

Post-transcriptional Inhibition of Luciferase Reporter Assays by the Nod-like Receptor Proteins NLRX1 and NLRC3*

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Background: A number of Nod-like receptors (NLRs) have been shown to inhibit signal transduction pathways using luciferase reporter assays (LRAs).

Results: Overexpression of NLRX1 and NLRC3 results in nonspecific post-transcriptional inhibition of LRAs.

Conclusion: LRAs are not a reliable technique to assess the inhibitory function of NLRs.

Significance: The inhibitory role of NLRs on specific signal transduction pathways needs to be reevaluated.

Luciferase reporter assays (LRAs) are widely used to assess the activity of specific signal transduction pathways. Although powerful, rapid and convenient, this technique can also generate artifactual results, as revealed for instance in the case of high throughput screens of inhibitory molecules. Here we demonstrate that the previously reported inhibitory effect of the Nod-like receptor (NLR) protein NLRX1 on NF- κ B- and type I interferon-dependent pathways in LRAs was a nonspecific consequence of the overexpression of the NLRX1 leucine-rich repeat (LRR) domain. By comparing luciferase activity and luciferase gene expression using quantitative PCR from the same samples, we showed that NLRX1 inhibited LRAs in a post-transcriptional manner. In agreement, NLRX1 also repressed LRAs if luciferase was expressed under the control of a constitutive promoter, although the degree of inhibition by NLRX1 seemed to correlate with the dynamic inducibility of luciferase reporter constructs. Similarly, we observed that overexpression of another NLR protein, NLRC3, also resulted in artifactual inhibition of LRAs; thus suggesting that the capacity to inhibit LRAs at a post-transcriptional level is not unique to NLRX1. Finally, we demonstrate that host type I interferon response to Sendai virus infection was normal in NLRX1-silenced human HEK293T cells. Our results thus highlight the fact that LRAs are not a reliable technique to assess the inhibitory function of NLRs, and possibly other overexpressed proteins, on signal transduction pathways.

Nod-like receptors (NLRs)⁷ represent an important class of intracellular pattern recognition molecules (PRMs), which are implicated in the detection and response to microbe- and danger-associated molecular patterns (MAMPs and DAMPs), respectively (1). NLRX1 represents the only NLR family member that localizes to the mitochondria (2, 3), and recent work demonstrated that this protein targets the mitochondrial matrix where it interacts with the matrix-facing protein UQCRC2 of the respiratory chain complex III (4), thereby modulating the production of mitochondrial reactive oxygen species, at least in an overexpression setting (3).

The exact role played by NLRX1 in innate immunity remains unclear. It was proposed that NLRX1 could inhibit cytosolic antiviral signaling by interacting with MAVS (2). However, these observations relied in part on the expression of an N-terminal tagged form of NLRX1, which failed to enter into mitochondria, likely because the NLRX1 N-terminal mitochondrial addressing sequence was masked by the added hemagglutinin (HA) tag (4). More recently, NLRX1-mediated inhibition of signal transduction pathways was shown to rely on the modulation of the more upstream signaling molecule TRAF6 rather than MAVS interaction directly (5, 6). TRAF6 is critical for numerous innate immune pathways, including Toll-like receptor (TLR)-dependent signaling. In agreement with this, NLRX1 was shown to inhibit TLR4-mediated innate immune response to lipopolysaccharide (5, 6). It remains unclear how NLRX1, which is found in the mitochondrial matrix, could inhibit TRAF6-dependent signaling modules present in the cytosol. It is conceivable that a pool of NLRX1 could be rerouted to the cytosol in response to specific signals, although this hypothesis has not been experimentally demonstrated. Finally, it must be noted that, using an independently generated NLRX1-deficient mouse strain, Tschopp and co-workers (7) did not identify MAVS-dependent antiviral signaling defects in NLRX1^{-/-} mice.

⁷The abbreviations used are: NLR, Nod-like receptor; LRA, luciferase reporter assay; TLR, Toll-like receptor; IRES, internal ribosome entry site; HCV, hepatitis C virus; LRR, leucine-rich repeat; MAVS, Mitochondria-Associated Viral Sensor.

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NLRX1 and NLRC3 Inhibit Luciferase Assays Nonspecifically

Luciferase reporter assays (LRAs) have been extensively used in the field of innate immunity to identify the role of PRMs in the modulation of specific signal transduction pathways. In these assays, cells are transfected with plasmids encoding for luciferase enzymes (most commonly from the firefly (*Photinus pyralis*) or from the Sea Pansy (*Renilla remiformis*)), and luciferase expression is under the regulation of specific promoters, the activity of which being correlated by measuring, in cell lysates, light emission following conversion of the substrate by the luciferase enzyme. In the case of the NLR proteins Nod1 and Nod2, NF- κ B-responsive luciferase constructs (NF- κ B Luc) have been used to demonstrate that these proteins activate the NF- κ B pathway and detect bacterial peptidoglycan (8–14). Remarkably, a number of NLR proteins have been shown instead to down-regulate signal transduction pathways in LRAs, and these include NLRX1 (2, 5, 6), NLRC3 (15), NLRC5 (16, 17), NLRP3 (18), NLRP12/Monarch-1 (19) and NLRP2 (20). At least in the case of NLRP3, it is clear that the initial function assigned to the protein (*i.e.* being a negative regulator of NF- κ B-dependent signaling) was incorrect, since NLRP3 is now known to act as a critical activator of caspase-1 inflammasomes (21).

In the present study, we aimed to better understand the role played by NLRX1 in the modulation of host signal transduction pathways, and used LRAs to do so. We were surprised to note that NLRX1 seemed to potently inhibit several unrelated pathways, and that inhibition was irrespective of whether the protein targeted the mitochondria or the cytosol. These observations suggested the existence of nonspecific effects of NLRX1 overexpression on the LRAs. This was confirmed by showing that overexpression of NLRX1 also inhibited luciferase activity of the enzyme expressed under a constitutive promoter, suggesting that inhibition was post-transcriptional. Further, by comparing luciferase activity and luciferase gene expression using quantitative PCR from the same samples, we unequivocally showed that NLRX1 inhibited luciferase post-transcriptionally. Likewise, we observed similar nonspecific effects on luciferase assays for NLRC3. Finally, we present evidence that upon Sendai infection, MAVS-dependent antiviral signaling is normal in NLRX1-silenced cells, thereby supporting the nonspecific effects of NLRX1 overexpression on LRAs. Our results highlight the difficulty and the risk of using LRAs to identify putative negative regulators of signal transduction pathways. We discuss possible mechanisms that could explain why some NLR proteins inhibit LRAs nonspecifically, and propose to systematically use qPCR to measure the expression of the *Luciferase* gene along with LRAs, in the case of studies aiming at identifying the potential inhibitory role of LRR-containing and possibly other overexpressed proteins.

EXPERIMENTAL PROCEDURES

Cell Culture—In LRA experiments, the epithelial cell line, HEK293T, was used as it offers optimal transfection efficiency and luciferase expression. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and penicillin-streptomycin.

Luciferase Transfection and Assay—Luciferase vectors were co-transfected with a β -galactosidase (pCMV- β gal, Clontech) vector for normalization of transfection efficiency. Cells were

seeded in 24-well tissue culture plates the day before to reach a confluency of at least 60%. Transfection mixes of the two vectors and other co-transfected vectors were normalized to contain the same amount of DNA per well using an empty pcDNA₃ expression vector with 300 ng DNA being the minimum for good transfection efficiency. The transfection reagent, polyethyleneimine, was added to the transfection mix in the ratio of 3 μ l per 1 μ g of DNA and the mix was incubated at room temperature for 20 min before adding to the cell medium. The next day, the medium was aspirated and 100 μ l of lysis buffer was added (freshly added 1 mM DTT, 25 mM Tris, pH 8.0, 8 mM MgCl₂, 1% Triton X-100, 15% glycerol, water). The plate was kept at 4 °C for subsequent analysis or –20 °C for storage.

For analysis, 10 μ l from each well was transferred to a 96-well white wall plate. Reading buffer was prepared (lysis buffer + 1 mM ATP, firefly luciferin). Reading was done using with automated injection in a Victor3 1420 multilabel counter (Perkin Elmer) set to measure 0.1s after injection of 100 μ l of reading buffer on the sample. In a separate clear wall plate, the β -gal assay was done by first adding 10 μ l of the lysate to a 96-well clear bottom plate. 100 μ l of development buffer was added (fresh development buffer is stock solution:dilution buffer = 1:9; dilution buffer: 0.06 M Na₂HPO₄, 0.04 M NaH₂PO₄, 0.01 M KCl, 1 mM M MgSO₄, pH 7; stock solution: 0.4% ONPG in dilution buffer, –20 °C storage). The mixture was incubated at 37 °C for 10 min or until yellow color appears. The absorbance was measured at 405 nm using the Victor3 1420. Luminescence data were then normalized with β -gal absorbance readings for data analysis purposes.

Luciferase and Other Expression Vectors—The expression vectors used were from the following sources: I κ B Luc (Dr. Luke O'Neill, Trinity College, Ireland), ISRE Luc (Dr. Kate Fitzgerald, University of Massachusetts), p53 Luc (Dr. Michael Ohh, University of Toronto, Canada), IFN β -Luc reporter plasmid (from K. Fitzgerald, UMass), pGL3 control (m⁷G-FF luc, Promega), pRL-TK (m⁷G-R luc, Promega), HCV IRES FF Luc (Dr. Jerry Pelletier, McGill, Canada), bicistronic R-luc-IRES-FF Luc (Dr. Jerry Pelletier, McGill, Canada), MyD88 (Dr. Fabio Martinon, UNIL, Lausanne), MAVS (Dr. Kate Fitzgerald, University of Massachusetts), p53 (Dr. Michael Ohh, University of Toronto, Canada). NLRX1 constructs (NLRX1 FL, Δ N-ter NLRX1, N-ter NLRX1, HA-NLRX1) have been described previously (3). NLRC3 was cloned from a human cDNA library and inserted into pEGFP-N1 (Clontech) to generate a GFP-NLRC3 fusion protein. After cloning, the construct was fully verified by sequencing.

Real Time Quantitative PCR—Following RNA extraction with Trizol (Invitrogen) or an RNA mini-prep kit, the RNA was treated with TURBO DNase (Ambion). The purified RNA was then reverse transcribed to cDNA using SuperScript III MMLV reverse transcriptase with random hexamer and oligo-dT primers (Invitrogen). The cDNA was diluted accordingly and used in PCR reactions for real time PCR using the Power SYBR Green 2X master mix reagent (ABI). Reactions were performed using ABI 7900HT real time PCR machine. Results were analyzed using the 2^{– Δ Ct} formula normalizing to the endogenous house-keeping control, β -actin, 18S or cyclophilin. The real time qPCR primers used are the following: 18S-For: GCAATTATT-CCCATGAACG; 18S-Rev: GGGACTTAATCAACGCA-

AGC; NLRX1-For: AACGGTGCTGGTGACACA; NLRX1-Rev: GCTCAGCTCATTGAAGTAGA GGT; IFN β -For: CAT-TACCTGAAGGCCAAGGA; IFN β -Rev: CAATTGTCCAGT-CCCAGAGG; LUC-For: GCGCGGAGGAGTTGTGTT (16); LUC-Rev: TCTGATTTTTCTTGCCTCGAGTT (16).

Western Blot and Cell Fractionation—Cell lysates or protein samples in SDS Laemmli blue solution were analyzed with SDS-PAGE. The gels were then transferred onto PVDF membranes using a semi-dry transfer apparatus and membranes were blocked with 5% skim milk in TRIS-buffered saline-Tween (TBS/T: 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% Tween 20). Primary antibodies were diluted in TBS/T and incubated with the membrane in rolling or shaking conditions. Membranes were washed in TBS/T for 1 h and then incubated in diluted secondary antibody. After washing once more, the membranes were incubated with chemiluminescence reagents (Millipore) according to manufacturer's protocol and exposed to radiographic film. Fractionation of cell lysates into cytosolic and heavy membrane fractions was previously described (4). Antibodies used included mouse mAb against FLAG (clone M2, Sigma; 1/2000 dilution) and AIF (clone E1, Santa Cruz Biotechnology; 1/1000).

Generation of Stable HEK293T Cell Lines Expressing siRNA against Human NLRX1—The pLKO.1 lentiviral knockdown system (Addgene, Cambridge, MA) was used to generate lentiviral shRNA particles against human NLRX1 and a non-targeting sequence (scramble). The siRNA sequences (sense strand) targeting NLRX1 and scramble were as follows. 5'-GAGGAG-GAC TACTACAACGAT-3' (NLRX1) and 5'-GTG CAG-GAGTAGGGCATTTAA-3' (scramble). Oligonucleotides containing these sequences were designed and ligated into the pLKO.1 vector as previously described (16). shRNA constructs were fully sequenced. Generation of lentiviral particles was performed in HEK293T cells and was previously described (16). Briefly, cells were co-transfected with the lentiviral shRNA vector (1 μ g) and the lentiviral packaging/envelope vectors psPAX2 (750 ng) and pMD2.G (250 ng). 48 h post-transfection, cell medium containing the lentiviral particles was collected and filtered through a 0.45- μ m syringe to remove any detached cells. HEK293T cells were transduced with purified lentivirus for 24 h and fresh medium containing 3 μ g/ml puromycin (Sigma) was added to select cells containing shRNAs. Stable cells expressing shRNAs were selected in puromycin for at least 5 days prior to performing experiments. Knockdown of endogenous NLRX1 was verified by Western blot using a polyclonal NLRX1 antibody (Cederlane, Canada), as well as by qPCR.

Viruses and in Vitro Infections—The Sendai H4 virus strain (SeV-H4) has been previously described (22, 23). In brief, HEK293T cells were seeded in 24-well tissue culture plates the day before and incubated in with virus for 1 h in serum-free medium. Cells were then rinsed with 1 \times PBS and fresh medium containing FBS was added for the duration of the infection.

RESULTS

We sought to determine the effect of NLRX1 overexpression (either full-length (FL) or deletion constructs, see Fig. 1A) on the activity of various reporter constructs expressing the firefly luciferase (FF Luc) under the control of different transcription

factor response elements. HEK293T cells were first transfected with a NF- κ B-responsive FF Luc (Ig κ -Luc) expression vector, together with a normalization plasmid that expresses β -galactosidase constitutively. Of note, all the subsequent LRAs presented below were also normalized for β -galactosidase activity. While overexpression of TLR adaptor MyD88 potentially up-regulated FF Luc activity, we noticed that overexpression of increasing amounts of full-length NLRX1-Flag (FL) resulted in a strong reduction of MyD88-driven FF Luc activity (Fig. 1B). This result is consistent with reports by other groups who proposed that NLRX1 blocked TLR signaling at the level of TRAF6 (5, 6), which acts downstream of MyD88 in TLR signaling. Similar inhibition was achieved when NLRX1 truncation constructs encoding for regions that included the LRR domain (Δ N-ter or LRR only) were overexpressed (Fig. 1B). This result was surprising because we previously reported that, while NLRX1 FL targets the mitochondrial matrix, Δ N-ter or LRR NLRX1 constructs remain completely cytosolic (3, 4). This suggested that NLRX1-mediated inhibition was either location-independent (occurring in the mitochondrial matrix or the cytosol) or that excess overexpression of NLRX1 FL resulted in the accumulation of a pool of the protein in the cytosol, likely as a result of the overloading of the mitochondrial import machinery. The latter hypothesis was supported by biochemical fractionation of cytosol *versus* heavy membranes, which showed that large overexpression of NLRX1 FL resulted in the cytosolic accumulation of the protein (Fig. 1C). In further support for this, overexpression of HA-NLRX1, an epitope-tagged form of NLRX1 that fails to target mitochondria because of the presence of an N-terminal HA tag masking the mitochondria localization signal (4), resulted in very potent down-regulation of MyD88-driven FF Luc activity (Fig. 1D). Interestingly, HA-NLRX1 seemed to repress luciferase activity more efficiently than NLRX1-Flag, again suggesting that LRA inhibition by NLRX1 overexpression was associated with cytosolic accumulation of the protein.

The above results imply that NF- κ B inhibition in NLRX1-overexpressing cells was unlikely to be physiologically relevant because it seemed to occur in a compartment (the cytosol) where the protein would only accumulate in enforced conditions. In addition, the effect of NLRX1 on NF- κ B-driven activity of the FF Luc was unaffected by the mutation of the lysine 172 (NLRX1 K172R) (Fig. 1E), a key amino acid of the P-loop region of the NACHT domain, which is conserved in all NLR proteins, and was shown to be critical for the function of all the NLR proteins in which it has been tested so far (24).

To better understand how NLRX1 overexpression resulted in apparent down-regulation of NF- κ B signaling in LRAs, we decided to characterize if transfection of NLRX1 also affected the activity of FF Luc expressed under the control of other transcription factor response elements. We observed that NLRX1 FL, Δ N-ter or LRR only, but not N-ter constructs, efficiently inhibited MAVS-dependent induction of the luciferase activity of FF Luc expressed under the control of an interferon-sensitive response element (ISRE) (Fig. 1F), in agreement with previous findings (2, 5, 6). NLRX1 also inhibited phorbol 12-myristate 13-acetate (PMA)-dependent induction of the luciferase activity of FF Luc expressed under the control of an activator protein

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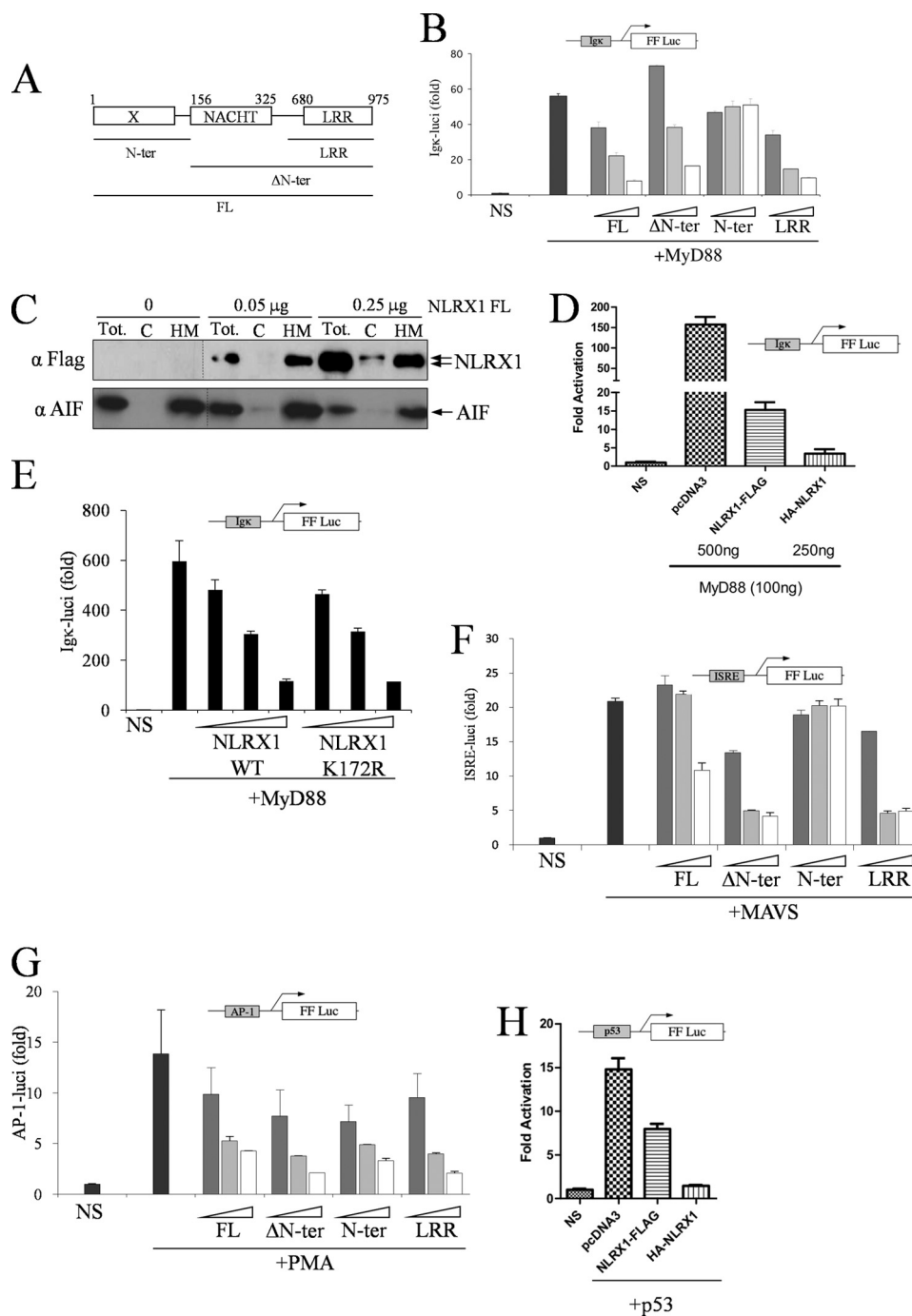


FIGURE 1. Effect of NLRX1 overexpression on inducible luciferase expression reporters. *A*, schematic representation of the NLRX1 deletion constructs used in the study: *FL*, full-length NLRX1 coding sequence; ΔN -*ter*, deletion of an N-terminal region; *N-ter* and the *LRR* refer to the domains by themselves. *B*, transfection of NLRX1 constructs in increasing amounts (5, 75, 250 ng of plasmid) in the NF- κ B LRA using co-transfection of MyD88 to stimulate the reporter. *C*, cells transfected to overexpress NLRX1 were separated into total cell lysates (*Tot.*), cytosolic (*C*), and heavy membrane fractions (*HM*) and were analyzed in Western blot for NLRX1 and AIF. *D*, effect of the overexpression of either N-terminal tagged HA-NLRX1 or NLRX1-FLAG in the NF- κ B LRA stimulated by overexpression of 100 ng expression vector encoding MyD88. *E*, wild type and K172R P-loop mutant NLRX1 overexpression in the NF- κ B LRA. *F* and *G*, Similar to the NF- κ B LRA, NLRX1 constructs were transfected in the (*F*) ISRE LRA with co-transfection of MAVS or (*G*) AP-1 LRA with PMA treatment. *H*, effect of the overexpression of either N-terminal tagged HA-NLRX1 or NLRX1-FLAG in the p53 LRA stimulated by overexpression of 100 ng expression vector encoding p53. *NS*, not stimulated.

1 (AP-1) response element (Fig. 1G). Because the three signal transduction pathways tested above (NF- κ B, type I interferon and AP-1) are regulated by TRAF6, we aimed to determine if NLRX1 could also block LRA when luciferase expression was under the control of very different signaling cascades. We first noticed that NLRX1 could potentially inhibit TNF-induced luciferase expressed under the control of NF- κ B (data not shown),

although it is well established that TNF signaling to NF- κ B is TRAF6-independent. In addition, cells were transfected with p53-responsive FF Luc expression vector, and as expected, overexpression of p53 stimulated LRA in these conditions (Fig. 1H). We observed that overexpression of either NLRX1-Flag or HA-NLRX1 resulted in inhibition of p53-driven induction of luciferase activity, although we noticed that the inhibitory

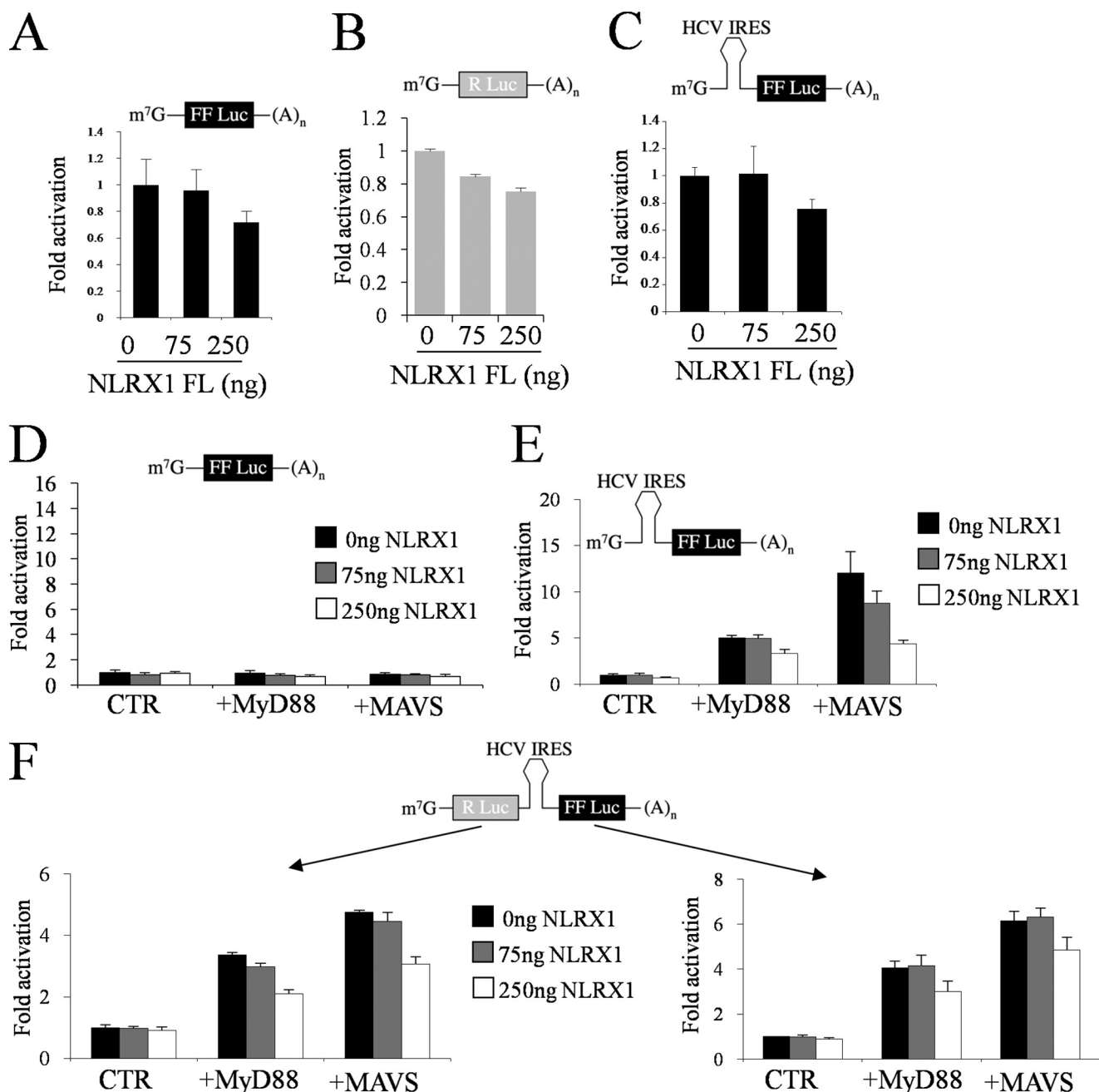


FIGURE 2. Effect of NLRX1 overexpression on constitutive luciferase expression reporters. A–C, cells were transfected with NLRX1 FL and one of three constitutive luciferase reporters: (A) firefly luciferase (m⁷G-FF Luc), (B) *Renilla* luciferase (m⁷G-R Luc), and (C) firefly luciferase downstream of an IRES site derived from the Hepatitis C virus (HCV IRES-FF Luc). D and E, NLRX1 was transfected with m⁷G-FF Luc (D) or IRES-FF Luc (E) reporter constructs and co-transfected with either MyD88 or MAVS. F, NLRX1 was transfected together with a constitutive bicistronic reporter that expresses *Renilla* luciferase with an m⁷G cap and firefly luciferase downstream of the IRES site (m⁷G-R Luc-HCV IRES-FF Luc), and co-transfected with either MyD88 or MAVS. *Renilla* and firefly luciferase data are shown on the left and right graphs, respectively. CTR, control.

effect of NLRX1-Flag on p53-Luc was reproducibly not as pronounced as on the other FF Luc constructs tested above. Once again, the inhibitory effect was much more dramatic for HA-NLRX1 than for NLRX1-Flag (Fig. 1H).

These results showed that NLRX1 overexpression inhibited FF Luc activation irrespective of the transcription factor response element driving the expression of the luciferase. Inhibition also occurred when NLRX1 was targeted to the cytosolic compartment, where the protein normally does not reside. Taken together, these observations thus strongly suggest that NLRX1-dependent inhibition of LRAs was nonspecific.

In light of the results above, we hypothesized that NLRX1 overexpression might inhibit the luciferase activity of Luc expressed under the control of a constitutive promoter, which would strongly support our contention that the inhibition observed was independent of transcription factor response elements driving Luc expression. We first noticed that increasing amounts of transfected NLRX1 FL vector had a minor effect (~20% inhibition at the highest concentration) on the luciferase activity of FF Luc (Fig. 2A) or *Renilla* Luc (R Luc) (Fig. 2B) expressed under the control of a constitutive promoter. Similar results were obtained with a FF Luc construct expressed under

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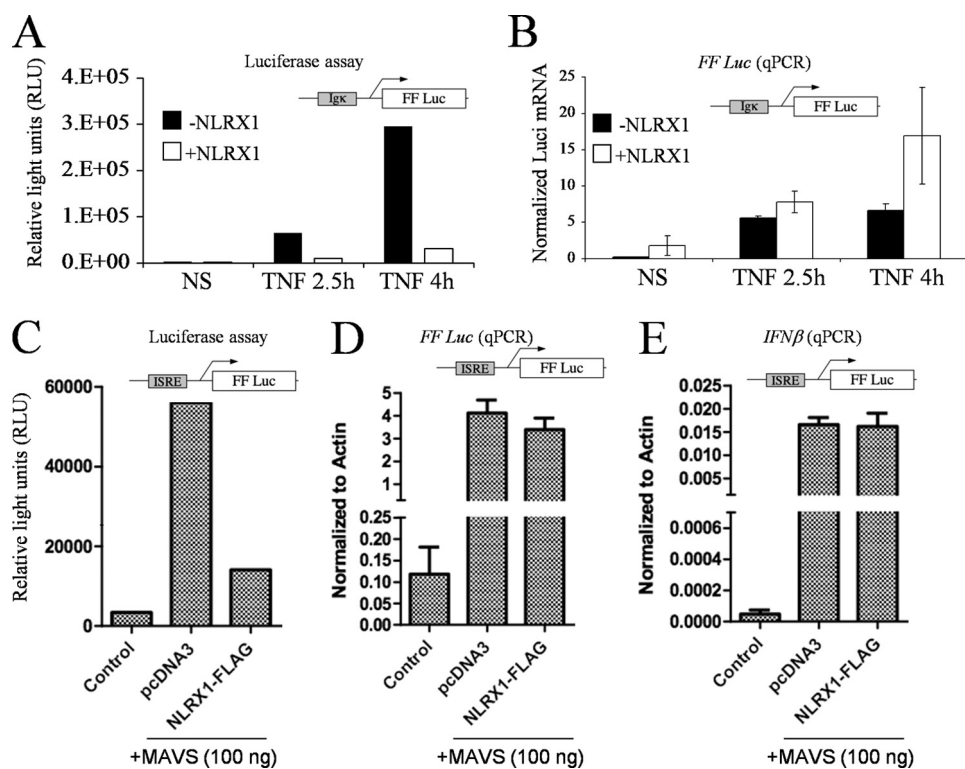


FIGURE 3. **NLRX1 inhibits LRAs post-transcriptionally.** pcDNA₃ transfected (negative control) or NLRX1-transfected cells were treated for 4 h with TNF (1 ng/ml) and assayed for (A) NF-κB-dependent luciferase activity and (B) luciferase mRNA expression by qPCR. C–E, cell transfected with ISRE Luc expression vector and MAVS (100 ng), in the presence or absence of overexpressed NLRX1 FL (400 ng) were analyzed in LRA (C) or in qPCR for FF Luc (D) and IFNβ (E). NS, not stimulated.

the control of a constitutive promoter, but whose mRNA 5' cap was replaced by an internal ribosome entry site (IRES) from hepatitis C virus (HCV), thus allowing cap-independent translation of the FF Luc gene (Fig. 2C). Together, these results suggest that, although there was a reproducible effect of NLRX1 overexpression on luciferase activity in these conditions, the inhibition capacity was clearly not as robust as when luciferase expression was driven by inducible factors.

We next reasoned that the contrasting results obtained when comparing luciferase expression under the control of inducible or constitutive promoters could be explained by either of the following: (i) NLRX1 overexpression inhibits LRAs only in inflammatory (MAVS, MyD88 expression or PMA stimulation) or stress (p53 expression) conditions; or (ii) NLRX1 overexpression blocks luciferase activity only if the enzyme is produced at a high dynamic rate. We first observed that ectopic expression of MAVS or MyD88 did not enhance the inhibitory capacity of NLRX1 over constitutively expressed FF Luc (Fig. 2D), thus suggesting that activation of inflammation/stress pathways is not a prerequisite for NLRX1-mediated inhibition of FF Luc activity. We next repeated this experiment with the HCV IRES FF Luc, whose expression is also under the control of a constitutive promoter. To our surprise, ectopic expression of MAVS or MyD88 resulted in a substantial up-regulation of basal luciferase activity (Fig. 2E), thus suggesting that inflammatory pathways might contribute to enhance the translation of IRES-containing mRNAs, as previously proposed (25). Interestingly, in these circumstances, NLRX1 overexpression resulted in a substantial inhibition of FF Luc activity (Fig. 2E),

thus suggesting that it is likely the dynamic inducibility of luciferase expression, rather than the action of specific signal transduction pathways, which represents a key factor for NLRX1 inhibitory effect on LRAs. We next observed that the effect of NLRX1 on Luc-expressing vectors under the control of constitutive promoters was not restricted to constructs carrying an IRES sequence in their 5'-untranslated region. Indeed, NLRX1 efficiently inhibited both R Luc- and FF Luc-dependent luciferase activities from genes encoded on a bicistronic mRNA sequence, in which R Luc translation was cap-dependent and R Luc was driven by an internal IRES sequence (Fig. 2F). As for the simple HCV IRES FF Luc construct, luciferase activity was up-regulated by overexpression of either MAVS or MyD88, and this affected both R Luc and FF Luc activity, suggesting that the presence of an IRES sequence likely stabilizes mRNAs in inflammatory conditions. This shows that the inhibitory effect of NLRX1 does not require that a given mRNA be translated under the control of an IRES element.

Together, the results obtained using Luc constructs expressing luciferase under the control of constitutive promoters clearly establish that NLRX1 can inhibit LRAs in a promoter-independent manner. However, we argue that the inhibitory effect of NLRX1 is usually more strongly highlighted with Luc reporter constructs driving luciferase expression in an inducible manner, in response to specific stimuli. The unexpected capacity of IRES elements to up-regulate luciferase activity in inflammatory conditions and in a promoter-independent manner allowed us to propose that it is expression inducibility in itself,

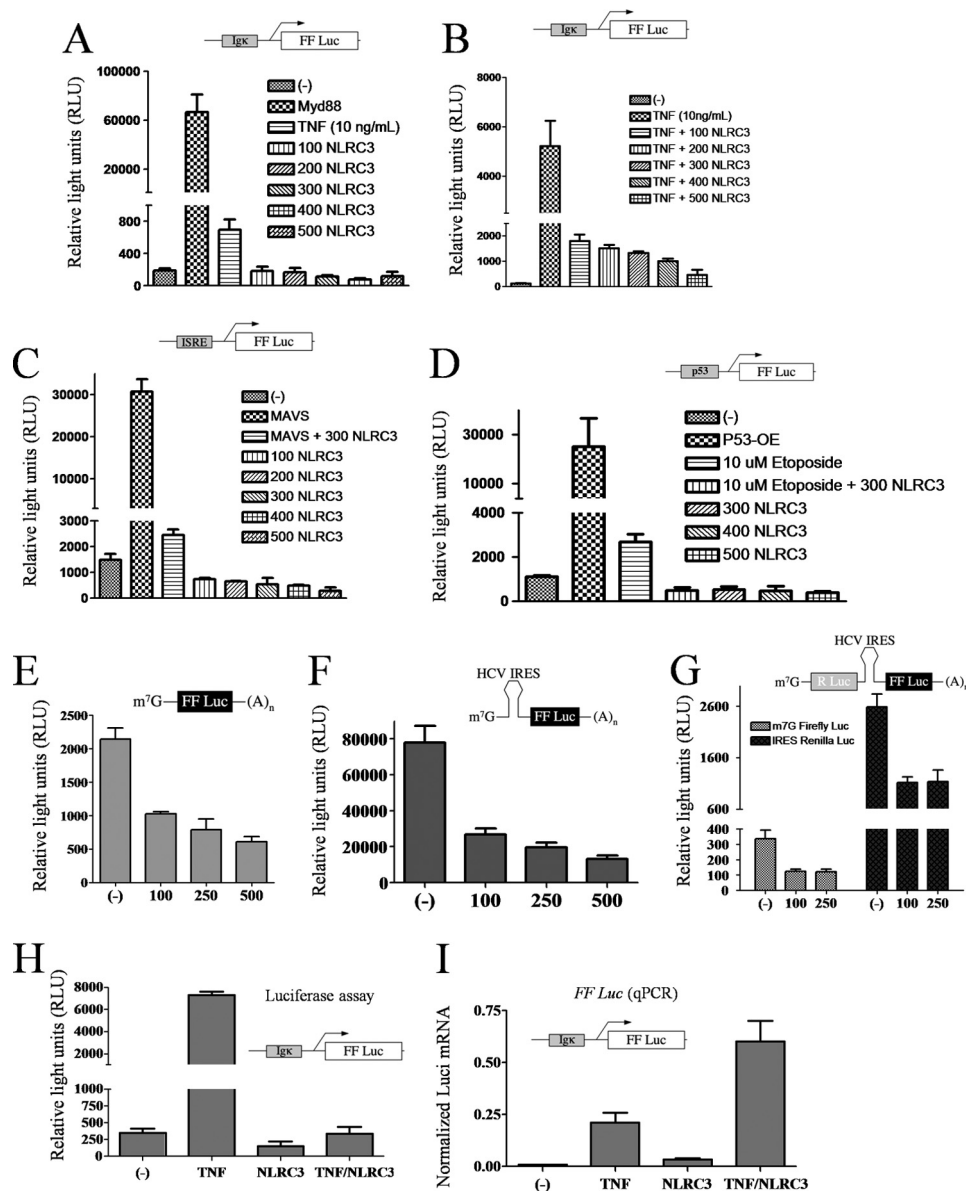


FIGURE 4. **NLRC3 also inhibits LRAs post-transcriptionally.** A, NLRC3 was transfected by itself in the NF- κ B LRA in increasing amounts compared with the effect of transfecting MyD88 or treating with TNF for 4 h. B–G, NLRC3 was transfected in the (B) NF- κ B LRA with MyD88, (C) ISRE LRA with MAVS, (D) p53 LRA with p53 transfection or etoposide treatment, (E) constitutive m⁷G-FF Luc assay, (F) constitutive HCV IRES-FF Luc, and (G) constitutive bicistronic m⁷G-R Luc-HCV IRES-FF Luc assay. H–I, control (–) or NLRC3-transfected cells were treated for 4 h with TNF (10 ng/ml) and assayed for (H) NF- κ B-dependent luciferase activity and (I) luciferase mRNA expression by qPCR.

rather than the activity of any particular pathway, which is critical for potentiating the inhibitory effect of NLRX1 in LRAs.

The above results, and in particular the differential effect of NLRX1 on Luc expressed in a cap- versus IRES-dependent manner, strongly suggested that the effect of NLRX1-dependent inhibition of LRAs was post-transcriptional. To test this hypothesis directly, we designed a procedure in which luciferase activity and expression of FF Luc by qPCR were measured from the same cellular lysates. In this experimental setup, HEK293T cells were first transfected overnight with an expression vector encoding for FF Luc under the control of NF- κ B, with or without NLRX1-expressing construct. Next, cells were stimulated for 2.5 or 4 h with TNF, in order to trigger NF- κ B-dependent signaling. As expected, overexpression of NLRX1

resulted in a very strong inhibition of luciferase activity, as determined in LRA (Fig. 3A). However, analysis of the same samples by qPCR revealed that the reduction of luciferase activity did not correlate with a decrease in the levels of FF Luc mRNA levels (Fig. 3B). Similarly, while NLRX1 FL overexpression efficiently inhibited MAVS-dependent induction of the luciferase activity when FF Luc was expressed under the control of ISRE (Fig. 3C), qPCR analysis from the same samples revealed that NLRX1 expression inhibited neither FF Luc (Fig. 3D) nor endogenous IFN β (Fig. 3E) expression induced by MAVS. Together, these results provide a direct demonstration that NLRX1 inhibits LRAs in a post-transcriptional manner, which likely makes it irrelevant to use this assay to probe the effect of NLRX1 overexpression on specific signal transduction pathways.

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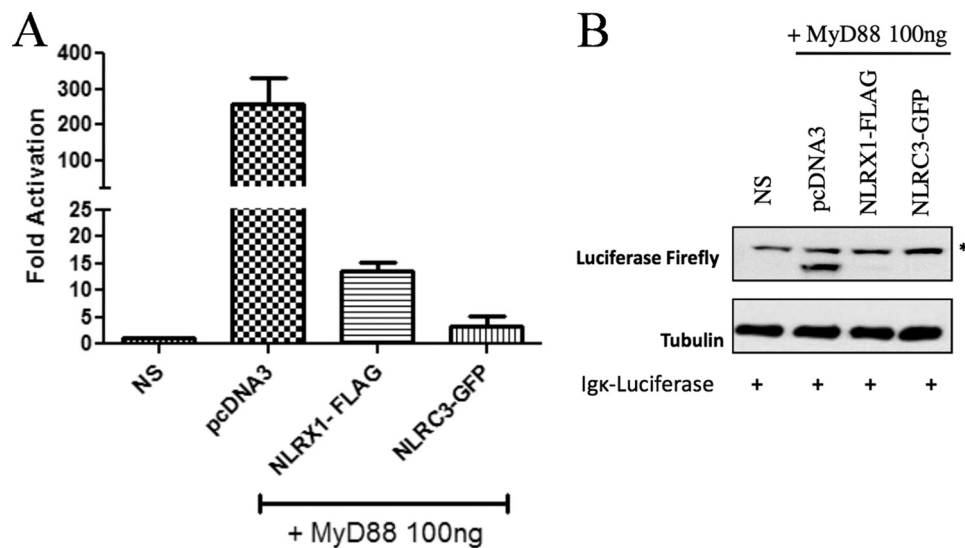


FIGURE 5. **Overexpression of NLRX1 or NLRC3 reduces luciferase protein levels.** A and B, HEK293T cells were transfected with Ig κ -Luc plus MyD88, in the presence or absence of either NLRX1-FLAG or NLRC3-GFP. Cell lysates were then separated and used for LRA (A) or Western blotting, using antibodies against FF Luc or tubulin, as a loading control (B). * indicates the presence of a nonspecific band.

We next aimed to determine if post-transcriptional inhibition of LRAs was specific to NLRX1 or was also observed with other previously reported inhibitory NLR proteins. We focused on NLRC3, a poorly characterized NLR protein that was shown to suppress T cell functions by inhibiting NF- κ B- and AP-1-dependent signaling, and this function was mainly inferred by using LRAs (15). We first observed that NLRC3 overexpression did not result in activation of NF- κ B-responsive luciferase activity (Fig. 4A), and potently inhibited TNF-induced luciferase activity (Fig. 4B). Similarly, NLRC3 overexpression inhibited luciferase activity triggered by MAVS in cells transfected with ISRE-responsive FF Luc construct (Fig. 4C) or by etoposide in cells transfected with p53-responsive FF Luc construct (Fig. 4D). As for NLRX1, ectopic expression of NLRC3 resulted in inhibition of luciferase activity when FF Luc was transcribed under the control of a constitutive promoter (Fig. 4, E–G), and the inhibitory effect was even more pronounced in the case of NLRC3 than it was for NLRX1 overexpression. Finally, we directly compared luciferase activity and qPCR expression of the FF Luc gene in cells transfected overnight with an expression vector encoding for FF Luc under the control of NF- κ B, with or without NLRC3-expressing construct, and stimulated with TNF. In this experimental setting, we demonstrated that the potent inhibitory effect of NLRC3 on LRAs was, similar to NLRX1, mediated at a post-transcriptional level. These results suggest that NLRX1 and NLRC3 likely inhibit LRAs by a similar nonspecific post-transcriptional mechanism.

To get insight into the step at which NLRX1 or NLRC3 overexpression inhibited LRAs post-transcriptionally, FF Luc expression was next followed by Western blotting. HEK293T cells were transfected with Ig κ -Luc plus MyD88, in the presence or absence of either NLRX1-FLAG or NLRC3-GFP, and luciferase activity measured (Fig. 5A). Interestingly, determination of FF Luc expression by Western blot on lysates from the same samples showed a strong correlation between LRAs and FF Luc protein levels (Fig. 5B), thus demonstrating that NLRX1- and NLRC3-dependent inhibition of LRAs must affect

either FF Luc mRNA translation or protein stability, but not enzymatic inhibition of luciferase activity.

Finally, to complement our NLRX1 overexpression studies and assess the proposed role of NLRX1 in antiviral signaling, NLRX1 was silenced in HEK293T cells. A dramatic reduction in NLRX1 endogenous protein levels was observed in cells expressing shRNAs targeting NLRX1, as compared with cells expressing shRNAs targeting a scramble sequence (Fig. 6A). To model the activation of MAVS-dependent antiviral signaling, a Sendai virus strain (strain H4) that is a potent activator of interferon- β (IFN β) production (22, 23) was used to infect scramble- and shNLRX1-expressing HEK293T cells. HEK293T cell lines expressing shRNAs were transfected with an IFN β -Luc reporter plasmid and infected with Sendai virus for various times. IFN β -Luc activation was comparable in Sendai-infected scramble and shNLRX1 cell lines (Fig. 6B). To confirm that IFN β mRNA expression was also unaffected in shNLRX1 cell lines during Sendai virus infection, qPCR was performed. In line with the luciferase reporter assays, levels of IFN β mRNA were unaffected in NLRX1-knockdown cells compared with scramble cells (Fig. 6, C and D). Taken together, these results strongly suggest that NLRX1 does not play a significant role in MAVS-dependent antiviral signaling in human epithelial cells, which fully supports the nonspecific effects of NLRX1 overexpression on the LRAs.

DISCUSSION

In this study, we present evidence that LRAs are not suitable for analyzing the potential inhibitory role played by NLRs, and possibly other proteins displaying a LRR domain, on specific signal transduction pathways. Our results are not the first to report nonspecific artifactual data in LRAs, although tracking these effects in the scientific literature is complicated by the fact that few studies highlight or aim to investigate the mechanism underlying what is considered as a technical issue. In the case of high-throughput screening of compounds with biological activity using LRAs, the importance of limiting artifactual false-

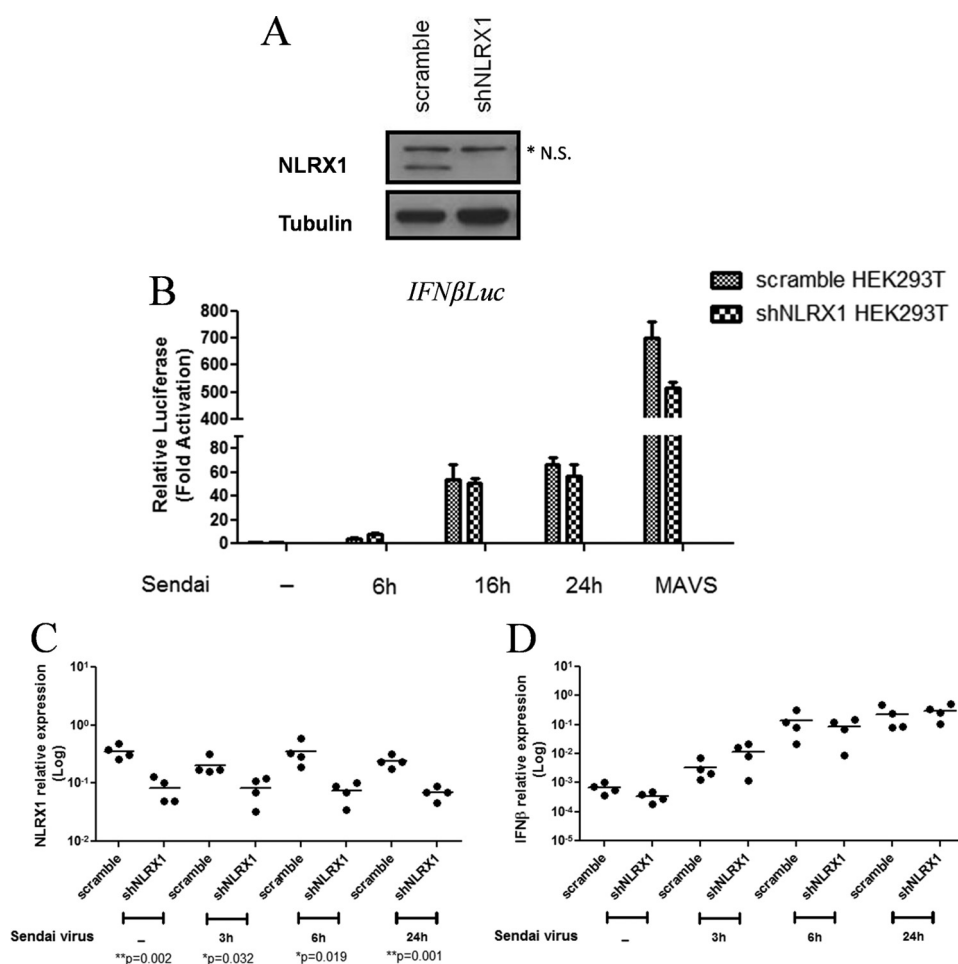


FIGURE 6. Knockdown of NLRX1 does not alter anti-viral signaling during Sendai virus infection. *A*, human embryonic kidney cells (HEK293T) cells were transduced with lentiviral particles expressing shRNAs against human NLRX1 or a non-targeting sequence (scramble) and cell lysates were analyzed through Western blot using anti-NLRX1 antibody. *N.S.* indicates a nonspecific band (human specific). *B*, cells as described in *A* were transfected overnight with an *IFNβLuc* reporter plasmid and infected with Sendai virus for various time points. Luciferase activity was measured at the time points indicated following virus infection and overexpression of MAVS was utilized as positive stimulus for *IFNβLuc* activity. *C* and *D*, cells described in *A* were infected with Sendai virus for 3, 6, or 24 h and total RNA was extracted. cDNA was prepared and gene expression levels of human *NLRX1* (*C*) and *IFNβ* (*D*) were measured using qPCR. Each data point was obtained from an independent experiment for that condition. Statistical analysis was performed using a two-tailed unpaired *t* test, and conditions for which $p < 0.05$ are indicated. *NLRX1* expression was statistically reduced in cells expressing a shRNA against human NLRX1 at basal conditions ($p = 0.002$) or when infected with Sendai virus for 3 h ($p = 0.032$), 6 h ($p = 0.019$) and 24 h ($p = 0.001$).

positive results prompted investigators to better understand the circumstances in which LRAs caused the occurrence of off-targets effects (26, 27). A first class of molecules that can interfere with LRAs are those inhibiting the enzymatic activity of luciferase, and these include quinolines, 1,2,4-oxadiazoles, and benzthiazoles, the latter displaying structural homology with D-luciferin, the FF Luc substrate (28). Interestingly, nonspecific accumulation of the enzyme on molecular aggregates has also been proposed as another possible mechanism of luciferase activity inhibition, and molecular aggregation seems to represent an important source of off-target effects in LRA-based screening of compound libraries (26, 27). The reason why enzymes such as luciferase accumulate at the surface of molecular aggregates, and how this results in inhibition of the enzymatic activity, remain unclear. However, it seems unlikely that overexpression of NLRX1 LRR domain would inhibit LRAs through a mechanism involving direct interference with luciferase substrates, because we found that inhibition of LRAs correlated with decreased levels of the luciferase protein in cell

lysates (see Fig. 5*B*). However, it is possible that nonspecific aggregation of luciferase on overexpressed LRRs from NLRX1 or NLRC3 would result in rapid degradation of the aggregates, resulting in overall decrease of luciferase protein levels. In support for this, large overexpression of NLRX1, and in particular the ΔN-ter construct, leads to the formation of discrete and bright cytosolic foci in transfected cells (3), which could accumulate protein aggregates. Further work is required to analyze if protein aggregation accounts for the inhibition of LRAs in NLRX1- or NLRC3-expressing cells.

In the LRAs presented here, plasmids encoding for luciferase were systematically co-transfected with a vector expressing β-galactosidase, and all results were normalized for β-galactosidase activity, to minimize effects due to transfection efficiency differences, a procedure that is commonly used in LRAs. While transfecting NLRX1 or NLRC3 inhibited luciferase activity, it is intriguing to note that overexpression did not affect the enzymatic activity of β-galactosidase. The reason for this differential effect on luciferase and β-galactosidase remains unclear

NLRX1 and NLRC3 Inhibit Luciferase Assays Nonspecifically

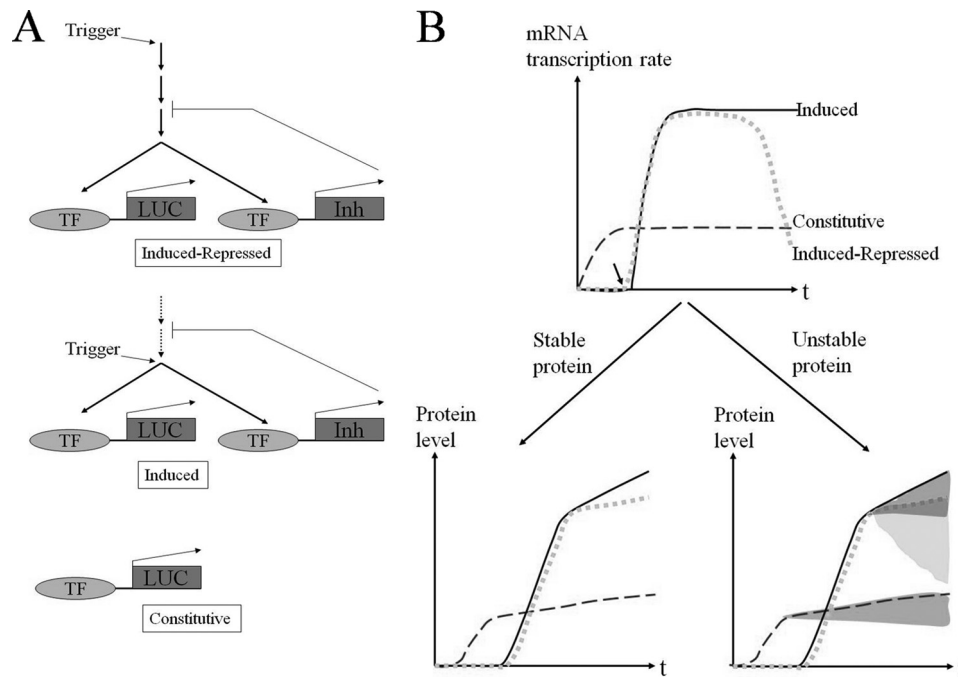


FIGURE 7. Model illustrating the possible impact of NLRX1 or NLRC3 overexpression on LRAs. *A*, schematic representation of the three main modes of *Luc* mRNA induction. In the Constitutive mode, *Luc* expression is dependent on a constitutive promoter, such as the one of the cytomegalovirus (*CMV*), which is commonly used. In the Induced and Induced-Repressed modes, *Luc* is under the control of inducible response elements (such as $\text{NF-}\kappa\text{B}$, ISRE, p53, AP-1, etc), and specific triggers (for instance TNF stimulation or MyD88 overexpression for $\text{NF-}\kappa\text{B}$ pathway, MAVS overexpression for ISRE) can activate both the exogenous *Luc* gene, as well as endogenous genes involved in negative feedback inhibition (for instance A20 for $\text{NF-}\kappa\text{B}$ signaling). Negative feedback loops efficiently inhibit the pathways in the Induced-Repressed mode, but not in the Induced mode. *B*, theoretical impact of protein destabilization on overall protein levels, whose genes are expressed under the Constitutive, Induced, or Induced-Repressed modes. Of note, similar theoretical results are expected if the protein is unstable or if the mRNA translation is decreased. *TF*, transcription factor; *Luc*, Luciferase; *Inh*, inhibitor.

but, according to the molecular aggregation hypothesis presented above, the results might be explained by a different affinity of the two enzymes for LRR-induced aggregates. Alternatively, it is possible that differences relate to the fact that, in these assays, β -galactosidase is produced at a constitutive, poorly dynamic rate, while efficient inhibition of luciferase was associated with the capacity of the enzyme to be dynamically regulated. Indeed, by comparing the effects of NLRX1 or NLRC3 expression on constitutive *versus* inducible luciferase constructs we concluded that, although these NLR proteins blocked LRAs at a post-transcriptional level, the level of inhibition correlated with the dynamic inducibility of the gene. It is possible that increasing the rate of luciferase gene transcription/translation above a certain threshold favors the accumulation of the enzyme on LRR-induced protein aggregates in the cell.

The dependence on gene dynamic inducibility for full-blown inhibitory effects of NLRX1 and NLRC3 in LRAs evoked above could also possibly explain why these NLR proteins differentially affect LRAs under the control of various transcriptional response elements, according to the following model (see Fig. 7). In this theoretical model, *Luc* gene expression can be induced following three main kinetics (Fig. 7A): 1) Constitutive, such as in the case of *Luc* downstream of a constitutive promoter. 2) Induced-Repressed, in the case of *Luc* downstream of a transcriptional response element, such as $\text{NF-}\kappa\text{B}$. In this case, the induction of *Luc* expression is inhibited at late stages because of the up-regulation of potent $\text{NF-}\kappa\text{B}$ -dependent negative feedback mechanisms, such as (but not restricted to)

induction of A20 from its endogenous promoter, triggered simultaneously to the exogenous $\text{NF-}\kappa\text{B}$ -dependent promoter of the *Luc* gene. 3) Induced, for *Luc* gene induced by a specific transcriptional response element, but refractory to endogenous negative feedback mechanisms, which could happen if specific pathways are not, or are only weakly, under the control of negative feedback loops. Alternatively, this induction profile could also happen if the *Luc* gene is controlled by a specific transcriptional response element and that the transcription factor regulating this regulatory element is overexpressed. Indeed, co-expression of p53 together with p53-*Luc* would likely bypass putative endogenous negative feedback mechanisms controlling p53 pathway upstream of p53 transcription factor itself. Similar scenario would likely occur with $\text{NF-}\kappa\text{B}$ p65 overexpressed together with *Luc* controlled by an $\text{NF-}\kappa\text{B}$ -dependent response element. Considering that NLRX1 or NLRC3 overexpression would alter the stability of *Luc* protein or translation of *Luc* mRNA, the outcome of the expression of these NLR proteins on *Luc* levels is expected to be dramatically different depending on whether *Luc* gene was expressed under Constitutive, Induced-Repressed or Induced conditions (Fig. 7B). In particular, down-regulation of *Luc* protein levels would be maximal in the Induced-Repressed case, because of the additive effect of having decreased transcription rate and poor protein stability or translation rate. We believe that this model offers a plausible scenario to explain why NLRX1 overexpression was found to inhibit much better MyD88/Igk-*Luc* or MAVS/ISRE-*Luc* (Induced-Repressed), than p53/p53-*Luc* (Induced), or *CMV-Luc* (Constitutive).

One might question why activation of the NF- κ B pathway by Nod1 and Nod2 can be readily observed using LRA, given the fact that these NLR proteins also have LRR domains that are predicted, from our study, to inhibit luciferase activity nonspecifically. It is likely that the very potent capacity of these molecules to activate NF- κ B signaling largely overrides the inhibitory effect on LRAs, resulting in an overall increase in NF- κ B-dependent induction of luciferase activity. However, it is interesting to consider that in this scenario, truncation molecules of Nod1 or Nod2 that lack the LRR domain would be predicted to display increased capacity to activate NF- κ B-driven luciferase reporter constructs, due to the lack of inhibitory activity of the LRR domain. Indeed, this effect has been reported, and serves as a basis to formulate the hypothesis that LRR domains are normally inhibitory for NLR activation, and that NLR activation requires unfolding of the LRR domain following detection of the PAMP or DAMP (29, 30). In light of our results, validation of this LRR unfolding model would require to use alternative techniques, distinct from LRAs, to measure Nod1 or Nod2 activation.

Besides NLRX1 and NLRC3, NLRC5 is another NLR protein that was suggested to act as a repressor of multiple signaling pathways (17), while convergent evidence now suggest that this protein is a positive regulator of MHC class I expression (31–36). Using several assays, including LRAs, Cui *et al.* reported that NLRC5 overexpression potently inhibited NF- κ B and MAVS-dependent signal transduction pathways in human epithelial cell lines (17). Moreover, the inhibition was mediated by NLRC5 LRR domain (17), a result that, in light of the result from the present study, could potentially be explained by a post-transcriptional effect on LRAs. In our hands, we also noted that overexpression of NLRC5 repressed pro-inflammatory signal transduction pathways in LRAs (16). In the case of NF- κ B-dependent signaling, we presented a direct comparison of Luc activity in LRA with Luc expression levels by qPCR, following the control procedures proposed in the present manuscript (16). This suggested that overexpression of NLRC5 in human epithelial cells had an effect, albeit modest, on NF- κ B signaling that was not attributable to post-transcriptional inhibition of LRAs.

Our results strongly suggest that caution should be taken when studying the potential role of NLR proteins as negative regulators of signal transduction pathways. In the case of NLRX1, the previously reported role of the protein as a negative regulator of NF- κ B and type I interferon responses relied in part on LRAs and NLRX1 overexpression (2, 5, 6). Here, we complement the LRAs studies by silencing NLRX1 in human epithelial cells and demonstrate that MAVS-dependent antiviral signaling is intact during Sendai virus infection (Fig. 6). These observations are in agreement with results obtained by the group of Dr. J. Tschopp that had independently generated NLRX1-deficient mice, and observed normal MAVS-dependent antiviral responses in NLRX1-deficient cells (mouse embryonic fibroblasts and bone marrow-derived macrophages) infected with Sendai virus (7). Therefore, the exact role played by NLRX1 in innate immunity remains uncertain.

Overall, our results suggest that LRA can generate undesirable off-target effects in the case of the overexpression of mol-

ecules containing a LRR domain, which could lead to erroneous identification of proteins acting as repressors of signal transduction pathways. When using luciferase-expressing constructs to monitor the potential inhibitory role of a given protein on signal transduction pathways, it is advisable to systematically confirm the results by measuring *Luciferase* gene expression in qPCR.

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