The corepressors GPS2 and SMRT control enhancer and silencer remodeling via eRNA transcription during inflammatory activation of macrophages

Highlights
- Corepressor recruitment is a genome-wide feature of inflammatory enhancers
- Corepressors control enhancer-silencer-promoter looping within the Ccl2 TAD
- Corepressors antagonize eRNA transcription and CBP-mediated H3K27 acetylation
- Ccl2 eRNA depletion in ob/ob mice supports eRNA function and targeting options

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In Brief
Huang et al. dissect epigenetic mechanisms underlying the anti-inflammatory action of macrophage corepressors. By studying chromatin structure and histone modifications, transcription factor and coregulator dynamics, and eRNA transcription at the mouse Ccl2 locus, they uncover a role of corepressors in controlling enhancer and silencer remodeling linked to inflammatory gene expression.
The corepressors GPS2 and SMRT control enhancer and silencer remodeling via eRNA transcription during inflammatory activation of macrophages

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SUMMARY

While the role of transcription factors and coactivators in controlling enhancer activity and chromatin structure linked to gene expression is well established, the involvement of corepressors is not. Using inflammatory macrophage activation as a model, we investigate here a corepressor complex containing GPS2 and SMRT both genome-wide and at the Ccl2 locus, encoding the chemokine CCL2 (MCP-1). We report that corepressors co-occupy candidate enhancers along with the coactivators CBP (H3K27 acetylase) and MED1 (mediator) but act antagonistically by repressing eRNA transcription-coupled H3K27 acetylation. Genome editing, transcriptional interference, and cistrome analysis reveals that apparently related enhancer and silencer elements control Ccl2 transcription in opposite ways. 4C-seq indicates that corepressor depletion or inflammatory signaling functions mechanistically similarly to trigger enhancer activation. In ob/ob mice, adipose tissue macrophage-selective depletion of the Ccl2 enhancer-transcribed eRNA reduces metaflammation. Thus, the identified corepressor-eRNA-chemokine pathway operates in vivo and suggests therapeutic opportunities by targeting eRNAs in immuno-metabolic diseases.

INTRODUCTION

The plasticity of macrophages in response to extracellular signals relies on rapid and reversible chromatin remodeling events coupled with transcriptional changes to control gene expression patterns (Glass and Natoli, 2016; Kuznetsova et al., 2020; Russell et al., 2019). These changes are coordinated by hierarchical networks of transcription factors (TFs) and coregulators, which cooperate to modify chromatin and to communicate with the basal transcription machinery, including RNA polymerase II (Pol II) (Heinz et al., 2010; Kaikkonen et al., 2013; Lam et al., 2013; Link et al., 2018; Romanoski et al., 2015). Although there are many candidate coactivators or corepressors involved in the control of inflammatory gene expression in macrophages, recent studies have highlighted the particular importance of an anti-inflammatory corepressor sub-complex containing the core subunits GPS2 and SMRT (hereafter referred to as GPS2 complex) (Barish et al., 2012; Fan et al., 2016; Huang et al., 2019b; Oberoi et al., 2011; Treuter et al., 2017), distinct from the pro-inflammatory NCOR-HDAC3 sub-complex (Emmett
and Lazar, 2019; Li et al., 2013; Mullican et al., 2011). Genome-wide transcriptome profiling of wild-type (WT) versus macrophage-specific Gps2 knockout (KO) mice has revealed that expression of Ccl2, encoding C-C motif chemokine CCL2 (alias MCP-1 [monocyte chemotractant protein-1]), is among the major inflammatory target genes that are repressed by the Gps2 complex in different macrophage populations. In humans with obesity and type 2 diabetes, Gps2 expression in adipose tissue macrophages and blood monocytes inversely correlates with the expression of CCL2 (Fan et al., 2016). Our previous cis-trome and epigenome profiling has revealed that the Gps2 complex occupies a majority of H3K27ac-marked macrophage enhancers (Fan et al., 2016). This includes several enhancers that potentially control the expression of Ccl2 but that have not yet been further characterized.

Enhancers are cis-regulatory elements of the epigenome that are located distally to the transcription start sites (TSSs) and function as binding sites for TFs and coregulators to transform signals from the cellular microenvironment into altered gene expression. Genome-wide studies suggest that one gene or co-regulated gene cluster (e.g., the Ccl2 locus) is controlled by multiple enhancers (Andersson and Sandelin, 2014; Andersson and Sandelin, 2020; Osterwalder et al., 2018), but whether they all are uniquely required or act in part redundant has not yet been elucidated in the context of inflammatory gene expression. The field has defined key features of enhancers (Andersson and Sandelin, 2020; Cramer, 2019; Gasperini et al., 2020; Madsen et al., 2020; Partridge et al., 2020; van Steensel and Furlong, 2019), such as open chromatin, presence of TFs, MED1 (mediator), CBP/p300, H3K27ac, eRNA transcription, and promoter looping. However, corepressors are commonly not integrated into recent models of enhancer function, despite growing evidence that they co-occupy enhancers along with the above factors (Coppo et al., 2016; Czimmerer et al., 2018; Fan et al., 2016; Hati et al., 2013; Huang et al., 2019b; Kim et al., 2018; Li et al., 2013; Pachristou et al., 2018; Siersbaek et al., 2017; Treuter et al., 2017). This becomes highly relevant in light of recent studies describing cis-regulatory elements that seemingly share features with enhancers but act as silencers to repress transcription of linked genes (Doni Jayavelu et al., 2020; Gisselbrecht et al., 2020; Huang et al., 2019a; Ngan et al., 2020; Pang and Snyder, 2020). How these silencers communicate with enhancers and whether corepressor binding is a distinctive feature of such silencers has not yet been addressed.

The function of enhancers and silencers involves dynamic three-dimensional (3D) chromatin structure changes, which facilitate direct interactions with promoters through looping structures within defined topologically associating domains (TADs) (Kim and Shendure, 2019; Robson et al., 2019; van Steensel and Furlong, 2019). In contrast to static pre-established TADs formed by CTCF/cohesins (Hanssen et al., 2017; Jin et al., 2013), the formation of sub-TADs and intra-TAD enhancer-promoter loops is more tightly linked to changes in gene expression and can be dynamically regulated by CTCF/cohesin, YY1, nuclear receptors, and inflammatory TFs (Brown et al., 2014; Daniel et al., 2014; Hsieh et al., 2020; Kim et al., 2018; Krietenstein et al., 2020; Phanstiel et al., 2017; Ren et al., 2017; Weintraub et al., 2017). Of particular relevance appear AP-1 family members, including c-Jun and JunB, which are enriched at inflammatory enhancers (Fonseca et al., 2019; Phanstiel et al., 2017; Wolter et al., 2008; Vangala et al., 2020) and which are key target TFs of the GPS2 complex in macrophages (Fan et al., 2016; Treuter et al., 2017). Compared with the evidence supporting the critical role of TFs and coactivators in intra-TAD enhancer-promoter looping linked to inflammatory gene expression in macrophages, the mechanistic involvement of silencer elements and corepressors remains to be characterized.

Enhancer activity correlates with the transcription of non-coding enhancer RNAs (eRNAs) (Andersson and Sandelin, 2020; Henriques et al., 2018; Kaikkonen and Adelman, 2018; Kaikkonen et al., 2013; Lai et al., 2020; Lam et al., 2013; Lee and Mendell, 2020). Although it has remained challenging to distinguish between the function of eRNAs and the process of enhancer transcription, there is in vitro evidence that eRNAs bind to CBP/p300 to modulate their histone acetyltransferase activity (Bose et al., 2017). As CBP/p300 are the major H3K27 acetylases at enhancers (Rainsner et al., 2018), there could be a functional link to eRNA transcription. Interestingly, GPS2 depletion increases H3K27ac levels at macrophage enhancers, which does not involve HDAC3 (Fan et al., 2016), suggesting a functional link to CBP/p300 and perhaps eRNA transcription.

In this study, we address these issues by characterizing the role of the GPS2 complex in controlling inflammatory gene expression in mouse macrophages, with an emphasis on the regulation of Ccl2. We provide evidence that GPS2 and SMRT prevent enhancer activation, which involves intra-TAD re-arrangements of promoter contacts with silencer and enhancer. We show further that these corepressors repress eRNA transcription and antagonize CBP-dependent H3K27 acetylation at enhancers. Finally, we demonstrate that the in vivo antisense targeting of a GPS2 complex-repressed Ccl2 eRNA in adipose tissue macrophages of obese (ob/ob) mice can partially reverse metaflammation and insulin resistance, highlighting the in vivo potential of therapeutically targeting inflammatory enhancers.

RESULTS

Identification of Ccl2 enhancer and silencer

To compare the genome-wide chromatin recruitment of the GPS2 corepressor complex to known enhancer marks in macrophages, we performed chromatin immunoprecipitation sequencing (ChIP-seq) for GPS2 along with H3K27ac, the macrophage lineage-determining TF PU.1, and the coactivators MED1 and CBP in the mouse macrophage RAW264.7 cell line (hereafter referred to as RAW cells). Genome-wide analysis revealed that GPS2 chromatin binding largely overlapped with MED1 and CBP both in basal condition (Figure 1A) and upon treatment with lipopolysaccharide (LPS; a TLR4 ligand) (Figure S1A). Although GPS2 was localized in both proximal (promoter) and distal (enhancer) regions along with H3K27ac, further analysis of its functional recruitment revealed that GPS2 bound more abundantly to enhancers than to promoters of repressed genes (Figure 1B). As MED1 marks also super-enhancers, we ranked the GPS2 peaks along with CBP and MED1 and identified the Ccl2 enhancer region as a super-enhancer (Figure S1B).
Figure 1. Mapping of GPS2/MED1/CBP/H3K27ac-positive macrophage enhancers and functional dissection at the Ccl2 locus
(A) Venn diagram of common GPS2, CBP, and MED1 peaks as determined using ChIP-seq in RAW cells (basal condition).
(B) Density plot indicating peak densities in relation to distance to the transcription start sites (TSSs). The blue line represents all genome-wide GPS2 peaks, the purple line represents all genome-wide H3K27ac peaks, and the red line represents all genome-wide H3K27ac peaks that significantly changed upon GPS2 depletion. The x axis indicates the distance of individual peaks to the TSS of the closest annotated genes, and the y axis indicates the peak density, corresponding to the relative occupancy of GPS2 and H3K27ac across the genome.
(C) Genome Browser tracks representing the distribution of H3K27ac, open chromatin, TFs, and coregulators across the Ccl2 locus in BMDMs and RAW cells.

The BMDM data were obtained from the Gene Expression Omnibus (GEO) database for H3K27ac (GEO: GSM1631858), PU.1 (GEO: GSM1533888), GPS2 (GEO: GSM1631866), SMRT (GEO: GSM665925), and ATAC-seq (GEO: GSM2974654). The ChIP-seq and ATAC-seq data in RAW cells were generated in this study.

(legend continued on next page)
We specifically looked into the organization of the Ccl2 locus as it is one of the most relevant GPS2-repressed genes in macrophages (Fan et al., 2016). We mapped the Ccl2 super-enhancer in both bone marrow-derived macrophages (BMDMs) and RAW cells using ChIP-seq. Co-occupancy of GPS2 and SMRT, H3K27ac, MED1, CBP, and PU.1 identified two major enhancer clusters (Figure 1C). Both clusters were located in open chromatin regions identified using assay for transposase-accessible chromatin using sequencing (ATAC-seq) (Figure 1C). We then performed H3K27ac ChIP-seq in both GPS2 and SMRT-knockdown RAW cells (Figure 1D). Genome-wide analysis revealed that more than 70% of the altered H3K27ac signals at target genes were co-regulated by GPS2 and SMRT (Figure S1C). Re-analysis of published RNA sequencing (RNA-seq) data in WT versus GPS2-KO RAW cells (Huang et al., 2019b) confirmed that Ccl2 is among these most significantly upregulated target genes (Figure S1D) and that this regulation is conserved between RAW cells and BMDMs (Fan et al., 2016). GPS2 and SMRT knockdown increased H3K27ac at both enhancers and promoter of Ccl2 at basal condition, consistent with gene expression changes (Figures 1D, S1E, and S1F), while nearby upstream genes were not affected (Figure S1G). Notably, knockdown of SMRT completely abrogated GPS2 recruitment at the Ccl2 locus and genome-wide (Figures S1H and S1I). This suggests that SMRT acts as the primary chromatin docking site of this corepressor sub-complex in macrophages, while GPS2 acts modulatory.

Further dissection of the two major Ccl2 enhancer clusters revealed their distinct function. We used CRISPR-Cas9 to delete the regions around the TF/coregulator binding centers (460 bp for the distal E1.1, 565 bp for the proximal E1.2, and 621 bp for E2) in RAW cells (for exact locations of the deleted regions, see STAR methods). Strikingly, we found that deletion of the distal enhancer (E1.1 or E1.2) abolished LPS-induced Ccl2 and Ccl7 expression (both genes share promoters), while removal of the proximal enhancer (E2) showed the opposite effect (Figures 1E and S1J). This regulation was gene specific, as the neighboring gene Asic2 was not affected (Figure S1J). As enhancer activation is regulated by multiple TFs that may differentially respond to individual inflammatory signals, we tested the responses of the enhancer-deleted RAW cells to KLA (TLR4), IFNα (STAT1), TPA (AP-1), IL-4 (STAT6), TNF-α (NF-κB) and IL-6 (STAT1/3, AP-1). We observed that the opposite function of E1 and E2 was universal and stimulus independent, as all signal responses were abrogated in E1-deleted cells but elevated in E2-deleted cells (Figure S1K).

We next performed H3K27ac ChIP-seq in WT and enhancer-deleted RAW cells (Figure 1F). Deletion of E1 enhancer largely reduced H3K27ac in E1, E2, and promoter regions, while deletion of E2 enhancer increased H3K27ac in these regions (Figure 1F), consistent with the changes in gene expression. This was confirmed by ChIP-qPCR (Figure S1L), and it was gene specific, as the enhancer deletions had no effects on the neighboring gene loci (Figure S1M). We continued to knock down GPS2 or SMRT in enhancer-deleted RAW cells to explore the function of the corepressor complex. In E1-deleted cells, knockdown of GPS2 or SMRT had no further effects on Ccl2 expression (Figures 1G and S1N). This suggests that E2, in the absence of E1, is not capable of driving Ccl2 transcription despite the loss of corepressors. In E2-deleted cells, however, knockdown of GPS2 or SMRT further increased Ccl2 expression. This suggests that E1, even in the absence of E2, is further de-repressed by loss of GPS2 or SMRT. Moreover, in SMRT-knockdown cells, E2 deletion did not further change Ccl2 expression, suggesting that SMRT participates in E2-mediated repression (Figure S1N). The combined results suggest that E1 acts as the critical enhancer for Ccl2 gene expression and for repression by GPS2 and SMRT, while E2 is dispensable for Ccl2 gene expression but instead has features of a silencer those deletion causes de-repression. The chromosomal positions of the corresponding deleted enhancer versus silencer regions along with key TF motifs are outlined in Figure S1O and specified in STAR methods. Hereafter, we refer to E1 as Ccl2 enhancer (E) and E2 as Ccl2 silencer (S).

**Differential role of enhancer and silencer in transcription complex assembly at the Ccl2 locus**

Gene transcription and H3K27ac are coordinated by TFs, coregulators, and Pol II, which can be mapped by ChIP-seq. However, ChIP-seq alone is not able to identify the primary binding sites of TFs and coregulators, as the cross-linking process fixes protein/DNA complexes in chromatin looping structures within TADs. The individual Ccl2 E/S deletions therefore represent a feasible tool to map the primary docking sites of TFs and coregulators along with Pol II.

First, we performed ChIP-seq of MED1, CBP, and Pol II in WT and E/S-deleted RAW cells in both LPS and basal condition (Figures 2A and S2). We found that in both conditions, in E-deleted cells the recruitment of these factors was abolished at all linked enhancer, silencer, and promoter regions. In S-deleted cells, however, occupancy of these factors at these regions was not abolished but rather increased (Figures 2A and S2A). These
data are consistent with the changes in H3K27ac and transcription. They indicate that E is the dominant enhancer to assemble the transcriptionally active MED1, CBP, and Pol II complex at the looping structures within the Ccl2 TAD.

Second, we analyzed the recruitment of relevant macrophage TFs (i.e., highly expressed and motifs enriched at GPS2, MED1, CBP-positive regions), that is, the lineage-determining TFs PU.1, RUNX1, and the signal-responsive AP-1 subunit JunB, in WT and E/S-deleted RAW cells. We found that deletion of E abrogated TF binding at E and promoter, but not at S in both basal and LPS condition (Figures 2B and S2A). This confirmed the E-promoter loop but also suggested that TF binding to S was non-functional, as it was disconnected from coactivator Pol II binding (Figure 2A).

In contrast, S deletion led to increased TF binding at E and promoter, which could be a consequence of the increased local concentration of these factors within the S-deleted TAD (Figure 2B). The binding differences of TFs and coactivators were Ccl2 gene specific, as their binding was not affected at neighboring genes (Figures S2B and S2C). These data suggest that both E and S are the primary docking sites for inflammatory TFs, but only the TFs at E are associated with the promoter.

Third, we analyzed the binding of GPS2 and SMRT in both basal and LPS condition and found distinctive binding patterns. In E-deleted RAW cells, GPS2 was lost at E and promoter but maintained at S, while S deletion increased GPS2 binding at the Ccl2 promoter (Figures 2C and S2A). Similar to GPS2, SMRT binding at E and promoter was abolished in E-deleted cells, while its binding to E and promoter was not affected in S-deleted cells (Figures 2C and S2A). The recruitment of SMRT and GPS2 at neighboring genes was not affected (Figures S2B and S2C). Strikingly, the binding pattern of GPS2 and SMRT resembles the pattern observed with RUNX1 and JunB (Figures 2B and S2A), suggesting that these TFs might be required for the enhancer recruitment of GPS2 and SMRT but not of CBP and MED1. This is supported by genome-wide motif enrichment analysis, identifying specifically AP-1 motifs to be more frequently enriched in GPS2 and SMRT peaks than in CBP and MED1 peaks (Figures S2D–S2G).

Fourth, we directly compared the recruitment profiles of the above factors in basal versus LPS condition in WT cells in a separate experiment (to minimize batch effects between the comparisons, including variation in cell viability, ChIP efficacy, library amplification, and sequencing). The ChIP-seq data suggest an increase of Pol II, MED1, CBP, PU.1, JunB, and RUNX1 binding in E, S, and promoter regions upon 2 h LPS treatment, while GPS2 and SMRT binding was decreased (Figure S2H). To confirm this and further study the binding dynamics, we performed ChIP-qPCR of GPS2 and JunB at different time points of LPS treatment along with monitoring mRNA expression (Figure S2I). The data reveal unique, in part adverse, chromatin binding dynamics for JunB and GPS2. The dynamics are compatible in the case of JunB, with its transient induction during...
inflammatory TLR4 activation (Fonseca et al., 2019), and in the case of GPS2, with the transient corepressor-coactivator switch and feedback mechanisms that terminate transcription despite continued inflammatory signaling (Treuter et al., 2017).

Last, to shed light on features the possibly distinguish the Ccl2 E and S regions, we mapped H3K27me3, a classic repressive histone modification linked to PRC-mediated chromatin silencing during development (Ngan et al., 2020), along with the mutually exclusive H3K27ac modification linked to activation, in WT and E/S-deleted cells (Figure S2J). Interestingly, H3K27me3 seemed specifically enriched around but not within the S region in WT cells, consistent with S being labeled by H3K27ac. In S-deleted cells, H3K27me3 was largely reduced in E, S, and promoter regions, consistent with the increase of H3K27ac. In contrast, in E-deleted cells, both H3K27me3 and H3K27ac patterns were erased. Collectively, these data depict how TFs, coactivators and corepressors coordinate the enhancer-silencer-promoter-gene interplay at the Ccl2 locus, they suggest that enhancer and silencer are rather determined by differential factor recruitment than by differential histone marks, and they suggest that corepressors are tightly linked to both enhancer and silencer-bound TFs.

**Chromatin dynamics within the Ccl2 locus upon inflammatory signaling and corepressor depletion**

We next explored the role of corepressors in chromatin dynamics within the Ccl2 TAD. Analysis of published Hi-C data (Link et al., 2018) revealed the TAD covering the mouse Ccl2 locus, but different signaling conditions were not studied, and the resolution was too low to further specify intra-TAD interactions between enhancers and promoters (Figure S3A). To further dissect these interactions under inflammatory conditions, we performed circular chromosome conformation capture sequencing (4C-seq) (van de Werken et al., 2012), which monitors all genomic regions that interact with a specific region of interest (bait) (Figure 3). In the absence of inflammatory signaling, promoter interactions were detected with S but not with E (Figures 3A, 3B, S3B, and S3C). Strikingly, this situation was reversed upon inflammatory stimulation with LPS (i.e., the S-promoter loop disappeared and instead the E-promoter loop was formed). Furthermore, we found that E and S require the promoter region to form the loops, as deletion of the promoter region abolished all looping structures as well as LPS-induced Ccl2 gene transcription (Figures 3A, 3B, S3B, and S3C). However, the promoter was not required for TF binding to the distal elements, as deletion of the promoter did not affect PU.1 binding to E and S (Figures S3D and S3E). Furthermore, deletion of either E or S had only marginal effects on promoter interactions of the remaining distal element, supporting the structural independence of enhancer and silencer regions (Figures S3F and S3G).

To address the role of the GPS2 complex in enhancer versus silencer interactions, we performed 4C-seq in corepressor-depleted RAW cells (Figures 3C–3E). We observed that knockdown of either GPS2 or SMRT induced E-promoter interactions, while S interactions with the promoter or E were not affected (Figures 3C–3E and S3H). As H3K27ac and Ccl2 expression were increased under similar conditions and required E, these data suggest that LPS activation and loss of corepressors have similar effects on the formation of enhancer-selective promoter interactions within the Ccl2 TAD to regulate gene transcription (Figure 3F), consistent with the TF and coregulator changes both at Ccl2 locus and genome-wide (Figures S2H and S3K). GPS2 depletion did not alter the chromatin accessibility (ATAC-seq) at the Ccl2 E, S, and promoter regions but significantly increased MED1 and Pol II binding at E and promoter regions (Figures S3I and S3J). Genome-wide analysis revealed that GPS2 depletion caused increased recruitment of co-activators (MED1 and Pol II) at upregulated genes including Ccl2 (Figure S3K), while TF binding (JunB and PU.1) was unaffected by GPS2 depletion (Figures S3L and S3M). This confirms that GPS2 controls TF activity but not the access of TFs to chromatin, as it was observed in other contexts (Jakobsson et al., 2009; Huang et al., 2019b).

**The Ccl2 enhancer-transcribed eRNA coordinates CBP-dependent H3K27 acetylation, Pol II recruitment, and enhancer-promoter interactions**

Enhancer-transcribed eRNAs correlate with enhancer activity and to the expression of adjacent genes, so we investigated how these events are mechanistically linked at the Ccl2 locus. We applied global run-on sequencing (GRO-seq) (Kaikkonen et al., 2013) to determine eRNA transcription in RAW cells upon inflammatory LPS stimulation. At the genome-wide level, eRNA expression correlated with gene expression, and eRNA expression plus H3K27ac served as an even better predictor of gene transcription than eRNA expression alone (Figure S4A). At the Ccl2 locus, eRNA expression was higher at enhancer E than at S (Figure S4B). In addition, enhancer E-derived eRNA (referred to as E eRNA hereafter) expression was increased by LPS or upon depletion of GPS2 or SMRT (Figures 4A and 4B). We next tested E eRNA dynamics in LPS-treated RAW cells (Figure S4C). LPS induction of E eRNA transcription started after 1 h treatment and reached the peak at 2 h. For comparison, Ccl2 and Ccl7 mRNA transcription were significantly delayed, with initial induction at 2 h and reaching the peak at 6 or 12 h, respectively (Figure S4C). Interestingly, S deletion further enhanced E eRNA transcription and Ccl2/Ccl7 mRNA expression (Figure S4D), which was consistent with the increased binding of TFs to enhancer E upon deletion of S (Figures 2A and 2B). Also, E eRNA expression did not require the Ccl2 promoter, as its depletion did not reduce the expression of E eRNA (Figure S4E). This indicates that functional transcription complexes are formed autonomously at Ccl2 enhancers to trigger eRNA transcription promoter-independently, in contrast to mRNA transcription which is promoter dependent.

We next applied CRISPRi using the dCas9/KRAB repressor system (Thakore et al., 2015) to stERICALLY interfere with enhancer-specific transcription at the Ccl2 locus (Figure 4C). We constructed specific guide RNAs (gRNAs) directing the dCas9/KRAB repressor to different genomic regions (both positive and negative strands) of E and S. Gene expression analysis revealed that blocking E eRNA transcription reduced Ccl2 and Ccl7 mRNA, while blocking S transcription had no significant effect (Figure 4C). To more directly test the role of the E eRNA transcript, without interfering with TF function, we targeted E eRNA using antisense locked nucleic acids (LNA-GapmeRs).
Figure 3. 4C-seq analysis of 3D chromatin structure and enhancer/silencer-promoter interactions at the Ccl2 locus

(A and B) 4C-seq upon LPS signaling: representative images showing the tracks of 4C-seq-based contact profiles at the Ccl2 locus using (A) E or (B) S as bait. 4C-seq was performed from untreated WT RAW cells (WT con), WT cells treated with LPS for 2 h (WT LPS), and in promoter-deleted cells treated with LPS for 2 h (ΔPro LPS). The upper panel of 4C-seq data shows the main trend of interaction profiles set to a 250 kb window using 20%–80% percentile values. Interaction frequencies were normalized to the strongest point (bait region). The black line shows the median interaction frequencies. The lower panel shows relative interactions within the 2–50 kb on the basis of a color-coded scale. Red dots indicate strong interactions, and gray dots indicate low interactions. In the lower panel, ChIP-seq data for CTCF (GEO: GSM918726), PU.1, and CBP were used to compare and mark the Ccl2 enhancers and the CTCF insulating regions (n = 3).

(C and D) 4C-seq upon corepressor depletion: representative images showing tracks of 4C-seq-based contact profiles at the Ccl2 locus using (C) enhancer E or (D) promoter as bait (n = 2). 4C-seq was performed from control RAW cells (shGFP), GPS2-depleted cells (shGPS2), and SMRT-depleted cells (shSMRT) under basal condition. The induced DNA interactions are highlighted with red arrows and the pre-formed interactions are highlighted with black arrows.

(E) Interaction strength by normalized 4C-seq tag counts for the Ccl2 promoter bait on Ccl2 enhancer E and silencer S regions upon depletion of GPS2 or SMRT. The data are represented as mean ± SEM.

(F) Model illustrating the 3D chromatin dynamics and altered enhancer/silencer-promoter interactions at the Ccl2 locus upon LPS signaling or corepressor depletion. See also Figure S3.

See also Figure S3.
We screened 22 different LNAs and identified 3 functional ones that efficiently reduced eRNA in LPS-stimulated RAW cells (Figure 4D). Gene expression analysis revealed that LNA-mediated silencing of eRNA reduced \( \text{Ccl2} \) and \( \text{Ccl7} \) mRNA, consistent with the dCas9/KRAB data but also extending them by suggesting a role of the eRNA itself (Figure 4D). We also found GPS2-mediated repression of \( \text{Ccl2} \) and \( \text{Ccl7} \) mRNA expression also required eRNA, as knockdown of eRNA reduced \( \text{Ccl2} \) and \( \text{Ccl7} \) expression in CRISPR-generated GPS2-KO cells (Figure 4D).
We further explored the mechanisms of how E eRNA controls Ccl2 promoter activation and gene transcription. In vitro, it has been shown that eRNAs physically bind to CBP and activate its histone acetyltransferase activity, which may link eRNA transcription to H3K27 acetylation during enhancer activation (Bose et al., 2017; Raisner et al., 2018). To test whether this can be recapitulated at the Ccl2 locus in RAW cells, we performed ChIP-seq of CBP and H3K27ac in WT versus LNA-treated cells (Figure 5A). Indeed, our data indicated that silencing of E eRNA reduced CBP-binding and H3K27ac at E, S, and promoter...
This effect was restricted to the Ccl2 TAD, as we observed no changes in the neighboring genes Zfp207 and Psmd11 (Figure 5E). The ChIP-seq changes of CBP and H3K27ac were confirmed by ChIP-qPCR analysis (Figure 5C), which additionally revealed that Pol II recruitment at both regions was reduced upon E eRNA silencing. Last, we performed 4C-seq using the Ccl2 promoter as bait and found that eRNA silencing abolished LPS-induced interactions between promoter and E (Figure 5D). The results were confirmed using E or S as bait (Figures S5A and S5B). Overall, these data support a functional role of the Ccl2 enhancer E-transcribed eRNA in mediating inflammatory enhancer activation (via CBP-H3K27ac) and sub-TAD formation (via enhancer-promoter looping) to facilitate Ccl2 mRNA transcription.

**Macrophage-specific Ccl2 eRNA knockdown reduces metaflammation and insulin resistance in ob/ob mice**

CCL2 is a major and well-studied chemokine that has been associated with adipose tissue macrophage infiltration and insulin resistance in the context of obesity, both in humans and in mouse models (see Introduction). We therefore tested whether in vivo depletion of the functional Ccl2 eRNA in white adipose tissue (WAT) macrophages of obese (ob/ob) mice would have beneficial effects. To achieve this, we generated glucan-encapsulated LNA particles (GeLP) based on the glucan-encapsulated small interfering RNA (siRNA) particle (GeRP) method, which delivers siRNAs (Aouadi et al., 2013; Barreby et al., 2019). Intra-peritoneal (i.p.) injection of these particles into 6-week-old ob/ob mice leads to macrophage phagocytosis specifically in the WAT, and with the help of the endo-porter peptide the LNAs escape the phagosomes and will silence E eRNA expression within the adipose tissue macrophages (Figure 6A). We observed that upon 10 days of GeLP injection, blood glucose levels of ob/ob mice treated with the Ccl2 eRNA LNA were significantly lower than in mice treated with scrambled control LNA (Figure 6B). The oral glucose tolerance test (OGTT) revealed improved glucose control upon 10 days of E eRNA silencing (Figure 6C), while baseline OGTT levels were not different between the two groups before treatment (Figure S6A). Furthermore, the total body weight and the weight of the different adipose tissue depots were not changed between the two groups upon 10 days of E eRNA silencing (Figures S6B and S6C). Importantly, E eRNA silencing reduced macrophage infiltration of visceral adipose tissue (VAT) by almost 50%, as determined by F4/80+CD11b+ fluorescence-activated cell sorting (FACS) analysis (Figure 6D). Consistent with this expression of the Ccl2 E eRNA, the Ccl2 mRNA, and the mRNAs of other inflammatory cytokines were reduced upon E eRNA silencing, both in the stromal vascular fraction (SVF) (Figure 6E) and in F4/80+ SVF macrophages (Figure 6F) of the VAT. This reduction seems specifically to occur in the macrophages, as it could not be detected in the whole adipose tissue (mainly adipocytes), that is, VAT (Figure 6G), subcutaneous adipose tissue (SAT) (Figure 6H), and brown adipose tissue (BAT) (Figure S6D), or in the liver (mainly hepatocytes) (Figure S6E). Collectively, these data demonstrate that GeLP-mediated silencing of the Ccl2 enhancer E-transcribed eRNA in an obese mouse model was effective in reducing Ccl2 mRNA expression and macrophage inflammation in the VAT and in partially reversing obesity-associated insulin resistance.

**DISCUSSION**

We systemically investigated the role of the GPS2/SMRT corepressor sub-complex in specifying the function of cis-regulatory elements and chromatin remodeling linked to inflammatory gene expression with an emphasis on the Ccl2 locus, both in cultured macrophages and in obese (ob/ob) mice. We identify a role of corepressors in antagonizing enhancer-promoter communication, MED1/Pol II action, CBP-mediated H3K27 acetylation, eRNA transcription, and pro-inflammatory gene expression. Our work thereby helps fill a current knowledge gap, because corepressors are mostly not yet integrated into models of enhancer function, in contrast to TFs and the enhancer-marking coactivators CBP/p300 and MED1.

An intriguing result is that Ccl2 expression is controlled by non-redundant, functionally distinct cis-regulatory regions that seemingly share common features of “active” enhancers, including high H3K27ac, Pol II binding, eRNA transcription, recruitment of shared sets of TFs (PU.1, AP-1, RUNX1), coactivators (MED1, CBP), and corepressors (GPS2, SMRT). The Ccl2 “super-enhancer” turned out to consist of a major enhancer (E) and a major silencer (S) region. Functional differences became only evident through the combined analysis of deletions (CRISPR-Cas9), inhibition of transcription (CRISPRi), eRNA depletion (antisense LNA GapmeRs), and promoter interactions (4C-seq) along with gene expression analysis. Our results are consistent with recent studies suggesting that silencers are much more common at the genome-wide level than anticipated (Doni Jayavelu et al., 2020; Gisselbrecht et al., 2020; Huang et al., 2019a; Ngan et al., 2020; Pang and Snyder, 2020). Notably, the classic “silencing mark” H3K27me3 (Wiles and Selker, 2017) seems not commonly associated with these silencers. Instead, they are positioned in open chromatin regions and share features with “weak” enhancers (Pang and Snyder, 2020). Key characteristics that distinguish such silencers from enhancers and thereby would allow to predict them have remained enigmatic. Silencers may enrich specific TF repressors, including AP-1 factors (Doni Jayavelu et al., 2020) and corepressors including NCoR (the SMRT homologs core subunit of the HDAC3 complex) (Pang and Snyder, 2020). However, these factors also occupy enhancers, and there is no experimental evidence yet that they are the determinants of silencer function.

Although our study does not identify genome-wide silencers, the insights into the Ccl2 regulatory landscape have delineated potential silencer features in inflammatory contexts. Regarding AP-1 factors, JunB is found at both silencer and enhancer, and Ccl2 silencer deletion had a strong de-repression effect upon treatment with TPA (a selective AP-1 activator). AP-1 members are the most abundant TFs at macrophage enhancers, many of which are induced by TLR4 ligands (Fan et al., 2016; Fonseca et al., 2019). Regarding corepressors, although we provide evidence in WT versus SMRT-depleted cells that SMRT is required for the Ccl2 silencer function, SMRT along with GPS2 also occupies and represses the Ccl2 enhancer, and most macrophage enhancers genome-wide. Possibly, as corepressors do not
Figure 6. In vivo depletion of the Ccl2 enhancer E-transcribed eRNA in adipose tissue macrophages of ob/ob mice

(A) Schematic representation of GeLP-mediated eRNA knockdown in ob/ob mice. Mice injected with GeLPs carrying scrambled LNA serve as control group (n = 7), and mice injected with GeLPs carrying Ccl2 E eRNA selective LNA serve as knockdown group (n = 8).

(B–D) Mice were characterized as follows: (B) random blood glucose (RBG), (C) OGTT, and (D) FACS analysis of F4/80+CD11b+ adipose tissue macrophages in visceral adipose tissue (VAT).

(E and F) qRT-PCR analysis of E eRNA and inflammatory genes in (E) stromal vascular fraction (SVF) and (F) F4/80+ SVF of VAT.

(G and H) qRT-PCR analysis of E eRNA and inflammatory genes in (G) whole VAT (H) whole subcutaneous adipose tissue (SAT). Unpaired t test was used to determine data significance. All data are represented as mean ± SEM.

*p < 0.05, **p < 0.01, and ***p < 0.001 versus control groups. See also Figure S6.
access DNA directly, their occupancy is a shared feature of silencers and enhancers, but the functional outcome of corepressor occupancy is determined by the underlying dynamic and transient TF networks at silencers versus enhancers. Specifically, the requirement of SMRT but not GPS2 for the Ccl2 silencer may be explained by the presence of distinct sets of target TFs at silencer versus enhancer, consistent with the assumption that SMRT has a broader TF-binding capacity than GPS (Oberoi et al., 2011; Treuter et al., 2017). Notably, SMRT was originally identified as a "silencing mediator of retinoid and thyroid receptors," which are metabolic sensors and dual repressors/activators within the nuclear receptor TF family (Chen and Evans, 1995). Therefore, our work indicates that the "silencing mediator" concept applies to inflammatory TFs and signaling contexts as well.

Our data support the model that SMRT and GPS2 modulate transcription under basal conditions but also under acute inflammatory conditions. In addition to the ChIP-based data, this is directly evidenced by the elevated LPS induction of inflammatory transcription upon GPS2 or SMRT depletion. This is biologically highly relevant for conditions of acute inflammation (this study) but also for chronic inflammation associated with obesity and type 2 diabetes (metaflammation), conditions that downregulate GPS2 expression (Fan et al., 2016). The combined data suggest that GPS2, along with SMRT within the sub-complex, functions under diverse conditions of inflammatory signaling. This can be understood from assuming a rather dynamic chromatin binding and release behavior of corepressors even under conditions of gene activation, thus expanding the common corepressor-coactivator exchange model (Kuznetsova et al., 2020; Perasi et al., 2010; Treuter et al., 2017).

Given the ongoing debate about whether eRNAs are functional (Kaikkonen and Adelman, 2018), our study supports their role in linking enhancer activity to inflammatory gene expression via modulating CBP-mediated H3K27acetylation (Bose et al., 2017). Specifically, we demonstrate that LNA-mediated knockdown of the Ccl2 enhancer E-derived eRNA led to reduced H3K27ac at E, S, and promoter, along with reduced Ccl2 gene expression. Furthermore, GPS2 depletion increases H3K27ac at enhancers in vivo (Fan et al., 2016) and in vitro (this study). By combining these two findings, we propose a model in which GPS2 and SMRT antagonize eRNA/CBP-dependent H3K27 acetylation at enhancers, presumably independent of HDAC3. Mechanistically, CBP/p300 antagonisms may involve (1) direct competition with CBP/p300 for binding to enhancer-bound TFs, (2) inhibition of eRNA transcription that in turn reduces CBP/p300 HAT activity catalyzing H3K27ac, or (3) interference with CBP/p300 modulation of paused Pol II and elongation (Boija et al., 2017), which may operate at promoter-enhancer loops. The latter mechanism is intriguing as many key inflammatory genes in macrophages are controlled at the level of Pol II pausing and elongation (Heinz et al., 2018; Kaikkonen et al., 2013; Yu et al., 2020). Regarding the question of which TFs are involved, AP-1 family members including c-Jun and JunB are strong candidates: they are direct targets for GPS2 and SMRT (Fan et al., 2016), they are among the most abundant enhancer-enriched TFs upon inflammatory macrophage activation (e.g., LPS/TLR4 signaling) (Fonseca et al., 2019; Glass and Natoli, 2016; Treuter et al., 2017), and they facilitate enhancer-promoter looping upon inflammatory signaling (Phanstiel et al., 2017; Vangala et al., 2020).

Finally, our work has implications for future therapeutic strategies that aim to target enhancers in the context of immune-metabolic disorders. CCL2 is of particular interest, as this macrophage-derived chemokine is a key mediator of metaflammation (Amano et al., 2014; Arner et al., 2012; Bonello et al., 2011; Bot et al., 2017; de Waard et al., 2010; Deshmukh et al., 2009; Haringman et al., 2006; Hildebrand et al., 2013; Hotamisilgil 2017; Kanda et al., 2006; Katakami et al., 2010; Kawano et al., 2016; Khyzha et al., 2019; Kulyte et al., 2014; Winter et al., 2018; Wolter et al., 2008). We show here that LNA-targeting the Ccl2 enhancer E-transcribed eRNA in adipose tissue macrophages of ob/ob mice could partially reverse metaflammation and insulin resistance. This not only verifies the corepressor-enhancer-eRNA-CCL2 pathway in vivo, it also suggests that the selective targeting of inflammatory enhancers/eRNAs in macrophages is possible in mice. As LNA-based therapeutic approaches seem to be safe in humans (Juliano, 2016; Lieberman, 2018; Roux et al., 2017), the feasibility of targeting human enhancer alterations linked to immuno-metabolic diseases (Allum et al., 2019; Liu et al., 2017; Sun et al., 2018) might become clinically relevant in the future.

Limitations of study
First, although in our study the direct function of corepressors at enhancers is well supported by the Ccl2 locus analysis and by genome-wide data, features of Ccl2-related silencers beyond corepressor binding remain poorly understood and require future genome-wide investigations. We imagine that there are unique TF-corepressor networks that need to be scrutinized along with histone marks. Second, to obtain insights into species conservation and relevance for human immuno-metabolic diseases, our analysis must be extended to human monocytes/macrophages. Third, chromatin recruitment of proteins via ChIP-seq must be interpreted with caution, as binding to a particular region does not necessarily mean functionality, and antibodies differ in affinity and specificity making quantitative comparisons difficult. Finally, long-range interactions remain difficult to identify using 4C-seq, so it is not known whether the Ccl2 enhancer/silencer is additionally engaged in such interactions.

STAR METHODS
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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

Z.H., R.F., and E.T. conceived the study. Z.H. and R.F. performed most experiments, with major input from N.L. and S.G., and A.D. analyzed the sequencing data. C.W. and A.P.B. contributed to the 4C-seq and data analysis. H.N. and M.U.K. performed the GRO-seq and data analysis, T.J. advised and provided tools for the CRISPR-Cas9 experiments. R.F., H.H., M.A., J.J., R.B., and N.V. contributed to the mouse experiments and tissue analysis. Z.H., R.F., and E.T. wrote the manuscript and further edited upon input from all co-authors.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies** | | |
| Anti-CBP, rabbit monoclonal | Cell Signaling Technology | Cat# (D6C5),7389s; RRID: AB_2616020 |
| Anti-CD11b, mouse monoclonal | Thermo Fisher | Cat# 17-0112-82; RRID: AB_469343 |
| Anti-F4/80, mouse monoclonal | Thermo Fisher | Cat# 25-4801-82; RRID: AB_469653 |
| Anti-GPS2, rabbit polyclonal | This lab | Jakobsson et al., 2009 |
| Anti-H3K27ac, rabbit polyclonal | Abcam | Cat# Ab4729; RRID: AB_2118291 |
| Anti-H3K27me3, rabbit polyclonal | Millipore | Cat# 17-622; RRID: AB_916347 |
| Anti-Jun B, rabbit polyclonal | Santa Cruz Biotechnology | Cat# Sc-46x; RRID: AB_2130022 |
| Anti-MED1, rabbit polyclonal | Bethyl Laboratories | Cat# A300-793A; RRID: AB_577241 |
| Anti-Pol II, mouse monoclonal | BioLegend | Cat# (BWS16),664906; RRID: AB_2565554 |
| Anti-PU.1, rabbit polyclonal | Invitrogen | Cat# PA5-17505; RRID: AB_10989141 |
| Anti-RUNX1, rabbit polyclonal | Abcam | Cat# ab23980; RRID: AB_2184205 |
| Anti-SMRT, rabbit polyclonal | Bethyl Laboratories | Cat# A301-147A; RRID: AB_2149145 |
| **Chemicals, peptides, and recombinant proteins** | | |
| IL4 | Sigma | Cat# SRP3211; |
| TPA | Sigma | Cat# P8139; |
| IFNγ | Sigma | Cat# SRP3058; |
| IL6 | Sigma | Cat# I2786; |
| KLA | Sigma | Cat# 699500P; |
| TNFα | Sigma | Cat# SRP3177; |
| LPS | Sigma | Cat# F8666; |
| FuGENE® HD Transfection Reagent | Promega | Cat# E2311 |
| Disuccinimidyl glutarate (DSG) | VWR | Cat# A7822.0001 |
| Formaldehyde | Thermo Fisher | Cat# 28906 |
| Puromycin | Sigma | Cat# P8833 |
| DNase | Invitrogen | Cat# EN0521 |
| Collagenase II | Thermo Fisher | Cat# 17101015 |
| BSA | Sigma | Cat# 05470 |
| Dpnl | NEB | Cat# R0543M |
| Niall | NEB | Cat# R0125L |
| T4 ligase | NEB | Cat# M0202M |
| Tn5 transposase | Illumina | Cat #FC-121-1030 |
| DNA Clean and Concentrator-5 Kit | Zymo Research | Cat# D4014 |
| Digitonin | Promega | Cat# G9441 |
| **Critical commercial assays** | | |
| QIAquick PCR purification kit | QIAGEN | Cat# 28104 |
| Expand long template PCR system | Roche | Cat# 17590600001 |
| Qubit Fluorometric Quantification kit | Thermo Fisher | Cat# Q33238 |
| SMARTer PicoPLEX library preparation kit | Takara | Cat# R400676 |
| SMARTer DNA unique dual index kits | Takara | Cat# R400660-R400663 |
| Clean & Concentrator Capped Zymo-Spin I kit | Zymo Research | Cat# D4013 |
| SYBR Green | KAPA Biosystems | Cat# 07959567001 |
| E.Z.N.A Kit | Omega | Cat# R8834-02 |

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Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Superscript III Reverse Transcriptase kit | Invitrogen | Cat# 18080093 |
| ChargeSwitch PCR Clean-Up Kit | Invitrogen | Cat# CS12000 |
| AccuPrime Pfx DNA Polymerase | Invitrogen | Cat# 12344032 |
| Dynabeads M-270 Streptavidin | Invitrogen | Cat# 65305 |

Deposited data

| NGS data for this study | This paper | GSE130383 |
|-------------------------|------------|-----------|
| BMDM Hi-C data | GEO | GSM2974676 |
| BMDM H3K27ac ChIP-seq | GEO | GSM1631858 |
| BMDM PU.1 ChIP-seq | GEO | GSM1533888 |
| BMDM CTCF ChIP-seq | GEO | GSM918726 |
| BMDM SMRT ChIP-seq | GEO | GSM665925 |
| BMDM GPS2 ChIP-seq | GEO | GSM1631866 |
| BMDM ATAC-seq | GEO | GSM2974654 |

Experimental models: cell lines

| RAW264.7 | ATCC | Cat# TIB-71; RRID: CVCL_0493 |
| HEK293 | ATCC | Cat# CRL-1573; RRID: CVCL_0045 |

Experimental models: organisms/strains

Mouse: ob/ob (JAX Mice Strain) | Charles River Laboratories; | Cat# JAX:000632; RRID: IMSR_JAX:000632 |

Oligonucleotides

| Primers for Gapdh; Forward: ATGGCCTTCCGTGTCCCTA; Reverse: GAACTCGAGAGACACACTT | This paper | N/A |
| Primers for Ccl2; Forward: CAGATGCAAGTTACGCCCCA; Reverse: TGAAGCTGTGAGCAAAAAACTACAG | This paper | N/A |
| Primers for Ccl7; Forward: GATCTCTCCACGCTTCTG; Reverse: TGCTTGAAGATAACAGCTTCCCA | This paper | N/A |
| Primers for Tnf; Forward: AGGCCACGCCTGCTGCAAAAACC; Reverse: GAGGAGCAGAGTGCAACAGG | This paper | N/A |
| Primers for Il1b; Forward: AAATACTCTGGCTCCTGAGGC; Reverse: CTTGGGATCCACACTCTTCCA | This paper | N/A |
| Primers for Il6; Forward: GTCTGGAGTACAGGAGATGCC; Reverse: TCTGACCACAGTGAGAATGTCCA | This paper | N/A |
| Primers for F4/80; Forward: CTTGGAGACTGAGGAGCTGAGAGT; Reverse: GCAGAGGGAGAGGAGGTATCTG | This paper | N/A |
| Primers for Asc2; Forward: ACTGTAACCTGCGCATCGCAC; Reverse: TTATCCGAGGAGGAGGAGG | This paper | N/A |
| Primers for Gps2; Forward: ACCAGCTTCCCGACTCTTCTTCT; Reverse: GAGGTTGGGGCTGAGCTTCTGT | This paper | N/A |
| Primers for Ncor1; Forward: TGGATGCTTGCTGCTGCTTACCT; Reverse: GGCTGCTTCTGCTGAGCAGT | This paper | N/A |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Primers for *Ncor2*; Forward: GCCCTTAGCTCTAGGTGGGG; Reverse: TTGTACAGAGCGTGTTGGGA | This paper | N/A |
| Primers for *Junb*; Forward: ATTCTGTGGGGAGGGGGA; Reverse: AGAGTGTGGAGGAGGTCC | This paper | N/A |
| ChIP primers for *Ccl2* E2 (S); Forward: GGCCACGGAAATGGGAAGGAGG; Reverse: GTTCAACAGAGGATGCT | This paper | N/A |
| ChIP primers for *Ccl2* E1(E); Forward: GTTCATTGCAAGCTCTTCTCTCTCTCT; Reverse: GCCATTGTGGGAAGGAGGCCA | This paper | N/A |
| ChIP primers for *Ccl2* C; Forward: GGCTTGAACTGTTTCTTAC; Reverse: TTTGTACCTCAAGATCGACATA | This paper | N/A |
| ChIP primers for *Ccl2* P; Forward: GTCCCTGTCTGCGTCTGGGG; Reverse: GGCTGTATGTGGGAGGAGGCCA | This paper | N/A |
| eRNA primers for *Ccl2* eRNA; Forward: AGCCACTGATTATACCCCAC; Reverse: GGGGAGCGTGTATATCTCAGG | This paper | N/A |
| Enhancer deletion oligonucleotides for gRNA 1; Forward: CACCCTTTATTTTCTCTTATTTGAATC; Reverse: AACATTCCAAAAAGGAGAAAATAAGC | This paper | N/A |
| Enhancer deletion oligonucleotides for gRNA 2; Forward: CACCCTTTTCTCTCAGTAGCATG; Reverse: AAACCATGTGGGAGGACTGACAGGCC | This paper | N/A |
| Enhancer deletion oligonucleotides for gRNA 3; Forward: CACCCTTAGACACATCATGAC; Reverse: AACACTCTGTACGTAGTGCTGAC | This paper | N/A |
| Enhancer deletion oligonucleotides for gRNA 4; Forward: CACCCTTAGACACATCATGAC; Reverse: AACACGTCTTTCTTGGTATATACC | This paper | N/A |
| Enhancer deletion oligonucleotides for gRNA 5; Forward: CACCCTTAGACACATCATGAC; Reverse: AACACGTCTTTCTTGGTATATACC | This paper | N/A |
| Enhancer deletion oligonucleotides for gRNA 6; Forward: CACCCTTAGACACATCATGAC; Reverse: AACACGTCTTTCTTGGTATATACC | This paper | N/A |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Enhancer deletion oligonucleotides for gRNA 7; Forward: CACCGCCTGGCCAGAGTAAGCAGC; Reverse: AAACAGTGCTTACTCGGAGGC | This paper | N/A |
| Enhancer deletion oligonucleotides for gRNA 8; Forward: CACCGCAGAGCGCTGGGTGGTCC; Reverse: AAAGGGAACACCGAGGGGCTCGTC | This paper | N/A |
| dCas9 oligonucleotides for KRAB-1-1+; Forward: AAACAGGACAGTGAGGAGCAG; Reverse: CACCTCCTCCTGTAACTGTCTC | This paper | N/A |
| dCas9 oligonucleotides for KRAB-1-1-; Forward: AAACGACTCCAAGTGAGTCTCCA; Reverse: CACCTTGAGACTCTTGAGTC | This paper | N/A |
| dCas9 oligonucleotides for KRAB-1-2+; Forward: AAACCAAGAGACCGGATCATTA; Reverse: CACCTAATGATCCGGTCTCTTG | This paper | N/A |
| dCas9 oligonucleotides for KRAB-1-2-; Forward: AAACGCACGAGTGTTGCGACAC; Reverse: CACCTGCTGTCGCAACACTCGTGC | This paper | N/A |
| dCas9 oligonucleotides for KRAB-2+; Forward: AAACGAAAGCCTTGCCCAATGTGT; Reverse: CACCACACTGGGAACGGCATCAT | This paper | N/A |
| dCas9 oligonucleotides for KRAB-2-; Forward: AAACACTGGGAGAACAGTTCATTT; Reverse: CACCAAATGAACTGTTCTCCGC | This paper | N/A |
| 4C-seq primers for Ccl2 enhancer bait (E); Forward: GATGAGGCCAAGCAGTAGATC; Reverse: CACCTAATGATCCGGTCTCT | This paper | N/A |
| 4C-seq primers for Ccl2 silencer bait (S); Forward: ACCAGACAGTAGTGATCGCAGCA; Reverse: CACCTGCTGTCGCAACACTCGTC | This paper | N/A |
| 4C-seq primers for Ccl2 promoter bait (P); Forward: GGTGAAACAGCTCGGATC; Reverse: CACCACACTGGGAACGGCATCAT | This paper | N/A |
| LNA oligonucleotides for scrambled control; Forward: AACACTGCTTTACCTA ACCATG | This paper | N/A |
| LNA oligonucleotides for Ccl2 eRNA; Forward: TTGGAATTGGAGAACA | This paper | N/A |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| shRNA oligonucleotides for GFP; Forward: CCGGGCAAGCTGACCCTGAAGTTTCACCTCGAGTGAACTTCA; Reverse: CTTTTTG; AATTCAAAAACAGTACCCTCTCTCTTTGCAGAACTCGAGTTCTGCA | This paper | N/A |
| shRNA oligonucleotides for GPS2; Forward: CCGGCTCTCGCTGGCCCTCTCATTATCTCGAGATAATTGAGGCCCAGCGAGGATTTTTG; Reverse: AATTCAAAAATCCTCGCCTGGCCCTCAATTATCTCGAGATAAATTGAGGGCCAGCGAGGA | This paper | N/A |
| shRNA oligonucleotides for SMRT; Forward: CCGGTCCTCGCTGGCCCTCAATTATCTCGAGATAATTGAGGGCCTCGAGTGAACTTCA; Reverse: CTTTTTG; AATTCAAAAACAGTACCCTCTCTCTTTGCAGAACTCGAGTTCTGCA | This paper | N/A |
| Recombinant DNA | | |
| PX458 | Addgene | Cat#: 48138 |
| PLKO.1-TRC | Addgene | Cat#: 10878 |
| psPAX2 | Addgene | Cat#: 12260 |
| pMD2.G | Addgene | Cat#: 12259 |
| hU6-sgRNA hUbC-dcas9-KRAB-T2a-Puro | Addgene | Cat#: 71236 |
| Software and algorithms | | |
| HOMER | http://homer.ucsd.edu/homer/ | RRID:SCR_010881 |
| Bowtie | http://bowtie-bio.sourceforge.net/index.shtml | RRID:SCR_005476 |
| Bioconductor | http://www.bioconductor.org/ | RRID:SCR_006442 |
| MACS2 | https://hbctraining.github.io/Intro-to-Chipseq/lessons/05_peak_calling_macs.html | RRID:SCR_013291 |
| GraphPad Prism; | https://www.graphpad.com/scientific-software/prism/ | RRID:SCR_002798 |
| edgeR | http://bioconductor.org/packages/release/bioc/html/edgeR.html | RRID:SCR_012802 |
| BWA | http://bio-bwa.sourceforge.net/ | RRID:SCR_010910 |
| UMass Medical School Flow Cytometry Core Lab, LSRII | https://www.scienceexchange.com/facilities/flow-cytometry-core-lab-umass | RRID:SCR_012630 |
| FlowJo | https://www.flowjo.com/solutions/flowjo | RRID:SCR_008520 |
| 4Cseqpipe | http://compgenomics.weizmann.ac.il/tanay/?page_id=367 | N/A |
| HiCExplorer/Galaxy | https://hicexplorer.usegalaxy.eu/ | RRID:SCR_006281 |
| Super-Enhancers (ROSE) algorithm | http://yonglab.wi.mit.edu/super_enhancer_code.html | RRID:SCR_017390 |
| DESeq2 | http://bioconductor.org/packages/release/bioc/html/DESeq2.html | RRID:SCR_015687 |
| RStudio | https://www.rstudio.com/ | RRID:SCR_000432 |
| Samtools | https://htslib.org/ | RRID:SCR_002105 |
| Deeptools | https://deeptools.readthedocs.io/en/develop/ | RRID:SCR_016366 |
| pipe4C-master | https://github.com/deLaatLab/pipe4C | N/A |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Eckardt Treuter (eckardt.treuter@ki.se).

Materials availability
Mice, antibodies, plasmids and oligonucleotides are listed in the key resources table.

Data and code availability
The published datasets GEO: GSM1631858, GSM1533888, GSM1631866, GSM665925, GSM918726, GSM2974676, GSM2974654 were re-analyzed in this study. RNA-seq, ATAC-seq, ChIP-seq, GRO-seq and 4C-seq data in this study have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GEO: GSE130383: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE130383

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals
ob/ob mice (JAX mice strain, stock no. JAX:000632) were obtained from Charles River Laboratories. All mice were maintained at the Center for Comparative Medicine at the Karolinska Institutet and the Karolinska University Hospital (PKL, Huddinge, Sweden), with a 12-h light/dark cycle and free access to diet and water. 6 weeks old male mice were used for the study.

Cell Culture Studies
HEK293 (ATCC, CRL-1573) cells and RAW264.7 (ATCC, TIB-71) were cultured in DMEM supplemented with 10% FBS and antibiotics at 37°C and 5% CO2.

METHOD DETAILS

Cell cultures and treatments
The mouse RAW264.7 macrophage cell line (ATCC stock no. TIB-71, hereafter referred to as RAW cells) was tested to be free of mycoplasma contamination. RAW cells were cultured in DMEM (GIBCO, 11995073) medium supplemented with 10% heat inactivated FBS (GIBCO, 26140079) and 1% P/S (GIBCO, 15140122). RAW cells were treated with 10 ng/ml LPS (Sigma, L4516) for RNA isolation (6 h) and 100 ng/ml for ChIP-qPCR or ChIP-seq (2 h). The CRISPR/Cas9-mediated GPS2 KO in RAW cells was described in our previous study (Fan et al., 2016). Additional treatments were applied using IL4 (20 ng/ml), TPA (5 nM), IFNγ (100 U/ml), IL6 (20 ng/ml), KLA (10 ng/ml), TNFα (20 ng/ml) for 6 h to induce the Ccl2 expression in GPS2 KO cells. RAW cells were transfected with Locked Nucleic Acid (LNA) GapmeRs as described previously (Roux et al., 2017) with minor modifications. LNA oligos were ordered from EXIQON with the following sequences: Scrambled control AACACGTCTATACGC, Ccl2 enhancer (E) eRNA TTGGAATTGGAGAACA. Briefly, 2 × 10^5 RAW cells were seeded one day before transfection in 24 well plates. The next day, RAW cells were changed with 100 μL antibiotic-free growth medium. 3 μL of 2 μM LNA and 5 μL FuGENE (Promega, E2312) were premixed in 100 μL serum- and antibiotic-free growth medium for 10 min. RAW cells were incubated with the transfection mix in 37°C incubator for 6-15 h (LNA final concentration was 30 nM). 400 μL full growth medium was added to the RAW cells and continued in culture for 24 h. The transfected cells were treated with 10 ng/ml LPS 2 h for RNA extraction. For ChIP-qPCR or ChIP-seq experiments, 2.5 × 10^6 RAW cells were seeded in 60 mm cell culture plate. Cells were transfected with 30 nM of LNA using FuGENE (Promega, E2312). The crosslink reaction was stopped with glycine at a final concentration of 0.125 M for 5 min. The harvested cells were used for ChIP-qPCR or ChIP-seq experiment (in next chapter). The Ccl2 LNA 4C-seq experiments were performed using 8 plates of 2.5 × 10^6 RAW cells (in total 2 × 10^7 cells for two biological replicates) with FuGENE transfection and followed with 4C-seq protocols (see details in the 4C-seq chapter).

ChIP-qPCR and ChIP-seq
ChIP (Chromatin Immunoprecipitation)-based experiments were performed as described previously (Fan et al., 2016; Huang et al., 2019b); H3K27ac ChIP-seq were performed using WT and enhancer KO RAW cells (ΔE1.1, ΔE1.2/ΔE and ΔE2/ΔS) without LPS treatment. H3K27ac ChIP-seq were performed in RAW cells using 1 h 100 ng/ml LPS treatment to check the enhancer activation of Ccl2. For TF and cofactor ChIP-seq, WT cells, enhancer KO cells (ΔE1.2/ΔE and ΔE2/ΔS), and promoter KO cells were treated for 2 h with/ without LPS (100 ng/ml). GFP, GPS2 and SMRT stable knockdown RAW cells were used for H3K27ac ChIP-seq without LPS treatment. CBP, Pol II and H3K27ac ChIP-qPCR and ChIP-seq were performed in Ccl2 eRNA LNA knockdown cells upon 2 h LPS treatment. Briefly, RAW cells crosslinked with 1% formaldehyde for 10 min for histone modification (H3K27ac) or double crosslinked with 2 mM disuccinimidyl glutarate (DSG) (VWR, A7822.0001) for 30 min, followed by 1% formaldehyde for 10 min. The crosslink reaction...
was stopped with glycine at a final concentration of 0.125 M for 5 min. The lysed RAW cell nuclei were sonicated for 30 min (30 s ON/30 s OFF) in Bioruptor Pico (Diagenode, B01060010). Protein A Dynabeads (Invitrogen, 100202D) were incubated with antibodies (specified in KEY RESOURCES TABLE). 2–4 μg antibody was incubated with 100 μg sonicated chromatin DNA sample at 4 °C overnight. The next day, beads were washed with wash buffer at least 6 times and followed by reverse-crosslinking at 65 °C overnight. The ChiP-ed DNA was purified using Clean & Concentrator Capped Zymo-Spin I (Zymo Research, D4013) kit. For library preparation and sequencing, 1–10 ng of ChiP-ed DNA was processed at the EMBL Genomics Core Facility (Heidelberg, Germany) and BEA Core Facility (Karolinska Institutet, Sweden) using standard protocols, and sequencing was performed in the Illumina HiSeq 2000 (Illumina, 505E reads) and NextSeq 550 (Illumina, 75E reads). ChiP-qPCR was performed with SYBR Green (KAPA Biosystems, 07959567001) to validate the ChiP-seq results. Primers for the ChiP-qPCR are listed in the KEY RESOURCES TABLE.

**GeLP-mediated eRNA silencing in adipose tissue macrophages of ob/ob mice**

Glucon-encapsulated LNA particle (GeLP)-mediated eRNA knockdown was performed in mice following previous protocols (Aouadi et al., 2013; Barreby et al., 2019) with modifications. Briefly, 3 nmol scrambled control LNA (AACACGTCTATACGC) or the LNA targeting the Ccl2 E eRNA (TTGGAATTGGAGAACA) were mixed with 50 nmol Endo-porter (GeneTools) and 30 mM acetate buffer for 15 min at room temperature. 1 mg gluanucitons were added to the mix buffer and incubated for 1 h at room temperature, in dark and without movement. PBS was added to the final volume (200 μl) of delivery dose for one mouse. The final mixed GeLP buffer was sonicated before i.p. injection. Ob/ob mice were divided into two groups (n = 7–8) and injected with control versus Ccl2 enhancer eRNA-specific GeLPs once every two days for ten days. The random blood glucose (RBG) was tested every day during GeLP injection, and glucose intolerance (OGTT) was tested after then days. After ten days of treatment, mice were sacrificed and adipose tissue macrophages were isolated by FACS. RT-qPCR was performed to determine Ccl2 and Ccl7 mRNAs expression, and Ccl2 E eRNA expression. All mouse procedures were performed in accordance with guidelines approved by the Swedish Ethical Committee (Jordbruksverket) Stockholm.

**Lentivirus-shRNA-mediated gene knockdown in RAW cells**

The lentivirus-shRNA-mediated knockdown system was described previously (Fan et al., 2016). Lentivirus-shRNAs were constructed for GFP (targeted sequence: GCAAGCTGACCCTGAAGTTCA), GPS2 (targeted sequence: ACCAGCTTCTCGGACTCATCTTCT), and SMRT (targeted sequence: CCAGTGTAAGAACTTCTACTT) and packaged in HEK293FT cells using PLKO.1-TRC (Addgene, 10878), for GFP (targeted sequence: GCAAGCTGACCCTGAAGTTCA), GPS2 (targeted sequence: ACCAGCTTCTCGGACTCATCTTCT), and SMRT (targeted sequence: CCAGTGTAAGAACTTCTACTT) and packaged in HEK293FT cells using PLKO.1-TRC (Addgene, 10878), for GFP (targeted sequence: GCAAGCTGACCCTGAAGTTCA), GPS2 (targeted sequence: ACCAGCTTCTCGGACTCATCTTCT), and SMRT (targeted sequence: CCAGTGTAAGAACTTCTACTT) and packaged in HEK293FT cells using PLKO.1-TRC (Addgene, 10878). 5 MOI viruses were used to infect RAW cells. Stable RAW cells were selected after 48 h virus transduction. Lentivirus-infected cells were selected in full culture medium supplemented with 5 μg/ml puromycin for 7 days. The stable knockdown cells were validated by RT-qPCR before further experiments. Stable GPS2 and SMRT knockdown cells were used for 4C-seq experiments.

**Deletion of Ccl2 enhancer, silencer and promoter using CRISPR/Cas9**

gRNAs for Ccl2 enhancer and promoter deletions were designed using the online gRNA design tool DNA Design 2.0 based on GPS2 ChiP-seq data to target the GPS2 peak center chr11: 81,818,327-81,818,802 (ΔE1.1); chr11: 81,820,266-81,820,810 (ΔE1.2/ΔE); chr11: 81,836,105-81,836,583 (ΔE2/ΔS) and chr11: 81,848,430-81,848,946 (ΔP). The following sequences of sgRNAs (sgRNA1: CTTATTTCCTCCTTTGGAAT, sgRNA2: CCCCTTCACTCCCTACATG, sgRNA3: TCAGCCACAGACTGACGAG, sgRNA4: GTATT AACCAAGAGAGAC, sgRNA5: GAGGCTTCAGAGCTTCTCTAG, sgRNA6: TGCCATGGCAGGTTGCTCG, sgRNA7: CCGGCCCCAGAGTACGACT, sgRNA8: CAGAGGCCCCTGGGTTTTCC) were inserted into PX458 vector (Addgene, 48138). The transfected GFP-expressing cells were sorted by FACS and followed by serial dilution. The single-cell colonies were validated by sequencing upon TA cloning. PX458 empty vector- transfected and sorted single cell clones were used as negative controls. DNA adjacent to the deleted regions was amplified and sequenced for verification. The deletions were located as follows (mm9): ΔE1.1: chr11:81,818,344-81,818,803, 460bp; ΔE1.2/E: chr11:81,820,237-81,820,801, 565 bp; and ΔE2/S: chr11:81,836,057-81,836,677, 621bp. TF motif enrichment analysis for the E1.2/E and E2/S core regions was performed with ‘findMotifs.pl’ by HOMER using default parameters.

**RNA isolation and RT-qPCR**

Total RNA from cells was isolated using E.Z.N.A Kit (Omega, R6834-02) followed by DNase (Invitrogen, EN0521) treatment according to the manufacturer’s instruction. 500 ng of total RNA was used for cDNA synthesis using Superscript III Reverse Transcriptase (Invitrogen, 18080093) with random hexamers. Quantitative transcript analysis was performed using SYBR green (KAPA Biosystems, 07959567001) and run on the Applied Biosystems 7500 Real-time PCR system. Values were normalized using GAPDH as internal control. Primers for the RT–qPCR are listed in the KEY RESOURCES TABLE.

**GRO-seq**

Global run-on sequencing (GRO-seq) was performed using cell nuclei as previously described (Kaikkonen et al., 2013). Briefly, 15 cm plates of both WT and GPS2 KO or shGFP and shGPS2 RAW cells were treated with vehicle or 100 ng/ml LPS for 1 h and cell nuclei were extracted. The nuclear run-on reaction was performed for 7 min and RNA hydrolysis was allowed to proceed for 10 min. RNA immunoprecipitation was performed by anti-BrdU-conjugated agarose beads (Santa Cruz Biotechnologies). Nascent RNA was
eluted from beads using 400 μl of elution buffer. All GRO-seq library preparations were performed in parallel to avoid batch effects.

ATAC-seq
ATAC-seq (Assay for Transposase-Accessible Chromatin using sequencing) was performed using cell nuclei as previously described (Buenrostro et al., 2015). Briefly, WT and GPS2 KO RAW264.7 cells were seeded with triplicates in the 12 wells plates with 2 x 10^5 cell number one day before the experiments. Cells were harvested (basal condition), washed with PBS and then resuspended in lysis buffer. Cell nuclei were spun down and transferred into the transposition reaction buffer and incubated at 37 °C for 30 min. Genomic DNA was extracted using QIAGEN MinElute PCR Purification Kit. ATAC-seq libraries were prepared and sequenced on NextSeq 550 (Illumina, 75 SE reads) in the BEA core facility (Karolinska Institutet, Huddinge, Sweden).

dCas9–KRAB repression of E eRNA in RAW cells (CRISPR)
The dCas9–KRAB repression plasmid pLV hu6-sgRNA hUbC-dCas9-KRAB-T2a-Puro was a gift from Charles Gersbach (Thakore et al., 2015) (Addgene, 71236). gRNAs were selected based on the two enhancer regions of Cc2 and cloned into the lentiviral plasmids. The gRNA-containing dCas9–KRAB lentiviruses were packaged in HEK293FT cells and RAW cells were infected and selected. The gRNA target sequences are listed in the Key Resources Table. Stable dCas9–KRAB-gRNA-expressing RAW cells were selected by 5 μg/ml puromycin for 7 days and then used for determining Cc2 E eRNA and mRNA expression upon 2 h LPS treatment.

Flow cytometry
Visceral adipose tissues from the ob/ob mice were collected and washed in 10 ml DPBS with 0.5% BSA (Sigma Aldrich). Tissues were then cut into small pieces and digested in 3 ml DPBS/BSA supplemented with 4 mg/ml collagenase II (ThermoFisher, 17101015) in 37% shaker (200 rpm) for 30 min. Samples were then filtered using a 100 μm cell strainer (Sigma Aldrich) and washed three times with DPBS/BSA. 1x10^6 purified cells from each mouse were resuspended in FACS buffer (ThermoFisher, 17101015) in 37% shaker (200 RPM) for 30 min. Samples were then transferred to 50 mL Falcon tubes with 700 μl of elution buffer. All GRO-seq library preparations were performed in parallel to avoid batch effects.

4C-seq
Ccl2 enhancer and promoter loops were characterized using chromosome conformation capture (3C) and high-throughput sequencing (4C-seq) as described previously (van de Werken et al., 2012) with slight modifications. In brief, RAW cells were cross-linked with 2% formaldehyde for 10 min and the reaction was stopped with 0.125 M glycine. Cells were counted and 10^7 cells were distributed per tube. WT or LNA control cells, CRISPR KO cells and Cc2 E1/E eRNA knockdown cells were re-suspended with 10 mL lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1% TX-100, for 10 min. Nuclei were re-suspended into 440 μL Milli-Q water and 60 μL of digestion buffer (15 μL 10% SDS and 75 μL 20% Triton X-100) was added. The cell lysis mixture was then incubated at 37 °C for 2 h in the shaker (900 RPM). 200U DpnII (NEB, R0543M) was added into the mixture three times. The lysis mixture was then incubated in the shaker overnight. The reaction mix was inactivated after overnight digestion by incubating at 65 °C for 20 min. The samples were then transferred to 50 mL Falcon tubes with 700 μL ligation buffer and 5.5 mL Milli-Q water. The samples were then supplemented with 100 U T4 ligase (NEB, M0202M) and incubated overnight at room temperature. After that, samples were de-crosslinked at 65 °C for 12 h. The ligated DNA was then purified by phenol-chloroform method, and further digested with Nialll (NEB, R0125L) at 37 °C overnight. The restriction enzyme was then inactivated for 20 min at 65 °C. The DNA was further ligated at room temperature overnight. After the second ligation, the DNA was purified by QiAquick PCR purification kit (QIAGEN, 28104). The 4C library was amplified using Cc2 4C bait primers (provided in the Key Resources Table) by expand long template PCR system (Roche, 117590600001). The PCR products were purified by QiAquick PCR purification kit. The DNA concentration was measured by Qubit Fluorometric Quantification kit (ThermoFisher, Q33238). 10 ng of DNA was used for the high-throughput sequencing library preparation. The library was prepared by SMARTer PicoPLEX library preparation kits (Takara, R400676) and SMARTer DNA unique dual index kits (Takara, R400661). The purified DNA library mix was sequenced on NextSeq 550 (Illumina, 75 SE reads) in BEA Core Facility (Karolinska Institutet, Huddinge, Sweden).

QUANTIFICATION AND STATISTICAL ANALYSIS
All replicate experiments were performed at least two times. ChIP-seq samples which had one biological replicate were confirmed by ChIP-qPCR at the specific target gene locus. D’Agostino and Pearson normality test were used to determine the normal distribution. All statistical tests were performed using GraphPad Prism 6.0b (GraphPad Software, Inc., La Jolla, CA), and all data were represented as mean ± s.e.m. Statistical tests were assessed after confirming that the data met appropriate assumptions (normality, homogeneous variance, and independent sampling). Gaussian distribution was tested using the Kolmogorov–Smirnov test. Group comparisons were assessed with Student’s t test to compare two groups, and one-way ANOVA test followed by Tukey’s or Dunnett’s post hoc test for multiple comparisons as appropriate. All statistical tests were two-tailed, and p < 0.05 was defined as significant. No statistical methods were used to predetermine sample size. No samples or animals were excluded from the analyses.
RNA-seq
We further analyzed our own published GPS2 KO RNA-seq data from RAW cells (Huang et al., 2019b). Data were aligned to the mouse mm9 genome by using HISAT2. Read counts were imported by HOMER and analyzed using analyzeRepeats with the option rna and parameters -roadj -condenseGenes and -count exons for four replicates per condition. Differential gene expression analysis was performed with DESeq2 using HOMER’s getDiffExpression.pl using default parameter. Transcripts with an adjusted p value < 0.05 were considered as differentially expressed genes. The tag counts were plotted by -rpkm for GPS2 KO significantly changed genes in both basal and LPS conditions.

ChIP-seq
Public ChIP-seq data were obtained from the Gene Expression Omnibus (GEO) database (H3K27ac: GSM1631858, PU.1: GSM1533888, CTCF: GSM918726, SMRT: GSM665925, GPS2: GSM1631865, ATAC-seq: GSM2974654) and aligned to the NCBI37/mm9 version of the mouse reference genome. Sequencing files (FASTQ files) were aligned to the NCBI37/mm9 version of the mouse reference genome using Bowtie2 on the Uppsala Multidisciplinary Centre for Advanced Computational Science (UP-PMAX) under project SNIC2018/8-122. Sequencing tags were read and imported to the HOMER (Hypergeometric Optimization of Motif EnRichment, http://homer.ucsd.edu/homer) package (Heinz et al., 2010). Peaks were identified using HOMER with default settings, and peak overlap was calculated by merging together all individual peak files for every experiment. The TF motif search was done in Homer by default settings. The statistical comparison of differential peak tag counts was performed using DEseq2 packages in R. Peaks with the adjusted p value < 0.05 were considered as differentially changed peaks. GPS2, MED1, CBP super-enhancer calling was done using the Rank Ordering of Super-Enhancers (ROSE) algorithm (Lovén et al., 2013). GPS2 ChIP-seq data in SMRT knockdown cells were further plotted using DeepTools (Ramírez et al., 2016).

GRO-seq
GRO-seq analysis of genome-wide gene expression was performed with HOMER (Heinz et al., 2010) followed by edgeR. Briefly, the sequencing reads were first trimmed to remove the low-quality reads, adapters and the poly-A sequences. The remaining reads were then aligned to mouse mm9 genome using bwa. For the genome browser visualization, bedGraph files were generated separately for plus and minus DNA strands. The minus strands were presented as negative values in the genome browser. Both gene body and intergenic transcription levels were quantified using HOMER analyzeRepeats.pl in each file. The comparison of differential tag counts in the gene bodies and intergenic regions, and the statistical analysis for differential expression, was performed using the edgeR (Robinson et al., 2010) package in R. Transcripts with an adjusted p value < 0.05 were considered as differentially expressed.

ATAC-seq
The paired-end FASTQ data was aligned to mice mm9 genome using Bowtie2. The ATAC-seq peak calling was done using MACS2 and ENCODE ATAC-seq pipeline (Feng et al., 2012). The statistical analysis for differential expression was further performed using the edgeR. Peaks changes with an adjusted p value < 0.05 were considered as differentially peaks.

4C-seq
The paired-end FASTQ data analysis was performed by the standard 4Cseqpipe protocol (van de Werken et al., 2012). The primer sequences were trimmed from the raw reads. The trimmed reads were mapped to the reference fragmented genome. The contact intensity was normalized by the fragment counts. The cis-interacting DNA contact profiles around the baits were plotted in the same chromosome window. Results from different groups were normalized to the same read counts to compare the significance. Further downstream analysis was performed to obtain the detailed tag counts using Pipe4C-master (Krijger et al., 2020). The FASTQ data were trimmed by bait primers and aligned to the mouse mm9 genome. Aligned bam files were then converted to .wig files by using 10^6 tags as normalize factor. Tag counts were extracted from wig files by using E1/E (chr1:81816382-81822382), E2/S (chr11:81834000-81839000), and promoter (chr11:81846000-81852000) coordinates, respectively, and then visualized in GraphPad software.

Hi-C
The public BMDM Hi-C data GSM2974676 (Link et al., 2018) was obtained from GEO database. The paired-end FASTQ data was mapped on Galaxy/HiCEXplorer using BWA-MEM tool. hicBuildMatrix tool was used to build the Hi-C contacts. The corrected Hi-C matrix was plotted using 50 kb and 5 kb contact matrix. The Hi-C contacts were integrated with the ChIP-seq data using the hicPlotTAD tool.

Flow cytometry
Samples were analyzed in a LSRII cytometer (Becton Dickinson) using FACS Diva (BD Biosciences) and FlowJo (Tree Star) softwares.