# Distinct functional modes of SUMOylation for retinoid X receptor alpha

Elsevier Editorial System(tm) for Biochemical and Biophysical Research Communications
Manuscript Draft

Manuscript Number:

Title: Distinct Functional Modes of SUMOylation for Retinoid X Receptor Alpha

Article Type: Full Length Article

Keywords: Retinoid X Receptor; transcription; SUMO; PIAS.

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**Cover Letter**

29th May 2015

Dear Editor

Please find attached our manuscript titled "**Distinct Functional Modes of SUMOylation for Retinoid X Receptor Alpha**" by Wai-Ping Lee *et al*. This work was the result of collaboration between the laboratories of Dr Paul Thompson (University of Ulster) and Dr Peter Jurutka (Arizona State University) and we would be delighted if you would consider this study for publication in *Biochemical Biophysical Research Communications*.

The report investigates the potential for human Retinoid X Receptor (hRXRα) as a substrate for SUMOylation and the novel factors that may modulate this process. RXRα is a pivotal member of the nuclear receptor superfamily and integral to the transcriptional regulation of embryogenesis, metabolism and homeostasis. Understanding how post translational events such as SUMOylation impact upon RXR functionality will be critical for delineating how this receptor imparts its regulatory effects in a cell and gene-specific fashion under different physiological and cellular challenges.

An earlier report by Choi *et al* (2006) detailed hRXRα to possess a SUMO acceptor site at lysine 108 within the AF-1 region of this receptor. This was an elegant study that focused on modification with SUMO1. In our present submission, we confirm and extend upon these findings to demonstrate hRXRα may be modified with all three tested SUMO isoforms and intriguingly find this event to be apparently reversed upon binding by the receptor of its cognate ligand.

Importantly, we identify lysine 245 within the omega loop region as a second acceptor site that is specifically modified with SUMO2 and in a PIAS4-dependent fashion. There are also intriguing indications that such modifications may be at least partially be achieved in a E3-ligase independent fashion.

While we confirm lysine 108 as the predominant site of SUMOylation with RXRα, the modification of lysine 245 is of equivocal functional relevance in terms of impact upon receptor activity. The complete loss of SUMOylation at both sites has striking effects upon ligand-induced transactivation by ligand in a fashion that may be dependent on DNA binding site context.

We acknowledge that these findings represent an initial stage of our research that will require further expansion. We do believe however that our present results are novel and given the considerable pharmacological relevance of RXR as a therapeutic target, will be of relevance for those researchers with a focus on metabolic/endocrine regulation in addition to treatment of Alzheimer's Disease and provide a basis for further investigation.

Our sincere thanks for your consideration of this manuscript

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S.J. Choi, S.S. Chung, E.J. Rho *et al*. Negative modulation of RXRalpha transcriptional activity by small ubiquitin-related modifier (SUMO) modification and its reversal by SUMO-specific protease SUSP1, J. Biol. Chem. 281 (2006) 30669-30677.

**Highlights (for review)**

- Human Retinoid X Receptor (hRXRα) can be modified with all three SUMO isoforms.
- SUMOylation preferentially occurs with the unliganded form of hRXRα.
- We confirm lysine 108 as the predominant SUMO acceptor site within hRXRα
- PIAS4 facilitates modification with SUMO2 at lysine 245 of hRXRα.
- Combined loss of SUMOylation at both sites highly potentiates hRXRα activity.

# Distinct Functional Modes of SUMOylation for Retinoid X Receptor Alpha

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**Abstract.**

The present study investigated human retinoid X receptor α (hRXRα) as a substrate for modification with small ubiquitin like modifier (SUMO) and how members of the protein inhibitor of activated STAT (PIAS) family may impact upon this process. In agreement with a previous study, we validate Ubc9 to facilitate SUMOylation of hRXRα at lysine 108 but note this modification to occur for all isoforms rather than specifically with SUMO1 and to preferentially occur with the unliganded form of hRXRα. SUMOylation of hRXRα is significantly enhanced through PIAS4-mediated activity with lysine 245 identified as a specific SUMO2 acceptor site modified in a PIAS4-dependent fashion. While individual mutations at lysine 108 or 245 modestly increase receptor activity, the combined loss of SUMOylation at both sites significantly potentiates the transcriptional responsiveness of hRXRα suggesting both sites may cooperate in a DNA element-dependent context. Our findings highlight that combinatorial effects of SUMOylation may regulate RXRα-directed signalling in a gene-specific fashion.

**Key words;**

Retinoid X receptor, transcription, SUMO, PIAS.

**Abbreviations;**

| | |
|---|---|
| STAT | Signal Transducers and Activators of Transcription |
| PIAS | Protein Inhibitor of Activated STAT |
| SUMO | Small Ubiquitin-Related Modifier |
| VDR | Vitamin D Receptor |
| RAR | Retinoic Acid Receptor |
| FXR | Farnesoid X Receptor |
| LXR | Liver X Receptor |
| PPAR | Peroxisome Proliferator Activated Receptor |
| RXRα | Retinoid X Receptor Alpha |
| Ubc9 | Ubiquitin-Conjugation Enzyme 9 |
| RING | Really Interesting Gene |

## Introduction

Retinoid X receptor (RXR) is a nuclear receptor (NR) represented by three isotypes (α, β and γ) for which 9-*cis* retinoic acid (9-*cis* RA), a stereoisomer of all-*trans* retinoic acid (ATRA), represents its most commonly noted ligand [1]. RXR plays a pivotal role within NR biology through its capacity to regulate transcription as a homodimer and also as a heteropartner for NRs such as the vitamin D receptor (VDR), retinoic acid receptor (RAR), peroxisome proliferator activated receptor (PPAR) and liver X receptor (LXR) [2]. Functional loss of the RXRα subtype has the most profound consequences which are lethal in homozygous knockout mice, with a heterozygous phenotype that closely reflects the biological functions of RXR-containing heterodimers [3]. Given its unique position within NR biology, RXR represents an attractive target of pharmaceutical interest with its most noted selective agonist Bexarotene (trade name Targretin) currently applied in the treatment of cutaneous T-cell lymphoma [4], and more recently implicated as a putative therapy for Alzheimer's disease [5]. A clear requirement exists for a detailed understanding of RXRα function and how its pleiotropic effects are impacted through metabolic challenges associated with disease, drug treatment or altered nutrient status, which can invoke changes in NR signalling through post-translational modifications [6]. Human RXRα (hRXRα) is known to be phosphorylated at serine 260, an event that underpins resistance of *Ras*-transformed keratinocytes to the growth-regulatory effects of both ATRA and the active vitamin D metabolite $1,25(OH)_2D_3$ (1,25D) [7,8]. hRXRα has also been reported to be modified with SUMO1 at lysine 108 (K108), an event which appears to diminish the transcriptional potency of RXRα [9], although the impact of other SUMO isoforms and RXR ligand(s) were not considered in this study. Given its unique and critical role within NR-directed signalling, we further explored hRXRα as a substrate for SUMO-modification and evaluated novel factors that may modulate this process.

## Materials and Methods

*Cell Culture and Ligands*

HEK293 cells were obtained from the European Collection of Cell Culture (ECACC) and maintained at 37ºC, 5% $CO_2$ in presence of DMEM + 10% FBS, 2mM L-glutamine, 50 units/ml penicillin G and 50μg/ml streptomycin. Media and supplements were purchased from GIBCO (Invitrogen, Carlsbad, CA) and 9-*cis* retinoic acid (9-*cis* RA) from Sigma. Bexarotene was synthesized as detailed by Wagner and co-workers [10].

*Plasmids*

Expression constructs encoding hRXRα (pSG5hRXRα and pcDNAV5-hRXRα), mouse PIAS proteins (pcDNAV5-PIAS, pcDNAGFP-PIAS and pcDNAHA-PIAS) and the Gal4-based hybrid expression vector (pCMVBD-RXRα) have been previously detailed [11]. Plasmids expressing His-tagged SUMO isoforms have been described by Dorval and Fraser [12] and were a generous gift of Prof Paul Fraser, University of Toronto. The pcDNAV5-UBC9 expression construct and its C93S mutant variant were provided by Prof Ron Hay, University of Dundee and used as a template to generate the vector encoding 'untagged' Ubc9 as previously detailed [13]**.** The firefly luciferase-based reporter construct pLUC-RXRE contains a retinoid X receptor responsive element (RXRE) based on a naturally occurring double repeat responsive element from the rat cellular retinol binding protein II gene [14]. The sequence used was AAAATGAACTGTGACCTGTGACCTGTGACCTG-TGAC. One copy of this double RXRE sequence was synthesized with an additional four base overhang for cloning into the HindIII and BglII sites of the pLUC-MCS reporter plasmid (Invitrogen). The transcriptional responses of Gal4-RXRα hybrid proteins were monitored through the luciferase signal generated from the pFLUC reporter (Stratagene) that contains five copies of the Gal4 response element.

*Cell-based SUMOylation assays*

Modification of hRXRα with SUMO was monitored through a cell-based protocol we have previously described [13]. Briefly, HEK293 cells were seeded in 60mm plates before transfection with the appropriate combination of constructs expressing hRXRα (2μg), SUMO1/2/3 (2μg), Ubc9 (1μg), PIAS (1μg) or mutated variant and appropriate parent vector control. Cells were incubated for a total of 48 hours post-transfection including a 24 hour period ± 9-*cis* RA (1μM) when considering effects of ligand. Cell lysates were processed as previously detailed before overnight incubation with V5 agarose beads (Abcam) followed by elution and subsequent analysis through western blotting.

*Western Blotting*

Protein lysates were separated on 4-12% NuPAGE Bis/Tris gels (Invitrogen), transferred onto Immobilon-P membranes (Millipore Corp) and then probed using the following antibodies: mouse monoclonal anti-V5 (Invitrogen) at a 1:5000 dilution; mouse monoclonal anti-GFP (Invitrogen) at a 1:1000 dilution. Target proteins were visualized using Supersignal®West Pico Chemiluminescent solution (Thermo Scientific) and development on autoradiographic film. All membranes were re-probed with a mouse monoclonal anti-β-actin antibody (Sigma) at 1:10,000 dilution. The secondary antibody was a rabbit anti-mouse IgG (whole molecule) peroxidase conjugate antibody (Sigma).

*Site-Directed Mutagenesis*

Synthesis of point mutations within the appropriate hRXRα encoding construct was achieved through the Quikchange XL site-directed mutagenesis system (Agilent Technologies) using the following mutagenic primer pairs;

RXRα K108R

5’-CAGCGAGGACATCAGGCCCCCCCTGGGC-3’

5’-GCCCAGGGGGGGCCTGATGTCCTCGCTG-3’

RXRα K245R

5’-GGCCGTGGAGCCCAGGACCGAGACCTACG-3’

5’-CGTAGGTCTCGGTCCTGGGCTCCACGGCC-3’.

*Transcriptional Activation Assays*

HEK293 cells were seeded unto a 24-well plate at 100,000 cells/well and maintained for a period of 24 hours before introduction of plasmid via calcium phosphate precipitation. At 16 hours post-transfection, cells were incubated in media supplemented with 1μM ligand (9-*cis* RA or bexarotene) or appropriate control for a further 24 hours, before recording of chemiluminescent signal using the Dual-Glo Luciferase Reporter Assay System (Promega). Transfection data were normalized relative to the luciferase signal produced from the constitutively active renilla vector (pRL-TK) and expressed as the means ± SEM from triplicate assays.

**Results**

We employed cell-based assays to evaluate the potential for hRXRα to be modified with different SUMO isoforms and how binding of its 9-*cis* RA cognate ligand may impact upon this process. Figure 1A details that under our specified conditions hRXRα can be conjugated with each tested SUMO isoform, with the major modified form of the receptor appearing as a distinct band migrating at ~90KDa (Fig. 1A, upper arrow) and the presence of 9-*cis* RA appearing to significantly diminish this process. To confirm these bands as specific products of the SUMO-enzymatic pathway, comparative assays were performed employing Ubc9 or its C93S variant possessing a deficient E2-SUMO conjugation function. As depicted in Figure 1B, hRXRα-SUMO2 could only be detected in the presence of the enzymatically intact Ubc9 (middle lane). Finally, as data detailed above were derived from transfected cell lysates

containing both RXRα and Ubc9 expressed as V5-'tagged' proteins, we wished to verify that higher migrating bands were not related to SUMO-modified forms of V5-Ubc9 that may have been co-precipitated in our assay system. Figure 1C illustrates that significantly increased levels of hRXRα-SUMO2 are detected when using a non-tagged version of Ubc9 which also elicited additional higher migrating bands (~110KDa/160KDa) signifying formation of multiple forms of hRXRα-SUMO2. Subsequent assays described in this manuscript incorporate the use of the non-tagged Ubc9.

Figure 2A describes the capacity of PIAS proteins to modulate RXRα SUMOylation. These experiments involved co-expression of V5-hRXRα with the indicated combination of Ubc9, His-SUMO and HA-PIAS1/ 2/ 3/ 4 or the PIAS4 (W356A) mutant that is defective in E3-SUMO ligase activity. The upper panel details the ability of PIAS1, 2, and 3 to increase the formation of SUMO1-hRXRα (~90KDa), however such effects are modest when compared to those achieved through PIAS4 for which the appearance of an additional band (~110KDa) suggests possible modification at more than one site. PIAS4 also enhanced RXRα conjugation with SUMO2 (Fig. 2A, middle panel and Fig. 2B) and SUMO3 (Fig. 2A, lower panel) with no such activity exhibited by the other PIAS proteins. As expected, the PIAS4 W356A mutant had no effect on modification with SUMO1 or SUMO2 but intriguingly retained a capacity to enhance formation of SUMO2-hRXRα. We probed this observation further, as well as the impact of PIAS4 when expressed in the absence of exogenous SUMO protein. While Figure 2C confirms a conjugated hRXRα to form through co-expression of SUMO2 and Ubc9 (lane 2), we note that even in the absence of exogenous SUMO, the combined activities of PIAS4 and Ubc9 generate a modified hRXRα (lane 3) that likely occurs with endogenous SUMO protein. The combined expressions of PIAS4, Ubc9 and SUMO2 (lane 4) dramatically enhanced receptor SUMOylation and invoked appearance of multiple SUMO-hRXRα conjugates. In contrast, the W356A mutant exhibited no

comparable effects when reliant upon endogenous SUMO as substrate (lane 5) but when co-expressed in combination with SUMO2 generated a single dominant band at ~90KDa (lane 6). Taken in combination, these data indicate PIAS4 to enhance RXRα modification with all three SUMO isoforms, an event which may involve formation of poly-SUMO chains on RXRα and/or modification at more than one acceptor site within the receptor. For one of these potential sites, PIAS4 appears to facilitate generation of RXRα-SUMO2 independently of its RING finger ligase function.

To investigate further if hRXRα may be modified at more than one site, we compared the SUMOylation patterns exhibited by the wild type receptor and a variant possessing a lysine (K) to arginine (R) substitution at amino acid 108 (K108R) previously reported to serve as a SUMO1 acceptor site within hRXRα [9]. The experiments detailed in Figure 3 included Ubc9 and PIAS4 within all tested parameters. Figure 3A verifies modification of wild type hRXRα with each SUMO isoform but also demonstrates hRXRα K108R capable of forming a conjugated species with SUMO2 with Figure 3B detailing that the presence of 9-*cis* RA will again inhibit or reverse this process. No conjugates were detected to form between the K108R mutant and SUMO1 or SUMO3. These results implicate the presence within hRXRα of at least one acceptor site additional to K108 that is specific for SUMO2. Analysis of the hRXRα protein sequence reveals SUMO consensus motifs at K201 and K245 which we then assessed for their relevance to receptor modification through generating K to R mutations at each site, alone and in combination with K108R. In Figure 3C we highlight experiments focusing on K245 and K108 as potential acceptor sites. While wild type hRXRα exhibited the expected pattern of modification, both the K245R and K108R mutants exhibit a single band corresponding to SUMO2-hRXRα. Intriguingly, the double (K108R/K245R) mutant RXRα demonstrated a complete loss of receptor modification verifying hRXRα to have two

acceptor sites represented by K108 and K245, with the latter apparently specific for modification with SUMO2.

We next evaluated the functional relevance of these acceptor sites upon hRXRα transactivation by its ligand using two different reporter assay systems. In Figure 4A, HEK293 cells received the appropriate expression constructs for V5-RXRα or mutant variants in combination with the pLUC-RXRE based reporter construct that contains two copies of a retinoid x response element (RXRE) sequence. The data is expressed as the fold increase (induction) of reporter activity that resulted from treatment with the synthetic RXR agonist bexarotene over that observed for vehicle control. As illustrated, all tested variants exhibited a fold induction of transactivation significantly higher than the wild type hRXRα and although all mutants are statistically equivalent, a clear trend is noted in which the K108R/K245R variant exhibits induction levels approximately 240% greater that of the wild type hRXRα compared to 98% and 128% for K108R and K245R, respectively. Consideration of such effects upon transcriptional activity should take consideration that we consistently note protein levels for the double mutation to be considerably lower in the presence of ligand when compared to the wild type hRXRα, suggesting even more pronounced functional differences may result if normalized based on equivalency of protein. In Figure 4B, each hRXRα was expressed as a hybrid protein fused to the gal4 DNA binding domain with transactivation monitored through the pFLUC reporter containing five tandem copies of the gal4 response element. The data are depicted as overall reporter activity and reveal the K108R/K245R mutant to exhibit a level of activity in the presence of ligand that is approximately 907% greater than that achieved using the wild type-based construct. In contrast, the individual K108R or K245R mutants do not exhibit significantly different activities from the intact receptor, at least in this reporter system.

**Discussion**

In this current study we probe hRXRα as a substrate for SUMOylation and demonstrate Ubc9 to facilitate the modification of hRXRα with all three isoforms tested in our experimental system, an event that is apparently reversed in the presence of the 9-*cis* RA RXR ligand. Ligand binding is known to invoke co-repressor release followed by ubiquitination and proteasome-mediated degradation as components within the RXRα transcriptional cycle [15, 16]. While we note hRXRα protein levels to be slightly reduced within precipitates from ligand-treated samples compared to vehicle control counterparts, this is not sufficient to explain the striking loss of receptor modification in the presence of 9-*cis* RA. It is possible that ligand-binding may elicit changes in receptor conformation that limit the accessibility of acceptor sites or introduce alternate modifications at the same or neighbouring residues. Choi and co-workers identified the removal of SUMO1 from RXRα to be mediated through sentrin/SUMO specific protease (SENP) activity, specifically that of SENP6, although the impact of ligand within this process was not defined [9]. We recently reported that ligand-bound VDR will recruit members of the SENP family to reverse its modification with SUMO2 [11]. It will be interesting to probe if such a model of ligand-driven deSUMOylation is also a modulatory component within the RXR transcriptional response.

The data discussed above considers hRXRα SUMOylation driven through Ubc9 activity alone. In this context, Choi and co-workers identified lysine 108 (K108) within the AF1 region of RXRα as a single and atypical acceptor site [9]. While Ubc9 can effectively mediate SUMOylation, other sites may be 'hidden', and require the additional presence of an E3-SUMO ligase for their modification [17]. As such an example, we recently reported PIAS4 to serve a specific SUMO ligase function for the modification of VDR with SUMO2 [13] and in the context of hRXRα we identify PIAS4 to also robustly enhance the

conjugation of this receptor with each co-expressed SUMO isoform and indeed increase the detectable levels of hRXRα modified with endogenous SUMO. Any effects noted for other PIAS proteins are considerably weaker and limited to a modest increase in receptor conjugated with SUMO1. Intriguingly, we find the PIAS4 variant deficient in E3-ligase activity retains a significant capacity to enhance formation of hRXRα-SUMO2, albeit at a level that is diminished compared to that achieved with enzymatically intact PIAS4. Similar observations have been reported for YY1 in which PIAS4 is proposed to facilitate interactions between substrate, Ubc9 and SUMO that serve to increase the accessibility of acceptor sites for conjugation [18]. Our results also highlight an intriguing possibility that hRXRα may possess acceptor sites that are alternately modified through distinct PIAS4-directed pathways which we seek to verify and further characterize.

Our data identify K245, a residue located within the omega loop region of RXRα as an additional SUMO acceptor site to K108. The pattern of modification exhibited by the K108R-RXRα mutant reveals it retains a capacity to be specifically modified with SUMO2 in the presence of PIAS4, , an event that is again reversed through the presence of ligand. Taken in combination our data indicate K108 to be the predominant acceptor site within RXRα that can be conjugated with all three SUMO isoforms, while K245 is specifically modified by SUMO2 in a PIAS4-dependent fashion.

SUMO-modified nuclear receptors typically exhibit decreased levels of transactivation and our data also imply SUMOylation to repress the transcriptional responsiveness of RXRα to its ligand. Our analysis focused on hRXRα signalling in a homodimeric context with consideration for how SUMOylation impacts upon its function as a heteropartner beyond the scope of this current study. We confirm the findings of Choi and co-workers that loss of the K108 as an acceptor site will result in a modest but significant increase in the transcriptional

potency of RXRα [9] but note similar effects with the K245R-RXRα variant signifying that although this site is not as extensively modified as K108, it is of at least equal importance in terms of functional relevance. The most significant changes in ligand response are displayed by the RXRα variant that is completely deficient in SUMOylation. When assessed through the RXRE-based reporter system, the heightened levels of transactivation exhibited by the K108R/K245R RXRα variant are an additive combination of the effects achieved by each individual mutation. More striking effects are noted when activity is monitored through the gal4-hybrid system that utilizes a reporter construct containing five tandem copies of the Gal4 response element. In this context, the levels of transactivation to ligand exhibited by the double mutant are profoundly increased and potentiated in a synergistic manner over those displayed by the intact receptor and its single mutant variants. These data suggest that SUMOylation may modulate transactivation of RXRα in a response element-specific fashion and imply that in certain promoter contexts the receptor may be subject to a functional interaction between its SUMO acceptor sites.

We acknowledge that these novel albeit initial studies for how SUMOylation impacts upon RXR-mediated function require expansion to consider other RXR isoforms, its relevance to heterodimeric complexes, and effects on endogenous gene targets. Nonetheless, our findings highlight possible mechanisms through which PIAS-directed activity, in response to stress or inflammatory stimuli, may modulate RXR transcriptional activity in a gene-selective fashion. Given the plethora of biological processes in which RXR participates, these are worthy of future analysis and a current focus of investigation within our laboratory.

**Acknowledgements**

Wai-Ping Lee and Sarita Jena were supported through the Vice Chancellor Research Scholarship of Ulster University.

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**Legends**

Figure 1

The unliganded form of hRXRα can be covalently modified with SUMO1, 2 and 3. **A.** HEK293 cells were transfected with expression constructs for V5-hRXRα and V5-Ubc9 in combination with His-SUMO1, His-SUMO2, His-SUMO3 or empty vector control (-) where indicated. After a 24 hour period of incubation ± 9-*cis* RA, lysates from each treatment group were processed as detailed in Materials and Methods and resulting immune-precipitated material subjected to western blot analysis using an antibody specific for the V5 epitope tag. The arrows highlight the detected SUMO-modified (~90KDa) and unconjugated (~ 62KDa) forms of V5-RXRα. **B.** SUMOylation of hRXRα is dependent upon an enzymatically intact Ubc9. Cell-based assay was performed to compare the hRXRα modification with SUMO2 when using constructs encoding V5-Ubc9 or its catalytically

inactive C93S variant. The lower panel confirms equal expression within precipitated lysates of both V5-Ubc9 forms. **C**. Detection of SUMO-hRXRα is increased using the native (untagged) form of Ubc9. Cell-based assays compared levels of SUMO2-hRXRα detected using the V5-tagged (left lane) and native (right lane) forms of Ubc9. The arrows highlight the V5-Ubc9 (left lane only) and hRXRα detected through western blot analysis of precipitated lysates with enhanced levels of SUMO2-hRXRα noted when untagged Ubc9 was employed.

Figure 2

PIAS4 facilitates SUMOylation of hRXRα. **A.** Depicted are cell-based SUMOylation assays in which HEK293 cells received expression constructs for His-SUMO1 (upper panel), His-SUMO2 (middle panel) or His-SUMO3 (lower panel) each in combination with V5-hRXRα and where indicated, Ubc9, HA-PIAS1, 2, 3, 4 or the catalytically inactive HA-PIAS4 W356A. Resulting immune-precipitated lysates were probed through western blot analysis using the V5-specific antibody. Arrows highlight detected V5-hRXRα and its slower migrating species conjugated with the different SUMO isoforms.

**B.** Confirmation of PIAS4 as a catalyst in modification of hRXR with SUMO2. HEK293 cells were co-transfected with the indicated combination of plasmids encoding V5-hRXRα, GFP-PIAS4, His-SUMO-2 and Ubc9. The upper panel denotes western blot analysis of immune-precipitated material processed as described above highlighting V5-hRXRα and its SUMO-modified form. The lower panels confirm the expression status of GFP-PIAS4, V5-hRXRα and His-SUMO2 within cell lysates derived from each treatment group using the appropriate antibodies specific for each epitope tag. **C.** PIAS4 can promote SUMOylation of hRXRα independent of its E3 ligase function. The abilities of PIAS4 and its enzymatically inactive W356A variant were assessed for their abilities to enhance modification of hRXRα

with SUMO2. HEK293 cells were transfected with the indicated combinations of expression constructs encoding V5-hRXRα, His-SUMO2, Ubc9, HA-PIAS4 (WT) or HA-PIAS4W356A with (-) representing inclusion of the appropriate parent vector control. Immune-precipitated lysates were subjected to the depicted western blot analysis that highlights the unconjugated and SUMO2-modified forms of V5-hRXRα.

Figure 3

Evidence for a second SUMO acceptor site within hRXRα. **A.** HEK293 cell-based SUMOylation assays were performed as previously described to compare modification profile exhibited by V5-hRXRα and its K108R variant that harbours a mutation of a previously identified SUMO1 acceptor site. Depicted are resulting western blot analysis using the V5-specific antibody of immune-precipitated cell lysates containing either wild type or mutant forms of V5-hRXRα in combination with the indicated construct for SUMO1, 2 , 3 or empty vector control. **B.** Confirmation that second acceptor site within RXRα is specific for SUMO2. HEK293 cells received expression constructs for V5-K108R RXRα mutant and the indicated SUMO isoform and incubated ± 9-*cis* RA. All treatment groups were processed and subjected to analysis as described above and in Materials and Methods. Arrows highlight both SUMO-conjugated and unmodified forms of the RXRα K108R protein. **C.** Identification of K245 as a SUMO2 acceptor site within hRXRα. Cell-based SUMOylation assays were performed to compare patterns of modification with SUMO2 exhibited by wild type V5-RXRα and its variants K108R, K245R and K108R/K245R. The upper panel depicts western blot analysis using the anti-V5 antibody of immune-precipitated lysates derived from each treatment group. The lower panels confirm the expression status of GFP-PIAS4, V5-RXRα and His-SUMO2 within lysates representing each treatment group.

Figure 4.

Loss of the K108 and K245 SUMOylation sites within hRXRα increases its transactivation by ligand. HEK293 cells received: **A.** the pMCS-RXRE luciferase based reporter construct in combination with the appropriate expression constructs for V5-hRXRα or its K108R, K245R or K108R/K245R variants; **B.** the pFLUC reporter vector together with pCMVBD-based expression vector encoding RXRα or the indicated mutant variants. Treated cells were dosed with bexarotene ($10^{-6}$ M) ligand or vehicle control for a period of 24 hours before measurement of luciferase activity. After normalization for transfection efficiency based on the activity of the pRL-TK control, results were expressed as fold-inductions (ratio of activity in the presence:absence of ligand) for **A**. or relative luciferase activity for **B**. All data within each figure represents means (± SE) of triplicate assays (n=3) where ns $p \geq 0.05$, * $p = 0.01$-$0.05$, *** $p = 0.0001$ - $0.001$, **** $p < 0.0001$.

Figure 1

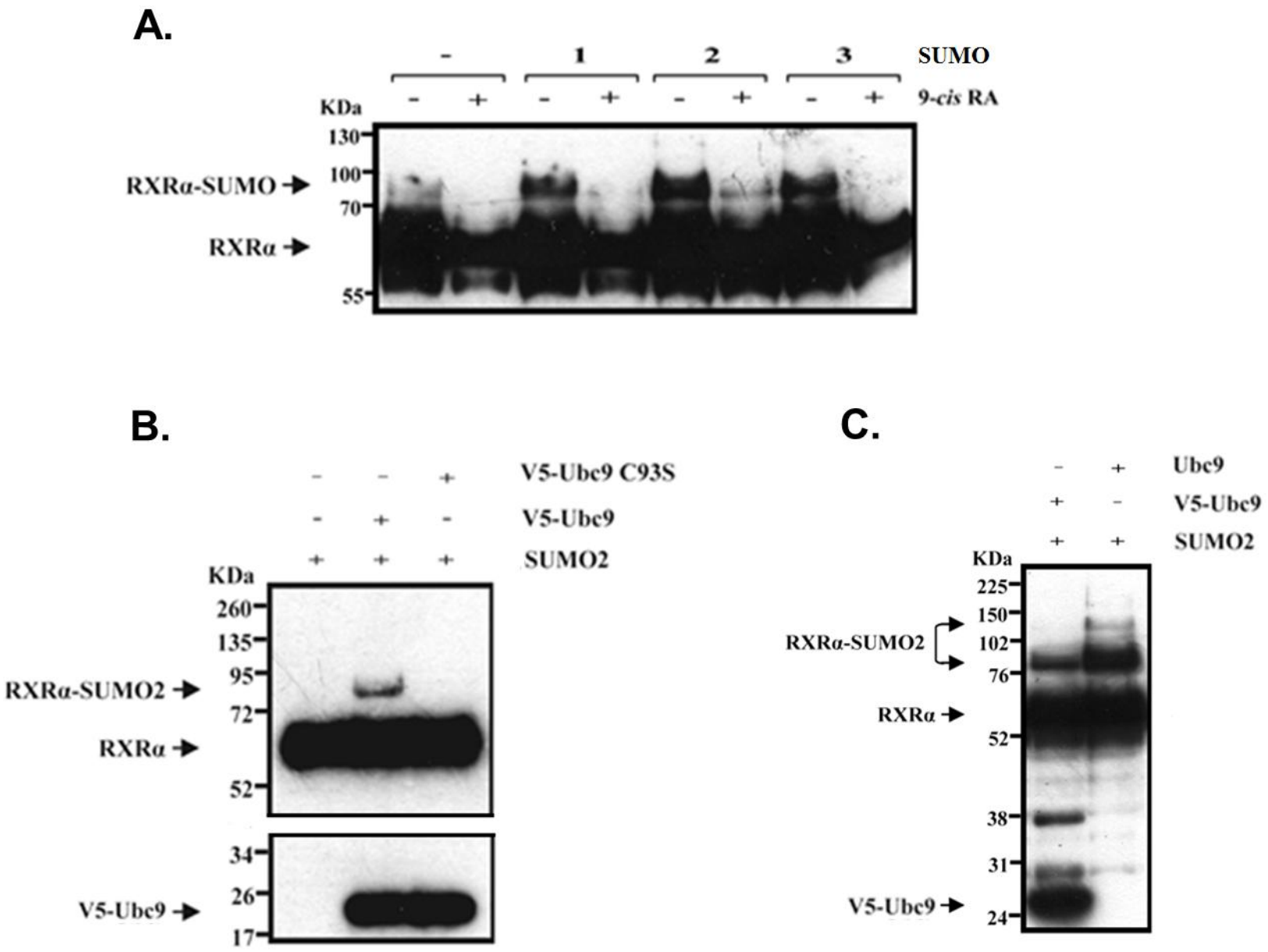

A.
SUMO
- 1 2 3
9-cis RA
- + - + - + - +
KDa
130
100
70
55
RXRα-SUMO
RXRα
B.
V5-Ubc9 C93S
V5-Ubc9
SUMO2
KDa
260
135
95
72
52
34
26
17
RXRα-SUMO2
RXRα
V5-Ubc9
C.
Ubc9
V5-Ubc9
SUMO2
KDa
225
150
102
76
52
38
31
24
RXRα-SUMO2
RXRα
V5-Ubc9

Figure 2

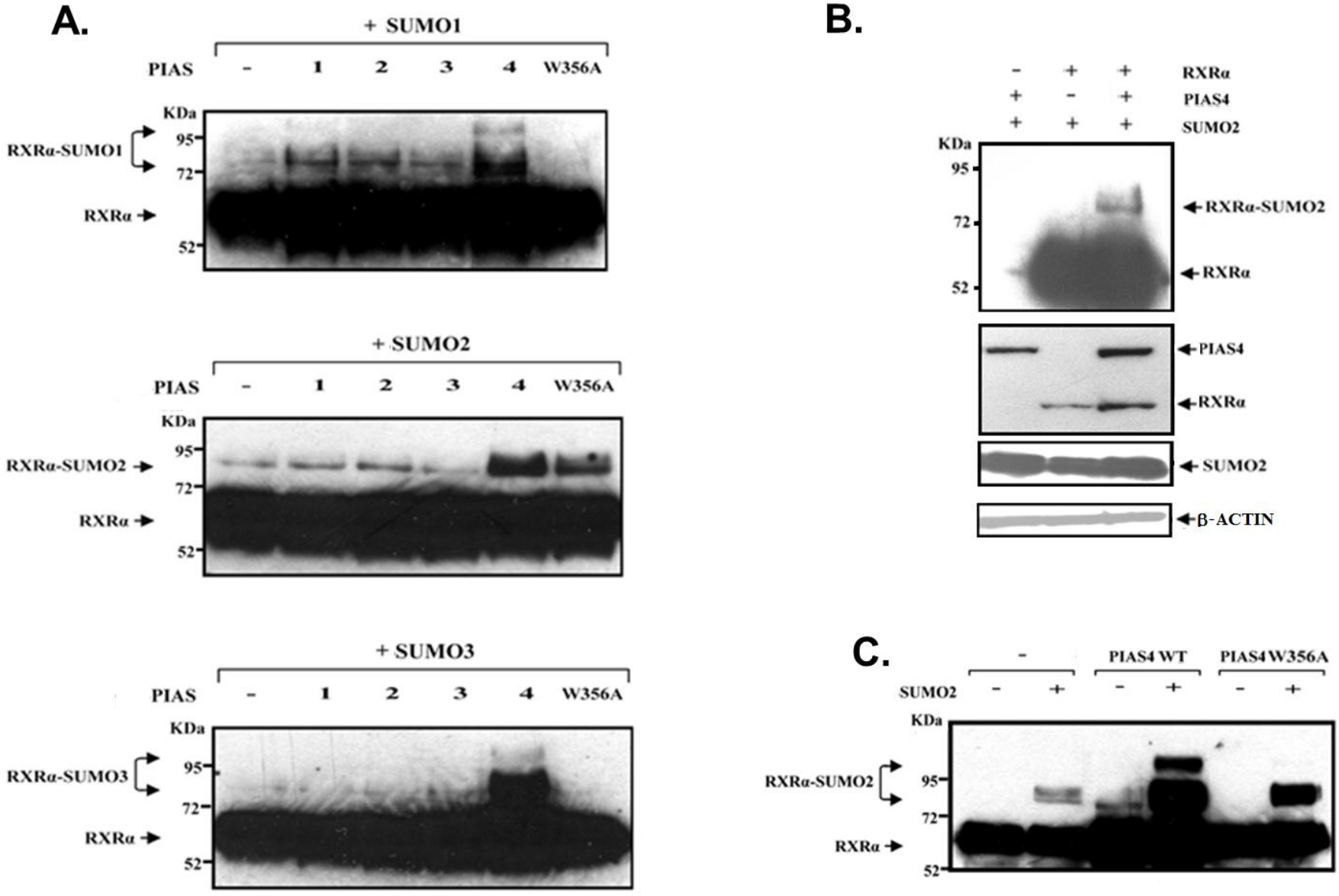

A.
+ SUMO1
PIAS - 1 2 3 4 W356A
KDa
95
72
52
RXRα-SUMO1
RXRα
+ SUMO2
PIAS - 1 2 3 4 W356A
KDa
95
72
52
RXRα-SUMO2
RXRα
+ SUMO3
PIAS - 1 2 3 4 W356A
KDa
95
72
52
RXRα-SUMO3
RXRα
B.
- + + RXRα
+ - + PIAS4
+ + + SUMO2
KDa
95
72
52
RXRα-SUMO2
RXRα
PIAS4
RXRα
SUMO2
β-ACTIN
C.
- PIAS4 WT PIAS4 W356A
SUMO2 - + - + - +
KDa
95
72
52
RXRα-SUMO2
RXRα

Figure 3

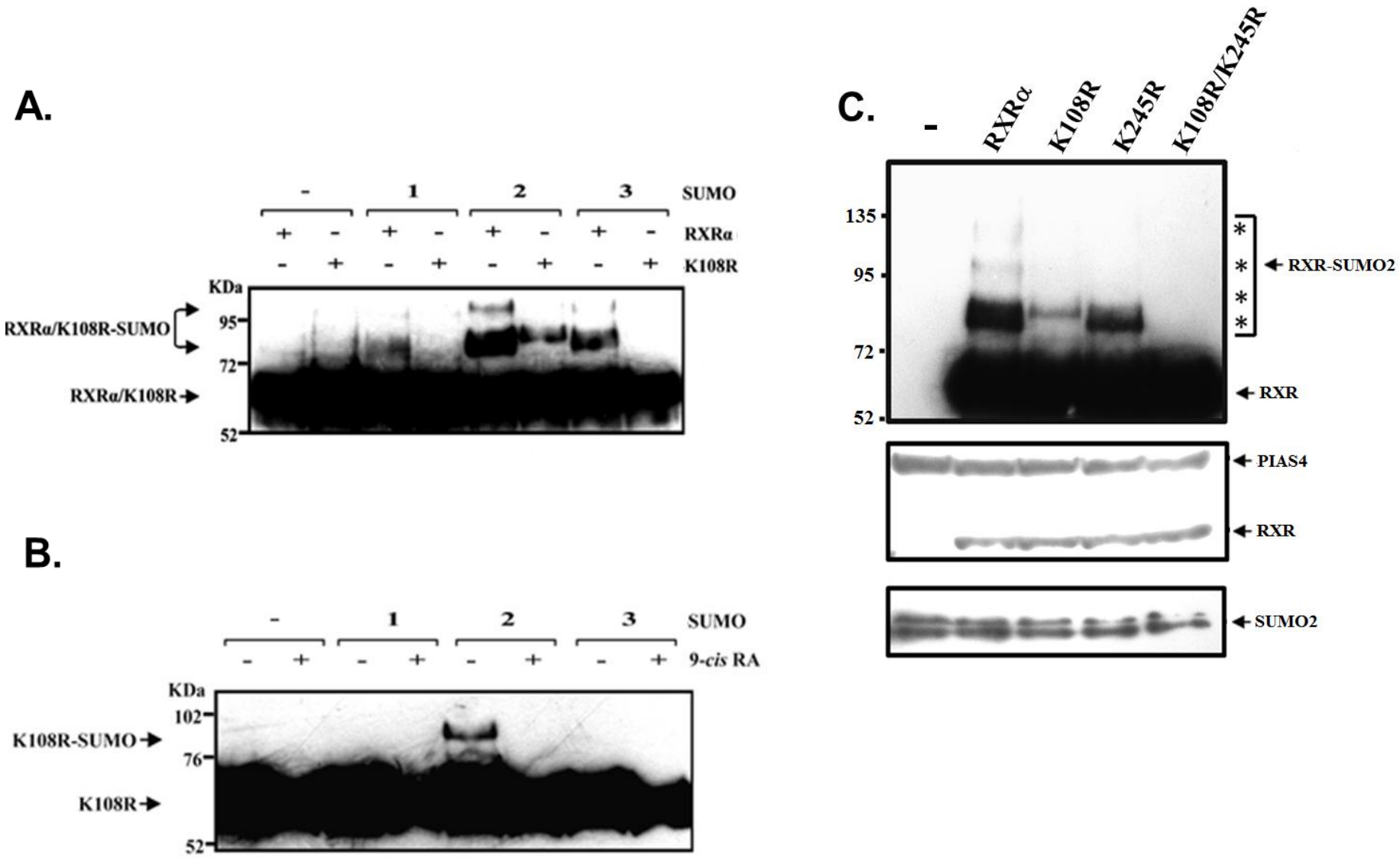
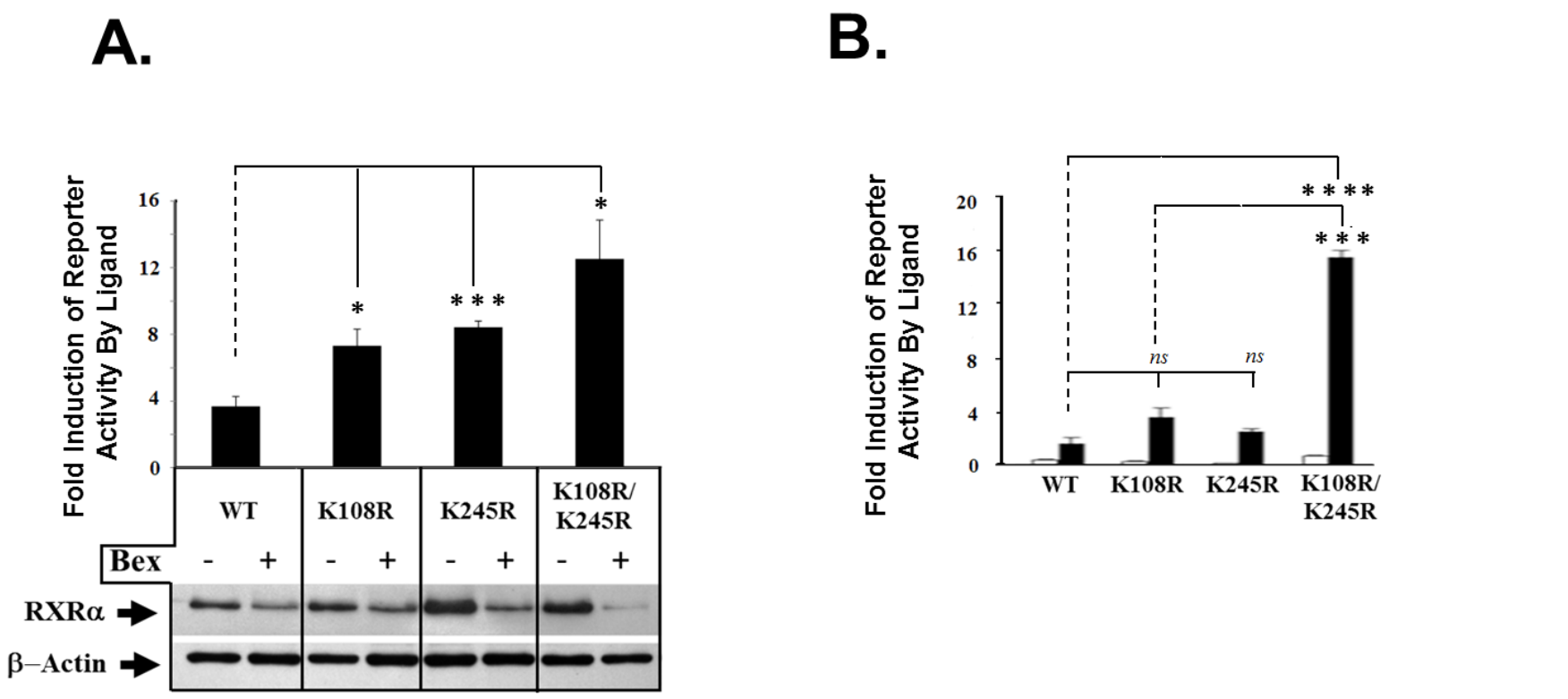

A.
SUMO
RXRα
K108R
KDa
95
72
52
RXRα/K108R-SUMO
RXRα/K108R
B.
SUMO
9-cis RA
KDa
102
76
52
K108R-SUMO
K108R
C.
RXRα
K108R
K245R
K108R/K245R
135
95
72
52
RXR-SUMO2
RXR
PIAS4
RXR
SUMO2

Figure 4


A.
Fold Induction of Reporter Activity By Ligand
16
12
8
4
0
*
*
***
*
WT
K108R
K245R
K108R/ K245R
Bex
- + - + - + - +
RXRα
β–Actin
B.
Fold Induction of Reporter Activity By Ligand
20
16
12
8
4
0
****
***
ns
ns
WT
K108R
K245R
K108R/ K245R

***Conflict of Interest**

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