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Noncoding RNAs and LRRFIP1 Regulate TNF Expression

Lihua Shi, Li Song, Michael Fitzgerald, Kelly Maurer, Asen Bagashev, and Kathleen E. Sullivan

Noncoding RNAs have been implicated in the regulation of expression of numerous genes; however, the mechanism is not fully understood. We identified bidirectional, long noncoding RNAs upstream of the TNF gene using five different methods. They arose in a region where the repressors LRRFIP1, EZH2, and SUZ12 were demonstrated to bind, suggesting a role in repression. The noncoding RNAs were polyadenylated, capped, and chromatin associated. Knockdown of the noncoding RNAs was associated with derepression of TNF mRNA and diminished binding of LRRFIP1 to both RNA targets and chromatin. Overexpression of the noncoding RNAs led to diminished expression of TNF and recruitment of repressor proteins to the locus. One repressor protein, LRRFIP1, bound directly to the noncoding RNAs. These data place the noncoding RNAs upstream of TNF gene as central to the transcriptional regulation. They appear to serve as a platform for the assembly of a repressive complex. The Journal of Immunology, 2014, 192: 3057–3067.

Tumor necrosis factor (TNF) is a cytokine primarily produced by myeloid cells, activated T cells, and NK cells. The major roles of TNF include killing of tumor cells, the induction of adhesion molecule expression at sites of inflammation, stimulation of bone resorption, induction of fever, and activation of B cells, neutrophils, and monocytes (1–3). TNF inhibition is used therapeutically for arthritis and inflammatory bowel disease, and inhibition is associated with an increased risk of infection (4–8). Collectively, these studies demonstrate that the regulation of TNF is rigorous and redundant, presumably to limit the adverse consequences related to under- or overexpression.

In our studies of chromatin at the TNF locus, we identified a region 300 bp upstream of the transcriptional start site (TSS) where the majority of the transcriptionally relevant histone modifications were found (19). We also identified a transcriptional repressor called LRRFIP1 (previously called GCF2) (35). Further evidence that this region might be important in the regulation of TNF came from a study of patients with systemic lupus erythematosus, which found that the histone modifications at this site were different in patients compared with controls (36). This led us to examine the upstream region of the TNF promoter more carefully. We found significant levels of noncoding RNAs (ncRNAs) that mapped to this region.

ncRNAs are common in the genome, with ~8000 identified (37). In general, their abundance, conservation, and correlation with transcription have argued for functionality, but there are relatively few specific examples known (38–41). The best-known ncRNA that regulates chromatin conformation is Xist, which coats the X-chromosome destined for inactivation (42, 43). Long noncoding RNAs have been implicated in pluripotency and innate immune responses (44–46). Several studies have found that chromatin-associated RNAs are bound to chromatin-modifying complexes on chromatin, suggesting a role in epigenetic regulation (47–49). In general, the ncRNA is thought to confer locus specificity and alter local histone modifications, but the specific mechanisms for each gene appear to be diverse and are largely not understood (50, 51).

Several groups have manipulated ncRNAs in an effort to dissect their exact function. The most common model is one in which the ncRNAs regulate H3K9me2 and H3K27me3 marks in cis and mediate transcriptional repression (52–58). Nevertheless, a recent study found that many ncRNAs regulate gene expression in trans, suggesting that there are many more mechanisms yet to be identified (45). Despite the rapid increase in our understanding of RNA-mediated transcriptional repression, much remains to be learned regarding the mechanisms of repression.

This study was undertaken to examine ncRNAs upstream of the TNF gene. We found a tightly linked choreography of ncRNAs and repressors on intergenic chromatin upstream of TNF. Further-

The online version of this article contains supplemental material.

Abbreviations used in this article: ChIP, chromatin immunoprecipitation; IRFI, IFN regulatory factor 1; LTA, lipoteichoic acid; ncRNA, noncoding RNA; qRT-PCR, quantitative RT-PCR; RIP, receptor interacting protein; RNA-IP, RNA immunoprecipitation; siRNA, short hairpin RNA; TSS, transcriptional start site.
more, we identified a novel function of the transrepressor LRRFIP1.

Materials and Methods

Cells, transfections, and reagents

All cell types are human. K562 is a hematopoietic stem cell–like line. THP1 is an immature monocytic leukemia line. MonoMac6 cells are a more highly differentiated monocytic cell line (59). Each was maintained in RPMI 1640 with 10% cosmetic calf serum (Fisher Scientific, Pittsburgh, PA). Primary monocytes were obtained from normal human donors and purified by elutriation at the Penn Center for AIDS Research and then further purified by adherence. They were cultured, as defined by mRNA expression. Transfection of cells was performed by electroporation with the Amaxa Cell Line Lona Nucleofector Kit (Amaxa Biosystems, Gaithersburg, MD). HPLC-purified LPS and PMA were from Sigma-Aldrich (St. Louis, MO). The SMART vector 2.0 Lentiviral LRRFIP1 short hairpin RNA (shRNA) or nontargeting negative control viral particles were purchased from Dharmacon (Chicago, IL). The target sequences of three LRRFIP1-shRNA used in the studies were as follows: 5′-GGUUAGCAACCCGAGAUAGA-3′, 5′-AAUGGAGGAGUCUCCGACA-3′, and 5′-GUAGGGAUACACGAAGA-3′. Three shRNAs were evaluated, and the most effective was used for the experiments. Transduction of cells and stable cell line development were done following the manufacturer’s protocol. The EZH2 small interfering RNA (siRNA) sequence was 5′-GGAGGUAGCAUUCAUUGATT-3′, and the SUZ12 sequence was 5′-GAUGAGAUGUUCUAUCAAT-3′ (Ambion Invitrogen, Grand Island, NY). The ncrRNA overexpression system used the PMK CMV vector with the ncrRNA ligated downstream of the CMV promoter.

RNA extraction, quantitative real-time PCR, and NanoString nCounter assay

RNA was prepared, DNase treated, and reverse transcribed using the Advantage RT for PCR Kit (Clontech, Mountainview, CA). Nuclear RNA was extracted with the PARIS Kit (Ambion, Grand Island, NY). Primer–probe combinations for all targets are listed in Supplemental Table I. Custom primers and labeled probes were synthesized by IDT (Coralville, IA). Spliced TNF mRNA was detected by proprietary gene-specific primers from Applied Biosystems on a TaqMan SDS 7090HT. Primers to amplify 18S RNA were included in each amplification and served as the internal standard for normalization.

NanoString nCounter analysis was performed using NanoString custom-synthesized probes (NanoString, Seattle, WA). Total RNA, nuclear RNA, or lysate was directly hybridized with gene-specific color-coded probes, and data collection was carried out in the nCounter Digital Analyzer, as described by the manufacturer. Transcript numbers for each gene were normalized to the mean of housekeeping genes. In addition, six positive-control and eight negative-control probes were added to each reaction. Normalization then used the GAPDH mean. All the reaction counts were within the linear dynamic range of the standard curve.

The 5′ and 3′ RACE were performed with the FirstChoice RLM-RACE Kit (Ambion), according to the manufacturer’s instructions. Total RNA was extracted from K562 and treated with DNase I. The PCR products were purified and cloned into a pGEM-T vector (Promega) for sequencing.

Subcellular fractionation, m⁶G-cap analysis, and poly A⁺ RNA purification

Subcellular fractions were prepared as described, with a few modifications (60, 61). A total of 5–10 × 10⁶ K562 cells was lysed in RSB-100 buffer (100 mM Tris-HCl [pH 7.4], 100 mM NaCl, 2.5 mM MgCl₂, 50 μg/ml digitinon, 100 U/ml RNasin, 1× phosphatase inhibitor mixture [Sigma-Aldrich]), followed by centrifugation at 2000 x g for 8 min. The supernatant was collected as the cytosolic fraction. The nuclear pellet was then resuspended in RSB-100 T (0.5% Triton X-100 in RSB-100). After centrifugation at 2000 x g for 8 min, the supernatant was collected as the nuclear fraction. The resulting chromatin pellet was resuspended in RSB-100T and sonicated. The soluble DNA-bound RNA fraction was collected after centrifugation at 4000 x g for 15 min. RNA was extracted with TRIzol (Invitrogen, Grand Island, NY) and treated with RNAse-free DNase I (Qiagen, Valencia, CA). Ab for the m⁶G-cap was from Synaptic Systems (Goettingen, Germany), and we performed the immunoprecipitation, according to the manufacturer’s instructions, with 30 μg total K562 RNA and protein G beads (GeneScript, Piscataway, NJ) (62, 63). The RNA-IP and the non–RNA-IP were reverse transcribed and analyzed by quantitative PCR. PolyA⁺ RNA was purified by oligo(dT)-cellulose (Sigma-Aldrich) with 150 μg K562 RNA, according to the manufacturer’s recommendation. The RNA extracted from the supernatant or wash buffer was used as a negative control. Controls using 18S (noncapped, nonpolyadenylated) and 18S (capped, polyadenylated) were used to confirm the appropriate recovery.

Northern blot, run-on assay, and RNA-binding assay

For Northern analysis, total RNA from K562 was isolated with TRIzol reagent (Invitrogen). A 674-bp 32p-labeled sense or antisense RNA probe was generated by in vitro transcription with the MAXiScript kit (Invitrogen). Hybridization was performed using QuickHyb (Agilent, La Jolla, CA), following the manufacturer’s instructions.

The modified run-on assay was performed, as described (64). Nuclei were isolated, and transcription was allowed to proceed in the presence of [3H]UTP (Roche, Penzberg, Germany). The resulting transcripts were collected on avidin magnetic beads (Dynal, Invitrogen) and AMV reverse transcriptase used to generate cDNA. The proximal cDNA was quantitated by quantitative RT-PCR (qRT-PCR) using custom primers with actin primers (Applied Biosystems) for normalization.

Three different-sized ncRNAs (674, 377, and 150) were produced by different primers (Supplemental Table I) and then ligated into the pGEM-T Easy vector. The sense and antisense ncRNAs were generated by in vitro transcription with T7 or SP6 polymerase using MaxiScript (Invitrogen). The 300-bp actin RNA derives from the 3' end of ACTB, and the GFP RNA derives from 735 bp of GFP cDNA. The RNA-binding assay was modified from that described (65). In brief, different-sized ncRNAs were incubated with purified protein in gel shift buffer (1 μM MOFS [pH 7.0], 50 mM KCl, 5 mM MgCl₂, 1 mM DTT, 10% glycerol, and heparin) at 4°C for 30 min. The binding reactions were loaded onto 0.5% agarose gel (prepared by TB buffer) and run in TB buffer (45 mM Tris, 45 mM boric acid).

Chromatin and nuclear RNA immunoprecipitations

Chromatin immunoprecipitation (ChIP) assays were carried out as previously described (66, 67) and used the Abs for SUZ12 (Abcam, Cambridge, MA), LRRFIP1 (Sigma-Aldrich), H3K27me3, and EZH2 (Millipore, Billerica, MA). A negative control Ab (anti-GST; Abcam) was always included but is omitted from some of the figures for simplicity. Duplicates or triplicates were analyzed from each experiment. ChIP data were normalized to input according to the following formula: 2 × (10⁻input Ct – sample Ct).

Nuclear RNA immunoprecipitation (RNA-IP) was prepared as described by Rinn et al. (68), with a few modifications. Briefly, 4 × 10⁶ cells were harvested and resuspended in 2 ml PBS, 2 ml nuclear isolation buffer (1.28 M sucrose, 40 mM Tris-HCl [pH 7.5], 20 mM MgCl₂, 4% Triton X-100, and 6 ml water on ice for 20 min. The nuclei were obtained by douncing with a mortar and pestle. Nuclei were pelleted by centrifugation at 2,500 x g for 15 min and resuspended in 1 ml receptor interacting protein (RIP) buffer (150 mM KCl, 25 mM Tris [pH 7.4], 0.5 mM EDTA, 0.5 mM DTT, 0.5% IGE-PAL, 9 μg/ml leupeptin, 9 μg/ml pepstatin, 10 μg/ml chymostatin, 3 μg/ml aprotinin, 1 mM PMSF, 100 μM RNasin). Nuclear membranes and debris were pelleted by centrifugation at 13,000 rpm for 10 min. Ab was added to supernatant and incubated for 2 h at 4°C. Protein A beads were added and incubated for 1 h at 4°C with gentle rotation. The beads were pelleted at 2,500 rpm for 30 s, resuspended in 500 μl RIP buffer, and repeated for a total of three RIP washes, followed by one wash in PBS. The beads were resuspended in 1 ml TRizol. Coprecipitated RNAs were isolated and reverse transcribed, as above. Real-time PCR was performed with TNF1–4 primers and normalized to 18S RNA.

Protein analysis

The TNF ELISAs used the BD Biosciences BD OptEIA TNF ELISA kit. A BIA3000 instrument (Biacore, Piscataway, NJ) was used to detect binding to the TNF promoter RNA or DNA sequences. Biotinylated probes centered on −308, consisting of 25-bp dsRNA or DNA or DNA:RNA and a 6-bp spacer, were captured to the CMG sensor chip via streptavidin. The proteins were resuspended in running buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, 0.005% Tween 20). The experiments were performed at 25°C with a flow rate of 30 μl/min. Kinetic constants were obtained by fitting the data to a simple one-to-one binding model using BIA EVALUATION 3.1 software.

The coimmunoprecipitations used EZH2 and SUZ12 Abs. Protein A beads were used for collection (Invitrogen). Protein was quantitated using a Bradford assay, equally loaded on 4–12% NuPAGE gel (Invitrogen Life Technologies, Carlsbad, CA), and blotted with LRRFIP1 Ab (BD Biosciences, Franklin Lakes, NJ).
Results

Identification of TNF upstream ncRNAs

We previously identified a transcriptional repressor of TNF, LRRFIP1, that was a known RNA-binding protein (35, 69). To understand potential mechanisms, UCSC genome browser was used to explore the existence of ncRNAs upstream of the TNF TSS. GM12878 and K562 tracks both showed low-abundance ncRNAs upstream of the TNF TSS. We also explored our own RNA-seq data for evidence of transcription upstream of the TNF TSS and found low levels of transcripts identified, similar to those seen in the UCSC tracks (Supplemental Fig. 1A). We examined the coding potential of these transcripts through in silico translation and found a very limited potential for peptide production.

To directly identify ncRNAs, we initially designed qRT-PCR amplimers across this region, as follows: TNF1, 2, 3, 4, and 5 (Fig. 1A). RNA species were detected in the lymphotoxin alpha (LTA)–TNF intergenic region in K562 cells after reverse transcription of RNA with directional primers. RNase treatment abrogated the signal, demonstrating that the amplification was truly RNA. The 3' end of the LTA gene lies only 1.24 Kb upstream of the TNF TSS, and RNA species were found throughout this region (Fig. 1B, 1C).

We wanted to confirm our detection of the ncRNAs without the potential confounder of skewing due to PCR amplification, and we therefore used a digital method called nCounter. Seven sense or antisense probes were designed across the intergenic region, as follows: S 1–7 and AS A–G (Fig. 1A). We compared the ncRNAs in several cell types of differing competence for TNF expression. The K562 cell line does not make TNF protein, although it produces very low levels of TNF message. HeLa cells are fully repressed for TNF expression. Jurkat, THP1, and primary monocytes do not produce TNF protein at baseline, but, in each case, the cells can be induced to express significantly more TNF protein and mRNA after stimulation. All probes detected ncRNA signal in five different cell types, confirming the presence of ncRNAs (Fig. 1D, 1E).

We further confirmed the presence of the ncRNAs by RACE, which showed heterogeneous lengths of sense and antisense ncRNAs. The shortest product was ~200 bp, and the longest product was >900 bp (Supplemental Fig. 1B). To better define the sizes of the RNAs, we used directional primers and RT-PCR (shown on the map in Fig. 1). This showed strong bands of 600 bp and smaller in both sense- and antisense-primed cDNAs (Fig. 2A). An 800-bp band was seen faintly in the sense-primed sample. Northern blot confirmation was attempted, but the signal was dispersed at ~700 bp, and no clear band was visualized (data not shown). Therefore, the ncRNAs are bidirectional and heterogeneous in length, with a peak length of ~600–700 bp.

The ncRNA structure

To examine the structure of the ncRNAs, we used an Ab for the 5' m7G-cap structure to determine whether the ncRNAs are capped and oligo(dT) cellulose to capture polyadenylated RNAs. These two strategies determined that the ncRNAs were largely capped and polyadenylated (Fig. 2B, 2C). Controls for the oligo(dT) and Ab capture demonstrated the appropriate modification patterns for 18S and IL-1β mRNAs (data not shown). Different species of ncRNAs localize to different cellular compartments and are associated with different posttranscriptional modifications. To lo-

FIGURE 1. ncRNAs identified upstream of TNF. RT-PCR and direct detection were used to characterize the ncRNAs upstream of TNF (A) A map demonstrates the location of the primers and probes to detect ncRNAs. Nanostring nCounter probes for digital detection are labeled S1-S7 (for sense targeting) and AS-A-G (for antisense targeting). TNF1–5 represent the qRT-PCR amplimers. The 674-bp ncRNA used for overexpression studies is shown near the bottom of the map. The 410 and 430 phosphorothioate oligonucleotides used for knockdown of sense and antisense ncRNAs, respectively, are shown. The LRRFIP1 binding site at -308 previously identified is indicated with an oval (37). The arrows indicate the direction of the oligonucleotide sequences. (B) Sense ncRNAs and (C) antisense ncRNAs were identified by qRT-PCR from total RNA with or without RNase A treatment. Directional reverse-transcription primers at exon 1 (for sense) and at -991 (for antisense) were used. The chart legend applies to both sense and antisense graphs. All the differences between K562 RNA and RNase A-treated K562 RNA and the No-RT K562 RNA (No RT) were significant (p < 0.05, n = 3). Error bars denote SE. NanoString nCounter technology was used to quantify the (D) sense or (E) antisense ncRNAs in five different types of cells.
calize the ncRNAs, we fractionated K562 cells into cytoplasm, nucleoplasm, and chromatin. The ncRNAs were detected by qRT-PCR and were found almost exclusively in the chromatin fraction (Fig. 2D). Thus, the ncRNAs are chromatin associated, capped, and polyadenylated.

Function of the ncRNAs

Knowing that the ncRNAs were chromatin associated, we hypothesized that the peaks of RNA at the 3' end of LTA and the 5' end of TNF represented “spill” from adjacent LTA and TNF mRNAs (Fig. 1). We therefore purified nuclear RNA to eliminate most mRNAs and were still able to detect signal across the region using nCounter, without the peaks near the ends of LTA and TNF (Fig. 3A, 3B). We noted that the repressed K562 nuclear RNA preparations had higher levels of the ncRNAs than the competent THP1 nuclear RNA preparations (Fig. 3A, 3B). When THP1 cells were stimulated with LPS, there was a rapid increase in TNF message (Fig. 3C), as expected. There was a concomitant decline of sense and antisense ncRNAs in the −300 region of TNF promoter (Fig. 3D).

To determine whether these ncRNAs function in the regulation of TNF expression, we designed a set of phosphorothioate oligonucleotides to knock down these ncRNAs. They had variable efficacy derepressing TNF mRNA and variable efficacy at knocking down the ncRNAs (Supplemental Fig. 2). We selected a pair of oligonucleotides (410, 430) that successfully targeted the ncRNA, derepressed TNF expression and were near the site where we had previously identified histone marks of repression (Fig. 1A). In the repressed K562 cell type, the 410 and 430 oligonucleotides effectively knocked down the ncRNAs and derepressed the TNF message (Fig. 4A, 4B). Although the level of TNF mRNA is quite low in these cells, the depletion of the ncRNAs clearly led to increased spliced message.

To further investigate the role of the ncRNAs, we made ncRNA overexpression constructs of 674 bp in both the sense and antisense orientation (Fig. 1A). Because K562 cells are already repressed cells, we treated the cells with low-dose PMA to induce low levels of TNF mRNA. We found that transfection of ncRNAs did successfully target chromatin (Fig. 4C). Transfection of antisense ncRNA led to significantly decreased TNF mRNA levels with transfection of sense ncRNA having a more modest effect (Fig. 4D). We additionally examined the effects in THP1 cells, a monocyte lineage competent for expression. We did not observe any effect of knockdown of the ncRNAs (data not shown), probably because the ncRNA levels are already so low in THP1 cells (Fig. 3D). Overexpression of the ncRNAs was associated with repression of TNF mRNA both in resting and stimulated THP1 cells (Fig. 4E). To demonstrate the specificity of the effect, we examined IFN regulatory factor 1 (IRF1), a transcription factor induced by LPS. Transfection of the ncRNAs had no effect on IRF1 transcript abundance in THP1 cells (Fig. 4F). Furthermore, the effect of the ncRNAs was demonstrable at the level of protein production (Fig. 4G). Therefore, knockdown of the ncRNAs derepressed expression of TNF mRNA, and overexpression led to repression of TNF mRNA. These studies suggested that these ncRNAs are mechanistically involved in repression of TNF.

Chromatin characteristics at the site of the ncRNAs

To better understand the chromatin environment at the site of the ncRNAs, we first examined histone modifications. One epigenetic mark of particular interest is trimethylation of lysine 27 on histone H3 (H3K27me3). EZH2 and SUZ12 are two important polycomb protein family members that have been implicated in ncRNA-mediated repression (70–72). EZH2 methylates H3K27, and SUZ12 regulates methyltransferase activity at both H3K9 and H3K27. We used ChIP assays to investigate chromatin marks of repression as well as the presence of EZH2 and SUZ12. We also examined a known repressor of TNF, LRRFIP1, which was of interest because of its reported RNA-binding function. As expected, H3K27me3, EZH2, SUZ12, and LRRFIP1 were all present on the TNF pro-
moter in repressed K562 cells but not competent THP1 cells (Fig. 5). They appeared to occupy a similar genomic space with a peak of amplification centered in the TNF3 amplimer site. These data suggested that a complex of proteins resided on the repressed promoter and that the repressed state was associated with H3K27me3. To determine whether LRRFIP1 might directly interact with EZH2 and SUZ12, noncross-linked coimmunoprecipitation was performed, demonstrating that EZH2 and SUZ12 both interacted with LRRFIP1 (Fig. 5E).

ncRNAs interact with LRRFIP1 and are required for LRRFIP1 binding to chromatin

LRRFIP1 has been previously described as a RNA-binding protein (69), and we hypothesized that it might interact with chromatin via interactions with specific RNA species. We therefore tested the hypothesis that ncRNAs interact with LRRFIP1 and that these interactions are required for its binding to chromatin.

**Figure 3.** ncRNA levels are inversely related to TNF mRNA expression. NanoString nCounter analysis indicated that there were more nuclear (A) sense and (B) antisense ncRNAs in K562 than THP1 cells. n = 2. (C) TNF mRNA levels greatly increased after LPS stimulation of THP1 cells. n = 3. (D) LPS stimulation led to decreased ncRNAs in THP1 cells. n = 2. Cells were treated with 1 µg/ml LPS for 30 or 60 min, and NanoString technology was used to measure nuclear ncRNAs and total TNF message. Error bars in panels denote SE. *p < 0.05, **p < 0.01, ***p < 0.001. (E) The purity of the cell fractions for the nuclear RNA analyses was confirmed by Western blot. Heat shock protein 90 was used as a marker for cytoplasm, and histone H3 was used as a marker for nuclear material.

**Figure 4.** The ncRNA regulation of TNF mRNA expression. Levels of ncRNAs were modulated to examine the effect. (A) K562 cells were transfected with the indicated 20-bp phosphorothioate oligonucleotides that knock down either the sense (410) or the antisense (430) transcripts. The ncRNAs were measured directly using qRT-PCR with random primers, demonstrating appropriate knockdown. n = 5. (B) Both sense and antisense ncRNA knockdown increased TNF mRNA in K562 cells. n = 5. (C) K562 cells were pretreated with 10 ng/ml PMA for 3 d to induce low levels of TNF expression and then transfected with the 674 ncRNA sense (S), antisense (AS) overexpression constructs or the empty vector alone (PMK). These exogenously transcribed ncRNAs successfully targeted chromatin. n = 3. (D) ncRNA overexpression decreased the TNF mRNA level in low-dose PMA-treated K562 cells. n = 3. (E) The effect of ncRNA overexpression was examined in THP1 cells. Both basal and LPS-induced TNF expression was diminished in the ncRNA-transfected cells compared with vector (PMK) alone. n = 4. (F) As a specificity control, we measured IRF1 transcript abundance from the same cultures. No effect was seen. n = 4. (G) THP1 cells were transfected with the overexpression constructs or the control vector. Cells were stimulated with LPS, and supernatants were collected. A TNF ELISA was used to quantitate the TNF protein production (n = 2). Error bars in panels denote SE. *p < 0.05, **p < 0.01, ***p < 0.001. Asterisks refer to the difference between the 410 or 430 and LacZ.
a RNA-mediated process. LRRFIP1 exists as two major RNA splice variants, which in turn lead to two very different proteins (Supplemental Fig. 3A). We made a full-length exon 2 start LRRFIP1 construct that expressed the 160-kDa LRRFIP1 protein, and we made a truncated LRRFIP1 construct that was predicted to lack the entire DNA-binding region and half of the RNA-binding domain (Supplemental Fig. 3A). Using a biosensor approach with 32-bp oligonucleotides centered on the LRRFIP1 binding site at -308 or an off-target sequence, we examined binding to different nucleic acid structures. We found that the full-length LRRFIP1 bound to dsRNA, ssRNA, DNA:RNA, dsDNA, and ssDNA, but it had higher affinity for dsRNA, ssRNA, and DNA:RNA compared with dsDNA (Table I). It recognized a dsRNA off-target structure (actin) similarly. In contrast, the truncated LRRFIP1 protein and GST did not exhibit any binding over background (data not shown).

To further confirm direct RNA binding, a RNA-binding assay was performed (Fig. 6A). A RNA species of 674 bp was produced by in vitro transcription (Fig. 1A) and was incubated with several different proteins, including LRRFIP1. The free RNA appeared as a low smear, whereas the LRRFIP1-RNA complex appeared as a shifted, higher band, confirming direct RNA binding. There was no binding to tRNA. We further examined the structural requirements of RNA binding of LRRFIP1 (Supplemental Fig. 3). Longer RNAs bound LRRFIP1 better than shorter species. dsRNA of all sizes bound LRRFIP1 better than ssRNA. Not all RNAs bound LRRFIP1 equally (Supplemental Fig. 3F), but there did not seem to be a strict sequence requirement as sense and antisense were equally capable of binding. MFold (73) was used to predict the secondary structure of the 674 antisense ncRNA and predicted a highly folded arrangement that would lead to extensive dsRNA structures even when transcribed as a single-stranded species (Supplemental Fig. 3H). Thus, this ncRNA would have the structural requirements that have been identified as ideal for LRRFIP1 binding.

Therefore, LRRFIP1 was found to bind dsRNA by both biosensor and RNA-binding analyses. To investigate LRRFIP1 binding to ncRNA in vivo, we performed a RNA-IP using K562 nuclei. ncRNAs were detected by qRT-PCR from LRRFIP1-immunoprecipitated nuclear RNA but not with H3K9me3 or GST-negative control Abs (Fig. 6B). Globin mRNA was also not recognized, thereby demonstrating specificity. These analyses demonstrated LRRFIP1 binding to RNA by in vitro and in vivo.

We wished to determine whether LRRFIP1 binding to chromatin was dependent on the ncRNAs. Transfection of both the reverse oligonucleotide 410 (targeting sense ncRNA) and forward oligonucleotide 430 (targeting antisense ncRNA) led to diminished LRRFIP1 binding to the upstream ncRNAs in K562 nuclei, with antisense targeting having slightly more of an effect. Mock-transfected cells and LacZ oligonucleotide-transfected cells were comparable, and immunoprecipitation with a GST Ab demon-

**Table I. Biosensor analysis of LRRFIP1 binding**

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<th>RNA Type</th>
<th>( K_a ) (1/MS)</th>
<th>( K_d ) (1/S)</th>
<th>( K_D ) (M)</th>
<th>( p ) Value (Compared with dsRNA)</th>
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<td>dsRNA</td>
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Average of five experiments for TNF oligonucleotides.
strated background levels of signal (Fig. 6C). These data demonstrated that the 410 and 430 oligonucleotides interfere with LRRFIP1 binding to the chromatin-associated ncRNA, presumably by depleting the ncRNAs. Having demonstrated that the 410 and 430 oligonucleotides diminished ncRNA interactions with LRRFIP1, we investigated whether that was sufficient to disrupt LRRFIP1 binding to chromatin. Transfection of the two phosphorothioate oligonucleotides led to decreased binding of LRRFIP1 to chromatin in a ChIP assay (Fig. 6D). Therefore, the ncRNAs are critical for LRRFIP1 targeting.

Overexpression of ncRNAs can drive repressive chromatin

We had demonstrated that overexpression of the ncRNA repressed TNF message levels (Fig. 4). We hypothesized that overexpression of the ncRNA could also drive marks of repression. The same overexpression constructs were used as before. We were restricted to using the TNF2 probe due to the high level of exogenous DNA after transfection. In this assay, we found increased levels of H3K27me3, EZH2, and SUZ12 in antisense 674–transfected cells compared with vector-transfected cells (PMK) (Fig. 7). For the 674 sense transfection, only EZH2 binding was found to be significantly increased. Overexpression of the ncRNA was sufficient to induce marks of repression, but the role of LRRFIP1 was still not clear. To identify reciprocal relationships between LRRFIP1, EZH2, and SUZ12, we knocked down EZH2 and SUZ12 (Fig. 8). Knocking down either EZH2 or SUZ12 compromised binding of EZH2, SUZ12, and LRRFIP1 to chromatin (Fig. 8B–D), demonstrating that the protein–protein interactions seen by coimmunoprecipitation are functionally significant and impact recruitment to chromatin.

LRRFIP1 regulation of TNF mRNA

Our data suggested that the ncRNAs, especially antisense ncRNAs, are involved in LRRFIP1 binding to the promoter as a transcriptional repressor. To confirm the function of LRRFIP1, we created stably transfected LRRFIP1 knockdown cells with short hairpin RNAs (shRNAs) in K562 and THP1 cells (Fig. 9A). We found that TNF mRNA was significantly increased in both the K562 and THP1 LRRFIP1 knockdown cells (Fig. 9B, 9C), and TNF protein was also increased in the LRRFIP1 knockdown THP1 cells after stimulation (Fig. 9D), consistent with its role as a repressor. We used PMA as an acute stimulus in THP1 cells and also found knockdown of LRRFIP1 was associated with increased transcription as defined by a modified run-on assay (Fig. 9E). Other genes, both induced or not by PMA, did not exhibit increased transcription on the LRRFIP1 knockdown cells. We also performed ChIP assays to define the effects of LRRFIP1 knockdown on chromatin marks of repressors; however, no changes were observed in H3K27me3, EZH2, and SUZ12 in both LRRFIP1 knockdown K562 and THP1 cells (data not shown). Therefore, LRRFIP1 binding to chromatin is dependent on EZH2 and SUZ12, but EZH2 and SUZ12 binding is not dependent on LRRFIP1. However, we found that knockdown of LRRFIP1 resulted in diminished ncRNA abundance, especially around the LRRFIP binding site (Fig. 9F). This suggests that the stability and function of ncRNA might depend on LRRFIP1 binding.

Discussion

Our data have defined a novel transcriptional regulatory pathway for the TNF gene. We first observed that the intergenic ncRNAs...
were tightly associated with chromatin and were of diverse lengths. K562 cells, repressed for TNF expression, had more abundant ncRNAs than THP1 cells, which are competent for expression of TNF protein. We used both knockdown and overexpression strategies and demonstrated that the more abundant the ncRNAs, the less TNF was expressed. The effects were small, suggesting that this mechanism may represent a fine-tuning strategy for transcriptional regulation. Alternatively, the knockdown strategy and the overexpression strategy may not have led to as effective targeting as the normal endogenous pathway, although we did show that the overexpressed ncRNA localized to chromatin. We believe the phenomenon is biologically relevant because effects on protein production were significant. We then pursued a strategy to define the mechanism of the ncRNA effect. We hypothesized that LRRFIP1, a known RNA-binding protein, which we had demonstrated acted as a repressor of TNF, might interact directly with the ncRNAs (35, 69). Our data demonstrated that not only did LRRFIP1 interact with the ncRNAs in vitro, but the interaction in vivo was required for localization of LRRFIP1 to the chromatin. Localization was also dependent on EZH2 and SUZ12.

LRRFIP1 (also known as TRIP, GCF2, and FLAP) was originally identified as a GC-rich–binding protein that repressed epidermal growth factor receptor expression and platelet-derived growth factor expression (74–76). It has also been described as a transcriptional repressor in other settings, a tumor suppressor, a platelet regulator, an early responder to foreign nucleic acids, and a β-catenin cofactor (69, 77–85). It exists as two major isoforms, a long 160-kDa isoform transcribed starting from exon 2 that includes RNA- and DNA-binding motifs and a shorter 120-kDa isoform transcribed from exon 1 that includes part of the RNA-binding motif but not the DNA-binding motif. The diverse functional descriptions may be in part due to isoform-specific effects. There is a homolog designated as LRRFIP2 that shares sequence homology only at the 5′ end (83). LRRFIP1 has no other homologs and no other defined motifs other than potential phosphorylation sites and a nuclear localization domain (86).

**FIGURE 7.** Overexpression of ncRNAs induced repressor recruitment to the TNF promoter. The effect of ncRNAs on chromatin conformation was examined. K562 cells were treated with 10 ng/ml PMA for 3 d to induce low levels of message and then transfected with the 674 ncRNA sense or antisense overexpression constructs or the vector (PMK). CHIP assays were used to investigate (A) H3K27me3, (B) EZH2, and (C) SUZ12 binding at the TNF promoter. Overexpression of the ncRNA led to increased marks of repression. n = 4. Error bars in panels denote SE. *p < 0.05.

**FIGURE 8.** Knockdown of SUZ12 and EZH2 impairs LRRFIP1 binding. Functional interactions of LRRFIP1, EZH2, and SUZ12 were examined. (A) A Western blot was used to determine the SUZ12 and EZH2 protein levels in the K562 cells transfected with siRNA-control, siRNA-EZH2, and siRNA-SUZ12. Knockdowns were effective. This is representative of three experiments. (B–D) ChIP assays demonstrated that knockdown of SUZ12 or EZH2 affected their own binding and that of LRRFIP1 at the TNF promoter. n = 3. Error bars in panels denote SE. *p < 0.05.
In our study, we demonstrated that LRRFIP1 interacts with the ncRNAs in a sequence-independent fashion. The sense and antisense sequences both bound LRRFIP1 and off-target dsRNA, supporting a model in which the structure is more important than the sequence. dsRNA bound more effectively than ssRNA, and longer species were also favored. These qualities are both found in the ncRNA upstream of TNF, and we speculate that the other chromatin sites of LRRFIP1 binding share similar structural qualities. We noted some asymmetry in the effects of the ncRNAs, with knockdown of antisense ncRNA slightly more effective at altering LRRFIP1 binding. Overexpression of antisense RNA also seemed to have a more robust effect on chromatin marks of repression and TNF mRNA abundance. It is not known whether the functions of the sense and antisense are different, and further studies will better define the structural requirement of the ncRNA for LRRFIP1 binding.

There are now numerous examples of ncRNAs regulating transcription, and their functional complexity, pervasive nature, and structural characteristics are just beginning to be understood. TSS, enhancers, and CpG islands all produce short transcripts of uncertain function. The ncRNAs described in this work were found to be strongly associated with chromatin, a population of RNAs initially described in 1975 (87). Several models have been posited to explain the role of ncRNAs in transcriptional regulation, as follows. 1) The ncRNA can serve as a decoy that titrates DNA-binding proteins away from genomic target sequences (88, 89). 2) The ncRNA can serve as a structural scaffold to facilitate interactions of multiple proteins (90–92). 3) Guide RNAs operating in cis or trans localize regulatory proteins and provide locus specificity (48, 93, 94). Our data clearly support the binding of LRRFIP1 to the RNA and via the RNA to the chromatin, consistent with a model in which the RNA provides locus specificity in cis. Our data are also consistent with a scaffolding function with EZH2, SUZ12, and LRRFIP1 assembling on a RNA tether to the region. These data suggest a model in which repressed cells express ncRNAs in a region where polycomb proteins bind. Once bound, LRRFIP1 is recruited to the site where it participates in the repressive complex. It is anchored to the site by the ncRNA. This model represents a hybrid of scenarios 2 and 3 above.

This study identified a novel transcriptional regulatory pathway. We presume that this pathway is not unique to TNF, although our studies did not extend beyond the one gene. Instead, we pursued a detailed analysis of one gene as an archetype. TNF is a relevant subject because of its importance in human disease states. Dissecting the mechanisms underlying the regulatory functions of ncRNAs is just beginning. This study adds to that fundamental knowledge by examining a gene with highly dynamic expression and by identifying the role of LRRFIP1 in ncRNA function.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.
References


The primers for making the constructs for ncRNA in vitro transcription

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<td>674F/674R</td>
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<tr>
<td>PGEM-T-377ncRNA</td>
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The primers for run on assay

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FIGURE S1. RNA is expressed between TNF and LTA. A) A primary monocyte RNA-seq library was prepared with the SOLiD™ whole transcriptome analysis kit (Applied Biosystems, Foster City, CA). Low levels of intergenic transcripts were seen. B) Sense and antisense ncRNA RACE results are shown. The arrows and arrowheads correspond to the Transcription Start Sites (TSSs) mapped by 5’ RACE. The asterisks represent the 3’ termini by 3’ race. The size of arrow or asterisk indicates the relative frequencies of the recovered clones.
FIGURE S2. Survey of knockdown oligonucleotides. K562 cells were transfected with the indicated 20bp phosphorothioate oligonucleotides which knocked down either the sense or the antisense transcripts. The ncRNA (TNF1-5 averaged) signal (A) and the TNF mRNA levels (B) were measured by qRT-PCR. The qRT-PCR signal was normalized to control transfected cells.
FIGURE S3. LRRFIP target characteristics. A) Full length and truncated LRRFIP1 cDNAs were ligated into pGEX-5X-1 vector for GST-fusion protein expression. Protein was purified according to the manufacturer’s instructions. The full-length LRRFIP1 construct started with exon 2 and expressed a 160kD LRRFIP1 protein. The truncated LRRFIP1 construct lacked the entire DNA-binding region and half of the RNA-binding domain. DNA binding is indicated with diamonds and RNA binding is indicated with four-point stars. The full length and truncated LRRFIP1 were codon-optimized to improve expression (GeneArt, Burlington, Calif.). B) Three different sizes of ncRNAs were ligated into the pGEM-T Easy vector and sense or antisense ncRNAs were transcribed using T7 or SP6 promoter. The map indicates the locations of the constructs. The 34bp nucleic acid structures were annealed oligonucleotides. C) Sense or D) antisense ncRNAs were incubated with or without LRRFIP1 and the mixture was run on a 0.5% agarose gel. t = tRNA. LRRFIP1 bound more of the longer ncRNA. E) LRRFIP1 bound to RNA species of various sequence. The 674 and 377 ncRNAs were derived from the TNF upstream region while the GFP and actin RNAs were of similar length and were produced in vitro from the same vector. LRRFIP1 prefered the TNF 674 RNA but demonstrated binding to the GFP and actin RNAs. F) LRRFIP1 bound to dsRNA better than ssRNAs. The same RNAs were prepared as sense (S), antisense (AS), or double stranded (DS) structures. G) LRRFIP1 showed higher affinity for dsRNA compared with dsDNA and RNA/DNA mixture. Oligonucleotides of different biochemical structure were synthesized and annealed as indicated. H) The proposed highly folded structure of the 674bp sense ncRNA. The structure was predicted based on physiologic salt and temperature by mFOLD.