A NIK–SIX signalling axis controls inflammation by targeted silencing of non-canonical NF-κB

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The non-canonical NF-κB signalling cascade is essential for lymphoid organogenesis, B cell maturation, osteoclast differentiation, and inflammation in mammals1,2; dysfunction of this system is associated with human diseases, including immunological disorders and cancer3–6. Although expression of NF-κB-inducing kinase (NIK, also known as MAP3K14) is the rate-limiting step in non-canonical NF-κB pathway activation6,7, the mechanisms by which transcriptional responses are regulated remain largely unknown. Here we show that the sine oculis homeobox (SIX) homologue family transcription factors SIX1 and SIX2 are integral components of the non-canonical NF-κB signalling cascade. The developmentally silenced SIX proteins are reactivated in differentiated macrophages by NIK-mediated suppression of the ubiquitin proteasome pathway. Consequently, SIX1 and SIX2 target a subset of inflammatory gene promoters and directly inhibit the trans-activation function of the transcription factors RELA and RELB in a negative feedback circuit. In support of a physiologically pivotal role for SIX proteins in host immunity, a human SIX1 transgene suppressed inflammation and promoted the recovery of mice from endotoxic shock. In addition, SIX1 and SIX2 protected RAS/P53-driven non-small-cell lung carcinomas from inflammatory cell death induced by SMAC-mimetic chemotherapeutic agents (small-molecule activators of the non-canonical NF-κB pathway). Our findings identify a NIK–SIX signalling axis that fine-tunes inflammatory gene expression programs under both physiological and pathological conditions.

Our investigation into mechanisms of cell-autonomous immunity revealed that the sine oculis (SO) homeobox family members SIX1 and SIX2 are integral components of the non-canonical NF-κB signalling cascade. In brief, we found that long-term exposure of U-2 OS human osteosarcoma cells with CD40 ligand (TNFSF5)8 restricted infection by two evolutionarily diverse intracellular pathogens, Gram-positive Listeria monocytogenes and Gram-negative Shigella flexneri (Fig. 1a, b). This cell-autonomous immune mechanism was dependent on signalling through the non-canonical NF-κB kinase NIK, but not the canonical NF-κB kinase TAK1 (also known as MAP3K7) (Extended Data Fig. 1a–g). In addition, ectopic expression of NIK, but not TAK1, potently inhibited bacterial infection (Fig. 1c, Extended Data Fig. 1h–j).

Fig. 1 | SIX proteins exhibit immunomodulatory functions.

a, b, Diagram and data showing lentiviral delivery of CD40L–RFP into U-2 OS cells and its effect on infection with GFP-expressing L. monocytogenes (LmGFP). a, Representative flow cytometry scatter plots showing (1) luciferase (Fluc)RFP-transduced uninfected cells; (2) untransduced LmGFP-infected cells; (3) FlucRFP-transduced cells infected with LmGFP; and (4) CD40L-RFP-transduced cells infected with LmGFP. b, Quantification of infection of CD40L-transduced cells with L. monocytogenes or S. flexneri (SF) as indicated. Bacterial infectivity was normalized to Fluc control (shown as 100%). Data are mean ± s.d. from six independent experiments; ****P < 0.0001 derived from biological replicates using one-way ANOVA (GraphPad). The same statistics were applied to later studies unless otherwise stated. c, Fibroblasts transduced with Fluc or NIK lentivirus and then infected with GFP-expressing L. monocytogenes or S. flexneri as indicated. Quantification of infection is presented as in b. Data are mean ± s.d. from six independent experiments, ****P < 0.0001. Representative flow cytometry scatter plots show infection efficiency as in a. d, Repeated trials of NIK-stimulated genes that inhibit (green) or enhance (red) infection by bacterial pathogens (L. monocytogenes or S. flexneri), positive single-stranded RNA viral pathogens (EAV, equine arteritis virus; WNV, West Nile virus; SINV, Sindbis virus), and negative single-stranded RNA viral pathogen (PIV3, parainfluenza virus type 3). The relative percentage of pathogen infection was normalized to Fluc control (black dotted line). Data are presented as box and whisker plots; box shows 25th and 75th percentiles, black line shows population median, whiskers show highest and lowest values (six independent experiments). *P < 0.05; **P < 0.01; ***P < 0.001; NS, no significant difference. e, Representative flow cytometry scatter plots showing L. monocytogenes infection efficiency in Fluc and SIX2 expressed fibroblasts as described in a. Data are representative of 6 independent experiments.

**NOTE: This paper contains corrections and updates to the original version, including changes to the abstract and some sections of the text. The corrections and updates are highlighted in the text.**
Previous studies have indicated that NIK also inhibits infection with both positive- and negative-sense single-stranded RNA viruses\(^1\), suggesting that activation of the non-canonical NF-κB signalling pathway is broadly antimicrobial.

To identify key genetic factors involved in the antimicrobial response to activation of non-canonical NF-κB, we generated a cDNA library encompassing 237 genes that were induced by ectopic expression of NIK (mimicking the antimicrobial conditions in Fig. 1c). We evaluated the rates of bacterial and viral infection in host cells transduced with each of the 237 NIK-stimulated genes in a one-gene to one-well format (Extended Data Fig. 2, Supplementary Tables 1, 2). A subset of NIK-stimulated genes reproducibly inhibited either bacterial or viral infection, including components of the non-canonical NF-κB signalling cascade (for example, CD40, MAP3K8, and RELB) and antiviral effectors (for example, IRF1, OAS2, and IFI6) (Fig. 1d). However, two homologous genes—SIX1 and SIX2—specifically caught our attention because, although these genes were activated by NIK, they induced an opposite phenotype to those induced by other NIK-stimulated genes, in that they potently enhanced bacterial and viral infection of host cells (Fig. 1d, e).

SIX1 and SIX2 are lineage-specific transcription factors that define progenitor cell identity in developing organs and are thought to be silenced in adult tissues\(^10,15\). We investigated whether endogenous SIX proteins are reactivated in terminally differentiated immune cells under conditions of physiological infection. Infection of mouse primary bone-marrow-derived macrophages (BMDMs) with \textit{L. monocytogenes} stimulated transcription of SIX1 (roughly twofold) and late-phase accumulation of SIX1 protein (Fig. 2a–c). Notably, protein expression of SIX1, but not mRNA induction, was potently suppressed in infected BMDMs isolated from Nik–/– mice (Map3k14 gene knockout; Fig. 2a, c, Extended Data Fig. 3a). De novo synthesis of SIX proteins was also observed in human fibroblasts stimulated with two distinct non-canonical NF-κB agonists: TWEAK, a TNF-family cytokine that signals through the FN14 receptor\(^13\), and BV6, a SMAC-mimetic compound that promotes rapid NIK protein accumulation by inhibiting cIAP1 and cIAP2\(^13,14\) (Fig. 2d, e). Notably, NIH–/– fibroblasts failed to express SIX proteins under these conditions. Finally, we found that long-lasting treatment (24 h) of cells with traditional canonical NF-κB pathway agonists (for example, TNF and lipopolysaccharide (LPS)) induced SIX1 and SIX2 through a mechanism that required signalling crosstalk with NIK\(^15\) (Extended Data Fig. 3b–e). These data indicate that NIK induces the expression of SIX proteins under a variety of inflammatory conditions.

We noted that recombinant SIX1 and SIX2 proteins were expressed at unusually low levels when driven by a strong cytomegalovirus (CMV) promoter in HEK293 cells (Extended Data Fig. 3f, g). Co-transfection of NIK or long-lasting treatment of cells with non-canonical NF-κB agonists induced CMV-driven expression of SIX proteins, revealing a post-transcriptional mechanism of control (Extended Data Fig. 3f–h). Application of the 26S proteasome inhibitor MG132 also induced rapid accumulation of CMV-driven SIX proteins in HEK293 cells and endogenous SIX proteins in BMDMs and human fibroblasts, suggesting that these proteins are constitutively marked for ubiquitin-mediated degradation\(^16\) (Extended Data Fig. 3i–k). Detailed kinetic analysis of endogenous SIX1 and SIX2 protein expression during non-canonical NF-κB pathway activation (Extended Data Fig. 3l) and investigation of the ubiquitination status of SIX2 (Extended Data Fig. 3m) revealed a concerted mechanism of SIX protein reactivation, involving the induction of SIX gene expression through secondary transcription followed by stabilization of SIX proteins through NIK-dependent inhibition of the ubiquitin–proteasome pathway (Extended Data Fig. 3n).

SIX family members regulate gene expression programs during development\(^17\). However, mutations that prevent the assembly of transcriptional co-activator complexes had no bearing on the immunological activity of SIX2, implying that it has an alternative mechanism of action\(^18\) (Extended Data Fig. 4a). We found that SIX proteins
suppressed NIK-mediated immunity, suggesting that they may negatively regulate non-canonical NF-κB (Extended Data Fig. 4b–f). We used whole-genome RNA sequencing (RNA-seq) to test this hypothesis. Chronic activation of non-canonical NF-κB by ectopic expression of NIK in fibroblasts induced the transcription of 891 genes, including those with primary and secondary inflammatory response signatures (Extended Data Fig. 4g, h, Supplementary Table 3). Remarkably, nearly 30% of these genes were potently suppressed by co-transduction of SIX2, including cytokines and chemokines that harbour consensus κB transcriptional binding sites or that are indirectly stimulated by NF-κB (Extended Data Fig. 4i). We confirmed that IL1B, IL8, IL13, IL33, CCL3, CCL19, CXCL1, and CXCL2 were inhibited by SIX2 using quantitative PCR with reverse transcription (qRT–PCR) (Extended Data Fig. 4j). In addition, SIX1–/–/SIX2−/− fibroblasts exhibited enhanced transcription of these genes after long-term cytokine stimulation (Fig. 2f) or viral transduction of NIK (Fig. 2g), indicating that endogenous SIX proteins negatively regulate inflammatory gene expression programs.

A subset of the SIX-regulated genes was induced by the canonical NF-κB subunit RELA (for example, IL1B and IL8) and others required non-canonical RELB (for example, IL13, IL33, CCL3, CCL19, CXCL1, and CXCL2)2 (Extended Data Fig. 5a–c). To determine whether SIX proteins inhibit multiple NF-κB isoforms, as these data suggest, we analysed luciferase reporter expression driven by five NF-κB binding sites (5 × κB). Transient transfection of fibroblasts and HEK293 cells with SIX family members inhibited the stimulation of 5 × κB-LUC by long-term cellular application of the canonical and non-canonical NF-κB agonists TNF and LTO.11,12, respectively (Extended Data Fig. 5d, e). The potency of SIX2 was equivalent to those of well-known inhibitors of NF-κB, including InhαB super repressor and A20, and was greater than either WIP1 or PIAS119–22 (Extended Data Fig. 5f). Direct studies on RELA−/− and RELB−/− fibroblasts confirmed that the SIX proteins suppressed transcription by both the canonical and non-canonical NF-κB isoforms (Extended Data Fig. 5g). Thus, SIX1 and SIX2 are negative regulatory components of the non-canonical NF-κB pathway by virtue of their NIK-dependent expression, and not by differential recognition of RELA or RELB target genes.

Mechanistic investigations suggested that SIX proteins exhibit gene-proximal inhibitory activities (Extended Data Fig. 6a–c). SIX1 bound promoter regions neighbouring the κB sequence(s) of the IL1B, IL8, and CCL3 genes, indicating that it was primed for transcriptional inhibition (Fig. 3a, Extended Data Fig. 6d, e). Cytokine treatment induced further recruitment of SIX1 to these genes (Fig. 3a, Extended Data Fig. 6d). Notably, the ability of SIX1 to occupy the promoters of inflammatory genes under both quiescent and stimulated conditions explains the observed increase in IL1B, IL8, and CCL3 mRNA expression in SIX1−/−/SIX2−/− cells (Fig. 2f).

SIX proteins formed a stable complex with nuclear RELA and RELB (Fig. 3b). Notably, this interaction did not affect the recruitment of NF-κB to target gene promoters (Extended Data Fig. 6f). In addition, SIX2 inhibited activation by both GAL4–RELA and GAL4–RELB of a 5 × GAL4 luciferase reporter gene, a reconstituted system that measures NF-κB transcriptional activity independent of its DNA binding preference23 (Fig. 3c, Extended Data Fig. 6g). These data suggest that SIX proteins inhibit the trans-activation function of NF-κB. In support of this conclusion, SIX2 interacted directly with the trans-activation domain (TAD; residues 473–522) of RELA, the functional region of NF-κB that recruits chromatin remodelling enzymes and basal transcriptional machinery, including RNA Pol II24 (Fig. 3d). Knockout of SIX1 and SIX2 increased the occupancy of 11.1B and IL8 by RNA Pol II in both basal and cytokine-treated fibroblasts (Fig. 3e). Collectively, these data support an inhibitory model in which SIX proteins regulate the trans-activation function of NF-κB at inflammatory gene promoters in a negative feedback loop (Extended Data Fig. 6b).

We next sought evidence that SIX proteins suppress inflammatory gene expression programs in vivo. Knockout of SIX1 or SIX2 causes embryonic lethality in mice25. We therefore adapted a doxycycline-inducible system for broad tissue expression of a human SIX1 transgene in adult mice26 (Extended Data Fig. 7a–d). As doxycycline is a powerful antibiotic, we monitored the inflammatory response and progression of disease in mice exposed to bacterial LPS. SIX1 suppressed the LPS-induced transcription of inflammatory mediators in peritoneal macrophages ex vivo, indicating that the human transgene maintains its function across species (Extended Data Fig. 7e). Remarkably, expression of SIX1 provided nearly complete protection of mice from a lethal LPS challenge as compared to littermate controls (Fig. 4a, Extended Data Fig. 7f). Whereas the clinical signs of septic shock were indistinguishable between genotypes six hours after LPS injection, SIX1-expressing mice made a near-complete recovery over the time course of the experiment (Fig. 4b). This recovery correlated with a reduction in inflammatory mediators in the serum of SIX1-expressing mice (Fig. 4c). Although these findings clearly indicate that SIX proteins promote inflammatory resolution in vivo, we suspect that reactivation of SIX1 or SIX2 will have cell-type-specific functions under physiological conditions associated with non-canonical NF-κB activation.
We then searched for a second line of evidence that SIX proteins regulate biological systems associated with non-canonical NF-κB function. Previous studies indicate that combinatorial application of SMAC-mimetic compounds (for example, BV6) and TNF promotes death of cancer cells, including non-small-cell lung cancer (NSCLC), through non-canonical NF-κB activation.13,14,26-28 However, many NSCLCs are resistant to death induced by BV6 and TNF, an observation that has limited the therapeutic efficacy of these compounds.27,29,30 A potential mechanistic explanation for the resistance of cancer cells to SMAC-mimetics emerged from our studies on SIX proteins. Specifically, we identified three RAS and P53-driven NSCLC cell lines (H1155, H1792 and H2087) that were refractory to combined BV6–TNF-induced cell death and exhibited high levels of endogenous SIX1 and SIX2 (Fig. 4d, e). CRISPR–Cas9-mediated knockout of SIX1 and SIX2 markedly sensitized these NSCLCs to BV6–TNF treatment (Fig. 4d, e). The anti-apoptotic function of SIX proteins was also observed in SV40-immortalized fibroblasts and U-2 OS cells (Extended Data Figs. 8, 9a–g). We confirmed that SIX1 and SIX2 suppressed caspase-8 mediated cell death in these cell lines (Extended Data Fig. 9h–i).

To broadly investigate whether the protective effects of SIX proteins arise from regulation of gene expression programs, we treated wild-type and SIX1−/−SIX2−/− H1792 NSCLC cells with BV6 and TNF and processed them for RNA-seq. More than 90% of the analysed transcripts were unaltered by BV6–TNF treatment. However, of the 1,024 genes that were induced greater than twofold (false discovery rate (FDR) < 0.05) by BV6–TNF treatment in wild-type cells, 120 were substantially upregulated in SIX1−/−SIX2−/− cells (cluster I, Fig. 4f, Extended Data Fig. 10a, Supplementary Table 4). Cluster I genes had a strong inflammatory response signature with enrichment of transcripts from cytokines and chemokines with experimentally verified κB-binding sites (Extended Data Fig. 10b). A large percentage of cluster I genes were also upregulated in unstimulated SIX1−/−SIX2−/− cells (Fig. 4f, Extended Data Fig. 10a, c), consistent with their promoter occupancy and inflammatory gene transcription profiles observed in non-cancer cells (Figs. 2f, 3a). In addition, SIX proteins suppressed only a subset of κB target genes, as 78% of transcripts induced by BV6–TNF were unaltered between wild-type and SIX1−/−SIX2−/− cells (cluster II, Fig. 4f, Supplementary Tables 5, 6). Together, these data provide an unbiased confirmation that SIX1 and SIX2 regulate gene-specific transcriptional responses induced by non-canonical NF-κB under both physiological and pathological conditions.

In summary, we have established that SIX family transcription factors function as immunological gatekeepers, dampening the promoter activity of inflammatory genes in response to persistent activation of the non-canonical NF-κB signalling cascade. In support of this mechanism, reactivation of SIX1 and SIX2 in immune cells is coupled to accumulation of SIX protein caused by chronic cytokine stimulation or pathogen infection. In addition, expression of SIX1 and SIX2 directly inhibits the trans-activation function of RELA and RELB in a negative feedback loop (Extended Data Fig. 6h). These findings not only connect the non-canonical NF-κB signalling cascade to a mechanism of transcriptional repression, but also indicate that disruption of this response circuit may have important consequences for the pathogenesis of human disease, including cancer.14,5

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41586-019-1041-6.

Received: 13 July 2017; Accepted: 18 February 2019;
Published online 20 March 2019.

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Acknowledgements We thank H. Ford (University of Colorado) for the Tet-on HA-SIX1 transgenic embryonic and for discussion in preparation of the manuscript; the UT Southwestern Medical Center transgenic core for reviving the frozen embryos; J. Minna (UTSW) for NSCLCs; and V. Tagliabucci, J. Mendell, D. Pan, I. D’Orso, N. Conrad, R. Brekken and members of the Alto Laboratory for discussions. This research was supported by grants from the National Institutes of Health (AI083359 to N.M.A., AI117922 to J.W.S., and 5K12 HD068369-09 to N.W.H.) and the Children’s Medical Center Foundation (N.W.H., and grants to N.M.A. from the Welch Foundation (I-1731), The Burroughs Welcome Fund, and the Howard Hughes Medical Institute and Simons Foundation Faculty Scholars Program.

Reviewer information Nature thanks Claus Scheidereit and the other anonymous reviewer(s) for their contribution to the peer review of this work.

Author contributions Z.L. and N.M.A. conceived and designed the study. Unless otherwise specified, Z.L. performed all experiments. K.B.M. assisted with mouse experiments. N.W.H. performed West Nile Virus infection. S.S.P. performed RNA-seq to identify NIK-stimulated genes. M.K. and C.X. performed bioinformatics analysis of RNA-seq data. J.W.S. designed the genetic screening platform for bacteria and viruses and provided critical input into project directions. Z.L. and N.M.A. analysed data and wrote the manuscript with editorial input from all authors.

Competing interests The authors declare no competing interests.

Additional information Extended data is available for this paper at https://doi.org/10.1038/s41586-019-1041-6.

Supplementary information is available for this paper at https://doi.org/10.1038/s41586-019-1041-6.

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To obtain primary bone-marrow-derived macrophages (BMDMs), bone marrow cells were collected from 6-week-old wild-type C57BL/6NJ, Nik−/−, or rtTA3/−/− or rtTA3/−/−SIX1 mice. Embryonic kidney and fetal liver tissue were perfused with PBS, treated with dispase IV to release adherent cells, followed by collagenase digestion to generate a single-cell population. Cells were then cultured in DMEM supplemented with 10% FBS, 1% NEAA, 1% glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO2 incubator. For cryopreservation, cells were resuspended in PBS containing 5% FBS and 10% DMSO and then stored at −80°C.

Cell lines. SV40-immortalized STAT1−/− fibroblasts (referred to as fibroblasts and described as WT in certain places to distinguish parental cells from the indicated gene edited cell lines) were obtained from J. Lian. The pIκBα−/− cell line was obtained from E. Olson (UT Southwestern Medical Center). Several cell lines were generated from murine embryonic fibroblasts or bone-marrow-derived macrophages (BMDMs). Langerhans cells, macrophages, and dendritic cells were generated as described below.

Lentivirus production and transduction. For lentivirus production, 4 × 105 HEK293T cells were seeded in each well of polylysine-coated 6-well plates. One microgram of indicated lentivirus-expressing genes, 0.8 μg HIV gag-pol, and 0.2 μg vesicular stomatitis virus glycoprotein (VSV-G) were transfected into HEK293T cells by polyethyleneamine (PEI) (Polyplus Transfection, Illkirch, France). The transduction medium was added at 16 h post-transfection and replaced with culture medium after 6 h. Transduced cells were split into duplicate 48 h post-transduction, followed by bacteria and virus infection assays.

Bacteria and virus infection. To generate GFP-expressing Shigella, S. flexneri M5 Zen was transformed with a pBBRMC51-GFP plasmid. GFP-expressing L. monocytogenes (10403s strain) was a gift from D. Portnoy (University of California, Berkeley). For Shigella infection, bacteria were grown in BHI broth medium (237500, BD Science) supplemented with 5 μg/ml chloramphenicol (CAM) at 37°C with 200 r.p.m. shaking overnight. Bacteria were then diluted 1:25 into BHI with 5 μg/ml CAM and incubated at 37°C for about 2 h (OD600 = 0.5). Bacteria were washed with PBS and then resuspended in PBS/0.03% Congo red (G6277, Sigma) and incubated at 37°C for 15 min. Bacterial suspensions (MOI of 10:1) were inoculated into each well of 24-well plates, followed by centrifugation at 1,000g for 10 min. Infected cells were then incubated at 37°C in a 5% CO2 incubator for 1.5 h. Extracellular bacteria were killed by replacing the medium with medium supplemented with 50 μg/ml gentamicin. After 8 h incubation, cells were collected for flow cytometry analysis.
FlowJo 10.0.6. For most analyses, we gated live cells, single cell populations from live cells, and then RFP-positive cells from the single-cell population. Finally, we gated GFP-positive units from the RFP-positive population. For RFP-, BFP-, or GFP-expressing experiments, we gated cells that expressed both RFP and GFP to analyse the GFP-expressing population.

**CRISPR–Cas9 gene editing cell lines.** RELA, RELB, NIK, TAK1, or SIX1/SIX2 guide RNA (the guide targets both SIX1 and SIX2 genes) was cloned into lenti-CRISPR v2 vector11 (S. Zhang, Addgene 52963) according to the manufacturer’s protocol. Lentivirus was produced as described above. We transfected 7 × 10^4 fibroblasts or 2 × 10^5 OS, H1155, H1792, or H2087 cells with the indicated lentivirus and incubated them for 48 h. Transduced cells were then selected with 2 μg ml^-1 (fibroblasts and U-2 OS) or 5 μg ml^-1 (H1155, H1792, and H2087) puromycin for 7 days. Single colony cells were sorted by flow cytometry. Homozygote knockout cells were then identified by genotyping and western blot. Knock out of SIX1 and SIX2 in H1155, H1792, and H2087 cells resulted from a single T insertion to both alleles (Extended Data Fig. 4c).

**Six protein accumulation induced by L. monocytogenes infection, cytokines, LPS or drug treatment.** Cells (1 × 10^5) wild-type, NIK^-/- or TAK1^-/- fibroblasts or 2 × 10^5 wild-type or NIK^-/- primary BMDMs were seeded in 12-well plates. Cells were then infected with L. monocytogenes (MOI = 0.1 (BMDMs)), or treated with 25 ng ml^-1 TNF, 50 ng ml^-1 LTP/12, 100 ng ml^-1 LPS, 50 ng ml^-1 TWEAK, 30 μM MG132 or 5 μM BV6 for 24 h or the indicated time. Whole cells were then lysed in lysis buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Triton X-100, and 1 μg protease inhibitor cocktail) along with 1 × laemmli sample buffer (160-0173, Biorad). Whole cell lysates were then separated by 8% SDS–PAGE and the proteins were detected by anti-HA or anti-Flag immunoprecipitation. Ubiquitinated SIX2 were loaded for anti-Flag immunoprecipitation. Western blot.

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**Immuno precipitation assay.** To test the interaction between SIX2 and RELA/RELB, 8 × 10^4 HEK293T cells were transfected with the indicated plasmids (6 μg total). After 48 h, cells were lysed in 1 ml lysis buffer, followed by 30 s on and 30 s off sonication for 7–10 cycles to break the nuclei. Anti-Flag immunoprecipitation was carried out using anti-Flag M2 affinity gel (A2220, Sigma) for 4 h. Beads were then washed four times with lysis buffer. Co-immunoprecipitated proteins were separated by SDS–PAGE and the proteins were detected by anti-HA or anti-Flag western blot.

**For ubiquitination of SIX2, Flag–SIX2 and HA–Ub were co-transfected with empty vector or GFP–NIK into HEK293T cells. After 48 h, equal amounts of Flag–SIX2 proteins were loaded for anti-Flag immunoprecipitation. Ubiquitinated SIX2 was detected by anti-HA or anti-Flag western blot.**

**Yeast two-hybrid assay.** To test the interaction between SIX2 and the RELA trans-activation domain, full-length SIX2 was cloned into the pACT2-AD vector (GAL4 activation domain (AD)). Amino acids 473–522 of RELA, which does not have the capacity to self-activate16, was cloned into pLexNA-BD vector (GAL4 DNA binding domain (BD)). Yeast transformation was performed using the standard LiAc-based method. Equal amounts of indicated yeast cells were placed on either minimal synthetic defined (SD) bases (Clontech) supplemented with yeast synthetic drop-out media supplements that without uracil, leucine, and tryptophan (SD/UWUL-, Clontech) or supplied with yeast synthetic drop-out media supplements that without tryptophan, histidine, uracil, leucine, and lysine (SD/WHULK-, Clontech) with 10 μM 3-amino triazole (3-AT) and grown for 2–3 days.

**RNA sequencing and data analysis.** RNA-seq was performed at UTSW McDermott Center Next Generation Sequencing Core and analysed at the Human Genome Sequencing Center (HgSCC, Houston, TX). Twenty-three million reads were generated from 10 ng of RNA using an Illumina HiSeq 2500 by reading paired-end 100-bp (base pair) reads. To consider the NIK-stimulated genes, each read was mapped to the human genome based on Human-hg19 by TopHat (v2.0.10) based on genome annotations (https://ccb.jhu.edu/software/tophat/). cufflinks/Cuffdiff (v2.1.1) was then used to calculate the expression value of each sample and to identify differentially expressed genes in the Fluc or NIK sample using a regularized t-test. Only genes with log2 (fold change) ≥ 1 or ≤ -1 and FDR < 0.05 were considered as up- or downregulated by NIK compared to Fluc control.

To analyse the SIX2-downregulated NIK-stimulated genes, Fluc^RELB^NIK/Fluc^NIK, Fluc^RELB^NIK/Fluc^BFP^, NIK^RELB^/ SIX2^BFP^, or Fluc^BFP^/SIX2^BFP^ lenti virus transductions were infected into fibroblasts. RNA purification and RNA sequencing were performed as described above. Comparison of NIK/SIX2 versus NIK/Fluc (comp I) or NIK versus Fluc (comp II) was carried out by considering log2 (FC) ≤ -1 or ≥ 2 and FDR < 0.01. The SIX2-downregulated NIK-stimulated genes were then adjusted by comparing comp I with comp II.

For BV6–TNF-induced gene transcription profiles in H1792 NSCLC cells, 3 × 10^4 wild-type or SIX1^-/- or SIX2^-/- cells were mock-treated or treated with 5 μM BV6 and 25 ng ml^-1 TNF for 24 h. The total RNA was extracted from the adherent cells. RNA-seq was performed as described above. The read length for this experiment was 75 bp single-end. to analyse the differential expression profiles, fastq files were checked for quality using fastqc (v0.11.2; http://www.bioinformatics. babraham.ac.uk/projects/fastqc) and fastq_screen (v0.4.4; http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen) and were then quality trimmed using fastx-mcf (ea-utils/1.2.806)30. Trimmed fastq files were mapped to btag9 (USCSC genome version, TOPOHat30) using TopHat30, duplicates were removed using picard tools (v1.127 https://broadinstitute.github.io/picard/), read counts were generated using featureCounts14 and differential expression analysis was performed using the generalized linear model likelihood ratio test implemented in edgeR42. For comparison of BV6–TNF-induced genes in wild-type cells, only genes that exhibited log2 (counts per million) ≥ 0.1, log2 (FC) ≥ 1, and FDR < 0.05 were considered upregulated. Genes that were differentially regulated between BV6–TNF-treated wild-type and SIX1^-/- or SIX2^-/- cells were identified by log2 (FC) ≥ 1 (up or ≤ -1 (down) and FDR < 0.05. The remaining genes were considered as no change. The expression levels of each of the 1,024 differentially expressed genes were normalized across conditions to generate z-scores and presented in Fig. 4f. Final gene lists were then used for pathway analysis with QIAGEN’s Ingenuity Pathway Analysis tool (QIAGEN Redwood City, http://www.qiagen.com/ingenuity). Trends in these gene lists were also plotted using various R packages (https://www.R-project.org/). The significance values for the canonical pathways were calculated using one-sided Fisher’s exact test.

**RNA-seq data validation and qRT–PCR.** To validate RNA-seq data, experiments were performed as described above. In brief, total RNA was isolated for synthesizing the cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen). The gene expression level was quantified using real-time PCR by detecting the SYBR green (Invitrogen). The gene expression level was determined by normalizing the computed signal across conditions to generate fold-change values and presented in Fig. 4f. The significance value for the canonical pathways was calculated using one-sided Fisher’s exact test.

For SIX1 and SIX2 gene expression stimulated by L. monocytogenes infection or TNF, 2 × 10^5 wild-type or NIK^-/- primary BMDMs were seeded into 12-well plates and then infected with L. monocytogenes (MOI = 0.1) or treated with 25 ng ml^-1 TNF for 24 h. For BV6–TNF-induced transcription profiles in H1792 NSCLC cells, 1 × 10^5 wild-type or SIX1^-/- or SIX2^-/- fibroblasts were treated with 2 μg ml^-1 TNF for 24 h. For TWEAK-induced transcription of IL8, IL8, and CCL3 in fibroblasts, wild-type and SIX1^-/- or SIX2^-/- fibroblasts were treated with 50 ng ml^-1 TWEAK for 24 h. For LPS-induced expression of inflammatory mediator genes in peritoneal macrophages, cells were treated with 100 ng ml^-1 LPS for 4 h. Relative gene expression was adjusted to that of the housekeeping gene Actb (mouse or human) and then normalized to experimental controls.

**NIK-stimulated genes library.** On the basis of RNA-seq data, we identified 273 genes as NIK-stimulated. Of these, 237 genes were cloned into the TRIP.CMV. IVS3.GENE.ires.TagRFP destination vector. We obtained 141 genes from the hORF Collection (Invitrogen), 35 from DNASTAR36 and 61 from the type I interferon (IIF) library. Lentiviruses were produced as described above.

**Luciferase reporter assay.** 1 × 10^4 HEK293T cells or 2 × 10^5 wild-type, RELA^-/-, or RELB^-/- fibroblasts were seeded into each well of 48-well plates. Indicated plasmids were transfected into cells along with LaiZ (as transfection control) and 5 × 10^5 B-LUC, Flp/L8-LUC, or 5 × GAL-A-LUC and incubated for 48 h. For cytokine treatment, after 24 h transfection, cells were treated with 25 ng ml^-1 TNF or 50 ng ml^-1 TWEAK for 24 h. Luciferase activity was measured according to the manufacturer’s protocol (Promega). The luciferase activity (normalized to β-galactosidase activity, pCMV. IVS3.GENE.ires.TagRFP buffer (2–nitrophenyl-5-β-galactopyranoside dissolved in 200 mM NaH2PO4, 2 mM MgCl2, and 100 mM β-mercaptoethanol) was used to measure LaiZ activity. The luminescence and absorbance units were measured using FLUOstar OPTIMA (BMG LABTECH).
Relative luciferase activity was quantified by adjusting to $Luc^Z$ control and normalizing to experimental control.

**Fluorescence microscopy.** To analyse the localization of truncated SIX2 fragments, 2 $\times$ 10^4 U-2 OS cells were seeded onto coverslips in 24-well plates. Indicated Flag-tagged SIX2 fragments were transfected into cells. After 48 h, cells were washed 2–3 times with PBS and were fixed by incubation in 500 µL PBS/3.7% formaldehyde for 10 min at 37°C, followed by washing three times with PBS and incubation in 500 µL PBS/50 mM NH$_4$Cl for 10 min. Cells were permeabilized in PBS/10% horse serum/0.5% Triton X-100 for 45 min. Cells were then incubated with primary antibodies (1:500 anti-Flag in PBS/10% horse serum/0.5% Triton X-100) for 45 min. After washing three times with PBS, secondary antibody (1:500 fluorescein-conjugated goat anti-mouse from Pierce and 1:1,000 DAPI in PBS/10% horse serum/0.5% Triton X-100) was added and incubated for 45 min. After washing three times with PBS and once with H$_2$O, the samples were mounted on slides and images were processed using a Zeiss Observer Z1 fluorescent microscope.

**ChIP.** A GFP–SIX1 stable cell line was generated by cloning GFP–SIX1 into a pScripSyb-blasticidin backbone. Lentiviruses were produced as described above. Fibroblasts and HCT116 cells were transduced and selected using 10 µg ml$^{-1}$ blasticidin. Positive cells were used for the following experiments. The ChIP assay was performed according to the manufacturer’s instructions (Millipore, 12–495). In brief, 1.0 $\times$ 10^5 cells were cross-linked with 1% PFA for 10 min at 37°C and 125 mM glycine was used to quench crosslinking. Cells were then washed with chilled PBS twice, and lysed in lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP-40, 1 mM PMSE, 1 x protease inhibitor). The nuclei were then pelleted down by spinning at 3,000 r.p.m. for 5 min and were resuspended in RIPA buffer (50 mM Tris-HCl pH 8.0, 1% NP-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 2.5 mM EDTA, 1 mM PMSE, 1 x protease inhibitor). Nuclei lysates were sonicated for 40 cycles (HCT116 cells, 70 cycles for fibroblasts) with 30 s on and 30 s off to yield fragments of 200–1,000 bp using Bioruptor (Diagenode). Six micrograms of IgG, SIX1, RELA or Pol II antibodies were conjugated with protein G beads (10004D, Invitrogen) for 1 h and blocked using 5% BSA for 1 h. After spinning down sonicated nuclei lysates at 13,000 r.p.m. for 10 min, we incubated an equal volume of supernatant of sonication products with the indicated antibodies for 3 h at 4°C, followed by washing three times with PBS/0.1% SDS, 2.5 mM EDTA, 1 mM PMSE, 1 x protease inhibitor. The bound protein–DNA complex was eluted using 500 µl elution buffer (100 mM NaHCO$_3$, 1% SDS). We added 20 µl 5 M NaCl to reverse crosslinks of protein and DNA by heating at 65°C for over 4 h or overnight. DNA was recovered by PCA and precipitated using ethanol in the presence of glycerol (AM9515, Invitrogen). Enrichment at the IL1B, IL8, and CCL3 promoters was measured by quantitative PCR (qPCR). Fold enrichment was normalized to experimental control. For RELA and Pol II ChIP experiments, fold enrichment was adjusted to ‘input DNA’ that was saved before immunoprecipitation and then normalized to experimental control.

**Cell viability assay.** The indicated cells (5 $\times$ 10^4 for 48 h treatment or 1 $\times$ 10^5 for 24 h treatment) were seeded into 96-well plates (Costar, black with clear flat bottom, 3603). After 19 h, the medium was removed and fresh medium supplemented with the indicated compounds was added and incubated at 37°C with 5% CO$_2$ for 24 or 48 h. Whole-cell ATP levels were measured using CellTiter Glo following the manufacturer’s instructions (G7572, Promega). The luminescence units were measured using FLUOstar OPTIMA (BMG LABTECH). For SIX2 complementation assays, 7 $\times$ 10^3 parental, NIK$^{-/-}$ or SIX1$^{-/-}$/SIX2$^{-/-}$ fibroblasts were seeded into 24-well plates. Then cells were transfected with Fluc, SIX2 or SIX2V$^{-/-}$ lentivirus (SIX2$^{-/-}$ has a silent mutation in the gRNA recognition sequence of the SIX2 cDNA that cannot be targeted by CRISPR–Cas9). After 50 h, transfected cells were seeded in 96-well plates and the experiments were performed as described above.

**Data availability**

All data generated during this study that support the findings are included in the manuscript or in its Source Data and Supplementary Information. All materials are available from authors upon reasonable request. The RNA-seq data associated with Fig. 4f and Extended Data Figs. 2b, 4g have been deposited in Gene Expression Omnibus (GEO) at NCBI (accession code: GSE126535).

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Extended Data Fig. 1 | CD40–NIK signalling axis mediates antibacterial function. 

**a**, **b**, Experiments were performed to exclude the possibility that the observed CD40L-induced antibacterial function was specific to a particular cell type or protocol of cytokine induction. We reconstituted the CD40L signalling pathway in HEK293 cells. These cells do not express CD40, the endogenous receptor for CD40L, or (a, left). HEK293 cells are also unable to be stimulated by CD40L (a, right). However, overexpression of CD40 strongly induced NF-κB pathway activation (a, right). Expression of CD40 restricted both *L. monocytogenes* and *S. flexneri* infection (b). The NF-κB activity was measured after 48 h and normalized to EV. Data are mean ± s.d. from six independent experiments.

**c**-**j**, Confirmation of genetic knockout of the MAP3K14 (NIK) and MAP3K7 (TAK1) genes in STAT1/−/− human fibroblasts. 

**c,** Schematic representation of In/Del base pairing and the sgRNA targets locus of exon 1 in NIK and TAK1. NIK/−/− contains deletion of (−)7 bp, insertion of (+)G, and +CTCAC alleles (top). TAK1/−/− contains + AT alleles, −2 bp, and −409 bp (bottom). 

**d,** Endogenous NIK and TAK1 expression in parental, NIK/−/− and TAK1/−/− cells. NIK is constitutively degraded by the cIAP-TRAF2/3 E3-ligase complex in quiescent cells. Wild-type, NIK/−/−, and TAK1/−/− fibroblasts were treated with 2.5 μM BV6 for 14 h and then probed with indicated antibodies using western blotting. 

**e,** NIK is necessary for restricting *L. monocytogenes* infection. Fibroblasts with the indicated genetic background were treated with vehicle control (DMSO) or 2.5 μM BV6 for 14 h and then infected with *Lm*Δ*mp*. Percentage bacterial infection was normalized to wild-type untreated control. Black asterisks denote the difference between wild-type and indicated cell lines; red denote the difference between DMSO and BV6 treatment. NIK/−/− cells exhibited much higher *L. monocytogenes* infection than either wild-type or TAK/−/− cells, consistent with the role of NIK in preventing infection after cellular stimulation. However, BV6 treatment—which suppressed *L. monocytogenes* infection of wild-type cells—had no effect on NIK/−/− cells, further indicating that NIK activation is necessary for the antibacterial response. Data are mean ± s.d. from nine independent experiments. 

**f,** NIK kinase activity is required for its antibacterial function. *Fluc* wild-type NIK or NIK-kinase dead mutant (NIKΔK429/430A referred to as NIKΔK) lentivirus was transduced into fibroblasts or U-2 OS cells as indicated. Cells were then challenged with *Sf*Δ*mp*. Quantification of bacterial infection is as in Fig. 1b. Data are mean ± s.d. from four independent experiments. 

**g,** NF-κB gene expression induced by NIK is kinase dependent. EV, NIK or NIKΔK was co-transfected with 5 × *κB*-LUC into HEK293T cells. NF-κB activity was measured after 48 h and normalized to EV. Data are mean ± s.d. from four independent experiments. 

**h,** Expression of NIK, but not TAK1, inhibits infection with *L. monocytogenes* and *S. flexneri*. Wild-type U-2 OS cells were transduced with combinational *Fluc*Δ*mp*/*Fluc*Δ*B*, NIKΔK/*Fluc*Δ*B*, or TAK1ΔKp/TAB1ΔKp lentivirus. Cells were then challenged with *Lm*Δ*mp* or *Sf*Δ*mp*. Infection efficiency was quantified by flow cytometry, gating GFP+ cells in both RFP+ and BFP+ cell populations. The relative percentage of pathogen infection was normalized to *Fluc* control. Data are mean ± s.d. from eight independent experiments. I, NIK protein expression levels corresponding to experiments in Fig. 1c. *Fluc*- and NIKΔK-transduced cells were lysed and probed with anti-NIK antibody. 

**i**, Previous studies have suggested that ectopic expression of NIK is cytotoxic in A549 cells. 

**j,** To test whether ectopic expression of NIK is cytotoxic in fibroblasts, we transduced fibroblasts with indicated lentivirus and measured cell viability after 72 h by measuring ATP. Data are mean ± s.d. from six independent experiments. *P* values were measured using one-way ANOVA (GraphPad); ***P < 0.001, ****P < 0.0001; ns, no significant difference. The same statistics were used in later figures unless otherwise stated. Western blot data are representative of three independent experiments. For gel source data, see Supplementary Fig. 1.
Extended Data Fig. 2 | Library screen for NIK-stimulated genes.

**a.** Schematic of NIK-stimulated gene library design, cloning, and multidimensional flow cytometry-based high-throughput screen. NIK-stimulated genes were identified by RNA-seq. The cDNAs of 237 NIK-stimulated genes were individually cloned into the lentiviral vector pTRIP upstream of IRES-tagRFP (see Methods). Fibroblasts were transduced with lentivirus in a one-gene to one-well format and were then infected with GFP-expressing pathogens, including *Lm*, *Sf*, EAV, WNV, SINV, or PIV3 in independent experiments. The effect of a single gene expression on infection was quantified by flow cytometry.

**b.** Relative expression levels of NIK-stimulated genes identified by RNA-seq. *Fluc* or NIK lentivirus was transduced into fibroblasts. Total RNA was isolated after 72 h and gene expression was determined by RNA sequencing. Graph shows expression of genes that were significantly stimulated (red, 237 genes) or downregulated (green, 84 genes) by NIK expression compared to *Fluc* control. Fold change of more than 2 (log2 ≥ 1) or less than 0.5 (log2 ≤ −1) and FDR < 0.05 (see Methods). Bars were ranked numerically from low to high (see Supplementary Table 1). The expression levels of *SIX1* and *SIX2* are indicated. Data are representative of two independent experiments.

**c.** Efficiency of lentiviral expression of NIK-stimulated genes used in the high-throughput bacterial and viral screen. NIK-stimulated genes were transduced into fibroblasts in a ‘one-gene to one-well’ format. Transduction efficiency measured as per cent RFP+ cells was determined by flow cytometry and was ranked numerically from low to high (see Source Data, values are the average of two technical replicates). Twelve out of 237 genes were poorly transduced (less than 20% RFP+) and were excluded from subsequent analyses.

**d.** Dot plots of *S. flexneri*, *L. monocytogenes*, EAV, WNV, SINV, and PIV3 infectivity in the presence of expressed NIK-stimulated genes (in c). Data were normalized to the average of each screen, indicated as the black dotted line. We chose to confirm hits in Fig. 1d on the basis of two criteria: (1) the effect of gene expression on inhibiting or enhancing pathogen infection by less than or greater than 50%; and (2) an adjusted z-score less than −2 or greater than 2 (see Supplementary Table 2). NIK-stimulated genes that reproducibly and significantly inhibited (green) or enhanced infection (red) by these criteria are indicated. The genes shown in black font are hits that were not reproduced in the confirmatory experiments (Fig. 1d). Data are mean ± s.d. from two (*S. flexneri*, *L. monocytogenes*) or one (EAV, WNV, SINV, and PIV3) independent experiments.
Extended Data Fig. 3 | NIK mediates reactivation of SIX proteins by inhibiting the ubiquitin–proteasome pathway. a, Control experiment for Fig. 2a, c showing that NIK is expressed in wild-type BMDMs but not in BMDMs isolated from Nik$^{-/-}$ mice. NIK is constitutively degraded under quiescent condition. We used the MG132 proteasome inhibitor to stabilize endogenous NIK protein expression. To validate NIK protein expression, wild-type and Nik$^{-/-}$ primary BMDMs were mock-treated or treated with 30 μM MG132 for 12 h and NIK was detected by western blot. b, c, Long-term treatment of cells with TNF (b) or LPS45 (c) stabilized SIX1 expression through activation of NIK in mouse primary BMDMs. Wild-type and Nik$^{-/-}$ primary BMDMs were treated with 25 ng ml$^{-1}$ TNF (b), quantification of SIX1 protein expression in TNF-treated cells, mean ± s.d. from three independent experiments as in Fig. 2c) or 100 ng ml$^{-1}$ LPS (c) for 24 h. d, e, Reactivation of human SIX1 and SIX2 by long-term treatment with both canonical (TNF) and non-canonical (LT$\alpha_1$$\beta_2$) NF-$\kappa$B agonists requires NIK but not TAK1. Wild-type, NIK$^{-/-}$ or TAK1$^{-/-}$ fibroblasts were mock-treated or treated with 25 ng ml$^{-1}$ TNF or 50 ng ml$^{-1}$ LT$\alpha_1$$\beta_2$ for 24 h. TAK1$^{-/-}$ cells were included as control to show that TNF and LT$\alpha_1$$\beta_2$ could induce SIX1 and SIX2 accumulation in a TAK1-independent manner (e). f, g, Ectopic expression of NIK induces expression of recombinant SIX1 and SIX2 driven by the strong CMV promoter in HEK293 cells. Plasmids encoding CMV-driven GFP–SIX1 or GFP–SIX2 were co-transfected into HEK293T cells with empty vector or Flag–NIK. Western blot (f) and fluorescence microscopy (g) assays were performed to detect expression of GFP–SIX1 and GFP–SIX2 48 h after transfection. We estimate that SIX1 and SIX2 protein are expressed in 5–10% of untreated cells and 60–70% of cells when co-transfected with NIK. Microscopy images were taken using a 10× objective, scale bars represent 150 μm (g). h, i, Activation of NIK by BV6 (h) or by inhibition of the proteasome with MG132 (i) stabilizes CMV–Flag–SIX2 expression in HEK293T cells. Flag–SIX2 was transfected into HEK293T cells for 24 h and cells were mock-treated or treated with 5 μM BV6 for 24 h or 30 μM MG132 for 12 h. j, Inhibition of the 26S proteasome with 30 μM MG132 induces endogenous SIX1 protein expression in primary BMDMs. k, Inhibition of the 26S proteasome promotes SIX1 and SIX2 expression in human fibroblasts, including NIK$^{-/-}$ fibroblasts (as in j). l, Kinetics of cIAP1 degradation and NIK, SIX1 and SIX2 accumulation in human fibroblasts treated with 5 μM BV6 for the indicated time. m, NIK suppresses SIX2 ubiquitination. HEK293T cells were co-transfected with HA–ubiquitin, Flag–SIX2 and GFP–NIK as indicated and cells were incubated for 48 h. SIX2 was immunoprecipitated with an anti-Flag antibody. The ubiquitination status of the protein was determined by anti-HA western blot. n, Reactivation mechanism of SIX proteins in response to non-canonical NF-$\kappa$B activation (see main text). All data are representative of three independent experiments. For gel source data, see Supplementary Fig. 1.
Extended Data Fig. 4 | SIX proteins oppose NIK-mediated antibacterial function by inhibiting NIK-stimulated gene expression. a, Previous studies have shown that SIX proteins assemble gene co-activator complexes through interaction with EYA family members17 and that SIX1 residues C16 and V17 are required for this interaction18. These residues are conserved in SIX2. We used mutant SIX2(C16RV17E) to show that SIX2 enhances L. monocytogenes infection independent of EYA interaction. Fluc wild-type SIX2, or SIX2(C16RV17E) lentivirus was transduced into fibroblast cells. Cells were then challenged with LmGFP. The percentage infection was normalized to Fluc control. Data are mean ± s.d. from four independent experiments. b, Expression of SIX2 suppresses the antimicrobial function of NIK and CD40. Fibroblasts were lentivirally transduced with a combination of cDNAs (FlucRFP/NIKRFP/FlucBFP, NIKRFP/FlucBFP, NIKRFP/SIX2BFP, CD40RFP/FlucBFP, or CD40RFP/SIX2BFP). After 72 h, cells were infected with LmGFP, RFP, BFP- and GFP-expressing cells were gated by flow cytometry. Infection was quantified as in Extended Data Fig. 1h. Data are mean ± s.d. from six independent experiments. ****P < 0.0001; ns, no significant difference. c, d, Characterization of SIX1−/−SIX2−/− fibroblasts generated by CRISPR–Cas9. c, Schematic representation of In/De l base pairing and the sgRNA that targets locus exon 1 in the SIX1 and SIX2 genes in fibroblasts. SIX1−/−SIX2−/− contains a single T insertion in both alleles of the SIX1 and SIX2 genes. d, Western blot shows endogenous SIX1 and SIX2 expression in parental and SIX1−/−SIX2−/− fibroblasts. Data are representative of three independent experiments. e, The antibacterial activity of NIK is enhanced in SIX1−/−SIX2−/− fibroblasts. Wild-type and SIX1−/−SIX2−/− fibroblasts were transduced with Fluc or NIK lentivirus. After 72 h, cells were challenged with LmGFP. Black asterisks denote the difference between WT (Fluc) and WT (NIK) or SIX1−/−SIX2−/− (Fluc or NIK). Red asterisks denote the difference between WT (NIK) and SIX1−/−SIX2−/− (NIK). Relative infectivity was normalized to WT (Fluc) control. These data indicate that SIX proteins oppose the function of NIK, potentially through suppression of non-canonical NF-κB gene expression (see f). Data are presented as mean ± s.d. from six independent experiments. ****P < 0.0001; ns, no significant difference. f, Relationship between NIK expression, SIX protein accumulation, and antimicrobial immunity. g, RNA-seq experiments used to identify NIK-stimulated genes that are suppressed by SIX2. The indicated combination of lentiviruses (group I: FlucBFP and Fluc; group II: NIKRFP and FlucBFP; group III: FlucBFP and SIX2BFP; group IV: NIKRFP and SIX2BFP) were transduced into fibroblasts. Total RNA was extracted for deep sequencing 72 h later (Supplementary Table 3). h, Group comparisons from data generated in g. In brief, NIK-stimulated genes that are suppressed by SIX2 were determined by comparing Group IV with Group II (log2 > 1) and then adjusted to NIK-stimulated genes from comparison of Group I with Group II (fold change > 4), FDR < 0.01 (see Methods). i, Representative raw data from RNA-seq experiments in g. h presented as fragments per kilobase of transcript per million mapped reads (FPKM) (bars show mean from two independent experiments indicated as circles). j, Validation of RNA-seq data. Experiments were performed as in g. Gene transcription level was determined by qRT–PCR and relative gene expression was normalized to Fluc control. Data are mean ± s.d. of three or two technical replicates and representative of three independent experiments. For gel source data, see Supplementary Fig. 1.
Extended Data Fig. 5 | SIX family proteins inhibit RELA- and RELB-mediated NF-κB activation. a, b, Characterization of RELA−/− and RELB−/− human fibroblasts. Schematic representation of In/Del base pairing and the sgRNA targeting locus of exon 3 in RELA and exon 1 in RELB. a, Top, RELA−/− cells contain +C, −371 bp, and a 109-bp fragment that was replaced with 122 bp containing GCGCTA with reverse complementarity (orange). a, Bottom, RELB−/− cells contain the indicated deletions. b, Western blot shows endogenous RELA and RELB expression in parental and RELA−/− or RELB−/− fibroblasts and response to stimulation by 24-h application of TWEAK. c, Contributions of the canonical and non-canonical NF-κB subunits RELA and RELB, respectively, to gene expression. Fluoc or NIK was transduced into wild-type, RELA−/−, and RELB−/− fibroblasts to stimulate the non-canonical NF-κB signalling pathway. mRNA expression of the indicated genes was evaluated by qRT–PCR. We conclude that IL1B is specifically stimulated by RELA because it is expressed in RELB−/− cells but not in RELA−/− cells. Similar logic was used to evaluate the seven additional genes shown. Experiments were performed as in Fig. 2g. Bars are means of two technical replicates (shown as circles) and representative of two independent experiments. d, The human SIX family consists of six unique isoforms. To determine which of these genes suppress NF-κB-mediated gene expression, empty vector (EV), SIX1, SIX3, SIX4, SIX5, or SIX6 cDNA was co-transfected with 5 × κB-LUC into HEK293T cells. After 24 h, cells were mock-treated or treated with 25 ng ml−1 TNF for 24 h. Luciferase activity was measured and normalized to EV untreated control. SIX1, SIX3, and SIX6 (SIX2 was not evaluated) inhibited both basal and inducible activity of NF-κB. Data are mean ± s.d. from seven independent experiments. e, SIX2 inhibits LTα1β2- and TNF-induced NF-κB activation. EV or Flag–SIX2 was co-transfected with 5 × κB-LUC into fibroblasts. After 24 h, cells were mock-treated or treated with 25 ng ml−1 TNF or 50 ng ml−1 LTα1β2 for 24 h. Data were analysed as in d. Data are mean ± s.d. from six independent experiments. f, The inhibitory potency of SIX2 is equivalent to those of A20 and IκBα SR. The Flag-tagged genes indicated were co-transfected with 5 × κB-LUC into HEK293T cells. After 24 h, cells were mock-treated or treated with 25 ng ml−1 TNF for 24 h. Data were analysed as in d. Data are mean ± s.d. from nine independent experiments. Anti-Flag western blot was performed to determine expression levels; IκBα regulation was included as a pathway activation control upon cellular stimulation with TNF. g, SIX2 inhibits the transcriptional activity of RELA and RELB. To evaluate the transcriptional activity of each NF-κB isoform independently, we transfected RELA cDNA into RELB−/− cells and RELB cDNA into RELA−/− cells along with SIX2 and 5 × κB-LUC. Transfection of either RELA or RELB potently induced NF-κB transcription of the indicated cells, and this transcription was suppressed by SIX2. Luminescence units were measured 48 h after transfection. Data are mean ± s.d. from twelve (left) and nine (right) independent experiments. All western blot data are representative of three independent experiments. For gel source data, see Supplementary Fig. 1.
Extended Data Fig. 6 | SIX family proteins inhibit NF-κB activation by occupying the target gene promoters. a, Wild-type and SIX1−/− SIX2−/− H1792 cells were treated with mock or 50 ng ml−1 TWEAK for 24 h. Cell lysates from cytoplasmic or nuclear fractions were analysed by western blot. Neither SIX1 nor SIX2 blocked RELA phosphorylation or p100/52 processing, or restricted NF-κB translocation to the nucleus. b, Top, SIX proteins are composed of a SIX domain (SD), homeobox domain (HD), and coiled-coil (CD) region. Full-length SIX1 and SIX2 have 80% identical amino acids over the protein-coding sequence. The SD and HD domains (residues 1–183) are 96% identical. The indicated Flag-tagged SIX2 fragments were transfected alone into U-2 OS cells and processed for microscopy (middle, scale bars represent 20 μm) or transfected with 5 × κB-LUC into HEK293T cells and processed for κB reporter activity (bottom). The highly conserved SD–HD domain was the minimal fragment that inhibited κB reporter activity. This fragment strictly localized to the nucleus, indicating that SIX proteins inhibit nuclear activity of NF-κB. Graph data analysed as in Extended Data Fig. 5d. Data are mean ± s.d. from nine independent experiments. c, SIX2 inhibits gene activation from the IL8 promoter. pIL8-LUC plasmid composed of the 1.5-kb promoter region of IL8 cloned upstream of LUC was co-transfected with indicated plasmids into HEK293T cells. After 24 h, cells were mock-treated or treated with 25 ng ml−1 TNF for 24 h. Luciferase activity was then measured and analysed as in Extended Data Fig. 5d. Data are mean ± s.d. from nine (left) and twelve (right) independent experiments. d, ChIP experiment providing additional evidence that SIX proteins bind to inflammatory gene promoters (Fig. 3a). Chromatin was prepared from stable GFP–SIX1 cell lines (HCT116 cells), mock-treated or treated with 25 ng ml−1 TNF for 2 h. Anti-SIX1 antibodies (or anti-IgG control) were used to immunoprecipitate SIX1 from nuclear extracts. Co-eluted DNA was amplified using primer sets as in Fig. 3a. Relative promoter occupancy was normalized to each experimental IgG control. Bars are means of three technical replicates (shown as circles) and data are representative of three independent experiments. e, Control experiments corresponding to Fig. 3a and Extended Data Fig. 6f showing GFP–SIX1 expression. Wild-type and stable GFP–SIX1 fibroblasts were stimulated with 50 ng ml−1 TWEAK for 24 h. GFP–SIX1 expression was measured by western blot. f, SIX1 expression does not affect recruitment of RELA to the IL8 promoter. Chromatin was prepared from wild-type or GFP–SIX1 stable fibroblasts and immunoprecipitated with anti-RELA. Bound DNA was amplified and quantified by qPCR. Results were adjusted to ‘input DNA’ that was saved before immunoprecipitation. Relative enrichment was then normalized to each group’s untreated control. Data are mean ± s.d. from three independent experiments. g, Control experiments corresponding to Fig. 3c showing that SIX2 inhibits 5 × κB-LUC activity induced by GAL4–RELA and GAL4–RELB. GAL4–RELA or GAL4–RELB construct was co-transfected with indicated plasmids into HEK293T cells. Forty-eight hours after transfection, the luminescence units were measured. Data are mean ± s.d. from nine (left) and twelve (right) independent experiments. h, Model showing NIK-mediated reactivation of SIX protein function in a negative feedback loop to control inflammatory gene expression by targeting gene promoters and inhibiting the trans-activation function of NF-κB. In quiescent cells (top), NIK and SIX are constitutively ubiquitinated and degraded by the proteasome. Non-canonical NF-κB agonists (for example, TWEAK, LTα1β2, or BV6) promote degradation of cIAPs, loss of NIK ubiquitination and subsequent NIK protein accumulation (middle). Under conditions of long-term cytokine exposure, NIK-mediated suppression of a currently unknown E3-ubiquitin ligase results in SIX protein accumulation (bottom). Consequently, SIX proteins suppress inflammatory gene expression by targeting gene promoters and directly inhibiting trans-activation by NF-κB in a negative feedback loop (bottom). All western blots and microscopy data are representative of three independent experiments. For gel source data, see Supplementary Fig. 1.
Extended Data Fig. 7 | Doxycycline-induced HA–SIX1 expression in mice. **a,** Doxycycline-induced HA–SIX1 bitransgenic mouse model system. CAG–rtTA3 mice were intercrossed with Tet-on driven HA–SIX1 mice to obtain rtTA3+/−/SIX1+ mice. In principle, doxycycline-bound rtTA3 targets the Tet-on operator and drives broad HA–SIX1 expression across multiple tissues. Primer sets used to genotype CAG–rtTA3 on chromosome 8 and Tet-on HA–SIX1 are shown. Electrophoresis gels show genotyping of a representative rtTA3+/−/SIX1+ mouse. **b,** Anti-SIX1 western blot of whole cell lysates from BMDMs isolated from rtTA3+/−/SIX1+ mice. HA–SIX1 is not expressed in the absence of doxycycline under quiescent condition (left). When TNF was administered to these cells, endogenous SIX1 was stimulated (right). **c,** Whole cell lysates from doxycycline-treated BMDMs isolated from rtTA3+/−/SIX1+ mice. BMDMs were stimulated with TNF as indicated and probed with anti-SIX1 antibody by western blot. Doxycycline induced HA–SIX1 expression (lane 1), and this induction was potentiated by TNF (lane 2). HA–SIX1 ran as a doublet, which potentially represents an unmodified and a mono-ubiquitinated form of SIX1. Neither endogenous SIX1 nor HA–SIX1 was detected in BMDMs isolated from Dox-treated rtTA3+/− mice (lane 3). In control experiments, TNF induced endogenous SIX1 in BMDMs isolated from Dox-treated rtTA3+/− mice (lane 4). **d,** rtTA3+/−/SIX1+ and rtTA3+/− mice were given 2 mg ml−1 doxycycline in drinking water for 10 days. Cell lysates from liver and spleen were used to probe HA–SIX1 expression by anti-SIX1 western blot. All western blot data are representative of three independent experiments. **e,** Peritoneal macrophages were isolated from rtTA3+/−/SIX1+ and rtTA3+/− littermate control mice. Adherent macrophages were incubated with 2 μg ml−1 doxycycline for 24 h and then mock-treated or treated with 100 ng ml−1 LPS for 4 h. Total RNA was isolated for qRT–PCR. Relative gene expression was normalized to rtTA3+/− untreated control. Bars show mean from two technical replicates (shown as circles). Data are representative of three independent experiments. **f,** Experimental procedures corresponding to Fig. 4a–c (see Methods). For gel source data, see Supplementary Fig. 1.
Extended Data Fig. 8 | NIK−/− and SIX1−/−SIX2−/− fibroblasts are sensitized to BV6–TNF-induced cell death. We validated the observation that SIX1−/−SIX2−/− sensitized NSCLC cells to cell death induced by BV6 and TNF in SV40 immortalized STAT1−/− fibroblasts. a, b, Fibroblasts of the indicated genotype were treated with BV6 (a) or TNF (b). Cell viability was determined by measuring ATP after 24 h. Cell survival rate was normalized to each genotype’s untreated control. Neither BV6 nor TNF (10 ng ml−1) alone induced fibroblast cell death. c, NIK−/− and SIX1−/−SIX2−/− fibroblasts are sensitized to BV6–TNF-induced cell death. Left, experiments and analysis as in a. Right, representative images showing the cell death phenotype induced by BV6–TNF in fibroblasts of the indicated genotypes. Scale bars represent 50 μm. d, Time course of combined BV6 (2.5 μM) and TNF (25 ng ml−1) treatment. NIK−/− and SIX1−/−SIX2−/− fibroblasts exhibited increased cleavage of poly ADP-ribose polymerase (PARP) and caspase-3 in BV6–TNF-treated fibroblasts. BV6–TNF induced NIK-dependent expression of both SIX1 and SIX2, suggesting that this cascade may be responsible for resistance to this treatment. e, f, We introduced a silent mutation in the gRNA recognition sequence of SIX2 cDNA that cannot be targeted by CRISPR–Cas9 (SIX2R, e, top). Expression of SIX2R in SIX1−/−SIX2−/− fibroblasts rescued the cell death phenotype (e) and suppressed both PARP and caspase-3 cleavage (f) induced by BV6–TNF. Wild-type and SIX1−/−SIX2−/− fibroblasts were transduced with Fluc or SIX2R lentivirus. After 72 h, cells were mock-treated or treated with 0.2 μM BV6 plus 10 ng ml−1 TNF for 24 h. Cell viability was determined by measuring ATP. Cell survival was normalized to each untreated control (e, bottom). For western blot, cells were treated with 2.5 μM BV6 plus 25 ng ml−1 TNF for 6 h (f). All quantified data are mean ± s.d. from nine independent experiments. **P < 0.01, ****P < 0.0001. Western blot data are representative of three independent experiments. For gel source data, see Supplementary Fig. 1.
Extended Data Fig. 9 | NIK−/− and SIX1−/−SIX2−/− sensitize U-2 OS cells to BV6–TNF-induced caspase-8-dependent cell death.

a–d, Knockout of NIK or SIX1 and SIX2 in U-2 OS cells using the CRISPR–Cas9 system. Western blots show endogenous NIK, SIX1, or SIX2 expression in parental, NIK−/− and SIX1−/−SIX2−/− U-2 OS cells (a, c). We used MG132 to stabilize endogenous NIK in wild-type and NIK−/− U-2 OS cells.

c, Schematic representation of In/Del base pairing and the sgRNA targeting locus of exon 1 in NIK in U-2 OS cells. NIK−/− contains +G, −5 bp, and −18 bp (disrupted alternative splicing site) alleles.

d, Schematic representation of In/Del base pairing and the sgRNA targeting locus of exon 1 in SIX1 and SIX2 in U-2 OS cells. SIX1−/−SIX2−/− contains +T of SIX1 and SIX2. e, SIX1−/−SIX2−/− and NIK−/− U-2 OS cells are sensitive to BV6–TNF-induced apoptosis. Wild-type, NIK−/−, or SIX1−/−SIX2−/− U-2 OS cells were treated with 25 ng ml−1 TNF alone or in the presence of indicated concentrations of BV6 for 48 h. Cell viability was measured by ATP. Cell survival was normalized to the absence of BV6 (control).

f, g, Expression of SIX2R (see Extended Data Fig. 8e) or SIX2 cDNA protected SIX1−/−SIX2−/− or NIK−/− cells, respectively, from BV6–TNF-induced apoptosis. Wild-type, NIK−/−, or SIX1−/−SIX2−/− U-2 OS cells were transduced with Flu, SIX2 or SIX2R lentivirus as indicated and treated with 2.5 μM BV6 plus 25 ng ml−1 TNF for 24 h.

h, i, Cell death was mediated by the extrinsic apoptotic pathway, as both the pan-caspase inhibitor z-VAD and specific caspase-8 inhibitor z-IETD blocked BV6–TNF-induced cell death in SIX1−/−SIX2−/− fibroblasts (h) and U-2 OS cells (i). Wild-type and SIX1−/−SIX2−/− fibroblasts were treated with 1 μM BV6 plus 10 ng ml−1 TNF alone or in the presence of 20 μM z-VAD or z-IETD for 24 h. U-2 OS cells were treated with 2.5 μM BV6 plus 25 ng ml−1 TNF alone or in the presence of 20 μM z-VAD for 48 h. Cell viability was measured by ATP. Cell survival rate was normalized to each untreated control.

j, Western blot showing the status of PARP and caspase-3 cleavage induced by BV6–TNF treatment in the absence of or the presence of caspase inhibitors in wild-type and SIX1−/−SIX2−/− fibroblasts. Cells were mock-treated or treated with 2.5 μM BV6 plus 25 ng ml−1 TNF alone or in the presence of 30 μM z-VAD or 40 μM z-IETD for 6 h. All quantified data are mean ± s.d. from nine (e, f, h, j) or six (g) independent experiments. *P < 0.05, ****P < 0.0001. Western blot data are representative of three independent experiments. For gel source data, see Supplementary Fig. 1.
Extended Data Fig. 10 | Validation and pathway analysis of RNA-seq in wild-type and SIX1−/−SIX2−/− H1792 NSCLC cells. a, To validate cluster I genes from RNA-seq data shown in Fig. 4f, wild-type and SIX1−/−SIX2−/− H1792 cells were mock-treated or treated with 5 μM BV6 plus 25 ng ml−1 TNF for 24 h. Total RNA was isolated for qRT–PCR. Gene expression was normalized to wild-type untreated control. Data are mean ± s.d. of three technical replicates and are representative of three independent experiments. b, Ingenuity pathway analysis (IPA) of cluster I genes was performed (see Methods). Pathway enrichment bar plots are shown. Data are from two independent experiments. Significance values for the canonical pathways were calculated by Fisher’s exact test (right-tailed). c, Inflammatory gene transcription is highly induced in SIX1−/−SIX2−/− H1792 cells (compared to wild-type controls) upon specific stimulation of the non-canonical NF-κB signalling pathway. Wild-type and SIX1−/−SIX2−/− H1792 cells were mock-treated or treated with 50 ng ml−1 TWEAK for 3 h. Total RNA was extracted for qRT–PCR. Gene expression level was normalized to wild-type untreated control. Data are mean ± s.d. of three technical replicates and representative of two independent experiments.
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

Flow cytometry (Stratedigm, model:S1000) was used to collect infectivity data by sorting GFP-, RFP-, or BFP-expressed cells. Illumina HiSeq 2500 was used to generate RNA-seq data. qRT-PCR data were generated by ABI 7500 fast real-time PCR machine. Luciferase reporter, ELISA, and cell viability data were measured by FLUOstar OPTIMA (BMG labtech).

Data analysis

FlowJo 10.0.6 was used to analyze FACS data; Graphpad prism 7 was used to perform statistical analysis; Tophat (v2.0.10) was used to map the RNA-seq reads; Cufflinks/Cuffdiff (v2.1.1) was used to calculate the gene expression value; fastqc (v0.11.2) and fastq_screen (v0.4.4) were used to check the quality of RNA-seq reads; fastq-mcf (ea-utils/1.1.2-806) was used to trim quality of reads; picard-tools (v1.127) was used to mark the duplicates; featureCounts was used to count the reads; edgeR was used to distinguish the differential gene expression; Ingenuity Pathway Analysis tool was used to perform pathway analysis; various R packages was used to plot the pathway enrichment.

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

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All figures associated raw data of graphs and western blot gels were deposited in Source data and in Supplementary figure. All data or materials associated this study are available from authors upon reasonable request.

RNA-seq data have been deposited to NCBI GEO. Accession number is GSE126535
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Although no statistical methods were used to predetermined sample size in vitro and in vivo analyses, we conducted preliminary experiments to estimate variances in each assay and determined sufficient sample size. The biological sample size is presented in the figure or stated in the figure legends section. |
| Data exclusions | No data were excluded from the analyses. |
| Replication | All attempts at replication were successful. The experiments number has been clearly stated in the figure legends. |
| Randomization | No randomization was used in this study. For in vitro experiments, indicated genotype cells were transduced with indicated lenti-virus, transfected with indicated plasmids, or treated with indicated drugs. For in vivo mice experiments, mice were grouped according to the genotype. |
| Blinding | For in vitro experiments, no blinding was done. For clinical score analysis, survival mice were evaluated blindly to reach clinical score. |

Behavioural & social sciences study design

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| Study description | Briefly describe the study type including whether data are quantitative, qualitative, or mixed-methods (e.g. qualitative cross-sectional, quantitative experimental, mixed-methods case study). |
| Research sample | State the research sample (e.g. Harvard university undergraduates, villagers in rural India) and provide relevant demographic information (e.g. age, sex) and indicate whether the sample is representative. Provide a rationale for the study sample chosen. For studies involving existing datasets, please describe the dataset and source. |
| Sampling strategy | Describe the sampling procedure (e.g. random, snowball, stratified, convenience). Describe the statistical methods that were used to predetermine sample size OR if no sample-size calculation was performed, describe how sample sizes were chosen and provide a rationale for why these sample sizes are sufficient. For qualitative data, please indicate whether data saturation was considered, and what criteria were used to decide that no further sampling was needed. |
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| Materials & experimental systems | Methods |
|----------------------------------|---------|
| Involved in the study n/a        | Involved in the study n/a |
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| ☐ Eukaryotic cell lines          | ☐ Flow cytometry |
| ☐ Palaeontology                  | ☐ MRI-based neuroimaging |
| ☐ Animals and other organisms    |         |
| ☑ Human research participants    |         |
| ☐ Clinical data                  |         |

#### Antibodies

We have listed indicated antibodies information used in this study as follows.

| Antibody Name; Purpose; Source; Manufacturer; Cat. No.; Clone No.; Lot No.; WB Dilution |
|----------------------------------------|---------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Flag; WB; HRP; Sigma; A8592; M2; 5LD09930; 1:10000 |
| Actin; WB; Rabbit; Sigma; A2066; N/A; 103M4826V; 1:5000 |
| HA; WB; Mouse; Covance; MMS-101P; 16812; D14F01308; 1:5000 |
| GFP; WB; Rabbit; Clontech; 632592; N/A; 1404005; 1:5000 |
| pIkBa; WB; Rabbit; Cell Signaling; #N/A; 14D4; 14; 1:2500 |
| IkBa; WB; Mouse; Cell Signaling; #N/A; 14D4; 14; 1:2500 |
| NIK; WB; Rabbit; Cell Signaling; #N/A; 14D4; 14; 1:2500 |
| SIX1; WB/Ch-IP; Rabbit; Cell Signaling; #N/A; 14D4; 14; 1:2500 |
| RelA; WB; Rabbit; Cell Signaling; #N/A; 14D4; 14; 1:2500 |
| RelA; Ch-IP; Rabbit; Santa Cruz; sc-372X; C-20; E0916; N/A |

Any manipulations. State what population the sample is meant to represent when applicable. For studies involving existing datasets, describe the data and its source.
Validation

All primary antibodies were purchased from the manufacturer's website: https://www.sigmaaldrich.com/catalog/product/sigma/a8592?lang=en&region=US

1. Flag, product citation is 640 from the manufacturer's website: https://www.sigmaaldrich.com/catalog/product/sigma/a8592?lang=en&region=US

2. Actin, Species (amoeba, chicken, wide range, vertebrates, slime mold, human), Application (IF, IHC(P), WB). Product citation is 1846 from the manufacturer's website: https://www.sigmaaldrich.com/catalog/product/sigma/a2066?lang=en&region=US

3. HA, Application (WB-Quality tested, IF, IP-Validated, Purification-Reported in Literature). Product citation is 102 from the manufacturer's website: https://www.biorad.com/en-us/products/purified-anti-ha-11-epitope-tag-antibody-11374

4. GFP, Application (WB, IF, Ch-IP seq etc.). Information is from the manufacturer's website: https://www.labome.com/product/Takara-Bio-Clontech/632592.html

5. plkB, Species (Human, Mouse, Rat, Monkey), Application (WB, IP). Product citation is 416 from the manufacturer's website: https://www.cellsignal.com/products/primary-antibodies/phospho-ikba-ser32-14d4-rabbit-mab/2859

6. IkBα, Species (Human, Mouse, Rat, Monkey, Bovine, Pig), Application (WB, IP etc.). Product citation is 338 from the manufacturer's website: https://www.cellsignal.com/products/primary-antibodies/iba-153a-mouse-antibody-3550

7. NIK, Species (Human, Mouse, Application (WB). Product citation is 59 from the manufacturer's website: https://www.cellsignal.com/products/primary-antibodies/nik-antibody-4994

8. SIX1, Species (Human, Mouse, Rat, Monkey, Application (WB, IP, etc.). Product citation is 2 from the manufacturer's website: https://www.cellsignal.com/products/primary-antibodies/six1-d4a8k-rabbit-mab/12891

9. RelA, Species (Human, Mouse, Rat, Hamster, Monkey, Dog), Application (WB, IP, Ch-IP etc.). Product citation is 739 from the manufacturer's website: https://www.cellsignal.com/products/primary-antibodies/ab-65-d4e12-xp-rabbit-mab/8242

10. RelA, Species (Mouse, rat and human), Application (WB, IP, etc.). Product citation is 1057 from the manufacturer's website: https://www.scbt.com/scbt/product/nf-kappa-b-antibody-c-20

11. pRelA, Species (Human, Mouse, Rat, Monkey, Bovine), Application (WB, IP, etc.). Product citation is 964 from the manufacturer's website: https://www.cellsignal.com/products/primary-antibodies/phospho-ikba-ser32-14d4-rabbit-mab/3033

12. RelB, Species (mouse, rat and human), Application (WB, IP, etc.). Product citation is 283 from the manufacturer's website: https://www.scbt.com/scbt/product/relb-antibody-c-19?requestFrom=search

13. p100/PARP, Species (human, rat and human), Application (WB, IP, etc.). Product citation is 123 from the manufacturer's website: https://www.cellsignal.com/products/primary-antibodies/p100/p52-antibody-c-57?requestFrom=search

14. H3, Species (Mouse, Rat, Chicken, Dog, Human etc.), Application (WB, IP, Ch-IP etc.). Product citation is 2428 from the manufacturer's website: https://www.abcam.com/histone-h3-antibody-nuclear-loading-control-and-chip-grade-ab1791.html

15. PARP, Species (Human, Mouse, Rat, Monkey), Application (WB). Product citation is 1581 from the manufacturer's website: https://www.cellsignal.com/products/primary-antibodies/parp-antibody-9542

16. Cleaved Casp 3, Species (Human, Mouse, Rat, Monkey, Bovine), Application (WB, IP, etc.). Product citation is 1504 from the manufacturer's website: https://www.cellsignal.com/products/primary-antibodies/cleaved-casp-3-antibody-5a112-16d4-rabbit-mab/491840

17. TAK1, Species (Human, Mouse, Rat, Monkey, Bovine), Application (WB, IP, etc.). Product citation is 1504 from the manufacturer's website: https://www.cellsignal.com/products/primary-antibodies/cleaved-casp-3-antibody-5a112-16d4-rabbit-mab/491840

18. TAK1, Species (Human, Mouse, Rat, Monkey, Bovine), Application (WB, IP, etc.). Product citation is 1504 from the manufacturer's website: https://www.cellsignal.com/products/primary-antibodies/cleaved-casp-3-antibody-5a112-16d4-rabbit-mab/491840

19. ciAP1, Species (Human, Mouse, Rat, Dog), Application (WB, IP, etc.). Product citation is 338 from the manufacturer's website: https://www.cellsignal.com/products/primary-antibodies/ciap-1-antibody-c-57?requestFrom=search

20. SIX2, Species (Human, Mouse, Rat, Dog), Application (WB, IP, etc.). Product citation is 338 from the manufacturer's website: https://www.cellsignal.com/products/primary-antibodies/ciap-1-antibody-c-57?requestFrom=search

21. CD40, Species (Human, Mouse, Rat, Dog), Application (WB, IP, etc.). Product citation is 338 from the manufacturer's website: https://www.cellsignal.com/products/primary-antibodies/ciap-1-antibody-c-57?requestFrom=search

22. Pol II, Species (Human, Mouse, Rat, Dog), Application (WB, IP, etc.). Product citation is 338 from the manufacturer's website: https://www.cellsignal.com/products/primary-antibodies/ciap-1-antibody-c-57?requestFrom=search

23. IgG, Application (WB, IP). http://www.emdmillipore.com/US/en/product/Normal-Rabbit-IgG_MM_NF-12-370
| Authentication | Common cell lines were identified by their morphology. Specific cell lines used in this study were authenticated by PCR or mutational status |
|----------------|---------------------------------------------------------------------------------------------------------------------------------|
| Mycoplasma contamination | All cell lines were tested and negative for mycoplasma contamination |
| Commonly misidentified lines (See ICLAC register) | No commonly misidentified cell line |

**Palaeontology**

- **Specimen provenance**: Provide provenance information for specimens and describe permits that were obtained for the work (including the name of the issuing authority, the date of issue, and any identifying information).
- **Specimen deposition**: Indicate where the specimens have been deposited to permit free access by other researchers.
- **Dating methods**: If new dates are provided, describe how they were obtained (e.g. collection, storage, sample pretreatment and measurement), where they were obtained (i.e. lab name), the calibration program and the protocol for quality assurance OR state that no new dates are provided.

Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.

**Animals and other organisms**

Policy information about **studies involving animals**: ARRIVE guidelines recommended for reporting animal research.

**Laboratory animals**: SIX1 transgene mice are FVB genetic background. CAG-rtTA3 mice are C57BL/6 background. rtTA3-SIX1 mice and their littermate controls (rtTA3) were obtained by intercrossing SIX1 mice with CAG-rtTA3 mice. 6-7 weeks old mice were given doxycycline water for 10 days prior to LPS administration experiments. Nk-/- mice are C57BL/6 background. 6-week-old mice were used for experiment. Both males and females were used for experiments.

**Wild animals**: The study did not involve wild animals.

**Field-collected samples**: The study did not involve samples collected from the field.

**Ethics oversight**: All animal experiments were performed according to the protocol that approved by UT Southwestern Medical Center IACUC.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

**Human research participants**

Policy information about **studies involving human research participants**.

**Population characteristics**: Describe the covariate-relevant population characteristics of the human research participants (e.g. age, gender, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write "See above."

**Recruitment**: Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.

**Ethics oversight**: Identify the organization(s) that approved the study protocol.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

**Clinical data**

Policy information about **clinical studies**. All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

**Clinical trial registration**: Provide the trial registration number from ClinicalTrials.gov or an equivalent agency.

**Study protocol**: Note where the full trial protocol can be accessed OR if not available, explain why.

**Data collection**: Describe the settings and locales of data collection, noting the time periods of recruitment and data collection.

**Outcomes**: Describe how you pre-defined primary and secondary outcome measures and how you assessed these measures.
ChIP-seq

Data deposition

- Confirm that both raw and final processed data have been deposited in a public database such as GEO.
- Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links

For "Initial submission" or "Revised version" documents, provide reviewer access links. For your "Final submission" document, provide a link to the deposited data.

Files in database submission

Provide a list of all files available in the database submission.

Genome browser session

Provide a link to an anonymized genome browser session for "Initial submission" and "Revised version" documents only, to enable peer review. Write "no longer applicable" for "Final submission" documents.

Methodology

Replicates

Describe the experimental replicates, specifying number, type and replicate agreement.

Sequencing depth

Describe the sequencing depth for each experiment, providing the total number of reads, uniquely mapped reads, length of reads and whether they were paired- or single-end.

Antibodies

Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and lot number.

Peak calling parameters

Specify the command line program and parameters used for read mapping and peak calling, including the ChIP, control and index files used.

Data quality

Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5-fold enrichment.

Software

Describe the software used to collect and analyze the ChIP-seq data. For custom code that has been deposited into a community repository, provide accession details.

Flow Cytometry

Plots

- Confirm that:
  - The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
  - The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

We seeded indicated cells in the 24-well-plate. Cells were then transduced with indicated lentivirus and then challenged with the GFP-expressing bacterial or viral pathogens. Cells were then detached by 37°C warmed Accumax (Sigma), followed by centrifugation at 800×g for 2 minutes. Cells were then fixed by suspending in PBS/1% PFA at 4°C for at least 30 minutes. Cells were then stored in PBS/3% FBS.

Instrument

Stratedigm, model:S1000

Software

Flowjo 10.0.6

Cell population abundance

We analyzed 30,000 single cells which are live

Gating strategy

For most part of analysis, we gated the live cells, single cells population from live cells, and then gated the RFP positive cells. Finally, we gated the GFP positive units from the RFP positive population.

For Extended Data Fig. 1h and 4b, we performed extra one step (BFP positive cells from RFP positive population) before we gated the GFP positive cells.

For, Extended Data Fig. 1e, we only performed GFP positive analysis in single cells.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.
## Magnetic resonance imaging

### Experimental design

| Design type          | Indicate or resting state; event-related or block design. |
|----------------------|-----------------------------------------------------------|
| Design specifications| Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials. |
| Behavioral performance measures | State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects). |

### Acquisition

| Imaging type(s) | Specify: functional, structural, diffusion, perfusion. |
|-----------------|-------------------------------------------------------|
| Field strength  | Specify in Tesla                                      |
| Sequence & imaging parameters | Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size, slice thickness, orientation and TE/TR/flip angle. |
| Area of acquisition | State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined. |
| Diffusion MRI   | Used | Not used |

### Preprocessing

| Preprocessing software | Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.). |
|------------------------|-------------------------------------------------------------------------------------------------|
| Normalization          | If data were normalized/standardized, describe the approach(es): specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization. |
| Normalization template | Describe the template used for normalization/transformation, specifying subject space or group standardized space (e.g. original Talairach, MN1305, ICBM152) OR indicate that the data were not normalized. |
| Noise and artifact removal | Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration). |
| Volume censoring       | Define your software and/or method and criteria for volume censoring, and state the extent of such censoring. |

### Statistical modeling & inference

| Model type and settings | Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects, drift or auto-correlation). |
|-------------------------|------------------------------------------------------------------------------------------------------------------|
| Effect(s) tested        | Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA or factorial designs were used. |
| Specify type of analysis: | Whole brain | ROI-based | Both |
| Statistic type for inference | Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods. |
| Correction              | Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo). |

### Models & analysis

| n/a Involved in the study | Functional and/or effective connectivity |
|---------------------------|------------------------------------------|
|                           | Graph analysis                           |
|                           | Multivariate modeling or predictive analysis |
|                           | Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information). |
| Functional and/or effective connectivity | Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.). |
| Graph analysis            | Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.). |
Multivariate modeling and predictive analysis

Specify independent variables, features extraction and dimension reduction, model, training and evaluation metrics.