

# Sumoylation of bZIP Transcription Factor NRL Modulates Target Gene Expression during Photoreceptor Differentiation\*

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Development of rod photoreceptors in the mammalian retina is critically dependent on the basic motif-leucine zipper transcription factor NRL (neural retina leucine zipper). In the absence of NRL, photoreceptor precursors in mouse retina produce only cones that primarily express S-opsin. Conversely, ectopic expression of NRL in post-mitotic precursors leads to a rod-only retina. To explore the role of signaling molecules in modulating NRL function, we identified putative sites of post-translational modification in the NRL protein by *in silico* analysis. Here, we demonstrate the sumoylation of NRL *in vivo* and *in vitro*, with two small ubiquitin-like modifier (SUMO) molecules attached to the Lys-20 residue. NRL-K20R and NRL-K20R/K24R sumoylation mutants show reduced transcriptional activation of *Nr2e3* and rhodopsin promoters (two direct targets of NRL) in reporter assays when compared with wild-type NRL. Consistent with this, *in vivo* electroporation of the NRL-K20R/K24R mutant into newborn *Nrl*<sup>-/-</sup> mouse retina leads to reduced *Nr2e3* activation and only a partial rescue of the *Nrl*<sup>-/-</sup> phenotype in contrast to the wild-type NRL that is able to convert cones to rod photoreceptors. Although PIAS3 (protein inhibitor of activated STAT3), an E3-SUMO ligase implicated in photoreceptor differentiation, can be immunoprecipitated with NRL, there appears to be redundancy in E3 ligases, and PIAS3 does not seem to be essential for NRL sumoylation. Our studies suggest an important role of sumoylation in fine-tuning the activity of NRL and thereby incorporating yet another layer of control in gene regulatory networks involved in photoreceptor development and homeostasis.

Spatiotemporal control of gene expression is critical for development and homeostasis (1). Cell type-specific expression patterns are established and maintained by transient or stable interactions between *cis*-regulatory elements in the target genes and *trans*-regulatory factors that together constitute gene regulatory networks (2). Signaling molecules, another key component of gene regulatory networks, can modify the activity of transcription factors by post-translational modifications (PTMs)<sup>3</sup> such

as phosphorylation, acetylation, ubiquitination, and sumoylation (3–6). Rapid and reversible modulation of the activity of transcription factors by PTMs is essential for adaptation to continuously changing cellular microenvironment(s) and is accomplished by altering protein stability, subcellular localization, and protein-DNA and/or protein-protein interaction (3, 6–8). Consequently, PTMs provide a higher level of control and complexity to gene regulation in a particular biological context.

The vertebrate retina exhibits a highly organized laminar structure that captures, integrates, and transmits visual signals to other parts of the central nervous system for further processing. Six neuronal cell types and Muller glia in the retina originate from pools of multipotent progenitor cells in a conserved sequential order (9, 10). The determination of specific cell fate and subsequent differentiation is dictated primarily by intrinsic control mechanisms; however, extrinsic signals modulate key steps in the developmental pathway (9–13). Rod and cone photoreceptors have a unique and specialized function and initiate the phototransduction process by converting photons into electrical signal (14). Differentiation and homeostasis of photoreceptors are tightly controlled by a set of key transcriptional regulatory proteins, which include nuclear receptors (such as ROR $\beta$  (15), thyroid hormone receptor  $\beta$ 2 (TR $\beta$ 2) (16), and NR2E3 (17–21)), homeodomain proteins (such as orthodenticle homeobox 2 (OTX2) (22) and CRX (cone-rod homeobox) (23, 24), signal transducers (including STAT3 (25), PIAS3 (26), glycogen synthase kinase 3 (GSK3) (27)), and NRL, a basic motif-leucine zipper (bZIP) protein of Maf subfamily (28).

The bZIP transcription factor NRL is a key regulator of rod *versus* cone photoreceptor cell fate in mammalian retina (28, 29). Targeted deletion of *Nrl* in mice leads to a retina with only cones that primarily express S-opsin (28), whereas ectopic expression of NRL in photoreceptor precursors leads to a rod-only retina (29). NRL expression is detected soon after the final mitosis and drives a photoreceptor precursor toward rod cell fate (30). NRL interacts with a number of transcription factors (including CRX, NR2E3, and SP4) and activates the expression of many rod-specific genes (23, 31–34). NRL is also the major regulator of NR2E3, an orphan nuclear receptor, and together these two proteins repress cone gene expression (19–21, 35).

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<sup>3</sup> The abbreviations used are: PTM, post-translational modification; NRL, neural retina leucine zipper; SUMO, small ubiquitin-like modifier; RAR, retinoic

acid receptor; MAF, musculoaponeurotic fibrosarcoma; ROR $\beta$ , RAR-related orphan receptor  $\beta$ ; NR2E3, nuclear receptor subfamily 2, group E; CRX, cone-rod homeobox; STAT, signal transducer and activator of transcription; PIAS, protein inhibitor of activated STAT; WT, wild type; Ub, ubiquitin; GFP, green fluorescent protein; GST, glutathione S-transferase; P, postnatal day.

## Regulation of NRL Function by Sumoylation

Loss of *Nr2e3* in mice results in rod photoreceptors that express cone genes and eventually degenerate (17, 18, 36, 37). CRX is another important modulator of photoreceptor maturation (38). Rods and cones do not fully differentiate in the *Crx* knock-out retina and lack outer segments (24). ROR $\beta$  and OTX2 control photoreceptor differentiation as well but act upstream of NRL and CRX in the transcriptional regulatory hierarchy (15, 22).

Differentiation of rod photoreceptors proceeds in a stepwise manner during the development of mammalian retina. In rodents, although some rods are born as early as embryonic day 12, a majority of rods are generated postnatally (9, 30, 39, 40). Interestingly, the expression of rod-specific visual pigment protein, rhodopsin, reveals a substantial “delay” with two distinct phases (41) despite the presence of key activator proteins, NRL, CRX, and NR2E3 (40). One can hypothesize that additional signals/factors are needed to modify the activity of one or more of these regulators and/or to stabilize the assembly of “enhanceosome” complex (42) before the transcription of photoreceptor-specific genes can be initiated. Recruitment of histone acetyl transferases by CRX is implicated in rod gene transcription (43). Recently, PIAS3, an E3-SUMO ligase, has been shown to interact with CRX and NR2E3 and play a significant role in rod differentiation by sumoylating the NR2E3 protein (26). Chromatin remodeling and post-translational modifications can therefore contribute to photoreceptor development by modulating cell type-specific transcription.

To gain insights into the role of extrinsic signaling molecules in guiding retinal development and homeostasis, we are exploring the impact of post-translational modifications on the transcriptional regulatory function of NRL. We have previously reported multiple phosphorylated isoforms of NRL (44) and demonstrated that a number of human retinopathy mutations in NRL alter its phosphorylation state and activity *in vitro* (45–47). Here, we show that the NRL protein is disumoylated *in vivo* and *in vitro* and that sumoylation of NRL modifies its activity toward two distinct target promoters, *Nr2e3* and rhodopsin. Although PIAS3 is part of a multiprotein complex with NRL, it does not appear to be the primary mediator of NRL sumoylation. Our studies further strengthen the growing role of post-translational mechanisms in influencing photoreceptor development and function.

### EXPERIMENTAL PROCEDURES

**Mice**—*Nrl*<sup>-/-</sup> mice on C57Bl/6J background were used for *in vivo* electroporation experiments. All animal studies followed approved institutional protocols.

**Antibodies**—The following antibodies were used: anti-NRL polyclonal antibody (44); rhodopsin monoclonal antibody, Rh4D2 (Dr. R. Molday, University of British Columbia, Vancouver, British Columbia, Canada); anti-PIAS3 and anti-FLAG monoclonal antibodies (Sigma); anti-SUMO1 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA); anti-cone arrestin polyclonal antibody (Chemicon, Billerica, MA); anti-rabbit and anti-mouse light chain specific horseradish peroxidase-conjugated anti-IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA); and goat-anti-rabbit and anti-mouse antibodies conjugated with Alexa Fluor 488, 568, and 633 (Molecular Probes, Invitrogen).

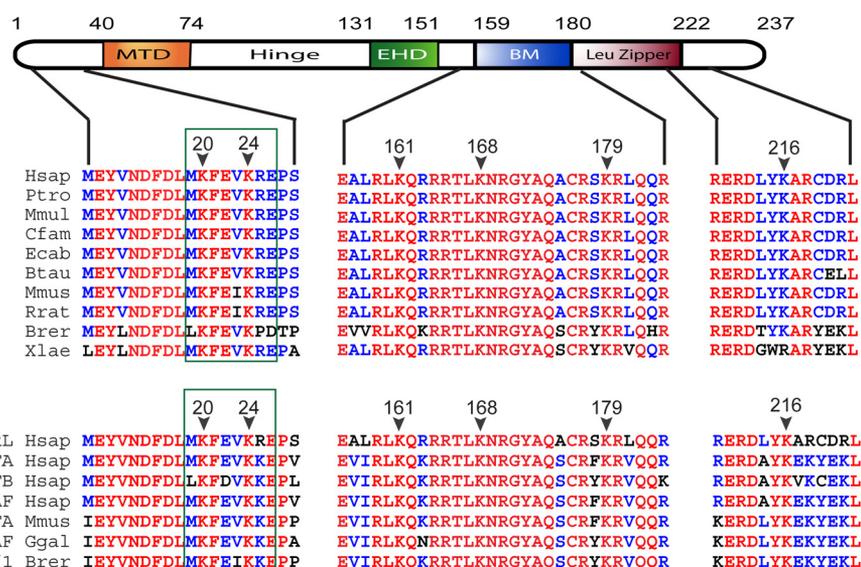
**Plasmid Construction and Mutagenesis**—Human wild-type (WT) NRL cDNA (714 nucleotides) was subcloned into pcDNA4c His/Max C vector (Invitrogen) (46), and mutants (NRL-K20R, K24R, K161R, K168R, K179R, K216R, and K20R/K24R) were generated using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). For *in vivo* electroporation, WT-NRL and NRL-K20R/K24R mutant were subcloned at EcoRI-NotI sites of the Ub-GFP vector after removing GFP (53). The ubiquitin (Ub) promoter used in this vector is transcriptionally active in all retinal cell types. The WT and mutant NRL proteins were expressed in *Escherichia coli* using pGex4T vector (GE Healthcare), containing an N-terminal glutathione *S*-transferase (GST) fusion tag under the control of the “tac” promoter. PIAS3 cDNA was subcloned into EcoRI-NotI sites of the pcDNA3.1/V5-His C vector (Invitrogen). pTag-FLAG-SUMO was a generous gift from Dr. Shiming Chen (Washington University, St. Louis, MO).

**Recombinant GST-NRL and *in Vitro* Sumoylation Assay**—For *in vitro* sumoylation assays, GST-NRL protein was expressed in bacteria using standard protocols and eluted from a GSTrap FF Sepharose column (GE Healthcare) with 50 mM Tris-HCl, pH 8.0, containing 20 mM reduced glutathione. This protocol yielded ~95% pure protein as assessed by SDS-PAGE analysis. Purified GST-WT-NRL or NRL mutants (0.5  $\mu$ g) were incubated at 30 °C for 3 h with E1 activating enzyme, E2 conjugating enzyme, and SUMO protein using the SUMOlink kit (Active Motif, Carlsbad, CA). Sumoylated proteins were assayed by SDS-PAGE and immunoblotting.

**Cell Culture and Transfection**—HEK-293 and HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 100 units/ml penicillin G, and 100  $\mu$ g/ml streptomycin. Cells at 80% confluence were transiently transfected with FuGENE 6 (Roche Applied Science).

**Dual-Luciferase Assay**—HEK-293 cells were seeded in 24-well plates (4  $\times$  10<sup>4</sup>/well) and co-transfected with 0.1  $\mu$ g of bovine rhodopsin promoter driving firefly luciferase (pBR130-luc (46)), 0.1  $\mu$ g of pcDNA4-CRX (46) and/or pcDNA4c-NR2E3 (46), 0.01–0.3  $\mu$ g of WT-NRL or NRL mutants, and 0.001  $\mu$ g of *Renilla* reporter pRL-TK (Promega, Madison, WI). Empty pcDNA4c was used to adjust the total amount of transfected DNA. Cells were harvested 48 h after transfections and lysed in 100  $\mu$ l of passive lysis buffer (Promega). Firefly and *Renilla* luciferase activities were determined using the Dual-Luciferase reporter assay system (Promega) and measured with the modulus microplate luminometer (Turner BioSystems, Sunnyvale, CA). *Renilla* luciferase activity was used as an internal control for transfection efficiency. All experiments were repeated three times. An analysis of variance test was performed for statistical analysis, and *p* value of < 0.05 was considered significant.

**Immunoprecipitation and Immunoblotting**—Transfected HEK293T cells were harvested after 48 h and lysed by sonication in radioimmunoprecipitation buffer supplemented with 20 mM *N*-ethylmaleimide (Sigma) and protease inhibitor (Roche Applied Science). Supernatants were used either for immunoprecipitation or for immunoblot analysis. For immunoprecipitation, lysates were incubated with anti-NRL anti-



**FIGURE 1. A schematic of the human NRL protein with the sequence alignment of NRL orthologs (upper panel) and MAF family proteins (lower panel).** Alignments were performed using AlignX from VectorNTI (Invitrogen). Amino acids conserved in all orthologs and across MAF family members are indicated in red, and less conserved residues are shown in blue. Arrowheads indicate lysine residues with their position in the human NRL protein. Sumoylation sites predicted with high probability are framed by a green rectangle. MTD, minimal transactivation domain; Hinge, hinge domain; EHD, extended homology domain; BM, basic motif; Leu Zipper, leucine zipper; Hsap, *Homo sapiens* (human); Ptro, *Pan troglodyte* (chimpanzee); Mmul, *Macaca mulatta* (rhesus monkey); Cfam, *Canis familiaris* (dog); Ecab, *Equus caballus* (horse); Btau, *Bos taurus* (cow); Mmus, *Mus musculus* (mouse); Rrat, *Rattus norvegicus* (rat); Brer, *Brachydanio rerio* (zebrafish); Xlae, *Xenopus laevis* (*Xenopus*); Ggal, *Gallus gallus* (chicken).

body for 6 h at 4 °C, and immunoprecipitate was collected on protein A-Sepharose beads for 1 h (GE Healthcare). Beads were washed in lysis buffer and boiled in 2× SDS-PAGE loading buffer (Invitrogen). Proteins were resolved by SDS-PAGE under reducing conditions and transferred to nitrocellulose membrane (Invitrogen). After 1 h of blocking with 5% skim milk in phosphate-buffered saline, 0.1% Tween 20 (PBT), the membrane was incubated overnight at 4 °C with antibody in PBT, 5% skim milk. After three washes in PBT, the membrane was then incubated with secondary antibody coupled to horseradish peroxidase for 1 h in PBT, 5% skim milk. After three washes in PBT, proteins were visualized by enhanced chemiluminescence plus (Thermo Scientific).

**In Vivo Electroporation**—Retina of P0 *Nrl*<sup>-/-</sup> pups was electroporated *in vivo* as described (53). Briefly, an equal amount of Ub-WT-NRL or Ub-NRL-K20R/K24R plasmid was mixed with Ub-GFP, and 0.2 μl (concentration, 1 μg/μl) was injected sub-retinally. Square electric pulses (80 V, 1 Hz, five pulses) were applied across the heads of pups with an ECM830 square wave electroporator using 10-mm diameter BTX Tweezertrode electrodes (Holliston, MA). Eyeballs were harvested at P21 for analysis.

**Immunohistochemistry**—Cryosections were probed with specific antibodies as described (66) and visualized using an Olympus FluoView FV1000 confocal laser scanning unit and Olympus BX61WI upright microscope (Olympus America Inc., Center Valley, PA).

## RESULTS

**Putative Sumoylation Sites Are Highly Conserved in Maf Subfamily Proteins**—SUMO (small ubiquitin modifier) is an 8-kDa protein that can be linked covalently to target proteins, usually

at a lysine residue within the consensus sequence  $\psi$ KX(E/D) ( $\Psi$  = hydrophobic residue) (48, 49). The human NRL protein contains six lysine residues that are evolutionarily conserved in other vertebrates and in Maf proteins (Fig. 1). SUMOplot analysis predicts two high probability sumoylation sites in NRL at Lys-20 and Lys-24 ( $p > 0.80$  and  $p > 0.93$ , respectively), close to the minimal transactivation domain (50).

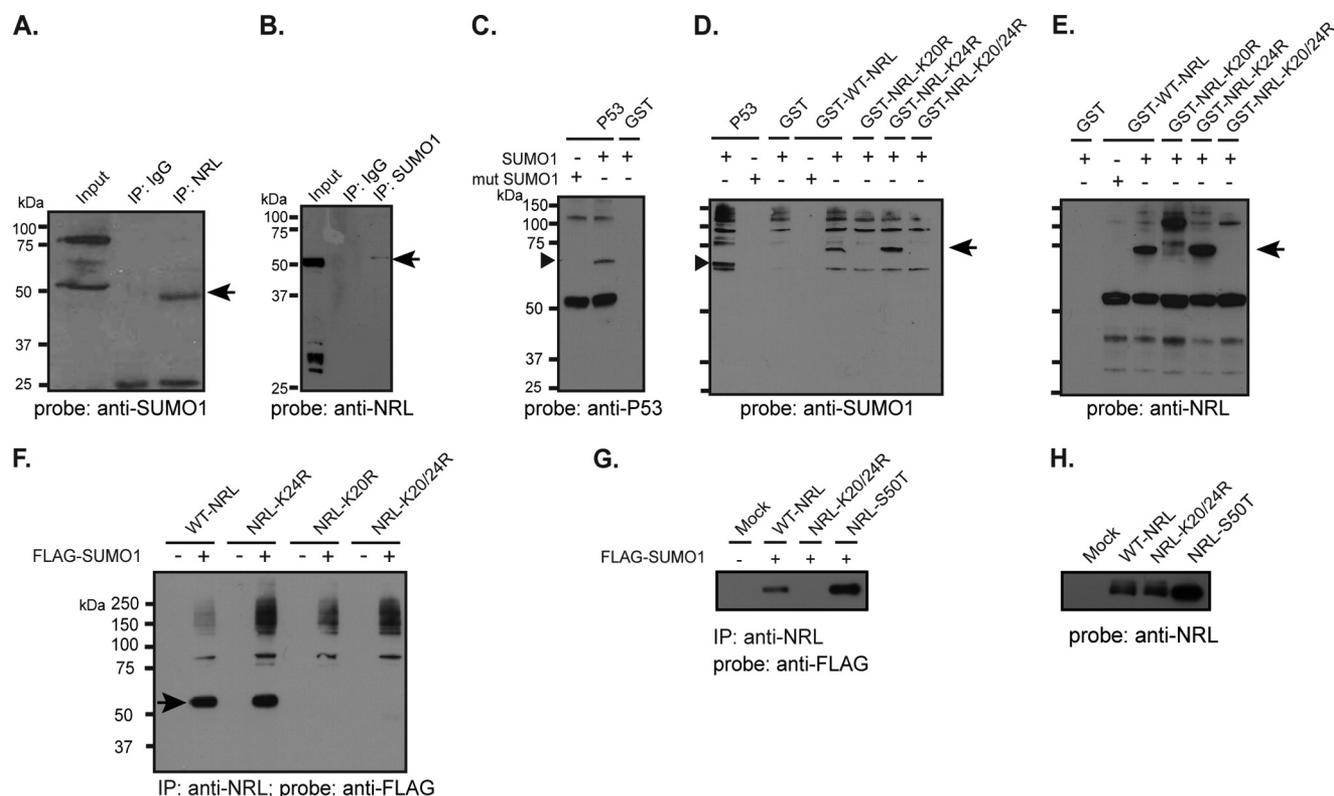
**NRL Is Disumoylated at Lys-20 Residue**—To examine NRL sumoylation *in vivo*, we performed immunoprecipitation from adult mouse retinal extract using an anti-NRL antibody (44) followed by immunoblot analysis with anti-SUMO1 antibody. Detection of a single protein of ~50 kDa (instead of phosphorylated NRL isoforms between 29 and 35 kDa (44)) is indicative of at least one sumoylated NRL isoform presumably with two linked SUMO1 molecules in the mature

retina (Fig. 2A). The reverse immunoprecipitation experiment (immunoprecipitation of all sumoylated proteins from adult retina with anti-SUMO1 antibody followed by immunoblotting with NRL antibody) further confirmed the disumoylation of NRL (Fig. 2B).

To validate and identify sumoylation sites in NRL, we performed *in vitro* sumoylation assays using *E. coli*-expressed GST-tagged WT and mutant NRL proteins. In this assay, p53 is used as a positive control and shows an expected molecular mass of 65 kDa (Fig. 2, C and D). WT-NRL and NRL-K24R proteins are sumoylated with SUMO1, E1, and E2 ligase; however, no sumoylation is detected with NRL-K20R and NRL-K20R/K24R mutants (Fig. 2, D and E). Consistent with *in vivo* data (Fig. 2, A and B), we observe a sumoylated GST-tagged WT-NRL protein of 70 kDa (Fig. 2D), indicating the addition of two SUMO1 proteins (note that the non-sumoylated GST-NRL is 52 kDa). Other observed bands correspond to SUMO1-conjugated E1 and E2 enzyme and are not detected in the samples with mutant SUMO1. Mutations in other lysine residues of NRL (K161R, K168R, K179R, and K216R) do not affect sumoylation in our assay conditions (data not shown). These results (Fig. 2, A–E) provide strong evidence for the presence of at least one Lys-20 disumoylated isoform of NRL *in vivo*.

To further establish the sumoylation of NRL, we co-expressed WT and mutant (K20R, K24R, and K20R/K24R) NRL proteins in HEK293T cells with a FLAG-tagged SUMO1 construct (Fig. 2F). Immunoprecipitation of transfected cell extracts with anti-NRL antibody followed by immunoblot analysis with anti-FLAG antibody reveals WT and NRL-K24R proteins of 55 kDa that correspond to the addition of two FLAG-SUMO1 molecules (Fig. 2F). NRL-K20R and NRL-K20R/K24R mutants do not show any sumoylation, further demonstrating

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**FIGURE 2. Sumoylation of NRL *in vivo* and *in vitro*.** *A* and *B*, adult mouse retina extracts were immunoprecipitated with anti-NRL IgG (*A*) or anti-SUMO1 (*B*) followed by immunoblotting with anti-SUMO1 antibody or anti-NRL IgG, respectively. The *arrow* indicates sumoylated NRL. *IP*, immunoprecipitation. *C*, p53 and GST control experiments in the presence of WT or mutant SUMO1. p53, used as a positive control, was conjugated with SUMO1 and not by mutant SUMO1 under our assay conditions. *D* and *E*, purified GST-tagged WT or mutant NRL proteins were sumoylated *in vitro* with E1 and E2 ligases in the presence of SUMO1 or mutated SUMO1 protein. *D* shows the immunoblot probed with anti-SUMO1, and *E* shows the immunoblot probed with anti-NRL antibodies. *Arrowheads* show sumoylated p53 control, and *arrows* indicate sumoylated NRL. *F*, HEK293T cells were co-transfected with plasmids expressing WT-NRL or lysine mutants with or without FLAG-SUMO1. Cell extracts were immunoprecipitated with anti-NRL IgG and immunoblotted with anti-FLAG antibody. The *arrowhead* shows sumoylated NRL. *G*, HEK293T cells were transfected with plasmids expressing WT-NRL, NRL-S50T, and NRL-K20R/K24R. After immunoprecipitation of cell extracts with anti-NRL IgG, immunoblot was probed with anti-FLAG antibody. *H*, HEK293T cells were co-transfected with plasmids expressing WT-NRL, NRL-K20R/K24R, and NRL-S50T with FLAG-SUMO1. Immunoblots of cell extracts were probed with anti-NRL IgG.

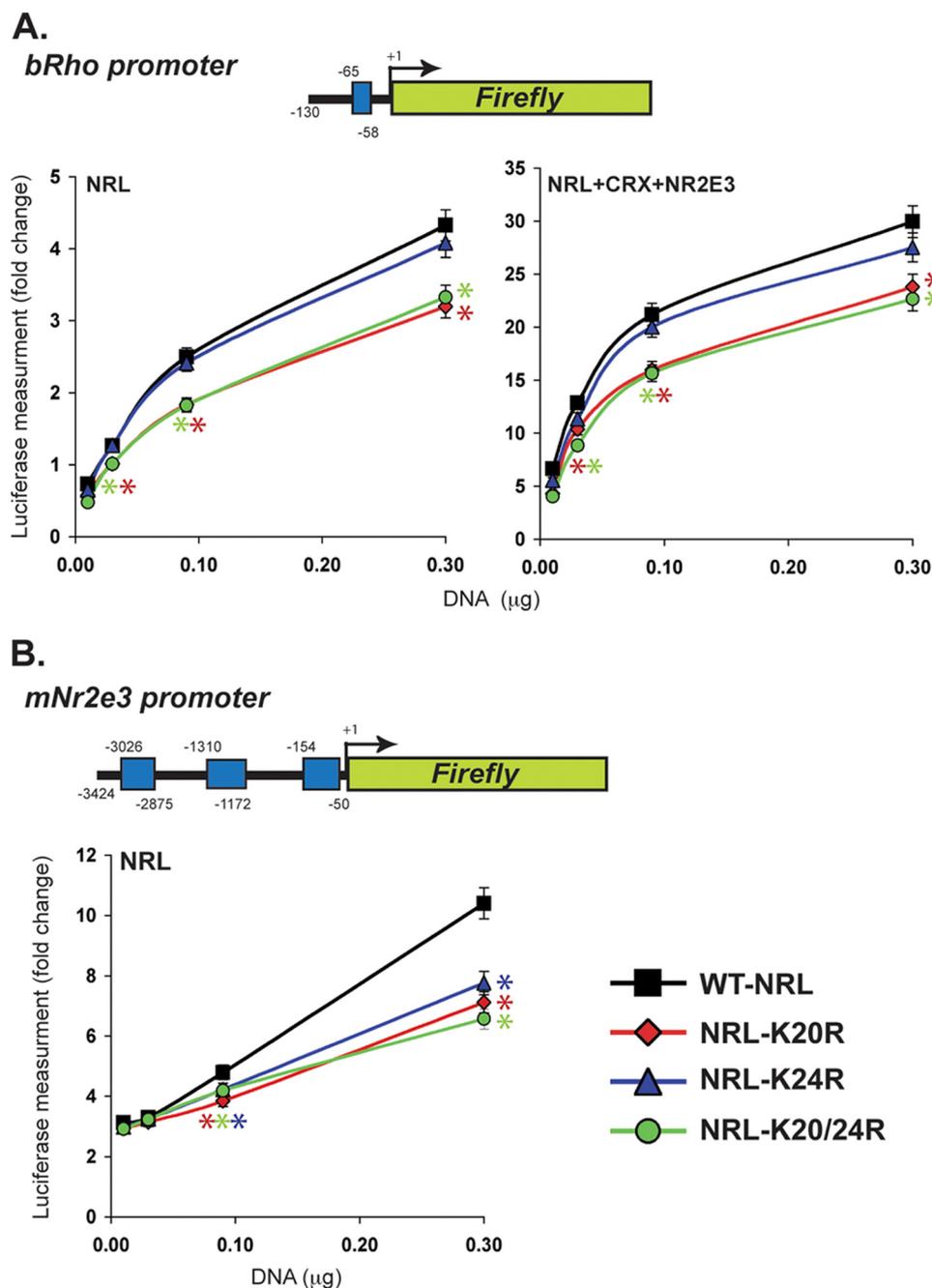
that WT-NRL is disumoylated at the Lys-20 residue. Immunocytochemical analysis of transfected HEK293T cells reveals that lysine mutations do not alter the nuclear localization of NRL (data not shown).

Because sumoylation can depend on the phosphorylation state of the target protein (51, 52), we examined the consequence of the S50T mutation, which affects NRL phosphorylation (45, 46). Sumoylation is not altered in the NRL-S50T mutant (Fig. 2*G*). Furthermore, K20R/K24R mutation in NRL (used as a control in this assay) does not affect the phosphorylation state (Fig. 2*H*).

**Sumoylation Modulates the Transcriptional Activity of NRL on *Rho* and *Nr2e3* Promoters**—We then investigated the effect of K20R, K24R, and K20R/K24R mutations on transcriptional activation of two known photoreceptor-specific target promoters of NRL—rhodopsin (*Rho*) (32) and *Nr2e3* (35). We performed Dual-Luciferase promoter activity assays to compare the transactivation ability of the WT and mutant NRL (either alone or with CRX and NR2E3 (18, 23, 35)) (Fig. 3). The NRL-K20R and NRL-K20R/K24R mutants, but not the NRL-K24R mutant, show statistically significant reduction of the *Rho* promoter activation when compared with WT-NRL (Fig. 3*A*). Correspondingly, similar and significant decrease in the induction of *Rho* promoter is observed when the NRL-K20R or NRL-K20R/

K24R mutant is co-expressed with CRX and NR2E3 (Fig. 3*A*). Interestingly, all three NRL mutants (K20R, K24R, and K20R/K24R) exhibit significantly lesser transactivation of the *Nr2e3* promoter when compared with WT-NRL (Fig. 3*B*). These data suggest that sumoylation fine-tunes the activity of NRL to activate promoters of specific target genes that contribute to rod development and function.

**NRL Sumoylation Is Required for Normal Rod Differentiation**—As expression of NRL can rescue the *Nrl*<sup>-/-</sup> phenotype in transgenic mice (29), we adopted an *in vivo* electroporation (53) assay to investigate the role of NRL sumoylation in the context of photoreceptor development. We performed *in vivo* transfection of newborn *Nrl*<sup>-/-</sup> mouse retina by electroporation using WT-NRL or NRL-K20R/K24R mutant construct and assessed photoreceptor development 3 weeks later (Fig. 4). Transfected retinal cells were monitored by co-injecting ubiquitin-GFP construct, and the untransfected portion of the retina was used as control (Fig. 4*A*). Following electroporation of WT-NRL at P0, rhodopsin expression is observed at P21 only in transfected GFP-positive cells but not in the untransfected region of the *Nrl*<sup>-/-</sup> mouse retina (Fig. 4, *A* and *B*). Consistent with the established role of NRL, transfected cells expressing rhodopsin did not express cone arrestin, a cone-specific marker that is highly expressed in *Nrl*<sup>-/-</sup> retina. Electroporation of the NRL-



**FIGURE 3. Modulation of transcriptional regulatory activity of NRL by sumoylation.** HEK293 cells were co-transfected with a construct containing bovine *Rho* (A) or mouse *Nr2e3* promoter (B) driving firefly luciferase reporter gene simultaneously with increasing concentrations (0.01–0.3  $\mu\text{g}$ ) of WT- or mutant NRL expression constructs, either alone or in association with CRX and NR2E3. -Fold change is relative to the mock expression vector control. The blue box indicates NRL-responsive element (NRE). Error bars show S.E. Asterisks indicate  $p$  value  $< 0.05$ . Black lines and red, blue, and green lines and asterisks correspond to WT-NRL, NRL-K20R, NRL-K24R, and NRL-K20R/K24R, respectively.

K20R/K24R mutant, however, results in a clearly distinct phenotype (Fig. 4, C and D). Rhodopsin expression is observed in lesser numbers of NRL-K20R/K24R-transfected cells ( $58 \pm 7\%$ ) when compared with WT-NRL ( $85 \pm 3\%$ ), and cone arrestin expression is also detected in some of the rhodopsin-positive cells transfected with the NRL-K20R/K24R mutant. The phenotype of *Nrl*<sup>-/-</sup> cells electroporated with NRL-K20R/K24R resembled that of the hybrid photoreceptors expressing both rod and cone markers in the *rd7* mutant mouse (36), where

*Nr2e3* function is abolished (54). Cells in the inner nuclear layer transfected with WT or mutant NRL construct (as revealed by Ub-GFP expression) do not show the expression of rod-specific genes (such as rhodopsin) (Fig. 4, B and C).

As the NRL-K20R/K24R sumoylation mutant resulted in decreased activation of the *Nr2e3* promoter *in vitro* (Fig. 3B) and produced photoreceptors expressing both rod and cone genes (as in *rd7* mouse) in electroporation assays (Fig. 4C), we examined whether NRL sumoylation is required for appropriate *Nr2e3* expression *in vivo*. *Nr2e3*, a direct target of NRL (35) is not expressed in *Nrl*<sup>-/-</sup> mice (28), as illustrated in the untransfected region of the retina that is used as a control (GFP-negative cells) in electroporation experiments (Fig. 4E). When WT-NRL is expressed,  $89 \pm 3\%$  of the GFP-positive cells in the outer nuclear layer strongly express NR2E3; however, the NRL-K20R/K24R mutant results in only  $42 \pm 6\%$  cells that show weak NR2E3 immunoreactivity (Fig. 4, F–H). These data demonstrate that sumoylated NRL is a stronger transcriptional activator of *Nr2e3* promoter.

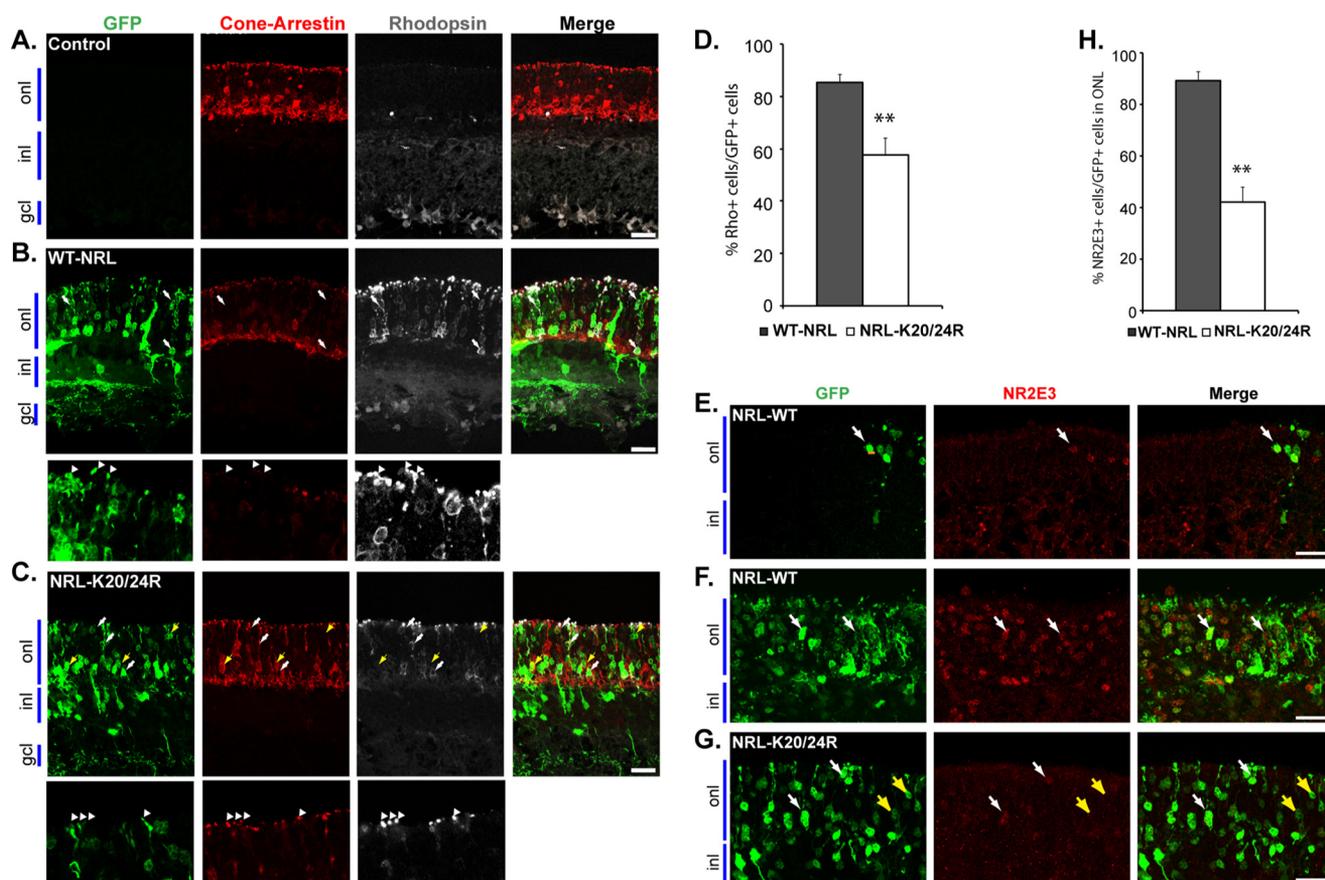
*Is PIAS3 Involved in NRL Sumoylation?*—As PIAS3 participates in sumoylation of NR2E3 (26), we investigated whether it can sumoylate NRL. We show that PIAS3 is indeed expressed in rod photoreceptors, isolated from the *Nrl*-GFP mouse retinas (30) that express GFP specifically in rods under the control of *Nrl* promoter (Fig. 5A). NRL and PIAS3 can be co-immunoprecipitated from P4, P10, and adult retinal extracts (Fig. 5B), suggesting their presence in a protein complex. However, although NRL is sumoylated in transfected HEK293T cells

(Fig. 2, C and D), PIAS3 is undetectable in these cells by immunoblot analysis (Fig. 5C). Furthermore, co-expression with PIAS3 does not alter NRL sumoylation pattern in transfected cells (Fig. 5D). It therefore appears that PIAS3 may not be the primary E3-SUMO ligase involved in NRL sumoylation.

## DISCUSSION

Selective covalent linkage of SUMO moieties can alter the function of conjugated proteins (5, 55, 56). Sumoylation of tran-

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**FIGURE 4. Partial rescue of the *Nrl*<sup>-/-</sup> phenotype and reduced expression of *Nr2e3* by NRL-K20R/K24R sumoylation mutant.** *A*, representative retinal photographs of P21 *Nrl*<sup>-/-</sup> mouse retinas: unelectroporated region. *onl*, outer nuclear layer; *inl*, inner nuclear layer; *gcl*, ganglion cell layer. *B*, *C*, *E*, *F*, and *G*, electroporated at P0 with Ub-GFP and either Ub-WT-NRL (*B*, *E*, and *F*) or Ub-NRL-K20R/K24R (*C* and *G*). *A*–*C*, GFP is green, cone arrestin is red, and rhodopsin is gray. White and yellow arrows show GFP+ electroporated cells with or without rhodopsin staining, respectively. Lower panels in *B* and *C* show higher magnification images with arrowheads indicating GFP- and rhodopsin-positive photoreceptors. Cone arrestin signal is observed in these cells only with NRL-K20R/K24R mutant. Scale bar: 20  $\mu$ m. *D*, quantification of GFP-positive cells expressing rhodopsin after electroporation of WT-NRL (gray) or NRL-K20R/K24R (white). Error bars show S.E. from seven independent electroporated retinas for each construct. \*\*,  $p < 0.01$  by Student's *t* test. *E*–*G*, GFP is green, and NR2E3 immunostaining is red. White and yellow arrows show GFP+ electroporated cells with or without NR2E3 staining, respectively. *E* and *F* show different regions of the retina electroporated with WT-NRL. Scale bar: 20  $\mu$ m. *H*, quantification of GFP-positive cells in ONL expressing NR2E3 after electroporation of WT-NRL (gray) or NRL-K20R/K24R (white). Error bars show S.E. from four independent electroporated retinas for each construct. \*\*,  $p < 0.01$  by Student's *t* test.

scription factors was originally associated with repression of gene expression (57), but like other reversible PTMs, SUMO modification can have diverse physiological consequences. Our studies demonstrate that at least one NRL protein isoform is disumoylated *in vivo* at Lys-20, and this modification has a positive impact on transcriptional activation of *Nr2e3* and rhodopsin expression. We propose that NRL sumoylation affects the assembly and/or stability of specific enhanceosome complexes that are needed for high level expression of critical rod-specific genes.

The transcriptional regulatory function of Maf subfamily proteins is modulated by PTMs, including phosphorylation and sumoylation (58–61). MAF-A sumoylation occurs at Lys-32, which corresponds to the non-sumoylated Lys-24 in NRL (Fig. 1). The sumoylation of MAF-A reduces its transcriptional activity particularly on the *Ins* gene without affecting its nuclear localization. Sumoylation of NRL, reported here, occurs on Lys-20, corresponding to Lys-28 in MAF-A, and does not affect its nuclear localization; however, unlike MAF-A, sumoylation of NRL positively impacts transcriptional activation of at least two downstream target genes. Notably, sumoylation of NR2E3 is

necessary for its ability to repress cone gene expression (26). Interestingly, NRL-K24 exhibits the highest likelihood of sumoylation based on *in silico* analysis but does not appear to be sumoylated *in vitro* or in transfected cells. However, we are unable to discriminate from our assays whether two SUMO1 proteins are added on Lys-20 exclusively or whether in some instances one SUMO1 is linked to Lys-20 and another to Lys-24. Interestingly, the Lys-24 mutation does not alter *Rho* promoter activation yet shows an effect on the *Nr2e3* promoter. It is possible that NRL is post-translationally modified at Lys-24 in a specific developmental context, but this requires Lys-20 sumoylation. Our data suggest that NRL disumoylation on Lys-20 is necessary for precise *Rho* promoter activation, but sumoylation at both Lys-20 and Lys-24 may be required for *Nr2e3* promoter activity. Distinct transcription factor PTMs may therefore exhibit different target specificity.

The sumoylated form of NRL promotes *Rho* and *Nr2e3* promoter activation to a greater extent than the non-sumoylated form. *In vivo* expression of the NRL-K20R/K24R mutant protein was not able to rescue the rod differentiation defects in *Nrl*<sup>-/-</sup> retina because of low NR2E3 expression, resulting in

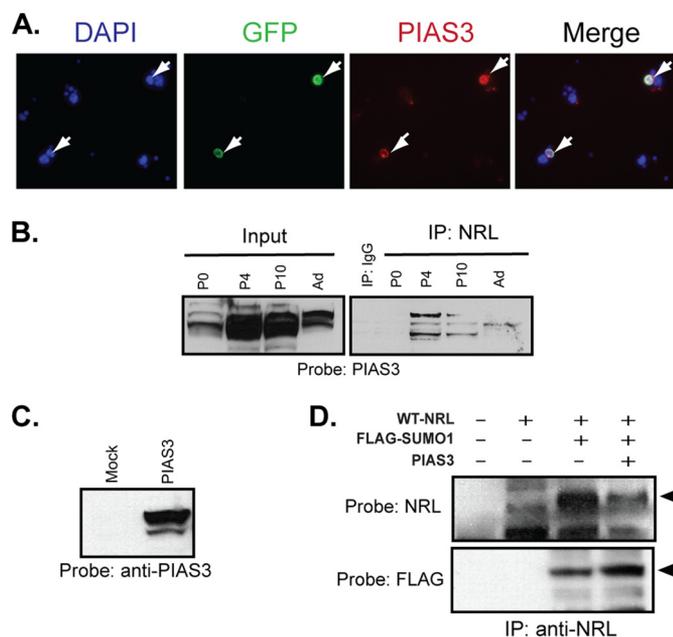


FIGURE 5. *A*, expression of *Pias3* in rod photoreceptors. Dissociated cells from adult Nrl-GFP mouse retina were stained with anti-PIAS3-antibody and 4',6-diamidino-2-phenylindole (DAPI). Arrows indicate colocalization of PIAS3 (red) in GFP-positive rods (green). *B*, co-immunoprecipitation of PIAS3 and NRL from retinal extracts. Immunoblots of anti-NRL immunoprecipitated (IP) proteins from P0, P4, P10, and adult (Ad) retina were probed with anti-PIAS3 antibody. Normal rabbit IgG served as negative control. *C*, immunoblot analysis of mock- and PIAS3-transfected HEK293T extracts with anti-PIAS3 antibody. *D*, conjugation of SUMO1 to NRL in the transfected cells in the absence or presence of PIAS3. HEK293T cell extracts transfected with WT-NRL, FLAG-SUMO1 and PIAS3 were immunoprecipitated with anti-NRL, and immunoblots were probed with anti-NRL or anti-FLAG antibody. Black arrows indicate sumoylated NRL protein.

incomplete inhibition of cone genes. However, we cannot rule out a direct repressor effect of sumoylated NRL on cone genes. Equally significantly, the NRL sumoylation mutant exhibited reduced activation of rhodopsin promoter either alone or with CRX and NR2E3 in transfected cells and by *in vivo* electroporation assay. As NRL is a major transcriptional activator of most, if not all, rod genes, we propose that sumoylation of NRL is utilized as a mechanism to produce quantitatively precise expression of specific genes during development.

Recent studies have linked sumoylation to oxidative stress and neurodegeneration (62). Daily renewal of outer segments puts an extreme stress on photoreceptor metabolic machinery, and any misregulation can lead to photoreceptor dysfunction and retinal degeneration. Continuous high expression of NRL in mature photoreceptors suggests its importance in rod homeostasis. Sumoylation and phosphorylation appear to be independent PTMs for controlling NRL activity. It is possible that the two participate in a transient shift between different NRL isoforms that may have unique gene regulatory functions. Circadian or light-induced changes in rod gene expression can be rapidly accomplished by tweaking the levels of sumoylation (and other PTMs) of NRL and to maintain homeostasis. We note that the circadian regulation of BMAL1 activity in the liver is mediated by sumoylation (63).

In contrast to ubiquitination, E3 ligase activity is not mandatory for protein sumoylation *in vitro* (7, 64), but it enhances it, as in the case of ROR $\alpha$  (65). PIAS3 sumoylates NR2E3 (26) and

interacts with NRL, but the addition of SUMO moiety on NRL is independent of PIAS3 at least in transfected cells. Our studies suggest a redundancy of SUMO ligases and that another E3 ligase may be involved in fine-tuning NRL activity by sumoylation. Although PIAS3 can sumoylate distinct transcription factors in enhanceosome complexes, additional investigations (such as conditional *Pias3*-knock-out) are necessary to decipher *in vivo* relevance of PIAS3 in NRL sumoylation and photoreceptor development.

Elucidation of gene regulatory networks that determine neuronal cell fate and function will require integration of signaling molecules to regulation of specific transcriptional target genes. Post-translational modifications, such as sumoylation, are critical components in delineating such networks. Together with a recent report (26), our study provides significant insights into the role of sumoylation in modulating the regulatory function of transcription factors that control photoreceptor differentiation and homeostasis.

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