susan G. Komen for the Cure and is a Hillman Foundation Fellow. The authors acknowledge support from the University of Pittsburgh Cancer Institute (UPCI) and UPMC.

Disclosure

The authors have nothing to disclose.

Acknowledgments

This work was supported by grants from the Department of Defense Breast Cancer Research Program DAMD17-02-1-0286 (AVL), W81XWH-06-1-0714 (AJC) and Susan G Komen PDF14301091 (SS). We thank Steffi Oesterreich for critical reading of this manuscript and John Katzenellenbogen and Carolyn Smith for advice and methodology on the whole cell ligand binding assay. We also thank ZaWaunyka Lazard, Curtis Thorne, Maia Ouspenskia, and Ping Zhang for assistance with cloning and preliminary experiments.

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