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RESEARCH ARTICLE

Upregulation of SIRT1 by 17β-estradiol depends on ubiquitin-proteasome degradation of PPAR-γ mediated by NEDD4-1

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ABSTRACT

17β-estradiol (E2) treatment of cells results in an up-regulation of SIRT1 and a down-regulation of PPARγ. The decrease in PPARγ expression is mediated by increased degradation of PPARγ. Here we report that PPARγ is ubiquitinated by HECT E3 ubiquitin ligase NEDD4-1 and degraded, along with PPARγ, in response to E2 stimulation. The PPARγ interacts with ubiquitin ligase NEDD4-1 through a conserved PPXY-WW binding motif. The WW3 domain in NEDD4-1 is critical for binding to PPARγ. NEDD4-1 overexpression leads to PPARγ ubiquitination and reduced expression of PPARγ. Conversely, knockdown of NEDD4-1 by specific siRNAs abolishes PPARγ ubiquitination. These data indicate that NEDD4-1 is the E3 ubiquitin ligase responsible for PPARγ ubiquitination. Here, we show that NEDD4-1 delays cellular senescence by degrading PPARγ expression. Taken together, our data show that E2 could upregulate SIRT1 expression via promoting the PPARγ ubiquitination-proteasome degradation pathway to delay the process of cell senescence.

KEYWORDS 17β-estradiol, PPARγ, senescence, SIRT1, ubiquitination

INTRODUCTION

SIRT1 (sirtuin 1) (Sir2 histone deacetylase) is an important determinant of longevity that regulates lifespan in diverse species (Kaeberlein et al., 1999). Caloric restriction (CR) can greatly increase life-span in mammals (Masoro, 2000), and the mammalian Sir2 orthologue, SIRT1, may mediate the life-span extending effect of CR by repressing peroxisome proliferator-activated receptor-γ (PPARγ) (Picard et al., 2004).

PPARγ is a transcription factor that belongs to the ligand-activated nuclear receptor superfamily. PPARγ plays an important role in the induction of cellular differentiation and the inhibition of cell growth by promoting arrest of the cell cycle (Chang and Szabo, 2000). In addition, PPARγ promotes cellular senescence by inducing $p16^{INK4\alpha}$ (CDKN2A), which is an important cell-cycle inhibitor (Gan et al., 2008).

Several nuclear receptors have been shown to be degraded by the ubiquitin-proteasome system, such as the retinoic acid receptor γ (Boudjelal et al., 2000; Kopf et al., 2000), the retinoic acid receptor α (Kopf et al., 2000), the thyroid hormone receptor (Dace et al., 2000) and PPARα (Blanquart et al., 2002). This degradation pathway is implicated in the regulation of many short-lived proteins involved in essential functions of the cells, including cell cycle control, transcription regulation, and signal transduction (Mimnaugh et al., 1999).

The females in many mammalian species live longer than the males, but critical questions remain to be clarified about the anti-aging mechanism of estrogen. In a preliminary experiment, we found that E2 elevated SIRT1 expression. Our previous study suggested that PPARγ could negatively regulate the transcription of SIRT1 (Han et al., 2010).

What role does PPARγ play in E2-stimulated SIRT1 gene expression? First, the PPARγ protein has a short half-life ($t^{1/2}$ = 2 h) and was found to be polyubiquitinated and degraded by the proteasome, which is a common mechanism to control protein levels posttranslationally (Hauser et al., 2000; Floyd and Stephens, 2002). In this study, we report that E2 stimulation enhances SIRT1 expression through inducing PPARγ degradation. This degradation is associated with ubiquitination of PPARγ.

Who could execute the degradation as the E3 ligase? Neu-

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ronal precursor cell-expressed and developmentally-downregulated protein NEDD4-1 is identified as the E3 ubiquitin ligase for ubiquitination of PPARγ. RNAi knockdown and overexpression analysis demonstrated that NEDD4-1 could delay cellular senescence. Furthermore, it is very important for the ability of E2 to enhance the ubiqutination degradation of PPARγ and increase the expression of SIRT1 during the aging process.

RESULTS

E2 enhances the expression of SIRT1 at the mRNA and protein levels and decreases expression of PPARγ at the protein level, but not at the mRNA level

First, we examined the expression of endogenous SIRT1 and PPARγ in response to E2 stimulation in Hela cells, an ERα-deficient cell line. In these experiments, Hela cells were grown in the absence of estrogen for at least 3 d, followed by either no treatment or treatment with saturating levels of E2 for 6 h. Fig. 1A shows, as expected, that treatment with E2 significantly enhanced the expression of SIRT1, accompanied by reduction of PPARγ.

Similarly, we compared the levels of SIRT1 and PPARγ in the absence or presence of E2 in tissues harvested by ovariectomy from Balb/c mice (4 months of age). The SIRT1 expression level increased and PPARγ expression level decreased in the hearts, brains and kidneys from these mice with the treatment of E2, compared with the levels in the organs of mice without E2 treatment.

To determine whether changes in the SIRT1 and PPARγ protein levels are due to changes in *SIRT1* and *PPARγ* transcription with the treatment of E2, we measured the levels of SIRT1 and PPARγ mRNA in the same cells and tissues via reverse transcriptase-polymerase chain reaction (RT-PCR). We found a significant correlation between the amount of SIRT1 transcript and translation (Fig. 1B). In contrast to protein levels, the level of *PPARγ* mRNA remained similar with either no treatment or treatment with E2 (Fig. 1B). Thus, the changes in the level of PPARγ protein occur post-transcriptionally, in response to E2 stimulation.

Our previous report (Han et al., 2010) suggested that PPARγ directly regulates SIRT1 transcription negatively. Subsequently, we performed the promoter activity experiment and determined that Hela cells were cotransfected with a negative control and either wild-type SIRT1-Luc containing PPARγ response elements or mutant SIRT1-Luc.

Cells were cultured and E2 was added to the culture medium 6 h prior to testing. Reporter activity was increased by treatment with E2; this activity was 50-fold higher in the presence of E2 compared with the activity in the absence of E2 (Fig. 1C). Mutation of SIRT1-Luc resulted in incomplete elevation of SIRT1 promoter activity in either the presence or absence of E2 (Fig. 1C). Thus, E2 directly regulates *SIRT1* transcription via the peroxisome proliferator response element (PPRE) cluster on the *SIRT1* promoter.

Using the chromatin immunoprecipitation (ChIP) assay, we determined the amount of PPARγ present on the estrogen target gene SIRT1 promoter. As shown in Fig. 1D, the quantity of PPARγ detected after 6 h of E2 stimulation had decreased significantly, compared to the control. Thus, E2-reduced PPARγ directly regulates the transcription of SIRT1.

E2-induced degradation of PPARγ is processed by proteasomes, not by lysosomes

Protein degradation can occur via proteasomes or lysosomes. It has been demonstrated that ubiquitination-mediated protein degradation is mediated via the proteasomal pathway (Hauser et al., 2000; Floyd and Stephens, 2002). To determine the mechanism of E2-induced PPARγ degradation, we used inhibitors of the proteasomal (MG132) or lysosomal (CHQ) pathways to assess the degradation route for PPARγ in Hela cells. As shown as in Fig. 2A, treatment with MG132 (lane 6) significantly inhibited E2-induced PPARγ degradation. The lysosomal inhibitor (CHQ) did not block the degradation of PPARγ (Fig. 2A, lane 2). Therefore, inhibition of E2-induced PPARγ degradation by MG132 in Hela cells might result from proteasomal inhibitory activity.

There are two possible causes for E2-induced reduction of PPARγ expression in Hela cells. One is suppression of the biosynthesis of PPARγ; the other is induction of the degradation of PPARγ. To determine the cause, after stimulating the Hela cells with E2, we treated the Hela cells with the protein translation inhibitor cycloheximide to eliminate protein biosynthesis, and then examined the effect of E2 stimulation on PPARγ decay. Fig. 2B (left panel) shows the decay of PPARγ under basal conditions; the PPARγ half-life was $\leqslant$ 2 h. Treatment with E2 accelerated the decay of PPARγ.

Because E2 affects PPARγ decay and this effect can be modulated by proteasome inhibitors, we hypothesized that there would be an increase in poly-ubiquitin-PPARγ conjugates after E2 treatment. To test this theory, we examined the formation of endogenous PPARγ-ubiquitin adducts in Hela cells. PPARγ proteins were immunoprecipitated from whole cell extracts that had been incubated in the presence or absence of E2 for the time indicated in Fig. 2. The immunoprecipitations were analyzed by immunoblotting using an anti-ubiquitin antibody (Fig. 2C). As shown in Fig. 2C (left panel), PPARγ was detected both in high molecular-mass forms that were present under basal conditions and again, with increased intensity, after E2 treatment.

The hypophosphorylated form of PPARγ, which often displays increased transcriptional activity (Lazennec et al., 2000), was found to be degraded more rapidly than the phosphorylated protein (Hauser et al., 2000; Floyd and Stephens, 2002), supporting a direct link between protein degradation and transcriptional activity (Muratani and Tansey, 2003). As shown in Fig. 2D, the phosphorylation level of PPARγ diminished dramatically after treatment with E2.

We further examined the effect of a PPARγ knockdown on the SIRT1 expression level in Hela cells in response to E2.

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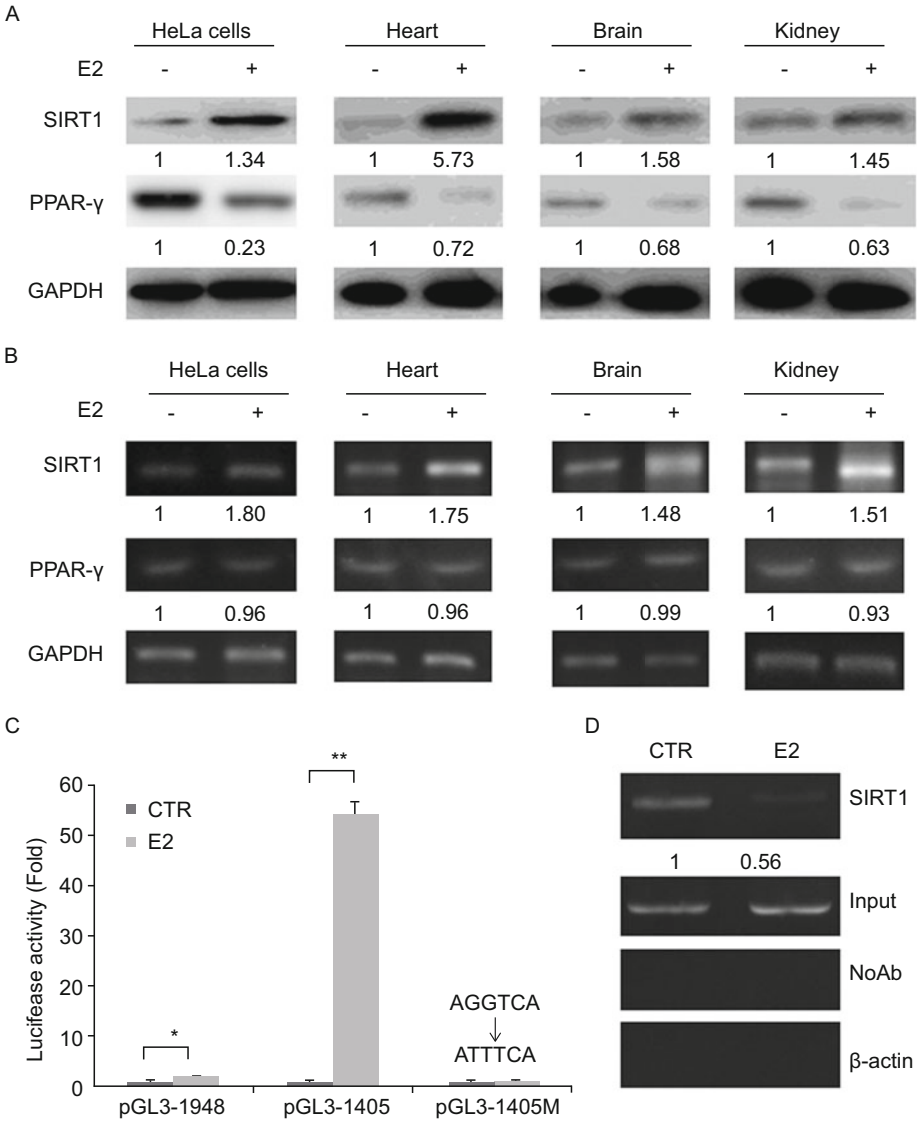

Figure 1. E2 induces degradation of PPARγ and enhances SIRT1 expression. Cells were grown in phenol red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% charcoal-dextran-stripped fetal bovine serum (FBS) for at least 3 d, and left untreated or treated with 100 nmol/L of E2 for 6 h. (A) Western blot of extracts from cells and animal tissues, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. Western blotting was performed using specific antibodies against SIRT1 and PPARγ, as indicated. (B) RT-PCR analysis of SIRT1 and PPARγ mRNA levels isolated from cells and animal tissues. GAPDH transcript was used as a control. The data presented are average values obtained from triplicate data points from a representative experiment (n = 3), which was repeated three times with similar results. (C) Normalized luciferase activity with the treatment of E2 (E2) for 6 h under the control of a series of the SIRT1 promoter containing peroxisome proliferator response element (PPRE) or PPRE-mutated fragments in Hela cells. Values are the mean ± s.d. of triplicate points from a representative experiment (n = 3), which was repeated three times with similar results. Values accompanied by different symbols are statistically significantly different from each other. (D) Occupancy of the SIRT1 promoter by PPARγ in Hela cells treated with E2 for 6 h, as measured by chromatin immunoprecipitation (ChIP). The PCR primers amplify the *SIRT1* promoter region from -592 bp to -163 bp.

As shown in Fig. 2E, the level of SIRT1 expression after the PPARγ knockdown was similar to the levels found after the E2 stimulation. To address the role of NEDD4-1 in regulating the E2-induced degradation of PPARγ, we compared the degradation of PPARγ in the NEDD4-1 knock-down cells with the control cells. Knockdown of NEDD4-1 significantly increased PPARγ expression levels (Fig. 2F, top panel, lanes 1 and 3). E2-induced PPARγ degradation was inhibited significantly by the NEDD4-1 knockdown (Fig. 2F, top panel, lanes 2 and 4).

Taking these results together, we conclude that E2-induced

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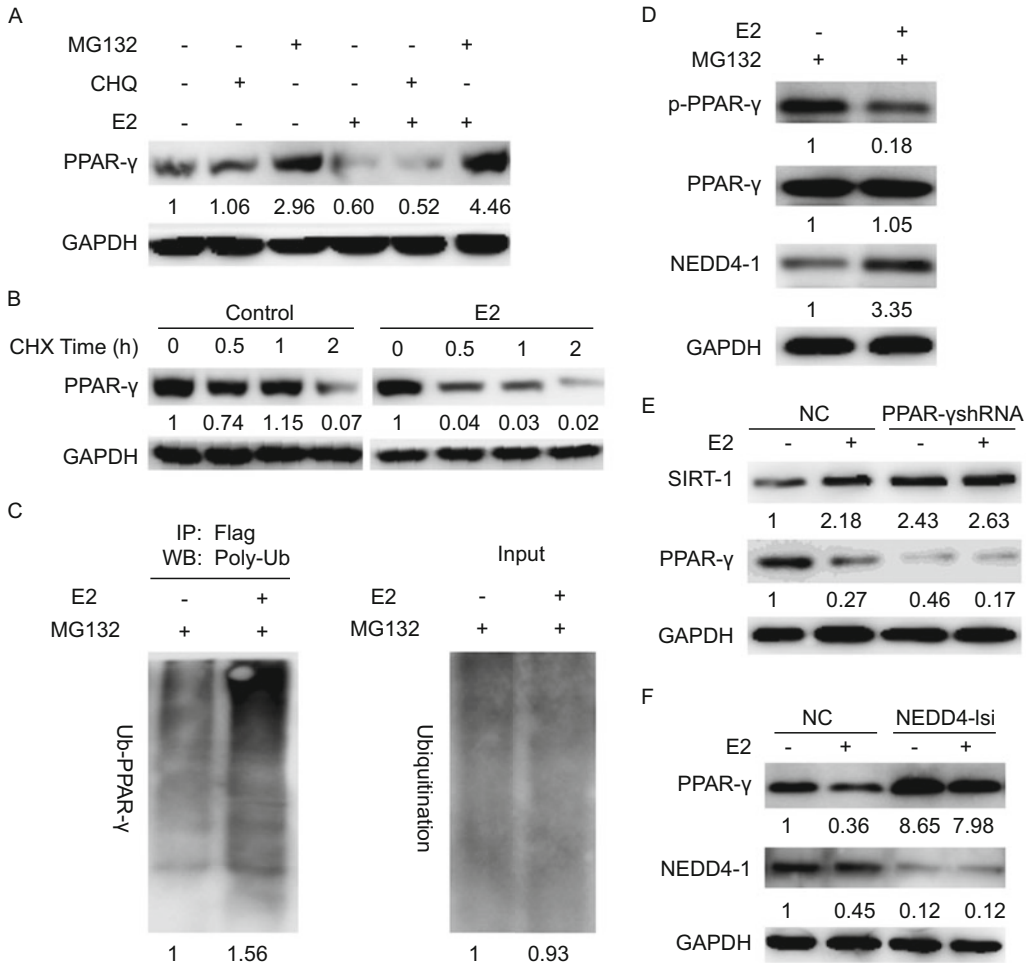

Figure 2. E2 treatment associated with an increase in PPARγ-ubiquitin conjugates. Cells were grown in phenol red-free Dulbecco's modified Eagle medium (DMEM) supplemented with 10% charcoal-dextran-stripped fetal bovine serum (FBS) for at least 3 days, and left untreated or treated with 100 nmol/L of E2 for 6 h. (A) The proteasomal and lysosomal inhibitors (20 μmol/L MG132 and 50 μmol/L CHQ) was added to the culture medium 1 h after E2 stimulation. After E2 stimulation for the indicated time, the cells were lysed. E2-induced degradation of PPARγ was determined by directly immunoblotting with an anti-PPARγ antibody. (B) Cycloheximide was added to the culture medium for various times after E2 stimulation. (C) Hela cells were treated with E2 (100 nmol/L) for 6 h subsequent to incubation with MG132 (20 μmol/L) for 5 h. (D) Western blot analysis of the phosphorylation levels of PPARγ in the presence or absence of E2. Total proteins were extracted, and western blotting was performed using specific antibodies PPARγ and phosphorylated PPARγ (p-PPARγ), as indicated. (E) The effect of E2 stimulation on SIRT1 after silencing the expression of PPARγ in Hela cells. (F) Knockdown of NEDD4-1 by RNAi enhances the expression of PPARγ and inhibits E2-induced degradation of PPARγ.

PPARγ degradation is processed by proteasomes and E2-elevated SIRT1 expression, which is mediated by PPARγ.

In vivo and *In vitro* PPARγ ubiquitination

To investigate whether PPARγ is ubiquitinated, we expressed either PPARγ, His-tagged ubiquitin or both in Hela cells. The cells were treated with or without MG132, a proteasome inhibitor proven useful in preserving short-lived ubiquitin conjugates. Immunoprecipitations were performed with an antibody against the Flag-tag, followed by a western blot analysis with the HA-tag antibody. In the absence of HA-tagged ubiquitin, no higher-weight molecular complexes were observed (Fig. 3A). Similarly, in the presence of both PPARγ and HA-tagged ubiquitin, but without the proteasome inhibitor, almost no high molecular-weight complex can be detected (Fig. 3A, lane 3). We conclude that the overexpressed PPARγ is polyubiquitinated and that the polyubiquitinated PPARγ is the substrate for proteasome, because treatment of cells with the proteasome inhibitor MG132 caused a robust increase of PPARγ polyubiquitination (Fig. 3A, lane 4).

Subsequently, we used recombinant glutathione S-transferase (GST)-tagged PPARγ as the substrate. After incubation with recombinant ubiquitin-activating enzyme (E1), ubiquitin-

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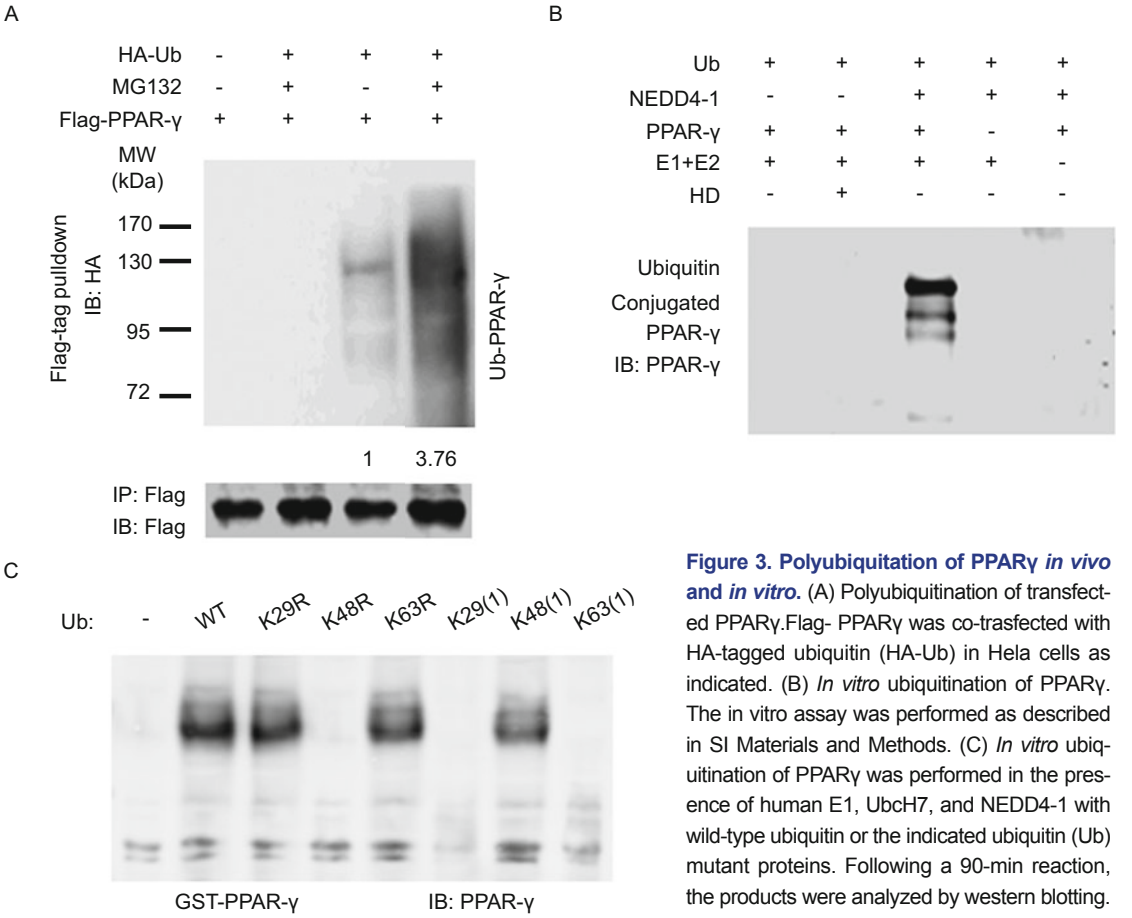

Figure 3. Polyubiquitation of PPARγ *in vivo* and *in vitro*. (A) Polyubiquitination of transfected PPARγ.Flag- PPARγ was co-trasfected with HA-tagged ubiquitin (HA-Ub) in Hela cells as indicated. (B) *In vitro* ubiquitination of PPARγ. The in vitro assay was performed as described in SI Materials and Methods. (C) *In vitro* ubiquitination of PPARγ was performed in the presence of human E1, UbcH7, and NEDD4-1 with wild-type ubiquitin or the indicated ubiquitin (Ub) mutant proteins. Following a 90-min reaction, the products were analyzed by western blotting.

conjugating enzyme UbcH7 (E2), ATP, ubiquitin, and GST-NEDD4-1 or HECT, the domain deletion-mutant of GST-NEDD4-1 (HD), as indicated in Fig. 3B, GST-PPARγ was pulled down by glutathione Sepharose, and polyubiquitination of GST-PPARγ was analyzed by immunoblotting against ubiquitin.

In the assay, we observed that NEDD4-1 was a dependent polyubiquitin of GST-PPARγ (Fig. 3B, lane 3), but not of GST or HD (Fig. 3B, lanes 1 and 2, respectively). The requirement of the HECT-domain for NEDD4-1 indicates that NEDD4-1 provides E3 ligase activity for PPARγ.

Finally, to establish the chain type specificities of NEDD4-1, *in vitro* ubiquitination reactions were performed with PPARγ, E1, UbcH7 and NEDD4-1 in the presence of wild-type or variant forms of ubiquitin. Ubiquitin variants contained either a single lysine-to-arginine mutation (e.g., K29R, K48R, K63R) or six lysine-to-arginine mutations, so that only a single lysine remained [K29(1), K48(1), K63(1)]. As shown in Fig. 3C, PPARγ was efficiently ubiquitinated by NEDD4-1 in the presence of wild-type ubiquitin, K29R, K63R, K29(1), K48(1) and K63 (1). These results indicate that NEDD4-1 has a strong preference for catalysis of K48-linked polyubiquitination *in vitro*.

NEDD4-1 can ubiquitinate PPARγ in cells

We confirmed the role of NEDD4-1 as PPARγ E3 in cells. Hela cells were contransfected with Flag-tagged PPARγ and HA-ubiquitin in the presence or absence of NEDD4-1 overexpression. Overexpression of NEDD4-1 in Hela cells caused an increase of PPARγ polyubiquitination in a NEDD4-1 dose–dependent manner (Fig. 4A). As expected, a high level of NEDD4-1 overexpression also caused a decrease of endogenous PPARγ protein in the Hela cells. Such a decrease of PPARγ protein level can be blocked by proteasomal inhibitor MG132 (Fig. 4B). Further, we showed that NEDD4-1 overexpression can improve polyubiquitination of endogenous PPARγ in Hela cells (Fig. 4C, lane 3), and that the NEDD4-1-catalyzed PPARγ polyubiquitination can be further enhanced by treatment with MG132 (Fig. 4C, lane 4). These results indicate that NEDD4-1 can polyubiquitinate endogenous PPARγ and thereby target PPARγ to proteasomal degradation.

Using the RNAi approach in a dose-dependent way, we demonstrated that endogenous NEDD4-1 negatively regulates PPARγ levels quantitatively following increased RNAi concentration (Fig. 4D).

We further showed that negative regulation of PPARγ steady-state level by NEDD4-1 indeed occurs through modulating PPARγ degradation. To examine the rate of PPARγ degradation, PPARγ was contransfected with plasmids for NEDD4-1 overexpression or RNAi knockdown. Subsequently, protein translation in cells was inhibited by treatment with cy-

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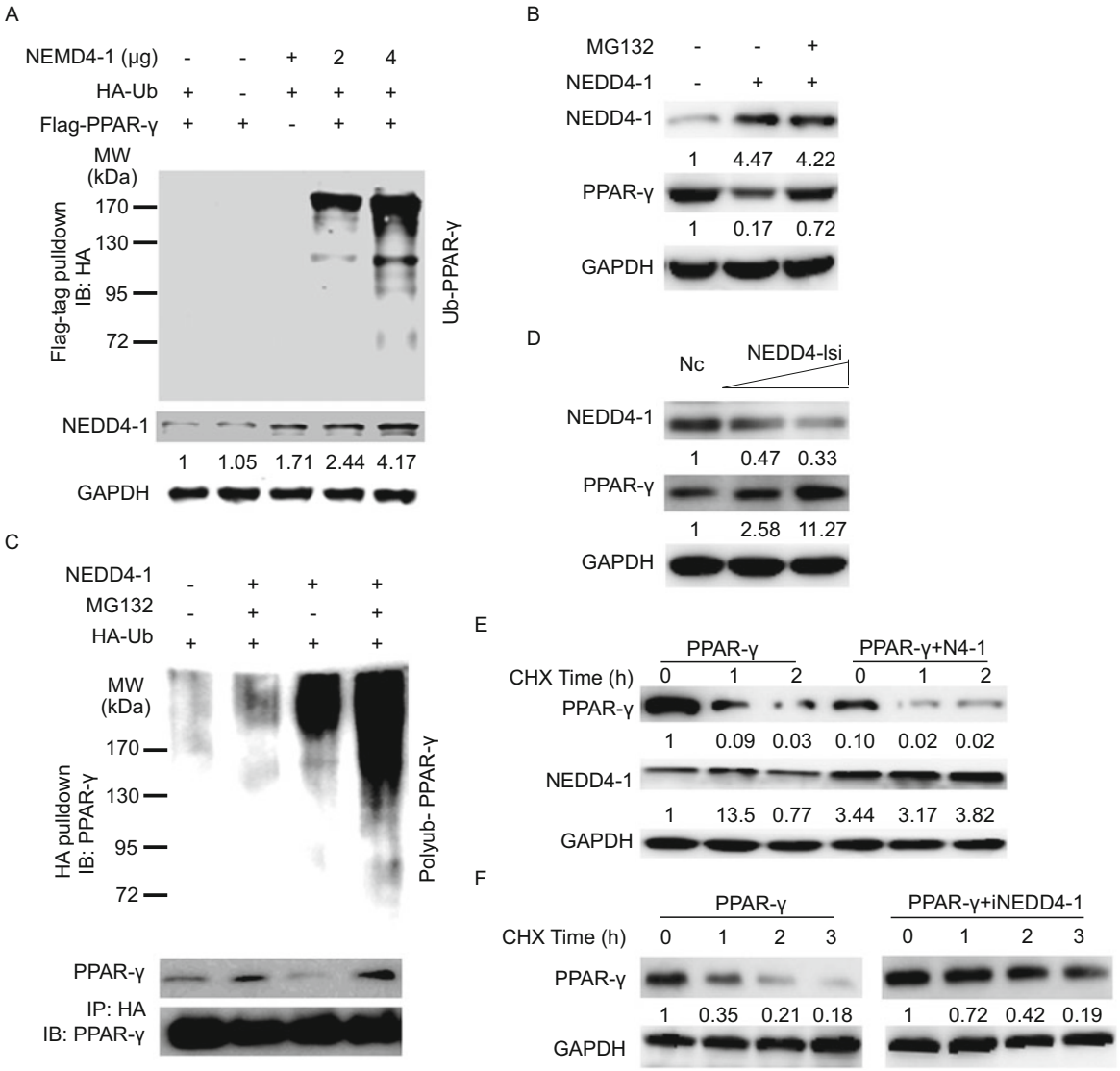

Figure 4. Regulation and degradation of PPARγ ubiquitination by NEDD4-1 in cells. (A) Overexpression of NEDD4-1 enhances polyubiquitination of PPARγ. Flag-tagged PPARγ, HA-Ub and increasing amount of NEDD4-1 were co-transfected into Hela cells, as indicated. (B) Overexpression of NEDD4-1causes a decrease in the PPARγ protein level. As indicated, NEDD4-1 with a C-terminal HA-tag or a vector alone was transfected into Hela cells. After 24 h, the cells were treated with or without 20 μmol/L MG132 for 5 h, as indicated. (C) NEDD4-1 polyubiquitinates endogenous PPARγ and targets it for proteasomal degradation, as described in the SI Materials and Methods. (D) Elimination of NEDD4-1 by RNAi increases the PPARγ level in cells. A gradually-increasing dose of NEDD4-1 RNAi was transfected into Hela cells, as indicated. (E) NEDD4-1 overexpression decreases PPARγ stability, as detailed in SI Materials and Methods. (F) NEDD4-1 RNAi increases PPARγ stability. PPARγ was cotransfected with NEDD4-1 RNAi or control plasmid, as indicated; cells were treated with cycloheximide and harvested at the indicated time points.

cloheximide, and PPARγ protein levels were detected at the times of treatment indicated in the figures. As shown in Fig. 4E and 4F, the overexpression of NEDD4-1 and the RNAi knockdown of NEDD4-1 caused an increased and a decreased rate of PPARγ degradation, respectively.

Taking these data together, we conclude that NEDD4-1 is a physiological E3 for PPARγ that targets PPARγ to proteasomal degradation.

The WW3 domain of NEDD4-1 interacts with PPARγ and is required for ubiquitination of PPARγ; PPARγ interacts with NEDD4-1 via a conserved WW domain–binding motif

To confirm the interaction of PPARγ with NEDD4-1 in cells, Flag-tagged PPARγ was expressed in Hela cells. Endogenous NEDD4-1 was coimmunoprecipitated with PPARγ after immunoprecipitation of PPARγ with an anti-Flag antibody (Fig. 5A).

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The full-length NEDD4-1 protein contains several functional motifs (Fig. 5B). In the N terminus, there is a proline-rich domain and a polyserine motif, followed by a C2 domain. NEDD4-1 also contains four WW domains in the middle and the signature E3 domain, the HECT domain, in the C terminus.

To determine the region in NEDD4-1 that interacts with PPARγ, we subcloned each of the four WW domains of human NEDD4-1 into a GST fusion protein expression vector (Fig. 5C) and performed the GST fusion fusion–protein pull-down assay by incubating purified bead-bound GST-WW domain fusion proteins with PPARγ of the product from the TNT Quick Coupled Transcription/Translation System. As shown in Fig. 5B, the bead-bound GST-WW3 precipitated PPARγ (lane 3, top panel), while the GST-WW1, GST-WW2 and GST-WW4 did not (lanes 2, 3 and 5, top panel, respectively), indicating that WW3 is the domain binding to PPARγ.

We next characterized the interaction of PPARγ with NEDD4-1, which belongs to the WW domain-containing family of E3 ubiquitin ligases (Rotin et al., 2000; Ingham et al., 2004) and has four WW-type domains that interact with the PPXY motif, in its ubiquitination substrates, or regulatory proteins (Sudol and Hunter, 2000).

The peptide sequence of PPARγ contains a PPXY motif located in a proline-rich region from amino acids 85–88 (Fig. 5D). To determine whether the PPXY motif is the site in PPARγ that interacts with NEDD4-1, we constructed a PPXY deletion mutant and designated this mutant the ΔPPARγ-3×Flag. As shown in Fig. 5A and 5E, endogenous PPARγ precipitated NEDD4-1 from Hela cell lysates, whereas ΔPPARγ did not, indicating that NEDD4-1 interacts with the PPXY domain of PPARγ.

NEDD4-1 delays cellular senescence

Cellular senescence is characterized by elevated levels of endogenous β-galactosidase activity at pH 6.0; this activity may be identified by assay with X-gal, which reacts with a blue color. Young (22 population doublings [PDs]) and senescent (61 PDs) cells from the 2BS cell line were analyzed as controls. We monitored morphological changes in cells through altered NEDD4-1 expression in Ras-induced senescent IMR-90 or 2BS cells. Young IMR-90 or 2BS cells infected with NEDD4-1 or NEDD4-1-shRNA were monitored (Fig. 6A and 6C, respectively). Infected IMR-90 cells were induced to express Ras protein into premature senescence by adding tamoxifen for 4–5 d. Whereas nearly all of the NEDD4-1-shRNA-infected cells displayed stronger levels of blue SA-β-gal staining similar to senescent cells, the control cells showed a lower frequency of SA-β-gal staining (Fig. 6B and 6D, respectively). However, only sporadic SA-β-gal-positive cells were seen in NEDD4-1 -transfected cells, although the corresponding vector control cells showed strong SA-β-gal staining (Fig. 6B and 6D, respectively). Taken together, these data indicate that NEDD4-1 caused resistance to cellular senescence.

DISCUSSION

Eukaryotic cells exhibit rigorous control over gene expression by tightly regulating the expression and activity of transcription-factor proteins. The concentration and activity of transcription regulators are controlled, at least in part, through proteasome-mediated protein degradation.

The first step, the ubiquitination of proteins which are subsequently degraded by the 26S proteasome complex, is a highly-regulated process leading to the modulation of transcription activity (Hodges et al., 1998). Recent data demonstrate that the ubiquitin-proteasome degradation system affects the activity of several nuclear receptors.

The novel observations in this study include: (1) the increased ubiquitin conjugation of PPARγ following the introduction of E2 to elevate SIRT1 expression, (2) evidence that PPARγ has been disclosed as a new substrate of the NEDD4-1 E3 ubiquitination ligase, and (3) evidence that NEDD4-1 can delay cellular senescence. These results open a new avenue to study the roles of ubiquitin-proteasome-mediated degradation of PPARγ and E2 in the process of aging.

Our results demonstrate that PPARγ is targeted by proteasome under basal conditions and following E2 treatment of Hela cells. We have also observed ubiquitin conjugation of PPARγ under basal conditions and demonstrated a substantial increase in ubiquitin conjugation of PPARγ after E2 exposure. Our results demonstrate that proteasome inhibitors reduce the effect of E2 on PPARγ expression. Furthermore, the results demonstrating the appearance of PPARγ-polyubiquitin conjugates indicate that E2 treatment results in the rapid degradation of PPARγ via the ubiquitin-proteasome pathway.

The rapid reduction in PPARγ protein levels following E2 treatment led us to predict that E2 treatment would suppress PPARγ activity in Hela cells. Our results showed that transcription activity of PPARγ was inhibited after E2 treatment to enhance SIRT1 promoter activity. Although E2 has not been shown to be a ligand for PPARγ, the activation of PPARγ is a ligand-dependent process (Rosen et al., 2000). Our data demonstrate that E2 treatment results in both the activation of PPARγ and the ubiquitin-proteasome-dependent degradation of PPARγ, suggesting that E2-mediated signaling in Hela cells may be associated with the binding of an endogenous ligand and the activation and subsequent degradation of PPARγ.

The phosphorylation of PPARγ by mitogen-activated protein kinases (MAPKs) has been described in various studies (Hu et al., 1996; Zhang et al., 1996; Adams et al., 1997; Camp and Tafuri, 1997; Camp et al., 1999). Phosphorylation plays an important role in targeting many substrates for ubiquitination and can either inhibit or increase the targeting of substrates to the ubiquitin-proteasome system (Hershko and Ciechanover, 1998; Weissman, 2001). The results in Fig. 2D demonstrate that phosphorylated PPARγ decreased dramatically following E2 treatment. The hypophosphorylated form of PPARγ, which often displays increased transcriptional activity (as described

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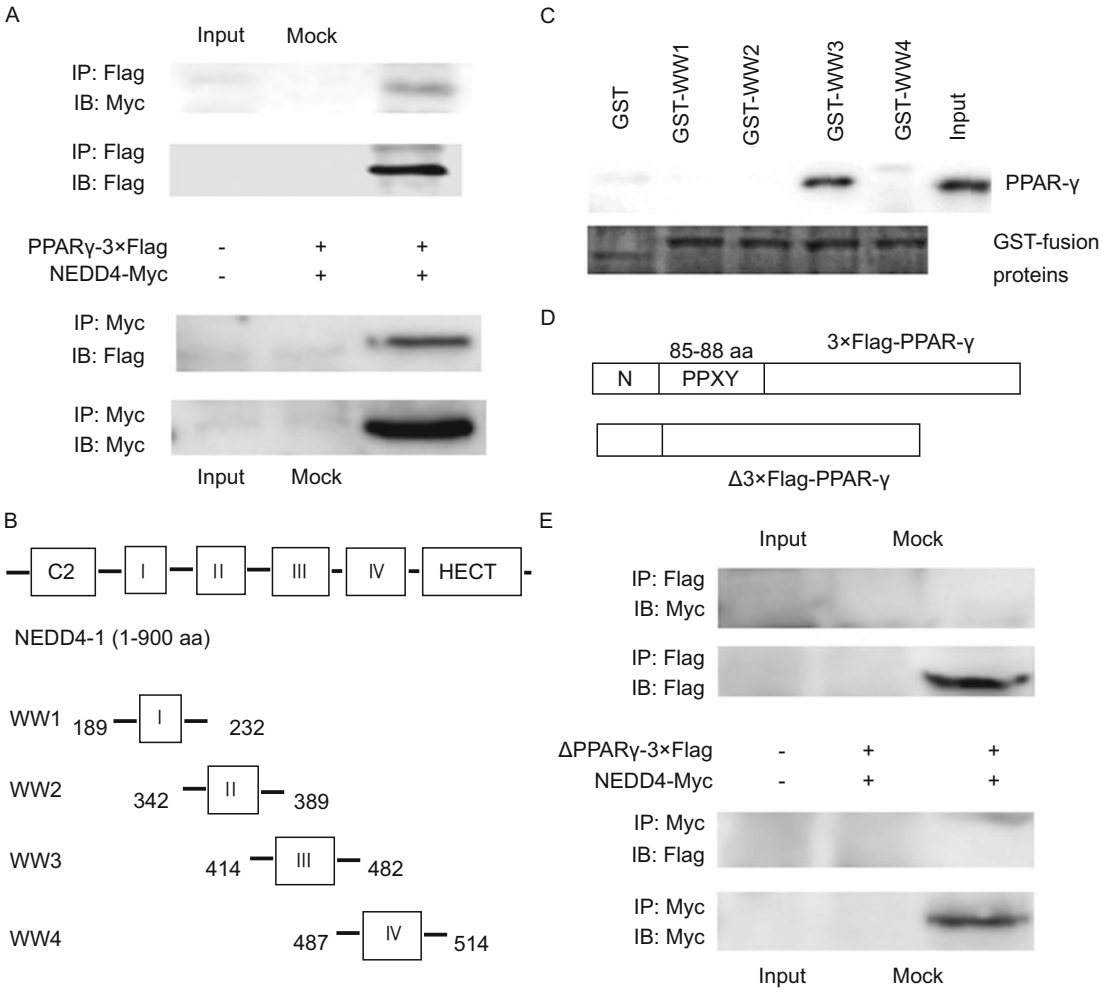

Figure 5. PPARγ interacts with NEDD4-1 through a conserved PPXY-WW-binding motif. (A) 3×Flag-tagged PPARγ and Myc-tagged NEDD4-1 cotransfected into Hela cells. (B) Schematic diagram showing the structural organization of the human GST NEDD4-1-WW domain constructs. The C2 domain translocates the protein to the membrane upon calcium binding. The four WW domains are known to bind substrate proteins containing PY motifs. The catalytic cysteine within the HECT domain is responsible for ubiquitin transfer. (C) Direct physical interaction of PPARγ with NEDD4-1 serial WW domain–deletion mutants, as determined by a GST pull-down assay. (D) Schematic representation of the PPXY-WW binding motif of PPARγ with the known NEDD4-1-binding site. (E) Myc-tagged NEDD4-1 and PPXY domain-defective mutant 3× Flag-tagged-ΔPPARγ were cotransfected into Hela cells. (F) ChIP-upon-ChIP assay showing the colocalization of PPARγ with NEDD4-1 at the *SIRT1* promoter.

earlier), was degraded more rapidly than phosphorylated protein (Hauser et al., 2000; Floyd and Stephens, 2002), supporting a direct link between protein degradation and transcriptional activity (Muratani and Tansey, 2003). Phosphorylation of PPARγ proteins may serve as an ubiquitin-proteasome targeting signal in which PPARγ is converted to the phosphorylated form prior to its degradation by the ubiquitin-proteasome pathway. It is not known currently how the phosphorylation status of PPARγ may regulate its ubiquitination and subsequent degradation.

Moreover, we demonstrated for the first time that PPARγ is a substrate of NEDD4-1. NEDD4-1 ubiquitinated PPARγ, which depended on the HECT domain.

Our data in Fig. 4 suggest an important role for NEDD4-1 in regulating PPARγ turnover. We observed a specificity of PPARγ ubiquitination, which was preferentially catalyzed by NEDD4-1 (Fig. 4). A proteasomal inhibitor such as MG132 is a cell-permeable compound that specifically blocks the activity of the 26S proteasome. Treatment using MG132 caused an accumulation of PPARγ ubiquitination (Fig. 4C). NEDD4-1 is required for PPARγ degradation, because knockdown of NEDD4-1 by RNAi significantly enhanced the protein expression level of PPARγ and suppressed degradation of PPARγ (Fig. 4F).

We need to determine further whether NEDD4-1–mediated ubiquitination of PPARγ is required for E2-induced PPARγ degradation. We can explore this issue by examining the dominant

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negative effects of the ubiquitination site mutants of PPARγ and the ligase-dead mutant of NEDD4-1 after PPARγ degradation.

On a positive note, we found that NEDD4-1 binds to PPARγ through a direct interaction between WW domain and the PPYY motif. Among the open research questions, we have not yet identified any ubiquitination acceptor lysines for the PPARγ protein.

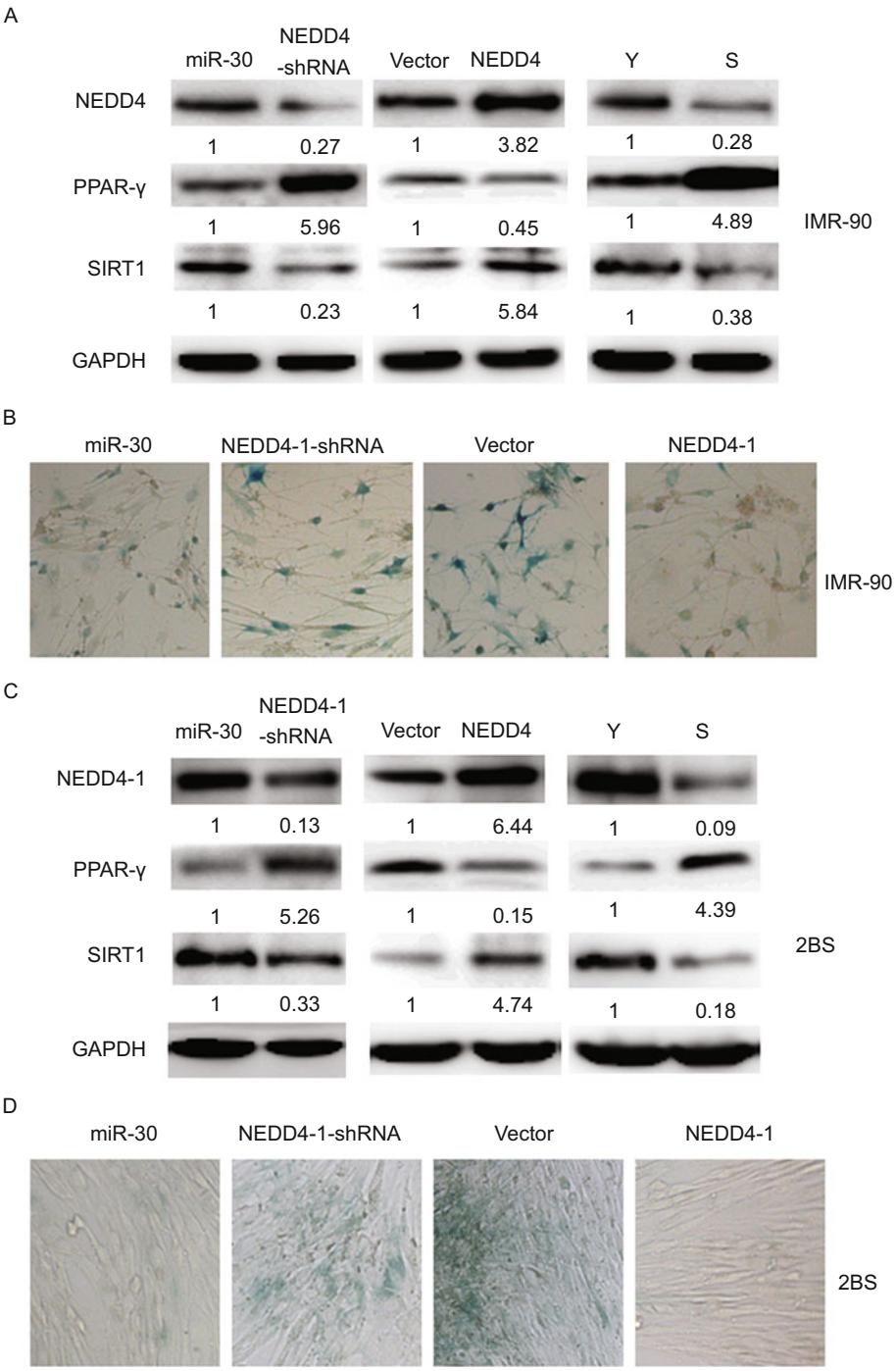

Figure 6. Changes in senescence-associated features in Ras-induced IMR-90 or 2BS cells were indicated by perturbations. (A and C) Western blot analysis of the NEDD4-1 protein levels in Ras-induced IMR-90 or 2BS cells exposed to the indicated perturbations, respectively. Western blotting was performed using specific antibodies against NEDD4-1 as indicated. The glyceraldehyde 3 phosphate dehydrogenase (GAPDH) lane is the loading control. (B and D) β-gal staining (blue) in Ras-induced IMR-90 or 2BS cells, exposed to the indicated perturbations, respectively.

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Senescence-associated beta-galactosidase (SA-*β*-gal) activity can distinguish senescent cells from quiescent or terminally differentiated cells and act as a biomarker of senescent cells (Dimri et al., 1995). We want to know whether the senescent-like phenotype (influenced by SA-*β*-gal activity) changes when we infect NEDD4-1 or NEDD4-1 shRNA plasmids into young 2BS cells or Ras-induced IMR-90 cells. Our results suggested that NEDD4-1 influenced the appearance of senescence-associated features (Fig. 6B and 6D).

In sum, our data demonstrate that E2 induces SIRT1 expression by enhancing the PPARγ ubiquitination. The PPARγ interacts with NEDD4-1 through its conserved PPXY motif. Through the interaction with PPARγ, NEDD4-1 was allowed, as the E3 ligase, to recognize and ubiquitinate the receptor. Analysis of RNAi knockdown or overexpression of NEDD4-1 in 2BS cells or Ras-induces IMR-90 cells demonstrated that NEDD4-1 could delay cellular senescence.

In light of our current findings and the studies cited above, we have formulated a model for the degradation of PPARγ proteins and the increase of SIRT1 expression following E2 during aging. This model, suggests that activation of PPARγ by E2 is followed by ubiquitin-proteasome–mediated degradation. This model also suggests that the E3 ligase of PPARγ ubiquitination is NEDD4-1 and that serine dephosphorylation contributes to PPARγ degradation.

Because only a few studies on E3 ligase–mediated senescence exist (Zhang and Cohen, 2004; Majumder et al., 2008), our results may offer an opportunity to investigate the interactions of the ubiquitin system that are associated with cellular senescence in aging-related diseases and suggest a new mechanism to regulate PPARγ degradation and cellular senescence.

MATERIALS AND METHODS

Plasmids , antibodies ,reagents and animals

pBABE-NEDD4-1 was kindly provided by Dr. Xuejun Jiang and cloned into pcDNA3.1-Myc, and pGEX-4T1-GST. The constructs HD (HECT domain deleted), WW1-4 (WW domains deleted) were cloned into pGEX-4T1-GST. PPXY deletion -mutant (85–88 aa) of PPARγ was constructed by overlapping PCR amplification of the pcDNA3.1-PPARγ plasmids and were cloned into pcDNA3.1-Flag. All clones were confirmed by DNA sequencing. The shRNA was designed according to the pMSCV instructionmanual (Clontech).

Antibodies which were used included: anti-SIRT1 (Abcam ab32441), anti-PPARγ (Upstate 07-466), anti-Multi Ubiquitin (MBL), anti-FLAG antibody (Sigma M2), anti-Myc (Santa Cruz TA-01), anti-GAPDH antibody (Tianjin Sungene Biotech Co., Ltd. KM9002) and anti-NEDD4-1 (Santa Cruz H-135).17β-estradiol(Sigma).The Super-Signal west picochemiluminescent substrate was obtained from Millipore.

Animals female BALB/C mice weighing 18–20 g (age 8–10 weeks) were employed in this study, and all animal studies were performed in accordance with the guidelines set forth by the Peking University Animal Ethics Committee.

Ovariectomy

Following 1-week habituation, mice were anesthetized with ketamine (100 mg/kg)/xylazine (10 mg/kg), and bilateral ovariectomies were performed using a dorsal midline incision inferior to palpated rib cage and kidneys. Ovaries were removed and 17 β-estradiol pellets (0.5 mg) 60-d time release or control pellets (Innovative Research, Sarason, FL) were inserted before closing the incision. These pellets have been used extensively in our laboratory and have resulted in physiological levels of plasma estradiol(Bake and Sohrabji, 2004).

Immunoprecipitation

The precleared cell lysate was incubated with primary antibody on ice for 30 min; then protein A beads were added, and the mixture was incubated at 4°C for 2 h with rotation. The beads were washed with lysis buffer three times, and the immunoprecipitation complexes were ready for directly dissolved in SDS-PAGE sample buffer for SDS-PAGE. Proteins were visualized using Chemiluminescent Substrate (Millipore) according to the manufacturer's instructions.

RT-PCR

Total RNA was isolated from Hela cells using a RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. First-strand cDNA was synthesized using the StarScript First-strand cDNA Synthesis Kit (GenStar Biosolutions Co. Ltd, Beijing, China).

Luciferase reporter assay

Hela cells were cultured in DMEM supplemented with 10% FBS and were transfected with the indicated plasmids by using Lipo transfection reagent (Invitrogen). Cell lysates were prepared with the Dual Luciferase reporter assay kit (Promega Corp., Madison, WI). A rennilla plasmid was included to control for efficiency of transfection.

Pulldown assays with GST fusion proteins

The GST fusion protein beads containing 15–30 μg of GST fusion protein were incubated with proteins translated in vitro using the TNT T7 Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's instructions.

In vitro ubiquitination assays

In vitro ubiquitination assays were carried out according to the manufacturer's instructions (Boston Biochem).

Chromatin immunoprecipitation

ChIP assays were performed using the Chromatin Immunoprecipitation Assay kit (Upstate, New York, NY) according to manufacturer's instruction.

RNA interference

Two 21-nucleotide small interference RNA (siRNA) sequences h-NEDD4-1 (GeneID:4734) TGGCGATTTGTAAACCGAATT (Wang et al., 2007) and h-PPARγ (GeneID:5468) GCCCTTCACTACTGTTGACTT (Kelly et al., 2004) corresponding to the coding region of hu-

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man NEDD4-1 or PPARγ was selected.

SA-β-gal analysis

SA-β-gal staining assays were performed according to the manufacturer's instructions (Dimri et al., 1995).

Statistical analysis

ANOVA was performed followed by the Student's *t* test. In all cases, $P < 0.05$ were considered significant.

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ABBREVIATIONS

ChIP, chromatin immunoprecipitation; E2, 17β-estradiol; E3, ubiquitin ligase; luc, luciferase; PPARγ, peroxisome proliferator-activated receptor-γ; SA-β-gal,senescence-associated beta-galactosidase; shRNA, small hairpin RNA; SIRT1, silent information regulator type1.

COMPLIANCE WITH ETHICS GUIDELINES

Limin Han, Pan Wang, Ganye Zhao, Hui Wang, Meng Wang, Jun Chen and Tanjun Tong declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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