Genetic Models Reveal cis and trans Immune-Regulatory Activities for lincRNA-Cox2

Roland Elling

University of Massachusetts Medical School

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Genetic Models Reveal *cis* and *trans* Immune-Regulatory Activities for lincRNA-Cox2

**Highlights**

- Study of lincRNA-Cox2 *in vivo* using recently generated KO and splicing mutant mice

- lincRNA-Cox2 functions through an enhancer RNA mechanism to regulate *Ptgs2* levels

- lincRNA-Cox2 has a *trans* regulatory role controlling many innate immune genes

- The lincRNA locus simultaneously regulates the expression of local and distant genes

**Authors**

Roland Elling, Elektra K. Robinson, Barbara Shapleigh, ..., John L. Rinn, Katherine A. Fitzgerald, Susan Carpenter

**Correspondence**

sucarpen@ucsc.edu

**In Brief**

Elling et al. utilize a number of lincRNA-Cox2 genetic models to show that lincRNA-Cox2 can regulate its neighboring gene *Ptgs2* (Cox2) through an enhancer RNA mechanism. They generate a lincRNA-Cox2 splicing-deficient mouse and confirm that lincRNA-Cox2 functions in *trans* to regulate immune genes following LPS-induced endotoxic shock.

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Genetic Models Reveal \textit{cis} and \textit{trans} Immune-Regulatory Activities for lincRNA-Cox2

Roland Elling,1,2,10 Elektra K. Robinson,3,10 Barbara Shapleigh,3 Stephen C. Liapis,4 Sergio Covarrubias,3 Sol Katzman,5 Abigail F. Groff,4 Zhaozhao Jiang,1 Shiuli Agarwal,1 Mona Motwani,1 Jennie Chan,1 Shruti Sharma,1 Elizabeth J. Hennessy,1 Garret A. FitzGerald,2 Michael T. McManus,7,8 John L. Rinn,4,9 Katherine A. Fitzgerald,1,11 and Susan Carpenter3,11,12,*

1Program in Innate Immunity, Department of Medicine, University of Massachusetts Medical School, Worcester, MA, USA
2Center for Pediatrics, Department of General Pediatrics, University of Freiburg, Freiburg, Germany
3Department of Molecular, Cell and Developmental Biology, University of California, Santa Cruz, Santa Cruz, CA, USA
4Harvard Stem Cell and Regenerative Biology Department, Harvard University, Cambridge, MA 02138, USA
5Center for Biomolecular Science and Engineering, University of California, Santa Cruz, Santa Cruz, CA, USA
6Department of Systems Pharmacology and Translational Therapeutics, Perelman School of Medicine, University of Pennsylvania, 3400 Civic Center Boulevard, Smonow, Philadelphia, PA 19104, USA
7Department of Microbiology and Immunology, University of California, San Francisco, San Francisco, CA, USA
8UCSF Diabetes Center, University of California, San Francisco, San Francisco, CA, USA
9Department of Biochemistry, BioFrontiers, University of Colorado Boulder, Boulder, CO 80301, USA
10These authors contributed equally
11Senior author
12Lead Contact
*Correspondence: sucarpen@ucsc.edu
https://doi.org/10.1016/j.celrep.2018.10.027

SUMMARY

An inducible gene expression program is a hallmark of the host inflammatory response. Recently, long intergenic non-coding RNAs (lincRNAs) have been shown to regulate the magnitude, duration, and resolution of these responses. Among these is lincRNA-Cox2, a dynamically regulated gene that broadly controls immune gene expression. To evaluate the \textit{in vivo} functions of this lincRNA, we characterized multiple models of lincRNA-Cox2-deficient mice. LincRNA-Cox2-deficient macrophages and murine tissues had altered expression of inflammatory genes. Transcriptomic studies from various tissues revealed that deletion of the lincRNA-Cox2 locus also strongly impaired the basal and inducible expression of the neighboring gene prostaglandin-endoperoxide synthase (Ptgs2), encoding cyclooxygenase-2, a key enzyme in the prostaglandin biosynthesis pathway. By utilizing different genetic manipulations \textit{in vitro} and \textit{in vivo}, we found that lincRNA-Cox2 functions through an enhancer RNA mechanism to regulate Ptgs2. More importantly, lincRNA-Cox2 also functions in \textit{trans}, independently of Ptgs2, to regulate critical innate immune genes \textit{in vivo}.

INTRODUCTION

Activation of myeloid cells is associated with differential expression of immune genes involved in host defense, tissue repair, and resolution of inflammation. Toll-like receptors (Tlrs) are germline-encoded receptors critical for the activation of signaling pathways controlling immune response genes. Dysregulation of these pathways can lead to deleterious autoimmune conditions, which can contribute to autoimmunity or cancer (Gajewski et al., 2013; Gierut et al., 2010; Masters et al., 2009). Tight control of these inflammatory signaling cascades is required to prevent host damage and is achieved both transcriptionally and posttranscriptionally.

The majority of mammalian genomes are pervasively transcribed, producing thousands of noncoding RNAs (ENCODE Project Consortium, 2012). Interestingly, the expression of these noncoding genes is highly cell type-specific (Morris and Mattick, 2014), and their function remains largely unknown. Long intergenic noncoding RNAs (lincRNAs) are a subclass of long noncoding RNAs (lncRNAs) that form the largest class of RNA produced in the genome. The tremendous number of newly annotated lincRNAs and their low evolutionary conservation has led to debates about their functionality (Bassett et al., 2014). However, the number of characterized lincRNAs is growing, and this class of gene has been shown to control various biological processes, including somatic tissue differentiation (Kretz et al., 2013), X chromosome inactivation (Carmona et al., 2018; Engreitz et al., 2013; Jégu et al., 2017), and organ development (Anderson et al., 2016).

LincRNAs function to control gene expression either in \textit{cis}, where they influence the expression and/or chromatin state of neighboring genes, or in \textit{trans}, where the lincRNA leaves the site of transcription and affects genes on different chromosomes (Kopp and Mendell, 2018; Liang et al., 2018; Neumann et al., 2018). These \textit{trans}-acting lincRNAs, such as FOXF1 adjacent non-coding developmental regulatory RNA (FENDRR), long-intergenic non-coding-erythroid prosurvival (linc-EPS), and Nettoie Salmonella pas Theiler’s (NeST), can function to regulate
chromatin states (Atianand et al., 2016; Gomez et al., 2013; Grote et al., 2013; Sauvageau et al., 2013), influence nuclear structure and organization (Rinn and Guttman, 2014), or interact with and regulate the behavior of proteins and/or other RNA molecules (Covarrubias et al., 2017; Kawasaki et al., 2018; Lee et al., 2016; Wang et al., 2017).

lincRNAs have recently been shown to regulate the development and function of immune cells (Kotzin et al., 2016; Wang et al., 2014, 2017). Previous work from our lab and others defined an immune-inducible lincRNA, lincRNA-Cox2 (synonym Ptgs2os2) with broad trans-regulatory activity on inflammatory responses (Carpenter et al., 2013; Covarrubias et al., 2017; Hu et al., 2016; Tong et al., 2016; Xue et al., 2018). In macrophages, where this lincRNA was highly induced upon inflammatory activation, lincRNA-Cox2 functioned to activate and repress distinct classes of innate immune genes (Carpenter et al., 2013). However, like most lincRNAs and protein-coding genes, the cis- and trans-regulatory elements encoded within the locus remain unstudied in vivo. Most recently, some lincRNAs have been discovered to have the ability to function both in cis and in trans (Carmona et al., 2018; Li et al., 2012; Yin et al., 2015).

Here we created a combination of different genetic deletion models to study the role of the lincRNA-Cox2 locus in macrophages and in murine models in vivo. Consistent with prior work, we found that lincRNA-Cox2-deficient macrophages had altered expression of numerous inflammatory genes (Carpenter et al., 2013; Covarrubias et al., 2017). In addition, we observed a profound cis function for lincRNA-Cox2: lincRNA-Cox2-deficient mice had severely reduced expression of the neighboring gene Ptgs2, a gene that encodes for cyclooxygenase-2 (Cox2), a central enzyme of the prostaglandin biosynthesis pathway, across several tissues. This finding provides evidence for a previously unrecognized cis function for lincRNA-Cox2. We have data that support lincRNA-Cox2 functioning through an RNA-mediated mechanism as an enhancer RNA (eRNA) to regulate Ptgs2 expression. Crossing our lincRNA-Cox2-deficient mice to wild-derived mice (MOLF) that have a distinct genetic background provides critical evidence that lincRNA-Cox2 indeed functions on the same chromosome to regulate Ptgs2 expression levels.

Finally, to distinguish the cis-regulatory element from the trans activity of the RNA transcript, we generated a “mutant” mouse model that retained the exonic sequences of the lincRNA but lacked the intron and splicing capabilities. This resulted in a mouse with a low basal expression level of lincRNA-Cox2, but the transcript is no longer inducible following inflammatory stimulation. In the mutant mouse, Ptgs2 expression is comparable with wild-type mice as the eRNA activity of the lincRNA-Cox2 locus is maintained. Using a conventional in vivo lipopolysaccharide (LPS) shock model, we identify an additional role for the lincRNA-Cox2 transcript in the trans regulation of a subset of immune genes as well as an organ-specific role independent of Ptgs2 biology. Collectively, these observations reveal a bimodal action of gene regulation by the lincRNA-Cox2 transcript: a trans-regulatory function controlling immune genes such as cytokines globally and a separate enhancer function acting to regulate prostaglandin biosynthesis via Ptgs2 (Cox2). Thus, lincRNA-Cox2 represents a regulator of the Ptgs2 pathway as well as an important mediator of immunity beyond the prostaglandin pathway.

RESULTS

Genetic Deletion of lincRNA-Cox2 Alters Immune Gene Expression in Macrophages

LincRNA-Cox2 is encoded on chromosome 1 and transcribed from the negative strand. The mature sequence has 2 exons and is 1.7 kb long. Its nearest protein coding gene, prostaglandin-endoperoxide synthase 2 (Ptgs2 or Cox2), is ~50 kb upstream and transcribed on the positive strand (Figure 1A). A lincRNA-Cox2 knockout (KO) mouse was generated by removing the entire genomic locus (5.9 kb), except for the promoter, and replacing it with a LacZ reporter cassette (Sauvageau et al., 2013; Figure 1B). KO mice were born at expected Mendelian frequencies with no obvious developmental abnormalities (Sauvageau et al., 2013). We and others have previously published that lincRNA-Cox2 acts to positively and negatively regulate the expression of distinct classes of innate immune genes (Carpenter et al., 2013; Covarrubias et al., 2017; Hu et al., 2016; Tong et al., 2016; Xue et al., 2018). In those studies, short hairpin RNA (shRNA)-based knockdown of lincRNA-Cox2 reduced the levels of pro-inflammatory cytokines like Interleukin-6 (Il6) in bone-marrow derived macrophages (BMDMs) activated with LPS, whereas a number of interferon-stimulated genes (ISGs) were expressed at higher levels. Here, using genetic approaches, we find that lincRNA-Cox2-deficient BMDMs (Figure 1C) produced less Il6 (Figure 1D) and more Chi3L as well as Ifi202b, an ISG, following LPS (Tlr4) and R848 (Tlr7) stimulation (Figures 1E and 1F).

Ptgs2 Levels Are Reduced in lincRNA-Cox2 KO Mice

The lincRNA-Cox2 KO mouse was generated so that the locus remains transcriptionally active and LacZ staining can be used as a surrogate for lincRNA-Cox2 expression (Sauvageau et al., 2013). Staining of several organs from these mice revealed the in vivo expression of lincRNA-Cox2 in both the brain (dorsal cerebral cortex) and the lung under steady-state conditions (Figure 2A). Further, RNA sequencing (RNA-seq) from a variety of organs confirmed that lincRNA-Cox2 was most highly expressed in the lung at steady state (Figure S1A). To identify the target genes of lincRNA-Cox2 at steady state, we performed RNA-seq on whole lung tissue, comparing wild-type and lincRNA-Cox2-deficient mice. Using differential expression sequencing 2 (DESeq2) with a cut off of 2.5-fold and a p value of 0.05, 476 genes showed altered expression (273 were upregulated and 203 were downregulated) (Table S1). Among the most significantly downregulated genes was Ptgs2 (Figure 2B, highlighted in red).

Next we wanted to investigate the lincRNA-Cox2 locus at higher resolution and across different tissues to understand whether this effect was specific to Ptgs2 or whether the genetic manipulation of the locus altered other neighboring genes. To that end, we compared the transcriptomes from WT and KO animal brains and lungs and generated cis region plots spanning a 1-Mb window in the vicinity of the lincRNA-Cox2 locus. In both the lung and brain, which have high expression of lincRNA-Cox2...
represented by LacZ staining in organs from KO mice (Figure 2A), the only gene affected in this region at steady state was Ptgs2 (Figures S2A and S2B). To determine whether the cis effect on Ptgs2 persisted after an inflammatory stimulus in vivo, a context in which the lincRNA transcript is highly induced, we measured the expression of Ptgs2 in the lung, spleen, and liver following intraperitoneal (i.p.) injection of E. coli LPS (20 mg/kg) for 6 hr. In WT mice, lincRNA-Cox2 and Ptgs2 were inducible in the spleen and liver (Figures 2C–2G) following LPS treatment. Ptgs2 levels were reduced in all tissues examined from lincRNA-Cox2 KO mice following LPS challenge (Figures 2D–2H). LacZ expression remained comparable with lincRNA-Cox2 expression in the tissues from KO mice following LPS treatment, confirming that the locus is actively transcribed (Figures S2C and S2D).

We also examined Ptgs2 levels in BMDMs from the lincRNA-Cox2 KO mice following stimulation with various Tlr ligands. In each case, the inducible transcriptional expression of Ptgs2 was greatly reduced (Figure 2I). We confirmed this effect at the protein level in lincRNA-Cox2-deficient BMDMs by immunoblotting for Ptgs2 (Figure 2J). Ptgs2 is the central enzyme of the prostaglandin pathway catalyzing the conversion of arachidonic acid to prostaglandins (Ricciotti and FitzGerald, 2011). Consistent with the reduced expression of Ptgs2 RNA and protein, there was reduced pro-inflammatory prostaglandin E2 (PGE2) production in lincRNA-Cox2-deficient cells as measured by mass spectrometry (Figure 2K). Together, all of these data indicate that the lincRNA-Cox2 locus controls the expression of Ptgs2 and, therefore, prostaglandin biosynthesis.

**Rescue of Mature lincRNA-Cox2 Fails to Restore Inducible Ptgs2 Expression in Macrophages**

LincRNA-Cox2 and Ptgs2 show parallel inducible expression kinetics following Tlr activation. In prior work, we showed that knocking down the lincRNA transcript using shRNA had no effect on the expression of Ptgs2 (Carpenter et al., 2013). This observation contrasts with the strong cis effect of the lincRNA locus observed in our KO model and could be consistent with a DNA-mediated enhancer effect, a mechanism that has previously been reported for other lincRNA loci (Engreitz et al., 2016; Groff et al., 2016; Joung et al., 2017; Paraikar et al., 2016). To gain more insight into the molecular basis for this cis
effect, we reconstituted lincRNA-Cox2-deficient primary BMDMs with the full-length spliced mature RNA transcript (Figure 3A) via plasmid electroporation. Despite restoration of lincRNA-Cox2 to a level of expression comparable with that seen in wild-type (WT) cells, Ptgs2 expression could not be rescued (Figure 3B). To control for possible effects mediated by knockin of the LacZ transgene, we also generated a BMDM line lacking lincRNA-Cox2 using CRISPR/Cas9 to validate these findings. We designed guide RNAs to the 5’ and 3’ ends of the lincRNA-Cox2 gene as outlined in Figure 3C, using two guide RNAs that remove the entire locus encoding the gene (while leaving the promoter intact). KO of lincRNA-Cox2 was confirmed by qRT-PCR (Figure 3D). Ptgs2 levels were also impaired in these Cas9-edited cells (Figure 3E). Again, reconstitution

Figure 2. lincRNA-Cox2 Expression in the Lung and Effects on Ptgs2 in cis
(A) Brain, lung, and liver were stained for LacZ expression.
(B) RNA sequencing was performed on lung tissue, comparing WT and KO samples. The volcano plot represents the top upregulated and downregulated genes, comparing KO-WT using DESeq (cutoff of 1.5 log2 fold change in expression with p > 0.05). Ptgs2 is labeled in red.
(C–H) WT and lincRNA-Cox2 KO mice were injected with LPS (20 mg/kg) for 6 hr, and spleens and lungs were extracted. The expression levels of lincRNA-Cox2 (C, E, and G) and Ptgs2 (D, F, and H) were tested by qRT-PCR and normalized to Gapdh. Each dot represents an individual animal. Error bars represent standard deviation of biological triplicates. Asterisks indicate statistically significant differences between mouse lines (Student’s t test with ***p ≤ 0.05). Student’s t tests were performed using GraphPad Prism to obtain p values.
(I) BMDMs were stimulated with Tlr ligands for 6 hr, and Ptgs2 levels were measured by qPCR and normalized against Gapdh.
(J) BMDMs were stimulated with LPS for the indicted times, and Ptgs2 levels were measured by western blot and quantified below.
(K) Phosphodiesterase (PDE) levels in WT and KO BMDMs was measured by mass spectrometry. The heatmap was generated by Morpheus (Broad Institute).
Figure 3. lincRNA-Cox2 Acts as an eRNA to Control Ptgs2
(A) Primary BMDMs from the lincRNA-Cox2 KO mice were reconstituted by plasmid electroporation with full-length lincRNA-Cox2. Expression of lincRNA-Cox2 was confirmed by qRT-PCR.
(B) Ptgs2 levels were determined by qRT-PCR in the lincRNA-Cox2-reconstituted cells. Data represent 2 combined biological replicates representative of 3 individual experiments.
(C) Schematic of the BMDM cell line using CRISPR/Cas9 to remove the lincRNA-Cox2 locus.
(D and E) Expression of lincRNA-Cox2 (D) and Ptgs2 (E) was determined by qRT-PCR in the lincRNA-Cox2 Cas9 KO BMDMs.

(legend continued on next page)
with the full-length lincRNA-Cox2 transcript by lentiviral expression, which localized to both the nuclear and cytoplasmic compartments (Figures S3A and S3B), failed to rescue this phenotype (Figures 3F and 3G).

**lincRNA-Cox2 Functions through an eRNA Mechanism to Regulate Ptgs2**

Recently, it has been shown that non-coding RNA loci have the potential to harbor enhancer activities (Engreitz et al., 2016; Groff et al., 2016; Kim et al., 2015; Kotzin et al., 2016; Melo et al., 2013; Paralkar et al., 2016; Ørom et al., 2010). Because the effect of lincRNA-Cox2 deficiency on Ptgs2 expression was not rescued by ectopic expression, we speculated that the lincRNA-Cox2 locus harbored a DNA enhancer element controlling Ptgs2 expression. LincRNA-Cox2 possesses two exons, with the majority of the sequence lying within exon 2. Using CRISPR/Cas9, we excised exon 2 from lincRNA-Cox2 in BMDMs (Figure 3H) and confirmed this deletion by qRT-PCR (Figure 3K). Ptgs2 was measured by qRT-PCR, and expression was markedly reduced in the KO BMDMs, again suggesting that either the lincRNA transcript or a DNA enhancer element within exon 2 is required for the activity on Ptgs2 (Figure 3L). These data confirm that there is no enhancer activity lying within exon 1, which remains intact in this model. To determine whether there are any enhancer marks present within exon 2 of lincRNA-Cox2, we studied chromatin immunoprecipitation sequencing (ChIP-seq) of histone marks associated with enhancers, including histone 3 lysine 4 (H3K4) mono-methylation, as well as p300 binding from the Mouse Encode project and from Lara-Astiaso et al. (2014) and Stamatoyannopoulos et al. (2012). We were surprised that there was no evidence of any enhancer within exon 2 of lincRNA-Cox2 and, instead, enhancer marks were identified upstream of the transcription start site of lincRNA-Cox2 (Figure 3I; Figure S4). These data suggest that this locus might function instead as an eRNA in which transcription of lincRNA-Cox2 functions to connect the enhancer region with the Ptgs2 locus to drive expression of the protein. The enhancer region within the lincRNA-Cox2 promoter remains intact in the KO mouse, and the locus is transcriptionally active, but there is a strong defect in Ptgs2 levels. Only the lincRNA-Cox2 transcript is absent in this model, and because there are no identifiable enhancer elements within this region, it suggests that specific transcription of the lincRNA-Cox2 sequence is driving this phenotype as an eRNA.

To test whether lincRNA-Cox2 can function as an eRNA, we used CRISPR interference (CRISPRi) as outlined in Figure 3J to inhibit transcription of the locus. We successfully knocked down lincRNA-Cox2 by over 95% using two independent guide RNAs in a clonal cell line (Figure 3M), and each led to a more than 95% decrease in Ptgs2 expression, as assayed using qRT-PCR (Figure 3N). This result was replicated in a second CRISPRi BMDM cell line (Figures S5C and S5D). These data strongly indicate that it is locus-specific transcription of lincRNA-Cox2 and not a DNA element within the exonic sequence of the gene that is critical to its function to control Ptgs2 levels.

To determine whether lincRNA-Cox2 regulates Ptgs2 on the same chromosome, one final experiment was performed using mice with distinct genetic backgrounds. We took advantage of the wild-derived mouse strain MOLF, which is genetically distinct from the common laboratory C57/Bl6 mice. MOLF mice possess numerous SNPs that are distinct compared with C57/Bl6 mice (Doran et al., 2016). Within the last exon of Ptgs2, MOLF mice have 5 distinct SNPs that can be used to distinguish the C57/Bl6 and MOLF alleles. As outlined in Figure 4A, we crossed WT C57/Bl6 or lincRNA-Cox2 KO C57/Bl6 mice with MOLF-WT mice to determine how loss of lincRNA-Cox2 affects Ptgs2. By qRT-PCR, we show that lincRNA-Cox2 expression is lost in the KO mice, whereas the native promoter is intact, represented by LacZ expression (Figures 4B and 4C). When examining the heterozygous mice, the MOLF-KO mice had reduced expression of lincRNA-Cox2 and Ptgs2 compared with MOLF-WT mice (Figures 4B and 4D). Using RNA-seq, we determined that only the lincRNA-Cox2 KO allele (C57/Bl6) had decreased expression of Ptgs2. The MOLF-WT allele, which has an intact lincRNA-Cox2 gene, was unaffected by the loss of lincRNA-Cox2 (Figures 4E and 4F). These results support the finding that lincRNA-Cox2 functions to regulate the expression of Ptgs2 on the same chromosome and is graphically summarized in Figure 4G.

**Ptgs2 Expression Is Not Required for Transcriptional Activity of lincRNA-Cox2**

Because lincRNA-Cox2 and Ptgs2 show parallel expression kinetics following an inflammatory stimulus (Carpenter et al., 2013), we wanted to explore whether the regulatory interaction between lincRNA-Cox2 and Ptgs2 was unidirectional or whether Ptgs2 itself affects the expression levels of the lincRNA. In addition, we wanted to understand whether the effect on Ptgs2 was responsible for the changes in inflammatory genes, such as Il6. To address this question, we crossed conditional Ptgs2 flox/flox mice (Wang et al., 2009) to Vavi-Cre mice to delete Ptgs2 in all hematopoietic cells. We generated BMDMs from these Ptgs2fl/fl-Vavi-Cre mice and confirmed deficiency of Ptgs2 by
immunoblotting and qRT-PCR in BMDMs stimulated with LPS (Figures 5A and 5B). The induction of lincRNA-Cox2 proceeded normally in Ptgs2-deficient BMDMs (Figure 5C). Importantly, the induction of Il6 and Ifi202b, which were both affected by lincRNA-Cox2 deficiency, was normal in Ptgs2-deficient cells (Figures 5D and 5E). Together, these results suggest that the lincRNA-Cox2 transcript can regulate genes in trans that are not normally regulated by Ptgs2. Thus, lincRNA-Cox2 may represent an important modulator within and outside of the Ptgs2 pathway.

Additionally, we used the BMDM CRISPRi clonal cell lines to target Ptgs2 using two independent guide RNAs, as represented in Figure 5F. CRISPRi-mediated knockdown of Ptgs2 by more than 90% had no effect on the transcription of lincRNA-Cox2 upon LPS stimulation. These data further confirm that Ptgs2, RNA or protein, is not necessary for transcriptional activity of lincRNA-Cox2 basally or after inflammatory stimulation (Figures 5G and 5H; Figures S6C and S6D), whereas lincRNA-Cox2 is required for Ptgs2 expression.

**A lincRNA-Cox2 Splicing Mutant Mouse Fails to Produce Any Inducible lincRNA-Cox2 Transcript**

To determine the trans functions of this lincRNA transcript independent of its role in regulating Ptgs2, we generated a mouse model with a deletion of the ~2.3-kb intronic region utilizing CRISPR/Cas9 (hereafter referred to as mutant mice). We

![Figure 4. lincRNA-Cox2 Influences the Expression of Ptgs2 in cis](image-url)
designed two guide RNAs targeting the 5' and 3' splice sites of lincRNA-Cox2. Exon 2 remained completely intact, whereas 50 bp were deleted from exon 1 (Figure 6A). PCR genotyping confirmed the mutant as described in Figure 6B. These mice were also born at expected Mendelian frequencies with no obvious developmental abnormalities. We generated BMDMs from the mutant mice and examined the expression of lincRNA-Cox2 in response to LPS challenge. Although the lincRNA-Cox2 transcript was inducible following LPS stimulation in WT BMDM cells, there was only very low basal expression of lincRNA-Cox2 in the mutant BMDM cells, as measured by qRT-PCR (Figure 6C).

In contrast to data we obtained with the full gene KO, Ptgs2 levels were comparable between the WT and mutant BMDMs (Figure 6D). We next examined Ptgs2 levels in tissues, specifically the lung, where lincRNA-Cox2 is most highly expressed, and the spleen, where the majority of white blood cells are stored (Figures 2A, 2C, and 2E, Figure S1A). In the mutant, intron-less mouse, lincRNA-Cox2 levels were dramatically reduced in the lung and spleen following LPS stimulation (Figures 6E and 6G). Again, there was no effect on Ptgs2 levels within the lung and spleen of mutant mice (Figures 6F and 6H). These observations support the hypothesis that this locus can function as an enhancer to regulate Ptgs2 and that this

**Figure 5.** Ptgs2 Does Not Affect the Expression of lincRNA-Cox2 or Its Target Genes

(A) BMDMs from WT, heterozygous (Het), and KO Ptgs2 cells were stimulated with LPS. The levels of Ptgs2 and β-actin were measured by western blot. (B–E) BMDMs from WT, Het, and Ptgs2 KO mice were stimulated with LPS for the indicated times, and the levels of Ptgs2 (B), lincRNA-Cox2 (C), Il6 (D), and Ifi202b (E) were measured by qRT-PCR and normalized to Gapdh.

(F) Schematic of where dCas9-Krab will sit at the transcriptional start site of Ptgs2.

(G and H) qRT-PCR was used to measure Ptgs2 (G), and lincRNA-Cox2 (H) levels were measured in 6-hr LPS-stimulated BMDMs following knockdown of lincRNA-Cox2 using CRISPRi clonal cell line 1. Data represent 3 combined biological replicates representative of 3 individual experiments. Error bars represent standard deviation of biological triplicates. Student’s t tests were performed using GraphPad Prism. Asterisks indicate statistically significant differences between mouse lines (**p < 0.005).
eRNA activity is maintained in this model. The enhancer located prior to the start site of the locus in conjunction with the basal transcript work together to mediate these effects on Ptgs2.

**lincRNA-Cox2 Regulates Distinct Sets of Genes in the Lung and Spleen**

Now having two separate mouse models, a complete deletion KO mouse, and an intron-less mutant mouse, we asked whether...
the absence of the lincRNA-Cox2 transcript has a trans and organ-specific role in vivo. lincRNA-Cox2 is expressed at high levels in the lung and very low in the spleen (Figures 2D and 2E; Figure S1A). The expression of lincRNA-Cox2 is inducible upon LPS challenge in the spleen and slightly reduced in the lung (Figures 2D and 2E). To define the global regulatory role of lincRNA-Cox2, we challenged WT, KO, and mutant mice by i.p. injection of E. coli LPS for 6 hr, followed by RNA-seq of the spleen and lung, as represented in Figure 6I.

DESeq2 analysis was used to determine the differentially expressed (DE) genes between WT versus KO, WT versus mutant, or KO versus mutant in both the lung and spleen (Figure 6J). The KO versus WT mice had 312 DE genes in the lung and 313 DE genes in the spleen (Figure 6J). The mutant versus WT mice had 115 DE genes in the lung and 179 DE genes in the spleen (Figure 6J). When comparing gene expression of mutant mice with KO mice, there were hundreds of significantly DE genes, reinforcing that these mice had very distinct phenotypes from one another because of the cis and trans functions of lincRNA-Cox2 (Figures 6J–6L). Interestingly, when comparing mutant lincRNA-Cox2-specific DE genes, there are only six that overlap between the spleen and lung (Figure 6M). Of these six genes, only one gene changed from being upregulated to downregulated from the spleen to the lung, Ptprh (synonym Sap-1) (Figure S7C; Bujko et al., 2017). Database for Annotation, Visualization, and Integrated Discovery (DAVID) analysis of the differentially expressed genes of the spleen and lung show similar pathways, such as glycoprotein and inflammatory response, as well distinct pathways, such as fibronectin and heparin binding in the spleen or cellular homeostasis and secretory granule pathways in the lung (Figures S7A and S7B). LincRNA-Cox2 is highly expressed in the lung in a very cell type-specific manner (Figures S1B and S1C). Using the Mouse Cell Atlas generated by Han et al., 2018, we can show the differences in cell type expression between Ptgs2 and lincRNA-Cox2 (Figure S1B). Overall, lincRNA-Cox2 has a higher expression in specific immune-related cells in the lung, whereas Ptgs2 has higher expression in epithelial and endothelial cells, providing additional evidence that lincRNA-Cox2 has distinct roles in trans independent of its regulation of Ptgs2.

lincRNA-Cox2 Controls Immune Genes in trans Independent of Ptgs2 following LPS Challenge In Vivo

The generation of the mutant intron-less lincRNA-Cox2 loss-of-function model allowed us to assess the contribution of the lincRNA transcript in vivo independent of the cis effect on Ptgs2. We asked whether the absence of the lincRNA-Cox2 transcript affects in vivo responses to LPS. We challenged the lincRNA-Cox2 mutant mice in vivo by i.p. injection of E. coli LPS. The temperature of the mice, a clinical parameter of septic shock, decreased over time in both strains (Figure 7A). Serum cytokines were then measured using multiplex assays. Interferon-stimulated genes, including Ccl5 (Rantes) and Ip10, were both found at elevated levels in the lincRNA-Cox2 mutant mice following LPS challenge (Figures 7B and 7C), whereas proinflammatory gene expression, including Il5, Lif, and Il17, was reduced (Figures 7D–7F). Tnfα expression levels were unchanged in control and mutant mice (Figure 7G). Since in vivo data are consistent with published in vitro data showing that lincRNA-Cox2 can act to both promote and inhibit the expression of innate immune...
genes. Because all genes affected in the mutant mice were located on different chromosomes to lincRNA-Cox2 (chromosome 1), these data confirm that lincRNA-Cox2 functions in trans to control immune responses in vivo.

DISCUSSION

lincRNAs remain an understudied class of genes specifically in the context of the immune system. Although there are a number of studies, both by our lab and others, showing that lincRNA-Cox2 promotes and restrains different classes of innate immune gene expression, none have shown the function of this gene in vivo (Carpenter et al., 2013; Covarrubias et al., 2017; Hu et al., 2016; Tong et al., 2016; Xue et al., 2018). Here we expand our knowledge of the role of lincRNA-Cox2 by generating two murine models and multiple macrophage cell lines, which strengthens our earlier findings that lincRNA-Cox2 can inhibit expression of ISGs and enhance pro-inflammatory gene expression. In addition, we now reveal that lincRNA-Cox2 also functions through an eRNA mechanism to control the expression of the neighboring gene Ptgs2 (Cox2) in vivo.

We initially characterized the lincRNA-Cox2 KO mouse, generated by replacing the locus with LacZ, by tracking LacZ expression in organs after LPS challenge. We observed that lincRNA-Cox2 was highly expressed in the lung, and further analysis of RNA-seq data confirmed this observation. To determine how loss of lincRNA-Cox2 expression affects gene expression at steady state, we performed RNA-seq on lung samples comparing WT and KO. We were intrigued to identify Ptgs2 among the most significantly altered transcripts in the KO lung. Our previous work using shRNA-mediated knockdown of lincRNA-Cox2 had not revealed any change in the expression levels of Ptgs2 in BMDMs (Carpenter et al., 2013), suggesting that this cis activity was carried out through an enhancer within the DNA of this locus. We were unable to rescue the expression of Ptgs2 by ectopic overexpression of lincRNA-Cox2, further suggesting that it is either the DNA responsible for the phenotype or that locus-specific expression of the transcript is required for this function. A caveat with the reconstitution experiment is that we express lincRNA-Cox2 off a plasmid using an EF1α promoter. Although the reconstituted lincRNA-Cox2 localizes to the nucleus and cytoplasm in a similar manner as the native transcript, it is no longer induced by its native promoter or undergoing splicing. Additional experiments, including ectopic expression of lincRNA-Cox2 with the native promoter or the entire locus, could help determine whether these features are critical for the function of lincRNA-Cox2 in controlling Ptgs2 in cis. The majority of the sequence for lincRNA-Cox2 lies within exon 2, and removing this exon using CRISPR resulted in a dramatic decrease in Ptgs2. However, when we examined the locus for enhancer elements, we did not identify any within exon 2 of the gene. Instead, we observed evidence for DNA enhancer elements that lie only within the promoter of lincRNA-Cox2, including H3K4 mono-methylation (H3K4me1) and p300 ChIP-seq. Interestingly, this enhancer region remained intact in the lincRNA-Cox2 KO mouse, and the locus was transcriptionally active, but Ptgs2 was downregulated. This indicates that locus-specific transcription of lincRNA-Cox2 is required to mediate the activity in cis, suggesting that lincRNA-Cox2 functions as a form of eRNA.

It has been reported that many lincRNAs act locally, explaining why lincRNAs, like lincRNA-Cox2, display a similar expression pattern as their neighboring protein-coding genes. A recent study by Engreitz et al. (2016) showed that lncRNA and protein-coding genes can function locally and affect each other’s expression levels. They show that 5 of 12 lincRNA loci they studied affected their neighboring gene in cis either through enhancer activity from within the promoter through the act of transcription or the act of splicing of the lincRNA (Engreitz et al., 2016).

eRNAs are produced from transcriptionally active enhancer regions, which are epigenetically defined by high levels of H3K4me1, low levels of H3K4 trimethyl (H3K4me3), and high levels of histone 3 lysine 27 acetylation (H3K27Ac) (Creighton et al., 2010; Heintzman et al., 2007; Visel et al., 2009). lincRNAs that function in cis to either enhance or suppress the expression of neighboring protein-coding genes can be classified as enhancer or repressor RNAs. Studies from Illott et al., 2014 identified over 40 canonical lincRNAs that act as eRNAs to regulate protein-coding genes upon LPS stimulation in human monocytes. Interestingly, although we found that knocking out lincRNA-Cox2 greatly affected Ptgs2, deletion of Ptgs2 itself did not affect basal or inducible levels of lincRNA-Cox2, showing that the effects are not reciprocal between these two loci. This result was further confirmed using CRISPR, which mediates heterochromatin formation and silencing of gene transcription. Knocking down the transcription of Ptgs2 by over 90% had no effect on the regulation of lincRNA-Cox2. The lincRNA-Cox2 locus has both high H3K4me1 and H3K4me3 marks, making it distinct from the typical definition of eRNAs. It is possible that, as the field and function of lncRNAs continues to grow, that the categories that define their functions will also expand.

To determine whether transcription of lincRNA-Cox2 is important to regulate Ptgs2, we knocked down expression of the gene using CRISPR. Our data show that we can knock down lincRNA-Cox2 expression more than 95%, which equally affects Ptgs2 expression by more than 95%. In addition, using active Cas9, we knocked out exon 2 of lincRNA-Cox2 (the majority of the lincRNA-Cox2 sequence), this perturbation also affected Ptgs2 expression. Because we did not identify any possible enhancer marks within exon 2, and all of the enhancer marks were within the promoter, we conclude that lincRNA-Cox2 is functioning through an eRNA mechanism to control Ptgs2. Although unlikely, it is possible that there is an unidentified enhancer mark within the DNA of exon 2. Future work aimed at inserting stop cassettes within exon 2 could confirm that, indeed, transcription of the gene is essential for Ptgs2 levels. The data we generated from our “intron-less splicing mutant” mice strongly suggest that this is the case. We used CRISPR/Cas9 to generate the mutant mouse. In this mouse, the exons of the lincRNA-Cox2 transcript are maintained; however, the intron and splice sites are removed. This mouse expressed basal levels of lincRNA-Cox2; however, the transcript is no longer inducible following LPS stimulation. It is possible that, because this transcript is no longer undergoing
splicing, that it is highly unstable and, therefore, rapidly degraded following LPS stimulation. In the future, northern blots should be performed to confirm there are no additional unexpected lincRNA fragments in this model. Effectively, this mouse functions as a transcript loss-of-function mouse model of lincRNA-Cox2. Our studies with BMDMs and mice in vivo suggest that splicing is not required for the observed effect on Ptgs2. Low basal expression of the spliced lincRNA-Cox2 transcript is sufficient to activate the Ptgs2 locus, further suggesting that, indeed, this gene can function through an eRNA mechanism.

A final experiment was performed to assess whether lincRNA-Cox2 is regulating Ptgs2 on the same chromosome. We crossed our C57/Bi6 WT and KO mice with MOLF mice, which contain millions of SNPs, allowing us to differentiate between the two alleles. Studying the Ptgs2 locus, we found that only expression from the cis (C57/Bi6) allele was affected when lincRNA-Cox2 was knocked out. These data confirmed that indeed lincRNA-Cox2 functions to regulate Ptgs2 on the same chromosome. We predict that lincRNA-Cox2 functions as an eRNA and the RNA is tethering the enhancer to the Ptgs2 locus and forming a topologically associating domain. Further work including performing 3C/Hi-C should confirm this.

The lincRNA-Cox2 mutant mice are a critical model enabling us to study the trans activity for lincRNA-Cox2 independent of its effects on Ptgs2. As mentioned previously, this mouse functions as a transcript loss-of-function mouse model of lincRNA-Cox2. The original lincRNA-Cox2 KO mouse not only attenuated the expression of lincRNA-Cox2 but also repressed the expression of Ptgs2, whereas this model only affects lincRNA-Cox2 levels, enabling us to use these models to distinguish between the cis and trans functions for this gene.

Distinct tissue-specific functions have been shown for several lincRNAs. One such example is NEAT1, which is critical for synapse formation in the brain (Sunwoo et al., 2017), whereas, in adipose tissue, NEAT1 is necessary for the differentiation of white adipocytes (Cooper et al., 2014). lincRNA-Cox2 KO and mutant mice were used to globally study the cis and trans role of lincRNA-Cox2 in vivo by performing RNA-seq on lungs and spleens from LPS-challenged WT, KO, and mutant mice. Not surprisingly, we observed over 300 genes that were differentially regulated when comparing KO with WT mice, whereas only half that number were affected when comparing mutants with the WT. We believe that this is due to the fact that the mutant mice reveal only the functions of lincRNA-Cox2 in trans, whereas the KO mice exhibit both cis effects on Ptgs2 and trans effects.

Interestingly, the mutant mice possessed differentially expressed genes in the lung and spleen, emphasizing a possible organ-specific role for lincRNA-Cox2. Indeed, we observe very high levels of lincRNA-Cox2 in the lung, whereas the transcript is only induced in the spleen following inflammatory stimulation. Further work will be needed to understand mechanistically how lincRNA-Cox2 affects inflammatory responses in the lung and spleen. From our LacZ staining within the lung, combined with data from the mouse cell atlas, we observe cell type-specific expression patterns for lincRNA-Cox2. In the future, it will be informative to isolate these specific cells for further mechanistic studies.

Finally, we wanted to determine whether lincRNA-Cox2 can affect gene expression in the periphery following LPS challenge in vivo. We observe an increase in Ccl5 and Ip10 (Cxc10) levels and a decrease in Il5, Il17, and Il1 levels in the serum of mice following endotoxic shock, suggesting that lincRNA-Cox2 can function in trans to control various innate immune genes. These data highlight two important features of this lincRNA’s function: the in vivo immune-regulatory activity of lincRNA-Cox2 on immune gene expression and the role of the lincRNA-Cox2 locus in controlling Ptgs2 (Cox2) levels. Together, these studies further our understanding of how lincRNAs can function both in cis and in trans to control gene expression, findings that are only evident through the use of multiple complimentary genetic perturbations. The drastic influence of non-coding genes on key immune signaling axes, such as the prostaglandin pathway as shown here, has broad translational implications because it indicates that protein-centered sequencing approaches are likely insufficient to fully understand genetically mediated autoimmune and auto-inflammatory conditions. Large-scale functional validations of noncoding mutations, facilitated by CRISPR/Cas9, will provide a promising approach to better understand the pathogenesis of inflammatory and non-inflammatory diseases. CRISPR/Cas9 technology has revolutionized the field of genome editing and will be a critical tool moving forward to enable rapid generation of animal models to fully characterize any lincRNA locus.

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Supplemental Information includes seven figures and seven tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.10.027.

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AUTHOR CONTRIBUTIONS

S. Carpenter and K.A.F. conceived and coordinated the project. S. Carpenter, K.A.F., and E.K.R. wrote the manuscript. R.E., S. Carpenter, and E.K.R. oversaw the majority of the work. R.E. performed qRT-PCR and western blotting. E.K.R. generated the CRISPRi data, performed qRT-PCRs, and carried out in vivo studies. B.S. and S. Carpenter performed qRT-PCRs and carried out in vivo studies. S.C.L. performed RNA-seq and LacZ staining. A.F.G. and S.K. performed analyses of RNA-seq data. Z.J. performed studies on Ptgs2 KO animals. J.L.R. generated lincRNA-Cox2 KO mice and provided intellectual input. S. Covarrubias assisted with the generation of KOIs using CRISPR/Cas9. E.J.H. and G.A.F. performed mass spectrometry analyses. S.A., M.M., J.C., and S.S. provided technical assistance for Figure 2. M.T.M. provided critical reagents and suggestions. All authors reviewed the results and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES


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CONTACT FOR RESOURCE AND SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead Contact, Susan Carpenter (sucarpen@ucsc.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Maintenance of mice
UMass Medical School, UCSC and the Institutional Animal Care and Use Committee maintained mice under specific pathogen-free conditions in the animal facilities of University of Massachusetts Medical School and University of California Santa Cruz (UCSC) in accordance with the guidelines.

LincRNA-Cox2 KO mice
LincRNA-Cox2 deficient mice were generated as previously published (Sauvageau et al., 2013). Briefly, the entire lincRNA-Cox2 locus was deleted (deletion size 5.9 kB, targeting vector coordinates in mm9 coordinates: chr1:152006173-152012078) using Velocigene technology and replaced with a lacZ reporter cassette, enabling reporter gene expression under the endogenous lincRNA-Cox2 promoter. Initially, the targeting constructs were electroporated into VGF1 hybrid mouse embryonic stem (ES) cells, derived from a 129S6S v/Ev female to a C57BL/6 male. Speed congenics were performed at Jackson laboratory to obtain a fully backcrossed (99%) C57/BL6 lincRNA-Cox2 line.

Ptgs2 (Cox2) f/f VavCre mice
For the generation of conditional Ptgs2 deficient BMDMs, floxed Ptgs2 mice which were generated on a C57/B6 and 129SV chimeric background (Wang et al., 2009) were crossed to mice expressing VavCre, thereby generating mice lacking Ptgs2 expression in all hematopoietic cells. Successful deletion of exons 6,7 and 8 of Ptgs2 was confirmed by genomic PCR, Western Blot and RT-qPCR using the primers published previously (Anderson et al., 2015).

CRISPR/Cas9-mediated generation of LincRNA-Cox2 intron-less mice
The guide RNA sequences targeting upstream and downstream of the intron of lincRNA-Cox2 are tabulated below. Guides were cloned using the same approach as described earlier. Once sgRNAs were confirmed by sequencing they were intro transcribed by inserting a T7 ahead of the guide sequence and using the megashortscript T7 kit from Ambion. The sgRNAs are purified using MEGAclear Transcription Clean-Up Kit (ThermoFisher) and eluted in elution buffer, followed by an additional ammonium acetate precipitation to concentrate the RNA. The concentrated sgRNA is re-suspended in nuclease-free water and prepared at a concentration approximate to 2 ug/ul. To prepare Cas9 we obtained the vector from Addgene 42229. We used this as a template for a PCR reaction.
to insert T7 using the following primers F: TAATACGACTCACTATAGGGAGAATGGGACTATAAGGACCACGAC, R: GCGAGCTCTAG GAATTCTTAC. The mRNA transcript was generated using mMESSAGE mMACHINE T7 ULTRA kit from Life Technologies according to the manufacturer’s protocol. Cas9 was used at a final concentration of 100 ng/μl and the guides at 25 ng/μl and were injected into the cytoplasm of fertilized oocytes at the injection facility at UCSF.

**Cell culture and BMDM differentiation**

Cells were cultured in D-MEM with 10% fetal bovine serum (FCS) supplemented with penicillin/streptomycin and ciprofloxacin. Primary BMDMs were generated by cultivating erythrocyte-depleted bone marrow cells in the presence of 20% L929 supernatant and the cells were used for experiments 6-9 days after differentiation. J2Cre virus (Blasi et al., 1989) was used on day 3/4 after isolation of bone marrow cells to establish transformed BMDM cell lines. BMDMs were cultured in the presence of J2Cre virus for 48 h and L929 was then gradually tapered off over 6-10 weeks depending on the growth pattern of transformed cells.

**METHOD DETAILS**

**LPS shock model**

Age- and sex matched wild-type and mutant mice (8-12 weeks of age) were injected i.p. with 20 mg/kg LPS (E.coli). For gene expression analysis and cytokine analysis, mice were euthanized 6 h post injection. Blood was taken immediately post mortem by cardiac puncture. Serum was isolated and sent to Eve technologies for cytokine analysis. Statistics were performed using GraphPad prism.

**In vitro stimulation of BMDMs**

Bone marrow cells were stimulated with Toll-like receptor (Tlr) ligands for the indicated time points using the following concentrations: Lipopolysaccharide (LPS) 100 ng/ml (Tlr4), Pam3CSK4 100 ng/ml (Tlr1/2), Pam2CSK4 100 ng/ml (Tlr2/6), Poly(I:C) 25 μg/ml (Tlr3), R848 1 μg/ml (Tlr7/8). For RNA and protein isolation, 1-2x10⁶ cells were seeded in a 12-well format, for cytokine measurement 1-2x10⁵ cells were plated in 96-well plates. Normalization of cell number for ELISA experiments was performed by CellTiter Glo Analysis.

**Transfection and stable lentiviral overexpression of lincRNA-Cox2**

The sequence of lincRNA-Cox2 (synonym: Ptgs2os2) has been previously published (Carpenter et al., 2013; Guttman et al., 2009). Stable lentiviral overexpression and trans-rescue was performed with a pMSCVneo retroviral vector containing the mature sequence of lincRNA-Cox2 (Carpenter et al., 2013). For generation of self-inactivating retroviral particles, HEK293T cells were transfected with packaging vectors VSVg (1 μg/gml), Gag-Pol (1 μg/gml) and pMSCVneo-lincRNA-Cox2 (3 μg/gml). Genejuice was used as a transfection reagent according to manufacturer’s instructions. Transfection media was removed 5 h after transfection. Viral supernatants were collected 48 and 72 hours after transfection, filtered and used for transduction of BMDMs. BMDMs were incubated with viral supernatants for 48 hours, and neomycin (100 μg/ml) was used for selection of transduced cells. For electroporation of primary BMDMs, cells were harvested from the bone marrow of WT and lincRNA-Cox2 KO mice and differentiated using MCSF from L929 cells as previously described. On day 4 of differentiation cells were transduced with 2 μg of lincRNA-Cox2 (pSico-vector) or with a control pSico-vector using the Lonza AD electroporation BMDM kit (VPA1009) as per the manufacturer’s description.

**CRISPR/Cas9 mediated deletion of lincRNA-Cox2 in immortalized BMDMs**

The Cas9 construct was constructed from a pSico lentiviral backbone with an EF1α promoter expressing T2A flanked genes: blasticidin resistant (blast), Blue fluorescent Protein (BFP), and humanized *Streptococcus pyogenes* Cas9. The single guide RNA (sgRNA) construct was also constructed from a pSico lentiviral backbone driven by EF1α promoter expressing T2A flanked genes: puromycin and cherry. sgRNAs were expressed from a mouse U6 promoter. Cloning of 20nt sgRNA spacer forward/reverse oligos were annealed and cloned via the AarI site. Immortalized bone marrow derived BMDMs (iMacs) were selected using blasticidin for 7 days. Control and lincRNA-Cox2 targeted cells were cloned out. Knockout of lincRNA-Cox2 was confirmed by RT-qPCR.

**CRISPRi mediated KO of lincRNA-Cox2 and Ptgs2 in immortalized BMDMs**

Similar to the Cas9 construct, our dCas9-krab plasmid was constructed from a pSico lentiviral backbone with an EF1α promoter expressing T2A flanked genes: blasticidin resistant (blast), Blue fluorescent Protein (BFP), and humanized *Streptococcus pyogenes* dCas9. The single guide RNA (sgRNA) construct was the same as described in the previous section. Immortalized bone marrow derived BMDMs (iMacs) were lentivirally infected with the dCas9-krab (described above) construct and was selected using blasticidin (1 μg/ml) for > 2 weeks to obtain dCas9-krab-expressing cells. Cells were then lentivirally infected with either non-targeting guide or gene targeted guide RNAs and were selected using puromycin (2 μg/ml) for 7-14 days. Knockdown of lincRNA-Cox2 or Ptgs2 was confirmed by qRT-PCR.
RNA isolation and cDNA synthesis and RT-qPCR

Total cellular RNA from BMDM cell lines or tissues was isolated using the Direct-zol RNA MiniPrep Kit (Zymo Research) according to manufacturer’s instructions. For tissue, RNA was isolated with the TRIZOL method after tissue homogenization. RNA was quantified and controlled for purity with a nanodrop spectrometer. (Thermo Fisher). For RT-qPCR, 500-1000 ng were reversely transcribed (iScript Reverse Transcription Supermix, Biorad) followed by RT-PCR (iQ SYBRgreen Supermix, Biorad) using the cycling conditions as follows: 50 °C for 2 min, 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 45 s. The melting curve was graphically analyzed to control for nonspecific amplification reactions.

RNA-Sequencing

For generation of RNA-Sequencing libraries, RNA was isolated as described above and the RNA integrity was tested with a BioAnalyzer (Agilent Technologies) or FragmentAnalyzer (Advanced Analytical). For RNA-Sequencing target RIN score of input RNA (500-1000ng) usually had a minimum RIN score of 8. RNA-Sequencing libraries were prepared with TruSeq stranded RNA sample preparation kits (Illumina), depletion of ribosomal RNA was performed by positive selection of polyA+ RNA. Sequencing was performed on Illumina HighSeq or NextSeq machines. RNA-seq 50bp reads were aligned to the mouse genome (assembly GRCm38/mm10) using TopHat. The Gencode M13 gtf was used as the input annotation. Differential gene expression specific analyses were conducted with the DESeq R package. Specifically, DESeq was used to normalize gene counts, calculate fold change in gene expression, estimate p values and adjusted p values for change in gene expression values, and to perform a variance stabilized transformation on read counts to make them amenable to plotting. Data was submitted to GEO GSE117379.

MiSeq

For the MOLF experiment, 5ul of cDNA from MOLF-WT or MOLF-KO (2 mice each) was used to template a PCR reaction using 1uM of primers: Ptgs2lastexon_fwd: gaaCCAcctGTGGGACAGGAGAGAAGGAAATGGC and Ptgs2lastexon_rev: CCAGCTTAGCCGCCTTTGATATTGACTCTGAGG and using the following cycle parameters: 1x (98°C for 30 s), 20x (98°C for 15 s, 56°C for 15 s, 72°C for 30 s) and 1x (72°C for 10 min) using phusion 2X master mix (ThermoFisher, F0531). PCR products (MOLF-WT, MOLF-WT, MOLF-KO, MOLF-KO) were purified using Macherey-Nagel Gel-extraction columns, were digested with BstXI/Bsp and were ligated into pU6-sgRNA vector (https://benchling.com/s/CXQ8OiQn/edit) (gift from the Weissman Lab, UCSF) using standard T4 ligase. Ligation was transformed, and coverage was determined to be > 200 colonies for each ligation. Using 1ul of the ligation reaction, we template a second PCR reaction using 1uM of the custom illumina primers (Liu et al., 2017).

Using the following cycle parameters: 1x (98°C for 30 s), 20x (98°C for 15 s, 56°C for 15 s, 72°C for 30 s) and 1x (72°C for 10 min). PCR products were gel extracted on a 1% agarose gel and were purified using Macherey-Nagel Gel-extraction columns. Amplicons were sequenced on the MiSeq using the custom sequencing. For each sample, counts were generated for MOLF or C57/B6l Ptgs2 alleles respectfully using sequences: and were normalized to total reads for analysis. Data was submitted to GEO GSE117379.

Measurement of prostaglandins by mass spectrometry

Prostaglandin E2 production was measured by liquid chromatography/mass spectrometry as previously described (16). Briefly supernatants were collected from Wild-Type or KO BMDMs following LPS stimulation for 6h. Systemic production of PGE2, determined by quantifying the major urinary metabolites 7-hydroxy-5,11-diketotetranorprostane-1, 16-dioic acid (PGEM). Results were normalized with creatinine.

Western Blot Analysis

For western blotting, BMDMs were scraped, washed and lysed buffer containing 20mM Tris-HCl (pH 7.4), 1% NP-40 and 5mM EDTA supplemented with Protease inhibitor (Promega). Clarified samples were separated by SDS-PAGE and transferred to PVDF membranes using the Trans-Blot® Turbo Transfer System (BioRad). After blocking of the PVDF membrane with PBS containing 5% (w/v) skim-milk powder and 0.1% Tween-20, the blots were probed with anti-COX2 (Cayman Item #160106) or anti-Actin (Sigma) antibodies. Visualization was performed with enhanced chemiluminescence substrate (ECL Pierce).

LacZ staining

For detection of reporter gene expression in lincRNA-Cox2 deficient mice, selected tissue was dissected and fixed in 4% paraformaldehyde (PBS) for 5 hours (4°C). Fixed tissues were washed 3x with wash buffer (2mM MgCl₂, 0.01%sodium deoxycholate, 0.02% NP-40) and subsequently incubated in staining buffer (1mg/ml X-Gal, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM MgCl₂, 0.01% sodium deoxycholate, 0.02% NP-40) in the dark overnight (room temperature). Staining solution was rinsed off with PBS 3x and tissue were frozen in OCT on dry ice and sectioned in 10μm sections.
QUANTIFICATION AND STATISTICAL ANALYSIS

For in vivo studies
All in vivo studies were carried out using n ≥ 5. In every figure, each dot represents an individual animal. Student’s t tests were performed using GraphPad Prism7. Asterisks indicate statistically significant differences between mouse lines (* = > 0.05, ** = > 0.01 and *** = > 0.005).

For in vitro studies
Data represents 3 combined biological replicates, representative of 3 individual experiments. Student’s t tests were performed using GraphPad Prism7. Asterisks indicate statistically significant differences between mouse lines (* = > 0.05, ** = > 0.01 and *** = > 0.005).

DATA AVAILABILITY
All sequencing data generated from Mi-Seq and RNA-seq reported in this paper have been deposited to GEO under the ID code GSE117379.