RNAi-Based Identification of Gene-Specific Nuclear Cofactor Networks Regulating Interleukin-1 Target Genes

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The potent proinflammatory cytokine interleukin (IL)-1 triggers gene expression through the NF-κB signaling pathway. Here, we investigated the cofactor requirements of strongly regulated IL-1 target genes whose expression is impaired in p65 NF-κB-deficient murine embryonic fibroblasts. By two independent small-hairpin (sh)RNA screens, we examined 170 genes annotated to encode nuclear cofactors for their role in Cxcl2 mRNA expression and identified 22 factors that modulated basal or IL-1-inducible Cxcl2 levels. The functions of 16 of these factors were validated for Cxcl2 and further analyzed for their role in regulation of 10 additional IL-1 target genes by RT-qPCR. These data reveal that each inducible gene has its own (quantitative) requirement of cofactors to maintain basal levels and to respond to IL-1. Twelve factors (Epc1, H2afz, Kdm2b, Kdm6a, Mbd3, Mta2, Phf21a, Ruvbl1, Sin3b, Suv420h1, Taf1, and Ube3a) have not been previously implicated in inflammatory cytokine functions. Bioinformatics analysis indicates that they are components of complex nuclear protein networks that regulate chromatin functions and gene transcription. Collectively, these data suggest that downstream from the essential NF-κB signal each cytokine-inducible target gene has further subtle requirements for individual sets of nuclear cofactors that shape its transcriptional activation profile.

Keywords: IL-1, corepressor, coactivator, NF-κB, transcription, chromatin, shRNA screen

INTRODUCTION

Inflammation is an evolutionary conserved reaction to the myriad of insults that can affect tissue homeostasis. At the cellular level, the inflammatory reaction is characterized by rapid reprogramming of gene expression. The transiently expressed genes encode multiple factors that regulate immune cell infiltration and activation, blood vessel tone, metabolic reactions, pain and wound repair (1).

The founding members of the IL-1 family of cytokines, IL-1α and IL-1β, are master coordinators of inflammation as they can induce the full spectrum of clinical signs of inflammation by upregulating the entire repertoire of inflammatory genes in most (if not all) cell types of the body during sterile or infectious inflammation (2, 3).

IL-1 binding to its heterodimeric receptor at the plasma membrane triggers the cytoplasmic activation of the canonical NF-κB pathway and activation of JNK and p38 MAP kinases through a series
functions related to IL-1  
ακ have identified across different species signaling components that  
ing chromatin accessibility but also serve as scaffolding platforms  
enzymatic activity to modify N-terminal histone tails for regulat-  
ment, or shut-down transcription (pressor or coactivator multiprotein complexes to support, aug-  
requires further nuclear factors that are organized as large core-  
endogenous IL-1 target gene, thus avoiding the introduction of  
gs with differential effects on basal or IL-1-induced mRNA  
transcription that allowed the identification of 22 nuclear cofac-  
screen with selected regulators of chromatin organization and  
In general, the quantitative and timely expression of genes  
strates or corepressors that modulate an endogenous IL-1-dependent  
11 endogenous highly regulated NF-kB target  
transcription of 11 endogenous NF-kB target genes confirmed that they were  
maximally induced between 30 and 180 min of IL-1 stimulation  
suggesting their onset of gene transcription might be coordi-  
cated by common nuclear mechanisms or cofactors (Figure S2 in Supplementary Material). We then selected a total of 170  
well-annotated nuclear cofactors that had been described by the  
Kouzarides and Shen groups (24, 25). The full list of the known  
nuclear functions according to Panther/GO classifications of  
these proteins is shown in Table S1 in Supplementary Material. To  
suppress the protein levels of these coactivators, 791 plasmid vec-  
tors prepared from the TRC.1 shRNA library based on the vector  
pLKO.1-puro were transfected into primary Mefs (26). Notably,  
each nuclear cofactor encoding gene was targeted multiple times  
and individually (791/170 = 4.65 shRNAs/gene on average,  
Table S1 in Supplementary Material) enabling higher probability  
of identifying true positive hits when knockdowns gave rise to  
similar phenotypic changes. All screens were performed in a  
48-well format, each well containing initially 3.5 × 10⁶ Mef cells.  
Transfection was performed with 270 ng plasmid DNA using  
lipofectamine LTX plus reagent under optimized conditions.  
Transfection rates were high as monitored using a GFP plasmid.  
In addition, cells were also selected with puromycin to eliminate  
the remaining non-transfected cells (Figure 1). On each plate,  
cells were also transfected with two different control plasmids,  
i.e., empty pLKO.1 or pLKO.1 containing a scrambled shRNA  
sequence. Then, cells were treated with IL-1 for 3 h (screen I) or  
1 h (screen II) or were left untreated and reverse transcription was  
performed in denatured total cell extracts following an adapted  
commercial protocol. To amplify specific cDNAs, a linear pream-  
plification PCR of 15 cycles was included using a pool of primer  
pairs contained in commercial Taqman assay kits. Aliquots of  
these cDNA mixtures were then further amplified, detected, and  
quantified by gene-specific quantitative PCR using the Taqman  
probes/primer pairs (Figure 1).

RESULTS

Considerations and Design of a shRNA Screen for Cofactors of IL-1 Target Gene Expression

We developed a shRNA-based screen to identify nuclear coactiva- 
tors or corepressors that modulate an endogenous IL-1-dependent  
gene response. Our set up combined (i) the knockdown of endog-  
enous proteins classified as nuclear cofactors and (ii) the analysis  
of the resulting phenotype at the level of mRNA expression of an  
endogenous IL-1 target gene, thus avoiding the introduction of  
reporter genes or other means of further (genetic) manipulation.  
We initially searched microarray data sets of different cell types of  
human or murine origin for IL-1-mediated activation which led  
to the identification of a common core set of strongly regulated  
inflammatory genes thereby providing suitable targets as read  
out for our screening pipeline. In murine embryonic fibroblasts  
(Mefs), microarray experiments revealed Cxcl2, Cxcl1, Cxcl5,  
Cxcl10, Ccl7, Ccl2, Ccl20, Il6, Nfkbia, Nfkbiz, and Icam1 as strongly  
regulated genes that were induced after 30 min of IL-1 stimula-  
tion (Figure S1A in Supplementary Material). We also screened  
a number of microarrays to identify genes that are not regulated  
by IL-1. We found no detectable changes for ActB, Ube2L3, Evi1,  
Mcm7, and Car13 in response to IL-1, as confirmed by RT-qPCR  
experiments (Figure S1B in Supplementary Material). These data  
revealed the usability of these genes as internal (“housekeeping”)  
controls for loading of equal RNA amounts in PCR reactions  
(Figure S1B in Supplementary Material). RT-qPCR experi- 
mements for 11 of the IL-1 target genes confirmed that they were  
maximally induced between 30 and 180 min of IL-1 stimulation  
transcriptional induction of inflammatory genes through initia- 
tion factors (TFs) of the NF-κB and AP-1 families that are  
substrates of the NF-κB and MAPK signaling pathways trigger  
transcriptional induction of inflammatory genes through initia-  
tion of mammalian genes at a large scale (10, 11). Mainly by using  
transiently transfected small interfering (si)RNAs, several groups  
have identified across different species signaling components that  
regulate transcriptional activity of NF-κB in response to a variety  
of immune stimuli including TNFα, a cytokine with inflammatory  
functions related to IL-1 (12–22). However, all these studies used  
artificial reporter systems to measure NF-κB activation as read- 
outs for the RNAi screens, resulting in surprisingly little overlap  
of identified NF-κB modulators. The stable integration of NF-κB  
cis-elements upstream of luciferase or GFP reporter genes facili- 
tates rapid, quantitative and high content assay screening but it  
gives little information on nuclear factors that regulate endogenous  
genomes in their native chromatin environment (23). Accordingly,  
there is a lack of functional studies describing the full repertoire  
of coactivator/corepressor complexes in a given biological context,  
including the IL-1-driven inflammatory cell reaction.  
Here, we report results from a small-hairpin (sh)RNA-based  
screen with selected regulators of chromatin organization and  
transcription that allowed the identification of 22 nuclear cofac- 
tors with differential effects on basal or IL-1-induced mRNA  
expression of 11 endogenous highly regulated NF-κB target genes.  
These data show that inflammatory genes require a gene- 
specific network of defined and interacting cofactors in addition  
to the canonical cytoplasmic signaling pathways that switch on  
their transcription.

Overview of Screening Results and Determination of shRNA Screening Hits

Using these setups, we performed two independent shRNA  
screens using the most strongly regulated IL-1 target gene Cxcl2  
as a readout (Figure S1A in Supplementary Material). Apart from  
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3.5 x 10^4 cells / 48-well plate

transfection (Lipofectamine LTX plus reagent®)

48 h selection with puromycin (1 µg/ml)

-/+ IL-1α 10 ng/ml (screen I 3 h, screen II 1 h)

denaturing cell lysis

reverse transcription

PCR

TaqMan® assays

data analysis based on ct values

40 PCR cycles

cDNA + TaqMan® assays for ActB, Ube2l3, Cxcl2

linear preamplification PCR

37°C/60min

95°C/5min

mRNA

5'3'

3'5'

5'3'

3'5'

3'5'

3'5'

40 PCR cycles

pLKO.1 vector backbone had a global suppressive effect on all ActB and Ube2l3 ct values showing that this condition was useful for monitoring transfection efficiency on the individual 48 well plates, but not for normalization or further analyses of changes in gene expression (Figure 2A). Compared to vector controls, shRNA transfections lowered the median ActB mRNA level in screen I (Figure 2A). In contrast, there were little effects of the shRNAs on the median Ube2l3 mRNA levels (Figures 2A,B). Therefore, we omitted ActB measurements as an internal control for the second screen and for all subsequent calculations of normalized mRNA levels. The IL-1 target gene Cxcl2 was expressed at lower abundance (i.e., higher ct values) in unstimulated cells but was strongly regulated after exposure of cells to IL-1 as expected (Figure 2A). This was also the case for screen II (Figure 2B).
As the goal of the screen was to identify changes of basal or inducible Cxcl2 expression levels by knockdown of nuclear cofactors, it was important to consider a suitable control situation for relative quantification of qPCR data. As shown in Figures 2A, B, cells transfected with empty pLKO.1 or the scrambled shRNA show a comparable regulation of Cxcl2 by IL-1, whereas expression...
of GFP reduced basal and inducible levels of Cxcl2 (Figure 2A). This increase in ct values suggested that GFP also had a suppressive effect on IL-1-mediated gene expression. Therefore, we decided to choose pLKO.1 as (vector) control for calculating relative differences in mRNA expression levels. In both screens, transfection of shRNAs resulted in a global increase in median ct values for Cxcl2 suggesting that multiple cofactors may be involved in regulating basal and inducible Cxcl2 levels (Figures 2A,B).

We then considered various types of changes of Cxcl2 mRNA levels that may occur in shRNA-treated cells. A schematic representation of equations reflecting these changes in basal conditions or in IL-1-treated cells as well as alterations in the extent of IL-1-mediated regulation in control versus shRNA-treated cells is shown in Figure S3 in Supplementary Material. The ΔΔct method was used to calculate these differences from ct values of Cxcl2 after normalization to Ube2I3 expression levels. The normalized values from screen I were merged to one expression (ct) value per shRNA target. Ratio values derived from the comparisons mentioned above were then ranked by Z-score (Figures 2C,D). Data from shRNAs with a Z-score value greater than 1 were then used to identify potential hits and to calculate the overlap of hits between screen I and screen II. To prove the validity of this selection procedure, individual fold changes for each shRNA target were visualized as heatmaps and are shown alongside all individual vector control values of the corresponding plates utilized in screens I and II (Figure S4 in Supplementary Material). With this strategy, 22 factors were identified whose suppression changed mRNA levels of Cxcl2 significantly (i.e., < 1 SD) in both screens (Figure 2E). Inspection of the data reveals that with a few exceptions (e.g., shKdm5a shRNA#4) the up to five shRNAs per gene changed ratios of Cxcl2 mRNA expression into the same direction (Figure S4 in Supplementary Material). These observations underscore the reliability of the TRC.1 selection procedure for shRNAs and show that our strategy enables identification of true positive hits with high probability. Although the extent of Cxcl2 regulation in the vector controls varied considerably between individual plates, the average regulation between both screens was comparable and showed strong induction by IL-1 (Figure S4 in Supplementary Material). We attribute this phenomenon to the relatively low basal mRNA expression level of Cxcl2 and to the lack of controlling input RNA or cDNA concentrations across samples during the miniaturized RT-PCR amplification procedure (see Figure 1). These confounding factors may cause changes in calculated ratio values. However, the inspection of all data values shown in Figure S4 in Supplementary Material clearly revealed shRNAs that suppressed (blue colors) or induced (red colors) Cxcl2 mRNA levels, thereby defining putative coactivators or corepressors of Cxcl2 expression in untreated or IL-1-stimulated conditions as summarized in Figure 2E.

Validation of Screening Hits and Identification of Positive or Negative Regulators of Cxcl2

To validate these findings, we performed conventional RT-qPCR experiments that allowed rigorous control of cell number, RNA purification and normalization for input total RNA in cDNA and qPCR reactions. Figure 3A demonstrates for two factors (Sin3a and Mbd3) the knockdown efficiency of pooled shRNAs and the suppressive or activating effect on basal and IL-1-inducible Cxcl2 expression, confirming the results from the screen as shown in Figure S4 in Supplementary Material. We then extended this analysis initially focusing on nine putative coactivators and later on seven putative corepressors of Cxcl2, respectively. As these shRNA transfections were performed in two independent series of experiments with some time lag between them, we chose to keep them separated also for subsequent data analysis to report the data as faithful as possible. All Cxcl2 mRNA expression data were normalized to Ube2I3 and to the maximal IL-1-inducible levels in vector control cells. As shown in Figure S5 in Supplementary Material, these experiments confirmed a role of all factors in basal or inducible Cxcl2 mRNA expression. Notable was a difference in the basal expression level of Cxcl2 in the vector controls between experiment series (1) and (2) (Figure S5 in Supplementary Material). While this effect corroborates the biological variation of the low abundant Cxcl2 transcript in cultured Mef cell lines, it does not affect the conclusion that knockdown of six factors (Ncoa1, Sin3a, Phf21a, Kdm6a, Padi4, Epc1) consistently suppressed basal Cxcl2 levels, while knockdown of eight factors (Suv420h1, Ruvbl1, Mbd3, Kdm5a, H2afg, Rpsi6a4, Mta2, Ncoa6) derepressed basal Cxcl2 levels (Figure S5 in Supplementary Material). Knockdowns of all of these factors except for Epc1 also affected the IL-1-induced mRNA level of Cxcl2 by more than 1.5-fold (Figure S5 in Supplementary Material). The ranked
Validation of screen results further defines coactivators and corepressors of Cxcl2. (A) Murine embryonic fibroblast (Mef) cells were transfected with empty pLKO.1 or with pooled shRNAs directed against Sin3a or Mbd3. After 48 h of selection in puromycin, cells were treated with IL-1 for 1 h or were left untreated. Then, total RNA was isolated and mRNA expression of Cxcl2, Sin3a, Mbd3 and Ube2l3 was determined by conventional RT-qPCR. Expression values of Cxcl2, Sin3a, or Mbd3 were normalized for expression of Ube2l3. The graphs show mean relative expression values ± SEM relative to the vector control from two independent series of experiments performed in duplicates. Asterisks indicate significant changes (p < 0.05) derived from Mann-Whitney Rank Sum t-tests. (B) The same RT-qPCR approach was used to determine the effects of 16 further knockdowns of nuclear cofactors on basal and IL-1-inducible Cxcl2 levels as described in detail in the legend of Figure S5 in Supplementary Material. The line graphs show the ranked mean fold changes of normalized Cxcl2 mRNA expression separately analyzed for basal and IL-1-induced conditions. Additionally, the effects on IL-1-mediated regulation (as defined in Figure S3 in Supplementary Material) are depicted. (C) Summarizing venn diagrams indicating the overlapping effects on Cxcl2 mRNA expression of shRNAs directed against 22 nuclear cofactors as identified by shRNA screens I and II. Asterisks indicate the 16 factors that were chosen for validation. (D) Overlap of functions of the shRNAs as found by conventional RT-qPCR. (A–D) Red or blue colors indicate at least 1.5-fold differences between shRNA versus empty vector transfections.

**FIGURE 3** Validation of screen results further defines coactivators and corepressors of Cxcl2. (A) Murine embryonic fibroblast (Mef) cells were transfected with empty pLKO.1 or with pooled shRNAs directed against Sin3a or Mbd3. After 48 h of selection in puromycin, cells were treated with IL-1 for 1 h or were left untreated. Then, total RNA was isolated and mRNA expression of Cxcl2, Sin3a, Mbd3 and Ube2l3 was determined by conventional RT-qPCR. Expression values of Cxcl2, Sin3a, or Mbd3 were normalized for expression of Ube2l3. The graphs show mean relative expression values ± SEM relative to the vector control from two independent series of experiments performed in duplicates. Asterisks indicate significant changes (p < 0.05) derived from Mann-Whitney Rank Sum t-tests. (B) The same RT-qPCR approach was used to determine the effects of 16 further knockdowns of nuclear cofactors on basal and IL-1-inducible Cxcl2 levels as described in detail in the legend of Figure S5 in Supplementary Material. The line graphs show the ranked mean fold changes of normalized Cxcl2 mRNA expression separately analyzed for basal and IL-1-induced conditions. Additionally, the effects on IL-1-mediated regulation (as defined in Figure S3 in Supplementary Material) are depicted. (C) Summarizing venn diagrams indicating the overlapping effects on Cxcl2 mRNA expression of shRNAs directed against 22 nuclear cofactors as identified by shRNA screens I and II. Asterisks indicate the 16 factors that were chosen for validation. (D) Overlap of functions of the shRNAs as found by conventional RT-qPCR. (A–D) Red or blue colors indicate at least 1.5-fold differences between shRNA versus empty vector transfections.

Effects of all knockdowns on Cxcl2 levels are summarized in Figure 3B. 11 of the 16 factors are also needed as supporting factors for IL-1-mediated regulation of Cxcl2 levels (Figure 3B, right panel). We define this signal-triggered difference as the ratio between basal and IL-1-induced levels (see Figure S3 in Supplementary Material). This ratio therefore reflects the IL-1 receptor-mediated signaling effect that activates Cxcl2 transcription. Only two knockdowns (shPhf21a, shEpcl1) further increase signaling because they regulate basal and inducible Cxcl2 expression in opposite directions. Thereby these two factors can amplify
the IL-1 response (Figure 3B, right panel). The overlapping hits between the combined screen results and the validation experiments are indicated by the Venn diagrams and are summarized in Figures 3C,D. By validation experiments, significant more factors were found to affect also the basal expression levels thus reducing the number of factors influencing IL-1 regulation compared to the screening results (Figures 3C,D). As outlined above, this can be attributed to the more reliable determination of changes in basal expression of Cxcl2 by conventional RT-qPCR. Collectively, these data corroborate the validity of our screening pipeline and underscore the importance of the identified genes in regulating an IL-1 mediated inflammation response.

Gene-Specific Requirements of the 16 Nuclear Cofactors for IL-1 Target Genes

These results raised the question if these cofactors affected the IL-1 response in a gene-specific manner. We therefore tested the expression of ten additional IL-1 target genes using the same RNA preparations as described above (Figures S6 and S7 in Supplementary Material). Figure 4A summarizes the ranked changes in mRNA expression for six further chemokine genes located on different chromosomes (chr.1, Ccl20; chr. 5, Cxcl1, Cxcl5, Cxcl10; chr.11, Ccl2, Ccl7). These data reveal that each inducible gene has its own quantitative requirement of cofactors to maintain basal levels and to respond to IL-1. Figure 4B displays heatmaps to visualize how each cofactor affects mRNA levels across the seven chemokine genes tested including Cxcl2 of chr. 5. Six factors (Ncoa1, Sin3a, Phf21a, Kdm6a, Epc1, Pad4) are required for basal and IL-1-induced expression for four out of seven chemokine genes; however, there are highly gene-specific variations. For example, Ncoa1 and Kdm6a knockdowns suppress expression of Cxcl2, Cxcl1, Ccl20, Cxcl5, and Cxcl10, but enhance expression of Ccl7. Likewise, five factors (Ncoa6, H2afz, Kdm5a, Mbd3, Ruvbl1) suppress expression levels of six chemokine genes, but have opposite effects on Cxcl10. We also tested the effects of these knockdowns on four additional IL-1 target genes that have different biological functions (Figure 5A). Most remarkably,
expression and regulation of the Nfkbia gene encoding the cytosolic IkBα inhibitor of NF-κB was almost unaffected by the knockdown of the 16 cofactors, whereas Il6 and Icam1 had similar positive cofactor requirements (compared to Cxcl2) but differed in the regulation by repressory genes (Figure 5B). A second inhibitor of NF-κB, Nfkbiz, varied significantly in the requirements of the 16 factors compared to all chemokine genes and to Icam1 or Il6 (Figure 5B). Bar graphs displaying the basal and inducible mRNA levels of all genes shown in Figures 4 and 5 are provided in Figures S6 and S7 in Supplementary Material.

Re-analysis of data previously published by us reveals that basal and inducible expression of 10 of these genes is lost in p65 NF-κB-deficient Mefs but can be restored by reconstitution of this NF-κB subunit proving that this pathway provides the essential signals for their expression [Figure S8 in Supplementary Material; (27)]. Ccl20 which was the only mRNA found not to be expressed in reconstituted Mefs was shown by others to be dependent on p65 NF-κB-deficient Mefs but can be restored by reconstitution of this NF-κB subunit proving that this pathway provides the essential signals for their expression [Figure S8 in Supplementary Material; (27)]. Ccl20 which was the only mRNA found not to be expressed in reconstituted Mefs was shown by others to be dependent on p65 NF-κB-deficient Mefs but can be restored by reconstitution of this NF-κB subunit proving that this pathway provides the essential signals for their expression [Figure S8 in Supplementary Material; (27)].

STRING database analysis revealed that 17 of the 22 identified factors are engaged in multiple protein:protein interactions based on experimental evidence, co-occurrence, and/or coexpression (Figure 7A) (30). They are predicted to form a multi protein network that shows many more interactions than expected compared to the whole genome as revealed by the network statistics (Figure 7A). Four factors have no yet known interactions within this network (Prmt6, Padi4, Dapk3, Rps6ka4). As the evidence contained in the STRING database reflects multiple biological conditions and species, we further asked if any of these factors have been previously implicated in IL-1, TNF, or NF-κB pathways. Table 1 summarizes the results from a detailed literature search indicating that 12 genes (Epc1, H2afz, Kdm2b, Kdm6a, Mbd3, Mta2, Phf21a, Ruvbl1, Sin3b, Suv420h1, Taf1, and Ube3a) have not been previously implicated in these responses. The others have some role in regulating NF-κB subunit function and some of them have also been related to TNF or IL-1 biology. However, a mechanistic role in regulation of IL-1 target genes has not been.
described so far for any of the 22 cofactors that we identified in this study. Thus, the experiments presented in Figures 4–7; Figures S5–S9 in Supplementary Material suggest that downstream of the NF-κB pathway each gene has further subtle requirements for nuclear cofactors that shape its transcriptional activation profile. Unlike NF-κB, however, none of these factors appears to be essential, but they are needed to fine tune mRNA expression levels. This hypothesis is summarized schematically in Figure 7B and provides a framework for future studies.

**DISCUSSION**

Inflammatory cytokines execute their biological functions through the transcriptional regulation of specific sets of genes (5). Gene transcription is a highly coordinated process involving multiple molecular machines and their interactions with chromatin to regulate removal of the chromatin barrier, RNA polymerase II recruitment, pre-mRNA processing, editing, and export (77). Unlike the cytoplasmic signaling pathways which have been elucidated at a considerable level of detail, the (combinations of) nuclear factors that are required for IL-1-mediated gene regulation have largely been elusive (4). Here, we report the results from a limited shRNA screen revealing a novel network of nuclear cofactors that shape the cytokine-driven gene response of 11 inflammatory target genes. shRNA screens are powerful tools to unravel gene functions on a broad scale. Usually, therefore, they are performed using streamlined assays in an automatized way (10, 11). Here, we describe in detail a smaller categorized system.
shRNA screen that can be performed by a single person using standard laboratory equipment. As a phenotypic readout, we chose to measure mRNA expression in response to IL-1. This more complicated multistep biological assay led us to limit the total number of investigated components to 170. By repeating the screen and by scoring the performance of individual shRNAs designed against the same target, we provide a thorough confirmation of the knockdown efficiency of individual shRNA constructs provided by the TRC consortium (26). As an example, we confirmed the knockdown of one factor, Sin3a, at the protein level, validated its differential effects on the expression of a set of chemokine genes and showed that Sin3a was recruited to chromatin. Ultimately, we identify 22 nuclear cofactors whose knockdown by several shRNAs suppresses or enhances expression of the target gene Cxcl2 in the same direction thus reducing the likelihood of off-target effects (78). Accordingly, the involvement in gene regulation of 16 cofactors was reproduced by thorough RT-qPCR validation. Our data analysis procedure further reveals that basal mRNA expression is the most variable biological factor influencing screening results when searching for factors modulating highly regulated genes as the real time PCR procedures used here were sensitive, efficient and highly specific (Figure S10 in Supplementary Material). We suggest that special care should be taken to calculate shRNA-dependent changes in mRNA expression for basal and inducible conditions. Furthermore, our data provide several examples of shRNAs that lower or enhance constitutive and IL-1-inducible mRNA in the same direction. In this case, a regulatory role of the shRNA target gene can be easily missed if relative cytokine-induced changes are calculated separately against vector control and shRNA transfection. Our global analysis of mRNA expression also revealed that care should be taken to use GFP-expressing vectors as a control as our results clearly reveal a dominant negative effect on Cxcl2 mRNA but also on Actb and Ube2l3 expression. This is in line with recent studies showing that GFP inhibits IL-2 expression in T cells and that NF-κB-driven GFP reporter constructs deteriorate expression of LPS- or TNF-induced endogenous IL6 and Cxcl1 genes (79, 80). This might be due to defective polyubiquitylation in GFP expressing cells which can perturb ubiquitin-based signaling cascades (81). In addition, free GFP has been reported to accumulate within the nucleus forming homotetramers or homohexamers (82). These GFP multimers might sequester chromatin complexes and cofactors that are required for endogenous regulation of genes, a phenomenon that might be missed in shRNA screens that use NF-κB-driven GFP reporter assays as global readouts for gene activation.

Further support for the strategy chosen here to identify relevant coactivators/corepressors for inflammatory gene expression can be derived from a limited number of published findings implicating these proteins in NF-κB activation or immune functions or by showing that their expression is regulated by TNF or IL-1 (Table 1). One example is the Clock TF that cooperates with the p65 NF-κB subunit to regulate the circadian expression of genes such as Dbp1 and Per1-3 (31, 83). These Clock target genes are downregulated in IL-1- or TNF-triggered cells (83). In
FIGURE 6 | Differential effects of Sin3a knockdown on the expression of seven chemokine genes. Mef cells were transfected with pooled shRNAs directed against SIN3A (shSin3a) or with empty pLKO.1 (vector). After 48 h of selection in puromycin, cells were treated with IL-1 for the indicated times or were left untreated. (A) Expression of SIN3A was analyzed in total cell extracts by immunoblotting. Anti tubulin antibodies were used to confirm equal loading. (B) Total RNA was isolated and mRNA expression of the indicated chemokine genes and of Ube2l3 was determined by conventional RT-qPCR. Expression values were normalized for expression of Ube2l3. The graph show relative expression values ± SEM from at least two independent experiments.

turn, the repressive Clock target gene Cryptochrome suppresses activation of NF-kB suggesting that the circadian-oscillator components and immune pathways are tightly intertwined (84, 85). Our results support these findings and show that conversely, Clock is required for full activation of inflammatory genes in the IL-1 pathway. Another factor is Rps6ka4 encoding the protein kinase MSK2. The closely related kinase MSK1 phosphorylates p65 NF-κB at S276 promoting its activation (60). Both kinases are also major histone H3S10 and CREB/ATF1 kinases and hence affect gene regulation at multiple levels (86). However, MSK1- and MSK2-double deficient mice show increased skin inflammation and activation of LPS target genes, an effect that was attributed to decreased levels of Dusp1 and IL-10, which are negative regulators of LPS signaling (61). Our results support a repressive and non-redundant role of MSK2 in inflammatory cell reactions by negative regulation of some but not all IL-1-target genes. The NCOA1 protein binds to the Il6 and Nfkbia promoters, coactivates Il6, Nfkbia, and NF-kB promoter luciferase constructs (together with p65) and is needed for angiotensin-II-mediated IL6 or for TNF-induced Nfkbia mRNA expression (52, 53). Here, loss of Ncoa1 severely suppressed mRNA expression of six chemokine genes (except Ccl7), Icam1 and Il6 but was without any effect on Nfkbia. In contrast, knockdown of the related Ncoa6 derepresses five chemokine genes plus Il6 but suppresses expression of Cxcl10. The latter is in contrast to one study which found that NCOA6 weakly stimulates p65-driven activation of NF-κB reporters (54). Thus, the effects of both coactivators are highly gene specific and partially opposing. Also, overexpressed PRMT6 was shown in one report to enhance TNF-mediated NF-κB nuclear translocation and activation of a NF-κB luciferase reporter. However, despite global activation of NF-κB in these assays, the ectopically expressed PRMT6 protein only enhanced...
TNF-mediated mRNA induction of *Il6*, *Tnf*, and *COX2* (*Ptgs2*) but not *MCP1* (*Ccl2*) or *Nfkbia*. In ChIP assays, PRMT6 also bound to the *IL6* and *TNF* promoters together with p65 (59).

This study nicely illustrates the different results obtained by using global reporter assays compared to investigating specific genes. In our screen, knockdown of PRMT6 enhanced *Cxcl2* expression,
sugestting that for this gene PRMT6 is a suppressor rather than an activator. Altogether these comparisons reveal the necessity to study the true function of nuclear cofactors at the endogenous mRNA or protein level.

The majority of factors identified in our screen have no established role in inflammatory gene expression (Table 1). Although some of them such as Sin3a, Padi4, Phf21a, Kdm6a, Ruvbl1, or Kdm5a are powerful activators or repressors of most of these genes, the overall result is that these cofactors execute gene specific functions. The mechanistic basis for this phenomenon awaits further investigations but is in line with conclusions derived from other systems, in particular the nuclear hormone receptor complexes (9). It will also be interesting to find out if any of these effects are specific to IL-1. So far, the only cellular molecule that is specific for the IL-1 pathway is the IL-1 type I receptor. All other pathway components including the entire NF-κB pathway are shared by other signaling systems (4, 87, 88). The 22 molecules that form the IL-1 coactivator network comprise different functional categories. There are many genes encoding enzymes that phosphorylate proteins (Dapk3, Rps6ka4), methylate or acetylate histone residues (Kdm5a, Kdm2b, Kdm6a, Prmt6, Sirt3, Suv420h1), remodel chromatin (Ruvbl1) or deiminate or ubiquitylate histones (Padi4, Ube3a). Several other genes encode scaffolds or components of corepressor complexes (Sin3a, Sin3b, Mbd3, Mta2, Phf21a, Epc1) (summarized in Table 1). It is therefore possible that these factors are components of cofactor complexes which have been remodeled from classical coactivator/corepressor complexes such as the NuRD, Sin3a, or CoRest complexes into IL-1-specific nuclear factories. These multiprotein complexes are predicted to assemble at chromatin to coordinate and synchronize the genome-wide IL-1-gene response in space and time. According to this concept, the specificity of the inflammatory gene response is encoded in the nuclear compartment rather than in the cytosolic signaling pathways.

In conclusion, the data derived from these screening experiments give raise to multiple new hypotheses. Proving a direct role of each of the factors in inflammatory gene expression will require a complex set of follow up studies including chromatin immunoprecipitation (ChIP) experiments. These experiments will be challenging as nuclear cofactors do not bind directly to DNA (unlike TFs) and are thus notoriously more difficult to crosslink and to immunoprecipitate. The cofactors may bind to distal enhancers or promoters or may regulate their target genes indirectly. Thus, ChIP-seq experiments are needed to determine their true sites of chromatin recruitment across the genome, provided suitable ChIP-grade antibodies are available. Further, there is no requirement for dynamic or IL-1-dependent recruitment of cofactors to execute their functions as regulation of their activity might occur entirely through posttranslational modifications. All these experiments are beyond the scope of our study, which reports new functional evidence derived from shRNA experiments.

**MATERIALS AND METHODS**

**Cells and Cell Culture Materials**

Mouse embryonic fibroblasts (Mef line 1, TA7) immortalized by the 3T3 protocol and carrying targeted alleles allowing conditional Cre-mediated deletion of HDAC3 (Hdac3<sup>−/−</sup>) were used for the shRNA screens and for all RT-qPCR validation experiments (89). These cells have normal HDAC3 protein levels in the absence of Cre activation and their IL-1 response has been extensively characterized in a previous study from our lab (27). These cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), complemented with 10% fetal calf serum, 2 mM l-glutamine, 1X non-essential amino acids, 100 U/ml penicillin and 100 µg/ml streptomycin. p65 NF-κB-deficient (Mefs p65<sup>−/−</sup>), reconstituted Mefs (p65<sup>−/−</sup> + p65 wt) (90) and Mef line 2 (MK2/5) (91) were cultured in Dulbecco’s modified Eagle’s medium (DMEM), complemented with 10% fetal calf serum, 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin.

**Plasmid Purification and Transfections for the shRNA Screens and Validation Experiments**

For the shRNA screens and validation experiments, mouse shRNA vectors, derived from TRC.1 library<sup>2</sup> as glycerol stocks, were purified by the PureYield<sup>TM</sup> plasmid miniprep system from Promega following the manufacturer’s instructions. pLKO.1-puro empty vector, pLKO.1-puro non-mammalian shRNA (scrambled shRNA) vector, and pLKO.1-puro GFP vector were used as negative controls or for transfection efficiency control by fluorescence microscopy. For transient transfection screening, 3.5 × 10<sup>5</sup> Hdac3<sup>−/−</sup> Mefs were seeded in 48-well plates. 270 ng plasmid DNA per well was transfected using Lipofectamine<sup>®</sup> LTX and Plus<sup>™</sup> reagent from Invitrogen<sup>™</sup> following the manufacturer’s instructions. 24 h post transfection, transfected cells were selected for 48 h by adding 1 µg/ml puromycin. For larger scale RT-qPCR validation experiments, 4 × 10<sup>5</sup> Hdac3<sup>−/−</sup> MEFs were seeded in 60-mm cell culture dishes and transfected with 5.5 µg plasmid DNA. After puromycin selection cells were left untreated.

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<sup>2</sup>https://portals.broadinstitute.org/gpp/public/
| Nuclear Cofactor (gene symbol) | Aliases | Principal function | Implication in IL-1 signaling | Implication in TNF signaling | Implication in NF-κB transactivation | Species, cell types | Reference |
|-------------------------------|---------|---------------------|-------------------------------|-------------------------------|-----------------------------------|---------------------|-----------|
| 1 Clock | BHLHE8, KAT13D | Heterodimeric TF factor (together with BMAL1), regulates circadian expression of genes | IL-1 suppresses Clock-dependent genes | TNF suppresses Clock-dependent genes; Clock enhances TNF-mediated transcription | Binds to p65, enhances NF-κB activity together with BMAL1 | Mouse, human; fibroblasts, epithelial cells | (31–33) |
| 2 Dapk3 | ZIPK, DLK | Serine/threonine kinase involved in apoptosis, autophagy, transcription, IFNγ expression, granulocyte migration | | Knockdown inhibits TNF-induced expression of VCAM-1 | | Rat; vascular smooth muscle cells (VSMC) | (34–36) |
| 3 Epc1 | Epl1 | Enhancer of polycomb homolog 1, component of the NuA4 histone acetyltransferase (HAT) complex | | | | (37, 38) |
| 4 H2afz | H2AZ | Histone variant at promoters/TSS of active and poised genes | | | | (39) |
| 5 Kdm2b | JHDM1B, FBXL10, JEMMA | H3K4 and H3K36 demethylase, component of polycomb repressive complexes | | | | (40, 41) |
| 6 Kdm5a | JARID1A, RBBP2 | H3K4 demethylase, enhances gene activation by CLOCK-BMAL1, required for IFNγ production of NK cells | Binds to p50 NF-κB | Mouse; splenocytes, NK cells | (42, 43) |
| 7 Kdm6a | UTX, KABUK2 | H3K27 demethylase, establishes active enhancers, involved in autoimmune syndromes and T-cell activation | | | (44–46) |
| 8 Mbd3 | | Histone variant at promoters/TSS of active and poised genes | Binds (un)methylated CpG-rich active promoters and enhancers, component of the NuRD complex, essential for mouse development | Ectopically expressed protein enhances p65 transactivation and IL6 promoter reporter activity | | (47, 48) |
| 9 Mta2 | MTA1L1, | component of NuRD and remodeling complexes, binds to GATA3 in TH2 cells, suppresses IL-2, IL-4, IFNγ expression and autoimmunity | | | | (49–51) |
| 10 Ncoa1 | BLHLE74, SRC1 | Transcription coactivator of nuclear receptors | TNF-induced recruitment to κBα promoter | | | | (52, 53) |
| 11 Ncoa6 | RAP250, AIB3, ASC-2, TRBP | Transcription coactivator of nuclear receptors | | | | | |
| 12 Padi4 | PADI-H | Protein-arginine deiminase, catalyzes the citrullination/deimination of histones/other proteins | Protein induced by TNF | | Mouse; VSMC | (55) |
| 13 Phf21a | BHC80a | Interacts with and inhibits LSD1 demethylase activity, binds unmethylated H3K4 and COREST | | | | (56–58) |

(Continued)
| Nuclear Cofactor (gene symbol) | Alias | Principal function | Implication in IL-1 signaling | Implication in TNF signaling | Implication in NF-κB transactivation | Species, cell types | Reference |
|-------------------------------|--------|---------------------|------------------------------|-----------------------------|-------------------------------------|---------------------|-----------|
| 14 Pmt6 | HRMT1L6 | Protein arginine methyltransferase, mediates H3R2, H3R42, H4R3, H4R3 methylation | Enhances TNF-mediated expression of IL6, binds to the IL6 promoter | Ectopic expression induces IL6, binds to p65 and activates NF-κB reporter | Mouse; tissues and Mefs | (59) |
| 15 Rps6ka4 | RSKB, MSK2, p90 RSK | Nuclear protein kinase downstream of ERK and p38 MAPK, phosphorylates H3S10, CREB1, ATF1, suppressor of inflammation | Increased levels of IL-1 in MSK1/2 ko mice in inflamed skin | Suppression of LPS-induced TNF secretion (together with MSK1) | Promotes phosphorylation of p65 at S276 and p65 transactivation, MSK1/2 ko increases skin inflammation | Mouse, human; macrophages, skin, epithelial cells, breast adenocarcinoma | (60–63) |
| 16 Ruvb1 | TIP49A, TAP54-Alpha, INO80H | Chromatin remodeler and ATPase involved in nucleosome sliding | Upregulation of IL1A/B, CXCL1/3, IL6, IL8 by Sin3a knockdown in cells with activated PI3K | Binds to NF-κB subunits during the cell cycle* | Human; U2OS sarcoma cells, genetically modified Myr-PIK3 transformed ovarian cancer cells | (64, 65) |
| 17 Sin3a | | Nuclear scaffold, transcriptional repressor, interacts with HDAC1/2 and multiple other proteins | | | | Human; | (66–68) |
| 18 Sin3b | | Nuclear scaffold, transcriptional repressor, interacts with MYC and HDAC1/2, binds H3K4me3/H3K36me3-enriched nucleosomes | | | | Human; | (69, 70) |
| 19 Sirt3 | SIR2L3 | NAD-dependent class III (histone) deacetylase | | | Binds to NF-κB subunits during the cell cycle* | Rat; cardiomyocytes | (71) |
| 20 Suv420h1 | KMT5B | methylates H4K20 | | | | | (72, 73) |
| 21 Taf1 | TAF11250, NSC12, CCG1 | Component of TFIID, primary mediator of downstream promoter binding of the preinitiation complex | | | | | (74) |
| 22 Ube3a | | Ubiquitin E3 ligase, interacts with polycomb protein Ring1B, linked to the Angelman syndrome | | | | | (75, 76) |

*Reported in reference 67, a publication that has been retracted.
or were treated with human recombinant IL-1α (10 ng/ml) (kind gift from Jeremy Saklatvala, London, UK) for the indicated periods of time.

**Measurements of mRNA Expression**

mRNA measurements were performed by conventional RT-qPCR or Agilent microarrays as described before (27, 92). For the shRNA screens, cDNA was synthesized in cell lysates and amplified using the TaqMan® PreAmp Cells-to-Ct Kit® and TaqMan® Gene-Expression Assays from Applied Biosystems following an adapted miniaturized protocol. The kit enables to perform gene expression analysis directly from limited or small numbers of cultured cells without RNA purification by including an intermediate amplification step (pre-amplification) between reverse transcription and real-time PCR. Cells were washed twice in cold PBS, transferred to tubes, and lysed in 12.5 µl lysis solution (DNase I was diluted at 1:100). After mixing five times the lysates were incubated for 5 min at room temperature. The reaction was stopped by adding 1.25 µl stop solution. After mixing five times, the samples were incubated for 2 min at room temperature. For reverse transcription, 4.5 µl of the lysate was used for a final reaction volume of 10 µl. 5 µl of 2x RT-buffer and 0.5 µl of 20x RT enzyme mix were added. The reaction tubes were incubated in a thermal cycler at 37°C for 60 min, then at 95°C for 5 min to inactivate the RT enzyme. In the following step, the cDNA was amplified using gene-specific primers contained in TaqMan® gene expression assays. The assays of interest were diluted in real-time PCR in 1x TE and 2.5 µl were used in a 10 µl reaction volume with 2.5 µl cDNA and 5 µl 2x TaqMan PreAmp MasterMix. The preamplification occurred in a thermal cycler at 95°C for 10 min, following 15 cycles at 95°C for 15 s/60°C for 4 min. Prior to real-time PCR, the preamplification products were diluted 1:5 with 1x TE. The following TaqMan® gene expression assays were used in this study: mUbe2l3 (Mm00784559_s1), mActB (Mm00607939_s1), mCcl1 (Mm00433859_m1), mCcl2 (Mm00436450_m1), mCxcl5 (Mm00784559_s1), mActB (Mm00607939_s1), mUbe2l3 (Mm00784559_s1), mGst1 (Mm00441238_m1), mCcl2 (Mm00441242_m1), mCcl7 (Mm00441213_m1), mCcl11 (Mm00441238_m1), mCcl20 (Mm00444228_m1), mNfkbia (Mm00607939_s1), mCar13 (Mm00617525_m1), mMcm7 (Mm0083349_g1), mEvl (Mm00468405_m1), and mkcam1 (Mm00516023_m1). The expression of the indicated target genes was determined by real-time PCR using the TaqMan® Fast universal PCR master mix and 7500 Fast Real-Time PCR System from Applied Biosystems. The following mRNA-arrays were detected by Fast SYBR® Green Master Mix: mSin3a (se: gccctgctcatctgacca; as: ttgttgaacagggcag) and mMBd3 (se: ggccacagctgtctttta; as: tttgttaagatgttgcatg). Representative examples for amplification and melting curves are provided in Figure S10 in Supplementary Material indicating the efficiency and specificity of PCR reactions.

Relative changes of mRNA expression compared to the unstimulated pLKO.1 vector control were normalized to the expression of mUbe2l3 and quantified using the 2−ΔΔCt method. Further ratio comparisons were calculated as shown in Figure S3 in Supplementary Material.

**Bioinformatics and Calculations**

Calculations, graphical representations, and statistical tests of data were performed using SigmaPlot11 and MS EXCEL2010. Z-scores were calculated according to the formula: \( z = (x - \mu) / \sigma (SD) \). Concerning box plots, the boundary of the box closest to zero indicates the 25th percentile, the line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers (error bars) above and below the box indicate the 90th and 10th percentiles. Points mark the remaining outliers. The STRING database and Cytoscape were used for network analysis and visualizations (30, 93). 18 non-regulated “housekeeping genes” were taken from RNA-seq data of 15 mouse tissues (94). For these and the ten NF-κB target genes (Ccl2, Ccl7, Cxcl1, Cxcl10, Cxcl2, Cxcl5, Icam1, Il6, Nfkbia, Nfkbi), 1000 bp of flanking DNA sequences were batch-extracted from the Ensembl database of the Mus musculus genome version GRCh38.p5 using Biomart software. Genomic sequences were searched for high quality NF-κB matrices using Match™ public 1.0 from TRANSFAC® public 6.0 database (95).

**Chromatin Immunoprecipitation**

2× 175-cm² flask of confluent MEF cells, untreated or treated for 1 h with IL-1α (10 ng/ml), was used for each condition. Proteins bound to DNA were cross-linked in vivo with 1% formaldehyde added directly to the medium. After 10 min incubation at room temperature, 0.1 M glycine was added for 5 min to stop the cross-linking. Then, cells were collected by scraping and centrifugation at 1,610×g (5 min, 4°C), washed in cold PBS containing 1 mM PMSF and centrifuged again at 1,610×g (5 min, 4°C). Cells were lysed for 10 min on ice in 3 ml ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.1, 1 mM PMSF, Roche protease inhibitor mix). The DNA was sheared by sonication (7 × 30 s on/30 s off, four times; Bioruptor, Diagenode) and lysates cleared by centrifugation at 16,100×g at 4°C for 15 min. Supernatants were collected and stored in aliquots at −80°C for subsequent ChIP. For determination of DNA concentration 20 µl of sheared lysate was diluted with 100 µl TE buffer including 10 µg/ml RNase A. After 30 min at 37°C, 3.8 µl proteinase K (20 mg/ml) and 1% SDS were added and incubated for at least 2 h at 37°C followed by overnight incubation at 65°C. Samples were resuspended in two volumes of buffer NTB (Macherey & Nagel) and DNA was purified using Nucleo Spin columns (Macherey & Nagel) according to the manufacturer's instructions. DNA was eluted with 50 µl 5 mM Tris pH 8.5, and concentration was determined by Nano Drop. For ChIP, the following antibodies were used: anti-NF-κB p65 (3 µg, Santa Cruz; sc-372), anti-RNA Pol II (1 µg, Millipore; 17-620), anti-P(S5)-Pol II (2 µg, Abcam; ab5131), anti-Sin3a (3 µg, Santa Cruz; sc-994), and IgG (2 µg, Cell Signaling; 2729).

Antibodies were added to precleared lysate volumes equivalent to 25 µg of chromatin. Then, 900 µl of ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris/HCl pH 8.1) were added, and the samples were rotated at 4°C overnight. Thereafter, 30 µl of a protein A/G sepharose mixture, 2 http://www.ensembl.org
pre-equilibrated in ChIP dilution buffer was added to the lysates and incubation continued for 2 h at 4°C. Beads were collected by centrifugation, washed once in 900 μl ChIP low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.1, 150 mM NaCl), once in 900 μl ChIP high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.1, 500 mM NaCl), once in 900 μl ChIP LiCl buffer (0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris pH 8.1) and twice in 900 μl ChIP TE buffer (10 mM Tris pH 8.1, 1 mM EDTA) for 5 min at 4°C. Beads were finally resuspended in 100 μl TE buffer including RNase A (10 mg/ml). In parallel, 1/10 volume (2.5 μg) of the initial lysate (input samples) were diluted with 100 μl TE buffer including 10 μg/ml RNase A. After 30 min at 37°C, 3.8 μl of the initial lysate (input samples) were diluted with 100 μl ChIP TE buffer (10 mM Tris pH 8.1, 1 mM EDTA) for 1% desoxycholate, 1 mM EDTA, 10 mM Tris pH 8.1) and twice 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.1, 500 mM NaCl, and immunoprecipitates were incubated for at least 2 h at 37°C followed by overnight incubation at 65°C. Samples were resuspended in two volumes of buffer NTB (Macherey & Nagel) and DNA purified using Nucleo Spin columns (Macherey & Nagel) according to the manufacturer’s instructions. DNA was eluted with 50 μl 5 mM Tris pH 8.5 and stored at −20°C until further use.

Quantification of ChIP DNA by Real-Time PCR

PCR products derived from ChIP were quantitated by real time PCR using the Fast ABI 7500 instrument (Applied Biosystems). The following primers were used as described in Ref. (29): Murine Ccl2 promoter (sense “ggagggctgtcaacctc” & antisense “tcagtgagagttggctggtg”), Ccl2 enhancer (sense “ccctgagagacgctgg” & antisense “gaggcttgacgaggata”) and gene-free negative control region upstream of Cxcl5 (sense “tttcatgcttctgtggtc” & antisense “tttcctggtcttgcccata”). The reaction mixture contained 2 μl of ChIP or input DNA (diluted 1:10 to represent 1% of input DNA), 0.25 μM of primers and 10 μl of Fast Sybr Green Mastermix (2×) (Applied Biosystems) in a total volume of 20 μl. PCR cycles were as follows: 95°C (20 s), 40× (95°C [3 s], 60°C [30 sec]). Melting curve analysis revealed a single PCR product. Calculation of enrichment by immunoprecipitation relative to the signals obtained for 1% input DNA was performed according to the following equation: percent of input = 2^−(Ct input−Ct ChIP).

Immunoblotting

Preparation of total cell extracts and immunoblotting was performed as described (92). The following antibodies were used: anti SIN3A (Abcam, ab3479, 1:2,000 in TBS/0.05% Tween, 5% BSA), and anti tubulin (Santa Cruz, sc-8035, 1:1,000 in TBS/0.05% Tween, 5% milk).

AUTHOR CONTRIBUTIONS

JM-S and DN performed experiments, JM-S, AW, and MK analyzed data. JK and TB provided the shRNA library. LJ contributed to ChIP experiments. JM-S and MK conceived the study. MK wrote the initial draft. MS, JK, JM-S, and MK finalized the manuscript. All authors approved the submitted version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00775/full#supplementary-material.

FIGURE S1 | Selection of IL-1 target genes and housekeeping genes in murine embryonic fibroblasts used in this study. (A) The 30 most strongly regulated IL-1-response genes of the immortalized fibroblast cell line used in this study were extracted from two previously published microarray experiments (27). Fold changes compared to untreated cells are presented as heatmap and are ordered according to mean regulation. Green colors represent genes selected for further analysis. (B) Displayed in the lower graph is the mRNA expression (as raw cycle threshold [ct] values) of five non-regulated housekeeping genes obtained using samples from untreated and IL-1-treated fibroblasts. Fold changes determined for Cxcl2 and Cc2 are shown in the upper graph for comparison. Shown are mean values ± SEM from a technical replicate.

FIGURE S2 | Validation and kinetics of IL-1-mediated gene regulation of the immortalized murine embryonic fibroblast lines used in this study. Mef cells were stimulated for different times with IL-1 or were left untreated and expression of the indicated genes was analyzed by RT-qPCR. Shown are mean relative fold changes ± SEM from two independent experiments. Blue colors indicate seven chemokine genes. Red colors mark the two time points chosen for the shRNA screens.

FIGURE S3 | Calculation of relative differences in mRNA expression across various biological conditions from cycle threshold (ct) values. The graph shows a hypothetical representation of Cxcl2 mRNA levels in the absence or presence of IL-1 in vector control cells or in cells after shRNA-mediated knockdown. Numbers in circles refer to the equations used to calculate relative differences (fold changes) in basal or IL-1-induced expression levels or in the IL-1-mediated regulation comparing vector controls with each of the shRNA samples.

FIGURE S4 | Heatmaps and ratio values of nuclear cofactors that repress or activate basal or inducible mRNA expression levels of Cxcl2. The tables and heatmaps show the computed fold changes for all shRNA samples of screen I and screen II that resulted in a Z-score below −1 or above 1 SD in the analyses shown in Figures 2C,D in both screens. The vector control values from the corresponding plates are shown for comparison. Colors indicate the direction of the shRNA effect (blue supression, red induction of mRNA levels) or IL-1-triggered expression in vector controls.

FIGURE S5 | Gene-specific requirements of the 16 nuclear cofactors for Cxcl2 mRNA expression. Murine embryonic fibroblast (Mef) cells were transfected with pooled shRNAs directed against 16 nuclear cofactors identified by the shRNA screens shown in Figures 1 and 2. After 48 h of selection in puromycin, cells were treated with IL-1 for 1 h or were left untreated. Then, total RNA was isolated and mRNA expression of Cxcl2 and Ube2d3 was determined by conventional RT-qPCR. Expression values of Cxcl2 were normalized for expression of Ube2d3. The graph shows relative expression values from two independent series of experiments (series 1 lanes 1–10, series 2 lanes 11–18). For each series all vector control values from IL-1-treated samples were pooled and set as 100%. Bars of shRNA samples represent mean values of at least three independent
experiments with exception of shEpc1 values which were derived from a biological duplicate experiment. Errors represent SEM. Controls of experiment series 1 (vector (1)) represent nine independent experiments, controls of experiment series 2 (vector (2)) represent three independent experiments. All data shown in Figures 3B and Figures S5–S7 in Supplementary Material are derived from these RNA preparations and the resulting ct values. Red or blue colors indicate at least 1.5-fold differences between shRNA versus empty vector transfections.

**FIGURE S6** | Gene-specific requirements of the 16 nuclear cofactors for 6 additional chemokine genes. The same RNA preparations described in Figure S5 were examined for the expression of additional chemokine genes by conventional RT-qPCR. Expression values were normalized for expression of Ube2l3. The graphs show relative expression values from two independent series of experiments (series 1, lanes 1–10; series 2, lanes 11–18). For each series, all vector control values from IL-1-treated samples were pooled and set as 100%. Bars of shRNA samples represent mean values of at least three independent experiments, shEpc1 experiments represent duplicates. Red or blue colors indicate at least 1.5-fold differences between shRNA versus empty vector transfections.

**FIGURE S7** | Gene-specific requirements of the 16 nuclear cofactors for 4 additional IL-1 target genes. The same RNA preparations described in Figure S5 in Supplementary Material were examined for the expression of additional genes representing adhesion molecules, cytokines or regulators of the NF-κB pathway by conventional RT-qPCR. Expression values were normalized for expression of Ube2l3. The graphs show relative expression values from two independent series of experiments (series 1, lanes 1–10; series 2, lanes 11–18). For each series, all vector control values from IL-1-treated samples were pooled and set as 100%. Bars of shRNA samples represent mean values of at least three independent experiments, shEpc1 experiments represent duplicates. Red or blue colors indicate at least 1.5-fold differences between shRNA versus empty vector transfections.

**RESULTS**

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**FIGURE S8** | Essential function of p65 NF-κB in basal and inducible expression of IL-1 target genes. (A) The heatmap shows results from two microarray experiments comparing p65-deficient cells (p65−/−) with knockout cells reconstituted with p65 NF-κB as previously published by us (27). Fold changes after 1 h of treatment with IL-1 compared to untreated cells are presented as heatmap and are ordered according to mean regulation. Green colors represent genes investigated in this study.

**FIGURE S9** | Recruitment of SINA to the murine Cc02 locus. (A) Schematic representation of the regulatory regions of the murine Cc02 locus as previously published (29) and the regions amplified by PCR in chromatin immunoprecipitation (ChIP) reactions. (B) Murine embryonic fibroblasts (Mefs) used for all experiments shown in this study (Tﬀ7, Mef line 2) and an additional immortalized Mef line (MK2/5, Mef line 1) were stimulated for 1 h with IL-1 or were left untreated. Chromatin was prepared and recruitment of p65 NF-κB, Ser5-phosphorylated or non-phosphorylated RNA polymerase (Pol II) or SINA to two different regions of the Cc02 locus and a gene-free control region upstream of Cc05 was examined by ChIP-PCR. IgG ChIP-PCRs were performed as controls. Shown is the mean enrichment of DNA fragments ± SEM from two (Mef line 2) or three (Mef line 1) independent experiments.

**TABLE S1** | Identifiers, annotations, and small-hairpin (sh)RNA sequences for the 170 nuclear cofactors examined in this study.

**TABLE S2** | Enriched NF-κB binding sites in eleven IL-1 target genes. Shown are NF-κB motifs in the 1000 basepair flanking sequences of the eleven IL-1 target genes compared to a set of non-regulated murine genes.
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