



## Distinct functional modes of SUMOylation for retinoid X receptor alpha

Lee, W.-P., Jena, S., Rodriguez, P., O'Donovan, S. P., Wagner, C., Jurutka, P. W., & Thompson, P. (2015). Distinct functional modes of SUMOylation for retinoid X receptor alpha. *Biochemical and Biophysical Research Communications*, 464(1), 195-200. <https://doi.org/10.1016/j.bbrc.2015.06.115>

[Link to publication record in Ulster University Research Portal](#)

**Published in:**

Biochemical and Biophysical Research Communications

**Publication Status:**

Published (in print/issue): 23/06/2015

**DOI:**

[10.1016/j.bbrc.2015.06.115](https://doi.org/10.1016/j.bbrc.2015.06.115)

**Document Version**

Author Accepted version

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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.

Corresponding Author: Dr Paul Thompson,

Corresponding Author's Institution: University of Ulster

First Author: Wai-Ping Lee, PhD

Order of Authors: Wai-Ping Lee, PhD; Sarita Jena, PhD; E. Patricia Rodriguez, PhD; Sinead P O'Donovan;  
Carl Wagner, PhD; Peter W Jurutka, PhD; Paul Thompson

29<sup>th</sup> 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 (hRXR $\alpha$ ) as a substrate for SUMOylation and the novel factors that may modulate this process. RXR $\alpha$  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 hRXR $\alpha$  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 hRXR $\alpha$  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 RXR $\alpha$ , 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  
Dr Paul Thompson  
University of Ulster

Dr Peter Jurutka  
Arizona State University

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

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

## **Distinct Functional Modes of SUMOylation for Retinoid X Receptor Alpha**

Wai-Ping Lee<sup>‡1</sup>, Sarita Jena<sup>‡1</sup>, E. Patricia Rodriguez<sup>1</sup>, Sinead P. O'Donovan<sup>1</sup>, Carl Wagner<sup>2</sup>, Peter W. Jurutka<sup>2</sup>, and Paul D. Thompson<sup>1\*</sup>

<sup>1</sup>School of Biomedical Sciences, University of Ulster, Coleraine BT52 1SA, Northern Ireland, UK and <sup>2</sup>School of Mathematical and Natural Sciences, Arizona State University, Glendale, Arizona, 85306, USA

‡ Both authors contributed equally to this work

\*Corresponding author: School of Biomedical Sciences, University of Ulster, Coleraine BT52 1SA, Northern Ireland, UK. Tel

+44 (0) 28 70323246

Email [p.thompson@ulster.ac.uk](mailto:p.thompson@ulster.ac.uk)

## **Abstract.**

The present study investigated human retinoid X receptor  $\alpha$  (hRXR $\alpha$ ) 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 hRXR $\alpha$  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 hRXR $\alpha$ . SUMOylation of hRXR $\alpha$  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 hRXR $\alpha$  suggesting both sites may cooperate in a DNA element-dependent context. Our findings highlight that combinatorial effects of SUMOylation may regulate RXR $\alpha$ -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
RXR $\alpha$	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 ( $\alpha$ ,  $\beta$  and  $\gamma$ ) 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 RXR $\alpha$  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 RXR $\alpha$  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 RXR $\alpha$  (hRXR $\alpha$ ) 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)<sub>2</sub>D<sub>3</sub> (1,25D) [7,8]. hRXR $\alpha$  has also been reported to be modified with SUMO1 at lysine 108 (K108), an event which appears to diminish the transcriptional potency of RXR $\alpha$  [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 hRXR $\alpha$  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 37°C, 5% CO<sub>2</sub> 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 hRXR $\alpha$  (pSG5hRXR $\alpha$  and pcDNAV5-hRXR $\alpha$ ), mouse PIAS proteins (pcDNAV5-PIAS, pcDNAGFP-PIAS and pcDNAHA-PIAS) and the Gal4-based hybrid expression vector (pCMVBD-RXR $\alpha$ ) 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 AAAATGAACTGTGACCTGTGACCTGTGACCTGTGAC. 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-RXR $\alpha$  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 hRXR $\alpha$  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 hRXR $\alpha$  (2 $\mu$ g), SUMO1/2/3 (2 $\mu$ g), Ubc9 (1 $\mu$ g), PIAS (1 $\mu$ g) or mutated variant and appropriate parent vector control. Cells were incubated for a total of 48 hours post-transfection including a 24 hour period  $\pm$  9-*cis* RA (1 $\mu$ M) 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<sup>®</sup>West Pico Chemiluminescent solution (Thermo Scientific) and development on autoradiographic film. All membranes were re-probed with a mouse monoclonal anti- $\beta$ -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 hRXR $\alpha$  encoding construct was achieved through the Quikchange XL site-directed mutagenesis system (Agilent Technologies) using the following mutagenic primer pairs;

RXR $\alpha$  K108R

5'-CAGCGAGGACATCAGGCCCCCCTGGGC-3'

5'-GCCCAGGGGGGGCCTGATGTCCTCGCTG-3'

RXR $\alpha$  K245R

5'-GGCCGTGGAGCCCAGGACCGAGACCTACG-3'

5'-CGTAGGTCTCGGTCCTGGGCTCCACGGCC-3'.

#### *Transcriptional Activation Assays*

HEK293 cells were seeded into 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 1 $\mu$ M 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  $\pm$  SEM from triplicate assays.

#### **Results**

We employed cell-based assays to evaluate the potential for hRXR $\alpha$  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 hRXR $\alpha$  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, hRXR $\alpha$ -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 RXR $\alpha$  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 hRXR $\alpha$ -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 hRXR $\alpha$ -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 RXR $\alpha$  SUMOylation. These experiments involved co-expression of V5-hRXR $\alpha$  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-hRXR $\alpha$  (~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 RXR $\alpha$  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-hRXR $\alpha$ . 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 hRXR $\alpha$  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 hRXR $\alpha$  (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-hRXR $\alpha$  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 RXR $\alpha$  modification with all three SUMO isoforms, an event which may involve formation of poly-SUMO chains on RXR $\alpha$  and/or modification at more than one acceptor site within the receptor. For one of these potential sites, PIAS4 appears to facilitate generation of RXR $\alpha$ -SUMO2 independently of its RING finger ligase function.

To investigate further if hRXR $\alpha$  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 hRXR $\alpha$  [9]. The experiments detailed in Figure 3 included Ubc9 and PIAS4 within all tested parameters. Figure 3A verifies modification of wild type hRXR $\alpha$  with each SUMO isoform but also demonstrates hRXR $\alpha$  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 hRXR $\alpha$  of at least one acceptor site additional to K108 that is specific for SUMO2. Analysis of the hRXR $\alpha$  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 hRXR $\alpha$  exhibited the expected pattern of modification, both the K245R and K108R mutants exhibit a single band corresponding to SUMO2-hRXR $\alpha$ . Intriguingly, the double (K108R/K245R) mutant RXR $\alpha$  demonstrated a complete loss of receptor modification verifying hRXR $\alpha$  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 hRXR $\alpha$  transactivation by its ligand using two different reporter assay systems. In Figure 4A, HEK293 cells received the appropriate expression constructs for V5-RXR $\alpha$  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 hRXR $\alpha$  and although all mutants are statistically equivalent, a clear trend is noted in which the K108R/K245R variant exhibits induction levels approximately 240% greater than that of the wild type hRXR $\alpha$  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 hRXR $\alpha$ , suggesting even more pronounced functional differences may result if normalized based on equivalency of protein. In Figure 4B, each hRXR $\alpha$  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 hRXR $\alpha$  as a substrate for SUMOylation and demonstrate Ubc9 to facilitate the modification of hRXR $\alpha$  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 RXR $\alpha$  transcriptional cycle [15, 16]. While we note hRXR $\alpha$  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 RXR $\alpha$  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 hRXR $\alpha$  SUMOylation driven through Ubc9 activity alone. In this context, Choi and co-workers identified lysine 108 (K108) within the AF1 region of RXR $\alpha$  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 hRXR $\alpha$  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 hRXR $\alpha$  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 hRXR $\alpha$ -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 hRXR $\alpha$  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 RXR $\alpha$  as an additional SUMO acceptor site to K108. The pattern of modification exhibited by the K108R-RXR $\alpha$  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 RXR $\alpha$  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 RXR $\alpha$  to its ligand. Our analysis focused on hRXR $\alpha$  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 RXR $\alpha$  [9] but note similar effects with the K245R-RXR $\alpha$  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 RXR $\alpha$  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 RXR $\alpha$  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 RXR $\alpha$  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.

## References

- [1] R.A. Heyman, D.J. Mangelsdorf, J.A. Dyck et al. 9-*cis* retinoic acid is a high affinity ligand for the retinoid X receptor, *Cell*. 68 (1992) 397-406.
- [2] R.M. Evans and D.J. Mangelsdorf, Nuclear Receptors, RXR, and the Big Bang, *Cell*. 157 (2014) 255-266.
- [3] A. Szanto, V. Narkar, Q. Shen et al. Retinoid X receptors: X-ploring their (patho) physiological functions, *Cell Death and Differentiation*. 11 Suppl 2 (2004) 126-143.
- [4] R. Gniadecki, C. Assaf, M. Bagot et al. The optimal use of bexarotene in cutaneous T-cell lymphoma, *Br. J. Dermatol.* 157 (2007) 433-40.
- [5] P.E. Cramer, J.R. Cirrito, D.W. Wesson et al. ApoE-directed therapeutics rapidly clear  $\beta$ -amyloid and reverse deficits in AD mouse models, *Science*. 335 (2012) 1503-1506.
- [6] W. Berrabah, P. Aumercier, P. Lefebvre et al. Control of nuclear receptor activities in metabolism by post-translational modifications, *FEBS Lett.* 585 (2011) 1640-1650.
- [7] N. Bruck, J. Bastien, G. Bour et al. Phosphorylation of the retinoid x receptor at the omega loop, modulates the expression of retinoic-acid-target genes with a promoter context specificity, *Cell Signal*. 17 (2005) 1229-1239.
- [8] M. Macoritto, L. Nguyen-Yamamoto, D.C. Huang et al. Phosphorylation of the human retinoid X receptor alpha at serine 260 impairs coactivator(s) recruitment and induces hormone resistance to multiple ligands, *J. Biol. Chem.* 283 (2008) 4943-4956.
- [9] 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.
- [10] C.E. Wagner, P.W. Jurutka, P.A. Marshall et al. Modeling, synthesis and biological evaluation of potential retinoid X receptor (RXR) selective agonists: novel analogues of 4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)ethynyl]benzoic acid (bexarotene), *J. Med. Chem.* 52 (2009) 5950-5966.
- [11] W.P. Lee, S. Jena, D. Doherty et al. Sentrin/SUMO specific proteases as novel tissue-selective modulators of vitamin D receptor-mediated signalling, *PLoS One*. 9 (2014) e89506.
- [12] V. Dorval, P.E. Fraser, Small ubiquitin-like modifier (SUMO) modification of natively unfolded proteins tau and alpha-synuclein, *J. Biol. Chem.* 281 (2006) 9919-9924.

- [13] S. Jena, W.P. Lee, D. Doherty et al. PIAS4 represses vitamin D receptor-mediated signaling and acts as an E3-SUMO ligase towards vitamin D receptor, *J. Steroid Biochem. Mol. Biol.* 132 (2012) 24-31.
- [14] D. J. Mangelsdorf, K. Umesono, S. A. Kliewer et al. A direct repeat in the cellular retinol-binding protein type II gene confers differential regulation by RXR and RAR, *Cell.* 66 (1991) 555-561
- [15] M. Boudjelal, J.J. Voorhees, G.J. Fisher, Retinoid signaling is attenuated by proteasome-mediated degradation of retinoid receptors in human keratinocyte HaCaT cells, *Exp. Cell. Res.* 274 (2002) 130-137
- [16] D.L. Osburn, G. Shao, H.M. Seidel, I.G. Schulman, Ligand-dependent degradation of retinoid X receptors does not require transcriptional activity or coactivator interactions, *Mol. Cell. Biol.* 21 (2001) 4909-4918
- [17] J.R. Gareau, C.D. Lima, The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition, *Nature Rev. Mol. Cell. Biol.* 11 (2010) 861-871.
- [18] Z. Deng, M. Wan, G.Sui, PIASy-mediated sumoylation of Yin Yang 1 depends on their interaction but not the RING finger, *Mol. Cell. Biol.* 27(2007) 3780-3792.

## Legends

### Figure 1

The unliganded form of hRXR $\alpha$  can be covalently modified with SUMO1, 2 and 3. **A.** HEK293 cells were transfected with expression constructs for V5-hRXR $\alpha$  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  $\pm$  9-*cis* RA, lysates from each treatment group were processed as detailed in Materials and Methods and resulting immunoprecipitated 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-RXR $\alpha$ . **B.** SUMOylation of hRXR $\alpha$  is dependent upon an enzymatically intact Ubc9. Cell-based assay was performed to compare the hRXR $\alpha$  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-hRXR $\alpha$  is increased using the native (untagged) form of Ubc9.** Cell-based assays compared levels of SUMO2-hRXR $\alpha$  detected using the V5-tagged (left lane) and native (right lane) forms of Ubc9. The arrows highlight the V5-Ubc9 (left lane only) and hRXR $\alpha$  detected through western blot analysis of precipitated lysates with enhanced levels of SUMO2-hRXR $\alpha$  noted when untagged Ubc9 was employed.

Figure 2

**PIAS4 facilitates SUMOylation of hRXR $\alpha$ .** **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-hRXR $\alpha$  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-hRXR $\alpha$  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-hRXR $\alpha$ , GFP-PIAS4, His-SUMO-2 and Ubc9. The upper panel denotes western blot analysis of immune-precipitated material processed as described above highlighting V5-hRXR $\alpha$  and its SUMO-modified form. The lower panels confirm the expression status of GFP-PIAS4, V5-hRXR $\alpha$  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 hRXR $\alpha$  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 hRXR $\alpha$

with SUMO2. HEK293 cells were transfected with the indicated combinations of expression constructs encoding V5-hRXR $\alpha$ , 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-hRXR $\alpha$ .

### Figure 3

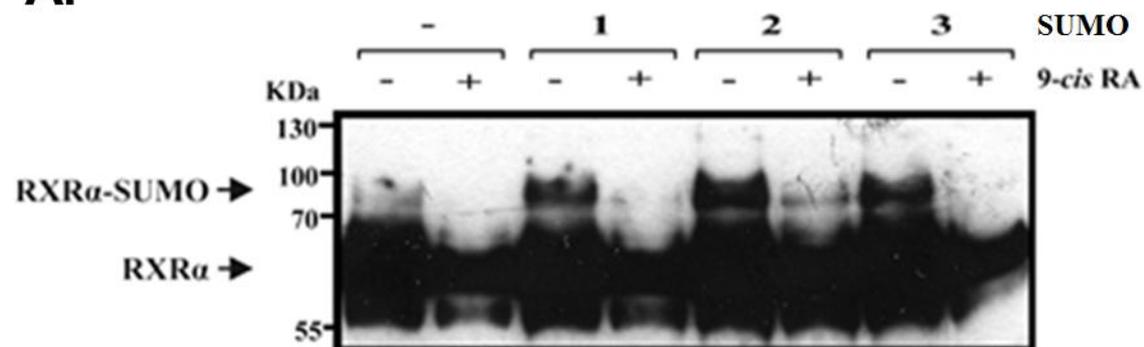
Evidence for a second SUMO acceptor site within hRXR $\alpha$ . **A.** HEK293 cell-based SUMOylation assays were performed as previously described to compare modification profile exhibited by V5-hRXR $\alpha$  and its K108R variant that harbours a mutation of a previously identified SUMO1 acceptor site. 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-hRXR $\alpha$  in combination with the indicated construct for SUMO1, 2, 3 or empty vector control. **B.** Confirmation that second acceptor site within RXR $\alpha$  is specific for SUMO2. HEK293 cells received expression constructs for V5-K108R RXR $\alpha$  mutant and the indicated SUMO isoform and incubated  $\pm$  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 RXR $\alpha$  K108R protein. **C.** Identification of K245 as a SUMO2 acceptor site within hRXR $\alpha$ . Cell-based SUMOylation assays were performed to compare patterns of modification with SUMO2 exhibited by wild type V5-RXR $\alpha$  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-RXR $\alpha$  and His-SUMO2 within lysates representing each treatment group.

Figure 4.

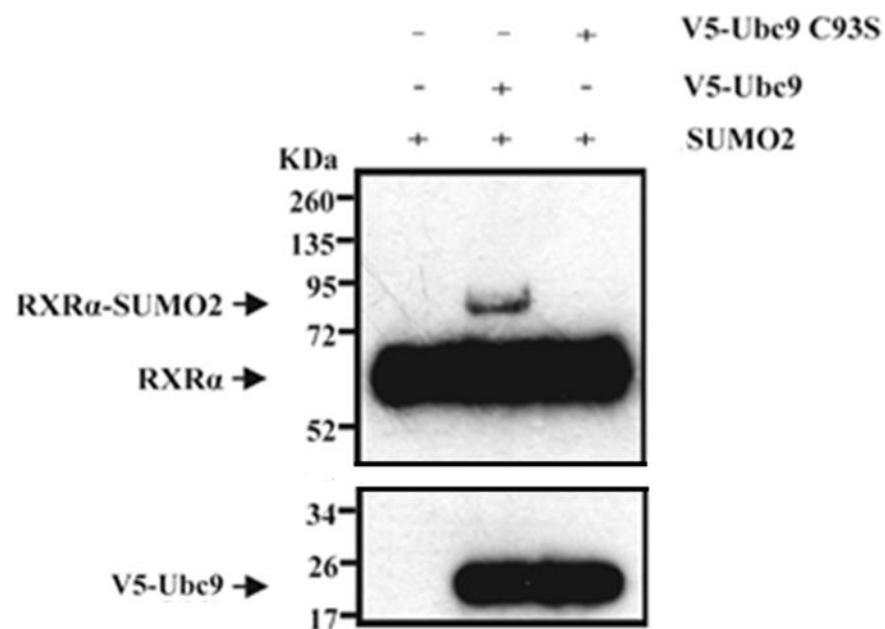
Loss of the K108 and K245 SUMOylation sites within hRXR $\alpha$  increases its transactivation by ligand. HEK293 cells received: **A.** the pMCS-RXRE luciferase based reporter construct in combination with the appropriate expression constructs for V5-hRXR $\alpha$  or its K108R, K245R or K108R/K245R variants; **B.** the pFLUC reporter vector together with pCMVBD-based expression vector encoding RXR $\alpha$  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 ( $\pm$  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.**



**B.**



**C.**

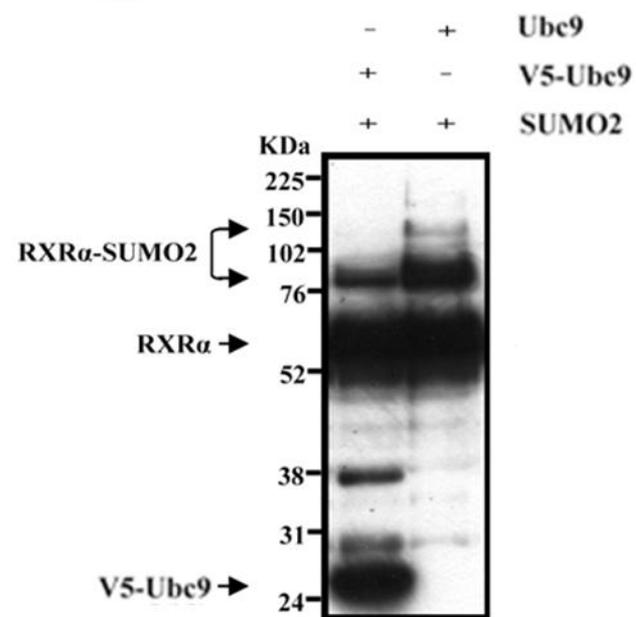
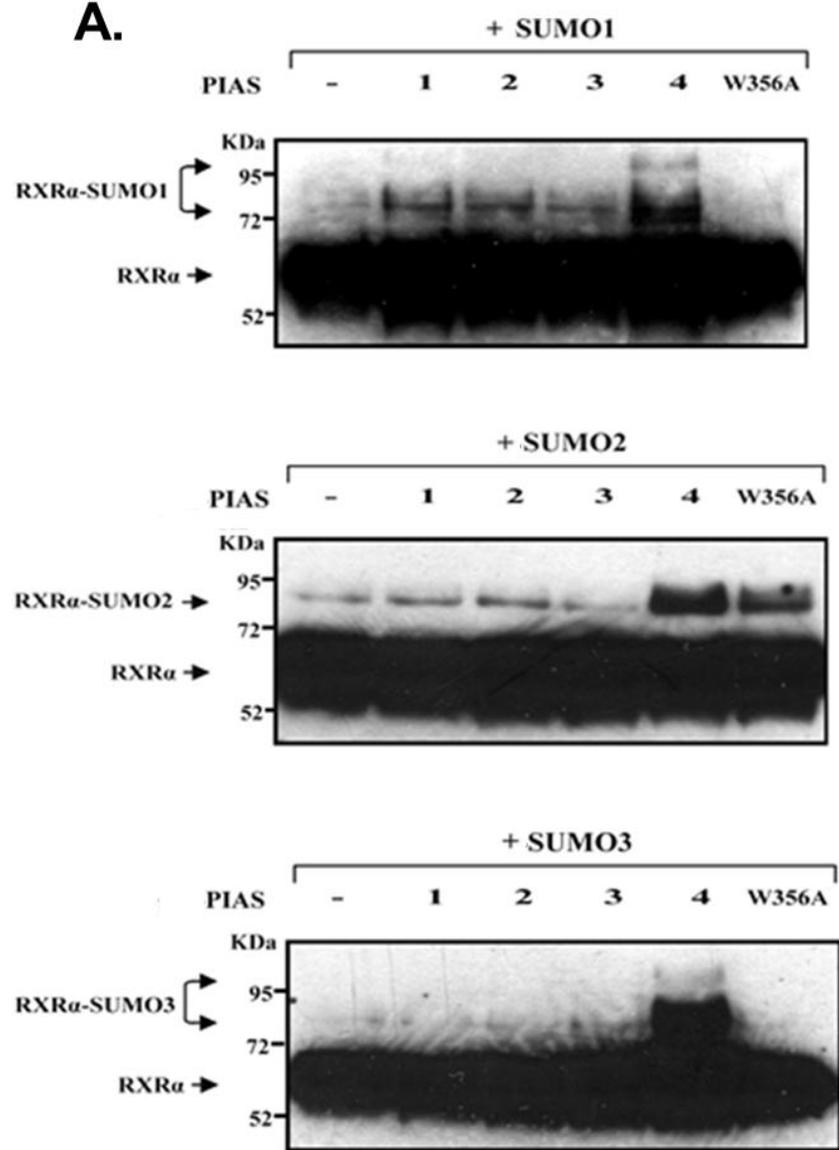
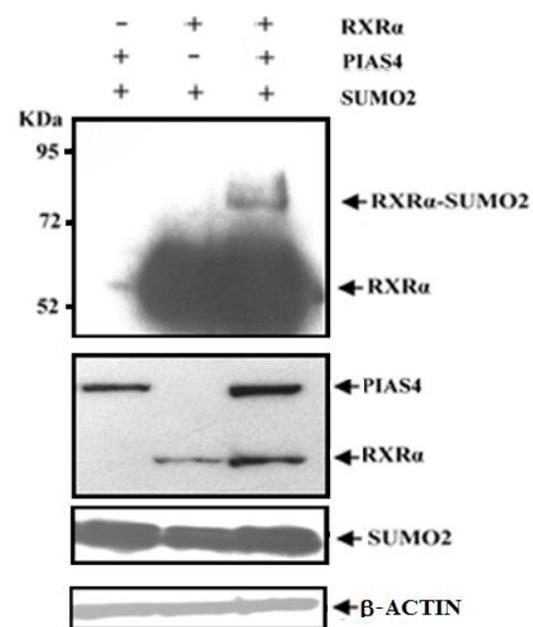


Figure 2

**A.**



**B.**



**C.**

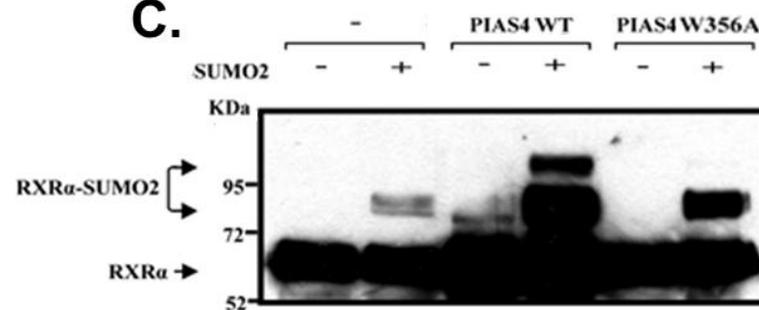
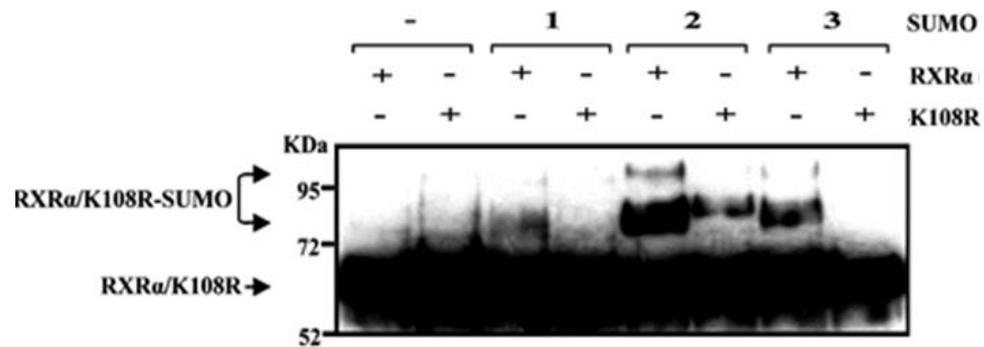
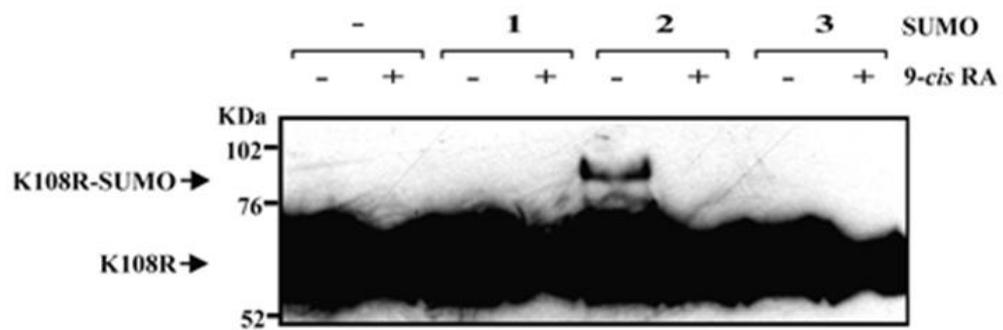


Figure 3

**A.**



**B.**



**C.**

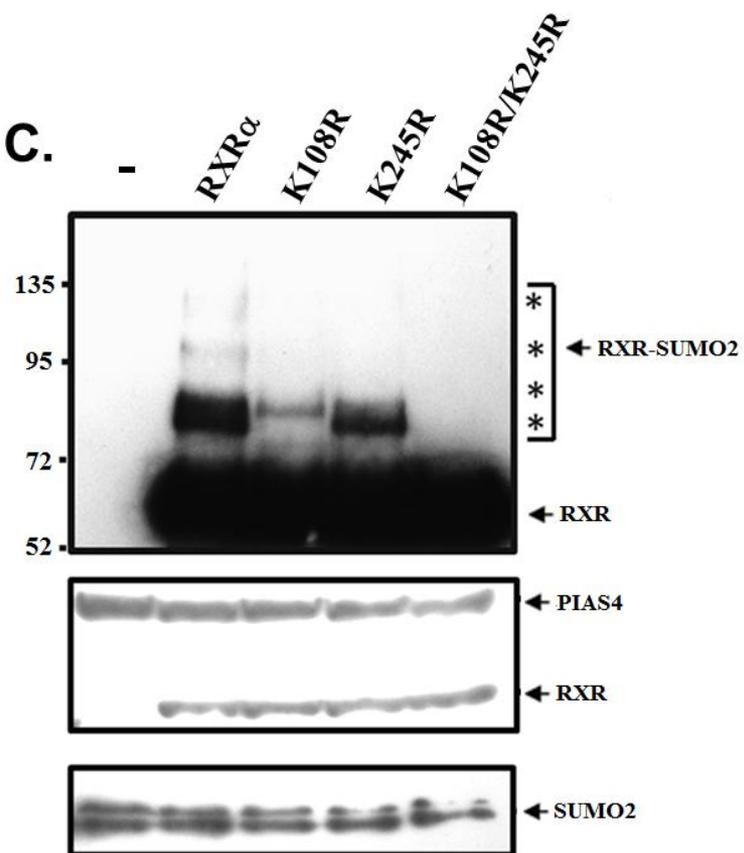
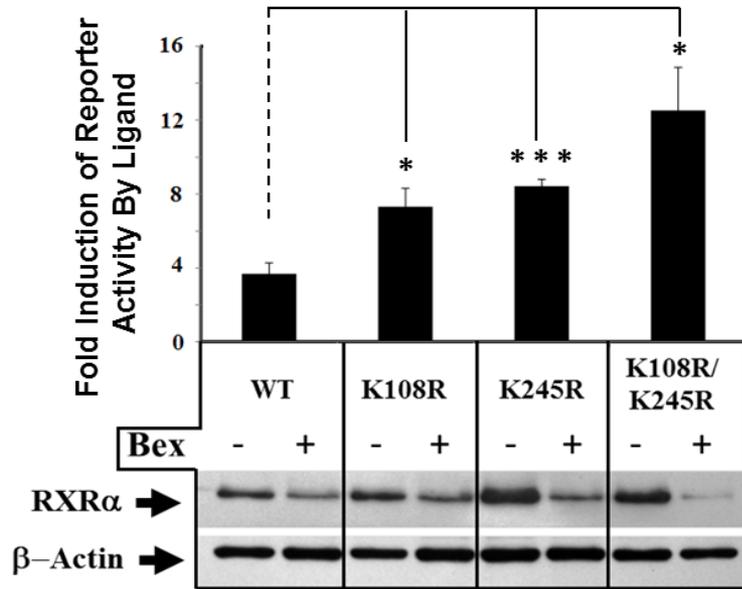
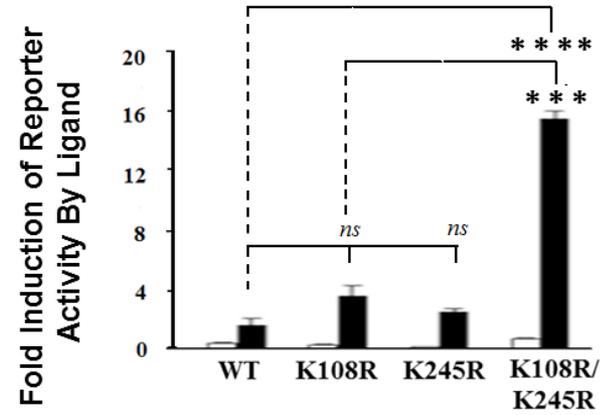


Figure 4

**A.**



**B.**



**\*Conflict of Interest**

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