Supplementary Material

Nascent transcript analysis of glucocorticoid crosstalk with TNF defines primary and cooperative inflammatory repression

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Running title: Glucocorticoid-mediated inflammatory repression

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Supplemental Methods

Cell culture

BEAS-2B cells (ATCC) were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Corning) containing L-glutamine and 4.5 g/L glucose and supplemented with 10% fetal bovine serum (VWR) and 1% penicillin/streptomycin (Corning). For lentiviral production, following transfection and 72 hr incubation, viral supernatant was collected from HEK cells, centrifuged at 1000 rpm for 10 min at room temperature and filtered with a sterile 0.45 um syringe filter. For transduction, BEAS-2B cells were plated on 10 cm tissue culture dishes and grown to confluence. Viral transduction was performed using a 1:1 ratio of viral supernatant to standard growth media along with 8 ug/ml polybrene. Cells were incubated for 24 hr and then transduced a second time, followed by antibiotic selection with puromycin (1 ug/ml) for 24-48 hr.

Reagents and Western blotting

Dexamethasone (dex; Sigma-Aldrich) was dissolved in sterile 100% ethanol (vehicle) and used at a final concentration of 100 nM. Recombinant Human TNF-alpha Protein purchased from R&D Systems was diluted in 1 × Dulbecco’s phosphate buffered saline (DPBS) containing 0.1% bovine serum albumin (BSA) and used at a final concentration of 20 ng/ml. Primary antibodies for Western blotting included mouse anti-TNFAIP3 [59A426] (ab13597), rabbit anti-NFKBIA [E130] (ab32518) and rabbit anti-Beta-Tubulin (ab52901) from Abcam, anti-GAPDH [FL-335] (sc-25778) from Santa Cruz Biotechnology, rabbit anti-CEBPB (PA5-27244) from Thermo Fisher Scientific, and anti-Lamin B1 [D4Q4Z] (12586S) from Cell Signaling Technology. Secondary antibodies were ECL sheep anti-mouse IgG-HRP (95017-332) and donkey anti-rabbit IgG-HRP (95017-330) from GE/Amersham. Western blotting and protein detection were performed using standard procedures (Sasse et al. 2016). Antibodies for GR ChIP-seq were GR-IA1 (Sasse et al. 2017) and GR-356, which was raised in rabbit against the identical epitope as IA1 (QPDSL KAVSLSMGYMETKVMGNDLG) and prepared by Covance.

Plasmids, transfection and reporter assays
Genomic enhancer regions of interest identified by GRO-seq were PCR-amplified, inserted into pCR2.1-TOPO (Life Technologies), then introduced into the pNL3.2 [NlucP/minP] minimal promoter-driven NanoLuc luciferase expression vector from Promega. Sequences of PCR primers used for cloning are as follows:

| Primer       | Forward Sequence (5’ to 3’) | Reverse Sequence (5’ to 3’) |
|--------------|-----------------------------|-----------------------------|
| pNL3.2-PNRC1 | CTACCACTGCTGGGTGTTTT        | GCCTGGGATTACTGGAGTTT        |
| pNL3.2-PGM3  | ATTCACTACCACGAGAAGTATGG     | TGCAGCTGCTGTCAATAAGT        |
| pNL3.2-IER3  | GTCTCCCCATGTTGCTCAA         | GGAAGAAATGACCAAGTGCTTG     |
| pNL3.2-NGF   | AGTGCCCTTTCCAGGGTAATAA      | GGAAGAAATTCCAGCTCTCATC      |
| pNL3.2-ANGPTL4 | CACAAGTGTCTGATGGTAAAG    | CACACAGGCCTAGAAGGTATAG     |
| pNL3.2-ZBTB16 | CCTCTTCCCTGAATCTGTGTATC   | CCCAGGATCCCCAATGACAAATAA   |

The pNL3.2-FKBP5 construct was generated by excising the insert from our previously described FKBP5 reporter in the pGL3-Promoter backbone (Altonsy et al. 2014) using KpnI/BglII and ligating into the pNL3.2 vector cut with KpnI/BglII. All constructs were verified by Sanger DNA Sequencing provided by Eton Biosciences and will be made available through Addgene.

For transfection experiments, BEAS-2B cells were plated in fresh complete medium on 6 cm tissue culture dishes at 3E6 cells/dish. The next day, each dish was transfected with an 11:1 ratio (8 ug total DNA) of NanoLuc luciferase construct to pGL4.13[luc2/SV40] firefly luciferase expression vector (Promega) as internal control, respectively, complexed with 20 ul Lipofectamine 2000 (Life Technologies). Medium was changed with fresh complete medium 4-6 hours later and cells were incubated overnight. The next day, cells were detached from each dish by trypsinization and plated at 75K cells/well in 48-well plates in fresh complete medium. Approximately 20 hours later, cells were treated with vehicle, TNF, dex, or TNF+dex in fresh complete medium for 1, 2, or 4 hours. Cells were briefly rinsed in 1 × DPBS and lysed in 1 × Passive Lysis Buffer (Promega) for at least 15 min prior to measuring firefly and NanoLuc luciferase activities with a Tecan Infinite M1000 plate reader using the Nano-Glo Dual-Luciferase Reporter Assay System (Promega) with the following modifications to the manufacturer’s protocol: 10 ul of lysate was combined with 40 ul of ONE-Glo EX Reagent for the first measurement, then 40 ul of NanoDLR Stop & Glo Reagent were added for the second measurement.
Global Run-on Sequencing (GRO-seq)

*Treatment and nuclear isolation.* BEAS-2B cells were plated on 15 cm tissue culture dishes at 3.75E6 cells/dish (2 plates per treatment per time-point) and grown to confluence (~3 days). Cells were treated with vehicle, TNF, dex, or TNF+dex for exactly 10 or 30 minutes. Plates were washed 3 times in ice-cold 1X PBS and cells were then scraped in ice-cold Lysis Buffer (10 mM Tris-HCl pH 7.5, 2 mM MgCl$_2$, 3 mM CaCl$_2$, 0.5% Igepal, 10% glycerol, 1 mM DTT, 1× EDTA-Free Protease Inhibitor Cocktail (Thermo Fisher Scientific), 4 U/ml SUPERase-In RNase Inhibitor (Invitrogen)). Cells from each treatment (2 plates per treatment per time-point) were pooled, transferred to 50 ml conical tubes, and centrifuged in a fixed-angle rotor at 1000 × g for 15 min at 4°C. Cell pellets were resuspended in fresh Lysis Buffer and centrifuged under the same conditions as above. Pellets were next resuspended in 1 ml of Lysis Buffer and transferred to 1.7 ml low binding microcentrifuge tubes (Corning). Suspensions were pelleted at 1000 × g for 5 min at 4°C, after which pellets were resuspended in 500 ul of Freezing Buffer (50 mM Tris-HCl pH 8.3, 5 mM MgCl$_2$, 40% glycerol, 0.1 mM EDTA pH 8.0, 4 U/ml SUPERase-In). Nuclei were pelleted at 2000 × g for 2 min, then resuspended in ~100 ul of fresh Freezing Buffer. Nuclei from each treatment were counted from 2 ul of suspension diluted in 1 × PBS. Freezing Buffer was then added to generate 4 × 100 ul aliquots containing 10E6 nuclei for each treatment, and samples were flash frozen in a dry ice/ethanol bath and stored at -80°C.

*Nuclear run-on and total RNA extraction.* After briefly thawing on ice, duplicate 100 ul aliquots of nuclei for each treatment and time-point were added to 100 ul of Reaction Buffer (10 mM Tris-HCl pH 8.0, 5 mM MgCl$_2$, 1 mM DTT, 300 mM KCl, 0.5 mM each rATP, rGTP, rCTP, and 5-bromouridine 5’-triphosphate (Br-UTP; Sigma), 1% Sarkosyl, 20 Units SUPERase-In) pre-heated to 30°C and incubated for exactly 5 min at 30°C. Reactions were stopped by addition of 1 ml of TRIzol Reagent.
(Invitrogen) and samples were completely homogenized by vortexing. To isolate RNA, 100 ul of chloroform were added and samples were centrifuged at max speed for 15 min at room temperature. The aqueous phase was transferred to a fresh tube and RNA was extracted with room temperature acid phenol/chloroform (Invitrogen) once, then chloroform-extracted once, and finally precipitated overnight at -20°C in 3 volumes 100% ethanol containing 1 ul GlycoBlue (Invitrogen).

**RNA fragmentation and DNase treatment.** RNA was pelleted by centrifugation at max speed for 20 min at room temperature, washed in 75% ethanol, then briefly air-dried prior to resuspension in nuclease-free water. RNA was fragmented to ~200 bp fragments by addition of NEBNext RNA Fragmentation Buffer (part of the Magnesium RNA Fragmentation Module from New England Biolabs) and incubation at 94°C for 5 min. Samples were purified over Bio-Rad P30 Buffer Exchange Columns as instructed by the manufacturer, then added to DNase reactions (1 × RQ1 DNase Buffer (Promega), 2 Units RQ1 RNase-Free DNase (Promega)) and incubated at 37°C for 10 min. Reactions were stopped by addition of DNase Stop Solution (Promega) and incubation at 65°C for 5 min, then tubes were chilled on ice.

**Br-UTP-RNA enrichment and nascent RNA extraction.** All anti-Br-UTP agarose bead (Santa Cruz) washes were performed in 500 ul of buffer for 5 min at room temperature with end-over-end rotation at ~15 rpm. Following each wash, samples were centrifuged at 1000 × g for 2 min at room temperature and supernatant carefully removed. Beads (60 ul/sample) were initially washed twice in Binding Buffer (0.25 × SSPE, 37.5 mM NaCl, 0.05% Tween-20, 1 mM EDTA, 0.004 U/ul SUPERase-In), after which 500 ul of Blocking Buffer (Binding Buffer supplemented with 0.1% PVP, 1 ug/ml BSA, and 0.04 U/ul SUPERase-In) were added and tubes were incubated for 1 hour with end-over-end rotation. Beads were pelleted as described above, washed twice in Binding Buffer, resuspended in Binding Buffer and then combined with RNA samples from above and incubated for 1 hr at room
temperature with end-over-end rotation. Beads now bound to nascent RNA were next washed in Binding Buffer, Low Salt Wash Buffer (0.2 \times \text{SSPE}, 1 \text{mM EDTA}, 0.05\% \text{ Tween-20}, 0.004 \text{ U/ul SUPERase-In}), High Salt Wash Buffer (0.25 \times \text{SSPE}, 1 \text{mM EDTA}, 0.05\% \text{ Tween-20}, 137.5 \text{ mM NaCl}, 0.004 \text{ U/ul SUPERase-In}; this wash only lasted \textasciitilde5 \text{sec}), and twice in TET Buffer (1 \text{mM EDTA}, 0.05\% \text{ Tween-20}, 10 \text{mM Tris-}\text{HCl pH 8}, 0.004 \text{ U/ul SUPERase-In}), after which nascent RNA was eluted from the beads 4 consecutive times by incubation in Elution Buffer (150 \text{mM NaCl}, 50 \text{mM Tris-}\text{HCl pH 7.5}, 20 \text{mM DTT}, 0.1\% \text{ SDS}, 1 \text{mM EDTA}, 0.004 \text{ U/ul SUPERase-In}) at 42^\circ\text{C}. RNA was then extracted with room temperature acid phenol/chloroform once, chloroform-extracted once, and finally precipitated overnight at -20^\circ\text{C} in 3 volumes 100\% \text{ ethanol containing 1 ul GlycoBlue and 300 mM NaCl. The next day, RNA was pelleted, washed in 75\% \text{ ethanol, then briefly air-dried prior to resuspension in nuclease-free water. Fresh aliquots of anti-Br-UTP beads were washed and blocked for each sample and then combined with resuspended RNA for a second round of nascent RNA enrichment and extraction, performed exactly as detailed above for the first round. RNA was again precipitated overnight at -20^\circ\text{C} in 3 volumes 100\% \text{ ethanol containing 1 ul GlycoBlue and 300 mM NaCl. The next day, RNA was pelleted, washed in 75\% \text{ ethanol, then briefly air-dried prior to resuspension in nuclease-free water.}

\textit{Strand-specific RNA library preparation and sequencing.} The NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs) was used according to the Protocol for use with NEBNext rRNA Depletion Kit provided by the manufacturer to generate libraries for sequencing. Specifically, random priming was performed by incubating nascent RNA with supplied random hexanucleotide primers at 65^\circ\text{C} for 5 min. Primed RNA was then added to First Strand cDNA Synthesis reagents (supplied) containing 0.5 \text{ug of freshly thawed Actinomycin D (Sigma-Aldrich) and incubated for 10 min at 25^\circ\text{C, 15 min at 42^\circ\text{C, and 15 min at 70^\circ\text{C. Reaction products were combined with Second Strand cDNA Synthesis reagents (supplied) and incubated for 1 hr at 16^\circ\text{C. Double-stranded cDNA}}}}

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was next purified using Mag-Bind RxnPure Plus Beads (Omega Bio-tek) at a relative volume of 1.8X, as instructed by the NEBNext Kit protocol, and eluted in 0.1 × TE Buffer. Purified double-stranded cDNA was then combined with supplied End Repair reagents and incubated for 30 min at 20°C followed by 30 min at 65°C in order to generate 5’-phosphorylated dA-tailed ends. The NEBNext Adaptor from the NEBNext Multiplex Oligos for Illumina Kit (New England Biolabs) was diluted 30-fold in 10 mM Tris-HCl containing 10 mM NaCl and added to the end-repaired products together with the supplied Blunt/TA Ligase Master Mix and incubated 15 min at 20°C. Adaptor-ligated products were then purified using Mag-Bind RxnPure Plus Beads at a relative volume of 1X, as instructed by the NEBNext Kit protocol, and eluted in 0.1 × TE Buffer. Purified adaptor-ligated cDNA was next combined with supplied PCR Enrichment reagents plus Universal PCR Primer and a single NEBNext Index Primer from the NEBNext Multiplex Oligos for Illumina Kit to uniquely barcode the libraries and cycled as follows: 1 cycle at 37°C for 15 min, 1 cycle at 98°C for 30 sec, 15 cycles of 98°C for 10 sec and 65°C for 75 sec, 1 cycle at 65°C for 5 min, then hold at 4°C. Amplified libraries were purified using Mag-Bind RxnPure Plus Beads at a relative volume of 0.9×, as instructed by the NEBNext Kit protocol, and eluted in 0.1 × TE Buffer. Libraries were then pooled and sequenced on an Illumina HiSeq instrument using 50 bp single-end reads by the Microarray and Genomics Core at the University of Colorado-Denver.

**GRO-seq computational analysis**

*Identifying regions with bidirectional transcriptional activity.* FStitch (v. 1.0) was first used to filter regions of inactive transcription in all samples to lessen downstream compute processing requirements. FStitch train was used with arguments ‘-r (sample.positiveStand.bedGraph) -t (trainingFile.bed) -o (outputTrainingParameters.hmminfo)’ using the IGV (v. 2.3.75) Region Navigator tool to produce a BED format training file generated from one of the 10 min dex-treated samples. This sample was chosen for training as it was determined to be most representative of the data in terms of
read coverage, complexity, and total reads mapped. All relevant analysis files are included in Supplemental File S7. FStitch segment was then run on all single-strand non-normalized bedGraph files with arguments ‘-r (sample.pos.bedgraph) -w (outputTrainingParameters.hmminfo) -o (sample.bed)’. Positive and negative strand files were then concatenated and sorted using sortBed in the BEDTools suite (v. 2.25.0). Following FStitch processing, Tfit (v. 1.0) was used to generate the predictions of bidirectionals/eRNAs. BED file annotations of preliminary bidirectional calls were generated using the Tfit bidir old module with arguments ‘-ij (sample.concatenated.bedGraph) -N (bidir old) -k (sample.concatenatedFStitch.bed) -mle 1 -o (output directory) -logout (output directory) -tss (hg38 RefSeq gene transcription start site file)’. Using the bidirectional predictions file, the Tfit model module was run to get higher confidence calls with arguments ‘-ij (sample.concatenated.bedGraph) -k (preliminaryBidirectionals.bed) -N (tfit mod) –logout (output directory) -o (output directory) -config (config file)’ to produce a final BED annotation file of predicted bidirectionals.

**ChIP-seq computational analysis**

*Data processing, visualization and peak calling*

For both antibodies (GR-IA1 and GR-356) at the 1 hour timepoint, two biological paired-end sequencing replicates for each treatment (veh, TNF, dex, and TNF+dex) for both shCtrl- and shGR-transduced cells were processed through a standardized ChIP-seq data pipeline. Samples were trimmed for adapters, minimum length, and minimum quality using the BBMap Suite (v. 38.05) bbduk.sh tool with arguments ‘ref = adapters.fa ktrim = r qtrim = 10 k = 23 mink = 23 hdist = 1 maq = 10 minlen= 20 tpe tbo’. Quality of each sample was assessed both pre- and post-trim by FastQC (v. 0.0.13). The FastQC reports suggested the ChIP samples may be contaminated with non-human DNA due to the appearance of two distinct G/C distribution peaks centered at 42% and 68% mean G/C content (Input control samples exhibited a normal distribution). To resolve these two peaks in the ChIP samples, bbsplit.sh (part of the BBMap Suite v. 38.05) was used with argument ‘ref = hg38.fa’ to
separate reads that mapped to hg38 from those that did not. NCBI BLAST was then used to
determine the organism responsible for the unmapped reads, which indicated that the majority of
these reads mapped to the Pseudomonas aeruginosa genome. To confirm P. aeruginosa was the
source of contamination, the P. aeruginosa genome strain F5677 was downloaded from NCBI (June-
29-2018, NCBI Reference Sequence: NZ CP026680.1) and included as an additional reference
genome in the bbsplit.sh module with the argument ‘ref = hg38.fa, p aeruginosa f5677’. Across all
ChIP samples, a mean number of 380706 reads mapped to the P. aeruginosa genome. While this
only accounts for a small fraction of total reads sequenced, there are 427 genome strain variants
reported by NCBI. Thus, the clear separation of G/C distribution peaks between reads mapping to
hg38 (centered at 42% G/C) and those either mapping to P. aeruginosa or remaining unmapped
(centered at 68% G/C) suggests the majority of the remaining unmapped reads likely correspond to a
different strain of P. aeruginosa. As samples were sequenced deeply in this study, an average of
22.7M reads for each paired-end run still remained even after contaminants were removed. Further,
two replicates per treatment condition were sequenced in this experiment, resulting in an average of
39.1M mapped reads per treatment condition, which was more than sufficient for downstream
sequencing analysis. The remaining hg38-specific reads were mapped to hg38 using hisat2 (v. 2.1.0)
with argument ‘--no-spliced-alignment’ using indexes built with hisat2-build (same version), which
resulted in over 91.6% mapping for all ChIP and Input control samples. The full data for percent G/C,
reads split to each genome, the remaining FastQC statistics, reads mapping to hg38, and complexity
and coverage information is included in a complete report generated by MultiQC (v. 1.6) in
Supplemental File S8. Resulting SAM files were converted to sorted and indexed BAM files using
samtools (v. 1.8) view, sort, and index, respectively. Complexity for each sample was determined
using Preseq (v. 2.0.3) c curve and lc extrap modules with argument ‘-B’. Read distributions across
the hg38 genome were calculated using RSeQC read distribution.py using the same RefSeq
reference annotation file that was used for differential transcription analysis. Sorted BAM files were
converted to bedGraph format using genomeCoverageBed from bedtools (v. 2.25.0) with arguments
‘-bg -ibam -g hg38’, read coverage was normalized to counts per million mapped, and bedGraphs were converted to TDF format using igvtools (v. 2.3.75) for visualization in IGV.

The 10 minute ChIP-seq data were processed using a Nextflow pipeline (https://github.com/Dowell-Lab/ChIP-Flow). A full pipeline report of the run as well as a quality control report generated by MultiQC (v. 1.7), including trimming, mapping, coverage, and complexity metrics, is included in Supplemental File S9.

For each ChIP-seq dataset, MACS2 (v. 2.1.1) callpeak with ‘--SPMR’ argument was applied to each biological replicate pair of ChIP and respective Input control samples to call significant peaks (Supplemental File S4 and S10 for 10 minute and 1 hour datasets, respectively).

qRT-PCR

shCtrl- and shGR-transduced BEAS-2B cells were plated at 500K cells/well in 6-well tissue culture dishes and treated with vehicle, TNF, dex, or TNF+dex on the following day for 1, 2, or 4 hours in biologic quadruplicate. RNA preparation and qRT-PCR were performed as previously described with normalization to RPL19 (Sasse et al. 2013). Primer sequences are listed in Supplemental Table S4.

ChIP-qPCR

shCtrl-transduced BEAS-2B cells were cultured, harvested, and immunoprecipitated in biologic quadruplicate using 12 ug of GR-356 antibody as described for ChIP-seq. Purified ChIP DNA was subjected to qPCR using primer sequences provided in Supplemental Table S5 and relative GR occupancy was calculated as described previously (Sasse et al. 2013).

REFERENCES

Altonsy MO, Sasse SK, Phang TL, Gerber AN. 2014. Context-dependent cooperation between nuclear factor kappaB (NF-kappaB) and the glucocorticoid receptor at a TNFAIP3 intronic enhancer: a mechanism to maintain negative feedback control of inflammation. J Biol Chem 289: 8231-8239.

Sasse SK, Altonsy MO, Kadiyala V, Cao G, Panettieri RA, Jr., Gerber AN. 2016. Glucocorticoid and TNF signaling converge at A20 (TNFAIP3) to repress airway smooth muscle cytokine expression. Am J Physiol Lung Cell Mol Physiol 311: L421-432.

Sasse SK, Kadiyala V, Danhorn T, Panettieri RA, Jr., Phang TL, Gerber AN. 2017. Glucocorticoid Receptor ChIP-seq Identifies PLCD1 as a KLF15 Target that Represses Airway Smooth Muscle Hypertrophy. Am J Respir Cell Mol Biol doi:10.1165/rcmb.2016-0357OC.
Sasse SK, Mailloux CM, Barczak AJ, Wang Q, Altonsy MO, Jain MK, Haldar SM, Gerber AN. 2013. The glucocorticoid receptor and KLF15 regulate gene expression dynamics and integrate signals through feed-forward circuitry. *Mol Cell Biol* 33: 2104-2115.
Table S2: t-test comparisons of the log2 normalized enrichment within GR-IA1 vehicle and dex ChIP-seq data subsets that co-localize with GRO-seq defined enhancer activity as indicated.

| ChIP-seq subset comparisons                             | t-statistic | p-value   |
|---------------------------------------------------------|-------------|-----------|
| IA1_shCtrl_V_down_vs_IA1_shCtrl_D_down*                 | 13.965      | 1.80E-37  |
| IA1_shGR_V_down_vs_IA1_shGR_D_down                      | -1.553      | 1.21E-01  |
| IA1_shCtrl_V_up_vs_IA1_shCtrl_D_up**                    | 4.164       | 3.31E-05  |
| IA1_shGR_V_up_vs_IA1_shGR_D_up                          | -10.379     | 3.59E-24  |
| IA1_shCtrl_V_up_vs_IA1_shCtrl_V_down                    | -7.497      | 1.48E-13  |
| IA1_shCtrl_D_up_vs_IA1_shCtrl_D_down                    | 6.601       | 6.58E-11  |

* “down” signifies co-localized enhancers that had decreased activity with dex.
** “up” signifies co-localized enhancers that had increased activity with dex.
Supplemental Table S3. Sequences of qPCR primers used to span enhancers of interest in MNase chromatin accessibility assay.

|          | **PTPRK** Forward Sequence (5' to 3') | **Reverse Sequence (5' to 3')** |
|----------|---------------------------------------|----------------------------------|
| Pair 1   | CTCTGACTAGTGGGTAGAGAAA                | GGAAACTCTCTGAGGATGGA             |
| Pair 2   | CTATTCTTGCCCTGTGAAA                   | GGGTATTTCGTGAGGAAACT             |
| Pair 3   | TCCATCCTCAGAGAGTTTCC                 | TCTTCAAGGATCTCCCTTC             |
| Pair 4   | AAGGGAGATCTCCAGAGTAAGAT              | GTCTCTTTGGGAAAGCTACAT            |
| Pair 5   | CTTTCTCAAAGAGACCTGAG                 | TTTCTATTCCCTGGCTAACA             |
| Pair 6   | GCTCTCTGGTCTTGAT                    | TGAGAGATATGCTGACAATGGA           |
| Pair 7   | GCATCATTTCCTCTATTGAG                 | CTGGAAGAGGAGATGTCTAGT            |
| Pair 8   | GGCAAGGAAGCTCATTG                   | AGGAAACGGAGACAAAGA              |
| Pair 9   | CAGGACAATGGTGAGGAGTTC               | AGAAAGAGGGTTTATCAAGCAG           |
| Pair 10  | TCTTGGTCTCCCTTCTCTT                 | CTGAGAGACATCAGATTTCTGTA          |
| Pair 11  | CTCTGCTTGAACTGAGTTTCT               | GAAGCTGAAGTACATTAGGCAAAG         |
| Pair 12  | TCTCAGTCCAGCTGACATTA                | CTGATAGGAGCTATGAGATTTCTGTA       |
| Pair 13  | CTGATACTCTATGACATTCC               | GATGGTCCTAGCAGAAGATT            |
| Pair 14  | AATCTTTGGTCTAGAGGACATTCTATC         | GGCAAGGTCAAACCTAGAAAGA           |
| Pair 15  | CTAGTTTGCAGTCTGCTTAT               | CGACCAATCCACGACAA               |
| Pair 16  | TGGATGGTGCCCTGAGG                   | AAAGCTATGGTACAGGCACTG           |
| Pair 17  | GTGGCAGACAGGGCTT                     | TTAGGCGATCATGATGCACTG            |
| Pair 18  | TGCACTATGGACTGGCTAAT                | TTTAATCTGTCCAGCGGCT             |

|          | **TMEM217** Forward Sequence (5' to 3') | **Reverse Sequence (5' to 3')** |
|----------|----------------------------------------|----------------------------------|
| Pair 1   | GATATGAGAAAGCATTGAGCATTAG             | GTAAATCTAAGGTGCGCTCAA            |
| Pair 2   | AGTTGACGGCACCCTTGA                    | GCAGCAACATATTTCTACGTAGAT         |
| Pair 3   | TCAATTTGCTCTCTACAGTTT                | CCCAGTGTTGCAAAGAT                |
| Pair 4   | TTGCAACACTGAGCCTTGC                  | CGGATAGATGACATTAGGCTG            |
| Pair 5   | AGAGCCTTTACATCTGATCTCAT              | CAGGCTGCTGCTGAAAC                |
| Pair 6   | AATAGGAGGATCATTACACTTGG              | GTCCCTGAGCCAAATG                 |
| Pair 7   | GCAAGGACAGAGACTCAA                   | CACTGCGAGATTTCTCGTGA             |
| Pair 8   | TCTTTTTCAACAGGAGATCTGG              | TCTTGCTCTCCCTCTCTCTTA            |
| Pair 9   | CTAAGAAGACGCGAGAGCA                  | GTGACCAATACAAGAGATGTCCAGG        |
| Pair 10  | AAGCCCTTTAAGTGTGAGTACT               | GTCTCAAGGTGTTGAGGTAATTTG         |
| Pair 11  | CTCTGTAACAAATTACCTCAAACC             | CCCACCTAGCTACTCTT                |
| Pair 12  | GAATCACAAGAGACTGCTGA                 | ACAAGTAGAGCCTTTAGATGG            |
| Pair 13  | GTTGCTTGGTCAAGGTTTCTC                | CTTCCCTCACAAGATAGA               |
| Pair 14  | GGCCTACTAAGACGCTCTACT               | GAAAGAATGCTGTCGCTCTT            |
| Pair 15  | TCTGACTGTGGCCAGGAA                   | CATGAGGAAATCAGGCTGACTA           |
| Pair 16  | TCTATAGTCTCAGCTTTGCTTC              | GACAGTGCAGCTTCCATT               |
| Pair 17  | AGCTGCACGTGTCTCGTATAGT              | CACCCTTATGTTGTCCTCTC             |
| Pair 18  | TAATTTGCTACACTCAGTGA                 | CCTCAAGTTGGCCTCATAATC            |
| Pair 19  | AAGCAACATAAAAGGGTTAAT                | ATACATACTAGAGATGTGAGGCGC         |
| Pair 20  | CAGGAGGCCAGGATT                      | CATGTTGCTCAAATACACAC             |
| Pair 21  | CCGGCTATCATCAGCTCTCTATGATG           | TGGAGAGGATATGAGTAAACA            |
| Pair 22  | CAAATGGTTGTTACTAATTCACCTCTCC        | ACCAAATGATAGCAGAAATGTA           |
| SH3RF3 | Forward Sequence (5' to 3') | Reverse Sequence (5' to 3') |
|--------|-----------------------------|-----------------------------|
| Pair 1 | GCTTACTTCAAGTCCCATGTT       | CGAGGACCAATCATCTTCCAGAG     |
| Pair 2 | TTCTTAGGAGCTCAGAGCA         | AGACGCTACACTTCTTGG          |
| Pair 3 | AGAGCTCGTGAAGATGATTGGG      | CTTCAGTGGCCAAAGGTG          |
| Pair 4 | CCACTTGGCAGAAGTGAGGTG       | TACCCTTCTCCTGGCTTTG         |
| Pair 5 | TTGGCAACTGGGAAGGCA          | TTCCCTTTCTCATTCTTTCCC       |
| Pair 6 | GAAAGAGGTTAAGGGAGAGAGA      | CTGTGTATCCGGTGCTTGG         |
| Pair 7 | AGAAGGTTAAGGGAGAGGGAAGG     | TTCCAGATGGTCTGAAGGG         |
| Pair 8 | ACACGGCATACACAGAGACAG        | ACACAAGTTCACAGCAAG          |
| Pair 9 | CCAGAATACTCACCACCCTCTC      | CAGACGAGTCTGGGATTTATG       |
| Pair 10| TGCAATTCTCCGTAACTCATGATA    | TGAATGGTCTGCTCCTG           |
| Pair 11| GGCAGAGCAGACATTATT          | GTTCAGGCTCTTGGTATT          |
| Pair 12| CAGGCAGACTAGGGAGTTG         | GCTGGATTAAAGCTCAACTCTCA     |
| Pair 13| GTCTCTGCGCCAGATTTGAG        | ACAGGCCCTATTTGCTAAATTA      |
| Pair 14| GTCTCACCTAGAAGCCTGAAA       | CACACTATTACACAGCAGAGG       |
| Pair 15| GCCTGTGCTGTGTAATAGTG        | CATTAGCTGGCCAGACTAG         |
| Pair 16| GACAGTGTCGCCAGTCAAA         | CCTGTGCTGCTCATAATG          |
| Pair 17| GGTCCAGCAGAATGATAACC        | TGAGCTGATCATCTACTCTTTA      |
| Pair 18| CAGGCCACCCACACATTA          | TCTGAGTTGGAAGGG            |
| Pair 19| AGGATTCGAAATAAGAAGCCCTTC    | TTGATAGCAATAAAGCTGCTG       |
| Pair 20| CTTCCTGGTGCTGCAATTT         | CATTACAAGGTGTGCTG           |

| ANGPLT4 enhancer | Forward Sequence (5' to 3') | Reverse Sequence (5' to 3') |
|------------------|-----------------------------|-----------------------------|
| Pair 1 | CTTCTGAGCTGCTGTGACTTT      | TCTCAACCTCAAGTCCGTT         |
| Pair 2 | CAGAGCTCAATTTATCTCCTTTC    | TTAGTGCCACCTTTTACACC        |
| Pair 3 | GATTACACAAGTGTCTGATGTTG     | AGGGCAGACAGCTATGAA          |
| Pair 4 | ATGTACGCTGCTACAGTTAT       | CTTCACAGTGGGAACCTTG         |
| Pair 5 | GAGGGTGGAAGGGAACCA         | CAAGAGCAGGACACTCAAC         |
| Pair 6 | CCCAGACTGGAAGGAATTTAG      | TCAGGCCAGAAAGGATGA          |
| Pair 7 | CCACTTCTCTGAGTCCTCCTTTC    | GCCACACTGTACTCACT           |
| Pair 8 | TGACCTGAGTGTGAGTACAG       | GGACTATGTAATGGCAGACTCG      |
| Pair 9 | GACACAGTGTCAGATTACA        | TCTCTCGGCTGAGACACT          |
| Pair 10| GGGCTTTGCTGTGAGAAGAATC     | CCTAGACAAAGTGTCAGGAAATA     |
| Pair 11| TGCAAGTCTCAAGGCAAGAGA      | GATGTGTCAGAGTCTAGA          |
| Pair 12| CTTGTGCTTGGAGCTTGTCAAAG    | AGATGTCAATCCCCATTAGGTAAG    |
| Pair 13| CTGCTCAAGCATTCTCAGTC       | ACAGGCCCTAGAAGGTATGAA       |
| Pair 14| GCTCCAGAATCTCTTACCTA       | CTAGATGGCTACATGTGCAA        |
| Pair 15| TCTAGGCCTGGTGTGTTAC        | GCTCATTGGCCACCATGAAT        |
| Pair 16| CCATTGCCAGCTAGGGAC         | GTATGATATCAGAGGACACAT       |
| Pair 17| ATGCTGGCAGCTGAAAGGACAA     | CCTTAAGAGGTGCTGGTATG        |
| Pair 18| ATCATAACGCTGAGAAGGACAC     | CATTGCTCTGATGAGAGTGGT       |
| Pair 19| GGGAGGAAACACCTCTGCTCA      | CCTGGCCAAACACTGATATT        |
| SMURF2 | Forward Sequence (5' to 3') | Reverse Sequence (5' to 3') |
|--------|-----------------------------|-----------------------------|
| Pair 1 | AATTTGTGGTACTTTGTCTCAGG    | CTGTATCGTATGTGATAAAGTATGGAA |
| Pair 2 | GAGGCCCTGGGAAATGAATA       |AACCTGTTGCAGAATGTGTTT |
| Pair 3 | AAACACATTCTGCACAAGGT      | GGGTGCATAAAATGGTCTTGGATGT |
| Pair 4 | CCAATTTGGTTTAAAGCATGGA    | TGCTTCATGCTATCGTGCTTTACAG |
| Pair 5 | TGCTGTAAGACGATAGCATGAA    | AGTAGTTGCAGTCAGTGATG |
| Pair 6 | CATCACTCGACTGCAACTAC      | TTGAGGATGATTGGATATGACTT |
| Pair 7 | GCAACATGAACAGGCTCAA       | CCATCTCGCCCTTAGAGAATTG |
| Pair 8 | AGTCATATCCATCCTCCTCAA     | CCATAAAGACTGCATCGTACA |
| Pair 9 | CAATTCTCTAAGGCGAGATGG    | TGGCCCATATTCTGCTGATTAC |
| Pair 10| GGGCAGGAAGTGGAAATA        | ATTAGCCACAGGGCTTATGA |
| Pair 11| TGTACGATGCAGCTTTATGGG    | AAGCAATGTTTGACATTGACC |
| Pair 12| CAGAGAATATGGGCCAGAATGA   | ATGACCGMCATGGAGAAT |
| Pair 13| GATCAACATGACTCATAGCCT    | TCTCTGGACCTGGTCCT |
| Pair 14| AATTCCCTCAATGGCTGCTCA    | GAGGAAATAAGGCTGAGG |
| Pair 15| CAGGACCCAGTCAGAGAA       | GGGCAGGAAGGAGGAATAAG |
| Pair 16| GAGAGCTTTGTGTGGCATTTC    | AGAGCTTTGAGTTTCTTGGTACAG |
| Pair 17| CTCCAGCCTATTTTCTCCTCCT   | GAGAAAAGACTGAGCTCAGGAC |
| Pair 18| CCTGTACCAAAGAACTCAAGACT  | GTGCCTACTTACTGACTGAGG |
| Pair 19| CTCAAGACTCTCCTCTCTTTGAGT  | GGCCAGACAGATGGGAAAG |
Supplemental Table S4. Sequences of primers used for qPCR experiment.

|              | Forward Sequence (5' to 3') | Reverse Sequence (5' to 3') |
|--------------|-----------------------------|-----------------------------|
| **RPL19** (internal control) | ATCGATCGCCACATGTATCA | GCGTGCTTCTTGGTCTTAG |
| **FKBP5**    | AGGCTGCAAGACTGCAGATC | CTTGGCCATTTGCTTTATGG |
| **TNFAIP3**  | GCCCAGGAATGCTACAGATAC | AGTGGAAACAGCTCGGATTTTC |
| **TNIP1**    | AACAAGCAGTGGGACCAGCATTTTC | TGCTTCTGCAATCAGGACCGCTTC |
| **IL1A**     | CATCCTCCACAATAGCAGACAG | GAGTTCCTGGCTATGGGATAAG |
| **CXCL8**    | CTTGGCAGCCTCTCCTGTATTT | GGGTGGAAAGGTTTGGAGTATG |
| **ICAM1**    | TGGGCAGTCAACAGCTAAA | GGTAGGGTTCTGGCCACT |
| **LIF**      | AAGGAGGCAGCAGAGTTGG | AGAGAGCGCAGAGGAAAGG |
| **CCL2**     | GCTCATAGCAGCCACTTCA | CTTGGCCACATGGGCTTTGA |

Supplemental Table S5. Sequences of primers used for ChIP-qPCR experiment.

|              | Forward Sequence (5' to 3') | Reverse Sequence (5' to 3') |
|--------------|-----------------------------|-----------------------------|
| Negative     |                            |                             |
| Control 1    | CGGCTACTAGCGGTTTACG | AAGAAGATGCAGGCTCTGT |
| Negative     |                            |                             |
| Control 2    | TGGCAGGATGAAATACTAAGCAAGTA | AGATGGGAAACTGAGAGACTTTAGTTAG |
| Negative     |                            |                             |
| Control 3    | GGCAAGGACAGAGAATACATA | CTCTGTGTTTCTGGGTAGTTAG |
| **FKBP5**    | TAACCACATCAAGCGAGCTG | GCATGGTTTAGGGGTCTTTG |
| **LIF**      | TGCCCTCTGTCCATCATCAG | GCCACTTCCCTCAGTAAATC |
| **ICAM1**    | GCTAGGCCTATAAAGGATCAC | CATAGCGAGGCTGAGTTG |
| **SMURF2**   | TGGGCAGAATGAGCATCAC | ATGAGCAGGCATTGAGGAAT |
Figure S1. Western blots for TNFAIP3, NFKBIA and CEBPB protein expression in BEAS-2B cells treated for indicated times with vehicle (ethanol (E)), TNF (T), dex (D), or TNF+dex (TD). GAPDH served as a loading control.
Figure S2. Volcano plots indicating differentially regulated nascent transcripts identified by GRO-seq.

30 min TNF vs TNF+dex

-log₁₀ p-value (adj)

log₂ Fold Change

-4 -2 0 2 4

TNF < TNF+dex; n = 609
TNF > TNF+dex; n = 228

30 min dex vs TNF+dex

-log₁₀ p-value (adj)

log₂ Fold Change

-4 -2 0 2 4

dex < TNF+dex; n = 856
dex > TNF+dex; n = 200
Figure S3. Genomic distances from transcripts in each cluster to the nearest eRNA in the analogous cluster. Clusters are based on $p_{\text{adj}} < .05$, the stringency of this cut-off likely impacts the frequency of close genomic associations.
Figure S4. MA plots illustrating statistically different (p < 0.0000001) MD scores amongst all 641 HOCOMOCO transcription factor binding motifs tested comparing Tfit-identified enhancers in cells treated for 30 minutes with vehicle or TNF.

MA for Vehicle vs. TNFα MD-scores
(p-value cutoff: 1.00E-07)
Figure S5. UCSC Genome Browser visualization of presumptive GR ChIP-seq peaks observed under basal culture conditions aligned with marks associated with open chromatin from ENCODE data.
Figure S6. Western blot of GR protein expression in nuclear vs cytosolic fractions isolated from Beas-2B cells treated as indicated for 1 hr. Fraction-specific markers also served as loading controls and included beta-Tubulin (cytosolic) and Lamin (nuclear).
Figure S7. qRT-PCR analysis of dex-induced control (A), TNF+dex cooperatively-induced (B), and TNF-induced, dex-repressed (C) loci in shCtrl and shGR Beas-2B cells treated with TNF (20 ng/ml) and/or dex (100 nM) for indicated times. *p < 0.05 vs. same treatment in shCtrl cells.
Figure S8. Western blot detection of GR protein with both GR ChIP antibodies in shCtrl- and shGR-transduced cells.
Figure S9. MA plots illustrating statistically different (p < 0.00001) MD scores amongst all 641 HOCOMOCO transcription factor binding motifs tested in dex-treated shCtrl vs shGR cells for each GR antibody. Motifs for glucocorticoid receptor (GCR) and progesterone receptor (PRGR), which recognize highly similar sequences, are highlighted in purple and exhibited the largest changes in MD-scores in both ChIP-seq datasets.
Figure S10. MD analysis of enrichment for indicated binding motifs in specified ChIP-seq datasets.

A

nGRE(1)

GR-IA1: shCtrl/veh

N = 24100; MD-score = 0.131

GR-IA1: shCtrl/dex

N = 24100; MD-score = 0.136

B

nGRE(2)

GR-IA1: shCtrl/veh

N = 32993; MD-score = 0.139

GR-IA1: shCtrl/dex

N = 22813; MD-score = 0.143

C

nGRE(2)

GR-IA1: shCtrl/veh

N = 11; MD-score = 0.000

GR-IA1: shCtrl/dex

N = 14230; MD-score = 0.137

D

GR-IA1: shCtrl/veh

N = 13253; MD-score = 0.219

NFkB1

GR-IA1: shGR/TNF

N = 24266; MD-score = 0.232

E

GR-IA1: shCtrl/TNF

N = 26529; MD-score = 0.221

NFkB2

GR-IA1: shCtrl/TNF

N = 20585; MD-score = 0.307

NFIA

N = 13062; MD-score = 0.283

TF65

GR-IA1: shGR/TNF

N = 14613; MD-score = 0.212

GR-IA1: shCtrl/veh

N = 10836; MD-score = 0.287

TEAD4

N = 9933; MD-score = 0.243

MAFK

N = 5516; MD-score = 0.235

FOS

GR-IA1: shCtrl/veh

N = 18156; MD-score = 0.506

GR-IA1: shCtrl/dex

N = 10996; MD-score = 0.418

FOSB

N = 13416; MD-score = 0.516

GR-IA1: shCtrl/veh

N = 17886; MD-score = 0.555

GR-IA1: shCtrl/dex

N = 8027; MD-score = 0.426

JUN

N = 1074; MD-score = 0.469
Figure S11. GR-356 ChIP-qPCR analysis in BEAS-2B cells treated for 1 hr with TNF and/or dex.
Figure S12. Integrated GRO- and ChIP-seq analysis reveals qualitatively different occupancy patterns between GR-repressed (A) and GR-induced (B) enhancers.
Figure S13. Relative magnitude of ChIP-seq peaks for IA1 vehicle and dex at different classes of enhancers.
Figure S14. UCSC Genome Browser visualization of dex-repressed enhancers associated with MACS2-called GR ChIP-seq peaks from 10 min GR-356 ChIP-seq experiment aligned with marks associated with open chromatin from ENCODE data. All ENCODE Transcription Factor ChIP data are visible in top screenshots but truncated in bottom screenshots due to space constraints.
Figure S15. H3K27Ac ChIP-seq in A549 cells at different classes of BEAS-2B enhancers defined by our GRO-seq data. A549 H3K27 acetylation data (ENCSR375BQN) were generated by the Reddy lab within the ENCODE consortium.
Figure S16. MNase chromatin accessibility assay for dex-repressed SMURF2 enhancer, as described in the legend for Fig. 6.
Figure S17. Aligned GRO- and ChIP-seq data for enhancers interrogated with MNase chromatin accessibility assay.
Figure S18. GRO-seq tracks and bar graphs showing relative genomic locations and normalized luciferase activity, respectively, of (A) Cluster 1 (TNF+dex cooperatively induced), (B) Cluster 2 (TNF-induced, dex-repressed) and (C) Cluster 3 (dex-induced, TNF-repressed) enhancers cloned into a destabilized, short half-life luciferase reporter vector and transiently transfected into BEAS-2B cells prior to treatment with vehicle, TNF, dex, or TNF+dex for 1, 2, or 4 hours. Luciferase activity of empty vector control (pNL3.2) is included in the inset of the pNL3.2-PNRC1 graph in Panel A. Bars indicate mean activity ± SD normalized to that of a firefly luciferase internal control. *p < 0.05 vs. veh; **p < 0.05 vs. TNF; ***p < 0.05 vs. dex.