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Phosphorylation of Activation Function-1 Regulates Proteasome-dependent Nuclear Mobility and E6-AP Ubiquitin Ligase Recruitment to the Estrogen Receptor β

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Abstract

The ubiquitin-proteasome pathway has been recognized as an important regulator in the hormonal response by estrogen receptor ERα, but its impact on ERβ function is poorly characterized. In the current study, we investigated the role of the ubiquitin-proteasome pathway in regulating ERβ activity and identified regulatory sites within the activation function AF-1 domain that modulate ERβ ubiquitination and nuclear dynamics in a hormone-independent manner. Whereas both ERα and ERβ were dependent on proteasome function for their maximal response to estrogen, they were regulated differently by proteasome inhibition in the absence of hormone, an effect shown to be dependent on their respective AF-1 domain. Given the role of AF-1 phosphorylation to regulate ERα and ERβ activity, we found that sequential substitutions of specific serine residues contained in MAPK consensus sites conferred transcriptional activation of ERβ in a proteasome-dependent manner through reduced ubiquitination and enhanced accumulation of mutant receptors. Specifically, serines 94 and 106 within ERβ AF-1 domain were found to modulate sub-nuclear mobility of the receptor to transit between inactive clusters and a more mobile state in a proteasome-dependent manner. In addition, cellular levels of ERβ were regulated through these sites by facilitating the recruitment of the ubiquitin ligase E6-associated protein in a phosphorylation-dependent manner. These findings suggest a role for ERβ AF-1 in contributing to the activation-degradation cycling of the receptor through a functional clustering of phosphorylated serine residues that cooperate in generating signals to the ubiquitin-proteasome pathway.

Introduction

Estrogen plays a central role in reproductive physiology but also in pathological events such as breast and endometrium cancers. Regulation of target gene expression by estrogen is mediated upon its interaction with the estrogen receptors ERα and ERβ, which belong to the nuclear hormone receptor family of ligand-activated transcription factors . Although ERα shares similarities in terms of structure and response to hormone with ERβ, it is considered a strong predictive factor for endocrine therapy of reproductive cancers (1,2), whereas ERβ was shown to display anti-tumorigenic properties (3-5).

The current model for transcriptional activation of estrogen responsive genes by ERs involves a conformational change upon ligand binding that favors interaction with their cognate estrogen response element (ERE) and combinatorial recruitment of transcriptional cofactor complexes that mediate chromatin reorganization and essential interactions with the basal transcription machinery (6,7). Increasing evidence indicates that upon activation of ERα by estrogen, the recruitment of cofactors involved in chromatin remodeling, post-translational modifications and transcriptional activity among others, occurs in an ordered fashion, thereby integrating the response elicitated by estrogen upon inducible promoters (8-11). Such concerted recruitment between cofactors is predicted to limit transactivation by ERα in response to changes in hormone levels.

Recent studies have integrated the ERα-mediated response to estrogen with proteasome-directed degradation of the receptor, thus supporting a mean by which target cells can sustain or limit a hormonal response through a continuous receptor turnover. ERα has been shown to be degraded through the 26S proteasome pathway in a ligand-dependent manner (12,13). Blocking proteasome activity with inhibitors such as MG132 impaired the ability of ERα to mediate a

hormone-dependent transcriptional response and contributed to immobilize ERα in an inactive state in the nucleus (14,15). Interestingly, components of the ubiquitin-proteasome system, such as the 19S proteasome regulatory subunit Trip1/SUG1 (16,17) and the E3 ubiquitin ligases MDM2 (18) and E6-AP (19), have been shown to enhance the transcriptional activity of several nuclear receptors including ERα. Moreover, these components were shown to cyclically reside with ERα on the pS2 responsive promoter in both a ligand-dependent and -independent manner, an effect abolished by MG132 resulting in the inhibition of pS2 gene transcription (10,11). These findings thus suggest that the proteasome-mediated degradation process is closely related to ERα transcriptional competence.

Ubiquination of ERα has been demonstrated to vary upon the presence of hormone or antiestrogens, and the extent of ubiquitination was shown to correlate with receptor degradation, suggesting that ligand-dependent conformational changes can modulate ERα ubiquitination (13,20). Interestingly, unliganded ERα was also demonstrated to be ubiquitinated (10,13), therefore providing evidence that mechanisms other than ligand binding may dictate ubiquitination. As such, other signaling events have also been proposed to regulate ERα and ERβ activity. Studies have reported that the ability of epidermal growth factor EGF to activate ERα involved a MAPK-mediated phosphorylation of serine-118 within ERα AF-1 domain (21,22). Such activation of ERα by phosphorylation was proposed to involve coactivator recruitment at the AF-1 domain of ERα (23). Similarly, we demonstrated that ERβ activity is also modulated by growth factor signaling through MAPK-directed phosphorylation of AF-1 (24). Such activation was found to involve a favored recruitment of coactivators SRC-1 and CBP upon phosphorylation of ERβ AF-1 serines 106 and 124 (25,26). However, the role of AF-1 in mediating ERβ response to non-hormonal stimuli remains poorly defined and although both ERα

and ERβ are regulated by growth factors, differences in terms of respective cofactor recruitment and AF-1 activity have been observed (27,28).

In the present study, we investigated the function of the ubiquitin-proteasome pathway in modulating the AF-1 dependent transcriptional response of ERβ. We identified specific MAPK sites within ERβ AF-1 domain that regulate ERβ activity in a proteasome-dependent manner by modulating receptor ubiquitination, sub-nuclear mobility, and recruitment of E6-AP ubiquitin ligase. Our results demonstrate a role for the AF-1 domain to regulate the activation-degradation process of ERβ, thereby integrating its response to changes in kinase-activated pathways.

Results

ERα and ERβ-mediated transcription are dependent upon proteasome function

In order to assess the role of the proteasome pathway of degradation on ERα and ERβ transcriptional function, we first tested the effects of proteasome inhibition on the hormonal response of both receptors in human embryonic kidney 293 cells transfected with either ERα or ERβ and the estrogen-responsive luciferase reporter EREbLuc construct. As also reported (14), ERα mediated transcriptional activation in the presence of estrogen was nearly abolished by MG132, an inhibitor of the 26S proteasome (Fig.1A). The hormonal response of ERα was also decreased using clasto-lactacystin, an irreversible proteasome inhibitor. Under the same conditions, we also observed that ERβ was subjected to a similar dependence on the proteasome pathway in its response to estrogen (Fig.1A). These results indicate that maximal activation of ERα and ERβ by estrogen requires proteasome function.

In order to test whether the activity of ERα and ERβ in absence of hormone was also dependent upon proteasome function, 293 cells were treated with increasing doses of MG132 in absence of estrogen and ER-driven luciferase activity was measured. We found that whereas basal ERα-mediated transcription was enhanced by MG132, similar doses of inhibitor had no significant effect on ERβ-dependent activity (Fig.1B). To ensure that this difference in response of ERα and ERβ to MG132 is not related to variations in their cellular content, both ERα and ERβ are shown to accumulate in cells treated with MG132 compared to untreated cells, consistent with the inhibition of their degradation (Fig.1C).

The AF-1 domain affects differently ERα and ERβ activity during proteasome inhibition

Given the apparent difference in the requirement of the proteasome function involved in ligand-independent activity of ERα and ERβ, we determined the relative contribution of each receptor AF-1 domain on ER activity in response to proteasome inhibition. Truncated forms of ERα and ERβ were transfected and tested for their activity in absence of hormone. We observed that for ERα, removal of the N-terminal AB domain (CDEFα construct in Fig.2A) did not greatly alter ERα response to proteasome inhibition, whereas removal of the AB region of ERβ (CDEFβ construct) resulted in a marked activation in the presence of MG132. These results suggest that each respective AF-1 domain may regulate differently the activity of ERα and ERβ in a proteasome-dependent manner. In support of such different role, cells expressing ABCα construct showed a stronger activation in response to proteasome inhibition compared to cells expressing ABCβ (Fig.2A). This apparent diverse contribution of each AF-1 was also transposable, as demonstrated with the use of chimeric forms of ER in which the AB region for each receptor was fused to the CDEF portion of the other, thus creating an ERαβ and ERβα fusion chimeras. As such, the addition of the ABα region to CDEFβ did not significantly modify the activation levels observed for CDEFβ with MG132 or clasto-lactacystin (compare ERαβ with CDEFβ in Fig.2A), whereas fusion of ABβ to CDEFα decreased the activation of CDEFα by both inhibitors, resulting in an activation profile more closely related to ERβ than to ERα. These results demonstrate a role for the AF-1 domain in regulating ERβ transcriptional activity which is dependent upon proteasome function, and further suggest that regulatory signals are contained within this region to mediate receptor turnover and activity. Consistent with these

observations, the N-terminal ABCβ construct was shown to strongly accumulate when expressed in cells treated with MG132 compared to untreated cells as determined by Western analysis, suggesting that this region itself is subjected to proteasome-dependent degradation (Fig.2B). Under similar conditions, ABCα also accumulated but to a lesser extent than ABCβ, whereas CDEFβ and CDEFα levels remained respectively unaffected or slightly increased upon MG132 treatment.

Contribution of MAPK consensus sites in proteasome-regulated transcription by ERβ.

Our earlier studies have determined that phosphorylation of AF-1 in response to MAPK activation resulted in ERβ transcriptional activation (24,25). More specifically, phosphorylation at positions 106 and 124 in mouse ERβ contributed in a favored recruitment of coactivators SRC-1 and CBP and subsequent ligand-independent activation (25,26). Besides serines 106 and 124, other serine residues are also contained within consensus MAPK phosphorylation sites in ERβ AF-1 domain and may thus also function as putative functional targets of MAPK-associated signaling pathways. We tested the contribution of several of these MAPK sites in the proteasome-dependent regulation of ERβ activity, using serine to alanine mutants. As shown in Fig.3A, single replacement of Ser-94, -106, or -124 conferred transcriptional activation of ERβ in the presence of MG132, while the Ser-69 substitution had no effect. Higher levels of activation were observed with the Ser-106 variant, reaching a near 4-fold increase. Interestingly, when Ser-94 was also substituted, thus creating a S94,106A double mutant, the increase in activity by proteasome inhibition was even stronger with a 7-fold increase compared to each respective single mutant. Other multiple variants containing the S106A mutation, such as the S106,124A and S94,106,124A variants, were also induced, although to a lesser extent than the

S94,106A mutant, in response to proteasome inhibition (Fig.3A). To ensure whether the apparent deregulation to proteasome inhibition in ERβ activity upon serine modifications resulted from the AF-1 domain itself, as suggested from the results presented in Fig.2A, we performed luciferase assay with corresponding serine mutants in which the DEF region of ERβ was removed. A comparable activation profile of the ABC constructs carrying successive substitutions of Ser-94, -106 and -124, was obtained (Fig.3B). In addition, removal of the DEF region conferred an even greater transcriptional potential to the S94,106A mutant in response to proteasome inhibition, reaching a 14-fold increase, compared to its corresponding full-length version (Figs.3A and B). A role for the AF-2 function in regulating transcription in a manner dependent on proteasome activity has already been described for ERα (13,14), and our results support a similar role for ERβ as well. However, coordinate removal of specific serine residues within ERβ AF-1 domain confers transcriptional activation when proteasome function is disrupted, raising the possibility that AF-1 regulatory sites participate in ERβ degradation by the proteasome.

To test how the AF-1 serine substitutions may affect the steady-state levels of ERβ, we performed Western analysis on cells expressing wild-type and mutated ERβ in response to proteasome inhibition. Whereas wild-type ERβ levels were markedly increased in cells treated with MG132, the ERβ variants carrying the Ser-94 and/or Ser-106 substitutions, in the context of full-lenght or DEF-truncated receptor, were less prone to accumulate (Fig.3C and D). Therefore, the changes in cellular levels of the S94,106A and S94,106,124A mutants did not account for the increase in their transcriptionnal activity following inhibition of proteasome function, but rather indicate an inverse relationship between receptor activity and cellular content. Such correlation

has also been described for ERα in response to hormone and shown to be required to achieve maximal receptor activation (13,14).

Contribution of AF-1 serine residues in ERβ stability and ubiquitination

Because we found that the S94,106A mutation rendered ERβ less prone to accumulate upon the presence of MG132, we performed a cycloheximide chase to evaluate the impact of the mutation on ERβ protein turnover. As shown in Fig.4A and B, the half-life of ERβ increased from ~8h to approximately 12h by disruption of Ser-94 and -106, indicating that at least these residues are involved in ERβ degradation. Ubiquitination is the post-translational process commonly used to tag and direct proteins to degradation by the 26S proteasome, and the pattern and the extent to which a protein is ubiquitinated determine its rate of degradation (29). We analyzed whether ERβ was ubiquitinated in absence of ligand, and how the S94,106A substitution may affect ERβ ubiquitination in vivo. By expressing ERβ in cells with tagged-ubiquitin, we observed several ubiquitinated forms of ERβ, which accumulated in the presence of MG132 (Fig.4C). Interestingly, reduced levels of ubiquitination were observed in cells expressing the S94,106A mutant, and treatment with MG132 had moderate effect on receptor ubiquitination, when compared to wild-type ERβ. The apparent lower levels of ubiquitination of the S94,106A mutant suggest it has a reduced ability to be targeted by ubiquitination, which may then result in enhanced stability and transcriptional activity. This indicates that specific residues within the AF-1 of ERβ modulate receptor ubiquitination in absence of ligand and therefore contribute to ERβ degradation by the proteasome.

AF-1 modifications affect the nuclear-cytoplasmic distribution of ERβ

Receptor redistribution between nuclear and cytoplasmic compartments has been described for many nuclear receptors in response to agonists and antagonists, and at least for ERα, such response was shown to affect receptor proteolysis (13,20,30). In order to test whether AF-1 substitutions may alter the nuclear-cytoplasmic distribution of ERβ in a proteasome-dependent manner, we determined the relative protein levels of ERβ compared to the S94,106A mutant in each cellular compartment. Both nuclear and cytoplasmic fractions were isolated and validated for the presence of selective markers PARP and GAPDH, respectively (Fig.5A). We found higher levels of the S94,106A variant in both nuclear and cytoplasmic fractions of untreated cells, when compared to wild-type ERβ (Fig.5B), supporting a less propensity of the mutant to be ubiquitinated and a prolonged half-life in conditions in which proteasome function is not impaired. In addition, the levels of nuclear S94,106A mutant were not significantly changed upon proteasome inhibition, while ERβ accumulated in the same conditions, suggesting that serines 94 and 106 contribute to modulate the nuclear-cytoplasmic distribution of ERβ in a proteasome-dependent manner (Fig.5B).

Sub-nuclear trafficking of ERβ is dependent on AF-1 modifications and proteasome function

Earlier studies using fluorescence recovery after photobleaching or FRAP, have revealed the dynamic nature of steroid receptor movement within the nucleus and their association with active chromatin templates to engage transcriptional regulation (15,31). Based on FRAP studies, the intranuclear mobility of ERα was found to be affected differently depending upon the addition of agonist or antagonist, indicating that ERα may associate to respectively

transcriptionally active domains within the nucleus or immobilized to inactive clusters referred to as the nuclear matrix (10,15,32). Inhibition of proteasome activity was also shown to immobilize ERα to components of the nuclear matrix in treated cells (20,33). We therefore evaluated how inhibiting proteasome activity may affect the mobility of ERβ and on the possible role of disrupting AF-1 activity under these conditions. FRAP analysis was performed on cells transfected with a YFP fusion of ERβ, which was initially validated for correct size by Western blot and for activity by luciferase assay (data not shown). In absence of ligand, ERβ was found highly mobile, reaching equilibrium in the bleached region within seconds, with a half-maximal recovery time (t½) of 1.7 ± 0.2 sec (*n* = 12 nuclei) (Fig.5C and D). Although slightly longer, this half-recovery time for ERβ was comparable to unliganded ERα (t½ ~ 1 sec, data not shown, and see ref. 15), indicating that both receptors reside in a highly dynamic state, presumably awaiting for activation signals. As also reported for ERα, inhibiting proteasome activity with MG132 resulted in a profound immobilization of unliganded ERβ, such that the half-recovery time could not be estimated (t½ > 300 sec, Fig.5D). Such proteasome-dependent immobilization has been suggested to result from the clustering and association to non-chromatin templates, which is consistent with the transcriptional activity we measured for ERβ in the presence of MG132. Remarkably, removal of Ser-94 and -106 in ERβ resulted in a more mobile receptor in the presence of MG132 (t½ = 39 ± 5 sec; *n* = 15 nuclei), suggesting that these sites were important to facilitate ERβ to immobilize in response to proteasome inhibition (Fig.5D). This behavior was also observed with the S94,106,124A mutant (t½ ~ 60 sec; data not shown). These results indicate that disruption of ERβ AF-1 activity may allow the receptor to escape from associating with inactive clusters during proteasome inactivation, and therefore become available for

transcriptional regulation. Consistent with these findings is the potent activation we observed for the S94,106A and S94,106,124A mutants by MG132 (Fig.3A and B).

The E6-AP ubiquitin ligase regulates ERβ degradation

E6-AP, an E3 ubiquitin-protein ligase proposed to mediate substrate specificity of the ubiquitin-proteasome degradation system (34), has been shown to participate in the degradation of ERα in response to estrogen (19,35,36). We tested whether E6-AP could also regulate the degradation of ERβ in transfected 293 cells. Coexpression of ERβ with E6-AP lead to a dramatic reduction in ERβ steady-state levels in absence of hormone as shown in Fig.6A, an effect relieved by the addition of MG132, indicating that E6-AP is involved in ERβ degradation through the proteasome pathway. The S94,106A was also subjected to E6-AP dependent degradation but to a lesser extent than wt ERβ. Similarly, Fig.6B shows a similar decrease in ERβ levels in response to increasing amounts of E6-AP in cells transfected with constitutive Mek1 and Erk plasmids, a condition that promotes ERβ phosphorylation and activity (25,26). Under the same conditions, the S94,106A mutant was less affected. These effects were dependent on the E3 ligase activity of E6-AP, as suggested with the use of a ligase defective C833A mutant, which did not decrease ERβ levels (Fig.6B).

The expression of E6-AP also reduced the half-life of ERβ in cells to approximately 4h, compared to ~8h in cells not transfected with E6-AP, whereas the turnover rate of the S94,106A mutant was less affected with a half-life of ~11h (compare Fig.6C; left panel and Fig.4B). Activation of Erk in cells expressing ERβ also contribute to accelerate its degradation with a half-life of ~2h, an effect shown to be highly dependent upon the integrity of Ser-94 and -106 (Fig.6C; right panel).

The E6-AP ubiquitin ligase is recruited to ERβ in a phosphorylation-dependent manner through serines 94 and 106

Based on the ability of E6-AP to regulate ERβ turnover, we then addressed whether E6-AP can be recruited to ERβ using co-immunoprecipitation assays. Fig.7A shows that E6-AP was detected in the ERβ immunoprecipitates, indicating that both proteins can interact. However, the extent by which E6-AP can be recruited to ERβ was severely diminished upon disruption of Ser-94 and -106, suggesting that these sites behave as important determinants in the interaction. More interestingly, the recruitment of E6-AP to ERβ was more pronounced in response to MAPK activation as shown in cells transfected with Mek1 and Erk compared to control cells (Fig.7B). The S94,106A mutation severely impaired the effect of MAPK activation on the ability of ERβ to efficiently recruit E6-AP (Fig.7B). These results suggest that the interaction of ERβ with E6-AP is regulated in a phosphorylation-dependent manner and involves Ser-94 and -106 of ERβ AF-1 domain.

Discussion

In this study, we identify an important and unrecognized function of the AF-1 domain in targeting ERβ to the degradation process mediated by the ubiquitin-proteasome system. The current model of ER degradation has mostly been described for ERα in response to estrogen. Studies using proteasome inhibitors have shown that the hormonal activation of ERα was dependent upon the ubiquitin-proteasome pathway, therefore establishing a correlation between receptor degradation and activity, an observation also made for other nuclear receptors (12,37). Similarly to ERα, we report that the response of ERβ to estrogen also required the proteasome function to achieve optimal receptor activation. However, in absence of hormone, while both ERs accumulated in cells treated for proteasome inhibition, the role of the proteasome in maintaining basal ER activity seemed to be different between the two receptor isoforms. Using truncated versions of ERα and ERβ, we observed that such difference was dependent on the N-terminal region of each receptor, and that at least for ERβ, the N-terminal domain was itself regulated by the proteasome in both its cellular content and activity. Using a similar deletion within ERα, it was demonstrated that ERα N-terminal region was stabilized during proteasome inhibition but this effect could not be correlated with its transcriptional activity (14). The significance of an apparent correlation between AF-1 activity and ER degradation is not completely understood, but it becomes clear that AF-1 activity could impact ER degradation and that this may occur in an isoform-dependent manner. Consistent with this, whereas the AF-1 region was shown to regulate the ligand-induced degradation of ERα (38), our results indicate that the role of ERβ AF-1 on receptor degradation can be uncoupled from the hormonal response.

The mechanistic activation of AF-2 function by ligand has been well detailed for nuclear receptors and in particular, crystallographic studies of their ligand binding domains, such as those for ERα and ERβ, have helped to identify the structural determinants involved (39-42). In contrast, modulation of AF-1 activity of estrogen receptors is intricate and variable, and mostly depends on post-translational modifications including phosphorylation. It is known that in response to growth factors, such as EGF, the activity of ERα and ERβ is associated with phosphorylation of their respective AF-1 (2,22,43). The AF-1 domain of ERβ contains many putative phosphorylation sites of which Ser-106 and -124 were described to be directly phosphorylated by MAPK resulting in AF-1 activation of the receptor in response to EGF or ras (24,25). Here, we show that these clustered serines along with Ser-94 are also involved in ubiquitination of ERβ, therefore providing evidence that the region within AF-1 that signals ERβ to degradation overlaps with the one involved in its activation by growth factor signaling pathways. This apparent paradoxical roles shared by the same domain within ERβ raised the possibility that both events, i.e. ubiquitination and transactivation, are required to mediate optimal AF-1 response of ERβ to non-hormonal stimuli. Such dual role for an activation domain to also signal to ubiquitination was reported to be important in limiting the activation potential of transcription factors (44). As opposed to other steroid receptors such as ERα and progesterone receptor, for which phosphorylation of their AF-1 was linked to hormone-dependent degradation and activity (38,45), our results indicate that the AF-1 of ERβ appears to be sufficient in mediating such response. Supporting this hypothesis, we have observed that the N-terminal region of ERβ can be ubiquitinated in a manner similar to the full-length receptor and that such ubiquitination was also dependent on the integrity of Ser-94 and -106 (data not shown). In addition, we found that ERβ missing the AF-1 domain (CDEFβ construct) did not accumulate in

response to MG132, an effect also observed by others (46). Our interpretation from these findings is that, in absence of hormone, Ser-94 and Ser-106 within the AF-1 domain provide the signal(s) that triggers ERβ ubiquitination. Recently, Tateishi et al. (46) have reported that the N-terminal domain of human ERβ corresponding to amino acids 1-37 was necessary for its degradation by estrogen. Whether the immediate N-terminal region of ERβ regulates ligand-dependent ubiquitination and/or receptor degradation in a manner similar to the region containing Ser-94 and -106 in absence of hormone is not known, but it can be predicted that while different stimuli, either hormonal or kinase-derived, regulate ERβ activity, they may also converge toward a common mechanism that dictates receptor turnover. In addition, given the significant impact of the N-terminal region of ERβ to regulate receptor ubiquitination, the exact site(s) of such ubiquitination remains however unknown. Ubiquitin are typically conjuguated to lysine residues in targeted proteins and many conserved lysines reside in the N-terminal region of ERβ from several species. However, attempts to delineate the ubiquitination sites in the N-terminal domain of ERα and ERβ in response to hormone have not resulted in the identification of the targeted lysines (38,46). So clearly, the exact mapping of the ubiquitination sites in the context of AF-1 activity of ERβ remains to be determined.

Recent studies based on fluorescent-based approaches have revealed the dynamic nature of ERα movement within the nucleus and its association with active chromatin templates to engage transcription in response to ligands, or its immobilization to inactive clusters referred to as the nuclear matrix during proteasome inhibition (10,15,32,33,47). We have therefore performed FRAP analysis to evaluate the mobility of ERβ in conditions in which proteasome activity was inhibited and on the possible role of disrupting AF-1 activity on ERβ nuclear dynamics. In absence of ligand, ERβ was found highly mobile, reaching equilibrium with a half-maximal

recovery time comparable to ERα, indicating that both ERs reside in a highly dynamic state, presumably awaiting activation signals. Remarkably, while addition of MG132 resulted in a profound immobilization of ERβ, likely reflecting its clustering to non-chromatin templates, removal of Ser-94 and Ser-124, concomitantly with Ser-106, resulted in a more mobile receptor. These results therefore suggest that disruption of ERβ AF-1 activity may allow the receptor to escape from associating to inactive clusters during proteasome inhibition, and to become or remain available for transcriptional regulation. Consistent with this hypothesis is the potent activation we observed for the S94,106A mutant in the presence of MG132 and its reduced ubiquitination, indicating that the absence of these residues may lead to a deregulated ERβ which would be less prone to proteasome-mediated degradation. The apparent inability of the AF-1 mutants to behave such as the wild-type ERβ and remained clustered and immobilized during proteasome inhibition raised the interesting question as to whether AF-1 phosphorylation could signal ERβ to transit from active to inactive chromatin compartments. Since these clustered residues are known to be targeted by MAPK and therefore participate in the response of ERβ to growth factor signaling (2,25), it would be expected that such intranuclear behavior may be shared with other nuclear receptors described to also be regulated by kinase-activated pathways. In that respect, we recently reported that activation of Akt modulated the intranuclear behavior and activity of ERβ, and its segregation with coactivator CBP through a conserved Akt site shared with other nuclear receptors (48).

The transcriptional deregulation of ERβ characterized by its enhanced activity during proteasome inhibition upon AF-1 disruption was rather intriguing and suggested a mechanism by which the AF-1 may participate in restraining or at least maintaining an adequate and regulated response of ERβ to cellular pathways involving MAPK activation. In an attempt to partly

elucidate such mechanism, the E6-AP ubiquitin ligase was found to be favorably recruited to ERβ in response to signals that promote ERβ phosphorylation. Notably, this interaction was strongly dependent upon the presence of Ser-94 and -106 which resulted in a more rapid degradation of ERβ in response to Erk activation. This suggests that phosphorylation at these sites regulates ERβ turnover through the functional recruitment of components of the proteasome degradation pathway. Many studies, mostly involving ERα, have depicted a role for estrogen to modulate ER cellular levels through the ubiquitin-proteasome pathway, thereby providing a functional link between receptor turnover and activity. As such, several proteins that exhibit E3 ubiquitin-protein ligase activity, such as Mdm2 (18), CHIP (49,50), BRCA1 (51) and E6-AP (19), have been shown to modulate the hormonal response of ERα. In that respect, E6-AP was described as a dual-function protein that associates with ERα to promote its degradation and coactivation by estrogen (19,35,36). This study extends the role of E6-AP to regulate ERβ degradation through the proteasome pathway and, with the increased ability of ERβ to recruit E6-AP compared to the S94,106A mutant in response to Erk activation, it further identifies a novel function for serines 94 and 106 in maintaining sub-optimal ERβ levels. These observations suggest a role for the AF-1 domain to provide the necessary signals through phosphorylation of critical residues in order to recruit E6-AP and dictate ERβ turnover under ligand-independent mechanisms. It was recently reported that the estrogen-dependent proteolysis of ERβ was mediated through the recruitment of the E3 ligase CHIP to the N-terminal region of ERβ (46). This raises the interesting possibility that a selective recruitment of specific E3 ligases may be dependent on the upcoming activation signal, whether it is hormonal or kinase-derived, to ERβ. Based on studies which have identified various E3 ligases associated with ERα degradation by estrogen Mdm2 (18,19,49-51), it is likely that the cellular context and/or other structural

determinants, such as phosphorylation, might dictate selectivity in recruiting components of the proteasome pathway. Consistent with this, the estrogen-induced recruitment of Mdm2 and E6-AP to ERα-responsive genes was shown to be dependent on the presence of S118, a phosphorylation site within ERα AF-1 (38), and on the context of ERα-targeted promoters (52).

The study presented here on the role of selective serine residues within ERβ AF-1 domain that participate in E6-AP recruitment and ubiquitination, combined with the apparent overlap of these sites to mediate coactivator recruitment in response to growth factor signaling pathways (2,25), raises the interesting possibility that these sites clustered within the AF-1 domain of ERβ could dictate apparent paradoxical functions in transcription and degradation by allowing the exchange or the sequential recruitment of coregulators to ERβ in a non-hormonal dependent process. Transcriptional activation of inducible promoters by ERα in response to estrogen was characterized by an ordered recruitment of cofactors that possess distinct enzymatic activities such as acetylation/deacetylation, chromatin remodeling and ubiquitination (8,10,11). Based on the current view that such coordinated recruitment is involved to control nuclear receptor response to ligands (53,54), it is expected that the response of ERβ to non-hormonal stimuli such as those that trigger Erk activation may depend on similar mechanisms. Our studies demonstrate a novel role for a distinct cluster of phosphorylated residues within the AF-1 of ERβ to regulate receptor ubiquitination and degradation, and further add to the intricate regulatory mechanisms involved in the control of nuclear receptor function by kinase signaling pathways.

Materials and Methods

Plasmids. Expression pCMX plasmids coding for mouse ERα, mouse ERβ, and related truncated (ABC, CDEF, and chimeric constructs) and serine (positions 106 and 124) mutants have been described previously (25,26,28). Other ERβ serine to alanine mutants (positions 69 and 94) were generated by PCR mutagenesis using Pfu polymerase (Stratagene) and confirmed by automated sequencing. Plasmids coding for constitutively active Mek1 and Erk2 (28), and HA-tagged ubiquitin (55) have been described. The coding region of E6-AP has been inserted into pCMX-HA to produce a N-terminal tagged protein and the C833A ligase-deficient mutant was generated by mutagenesis as above.

Cell culture, transfection and luciferase assay. Human embryonic kidney 293 cells were routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) supplemented with 5% fetal bovine serum, in a humidified atmosphere of 5% CO_2 at 37C. For transient transfection, cells were seeded in phenol red-free DMEM supplemented with charcoal dextran-treated serum and plasmid constructs were introduced into cells using the calcium phosphate precipitation method as described (28). Typically, for luciferase assay, cells were transfected with 500ng EREtkLuc reporter construct, 100ng ER expression plasmids, 50ng each of Mek1 and Erk2 expression plasmids, and 250ng pCMX-βgal in a total of 1.5µg DNA per well. After 5-6 h, the medium was changed and cells were treated for 16hrs with 10nM 17β-estradiol (E2) and/or 1µM proteasome inhibitor MG132 (Sigma) or clasto-lactacystin β-lactone (BioMol), unless otherwise stated. Cells were then harvested in potassium phosphate buffer containing 1% Triton X-100 and lysates analyzed for luciferase activity using a luminometer (Wallac). Luciferase values were normalized for transfection efficiency to β-galactosidase activity and

expressed as relative fold response compared to controls. Luciferase assays are performed in duplicates from at least three independent experiments.

Cell lysates, immunoprecipitation and immunoblotting. Determination of ERα and ERβ cellular content by Western analysis has been described (28). Briefly, transfected cells were treated with 10nM E2 or 1μM MG132 for 16 hrs, washed with ice cold PBS and lysed in PBS containing 1% Triton X-100, 0.5% deoxycholate acid, 0.1% SDS, 1mM sodium orthovanadate, 1mM sodium fluoride, 1mM PMSF, and protease inhibitor cocktail (Roche). Cell lysates were then subjected to SDS-PAGE and proteins transferred to nitrocellulose for immunoblotting. Membranes were incubated at 4°C with blocking reagent (Roche) in TBS, probed with antibodies against ERα or ERβ, and signals revealed by ECL using appropriate HRP-conjuguated secondary antibodies. For cells transfected with HA-tagged ERβ constructs (truncated forms and serine mutants), an anti-HA antibody (12CA5) was used for immunoblotting. In each experiment, total protein content was normalized using an anti-β-actin antibody (Abcam). To detect ubiquitinated forms of ERβ, cells were transfected with ERβ (wt or mutated) in the presence or absence (control) of HA-tagged ubiquitin plasmid. Cells were treated with vehicle or 1μM MG132 for 16hrs, washed with ice cold PBS and lysed with 1% NP-40, 0.5% deoxycholate acid, 0.1% SDS, 1mM sodium orthovanadate, 1mM sodium fluoride, 1mM PMSF, and protease inhibitors in PBS. Cell lysates were pre-cleared before incubation with 1-2μg of anti ERβ antibody (Santa Cruz Biotech) overnight at 4°C with gentle agitation. Immune complexes were recovered with protein A/G-PLUS agarose, washed three times in lysis buffer and subjected to SDS-PAGE and immunoblotting as described above using anti-HA antibody. Co-immunoprecipitation analysis was performed to detect ERβ/E6-AP interaction in cells

transfected with wt or mutated ERβ (myc-tagged) in the presence of HA-tagged E6-AP. Immunoprecipitation of ERβ was performed as above with an anti-myc antibody (9E10), except that salt concentration was raised to 0.7M and no SDS was added in the lysis buffer. The anti-HA antibody (12CA5) was used for immunoblotting.

Cycloheximide chase. 293 cells were transiently transfected with plasmids expressing HA-tagged wild-type or S94,106AERβ in absence or presence of E6-AP or Mek1/Erk2 plasmids. At 12 hours after transfection, cycloheximide (Sigma) was added at a concentration of 50μM and cells were lysed for Western blot analysis at the indicated time points. Each signal intensity derived from two separate experiments was quantitated using an image analyzer (Alpha Innotech, San Leandro, CA) and expressed relative to β-actin levels.

Preparation of nuclear and cytoplasmic extracts. To prepare nuclear and cytoplasmic extracts, cells were pelleted by centrifugation, resuspended in hypotonic buffer containing 10mM Hepes, pH7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 1mM sodium orthovanadate, 1mM sodium fluoride, 0.5mM PMSF and protease inhibitors, and lysed by three freeze-thaw cycles. Nuclei were separated from the cytosolic fraction by centrifugation at 12000g for 5min at 4°C. The nuclear pellet was resuspended in the same hypotonic buffer except that salt concentration was raised to 420mM KCl and 25% (v/v) glycerol was added. Equivalent amounts of cytoplasmic and nuclear fractions were then subjected to SDS-PAGE for Western analysis. The content of selective markers for nuclear (anti-PARP; Santa Cruz Biotech) and cytoplasmic (anti-GAPDH; Santa Cruz Biotech) compartments was tested by immunoblotting to ensure for the qualitative purity of the prepared fractions.

Fluorescence recovery after photobleaching (FRAP). FRAP analysis was carried out on live 293 cells transfected with YFP fusions of wild-type or S94,106A mutant of ERβ. Cells were grown on chambered slides (LabTek) in phenol red-free DMEM containing 5% charcoal dextran-treated serum, and transfected by the calcium-phosphate procedure as descrived above. FRAP experiments were performed on a Zeiss LSM510 confocal microscope equipped with an Argon 514nm laser and a 530nm LP filter. A single z-section image of whole cell nuclei was captured before the bleach and at the indicated time points after. The bleached regions correspond to <10% of total nucleus using 100% laser intensity while scanned images were taken at 0.1% with an open pinhole and a numerical aperture of 0.8 to ensure that diffusion in the z dimension is avoided. Fluorescence intensities were analyzed using Zeiss Physiology sofware 3.2 and averaged from at least 10 nuclei.

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Figure legends

Figure 1. Transactivation by ERα and ERβ is variably dependent upon proteasome function. A- Human 293 cells were transfected with an EREbLuc reporter gene and ERα or ERβ expression plasmid, and treated with vehicle, 10nM 17β-estradiol (E2), and proteasome inhibitors MG132 (1μM) or clasto-lactacystin (1μM) for 16hrs. Cells were then harvested for luciferase activity measurements, and results normalized to β-galactosidase activity are expressed as relative luciferase units (RLU). **B-** 293 cells were transfected with EREbLuc reporter with ERα or ERβ expression plasmid and treated with increasing amounts of MG132 for 16hrs. Values are expressed as % change in activity compared to untreated cells set at 100%. **C-** 293 cells were transfected with ERα or ERβ constructs and treated with 10nM E2 or 1μM MG132 for 16hrs. Whole cell extracts were analysed by Western blot using ER specific antibodies. Sample loading was normalized with β-actin content for each sample.

Figure 2. Contribution of the AF-1 region to the proteasome-dependent activity of ERα and ERβ. A- The transcriptional activities of full-length versions or truncated forms lacking the N-terminal (CDEF constructs) or the C-terminal region (ABC constructs) of ERα or ERβ were determined on an EREbLuc in 293 cells, in response to 1μM MG132 or 1μM clasto-lactacystin. Also tested were chimeric forms of ERα and ERβ in which the AB regions were swapped between each ER isoforms. Values are expressed as fold response in luciferase activity compared to untreated cells set at 1.0 for each ER construct used in transfection. **B-** HA-tagged forms of C-terminal (ABC) or N-terminal (CDEF) truncated forms of ERα and ERβ were expressed in 293

cells and analyzed by Western blot using an anti-HA antibody. Cells were treated with 10nM E2 or 1μM MG132 for 16hrs prior to harvest. Protein amounts were normalized to β-actin content.

Figure 3. MAPK consensus sites within the AF-1 modulate ERβ activity upon proteasome inhibition. A- 293 cells were transfected with an EREbLuc reporter in the presence of wild-type ERβ or various serine to alanine substituted mutants at the indicated positions within the AF-1 region of ERβ. After transfection, cells were treated with 1μM MG132 for 16hrs and harvested for luciferase assay. Values are expressed as fold response compared to untreated cells set at 1.0 for each ERβ construct used in transfection. **B-** Similar experiment as in (A) except that wt and serine mutants of ERβ C-terminal truncated ABC constructs were used in transfection. **C-** Western analysis of wt and serine mutated ERβ expressed in cells treated with vehicle or 1μM MG132 for 16 hrs. Protein amounts were normalized to β-actin content. **D-** Similar as in (C) except that ABC truncated forms of ERβ were expressed.

Figure 4. Serines 94 and 106 regulate ERβ stability and ubiquitination. A- Cycloheximide chase experiment using 293 cells expressing ERβ or the S94,106A mutant. Transfected cells were treated with 50μM cycloheximide and lysed at the indicated time points for Western analysis. β-actin was used as a loading control. **B-** The S94,106A mutation confers an increased stability to ERβ. Quantitation of signal intensity of ERβ and S94,106A mutant derived from two separate experiments of cycloheximide chase described in (A). Results are normalized to β-actin content and expressed as the % change of time zero which was set at 100%. **C-** 293 cells were transfected with either wt or the S94,106A mutant of ERβ in the presence of a HA-ubiquitin

plasmid. Cells were treated with vehicle or 1μM MG132 for 16hrs, and harvested for immunoprecipitation using an anti ERβ antibody. Immunoprecipitates were analyzed by Western blot with an anti-HA antibody.

Figure 5. Proteasome function and serine modifications affect the nuclear dynamics of ERβ. A- Validation of the separation into cytoplasmic (C) and nuclear (N) protein extracts by Western analysis using antibodies against respectively PARP and GAPDH markers. **B-** 293 cells were transfected with HA-tagged wild-type or the S94,106A ERβ construct, and treated with vehicle or 1μM MG132 for 16hrs. Both cytoplasmic (C) and nuclear (N) fractions were isolated and analyzed by Western blot using an anti-HA antibody. **C-** Untreated and MG132-treated 293 cells transfected with wild-type or S94,106A mutant of YFP–ERβ were subjected to fluorescence recovery after photobleaching (FRAP) analysis. Images show single z-sections of whole cell nuclei obtained before and at the indicated time points after bleaching. Bleached images were obtained ~0.5 sec after the actual bleach corresponding to the rectangular box which represents 10-15% of the total cell volume. Scanned images were taken at 0.1-0.5% laser intensity, while bleach was at 100%. After bleach, total nuclear fluorescence reaches equilibrium faster for untreated compared to MG132 treated cells, indicating a restrained mobility of ERβ during proteasome inhibition. Scale bar represents 10 μm. **D-** Recovery curves from cells treated with vehicle (open squares) or 1μM MG132 (filled squares). Fluorescence intensity values were averaged (n=10 nuclei) and plotted over time. Values before bleach are shown to control for fluctuations of fluorescent signals.

Figure 6. The E6-AP ubiquitin ligase enhances ERβ degradation. A- Western analysis of wt and S94,106A mutated ERβ in response to E6-AP expression. After transfection, cells were treated with vehicle or 1μM MG132 for 16 hrs and analyzed for ERβ content. Protein amounts were normalized relative to β-actin. **B-** The S94,106A mutation impairs the E6-AP dependent decrease of ERβ levels in Erk-activated cells. Cells were transfected with ERβ or S94,106A mutant in the presence of increasing amounts of E6-AP construct, and analyzed by Western. Erk was activated by coexpressing constitutive Mek1 and Erk plasmids. Cells were also transfected with the C833A ubiquitin ligase-deficient form of E6-AP. **C-** The S94,106A ERβ mutant is less prone to degradation by E6-AP and Erk activation. Cells were transfected with the respective constructs as in (B) and subjected to a cycloheximide chase. Representative blots are shown for ERβ and S94,106A mutant in cells expressing E6-AP (left panel) or Mek1 and Erk (right panel). Corresponding quantitation of signal intensity of ERβ and S94,106A mutant derived from two separate experiments of cycloheximide chase is also shown. Results are normalized to β-actin content and expressed as the % change of time zero which was set at 100%.

Figure 7. Phosphorylation-dependent recruitment of E6-AP to ERβ through serines 94 and 106. A- E6-AP coimmunoprecipitates with ERβ. 293 cells were tranfected with myc-tagged ERβ or S94,106A mutant in absence or presence of HA-E6-AP, and then treated with 1μM MG132 for 16hrs. Immunoprecipitation was carried out with an anti myc antibody, and E6-AP was detected by Western analysis using an anti HA antibody. ERβ was also monitored in each sample using an ERβ antibody. **B-** E6-AP is recruited in a phosphorylation-dependent manner involving Ser-94 and -106. Coimmunoprecipitation assay as described in (A) except that Mek1/Erk plasmids were used in transfection to promote Erk activation and no MG132 was added to cells.

Fig.1

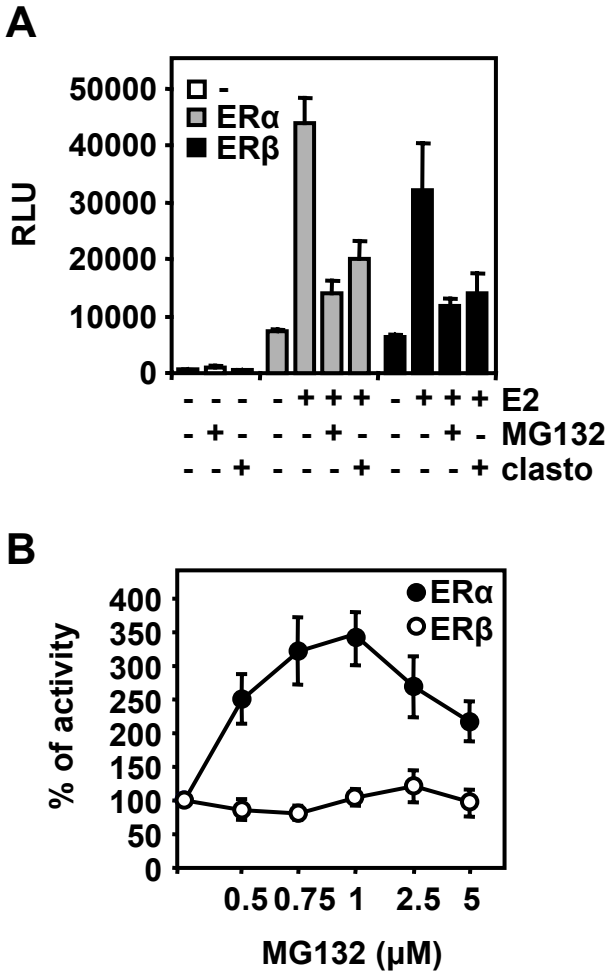

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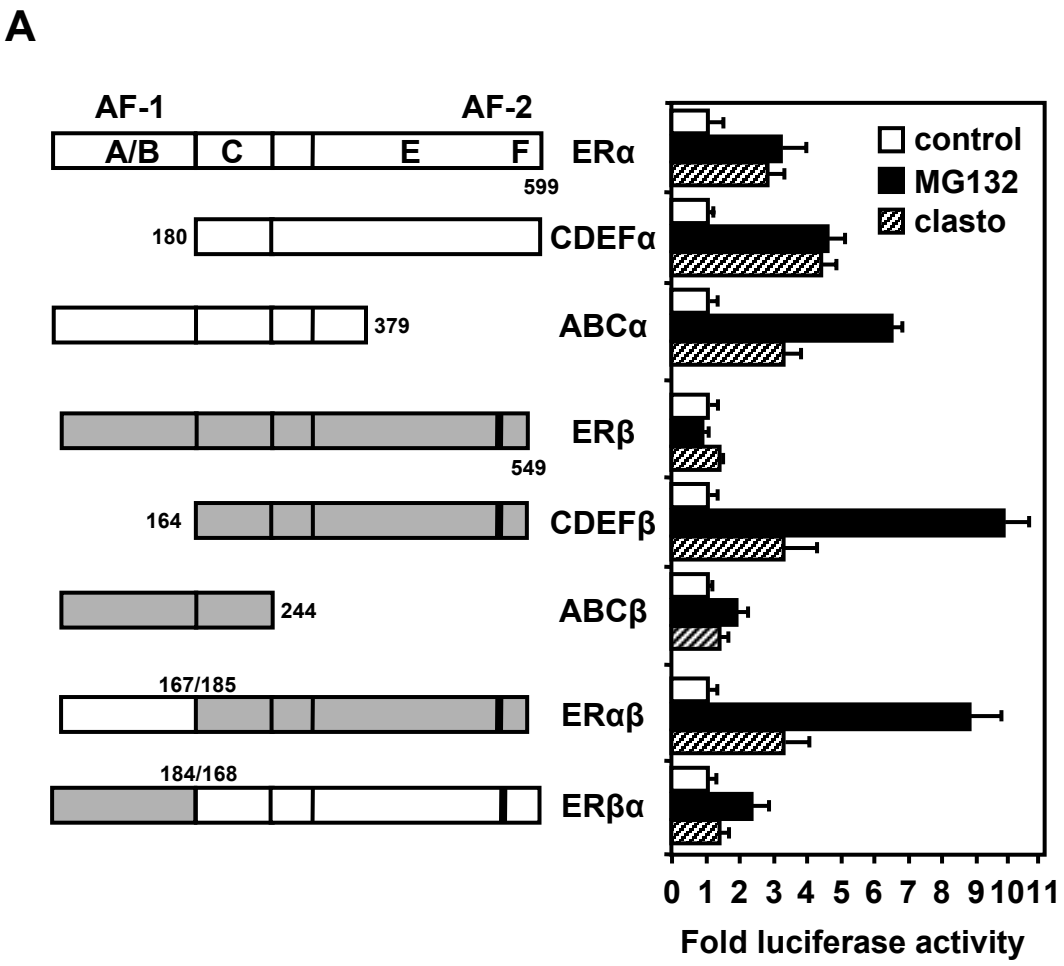
Fig.2



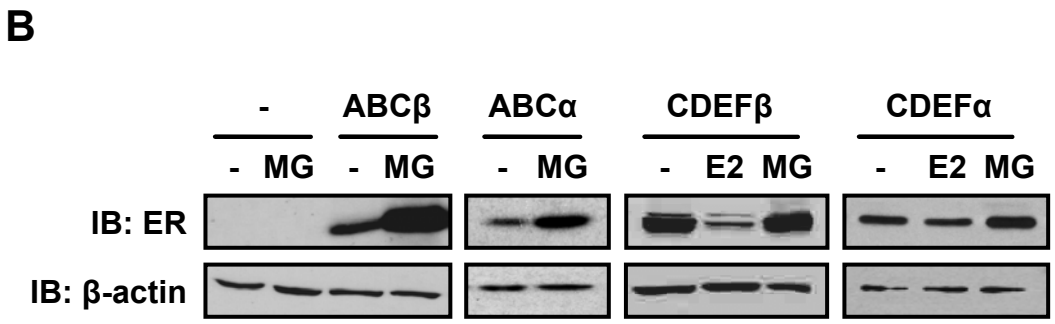
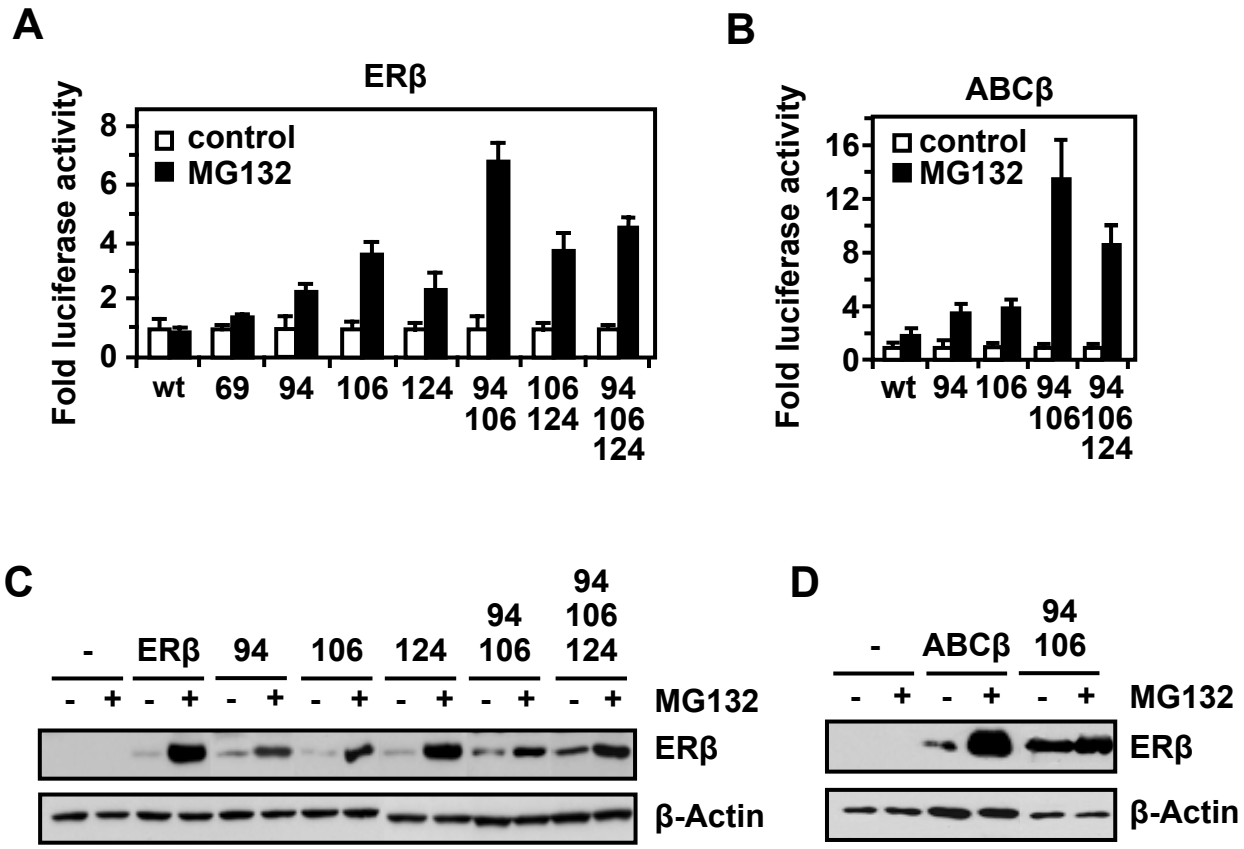


Fig.3



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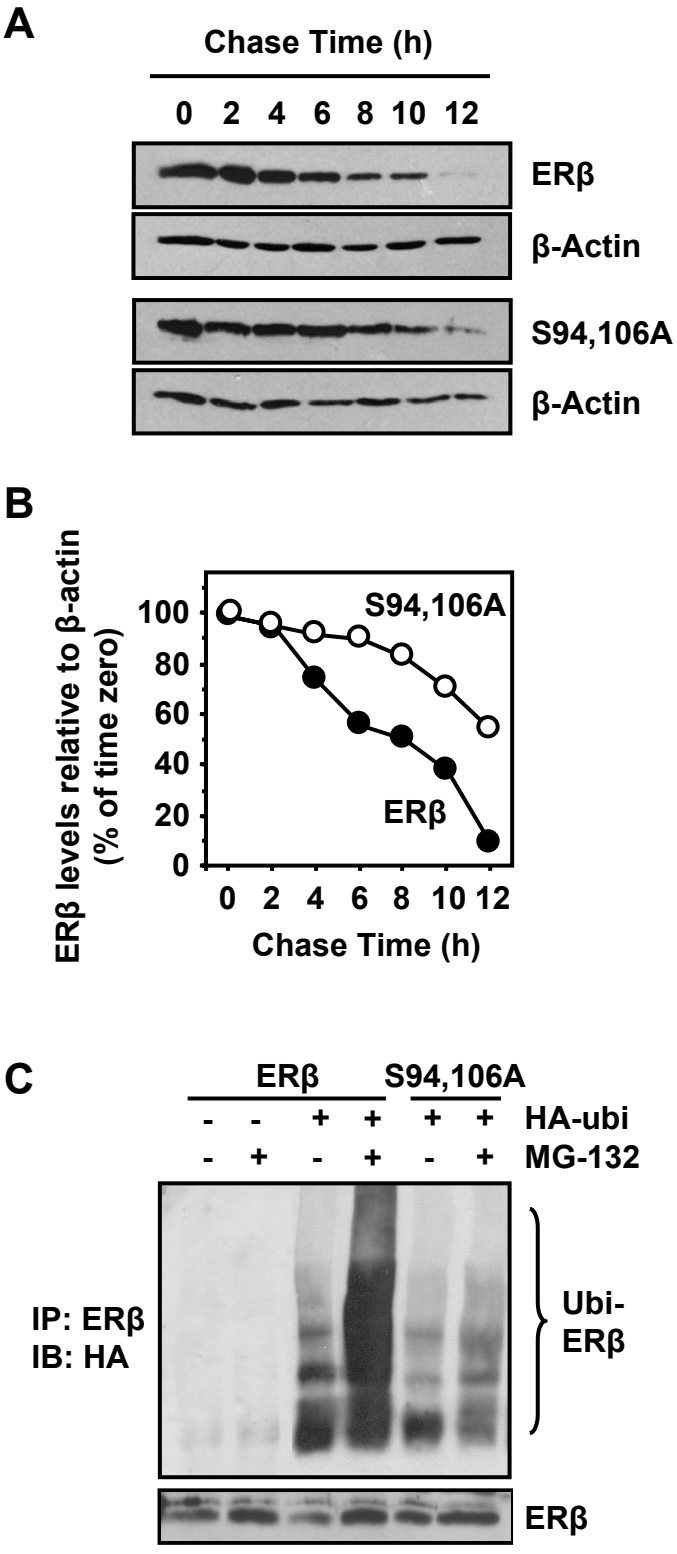

Fig.5

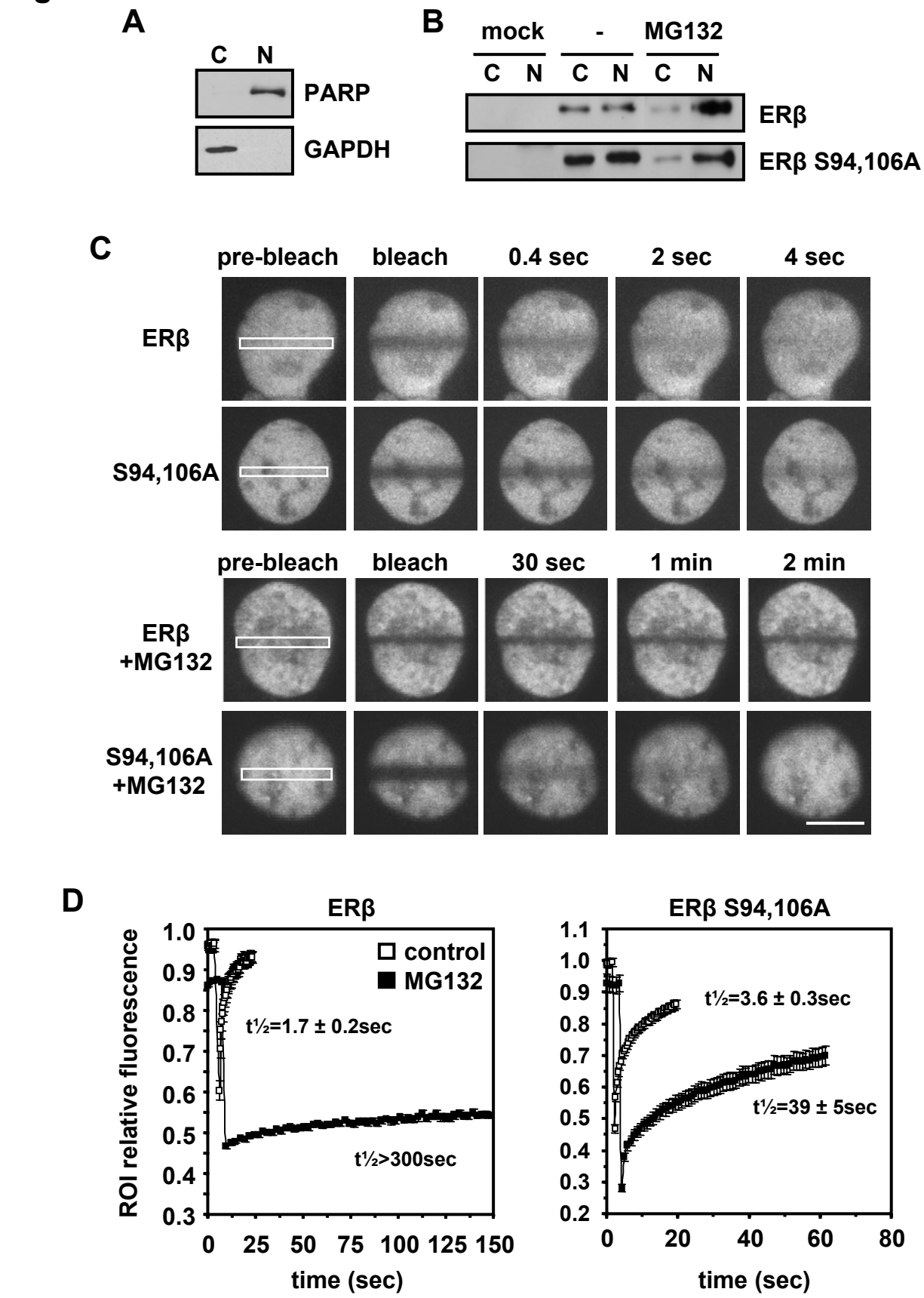

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Fig.6

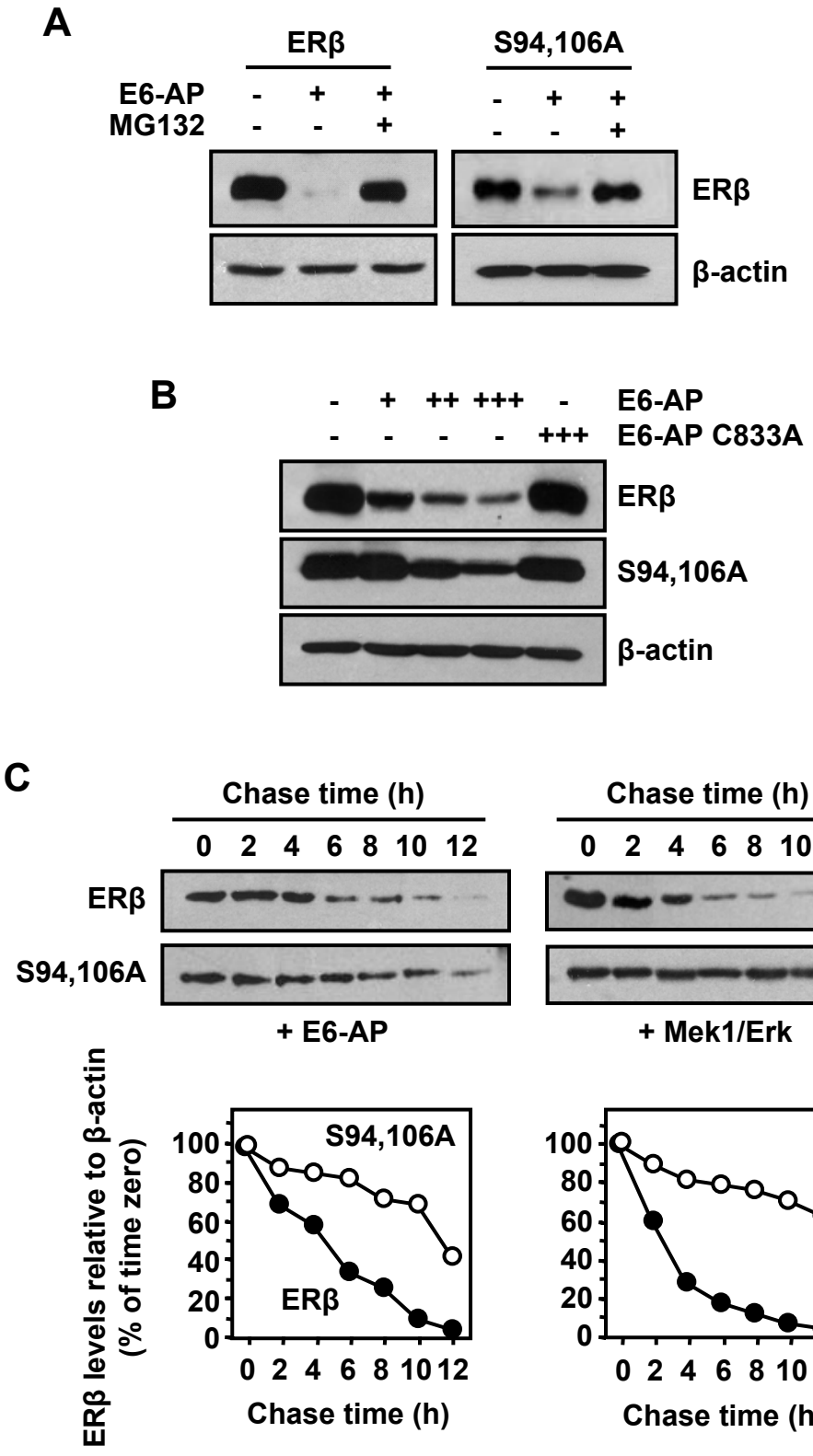

Fig.7