

# PIAS $\gamma$ Enhanced SUMO-2 Modification of Nurr1 Activation-Function-1 Domain Limits Nurr1 Transcriptional Synergy

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## Abstract

Nurr1 (NR4A2) is a transcription factor that belongs to the orphan NR4A group of the nuclear receptor superfamily. Nurr1 plays key roles in the origin and maintenance of midbrain dopamine neurons, and peripheral inflammatory processes. PIAS $\gamma$ , a SUMO-E3 ligase, represses Nurr1 transcriptional activity. We report that Nurr1 is SUMOylated by SUMO-2 in the lysine 91 located in the transcriptional activation function 1 domain of Nurr1. Nurr1 SUMOylation by SUMO-2 is markedly facilitated by overexpressing wild type PIAS $\gamma$ , but not by a mutant form of PIAS $\gamma$  lacking its first LXXLL motif (PIAS $\gamma$ mut1). This PIAS $\gamma$ mut1 is also unable to interact with Nurr1 and to repress Nurr1 transcriptional activity. Interestingly, the mutant PIAS $\gamma$ C342A that lacks SUMO ligase activity is still able to significantly repress Nurr1-dependent transcriptional activity, but not to enhance Nurr1 SUMOylation. A SUMOylation-deficient Nurr1 mutant displays higher transcriptional activity than the wild type Nurr1 only in promoters harboring more than one Nurr1 response element. Furthermore, lysine 91, the major target of Nurr1 SUMOylation is contained in a canonical synergy control motif, indicating that SUMO-2 posttranslational modification of Nurr1 regulates its transcriptional synergy in complex promoters. In conclusion, PIAS $\gamma$  can exert two types of negative regulations over Nurr1. On one hand, PIAS $\gamma$  limits Nurr1 transactivation in complex promoters by SUMOylating its lysine 91. On the other hand, PIAS $\gamma$  fully represses Nurr1 transactivation through a direct interaction, independently of its E3-ligase activity.

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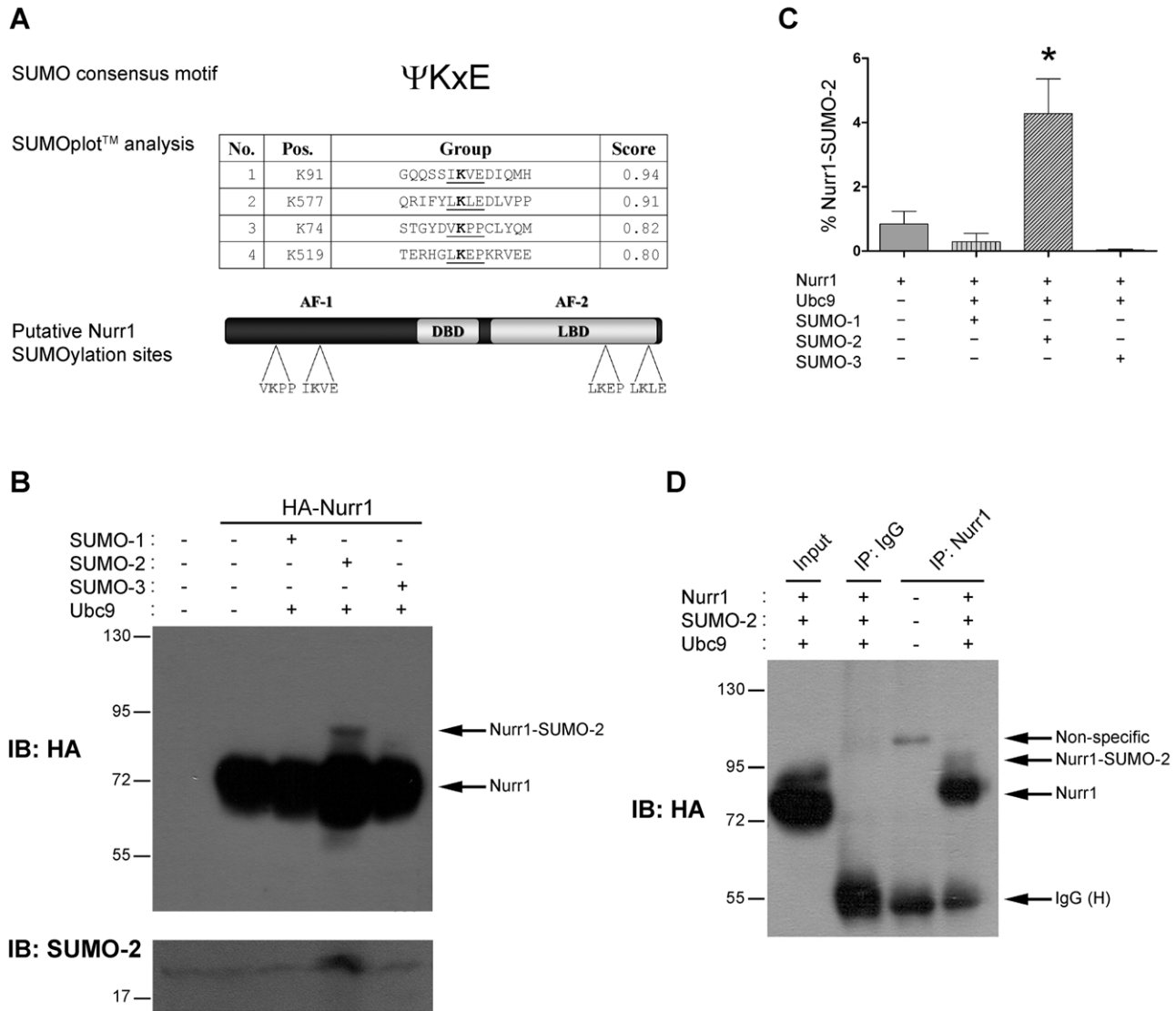
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## Introduction

Nurr1 (NR4A2) is a transcription factor with several functions, but highlights for its key role inducing and maintaining midbrain dopamine neurons of the mammalian central nervous system [1]. Nurr1 together with Nur77 (NR4A1, NGFI-B) and Nor1 (NR4A3) conform the NR4A group of the nuclear receptor superfamily [2]. Nurr1 shares with the other NR4A members a highly conserved structural organization. This structure consists of an almost identical DNA-binding domain (DBD); a moderately conserved C-terminal region, which encloses both the ligand-binding domain (LBD) and the transcriptional activation function-2 (AF-2); and the N-terminal region containing the AF-1, which is the most divergent domain [3]. Nurr1 binds DNA as monomer to the NGFI-B response element (NBRE, 5' AAAGGTCA 3') and as homo or heterodimer with Nur77 to the NurRE elements. Furthermore, Nurr1 and Nur77 can form heterodimers with the retinoid X receptor binding to DR5 elements [1]. Traditionally, nuclear receptors regulate transcription in a ligand-dependent manner, but the three members of the NR4A subfamily are classified as "orphan" because they are not associated with ligands [3]. Structural studies have shown that the LBD domain of Nurr1

lacks the cavity to accommodate a ligand, and its AF-2 adopts naturally a stable transcriptional active conformation [4]. In addition, current data indicates that Nurr1 is not regulated by traditional transcriptional coactivators [5]. Since Nurr1 is not regulated by endogenous ligands, post-translational modifications are one of the most significant mechanisms to regulate Nurr1 transcriptional activity.

Previously, we suggested that Nurr1 is SUMOylated since we probed that Nurr1 interacts with PIAS $\gamma$  a SUMO-E3 ligase [6–7] and that this interaction inhibits Nurr1-dependent transcriptional activity [8]. SUMOylation is a post-translational modification of proteins that involves the attachment of the small ubiquitin-like modifier (SUMO) peptide to the target protein. In mammals there are four SUMO peptides: SUMO-1, SUMO-2, SUMO-3 and SUMO-4. The SUMO modification process requires the action of an E1 activating enzyme (SAE1/SAE2), the E2 conjugation enzyme (Ubc9) and an E3-ligase enzyme [9]. The conjugation of SUMO to proteins is through an isopeptide bond between the C-terminus of SUMO and a  $\epsilon$ -amino group of a lysine residue in the target protein; this lysine residue is often located in a consensus sequence composed of a characteristic



**Figure 1. Nurr1 is SUMOylated by SUMO-2.** (A) Schematic representation of Nurr1 (bottom) showing the position of four putative SUMOylation sites according with SUMOplot™ software analysis (middle). The table shows the sequences of the potential SUMOylation sites of rat Nurr1 sorted from the highest score. Putative SUMO acceptor lysines (K) are highlighted, and potential SUMO sites are underlined. (B) COS-7 cells were transfected with plasmids expressing HA-Nurr1, Ubc9, and SUMO-1, SUMO-2 or SUMO-3. Cells were harvested 48 hours post-transfection and lysed directly in loading buffer containing the SUMO-isopeptidase inhibitor N-ethylmaleimide 20 mM, and fractionated in SDS-PAGE. Representative western-blot with anti-HA (upper) and anti-SUMO-2 (bottom) antibodies. (C) Quantitative densitometry analysis of Nurr1-SUMO-2 signal described in (B), using Image J software. Data correspond to the mean  $\pm$  S.E.M. of 3 independent experiments for each condition. Statistical significance was estimated by the non-parametric Mann-Whitney U-test. \*  $p < 0.05$  (Nurr1+Ubc9+SUMO-2 v/s Nurr1). (D) Total lysates from COS-7 cells transfected with HA-Nurr1, Ubc9 and SUMO-2 were immunoprecipitated with an anti-Nurr1 antibody or control IgG. The immunoprecipitates were analyzed in western blots with anti-HA antibody. Bands for immunoglobulin are indicated as IgG heavy chain (H). doi:10.1371/journal.pone.0055035.g001

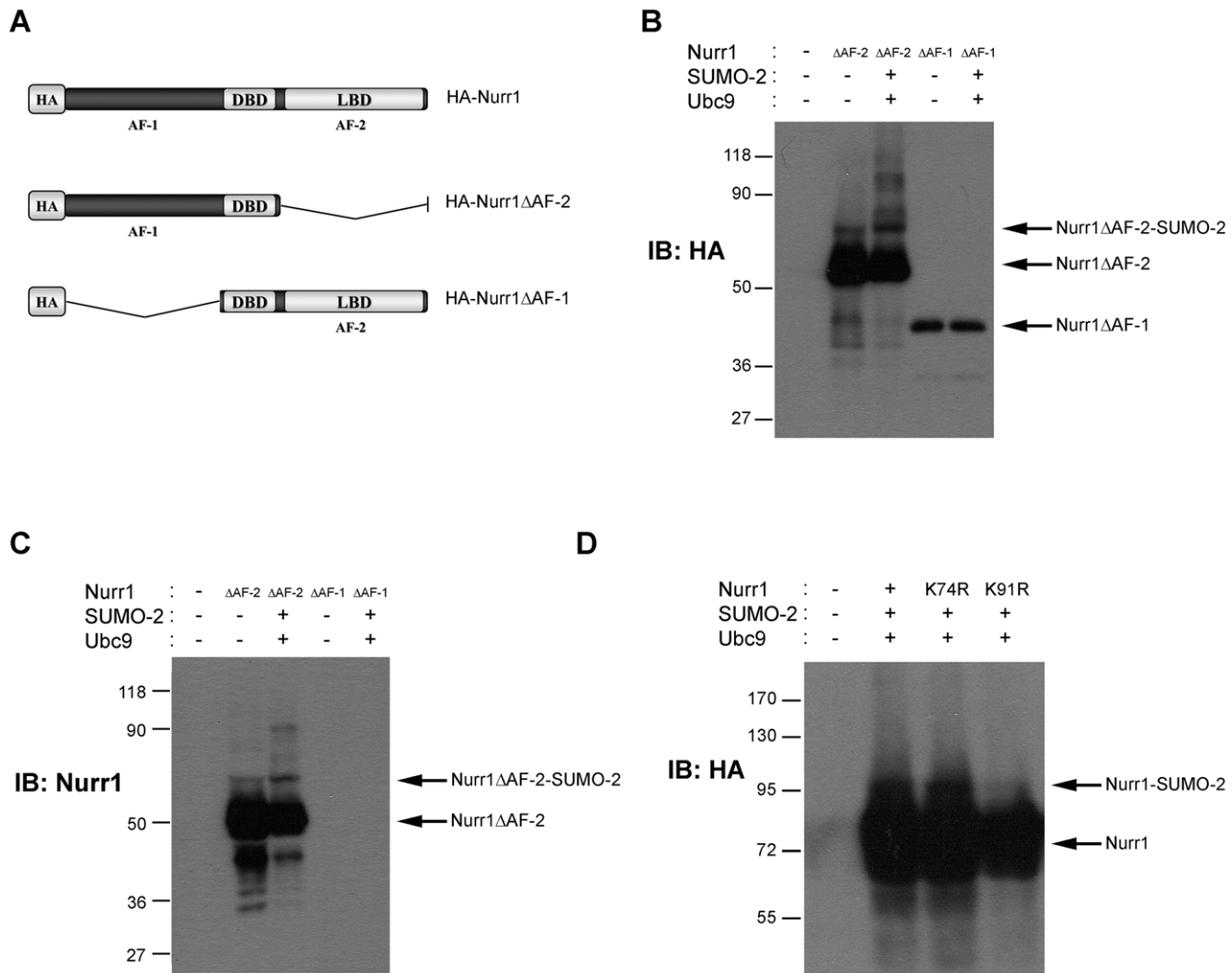
$\Psi$ KXE motif [9]. SUMOylation is a reversible process, in which the de-SUMOylation is exerted by SUMO-specific proteases (SENPs) [10]. SUMOylation of transcription factors regulate their half-life, the subcellular location and the transcriptional activity, among other features [11–12]. Interestingly, SUMOylation of several transcription factors as the glucocorticoids, androgen and estrogen nuclear receptors, restricts their transcriptional activity in promoters with several response elements arranged in tandem [13]. This SUMOylation occurs in lysines overlapping with a synergy control (SC) motif [14]. Here, we report that Nurr1 is SUMOylated by SUMO-2 at the lysine 91 located in a functional SC motif. Thus, SUMOylation of Nurr1 in the lysine

91 restricts its transcriptional activity in promoters with more than one response element. We show also that PIAS $\gamma$  enhances Nurr1 SUMOylation on lysine 91. Thus, we conclude that PIAS $\gamma$  exert two mechanisms of repression over Nurr1 transactivity, one dependent and other independent of Nurr1 SUMOylation.

## Materials and Methods

### Plasmid constructions

The hemagglutinin (HA)-Nurr1 expression vector (pCGN-Nurr1) encoding full-length rat Nurr1, the mutant HA-Nurr1-K91R and Myc-Nurr1<sub>363–598</sub> were described previously [8].

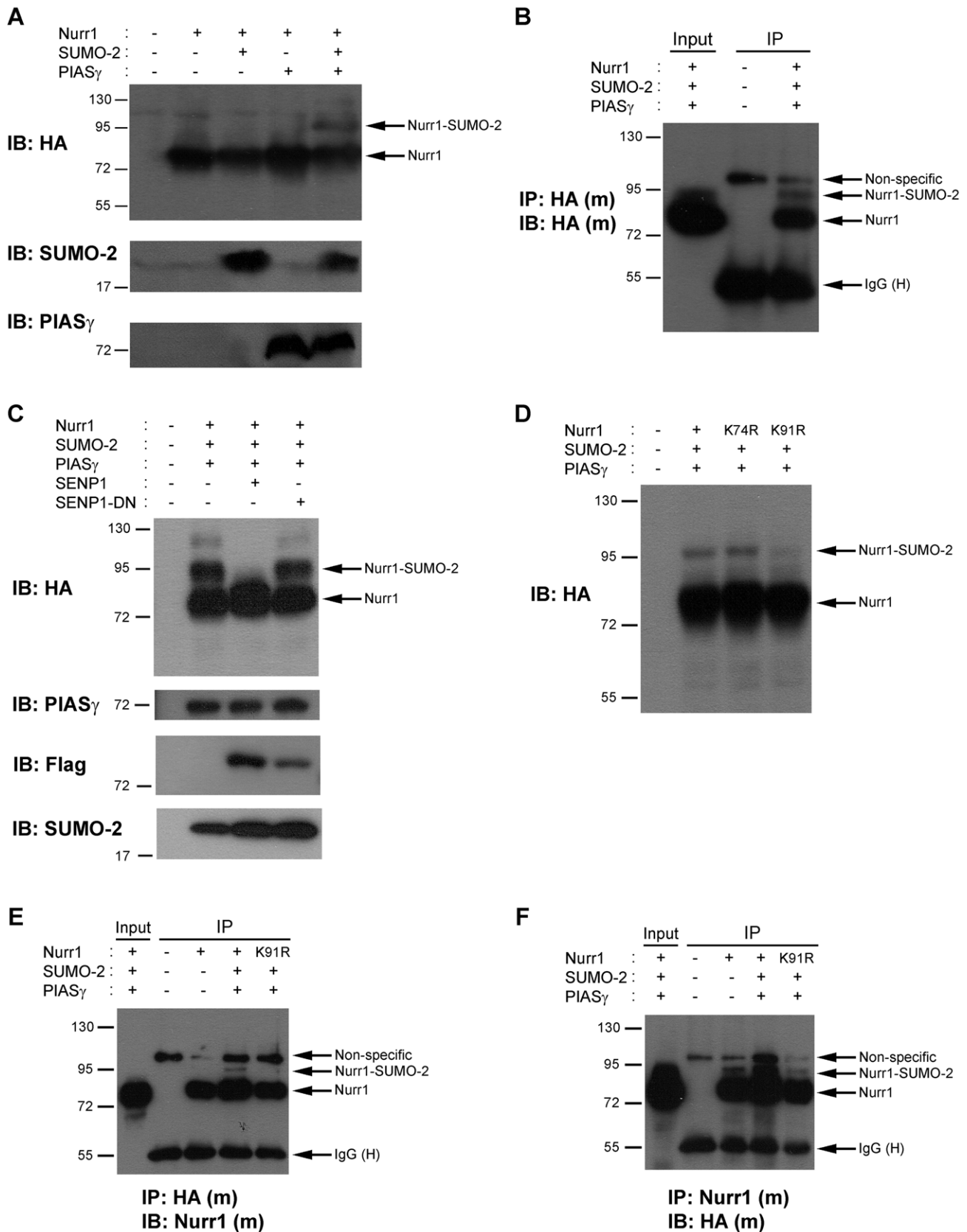


**Figure 2. Lysine 91 is the main SUMOylation site of Nurr1.** (A) Schematic representation of full-length HA-Nurr1, and truncated isoforms HA-Nurr1 $\Delta$ AF-2 (amino acids 1–353) and HA-Nurr1 $\Delta$ AF-1 (amino acids 262–598). (B, C) Total extracts of COS-7 cells transfected with HA-Nurr1 $\Delta$ AF-2 or HA-Nurr1 $\Delta$ AF-1, plus Ubc9 and SUMO-2 were fractionated in SDS-PAGE and proteins analyzed with anti-HA (B) and anti-Nurr1 (C) antibodies. (D) Total extracts of COS-7 cells transfected with wild type HA-Nurr1 or the point mutants HA-Nurr1-K74R or HA-Nurr1-K91R were fractionated in SDS-PAGE. Western blot was performed with an anti-HA antibody.  
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Point mutation HA-Nurr1-K74R was generated by overlapping PCR and the mutated fragment was cloned into pCGN-Nurr1 using XbaI/PstI sites. Point mutations of HA-Nurr1-K558R and PIAS $\gamma$ C342A were generated by site-directed mutagenesis, using mutagenic primers and pfu polymerase in a PCR reaction followed by DpnI digestion for 1 hour. To generate HA-Nurr1 $\Delta$ AF-2 (residues 1–353), HA-Nurr1 was cut with BamHI to get rid of the fragment 354–598 and re-ligated. HA-Nurr1 $\Delta$ AF-2-K91R was similarly generated from HA-Nurr1-K91R. HA-Nurr1 $\Delta$ AF-1 (residues 262–598) was obtained by PCR using specific primers spanning the coding region for amino acids 262–598 and cloned in frame with HA in pCGN plasmid. pcDNA3.1-HA-Nurr1 expression vector was generated from pCGN-Nurr1 using PCR to generate an HA-Nurr1 fragment that was inserted in pcDNA3.1+ within EcoRV/NotI sites. GST-PIAS $\gamma$  was generated by cloning the human cDNA of PIAS $\gamma$  coding for amino acids 1–158 in frame with glutathion-S-transferase in the pGEX-4T3 vector. GST-PIAS $\gamma$ mut1 y GST-PIAS $\gamma$ mut2 were generated by overlapping PCR using primers codifying alanine instead leucine in the sequence coding for each

LXXLL (for mut1 *gac-ctt-cag-atg-ctc-ctg-ggt* was changed by *gac-gct-cag-atg-gcc-gcg-ggt* and for mut2 *atg-ctg-gat-gag-ctg-ctg-aag* was changed by *atg-gcg-gat-gag-gcg-gcg-aag*). All mutagenesis and constructs were confirmed by sequencing and correct protein expression was checked by western blot.

The reporter plasmid NBRE-3X-tk-Luciferase [5] that contains three mer NBRE elements was kindly donated by Dr. T. Perlmann (Karolinska Institute, Sweden). pcDNA3.1-HIS-SUMO-1, pcDNA3.1-HIS-SUMO-2 and pcDNA3.1-HIS-SUMO-3 were kindly donated by Dr. R. Hay (Center for Biomolecular Sciences, Scotland). 1NBRE-LUC and 3NBRELUC [15] were kindly donated by Dr. A. Winoto (University of California, Berkeley, California, USA). pcDNAPIAS $\gamma$  was kindly donated by Dr. F. White (Indiana University School of Medicine, Indianapolis, USA). The expression vectors for SENP1 and SENP1-dominant negative (SENP1-DN) [16] were donated by Dr. O'Hare (Marie Curie Research Institute, UK).



**Figure 3. PIAS $\gamma$  enhances Nurr1 SUMOylation.** (A) Total extracts of COS-7 cells transfected with plasmids encoding HA-Nurr1, SUMO-2 and PIAS $\gamma$  were fractionated in SDS-PAGE and western blots were performed with anti-HA (upper), anti-SUMO-2 (middle) and anti-PIAS $\gamma$  (bottom) antibodies. (B) Total extracts of COS-7 cells transfected as in (A) were immunoprecipitated and immunoblotted with a monoclonal (m) anti-HA antibody. (C) Total extracts of COS-7 cells transfected with HA-Nurr1, SUMO-2, PIAS $\gamma$  and Flag-SENP1 or Flag-SENP1-DN were fractionated in SDS-

PAGE and western blots were performed with the indicated antibodies. (D) Total extracts of COS-7 cells transfected with HA-Nurr1 or the point mutants HA-Nurr1-K74R or HA-Nurr1-K91R were fractionated in SDS-PAGE. Western blots were performed with an anti-HA antibody. (E, F) Total extracts of COS-7 cells transfected with HA-Nurr1 or HA-Nurr1-K91R plus SUMO-2 and PIAS $\gamma$  were immunoprecipitated with anti-HA antibody and western blot carried out with anti-Nurr1 antibody (E) or immunoprecipitated with anti-Nurr1 antibody and western blot carried out with anti-HA antibody (F). (m): monoclonal, (H): IgG heavy chain.  
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### Cell culture and transfections

COS-7, COS-1 and HEK293 cell lines obtained from American Type Culture Collection (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and maintained at 37°C and 5% CO<sub>2</sub>, and supplemented with 1% penicillin/streptomycin. PC12 cell line also was obtained from ATCC and cultured in DMEM supplemented with 5% FBS and 10% horse serum, and maintained in 10% CO<sub>2</sub>. Transfections were carried out using Lipofectamine2000 reagent (Invitrogen).

### Immunoprecipitation and western blot

Cells were grown and transfected in 100-mm plate and lysed with 0.9 mL of lysis buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 1% NP40), supplemented with a cocktail of protease inhibitors and 20 mM of N-ethylmaleimide. Cell extracts were immunoprecipitated as follow: pre-cleared with 10  $\mu$ L of protein A/G PLUS agarose beads (Santa Cruz Biotechnology) plus 0.5  $\mu$ g of preimmune mouse IgG, during 1 hour at 4°C. The extracts were recovered by centrifugation and incubated with 1  $\mu$ g of specific antibody during 3 hours and then with 15  $\mu$ L of protein A/G PLUS agarose beads for an additional 1.5 hours. The beads were pelleted, and washed two times with lysis buffer and two times with PBS. Finally, bound proteins were eluted by boiling in 2X Laemmli sample buffer. Immunoprecipitated proteins were fractionated by SDS-PAGE and western blots performed with a monoclonal anti-HA antibody (Covance) or a monoclonal anti-Nurr1 antibody (447C2a, Santa Cruz).

### GST-pull-down assays

GST-Pull-down assays were performed essentially as we have described [17]. Protein concentration and purity of recombinant GST-PIAS $\gamma_{1-158}$ , wild type and mutants, were determined by densitometry on 12% polyacrilamida-SDS gel stained with coomassie blue and compared to a BSA concentration curve. All recombinants GST-PIAS $\gamma$  migrated at the expected molecular weight of about 46 kD (26 kD of GST and 20 kD PIAS $\gamma_{1-158}$ ) and a minor band of about 39 kD. Both bands were recognized by anti-PIAS $\gamma$  antibody. To test the interaction between GST-PIAS $\gamma_{1-158}$  (wild type and mutants) and Nurr1, whole extracts of COS-1 cells transfected with Myc-Nurr1<sub>363-598</sub> were incubated with GST-PIAS $\gamma$  fusion proteins or GST alone (1.5  $\mu$ g) bound to glutathione-agarose for 1 h at 4°C. After extensive washing, the retained proteins were eluted with sample buffer and resolved on 12% SDS-PAGE. Immunoblots were incubated with monoclonal anti-Myc (9E10).

### Mammalian reporter gene assays

Cells were growing in 24-well plates and transfected with 100 ng of the reporter plasmid and an equivalent molar amount of expression plasmids or empty vectors (pCGN or pcDNA3.1+). Total amount of DNA (400 ng) was kept constant by adding pBluescript SR (Stratagene). A reporter gene expressing the  $\beta$ -galactosidase cDNA driven by the cytomegalovirus promoter was cotransfected (20 ng) in all experiments as an internal control for transfection efficiency. Cells were harvested 48 hours after transfection. Luciferase activities were normalized to the activity

of the internal control  $\beta$ -galactosidase. Each set of experiments was performed in triplicate and repeated at least three times.

### Immunofluorescence and confocal analysis

The immunofluorescence and co-localization analysis were performed essentially as we have described [18]. Briefly, fixed and permeabilized cells were incubated with polyclonal anti-PIAS $\gamma$  1/500 and monoclonal anti-HA 1/500 overnight. After exhaustive washing cells were incubated with following secondary antibodies: donkey anti-goat Alexa Fluor 488 (green) and donkey anti-mouse Alexa Fluor 594 (red). Immunofluorescence images were captured with a confocal microscope (Olympus®, Fluoview 1000). Quantification of fluorescence colocalization was done using the method described by van Steensel et al. [19]. Cross correlation function (CCF) of dual labeling images was calculated by shifting the green image with respect to the red image or vice versa.

*Statistical analyses* – Results are expressed as mean  $\pm$  S.E.M. from at least three independent assays. Statistical analyses were performed using the non-parametric Mann-Whitney U test.

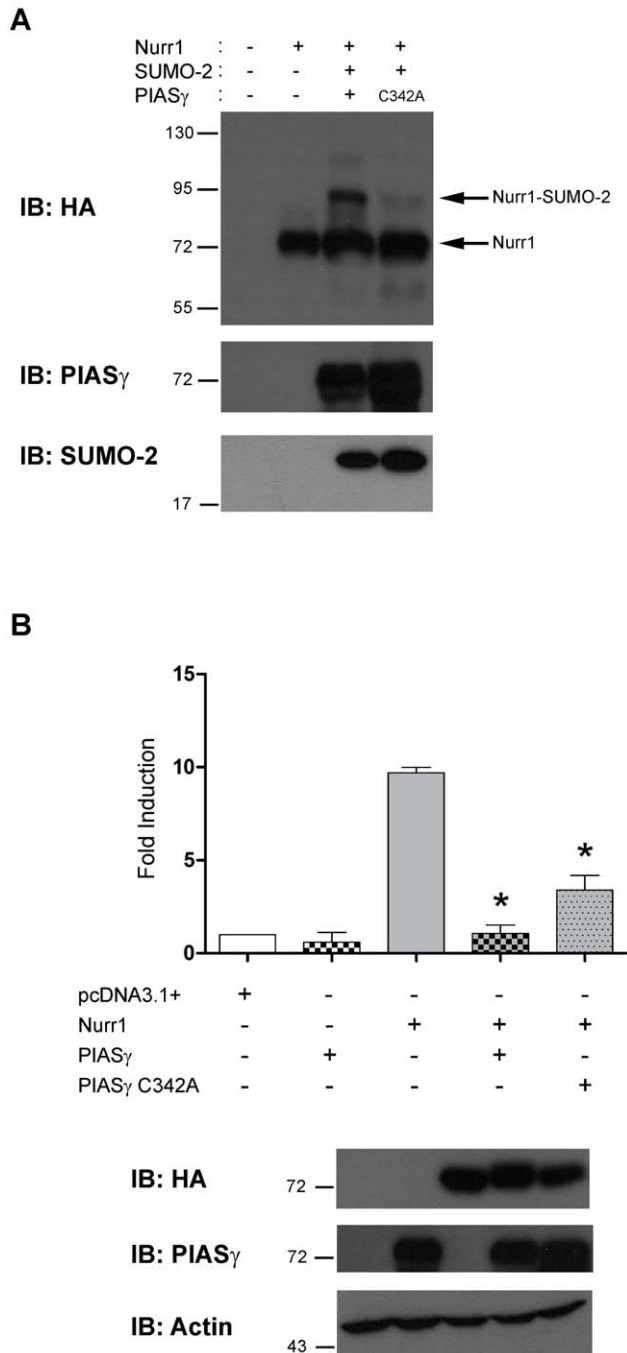
## Results

### Nurr1 is SUMOylated

Previously, we identified PIAS $\gamma$  as an interacting partner of Nurr1 [8]. This result suggested us that Nurr1 is a SUMO target protein. According with SUMOplot<sup>TM</sup> software, Nurr1 has four lysines with high score to be SUMO target: lysine 91 and lysine 577 that are in canonical  $\Psi$ KXE motifs, and lysines 74 and 519 located in non-canonical SUMO sequences, but showing a high score for SUMOylation (Fig. 1A). To determine if Nurr1 is a target of SUMO peptide, we overexpressed Nurr1 along with each of the SUMO peptides (SUMO-1, SUMO-2 or SUMO-3) plus Ubc9 in COS-7 cells. Immunoblotting of the cell extracts showed two immunoreactive bands, a major band of about 72-kDa corresponds to the expected unmodified HA-Nurr1 and a band of lesser intensity and more slowly migrating of about 95-kDa. This 95-kDa band is observed only in the condition when SUMO-2 and Ubc9 were overexpressed (Fig. 1B). The quantification of the 95-kDa slower migrating band indicates that only around a 3% of HA-Nurr1 is significantly SUMOylated (Fig. 1C). To further confirm that Nurr1 is a target of SUMO-2, we carried out coimmunoprecipitation assays. As shown in figure 1D, western blot with anti-HA antibody detected two bands, one corresponding to the unmodified HA-Nurr1 and a slower migrating diffuse signal specifically found in precipitates with anti-Nurr1. These results indicate that Nurr1 is a target of SUMO-2 posttranslational modification.

### Identification of major SUMO sites in Nurr1

The lysines with high score for SUMOylation in Nurr1 are within the transcriptional activation domains of this transcription factor. Lysines 74 and 91 are within the AF-1 domain, while lysines 519 and 577 are within the AF-2 domain of Nurr1 (Fig. 1A). In order to determine faster the segment of Nurr1 that is target of SUMO-2 we worked with truncated forms of Nurr1 (HA-Nurr1 $\Delta$ AF-1 or HA-Nurr1 $\Delta$ AF-2) containing only one of the transcriptional activation domains plus the DBD (Fig. 2A). Western blot assays performed either with anti-HA (Fig. 2B) or



**Figure 4. Point mutant PIAS $\gamma$ C342A fails to SUMOylate Nurr1.**

(A) Total extracts of COS-7 cells transfected with HA-Nurr1, SUMO-2 and PIAS $\gamma$  or the point mutant PIAS $\gamma$ C342A were fractionated in SDS-PAGE and western blot assays performed with anti-HA (upper), anti-PIAS $\gamma$  (middle) and anti-SUMO-2 (bottom) antibodies. (B) HEK293 cells were transfected with 100 ng of NBRE-3X-tk-LUC reporter and equimolar amounts of HA-Nurr1, PIAS $\gamma$  or PIAS $\gamma$ C342A. Cells were harvested 48 hours post transfection and lysates assayed for luciferase activity. Results are expressed as fold of induction related to control (pcDNA3.1+) and correspond to the mean  $\pm$  S.E.M. of three independent assays performed each in triplicate. Statistical significance was estimated by the non-parametric Mann-Whitney U test. \* $p < 0.05$  (Nurr1+PIAS $\gamma$  v/s Nurr1) and (Nurr1+ PIAS $\gamma$ C342A v/s Nurr1). In the bottom, western blots showing the expression of recombinant proteins and actin used as loading control.

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anti-Nurr1 (Fig. 2C) showed a slower migrating band of Nurr1 only when HA-Nurr1 $\Delta$ AF-2 was overexpressed (Fig. 2B, C). When HA-Nurr1 $\Delta$ AF-1 was overexpressed, we detected only the band corresponding to the unmodified HA-Nurr1 $\Delta$ AF-1 fragment of Nurr1 (Fig. 2B). Overexposure of this film failed to show additional bands that could suggest post-translational modification of this fragment of Nurr1 (data not shown). HA-Nurr1 $\Delta$ AF-1 fragment was not detected with the anti-Nurr1 antibody used, because this antibody was raised against the N-terminal domain of Nurr1 (Fig. 2C). Together the data indicate that Nurr1 is SUMOylated by SUMO-2 in the AF-1. Therefore, we continue with lysines 74 and 91 as the putative SUMOylation targets in Nurr1. We replaced lysines 74 and 91 with arginines (Nurr1-K $\Delta$ R mutants) and overexpressed each HA- epitope tagged Nurr1-K $\Delta$ R mutant along with SUMO-2 and Ubc9 in COS-7 cells. Immunoblotting of extracts of cells expressing these constructs revealed that the HA-Nurr1-K91R mutant was only weakly SUMOylated, but HA-Nurr1-K74R was SUMOylated similarly to wild type HA-Nurr1 (Fig. 2D). This evidence indicates that lysine 91 is the major target of SUMO-2 in Nurr1.

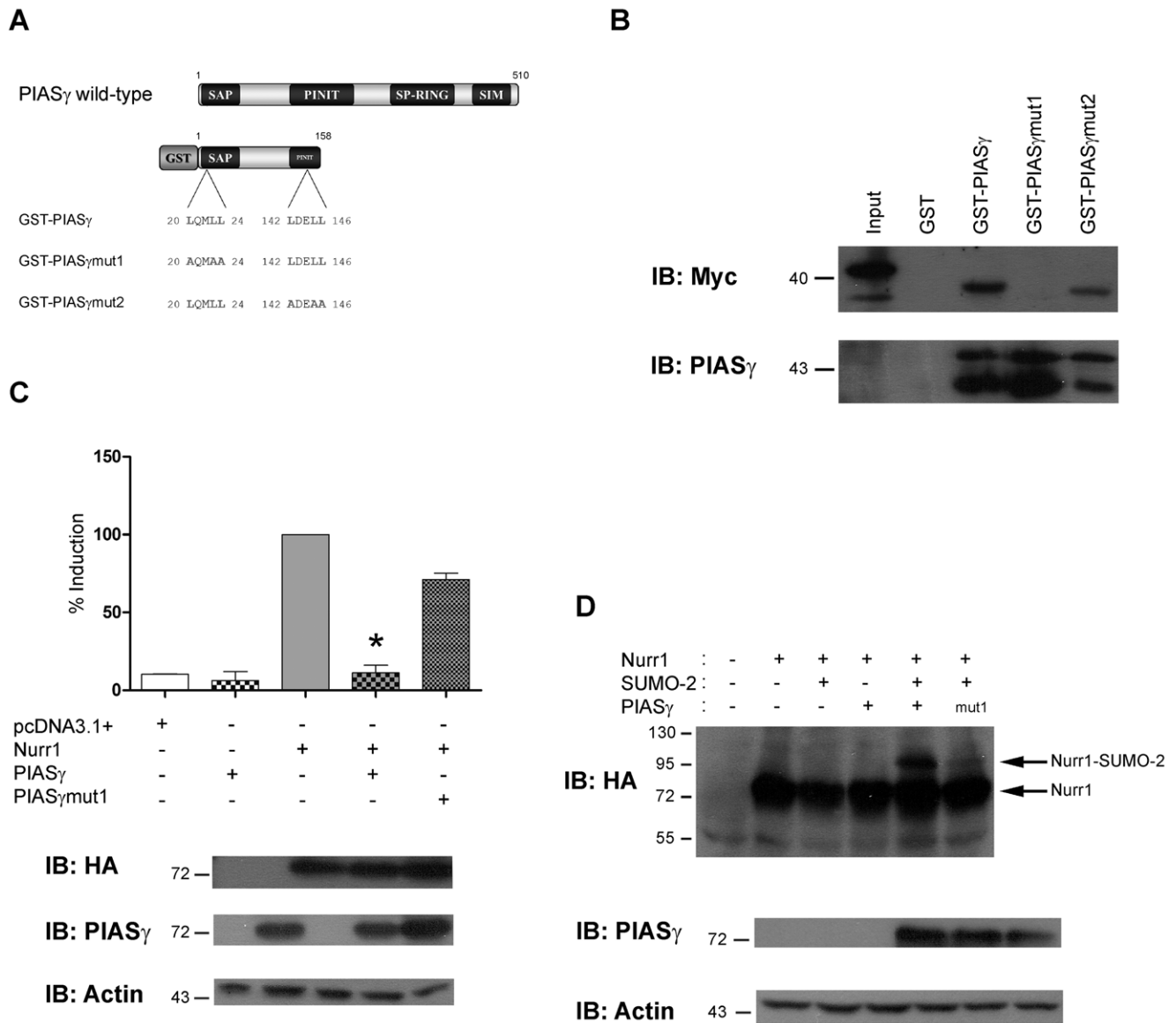
### PIAS $\gamma$ enhances SUMOylation of Nurr1

Because PIAS $\gamma$  interacts and regulates Nurr1 [8], we tested the hypothesis that PIAS $\gamma$  is the SUMO-E3 ligase in Nurr1 SUMOylation process. To this end, we overexpressed PIAS $\gamma$  instead Ubc9, along with Nurr1 and SUMO-2 in COS-7 cells. The immunoblot of these cell extracts showed the slower 95-kDa migrating band (Fig. 3A) corresponding to Nurr1-SUMO-2. The quantification of the slower migrating band of 95-kDa indicates that about an 8.56  $\pm$  3.90% ( $p < 0.03$ ) of the total Nurr1 became SUMOylated in the presence of PIAS $\gamma$ . In addition, coimmunoprecipitation assays performed from extracts of COS-7 cells overexpressing PIAS $\gamma$  instead of Ubc9, along with SUMO-2 and HA-Nurr1 showed a clear slower migrating 95-kDa band corresponding to HA-Nurr1-SUMO-2 (Fig. 3B). The 95-kDa band was totally lost when the de-SUMOylating enzyme SENP1 was overexpressed (Fig. 3C), but not when the variant dominant negative SENP1-DN [16] was overexpressed in the same conditions (Fig. 3C). These evidences indicate that PIAS $\gamma$  behaves as a SUMO-E3 ligase for Nurr1 SUMOylation.

Then, we studied whether lysine 91 is also the major SUMOylation site of Nurr1 when PIAS $\gamma$  is overexpressed. Immunoblots showed that the slower migrating 95-kDa band is significantly decreased in cell extracts of HA-Nurr1-K91R mutant, but not in the HA-Nurr1-K74R mutant (Fig. 3D). To support further lysine 91 as the SUMO target of Nurr1, coimmunoprecipitation assays were carried out. As shown in figure 3E, immunoblotting of immunoprecipitated Nurr1 with anti-HA (Fig. 3E) or anti-Nurr1 (Fig. 3F) antibodies showed the HA-Nurr1 band and a well-defined 95-kDa slower migrating band corresponding to the SUMOylated form (Fig. 3D). The slower 95-kDa band decreased significantly in the mutant HA-Nurr1-K91R compared to wild type HA-Nurr1 immunoprecipitates (Fig. 3E, F).

To test whether the SUMO E3-ligase enzymatic activity of PIAS $\gamma$  is required to SUMOylate Nurr1, we replaced the cysteine 342 by alanine, PIAS $\gamma$ C342A, which revokes SUMO ligase activity of PIAS $\gamma$  [20]. As shown in figure 4A, the mutant PIAS $\gamma$ C342A was not able to enhance Nurr1 SUMOylation, albeit the amount of overexpressed wild type and mutant PIAS $\gamma$  is similar in the cell extracts (Fig. 4A). This set of experiments show that PIAS $\gamma$  mediates SUMO-2 conjugation on lysine 91 of Nurr1 as a SUMO-E3 ligase.

As previously mentioned, PIAS $\gamma$  not only interacts with Nurr1, but also inhibits its transcriptional activity [8]. Thus, we asked

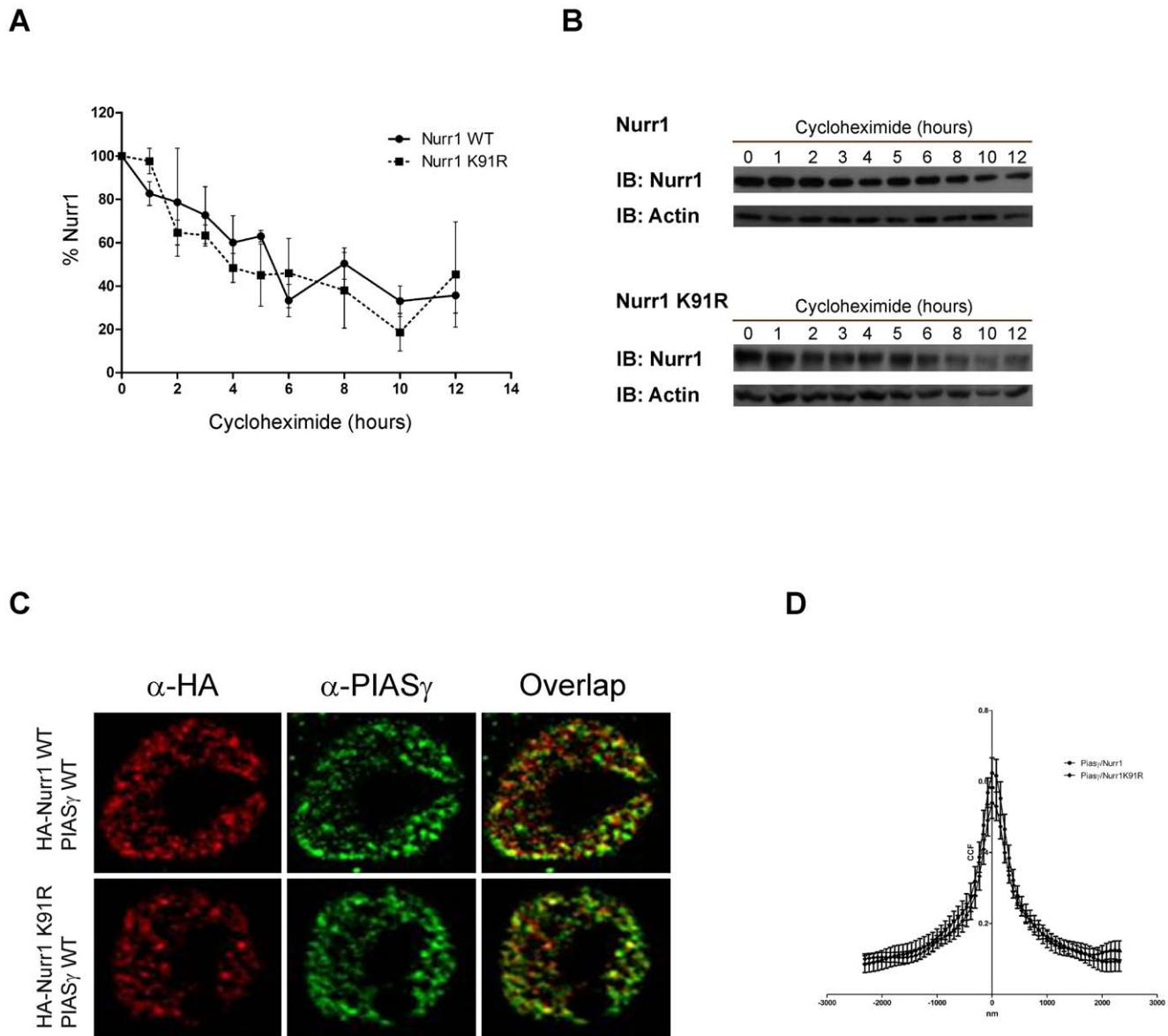


**Figure 5. PIAS $\gamma$  requires the  $_{20}$ LXXLL $_{24}$  motif to interact, SUMOylate and repress Nurr1.** (A) Schematic representation of full-length PIAS $\gamma$  indicating functional domains SAP: Scaffold Attachment factor-A/B acinus and PIAS; the PINIT domain; SP-(Siz/PIAS)-RING and SIM: SUMO Interaction Motif. PIAS $\gamma$  has two LXXLL motifs. Mutations changing leucines (L) by alanines (A) in each LXXLL domain are depicted for GST-PIAS $\gamma$ mut1 and GST-PIAS $\gamma$ mut2. (B) GST, GST-PIAS $\gamma$ , GST-PIAS $\gamma$ mut1 and GST-PIAS $\gamma$ mut2 retained in glutathione-agarose beads were incubated with extracts from COS-1 cells transfected with Myc-Nurr1 $_{363-598}$ . Retained proteins were fractionated in SDS-PAGE and western blot developed with anti-Myc monoclonal antibody, revealing that only GST-PIAS $\gamma$  and GST-PIAS $\gamma$ mut2 are able to interact with Nurr1. Recombinant GST-PIAS $\gamma$  proteins were equally loaded in each GST-pull down assays as shown by western blot using anti-PIAS $\gamma$  antibody (bottom). (C) Luciferase reporter assay showing that full-length PIAS $\gamma$ mut1 lose repressor capacity over Nurr1 transactivity. HEK293 cells were transfected with the NBRE-3X-tk-LUC along with HA-Nurr1, PIAS $\gamma$  or PIAS $\gamma$ mut1. After 48 hours, cells extracts were assayed for luciferase activity. Data are expressed as percentage of Nurr1 transactivation and correspond to the mean  $\pm$  S.E.M. of 4 independent experiments each performed in triplicates. Statistical significance was estimated by the non-parametric Mann-Whitney U test. \* $p < 0.05$  (Nurr1+PIAS $\gamma$  v/s Nurr1+PIAS $\gamma$ mut1). In the bottom, western blots showing the expression of recombinant proteins and actin used as loading control. (D) Total extracts of COS-7 cells transfected with HA-Nurr1, SUMO-2 and PIAS $\gamma$  or PIAS $\gamma$ mut1 were fractionated in SDS-PAGE and western blots performed with anti-HA, anti-PIAS $\gamma$  and actin (load control) antibodies.  
doi:10.1371/journal.pone.0055035.g005

whether the SUMOylation activity of PIAS $\gamma$  is required for its inhibitory action over Nurr1. Surprisingly, the mutant PIAS $\gamma$ C342A was still able to repress significantly Nurr1 transcriptional activity (Fig. 4B). However, the repression exerted by PIAS $\gamma$ C342A was lower than wild type PIAS $\gamma$  (Fig. 4B).

To further characterize the role of PIAS $\gamma$  regulating Nurr1 SUMOylation and its transcriptional activity, we determined the domain of PIAS $\gamma$  needed to interact with Nurr1. As shown in

figure 5A, PIAS $\gamma$  has two LXXLL motifs located between amino acids 20–24 and 142–146. LXXLL motifs are essential for some coregulators to interact with transcription factors [21]. Thus, we replaced leucines by alanines in each LXXLL motif (Fig. 5A) of PIAS $\gamma$  to learn whether any or both of these motifs mediate the interaction between Nurr1 and PIAS $\gamma$ . As shown in figure 5B, PIAS $\gamma$  encompassing the first 158 amino acids of the protein was able to retain specifically the recombinant Myc-Nurr1 $_{363-598}$  [8] in



**Figure 6. SUMOylation on lysine 91 does not modify Nurr1 half-life and location.** (A) HEK293 cells were transfected with HA-Nurr1 or HA-Nurr1-K91R. Twelve hours after transfection the cells were treated with cycloheximide and harvested at the indicated hours. Total extracts were fractionated in SDS-PAGE and western blots developed with anti-HA and anti-actin (load control) antibodies. Densitometric analysis of 3 independent experiments was performed with Image J software. Data is expressed as percentage of HA-Nurr1 or HA-Nurr1-K91R expression at zero time and correspond to the mean  $\pm$  S.E.M. (B) Representative western blots of HA-Nurr1 and HA-Nurr1-K91R during cycloheximide treatment. (C) PC12 cells were transfected with the indicated plasmids. Fixed cells were subjected to double immunofluorescence protocols using HA and PIAS $\gamma$  antibodies. Alexa 594 (red) second antibody was used to visualize HA and Alexa 488 (green) second antibody was used to visualize PIAS $\gamma$ . Cells were examined by deconvolution microscopy. (D) Colocalization of Nurr1 or the mutant Nurr1-K91R with PIAS $\gamma$  using van-Steensel analysis [19]. doi:10.1371/journal.pone.0055035.g006

GTS-pull down assays. GTS-PIAS $\gamma$ mut1, lacking the first LXXLL motif lost the capacity to interact with Myc-Nurr1<sub>363–598</sub> (Fig. 5B); however, the GST-PIAS $\gamma$ mut2 was able to retain it (Fig. 5B), indicating that PIAS $\gamma$  requires only the first <sub>20</sub>LXXLL<sub>24</sub> motif for the interaction with Nurr1. The lack of interaction between Nurr1 and PIAS $\gamma$ mut1 was not due to changes on the amount of recombinant GST-PIAS $\gamma$  constructs, since similar amount of GST-PIAS $\gamma$  constructs were observed with an anti-PIAS $\gamma$  antibody (Fig. 5B, lower panel). These results prompted us to study the effect of full-length PIAS $\gamma$ mut1 lacking the first LXXLL motif on Nurr1 transcriptional activity. As shown in figure 5C, full-length PIAS $\gamma$ mut1 was unable to repress Nurr1-dependent

transcriptional activity, compared to the total transcriptional repression exerted by wild type PIAS $\gamma$  (Fig. 5C). PIAS $\gamma$ mut1 showed similar expression than wild type PIAS $\gamma$  (Fig. 5C lower panel) indicating that the lack of repressive effect is due to the lack of interaction with Nurr1 and not to a wrong localization and/or lower expression. In addition, overexpression of PIAS $\gamma$ mut1 did not enhance Nurr1 SUMOylation by SUMO-2 as compared with wild type Nurr1 (Fig. 5D). Thus, PIAS $\gamma$  requires its first LXXLL motif to interact, repress and SUMOylate Nurr1.

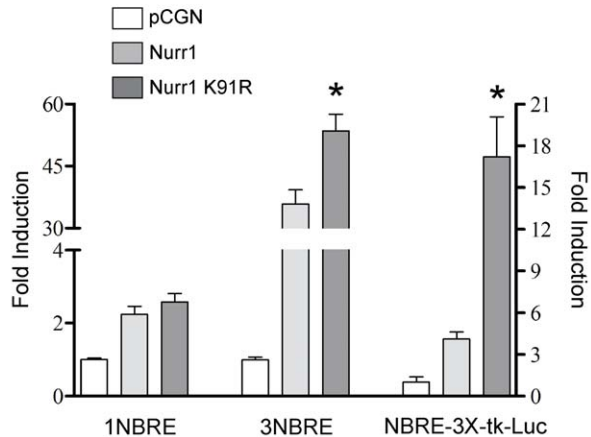
SUMOylation can regulate nuclear receptor localization, stability and transcriptional activity, among other functions [9] [11–12]. The amount of recombinant Nurr1 and Nurr1-K91R in



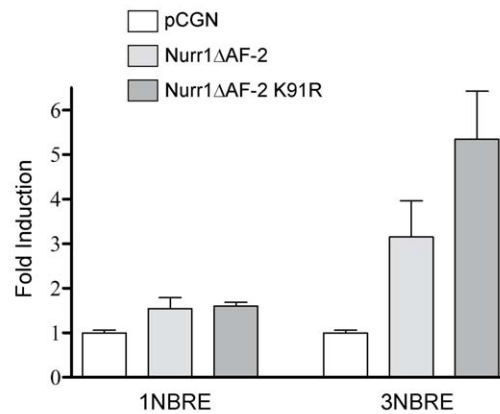
A

Consensus SC motif (P/G) - X<sub>(0-4)</sub> - ΨKXE - X<sub>(0-4)</sub> - (P/G)Nurr1 SC motif G<sup>85</sup> - X<sub>(4)</sub> - IKVE

B



C



**Figure 7. Lysine 91 of Nurr1 is in a synergy control (SC) motif.** (A) Consensus SC motif [14] and putative Nurr1 SC motif. (B) HEK293 cells were transfected with 100 ng of 1NBRE-Luc (one NBRE element), 3NBRE-Luc (three NBRE elements) [15] or NBRE-3X-tk-LUC (three NBRE elements) [5] reporters and equimolar amounts of HA-Nurr1 or point mutant HA-Nurr1-K91R. Cells were harvested 48 hours post-transfection and lysates assayed for luciferase activity. Results are expressed as fold of induction related to control (pCGN, empty vector) and correspond to the mean  $\pm$  S.E.M. of three independent assays performed each in triplicate. Scale in left axis is for 1NBRE-Luc and 3NBRE-Luc reporters' activity, and scale in right axis is for NBRE-3X-tk-LUC reporter activity. Statistical significance was estimated by the non-parametric Mann-Whitney U test \* $p < 0.05$  (HA-Nurr1-K91R v/s HA-Nurr1). (C) HEK293 cells were transfected with 100 ng of 1NBRE-Luc or 3NBRE-Luc reporters and equimolar amounts of HA-Nurr1 $\Delta$ AF-2 or point mutant HA-Nurr1 $\Delta$ AF-2-K91R. Cells were harvested 48 hours post-transfection and lysate assayed for luciferase activity. Results are expressed as fold of induction related to control (pCGN) and correspond to the mean  $\pm$  S.E.M. of three independent assays performed each in triplicate. doi:10.1371/journal.pone.0055035.g007

HEK293 cell extracts observed at different time after cycloheximide treatment show that both HA-Nurr1 and the mutant HA-Nurr1-K91R have a similar half-life (Fig. 6A, B) of about six hours [22], suggesting that SUMOylation of lysine 91 is not involved in Nurr1 stability. Previously, we showed that Nurr1 and the mutant Nurr1-K91R are located in the nuclei of cells [8] suggesting that Nurr1 SUMOylation in K91 does not modify its subcellular localization. Here we show, by confocal immunofluorescence analysis, that the mutant Nurr1-K91R has a similar subnuclear localization than the wild type Nurr1 (Fig. 6C). In addition, Van Steensel coefficient quantification [19] indicates that PIASy colocalize equally well with HA-Nurr1 and the mutant HA-Nurr1-K91R (Fig. 6D), suggesting a similar capacity of interaction, a result that reinforces the idea that PIASy is able to repress equally well the transcriptional activity of wild type Nurr1 and the mutant Nurr1-K91R.

#### SUMO consensus motif of Nurr1 overlaps with a transcriptional synergy control motif

Some years ago, Iníguez-Lluhi and Pearce [14] identified a motif in several transcription factors that restricts transcriptional activation in promoters with more than one response element for the transcription factor, denominated complex promoters. The motif was called "synergy control" (SC) and its consensus sequence includes a SUMO motif flanked by proline [14] or glycine [23] residues (Fig. 7A). Because the lysine 91 of Nurr1 lies within a

putative SC motif (Fig. 7A), we tested the hypothesis that SUMOylation of lysine 91 restricts synergic Nurr1-dependent transcription. To this end, we compared the transcriptional activity of HA-Nurr1 and the mutant HA-Nurr1-K91R using luciferase reporter constructs containing one mer (1NBRE) and three mer (3NBRE; NBRE3X-tk). As expected, wild type HA-Nurr1 synergistically activated transcription from the three mer NBRE reporters, leading to more luciferase activity compared with the one mer NBRE construct (Fig. 7B). As we have previously shown [8], HA-Nurr1-K91R mutant induced a significant higher luciferase activity than wild type HA-Nurr1 using NBRE3X-tk-Luc reporter (Fig. 7B). HA-Nurr1-K91R mutant also showed a higher transcriptional activity than wild type HA-Nurr1 using another luciferase reporter that also contains three mer NBRE elements [15], but that showed a stronger induction with Nurr1 overexpression (Fig. 7B). Remarkably, HA-Nurr1-K91R showed similar transcriptional activity compared with wild type HA-Nurr1 in the reporter containing only one mer NBRE element (Fig. 7B). Similar results were obtained when we transfected the wild type and K91R mutant forms of truncated Nurr1 lacking AF-2 domain. As shown in figure 7C, similar slight transcriptional activation was induced by wild type HA-Nurr1 $\Delta$ AF-2 and HA-Nurr1 $\Delta$ AF-2-K91R from one mer (1NBRE) reporter, while the mutant HA-Nurr1 $\Delta$ AF-2-K91R induced a higher luciferase activity than HA-Nurr1 $\Delta$ AF-2 from three mer (3NBRE) reporter. These results indicate that the lysine 91 of Nurr1 is in a true SC motif, whose

SUMOylation control synergic transcription activity from promoters containing multiple NBRE elements.

## Discussion

We have previously shown that PIAS $\gamma$  interacts and inhibits Nurr1 transcriptional activity [8], leaving as an open question whether Nurr1 is, in fact, SUMOylated. Here, we show that Nurr1 is a target of SUMO-2 peptide, and that lysine 91 is its major SUMOylation acceptor site. The results also indicate that lysine 91 of Nurr1 is within a functional SC motif. Lysine 91 in this SC motif restricts Nurr1 transcriptional activity in promoters containing more than one NBRE element. In addition, we report that PIAS $\gamma$  behaves as a SUMO-E3 ligase for Nurr1.

We observed a small, but significant percentage of Nurr1 SUMOylated (about 3% in the presence of Ubc9 and 8% in the presence of PIAS $\gamma$ ) in our experimental conditions. This small amount of Nurr1 SUMOylated does not match with the 30–60% increment observed in the transcriptional activity induced by the mutant Nurr1-K91R compared with the wild type Nurr1 (Fig. 7). This paradox known as the “SUMO enigma” has been observed for several transcriptional factors, allowing the proposal that SUMOylation is required to initiate a response but not to maintain it [9] [11–12]. The “SUMO enigma” hypothesis [9] [11–12] sustains that SUMOylation is a habilitation mark to be incorporated to a transcription repressor complex, but it would be not necessary to be kept in the complex. This hypothesis is also consistent with the broad regulatory repertoire that a transcription factor can exert depending on the promoter used. For example, our data show that wild type Nurr1 induces 30 times one promoter (3NBRE), [15] and only 3 times the other promoter (NBRE-3x-tk-Luc) [5], in circumstances that both promoters have 3 in tandem NBRE elements. In addition, no differences are observed between wild type Nurr1 and K91R mutant when the promoter has only one NBRE element. By SUMO-modifying the entire pool of Nurr1, the transactivation of each promoter would be proportional to the initial transcription effect and our data suggest that this is not the case. These data suggest that Nurr1 SUMOylation should occur in a promoter dependent manner to give a fine, differential response for each case.

We found that SUMO-2 is the only SUMO peptide post-translational modifying Nurr1. We cannot exclude that Nurr1 is a target of SUMO-1 or SUMO-3 in this or other cellular contexts. However, preferential SUMOylation by one of the SUMO peptides is increasingly being reported [24]. Our results indicate that lysine 91 is the major SUMO acceptor site in Nurr1. Since a faint signal of the slower migrating 95-kDa band remains in immunoblots with the mutant K91R, it is possible that another minor SUMOylation site is present in Nurr1. Saijo et al [25] showed that Nurr1 is SUMOylated by SUMO-2 and SUMO-3 in the lysine 558, in the context of an anti-inflammatory response in microglia. We replaced the lysine 558 by arginine (HA-Nurr1-K558R), and immunoblots of extracts from COS-7 cells transfected with this mutant showed the slow migrating 95-kDa band of similar intensity compared to wild type HA-Nurr1 immunoblots, indicating that lysine 558 is not a target of SUMOylation in our experimental conditions (data not shown). In addition, the truncated Nurr1 (HA-Nurr1 $\Delta$ AF-1) did not show any slower migrating band in the presence of SUMO machinery, discarding that the LBD/AF-2 of Nurr1 are target of SUMOylation by SUMO-2. Therefore, we conclude that the SUMOylation of Nurr1 is principally occurring in the N-terminal region with lysine 91 as the main acceptor site of SUMO-2. It is worth to mention that nuclear receptor SUMOylation has been shown mainly in the

N-terminal domain, for instance progesterone receptor [26], androgen, glucocorticoid and mineralocorticoid receptors [13].

Two pieces of information allow us to indicate that PIAS $\gamma$  behaves as the SUMO-E3 ligase of Nurr1. First, overexpressing PIAS $\gamma$ , but not PIAS $\gamma$ mut1 that is unable to interact with Nurr1, significantly enhanced Nurr1 SUMOylation. Second, Nurr1 SUMOylation was no longer observed, when PIAS $\gamma$ C342A, mutant that lacks SUMO ligase activity, was overexpressed. In addition, we suggest that PIAS $\gamma$  gives specificity and more efficiency to Nurr1 SUMOylation process, since a stronger and better defined band corresponding to Nurr1-SUMO-2 in immunoblots was observed in the presence of PIAS $\gamma$ . Our results allow us to propose that PIAS $\gamma$  exerts two inhibitory mechanisms over Nurr1 transcriptional activity. One mechanism requires its SUMO ligase activity. The SUMOylation of Nurr1 in the lysine 91 by PIAS $\gamma$  would limit Nurr1 transactivity in complex promoters (see below). Indeed, the mutant PIAS $\gamma$ C342A exerted lesser repression than wild type PIAS $\gamma$  in reporter assays using complex promoters. The other repressive mechanism is independent of PIAS $\gamma$  SUMO ligase activity; because the mutant PIAS $\gamma$ C342A exerted still a strong inhibition of Nurr1-dependent transcriptional activity, and we have shown previously [8] that the mutant Nurr1-K91R, which is resistant to SUMOylation is fully repressed by PIAS $\gamma$ . The immunofluorescent assays showing a similar strong colocalization of Nurr1-K91R with PIAS $\gamma$  compared to the wild type Nurr1, further support that PIAS $\gamma$  inhibition of Nurr1 is independent of its SUMOylation. Further work is required to reveal the mechanism by which PIAS $\gamma$  exert this dominant inhibition of Nurr1-dependent transcriptional activity and in what conditions only modulate its activity in the different type of promoters.

Data from literature indicates that SUMOylation can regulate several features of the transcription factors among them, the nuclear and/or subnuclear localization, stability and/or transcriptional activity [9] [11–12]. Our data suggest that SUMOylation of Nurr1 at lysine 91 does not modify its stability, since the SUMOylation-deficient Nurr1 mutant displays equal half-life than wild type Nurr1. Our half-life data for Nurr1 is similar to the half-life reported previously for Nurr1 [22]. The analysis of immunofluorescence data of Nurr1 and the mutant Nurr1-K91R and their colocalization with PIAS $\gamma$  indicates that there is no a relocation of the mutant K91R suggesting that SUMOylation does not regulate Nurr1 nuclear localization. Similarly, Belaguli and collaborators [27] showed that the SUMOylation of the transcription factor GATA4 did not modify its stability or the nuclear localization. Transcriptional repression induced by SUMOylation of transcription factors has been correlated with relocation of the transcription factor towards Promyelocytic Leukemia Protein bodies in the nucleus [28]. Additional work is required to learn whether the mechanism by which PIAS $\gamma$  represses Nurr1 is due to a relocation to silence sectors in cell nuclei.

Transcription factors interact among them giving synergic responses when they recognize multiple copies of their response element in the target promoters. This synergic regulation of transcriptional activity is restricted by SUMOylation of lysines within SC motifs present in some transcription factors [14] [29]. According with previous [8] and current results, SUMOylation decreases Nurr1 transcriptional activity. The lysine 91 of Nurr1, target of SUMO-2 is in a SC motif. This synergic control of transcriptional activity has been described for other nuclear receptors as progesterone [30], androgen [13], glucocorticoid [31] and estrogen-related receptors [32]. Nurr1 and Nurr1-K91R mutant showed similar transcriptional activity in the promoter with one NBRE element; however, Nurr1-K91R showed an

enormous transcriptional activity compared with wild type Nurr1, when recognized three NBRE elements, demonstrating the classical behavior of transcriptional activity mediated by SC motif. Besides, Nurr1 has a glycine residue in position 85, preceding the SC motif core, further evidence of the presence of a functional SC motif [23] [29].

Our study provides evidence that Nurr1 SUMOylation controls its transcriptional synergy in complex promoters. Dopaminergic gene targets of Nurr1, such as tyrosine hydroxylase [33], RET [34] and dopamine transporter [35] harbor several NBRE elements

combined with multiple elements for other transcription factors in their promoters. Future work is needed to reveal the regulatory role of Nurr1 SUMOylation in the control of the expression of genes of the dopaminergic system.

### Author Contributions

Conceived and designed the experiments: CA AV MEA. Performed the experiments: CA AV LAP MO LG MEA. Analyzed the data: CA MEA. Wrote the paper: CA MEA.

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