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Coronin2A mediates actin-dependent de-repression of inflammatory response genes

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Abstract

Toll-like receptors (TLRs) function as initiators of inflammation through their ability to sense pathogen-associated molecular patterns and products of tissue damage[1,2]. Transcriptional activation of many TLR-responsive genes requires an initial de-repression step in which NCoR co-repressor complexes are actively removed from target gene promoters to relieve basal repression[3,4]. Ligand-dependent SUMOylation of liver X receptors (LXRs) potently suppresses TLR4-induced transcription by preventing the NCoR clearance step[5–7], but the underlying mechanisms remain enigmatic. Here, we provide evidence that Coronin 2A (Coro2A), a component of the NCoR complex of previously unknown function[8,9], mediates TLR-induced NCoR turnover by a mechanism involving interaction with oligomeric nuclear actin. SUMOylated LXRs block NCoR turnover by binding to a conserved SUMO2/3 interaction motif in Coro2A and

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Author contributions

W.H., S.G., K.S., M.C., B.L.G., M.G.R. and C.K.G. conceived the project and planned experiments and analysis, which were performed by W.H., S.G., K.S., M.G., M.A., G.T., D.Z and J.Y. The entire project was supervised by C.K.G., who wrote the manuscript with W.H.

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preventing actin recruitment. Intriguingly, the LXR transrepression pathway can itself be inactivated by inflammatory signals that induce CaMKIIγ-dependent phosphorylation of LXR, leading to its deSUMOylation by the SUMO protease SENP3 and release from Coro2A. These findings reveal a Coro2A/actin-dependent mechanism for de-repression of inflammatory response genes that can be differentially regulated by phosphorylation and nuclear receptor signaling pathways that control immunity and homeostasis.

To delineate mechanisms by which SUMOylated LXRs block signal-dependent clearance of NCoR complexes from TLR4-inducible promoters, we searched for potential SUMO interaction motifs (SIMs) within proteins associated with the NCoR complex that might mediate interactions with SUMOylated LXRs. This search identified a conserved motif in the carboxyl-terminus of Coro2A (Fig. 1a), that matches a recently identified SIM that is specific for SUMO2/3[10]. Coro2A is a member of the Coronin family of actin binding proteins that was identified as a component of the NCoR complex in nuclear extracts of HeLa cells[8,9] and is highly expressed in cells of the hematopoietic lineage[11,12]. All other members of the Coronin family have thus far been found to play roles in regulation of the actin cytoskeleton through interactions with F-actin[13,14], but functional roles of Coro2A in the NCoR complex have not been established. We confirmed Coro2A/NCoR interactions in primary macrophages by co-immunoprecipitation (Co-IP) assay and found that In contrast to other Coronin family members, Coro2A is primarily localized to the nucleus in primary bone marrow-derived macrophages (BMDMs) (Supplementary Fig. 2a, b). In addition, immunofluoresence microscopy demonstrated that Coro2A co-localizes to NCoR-rich regions of the nucleus (Fig. 1b). Chromatin immunoprecipitation (ChIP) studies further demonstrated that Coro2A is localized to NCoR target promoters such as the *Nos2* and *Ccl2* promoters in resting macrophages and its occupancy was reduced by treatment with the TLR4 ligand lipopolysaccharide (LPS) (Fig. 1c). Sequential ChIP experiments indicated that Coro2A and NCoR reside together on the *Nos2* promoter (Supplementary Fig. 2c). Reduction of NCoR expression in primary macrophages using specific siRNAs resulted in a corresponding reduction in Coro2A occupancy on the *Nos2* and *Ccl2* promoters without affecting total Coro2A protein expression (Supplementary 2d, e). In contrast, Coro2A was not found on the *Csf3* promoter (Supplementary Fig. 2d), which is not a target of NCoR repressison[15].

To investigate the potential role of Coro2A as a molecular beacon for SUMOylated LXRs, co-immunoprecipitation (Co-IP) studies were performed in which HeLa cells were transfected with Flag-tagged wild type LXRβ or a mutant of LXRβ (K410/448R) that cannot be SUMOylated[5] and treated with the synthetic LXR agonist GW3965 (GW). The mutant form of LXRβ did not co-precipitate with Coro2A, whereas wild type LXRβ was co-precipitated with Coro2A and migrated at the expected molecular weight for SUMO-LXRβ (Fig. 1d). This interaction was largely dependent on treatment with GW3965 (Supplementary Fig. 3a). In addition, in vitro transcribed and translated Coro2A preferentially interacted with recombinant GST-LXRβ conjugated to SUMO3 in vitro, as compared to unconjugated GST-LXRβ or de-SUMOylated LXRβ (Supplementary Fig. 3b). Next, ChIP experiments were performed in primary macrophages following transfection with control or Coro2A-specific siRNAs. Knockdown of Coro2A expression resulted in an

almost complete loss of recruitment of LXR to the *Nos2* and C*cl2* promoters in response to GW3965 (Fig. 1e). Although a recent study reported that GPS2 was required for recruitment of SUMOylated LXRβ to the *Crp* and *Hp* promoters in liver[7], GPS2 is not present above IgG background on the *Nos2* or *Il1b* promoters as determined by ChIP assay, and GPS2 knockdown had no impact on LXR transrepression of *Nos2* or *Il1b* in primary macrophages (Supplementary Fig. 4a). Point mutations were therefore introduced into the SIM of Coro2A to evaluate its potential importance in recruiting SUMO-LXRs. HeLa cells were transfected with Flag-tagged wild type or SIM mutant versions of Coro2A and treated with vehicle or GW3965. Both WT and SIM mutant Coro2A were immunoprecipitated by anti-NCoR antibody, but only WT Coro2A interacted with LXR, and this interaction was enhanced by treatment with GW3965 (Fig 1f). In addition, a mammalian two-hybrid assay indicated that LXR interacts with Coro2A in a ligand- and SIM motif-dependent manner (Supplementary Fig. 3c). When over-expressed in RAW264.7 macrophages, this Coro2A SIM mutant occupies the NCoR-residing *Nos2* promoter (Supplementary Fig. 4b). However, ligand-mediated recruitment of LXR and its repression function was significantly reduced by overexpression of this mutant, consistent with a dominant-negative function (Fig. 1g and Supplementary Fig. 4c). In contrast, dexamethasone repression mediated by the glucocorticoid receptor, via an NCoR and SUMOylation independent mechanism[16], remained unaffected (Fig 1g).

Unexpectedly, knockdown of Coro2A expression resulted in impaired responsiveness of a subset of TLR inducible NCoR target genes, including *Nos2*, *Il10*, *Il1b* and *Ccl2*, but not of non-NCoR-regulated promoters, such as *irf1* (Fig. 2a). ChIP experiments further revealed that knockdown of Coro2A resulted in a failure of LPS to promote clearance of NCoR from the *Nos2* and *Il1b* promoter (Fig. 2b and Supplementary Fig. 5a). As there is increasing evidence for diverse transcriptional roles of oligomeric nuclear actin[17], we considered the possible role of nuclear actin in NCoR clearance. We confirmed that Coro2A interacts with actin and using conserved sequence features required for the interaction of murine Coronin1 with actin[14,18,19], introduced point mutations in Coro2A (K11A/R13A) that severely compromised actin binding (Supplementary Fig. 5b). Overexpression of either an N-terminal deletion mutant of Coro2A lacking the actin-binding domain (ΔN), or the Coro2A K11A/R13A mutant blocked LPS-induced NCoR clearance and impaired activation of the *Nos2* promoter in RAW264.7 macrophages (Fig. 2c, d). ChIP assays using an antibody (2G2) that specifically recognizes nuclear actin[20] revealed transient recruitment of actin to the *Nos2* (Fig. 2e) and *Il1b* (Supplementary Fig. 5c) promoters coincident with the timing of LPS-induced NCoR clearance. Actin was not recruited to the TLR4-inducible, NCoR-independent *Csf3* promoter or the *Abca1* promoter, which is a positive transcriptional target of LXRs that is occupied by NCoR in the absence of LXR agonists[4,21] (Supplementary Fig. 5c). Actin recruitment was dependent on both NCoR and phosphorylation of cJun, as it was abolished in primary macrophages by NCoR knockdown and in RAW264.7 macrophages by overexpression of a mutant form of c-Jun that cannot be phosphorylated (Supplementary Fig. 5d-e). LPS-induced actin recruitment to the *Nos2* promoter in macrophages was also abolished by siRNA-mediated knockdown of Coro2A (Fig. 2e). Short-term treatment of primary macrophages with latrunculin A, which inhibits actin polymerization[22], similarly blocked LPS dependent NCoR clearance and induction of *Nos2* mRNA (Supplementary Fig.

6a, b). In contrast, latrunculin A did not affect TLR4 activation of the NCoR-independent *Irf1* gene, LXR activation of the *Abca1* gene (Supplementary Fig. 6c-e) or TLR4-dependent phosphorylation of cJun (Supplementary Fig. 6f, g). Notably, treatment of primary macrophages with the LXR ligand, GW3965, blocked LPS-induced actin recruitment (Fig. 2f), but not cJun phosphorylation (Supplementary Fig. 6h), suggesting that SUMOylated LXRs may exert anti-inflammatory effects by blocking the actin-dependent step required for NCoR clearance.

LXRs are unable to suppress transcriptional responses of NCoR target genes to pro-inflammatory signals that induce CaMKII activation, including the TLR1/2 agonist Pam3CSK4[21], the TLR2/6 agonist FSL-1, and *Salmonella typhimurium* [23] (Supplementary Fig. 7a, b). Co-IP studies in RAW264.7 macrophages revealed that Pam3CSK (Pam3) signaling significantly reduced interaction of SUMO-LXRβ with Coro2A in a CaMKIIγ-dependent manner (Fig. 3a). Because interaction of LXR with Coro2A requires SUMOylation, we considered the possible involvement of deSUMOylating enzymes in negatively regulating LXR repression functions. Using siRNAs to knock down SUMO proteases expressed in primary macrophages, we found that knockdown of SENP3 restored the ability of LXRs to suppress TLR1/2-dependent *Nos2* expression (Fig. 3b), interact with the Nos2 promoter, and prevent NCoR clearance (Fig. 3c). ChIP experiments further demonstrated that SENP3 was rapidly recruited to the *Nos2*, but not the *Csf3*, promoter following Pam3 stimulation, and this recruitment was dependent on CaMKIIγ (Fig. 3d). Knockdown of CaMKIIγ also restored LXR transrepression of *Il1b* and *BIC* (precursor of miR-155) as well as abolished SENP3 recruitment to these gene promoters (Supplementary Fig. 7c–f).

Co-immunoprecipitation experiments suggested a sequence in which Pam3 induced CaMKII-LXR interaction, followed shortly thereafter by SENP3-LXR interaction (Supplementary Fig. 8a). As both LXRα and LXRβ contain a conserved motif that conforms to a CaMKII phosphorylation site (S427 in LXRβ, Fig. 3e), we considered the possibility that LXRs were themselves the targets of CaMKIIγ kinase activity and that phosphorylation of LXR might promote its interaction with SENP3. Consistent with this possibility, TLR1/2 activation was found to result in robust serine phosphorylation of LXRβ in a CaMKIIγ-dependent manner (Fig. 3e). WT-Flag-tagged LXRβ was a substrate for recombinant CaMKIIγ in vitro, but Flag-tagged LXRβ with a serine to alanine point mutation at the 427 residue was not (Fig. 3f and Supplementary Fig. 8b). In addition, the phospho-null mutant of LXR continued to associate with Coro2A-NCoR bound *Nos2* promoter and retained transrepression activities upon TLR1/2 signaling (Fig. 3g and Supplementary Fig. 8c), recapitulating the effect of knocking down CaMKIIγ[21] and suggesting that LXR S427 is likely the major or only substrate of CaMKII relevant to inactivation of the LXR transrepression pathway. In contrast, a phospho-mimic mutant of LXR (S427D) was not able to interact with Coro2A to promote transrepression of the *Nos2* luciferase reporter in RAW64.7 macrophages (Supplementary Fig. 8d, e).

To investigate whether CaMKII phosphorylation of LXRβ created a docking site for SENP3, RAW264.7 macrophages were transfected with expression vectors for HA-LXRβ, Flag-SENP, and control or CaMKIIγ-specific siRNAs and treated with GW3965 in the absence or

presence of Pam3. Immunoprecipitation assays demonstrated that TLR1/TLR2 activation induced rapid interaction of SENP3 with LXRβ that was dependent on CaMKIIγ (Fig. 3h). This interaction was not observed with the LXRβ S427A mutant (Fig. 3i).

We next used a thioglycollate-induced sterile peritonitis model [24] to investigate potential roles of the Coro2A-NCoR clearance pathway *in vivo*. Injection of thioglycollate into the peritoneal cavity leads to massive infiltration of activated macrophages, which by three days following injection account for more than 90% of the total peritoneal cavity cell population. *In vivo* knockdown of Coro2A expression in this macrophage population using two independent methods significantly reduced expression of *Ccl2*, *Il1b*, and *Cxcl10,* but not *Abca1* (Fig. 4a and Supplementary Fig. 9a) and resulted in significantly increased NCoR occupancy (Fig. 4b). The presence of phosphorylated (active) CaMKII in the elicited macrophage population (Supplementary Fig. 9b), suggested that an endogenous LXR repression pathway in these cells might be inactivated. Consistent with this possibility, CoIP studies demonstrated that LXRα/β were not associated with Coro2A in elicited macrophages in control animals, but became associated with Coro2A in animals that were treated with the CaMKII inhibitor, KN93, (Supplementary Fig. 9b). In addition, KN93 treatment increased NCoR occupancy on the *Ccl2*, *Cxcl10* and *Il1b* promoters in macrophages in WT mice, but not in LXRα/β double knockout mice (Fig. 4c). Finally, KN93 treatment reduced the expression of *Ccl2*, *Cxcl10* and *Il1*β in wild type mice, but not in LXR double knockout mice (Fig. 4d), suggesting that pharmacological inhibition of CaMKII ‘rescues’ endogenous LXR transrepression activity in this inflammatory context.

Collectively, these findings provide evidence that Coro2A functions as an NCoR exchange factor that is required for de-repression of TLR target genes in the macrophage (Supplementary Fig. 1). Recent findings providing evidence for roles of nuclear actin in movements of specific chromosomal loci to transcriptional ‘hubs’ [25,26] raise a number of interesting possibilities with respect to dynamic regulation of the localization of repression machinery. The ability of other Coronins to mediate disassembly of actin polymers in the cytoplasm[14,27] suggests that Coro2A may function to release inflammatory response genes from localized actin networks dedicated to repression functions. The integrated functions of Coro2A as both a docking site for SUMOylated LXRs and as an actin-dependent NCoR exchange factor suggest a parsimonious transrepression model in which SUMOylated LXRs prevent NCoR turnover and repress responses to LPS by blocking actin recruitment. The ability of CaMKIIγ to inactivate the LXR transrepression pathway by creating a docking site for SENP3 further identifies a new transcriptional role for SUMO proteases and reveals an entry point for negative regulation of the LXR repression pathway that could be engaged by numerous other signaling pathways that mobilize intracellular calcium, such as purinergic receptors[21]. It is notable that the SUMO2/3-specific SIM in Coro2A and the CaMKIIγ phosphorylation sites in LXRs are highly conserved across vertebrate species, suggesting evolutionary pressure to maintain these regulatory pathways. While such pressures presumably relate to key functions of the innate immune system in responding to infection and injury, signal-induced inactivation of the LXR transrepression pathway may also contribute to pathological forms of inflammation.

Methods Summary

BMDM were generated from 6-wk old wildtype C57BL/6 (Harlan) and LXRα/β$^{-/-}$ mice as described in [15]. RAW264.7 cells were cultured as described in [5]. LPS, Pam3CSK4, and GW3965 were used at a concentration of 100ng/ml, 300ng/ml, and 1μM respectively. GW3965 was used to pre-treat macrophages for 1hr. LPS and Pam3CSK4 treatment was 1hr for ChIP assays and 6hrs for luciferase and mRNA expression analysis unless otherwise noted in the figure. ChIP assays were performed as described in detail in [15]. Proximal promoter regions of *Nos2, Ccl2, Il1b, Csf3, Abca1, and BIC* were amplified by real-time PCR. mRNA values determined by real time PCR are normalized to GAPDH mRNA content. Data are represented as mean +/− SD of three independent experiments in duplicates. RAW264.7 or HeLa cells were transfected with wildtype or mutant expression vectors for LXR and Coro2A as described in [21]. For RNAi experiments in cultured primary macrophages, scrambled control or smart-pool siRNAs (Dharmacon) against Coronin2A, SENP3, CaMKIIγ, and NCoR were transfected into primary macrophages using lipofectamine 2000 (Invitrogen) as described [15]. For in vitro CaMK kinase assays, 0.5μg recombinant CaMKIIγ (Upstate) was incubated with bacterially purified GST-LXRβ (full-length) fusion proteins bound on glutathione beads. Thiogycollate elicited sterile peritonitis was induced as previously described [28]. Briefly, 2ml of thiogycollate medium was delivered to in each animal by i.p. injection and elicited macrophages in the peritoneal cavities were harvested with 10ml of PBS after three days. For in vivo RNAi experiments, 100μg scrambled or Coronin2A specific siRNA were complexed in lipofectamine 2000 and serum depleted medium in a final volume of 1ml and delivered to animals by i.p. injection as previously described in [29]. Alternatively, GeRP capsulated scrambled and Coronin2A specific siRNA were prepared and delivered to animals by i.p. injection for 5 consecutive days as described in [30].

Methods

Reagents and plasmids

LPS and Flag M2 antibodies were obtained from Sigma. Pam3CSK4 was from InvivoGen. GW3965 was kindly provided by GlaxoSmithKline. 22R and T0901317 were purchased from Sigma and 25EC from Biomol. KN93 was purchased from CalBiochem. Goat anti-Coro2A, rabbit anti-Coro2A, CaMKII, SENP3, LXRa/b, Myc, HDAC4, goat anti-NCoR and normal rabbit/goat antibodies were from Santa Cruz Biotech. Rabbit anti-NCoR antibodies were from ABR. α-tubulin and phospho-CaMKII antibodies were from Cell Signalling and phosphor-serine antibodies were from Chemicon. The reporter plasmid *nos2*-luc has been previously described[4] . Expression vector for VP16LXRb was obtained from Xceptor. Expression constructs, Myc-SUMO2, FlagLXRb, HaLXRb had been previously described[5]. Mutations in expression vectors were made using the QuickChange site-directed mutagenesis kit (Stratagene).

Plasmid and siRNA Transfection

Transient transfections in RAW264.7 cells were performed as described in[5] using Superfect reagent (Qiagen). For siRNA experiments in RAW264.7 cells, transfections were carried out

with (100nM) using Superfect reagent for 48 h before activation with TLR ligands. Transfection data are represented as mean +/− SD of three independent experiments in triplicates. For RNAi experiments in cultured primary macrophages, scrambled control or smart-pool siRNAs (Dharmacon) against Coronin2A, SENP3, CaMKIIγ, and NCoR were transfected into primary macrophages using lipofectamine 2000 (Invitrogen) as described[15].

siControl Non-targeting siRNA Pool(Dharmacon, D-001206-13): AUGAACGUGAAUUGCUCAA, UAAGGCUAUGAAGAGAUAC, AUGUAUUGGCCUGUAUUAG, UAGCGACUAAACACAUCAA; siGenome SMART pool for mouse Coro2A (Dharmacon, D-058157): GAAAGUUGGACCCUCACUA, GCAAGAUUCGGAUUGUUGA, GGAUCGGUAUCAUGCCAAA, GAGGGAACGUCUUGGACAU; siGenome SMART pool for mouse SENP3 (Dharmacon, D-057149): GGACCACAGUGCCAACUAG, CGACGUACCAUCACCUAUU, GCUCUCCGACCCUCUCAUA, CAAACUCCGUCGUCAGAUC; siGenome SMART pool for mouse CaMKIIg (Dharmacon, D-040009): GGAAAGAUCCCUAUGGAAA, GUAGAGUGCUUACGCAAAU, GGAGUUGUUUGAAGACAUU, AAACAUCUACGCAGGAAUA; siGenome SMART pool for mouse NCoR1 (Dharmacon, D-058556): GAAAUCCCACGGCAAGAUA, CCAGGUCGAUGACAAGUGA, GCAGUGGAAGGAAGUAUAA, GAUCAUCACCCGGCAAAUU. Note that siRNA in pools of four were used in in vitro studies and the siRNAs underlined were used for the in vivo experiments.

Preparation of GeRPs containing Coro2A siRNA

β1,3-d-glucan particles were purified from baker's yeast by a series of alkaline and solvent extractions, hydrolysing outer cell wall and intracellular components and yielding purified, porous 2 4 μm β1,3-d-glucan particles, as previously described[30]. To load siRNA into glucan shells for 1 mouse injection, 5 nmoles Coro2A siRNA was incubated with 50nmoles Endo-Porter peptide (Gene Tools) in acetate buffer pH 4.8 for 15 min at 21 degrees C. The siRNA/Endo-Porter solution was added to 1 mg glucan shells and then vortexed and incubated for 1 hour. TRIS-EDTA buffer pH 8.4 was added to the particles and incubated for 15 min at RT to adjust the pH. The resulting glucan encapsulated siRNA Particles (GeRPs) were resuspended in PBS for intraperitoneal (i.p.) injections.

RNA isolation and real time PCR

Total RNA (isolated by RNeasy kit, Qiagen) was prepared from MEFs or macrophages treated as indicated in each legend. One μg of total RNA was used for cDNA synthesis, and 1 μl of cDNA was used for real time PCR analysis was performed on an Applied Biosystems 7300 Real-time PCR system. Values are normalized with GAPDH content. Data are represented as mean +/− SD of at least three independent experiments in duplicates.

Primers for mRNA-QPCR analysis—*Gapdh* (AATGTGTCCGTCGTGGATCT, CATCGAAGGTGGAAGAGTGG); *Nos2* (AGCCTTGCATCCTCATTGG, CACTCTCTTGCGGACCATCT); *Irf1* (AGGCCGATACAAAGCAGGAGA, GCTGCCCTTGTTCCTACTCTG); *Il1b* (GCAACTGTTCCTGAACTCAACT, ATCTTTTGGGGTCCGTCAACT); *Ccl2* (CCCAATGAGTAGGCTGGAGA,

TCTGGACCCATTCCTTCTTG); *Senp3* (TTTGACTCCCAGCGAACTCT, AAAGAGACTATACAGGGGACCG); *Coro2A* (AGGGGATCGGTATCATGCCAA, CATGGACACTGGCTCGATGAG); *NCoR* (CTGTGCATGAGAAGCAGGAC, TGGTGAAGAATGAAGGCAAG); *Abca1* (AAAACCGCAGACATCCTTCAG, CATACCGAAACTCGTTCACCC); *BIC* (AAACCAGGAAGGGGAAGTGT, GTCAGTCAGAGGCCAAAACC); *CaMKII*γ (AACAAAAACAGTCTCGTAAGCCC, GCCCTTGATCCCATCTGTAGC) *Gps2* (AGGCGAAAGGAACAGAGTGA, GAGTACCTGGGCGATTGTGT).

Primers for promoter ChIP-QPCR analysis—*Nos2* (GGAGTGTCCATCATGAATGAG, CAACTCCCTGTAAAGTTGTGACC); *Il1b* (GGACAATTGTGCAGATGGTG, CCTACCTTTGTTCCGCACAT); *BIC* (GCTGGAGACAAGTCCCTTGA, TCCTCCAGTGATTCCTTTGG); *Ccl2* (TCCAGGGTGATGCTACTCCT, AGTGAGAGTTGGCTGGTGCT); *Hp* (CACTAAGGCAGCATGGTTGA, TGGGAGAGGGTGTTGGTTAG); *Csf3* (CCTACCTAGGGTGCTGTGGA, GGACAAACATCCCGAGAGAA), *Abca1* (GGGGCTGAGCAAACTAACAA, TTGTGGGTGTTGCTTTTTGA).

Co-IP assay

RAW264.7 or HeLa cells were transfected with wildtype or mutant expression vectors for LXR and Coro2A. Cells were lysed in 10 mM Tris-HCl ph 7.5, 450 mM NaCl, 0.5% NP40, 1mM EDTA with protease inhibitors and 10uM NEM. Co-IPs were carried out with anti-SENP3, LXR, CaMKII, NCoR, or Flag antibodies and detected by western blotting.

Kinase Assays

For in vitro CaMK kinase assays, 0.5ug recombinant CaMKIIγ (Upstate) was incubated with bacterially purified GST-LXRb (full-length) fusion proteins attached to glutathione beads. The beads were washed, boiled, and then subjected to SDS-PAGE. Serine phosphorylations were detected by anti-p-Serine antibody (Chemicon).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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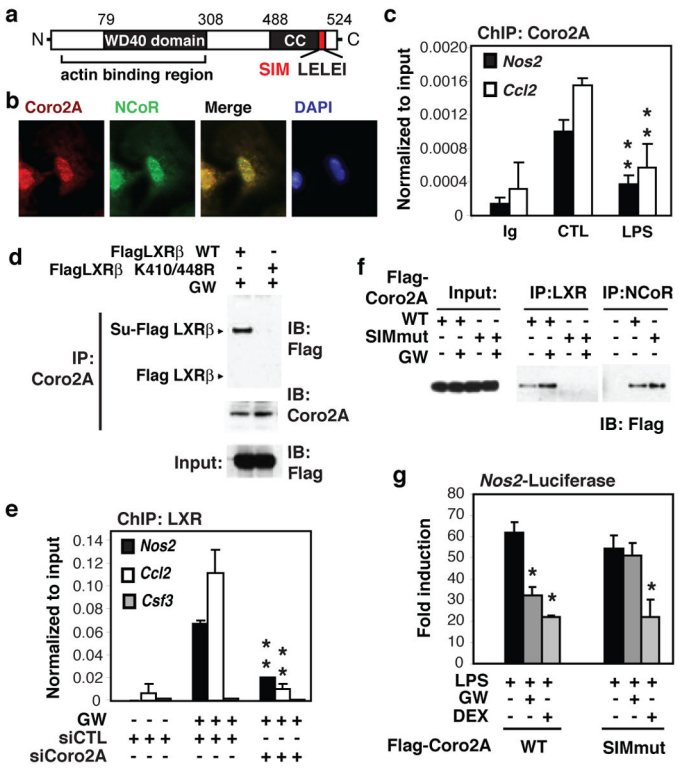

Figure 1. SUMO interaction motif of Coronin2A is required for recruiting SUMOylated LXR to NCoR-residing proinflammatory gene promoters

a, Known and predicted domain structure of mCoro2A. (CC: coiled-coil domain; SIM: SUMO2/3 interaction motif). **b,** Bone marrow-derived macrophages (BMDMs) were analyzed for Coro2A and NCoR expression and localization using fluorescence microscopy (magnification ×60). c, ChIP for Coro2A-binding to *Nos2* and *Ccl2* promoters in BMDMs. **d,** Lysates from HeLa cells transfected with the indicated LXRβ expression vectors and treated with GW3965 were subjected to immunoprecipitation (IP) with Coro2A-specific antibody and analyzed by immunoblotting (IB) against Flag. Su-Flag LXR; expected migration position for SUMO-LXR. **e,** ChIP for LXR-binding to the *Nos2*, *Ccl2* and *Csf3* promoters in BMDMs after siRNA transfection treated with or without GW3965. The *Csf3* promoter serves as a negative control. Knockdown efficiencies are provided in Supplemental Fig. 10. **f,** Lysates from HeLa cells transfected with the indicated vectors and treated with GW3965 were subjected to IP with LXR or NCoR-specific antibodies and analyzed by IB against Flag. **g,** Luciferase assays were performed in RAW264.7 cells transfected with *Nos2*-luc reporter plasmids and the indicated Coro2A expression vectors, and stimulated with LPS in the presence of GW3965 or dexamethasone (DEX). (Bar graphs are averages ± s.e.m., **P<0.04 versus control *P<0.02 versus LPS).

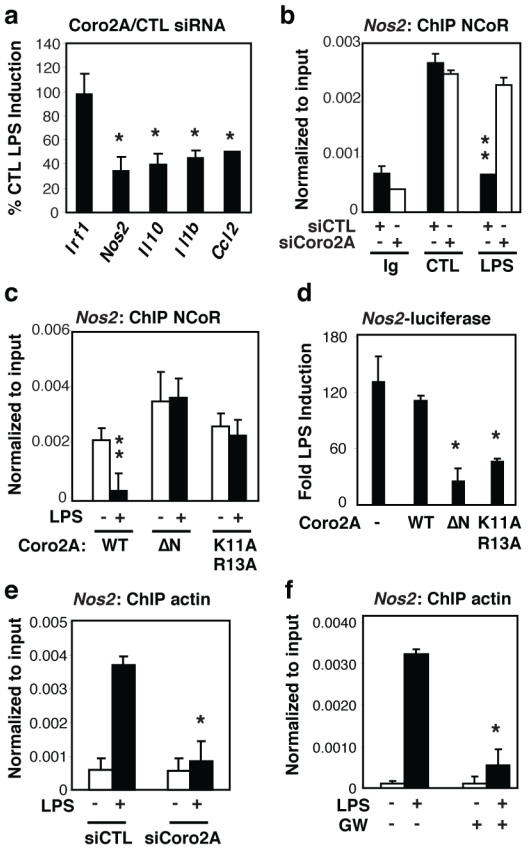

Figure 2. The actin-binding domain of Coronin2A is required for LPS de-repression of inflammatory gene promoters

a, BMDMs were transfected with Coro2A or control siRNAs and treated with or without LPS. Total RNA was used for assay of the indicated transcript levels by Q-PCR. Results are plotted as fold LPS induction in siCoro2A cells/fold LPS induction in siCTL cells × 100. **b,** ChIP for NCoR-binding to the *Nos2* promoter in BMDMs after siRNA transfection followed by 1hr of LPS stimulation. **c,** ChIP for NCoR-binding to, *Nos2*-luc promoter in RAW264.7 cells transfected with *Nos2*-luc reporter plasmids and the indicated expression vectors and stimulated with LPS. **d,** Luciferase assays were performed in RAW264.7 cells transfected with *Nos2*-luc reporter plasmids and the indicated expression vectors and stimulated with LPS. Luciferase activities in cells not treated with LPS were normalized to a value of 1.0. **e,** ChIP for actin-binding to *Nos2* promoter in BMDMs after siRNA transfection followed by 5min of LPS stimulation. **f,** ChIP for actin-binding to the *Nos2* promoter in BMDMs treated with LPS for 5min pretreated with or without GW3965. (Averages ± s.e.m., **P<0.05 versus control *P<0.05 versus LPS).

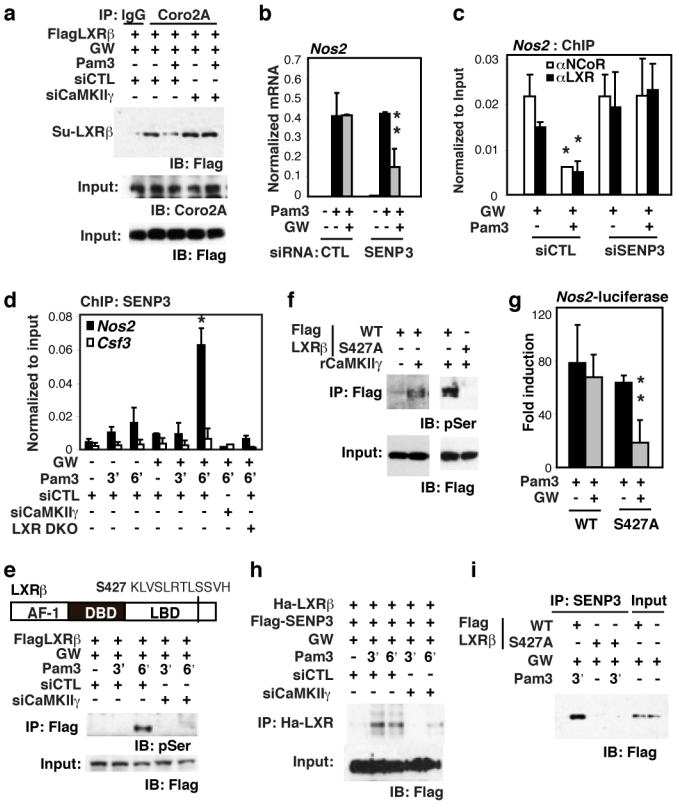

Figure 3. LXR-Coro2A axis is disrupted by CaMKII γ phosphorylation and SENP3 deSUMOylation of LXR.

a, RAW264.7 cells were transfected with Flag-LXRβ expression vector and siRNAs as indicated. Cells were pretreated with GW3965 and challenged with Pam3CSK4. Lysates were subjected to IP with Coro2A antibodies and analyzed by IB against Flag. Knockdown efficiencies for CamKIIγ are provided in Supplemental Fig. 10c. **b,** BMDMs were transfected with control or SENP3 siRNAs and pretreated with or without GW3965 followed by Pam3CSK4. RNA transcript levels were assayed by Q-PCR. Knockdown efficiencies for SENP3 are provided in Supplemental Fig. 10d. **c,** ChIP for LXR and NCoR-binding to the *Nos2* promoter in BMDMs after siRNA transfection. **d,** ChIP for SENP3 occupancy of the *Nos2* promoter in siRNA transfected wildtype or LXRα/β$^{-/-}$ BMDM. Cells were pretreated with GW3965 and then stimulated with Pam3CSK4. **e,** Domain structure of LXR. (AF-1: Activation Function-1 domain; DBD: DNA binding domain; LBD: ligand binding domain). A predicted CaMKII phosphorylation site at Serine (S) 427 in the LBD is highlighted. RAW264.7 cells were transfected with Flag-LXRβ expression vector and control or CaMKIIγ-specific siRNAs. Cells were treated with GW3965 and challenged by Pam3CSK4 for 3 and 6 min. Lysates were subjected to IP with Flag antibody and IB for phosphoserine (top) or Flag (bottom). **f,** Flag-LXRβ WT or S427A mutant were expressed in HeLa cells, captured on anti-Flag-agarose, and incubated with 0.5μg of activated rCaMKIIγ. Anti-phospho-serine antibody was used to detect phosphorylated proteins by IB. **g,** RAW264.7 cells were transfected with *Nos2*-luc reporter plasmids and the indicated expression vectors and treated with GW3965 followed by Pam3CSK4. Luciferase activity

was represented as fold induction compared to untreated cells. **h,** RAW264.7 cells were transfected with expression vectors for Flag-SENP3 and Ha-LXRβ, and control or CaMKIIγ specific siRNAs as indicated. Lysates were subjected to IP with anti-Ha antibody, and SENP3 was detected by Flag antibody. **i,** RAW264.7 cells were transfected with the indicated Flag-LXRβ expression vectors, treated with GW3965, and challenged with Pam3CSK4. Lysates were used in IP assays with SENP3 specific antibody, and analyzed by IB against Flag. (Averages ± s.e.m., **P<0.04 versus Pam3 *P<0.04 versus control).

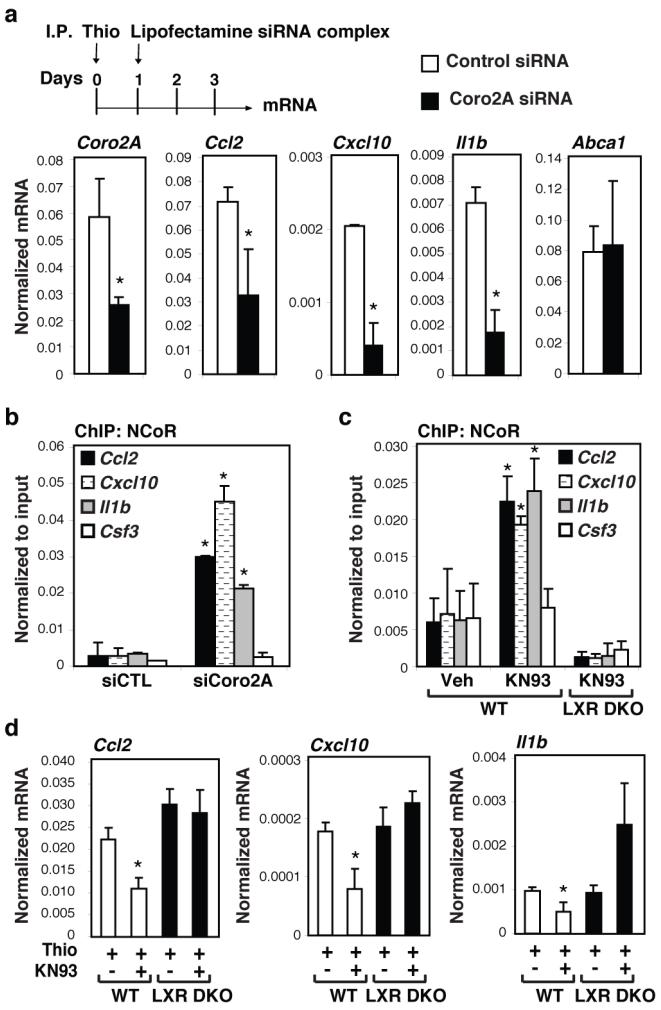

Figure 4. Function of the Coro2A-LXR-CaMKII pathway in thioglycollate-elicited peritonitis

a, Sterile peritonitis was initiated by intraperitoneal (I.P.) injection of thioglycollate (Thio) on day 0. Mice were injected with lipofectamine-siRNA complexs on day one, and peritoneal exudate cells were recovered for analysis of *Coro2A*, *Ccl2*, *Cxcl10*, *Il1*β and *Abca1* mRNA on day 3 (n=4 per condition). (Values are normalized to *Gapdh*. Averages ± s.e.m., *P<0.05 versus siCTL). **b,** ChIP for NCoR-binding to the *Ccl2*, *Cxcl10*, *Il1b*, and *Csf3* promoters in peritoneal exudate cells described in **a** (n=3 per condition). (Averages ± s.e.m., *P<0.05 versus siCTL). **c,** ChIP for NCoR-binding to the *Ccl2*, *Cxcl10*, *Il1b*, and *Csf3* promoters in peritoneal exudate cells derived from wild type (WT) or LXRα/β double knockout (DKO) mice treated with KN93 as indicated (n=3 per condition). (Averages ± s.e.m., *P<0.05 versus non-KN93 treated wildtype animals). **d,** Expression of *Ccl2*, *Cxcl10*, and *Il1b* expression in peritoneal exudate cells derived from WT (n=5 per condition) or LXRα/$\beta^{-/-}$ (n=3 per condition) mice treated with KN93 as indicated. (Averages ± s.e.m., *P<0.01 versus non-KN93 treated wildtype animals). Statistical significance was determined by a two-tailed Student's t-test.