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Figures and figure supplements

p50-associated COX-2 extragenic RNA (PACER) activates *COX-2* gene expression by occluding repressive NF-κB complexes

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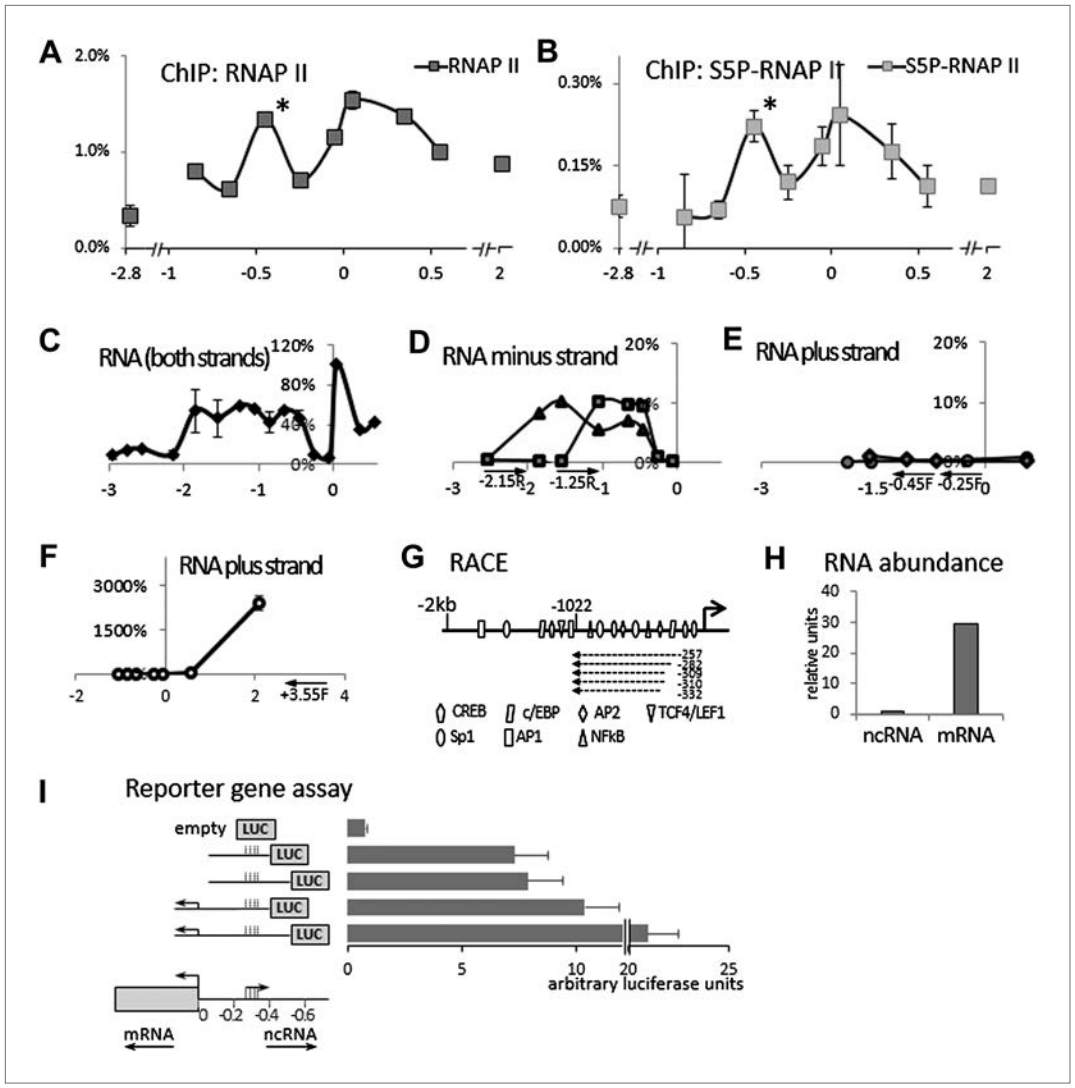

Figure 1. Identification of an antisense long non-coding RNA in the upstream region of the *COX-2* gene. (**A** and **B**) ChiP experiments were performed with chromatin extracts from HMEC cells with antibodies directed against RNAP II or phospho-serine 5 modified RNAP II, respectively, and analyzed by qPCR. Each histogram represents one qPCR data point (amplicon). Non-COX-2 promoter RNAP II peak is marked with an asterisk. X-axis scale is relative to COX-2 transcription start site. (**C**) Transcript abundance in the *COX-2* locus in HMECs was measured by RT-qPCR. cDNA was primed with random oligos and therefore provide no information on strand specificity. Each diamond represents one RT-qPCR data point (amplicon). Y-axis values are relative to the signal obtained with the 5′ UTR amplicon. (**D** through **F**) Strand specific RT-qPCR. cDNA synthesis was performed with two sense oligos (m2R and jR) measuring antisense transcription or three antisense oligos (jF, eF, and control −3.55F) measuring sense transcription. (**G**) A schematic representation of lncRNA ends mapped with RACE. (**H**) Relative abundance of COX-2 mRNA and non-coding RNA. (**I**) A reporter gene assay used to measure the strength of the ncRNA promoter. Luciferase-only construct (empty) or constructs containing indicated fragments of COX-2 upstream region were transfected into 293T cells and luciferase activities were measured 48 hr post-transfection. Activity units reflect the ratios between Firefly and control Renilla luciferases.

DOI: 10.7554/eLife.01776.003

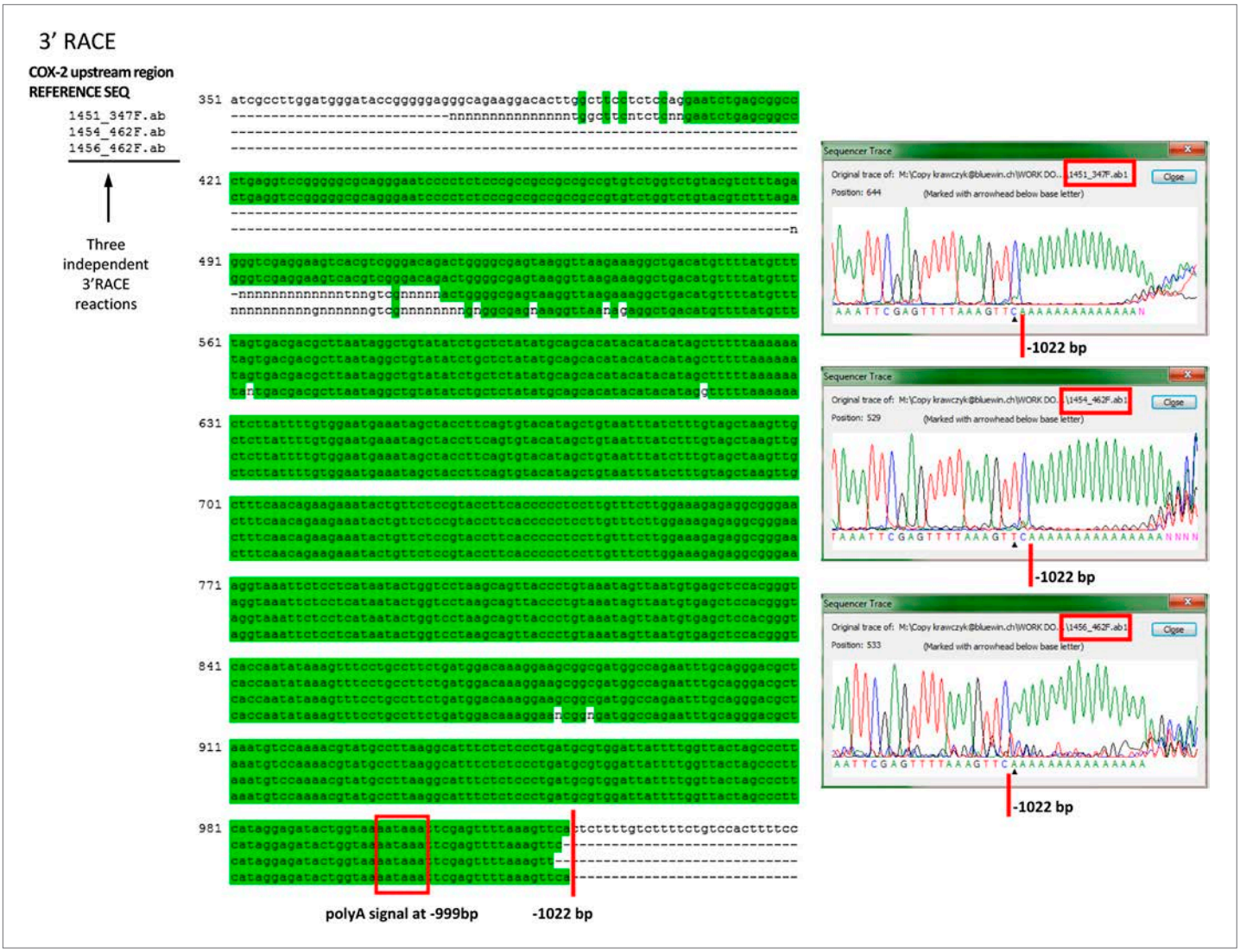

Figure 1—figure supplement 1. Mapping the 3' end of the non-coding RNA transcript within the COX-2 upstream region by RACE.

DOI: 10.7554/eLife.01776.004

Figure 1—figure supplement 2. Mapping the 5′ end of the non-coding RNA transcript within the COX-2 upstream region by RACE.
DOI: 10.7554/eLife.01776.005

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  1  GAGTTCCACC GCCCCAGGCG CACAGGTTTC CGCCAGATGT CTTTTCTTCT TCGCAGTCTT TGCCCGAGCG CTTCCGAGAG CCAGTTCTGG ACTGATCGCC
101  TTGGATGGGA TACCGGGGGA GGGCAGAAGG ACACTTGGCT TCCTCTCCAG GAATCTGAGC GGCCCTGAGG TCCGGGGGCG CAGGGAATCC CCTCTCCCGC
201  CGCCGCCGCC GTGTCTGGTC TGTACGTCTT TAGAGGGTCG AGGAAGTCAC GTCGGGACAG ACTGGGGCGA GTAAGGTTAA GAAAGGCTGA CATGTTTTAT
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401  ACCTTCAGTG TACATAGCTG TAATTTATCT TTGTAGCTAA GTTGCTTTCA ACAGAAGAAA TACTGTTCTC CGTACCTTCA CCCCCTCCTT GTTTCTTGGA
501  AAGAGAGGCG GGAAAGGTAA ATTCTCCTCA TAATACTGGT CCTAAGCAGT TACCCTGTAA ATAGTTAATG TGAGCTCCAC GGGTCACCAA TATAAAGTTT
601  CCTGCCTTCT GATGGACAAA GGAAGCGGCG ATGGCCAGAA TTTGCAGGGA CGCTAAATGT CCAAAACGTA TGCCTTAAGG CATTTCTCTC CCTGATGCGT
701  GGATTATTTT GGTTACTAGC CCTTCATAGG AGATACTGGT AAAATAAATT CGAGTTTTAA AGTTCA
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Figure 1—figure supplement 3. Entire sequence of PACER, corresponding to the longest 5′ variant mapping to nucleotides −257 to −1022.
DOI: 10.7554/eLife.01776.006

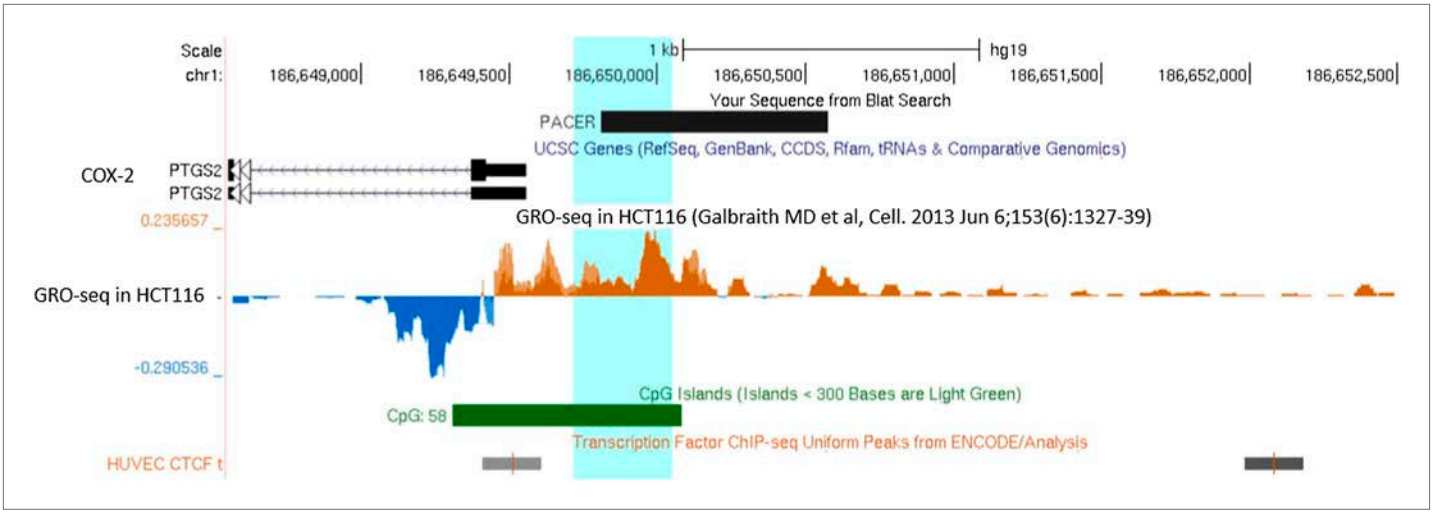

Figure 1—figure supplement 4. GRO-seq data supports the existence of a long non-coding RNA species within COX-2 upstream region.
DOI: 10.7554/eLife.01776.007

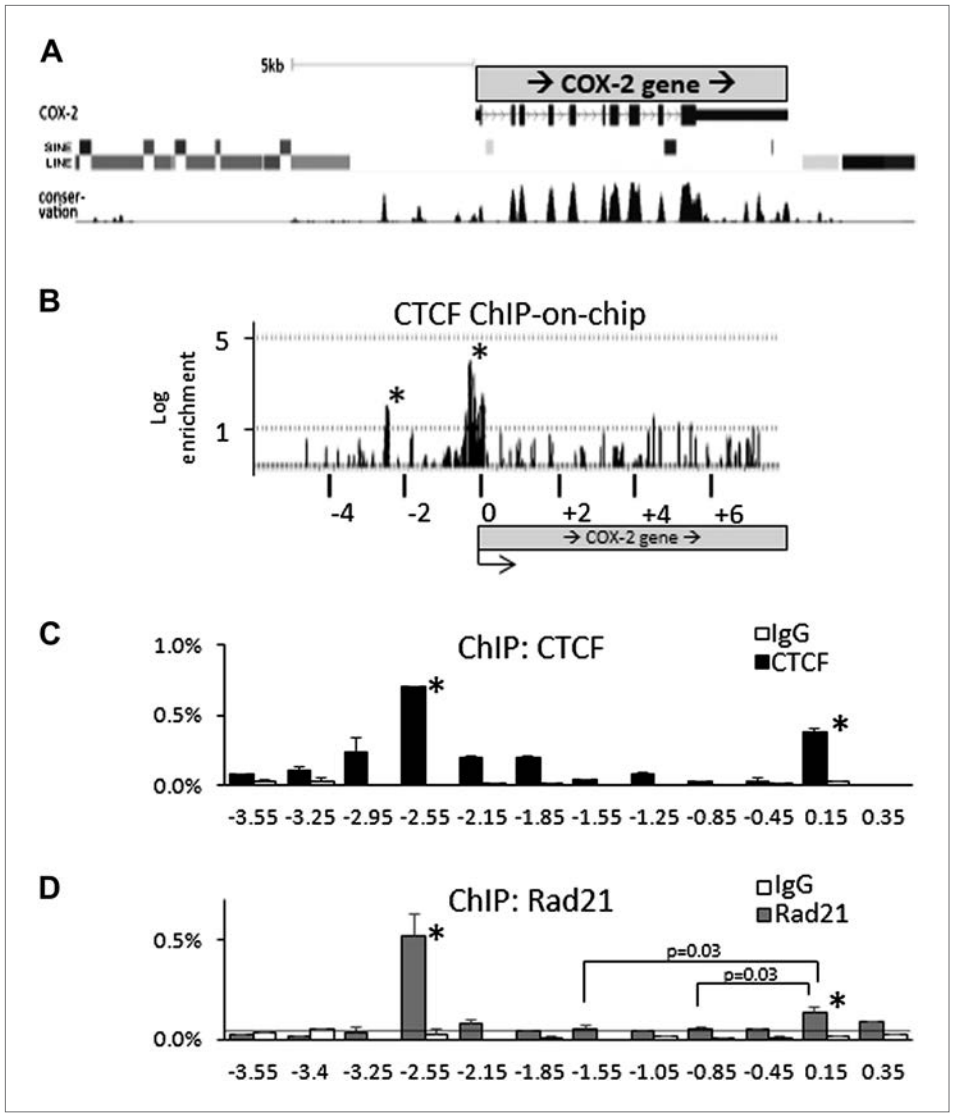

Figure 2. CTCF/cohesin complexes bind to two sites within the *COX-2* locus, encompassing the promoters of mRNA and lncRNA. (**A**) Genomic neighborhood of the human *COX-2* gene. Note the repeat-DNA-free domain extending up to approximately −3 kb upstream of the COX-2 promoter. (**B**) ChIP-on-chip analysis of CTCF binding within the *COX-2* genomic domain in human mammary epithelial cells (HMECs). Two CTCF peaks are evident at approximately −2.5 kb and the proximal promoter. Numerous other loci on the array served as negative controls (***Figure 2—figure supplement 1***). Results are represented as log2ratios between hybridization signals obtained with CTCF-ChIP and input DNA samples. Each vertical line represents one probe on the array. X-axis kb scale is relative to the COX-2 transcription start site. Significant signals are marked by asterisks. (**C** and **D**) Mapping of CTCF and cohesin binding in HMECs analyzed by ChIP-qPCR primer-walking. Amplicon coordinates are relative to the COX-2 transcription start-site.

DOI: 10.7554/eLife.01776.008

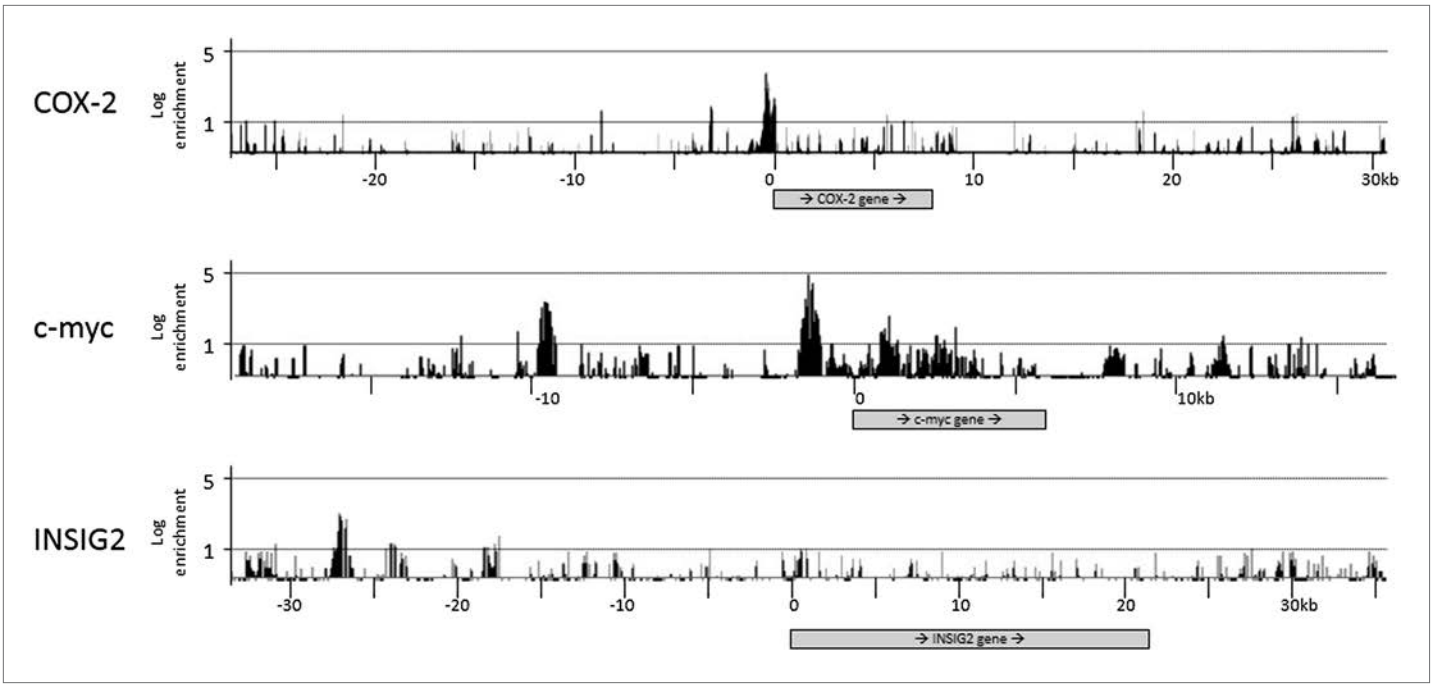

Figure 2—figure supplement 1. Selected results of CTCF ChIP-on-chip experiments.

DOI: 10.7554/eLife.01776.009

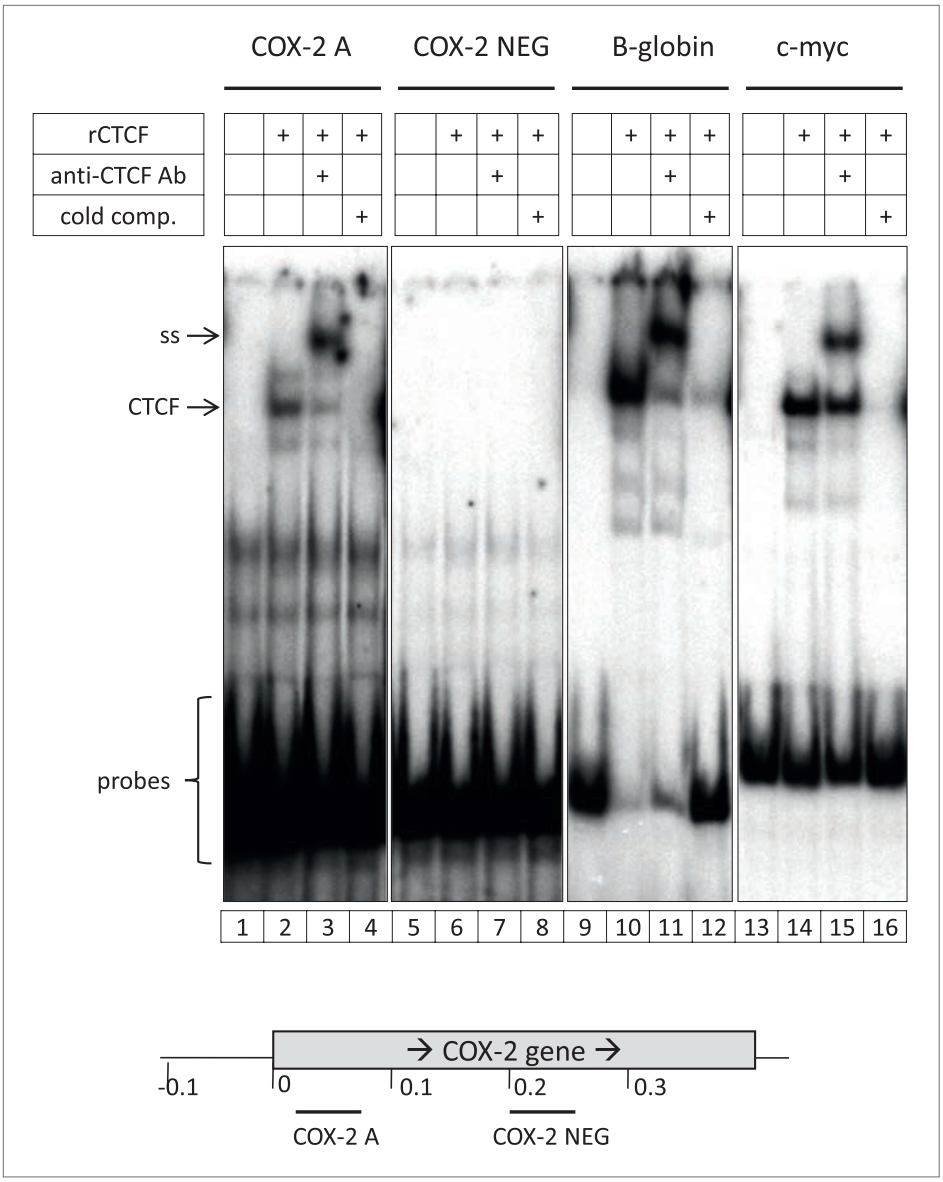

Figure 2—figure supplement 2. Identification and characterization of the 5′ UTR CTCF binding site in the *COX-2* gene.

DOI: 10.7554/eLife.01776.010

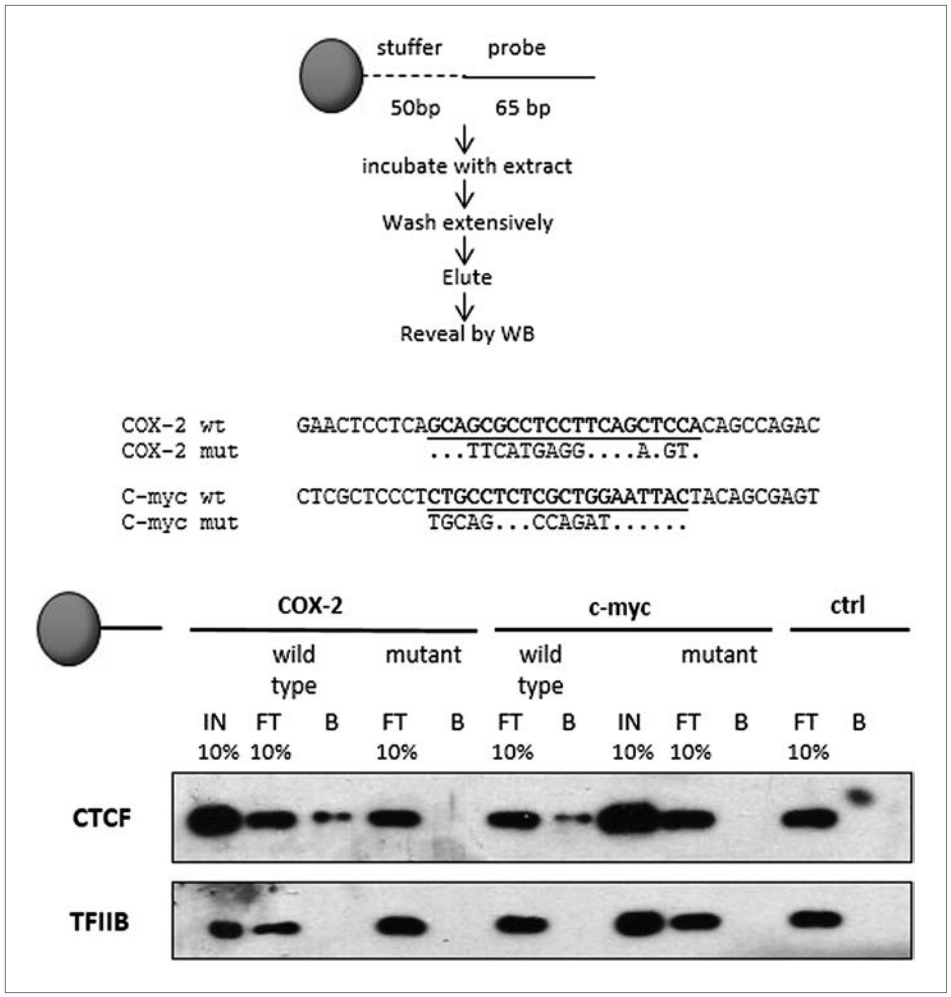

Figure 2—figure supplement 3. Mutations in predicted CTCF sites abolish CTCF recruitment to DNA templates.
DOI: 10.7554/eLife.01776.011

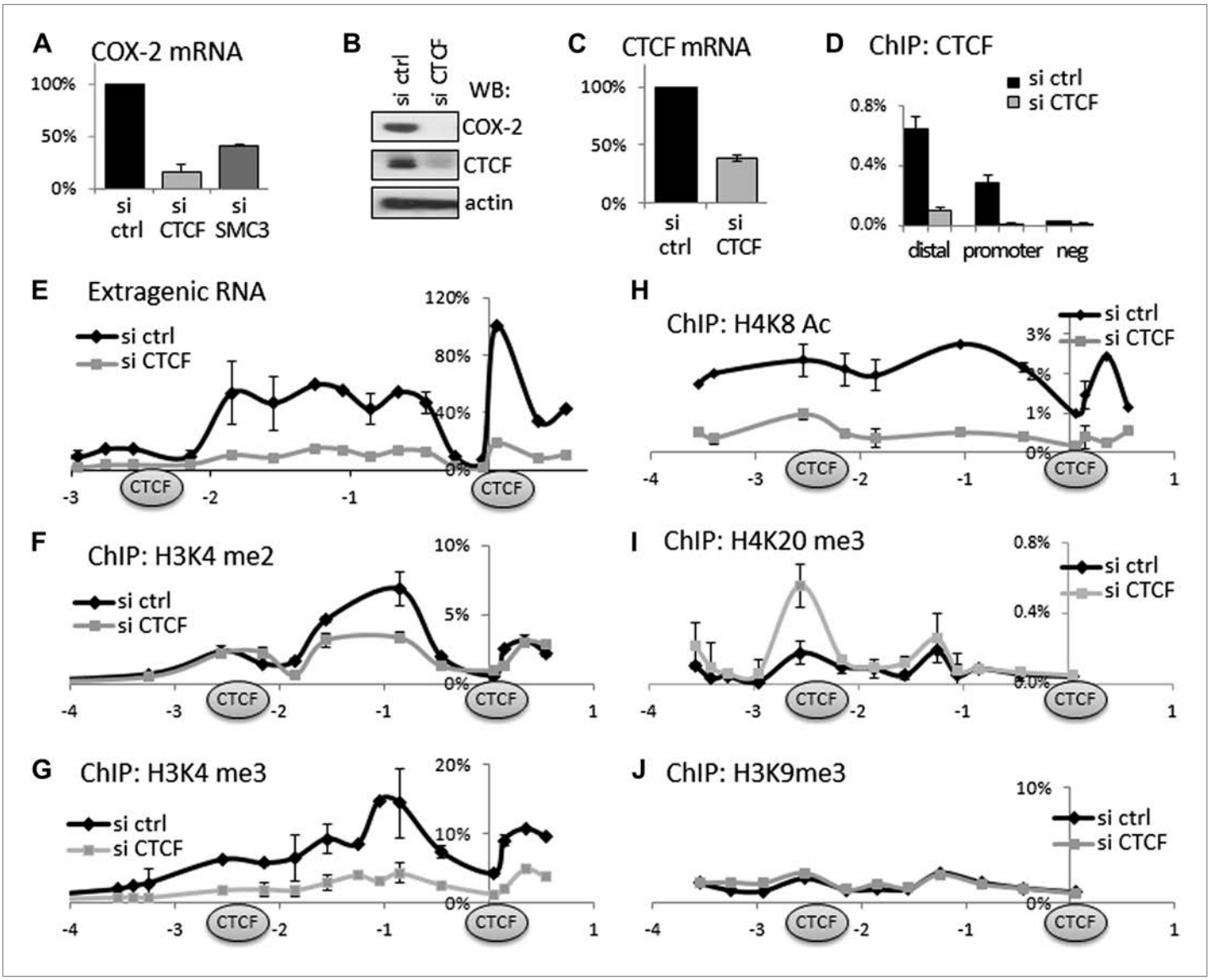

Figure 3. CTCF and cohesin maintain COX-2 mRNA and PACER lncRNA expression by demarcating a chromatin domain that is characterized by decreased H4K20 trimethylation, increased H3K4 di- and tri- methylation and increased histone acetylation. (**A**) siRNA-mediated knockdown of CTCF or a cohesin subunit SMC3 reduces COX-2 mRNA levels in HMECs. RT-qPCR was performed with RNA prepared from HMECs 72 hr after transfection with the indicated siRNAs. Signals were normalized with 18S rRNA, TBP and GAPDH genes. (**B**) Western blot analysis of COX-2 and CTCF levels upon siRNA-mediated knockdown of CTCF. (**C**) CTCF mRNA levels in control and siRNA-CTCF transfected HMECs. (**D**) Binding of CTCF to the COX-2 promoter and distal sites were analyzed by ChIP-qPCR in control and CTCF knockdown cells. (**E**) Transcription across the *COX-2* locus was measured by RT-qPCR in control (black diamonds) or siCTCF-transfected HMECs (grey squares). Signals were normalized as above and re-normalized using genomic DNA. (**F**–**J**) Levels of activation-associated (H3K4me2 and me3, H4K8Ac) and repression-associated (H4K20me3, H3K9me3) histone modifications were measured using ChIP-qPCR in HMECs transfected with control siRNAs (black lines) or siRNA against CTCF (grey lines). Each diamond or square denotes a qPCR amplicon. CTCF/cohesin binding sites are marked with oval shapes. X-axis scale is relative to the COX-2 transcription start site.

DOI: 10.7554/eLife.01776.012

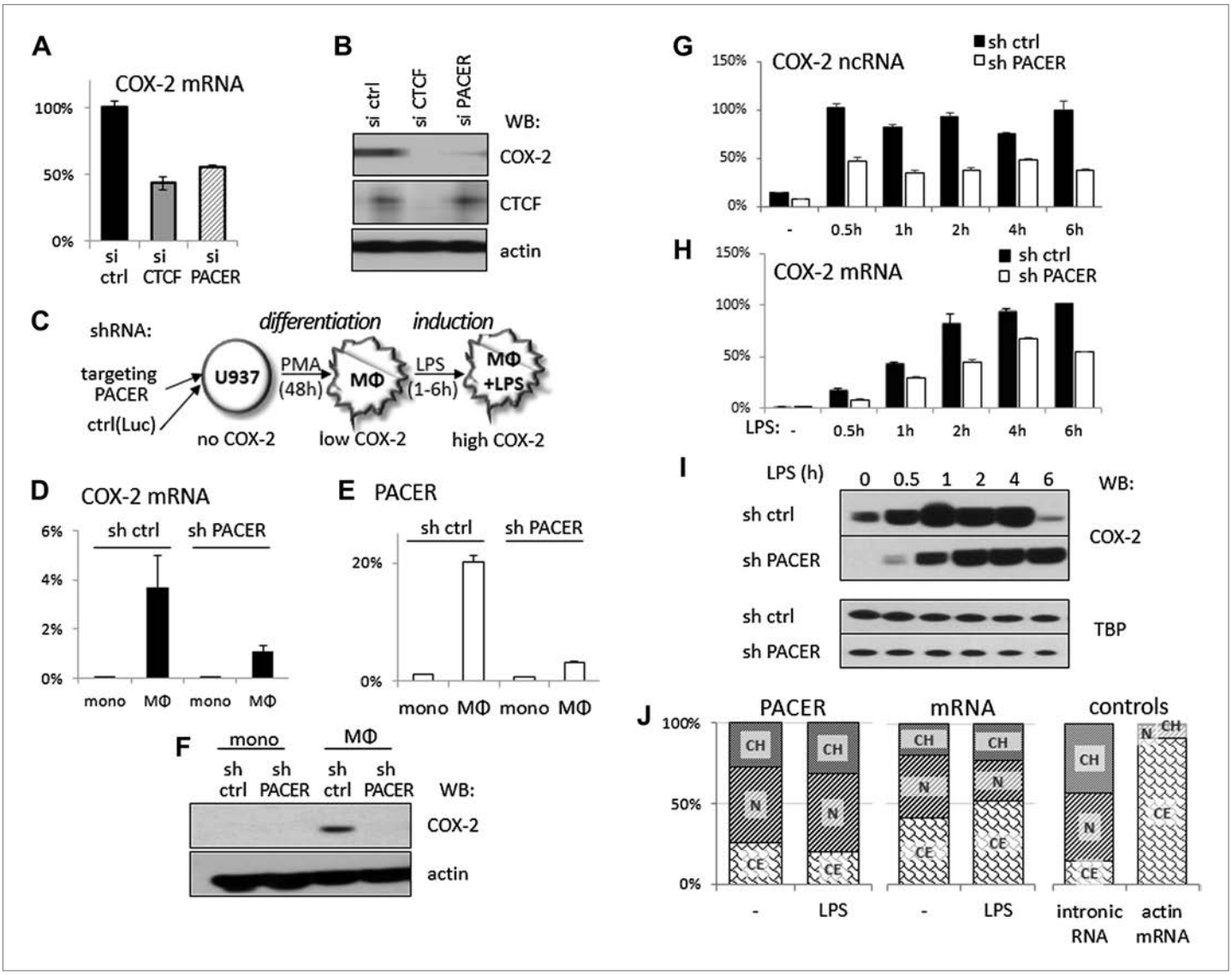

Figure 4. The antisense PACER lncRNA is a positive regulator of COX-2 expression in human mammary epithelial cells and in monocyte-derived macrophages before and after LPS stimulation. Levels of COX-2 mRNA (**A**) and protein (**B**) were measured by RT-qPCR and Western blotting in control-scrambled siRNA, siRNA-CTCF, and siRNA-lncRNA transfected HMECs 72 hr post transfection. (**C**) Schematic representation of the monocyte-macrophage system used in this study. The human monocyte cell line, U937, was differentiated to macrophages with PMA and induced to express high levels of COX-2 by LPS stimulation. (**D** and **E**) COX-2 mRNA (**D**) and PACER (**E**) levels were measured by RT-qPCR in U937 lines carrying stably integrated control shRNA or shRNA-PACER expressing constructs before and after differentiation into macrophages. Values were normalized using 18S rRNA, TBP, and ß-globin genes. (**F**) COX-2 protein expression analyzed by Western blotting in the same experiment. (**G** and **H**) COX-2 ncRNA and mRNA levels were measured in a time course of LPS stimulation in control U937 (black bars) and PACER knockdown U937 macrophages (white bars). Values are relative to maximal COX-2 expression in control U937 cells after 6 hr LPS stimulation. (**I**) COX-2 protein expression analyzed by Western blotting in the same experiment. TBP served as a control. (**J**) Subcellular localization of COX-2 mRNA, PACER lncRNA and controls: nuclear intronic RNA and exclusively cytoplasmic actin mRNA. Relative levels in each fraction were measured by RT-qPCR and plotted such that they add up to 100%. CH, chromatin-bound fraction, N, nucleoplasm, CE, cytoplasm.

DOI: 10.7554/eLife.01776.013

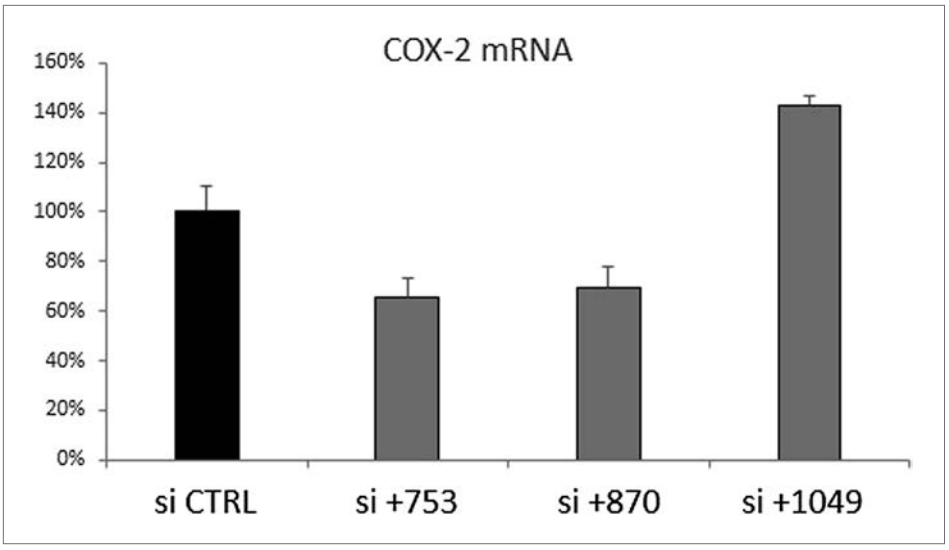

Figure 4—figure supplement 1. Knockdown of COX-2 mRNA with siRNA targeting PACER (si+753 and si+870) or positioned outside PACER (+1049).

DOI: 10.7554/eLife.01776.014

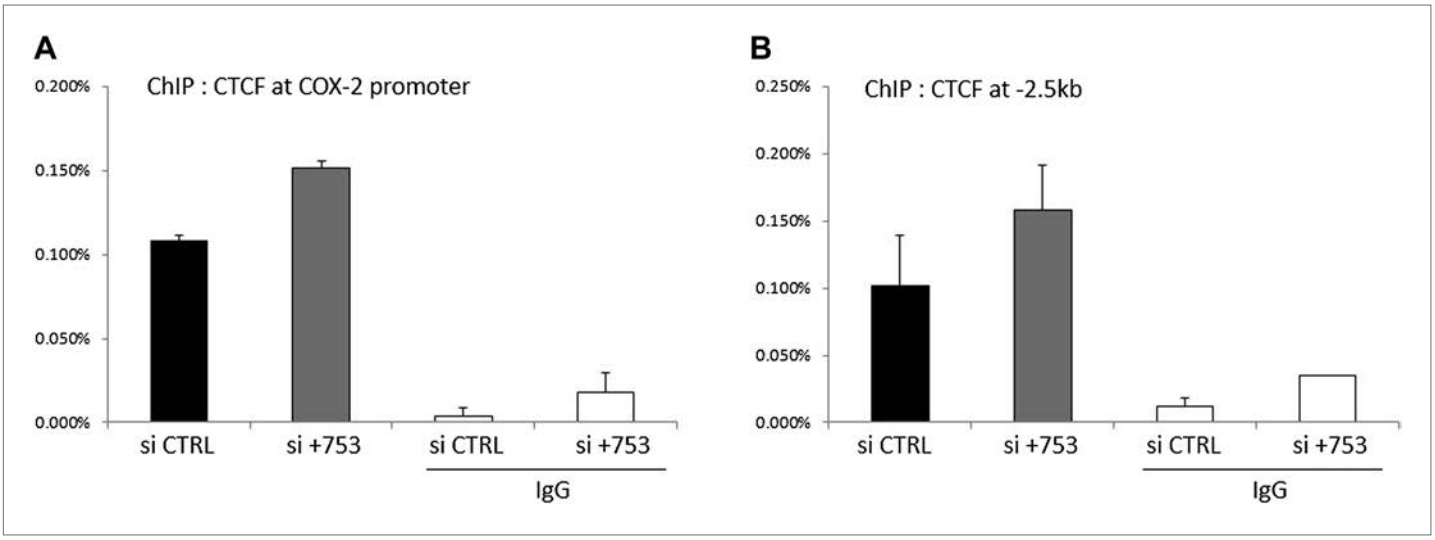

Figure 4—figure supplement 2. Binding of CTCF to the COX-2 promoter upon PACER knock-down in HMECs.

DOI: 10.7554/eLife.01776.015

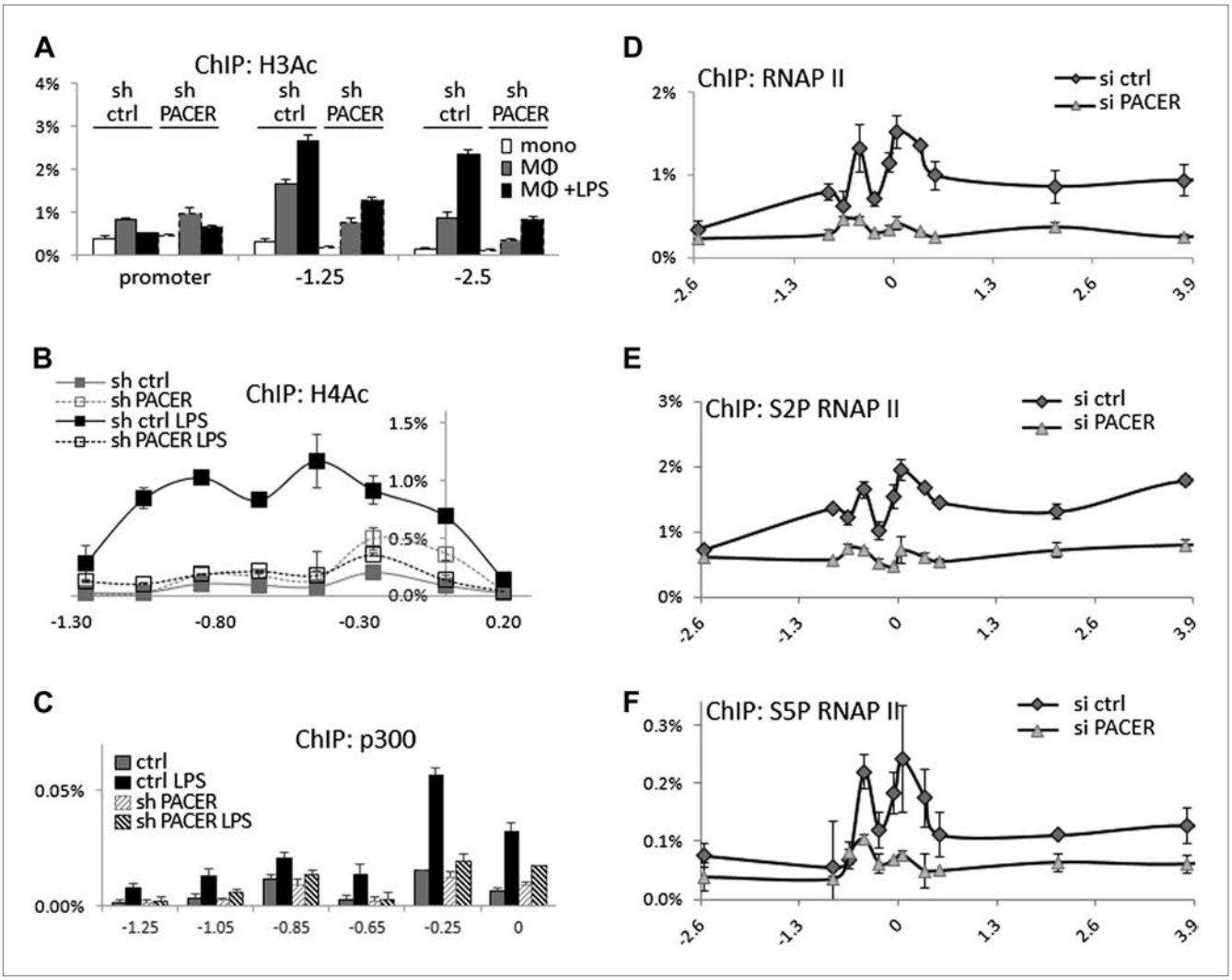

Figure 5. PACER facilitates recruitment of p300 HAT and RNAP II pre-initiation complexes to increase histone acetylation and induce *COX-2* transcription. (**A**) ChIP-qPCR was used to measure levels of histone H3 acetylation in control U937 and sh-lncRNA U937 monocytes (white bars), macrophages (grey-bars) and LPS-stimulated macrophages (black bars) at the COX-2 promoter, −1.25 kb upstream and −2.5 kb upstream of the COX-2 transcription start site. (**B**) ChIP-qPCR analysis of histone H4 acetylation across the COX-2 upstream region in control and sh-PACER U937 macrophages before and after LPS stimulation. (**C**) Association of p300 with the COX-2 upstream region was analyzed by ChIP-qPCR in control (filled squares) and sh-PACER (open squares) U937 macrophages before and after LPS stimulation. (**D**–**F**) Association of bulk RNAP II (**D**), S2-phosphorylated (**E**) and S5-phosphorylated (**F**) RNAP II across the *COX-2* locus analyzed by ChIP-qPCR in control HMECs (diamonds) or PACER-knockdown (triangles) HMECs. Association of RNAP II with the *COX-2* locus in control or siRNA-PACER transfected HMECs was assayed using ChIP-qPCR 72 hr post transfection. X-axis scale is relative to the COX-2 transcriptional start site.

DOI: 10.7554/eLife.01776.016

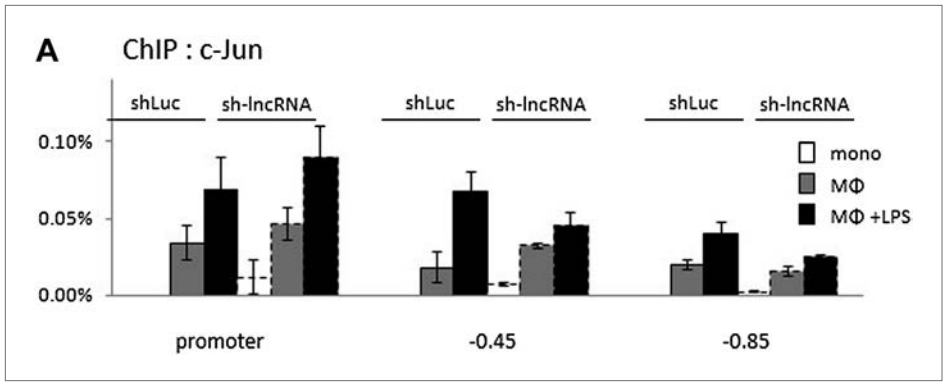

Figure 5—figure supplement 1. Binding of c-Jun to the COX-2 region upon PACER knock-down in U937 monocytes, macrophages, and LPS-stimulated macrophages analyzed by ChIP.

DOI: 10.7554/eLife.01776.017

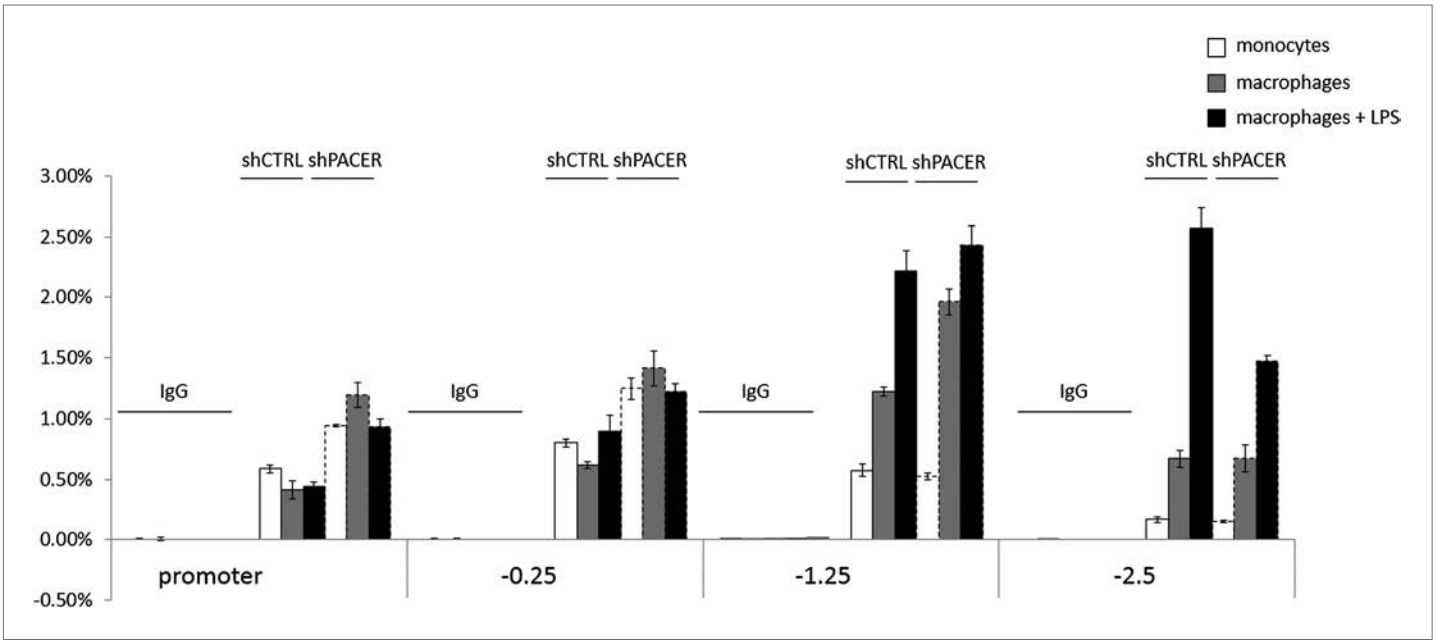

Figure 5—figure supplement 2. Levels of H3K4 dimethylation at COX-2 region upon PACER knock-down in U937 monocytes, macrophages, and LPS-stimulated macrophages analyzed by ChIP.

DOI: 10.7554/eLife.01776.018

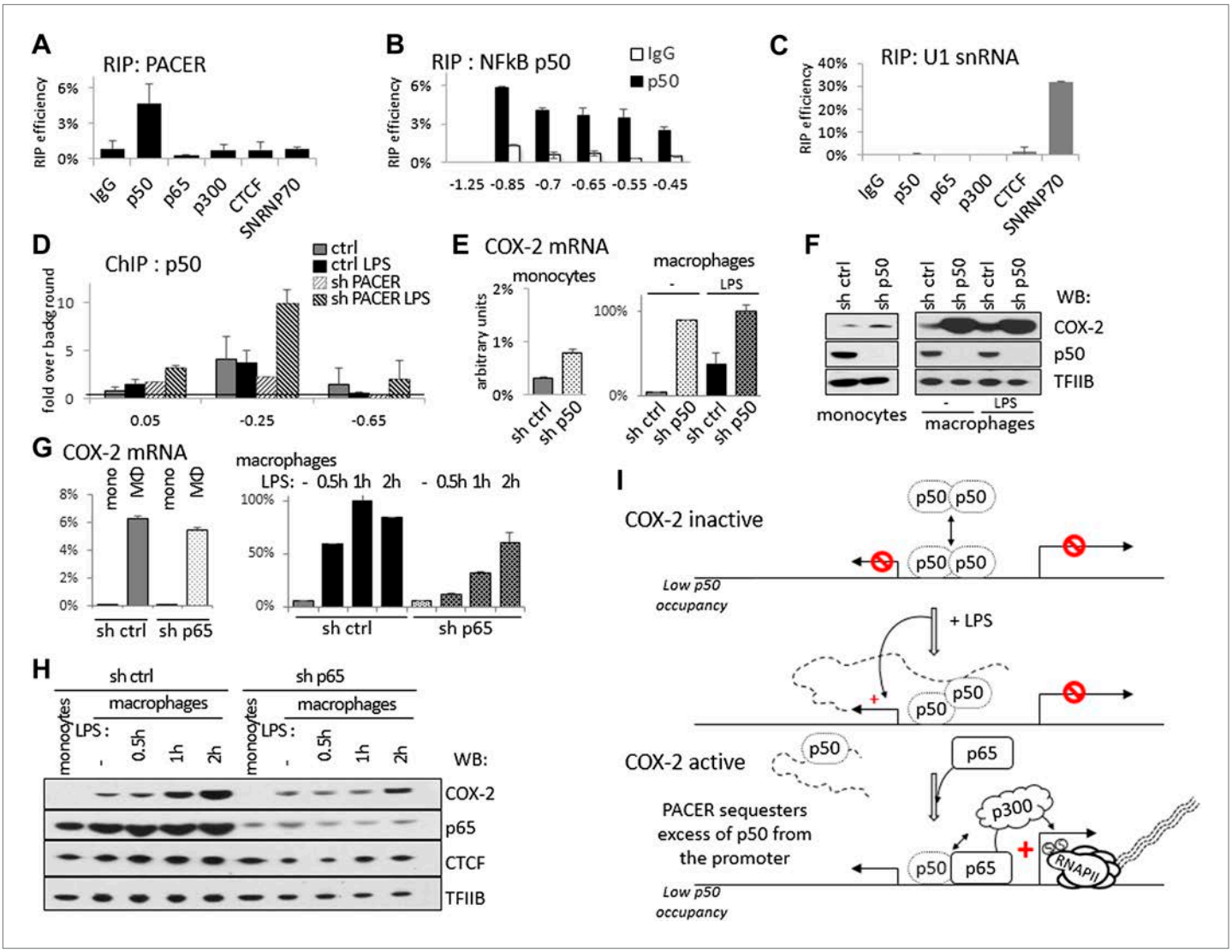

Figure 6. PACER controls *COX-2* expression through binding of the repressive NF-κB subunit p50. (**A**) Direct association of p50, p65/RelA, p300, and CTCF with PACER analyzed by RNA immunoprecipitation (RIP) in U937 macrophages. Primers for amplicon −0.85 kb were used. Signal is detected only in p50 immunoprecipitates. (**B**) Direct association of p50 with PACER analyzed by RNA immunoprecipitation (RIP) in U937 macrophages. Sites −0.45 kb through −0.85 kb lie within the lncRNA, while site −1.25 kb is outside and shows no detectable signal. IgG immunoprecipitation is shown as a control. (**C**) Control RIP experiments using the same antibodies as in (**A**) to analyze association with U1 snRNA. (**D**) Association of a small NF-κB subunit p50 with the COX-2 control region was analyzed by ChIP-qPCR in control and sh-lncRNA U937 macrophages. Binding is evident at the −0.25 kb site. (**E** and **F**) Expression of COX-2 mRNA (**E**) and protein (**F**) analyzed in control U937 monocytes and in mock and LPS-stimulated U937 macrophages and upon p50 knockdown. (**G** and **H**) Expression of COX-2 mRNA (**G**) and protein (**H**) analyzed in control U937 monocytes and in mock and LPS-stimulated U937 macrophages and upon p65 knockdown (**I**). A model of PACER-controlled COX-2 activation involving restricting promoter interaction of repressive p50 to facilitate recruitment of p300 HAT and RNAP II pre-initiation complexes to activate mRNA transcription.

DOI: 10.7554/eLife.01776.019