Spatial proximity of homologous alleles and long noncoding RNAs regulate a switch in allelic gene expression

Kalliopi Stratigi[a,b], Manouela Kapsetaki[a], Michalis Aivaliotis[a], Terrence Town[c], Richard A. Flavell[d,e,1], and Charalampos G. Spilianakis[a,b,1]

[a]Institute of Molecular Biology and Biotechnology–Foundation for Research and Technology Hellas, GR70013 Heraklion, Greece; [b]Department of Biology, University of Crete, GR70013 Heraklion, Greece; [c]Department of Physiology and Biophysics, Zilkha Neurogenetic Institute, Keck School of Medicine of the University of Southern California, Los Angeles, CA 90089; and [d]Department of Immunobiology and [e]Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT 06520

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Physiological processes rely on the regulation of total mRNA levels in a cell. In diploid organisms, the transcriptional activation of one or both alleles of a gene may involve trans-allelic interactions that provide a tight spatial and temporal level of gene expression regulation. The mechanisms underlying such interactions still remain poorly understood. Here, we demonstrate that lipopolysaccharide stimulation of murine macrophages rapidly resulted in the actin-mediated and transient homologous spatial proximity of *Tnfα* alleles, which was necessary for the mono- to biallelic switch in gene expression. We identified two new complementary long noncoding RNAs transcribed from the TNFα locus and showed that their knockdown had opposite effects in *Tnfα* spatial proximity and allelic expression. Moreover, the observed spatial proximity of *Tnfα* alleles depended on pyruvate kinase muscle isoform 2 (PKM2) and T-helper-inducing POZ-Krüppel-like factor (ThPOK). This study suggests a role for lncRNAs in the regulation of somatic homologous spatial proximity and allelic expression control necessary for fine-tuning mammalian immune responses.

homologous spatial proximity | lncRNAs | *Tnfα* | macrophages

Tight control of total mRNA levels in a cell is essential for cellular homeostasis and normal physiology. The mRNA levels of a gene are regulated at multiple levels, and in addition to mRNA splicing, turnover, and translation, they also involve the epigenetic regulation of gene transcription (1). Transcriptional control in eukaryotic cells involves the tissue-specific activation and binding of transcription factors, which mediate their mode of action on the chromatin fiber, determined by both histone and DNA modifications (2). Another defining principle of transcriptional regulation is provided by the levels of chromatin and genome organization, which are also affected by discrete subnuclear entities, being regions of increased local concentration of protein or RNA molecules (3, 4). On the basis of the tremendous advancement of technologies that depict the genome's organization in diverse cell types and tissues, we now know that the regulatory mechanisms involved in gene transcription include the formation of transcription networks mediated by long-range chromatin interactions (3, 5–7).

The chromosome conformation capture-based approaches in combination with fluorescence in situ hybridization to DNA (DNA FISH) have shown that a gene locus may be involved in a chromatin network formed by either intrachromosomal or interchromosomal interactions. In mammals, examples of long-range chromatin interactions have been described for the alpha and beta globin loci (8, 9), imprinted loci (10, 11), the two homologous X chromosomes (12–15), the olfactory receptor genes and the H enhancer (16, 17), and the IFNγ and T-helper-type 2 cytokine gene loci expressed in alternate cell fates of $CD4^+$ T cells (18). The public research consortium ENCODE (the Encyclopedia of the DNA Elements) was recently launched in an attempt to identify all functional elements in the human genome (19). Systematic integrated analysis of the genome-wide chromatin interactions, which emerged from the project's data (20–22), showed that long-range chromatin interactions are more prominent than previously thought.

Most long-range interchromosomal interactions that have been functionally characterized in interphase nuclei so far involved loci localized on nonhomologous chromosomes, with some exceptions (13, 23–25). During meiosis, most organisms go under the process of pairing their homologous chromosomes, which is usually restricted in the germ line. Somatic homologous pairing, however, has been extensively observed in dipteran insects, where it is evident in diverse cell types. In 1954, E.B. Lewis introduced the term transvection to describe cases in *Drosophila melanogaster* in which homologous pairing influenced gene expression involving the action of enhancers *in trans* (26). These interchromosomal interactions have been studied extensively in several systems, pointing out that such a mechanism regulating gene expression *in trans* may be a general phenomenon.

All these transsensing regulatory mechanisms ultimately point to the complex regulation of physiological processes in a cell. Innate immune responses, although tightly regulated, lack such mechanistic insight regarding the dynamic regulation of chromatin and genome organization. Macrophages, as crucial mediators of an innate immune response, can be activated by lipopolysaccharide (LPS) of Gram-negative bacteria via Toll-like receptor 4

Significance

In diploid organisms, trans-allelic interactions control gene expression, providing a tight spatial and temporal level of transcription regulation. Although homologous trans-allelic interactions are quite abundant in various organisms such as Drosophila, plants, and fungi, they have not been widely reported in mammals. This article demonstrates that such a trans-allelic association is evident in mammals and involves the homologous spatial proximity of *Tnfα* alleles as a prerequisite for the biallelic expression of the *Tnfα* gene. We believe the phenomenon we describe here provides mechanistic insights for the regulation of gene allelic expression and mRNA dosage control necessary for fine-tuning physiological processes in mammals.

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[1]To whom correspondence may be addressed. Email: spiliana@imbb.forth.gr or richard.flavell@yale.edu.

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that ultimately leads to the activation of several classes or responsive genes, such as the cytokine tumor necrosis factor alpha (TNFα) (27). TNFα is a proinflammatory cytokine with a critical role in the initiation of innate and adaptive immune responses. Although TNFα deficiency causes increased susceptibility to infection, resulting in complete lack of B-cell follicles or causing tuberculosis, prolonged high concentrations of TNFα can result in severe tissue damage, autoimmunity, and cancer (28–30). It is evident that a tightly regulated balance of TNFα levels is of critical importance. *Tnfα* gene transcription is controlled in a cell type-specific and stimulus-specific manner. Nonetheless, it also requires a tight spatial and temporal level of regulation (31, 32).

In our study, we explored the subnuclear interacting events that regulate *Tnfα* gene expression. We focused on the identification of factors (proteins or RNA molecules) capable of mediating its subnuclear localization pattern and investigated the mechanisms involved in the fine-tuning of *Tnfα* mRNA levels.

Results

Homologous Spatial Proximity of *Tnfα* Alleles Is Detected Early upon LPS Stimulation of Macrophages. To study the subnuclear localization of the two *Tnfα* alleles, we performed experiments in thioglycollate-elicited and LPS-stimulated murine peritoneal macrophages. The lymphotoxin/TNFα (LT/TNF) locus includes three genes; namely, the LT beta (*Ltb*), TNFα (*Tnfα*), and LTα (*Lta*) genes (Fig. 1*A*). Quantitative reverse transcription coupled to PCR (qRT-PCR) experiments performed in primary macrophages revealed that the *Tnfα* gene is expressed on a time course of LPS stimulation, whereas no mRNA was detected for the *Lta* and *Ltb* genes (Fig. 1*B*). Subsequently, we performed DNA FISH, using fluorescently labeled bacterial artificial chromosome-DNA probes encompassing the LT/TNF locus. Interallelic distances were measured and normalized to the cell nuclear volume [normalized distance (ND) defined in Fig. 1*C*] to control for differences in the volume between individual cells. We found a considerable decrease of the *Tnfα* allele mean ND after 1 h of LPS stimulation (ND = 0.265), representing shorter *Tnfα* alleles distance in average. The homologous spatial proximity of the *Tnfα* alleles that was detected in wild-type macrophages was abolished in macrophages isolated from MyD88$^{-/-}$ mice, indicating the necessity for LPS signaling. More importantly, we found that in Delta3$^{-/-}$ macrophages, harboring an 8.4-kb deletion in the LT/TNF locus (Fig. 1*A*), the *Tnfα* alleles do not undergo homologous association, indicating the specificity of association to the LT/TNF locus (Fig. 1 *D* and *E*).

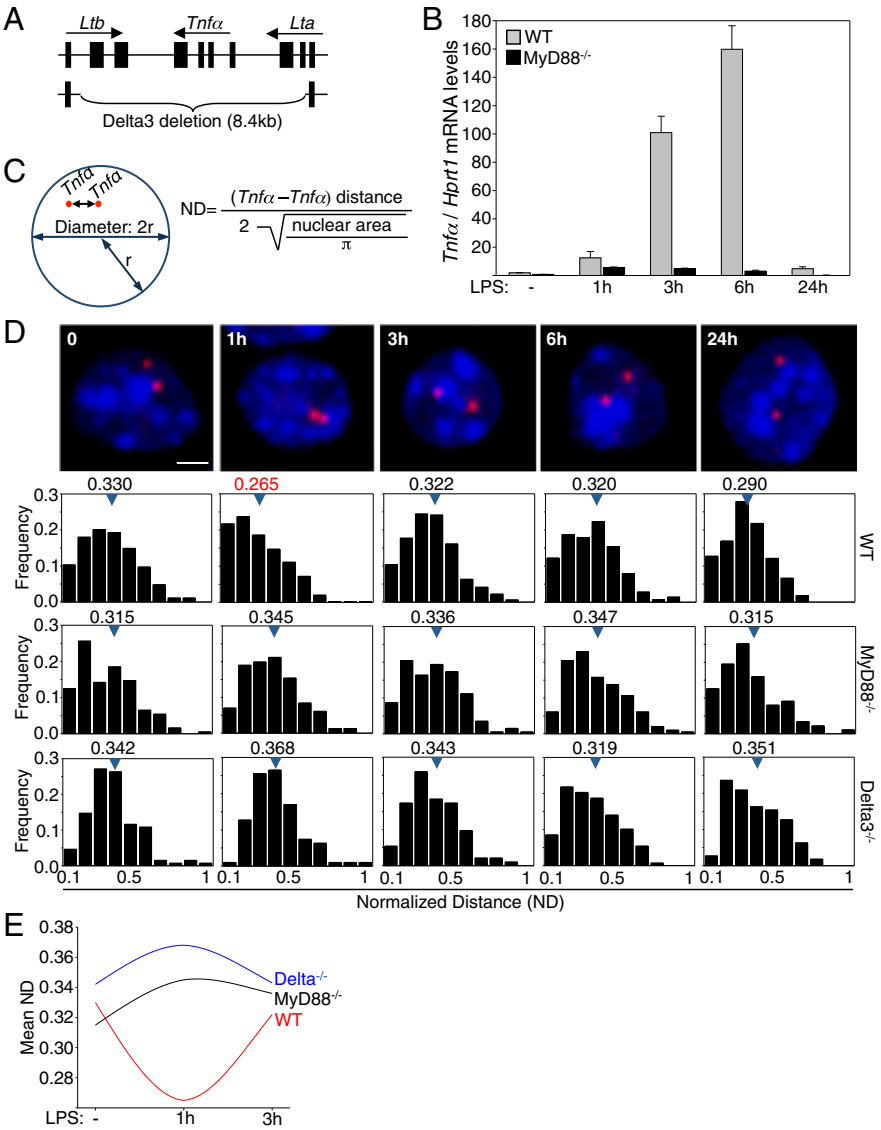

Fig. 1. LT/TNF locus subnuclear relocalization upon LPS stimulation of macrophages. (*A*) The LT/TNF locus on murine chromosome 17. (*B*) qRT-PCR analysis of the total *Tnfα* mRNA levels. (*C*) Calculation formula for the ND of *Tnfα* alleles. (*D*) Single z-sections of DNA FISH analysis for the LT/TNF locus in LPS-stimulated primary macrophages. The *Tnfα* alleles distance was normalized for the volume of each cell, and the frequencies of cells with a normalized distance from 0 to 1 were plotted. Mean ND, blue triangle. (Scale bar, 2 μm.) Sample size, 2,931 cells. (*E*) Mean ND of total number of cells. The lowest mean ND was detected for the wild-type macrophages.

To meet the demands of the subsequent biochemical experiments, we have then used the RAW 264.7 monocyte-derived murine macrophages. We performed DNA FISH experiments in a time course of LPS stimulation. We found a considerable decrease of the *Tnfα* allele mean ND after 30 min of LPS stimulation (ND = 0.375), representing shorter *Tnfα* allele distance on average. This decrease in the interallelic distance between the two *Tnfα* alleles was progressive, comparing the macrophage cell population before LPS stimulation with the cell populations stimulated with LPS for 10, 20, or 30 min. A subsequent increase in the mean ND places the longest interallelic distances after 1 h of LPS stimulation of macrophages, which then decreased in the time course of LPS stimulation (Fig. 2 *A* and *B*). Furthermore, cumulative frequency curves for the *Tnfα* alleles NDs displayed that the macrophage population stimulated with LPS for 30 min was clearly differentiated from the other points during the course of LPS stimulation, as well as the untreated cells (Fig. 2*C*). This finding was corroborated by statistical analysis for the randomness of the distance distributions for each point of LPS stimulation (Kolmogorov-Smirnov test), portraying a normal distribution for the interallelic distances in untreated cells, but not for the 30-min LPS-stimulated macrophages ($P < 0.001$; Fig. 2*D*). This increase in the proximity of the *Tnfα* alleles could not be attributed to a change in the macrophage cell volume upon LPS stimulation, as the mean nuclear diameter of the cells during the course of activation did not significantly change (*SI Appendix*, Fig. S1*A*). The close proximity of the *Tnfα* alleles in macrophages, after 30 min of LPS stimulation, taken together with the known rarity of somatic homologous pairing in interphase nuclei of mammalian cells, suggested that the homologous spatial proximity of the *Tnfα* alleles would be temporally transient. Interestingly, we found that this proximity was explained by the fact that the *Tnfα* alleles paired (ND < 0.1, allele distance shorter than 0.6 μm, given the fact that the mean nuclear diameter is 6 μm; *SI Appendix*, Fig. S1*A*) in 18.3 ± 3.3% of the cells after 30 min of LPS stimulation compared with 4.93 ± 3% of untreated macrophages (Fig. 2*E*).

To determine whether genomic regions flanking the LT/TNF locus were also drawn into close proximity by the homologous spatial proximity of the *Tnfα* alleles, we measured the interallelic distances of the *E4f1* locus, which is mapped 10.7 Mb upstream of the *Tnfα* gene on mouse chromosome 17 (*SI Appendix*, Fig. S2*A*). As an additional control, we performed DNA FISH experiments and measured the interallelic distances for the gene loci mapped on different mouse chromosomes, such as the *P2rx4* locus mapped on chromosome 5 and the *Arrb1* locus mapped on chromosome 7 (*SI Appendix*, Fig. S1 *B* and *C*). The cell volume of the

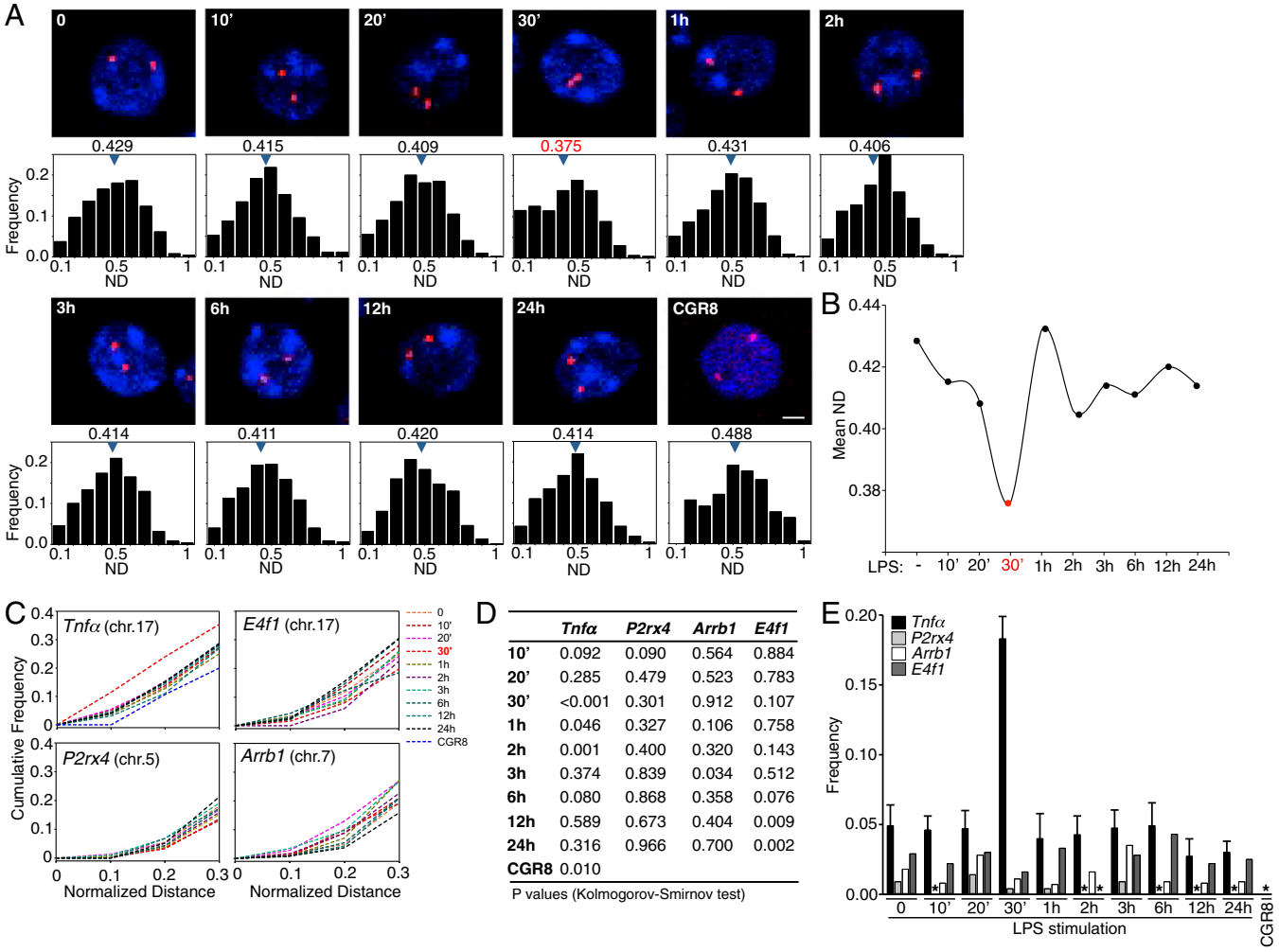

Fig. 2. Homologous spatial proximity of the *Tnfα* locus. (*A*) Z-sections of DNA FISH analysis. (Scale bar, 2 μm.) Sample size, 441–1104 cells for each time. (*B*) Mean ND of total number of cells. (*C*) Cumulative frequency curves for *Tnfα*, *E4f1*, *P2rx4*, and *Arrb1* allele pairs. (*D*) Kolmogorov-Smirnov test. (*E*) Frequency of cells with an allele ND < 0.1. Bars depict the mean value with SDs from 14 independent experiments. *Value not detected. CGR8, mouse embryonic stem cell line.

mouse macrophage cells did not alter during the course of LPS stimulation for these DNA FISH experiments (*SI Appendix*, Fig. S1*B*). The cumulative frequency curves and the frequency of cells with ND < 0.1 for the *E4f1* locus, as well as the *P2rx4* and *Arrb1* gene loci, did not show any evident allelic spatial proximity (Fig. 2 *C*–*E*), although they are activated by LPS (*SI Appendix*, Fig. S3).

In summary, our data highlight a transient homologous spatial proximity event of the *Tnfα* alleles in mouse macrophages, early upon LPS stimulation, which was specific to *Tnfα* and was not observed in LT/TNF proximal loci or the tested loci mapped on other mouse chromosomes.

The *Tnfα* Gene Has a Distinct Pattern of Allelic Gene Expression. Taking into account that the subnuclear localization of gene loci has a direct effect on the gene's expression kinetics, we wanted to study whether the transient homologous spatial proximity of the *Tnfα* alleles had any functional effect on *Tnfα* gene expression. We performed semiquantitative RT-PCR analysis for the *Ltb*, *Tnfα*, and *Lta* genes of the LT/TNF locus and found that only the *Tnfα* gene was highly expressed in macrophages upon LPS stimulation (Fig. 3*A*), in agreement with the data obtained for the primary macrophages. We then performed qRT-PCR experiments and found that *Tnfα* reached maximal mRNA levels after 1 h of LPS stimulation (Fig. 3*B*). To analyze the allelic expression pattern of the *Tnfα* gene at the single-cell level, we performed RNA-DNA FISH experiments, with the simultaneous detection of both the newly synthesized *Tnfα* mRNA and the DNA of the two *Tnfα* alleles. The analysis of the RNA-DNA FISH experiments we have performed in macrophages on a time course of LPS stimulation revealed that the highest frequency of *Tnfα*-expressing cells (74 ± 9.6%) was detected after 1 h of LPS stimulation (Fig. 3*C*), in agreement with the qRT-PCR results (Fig. 3*B*). We found that nascent *Tnfα* mRNA was not detected in resting macrophages, but the gene was rapidly activated upon LPS induction and expressed

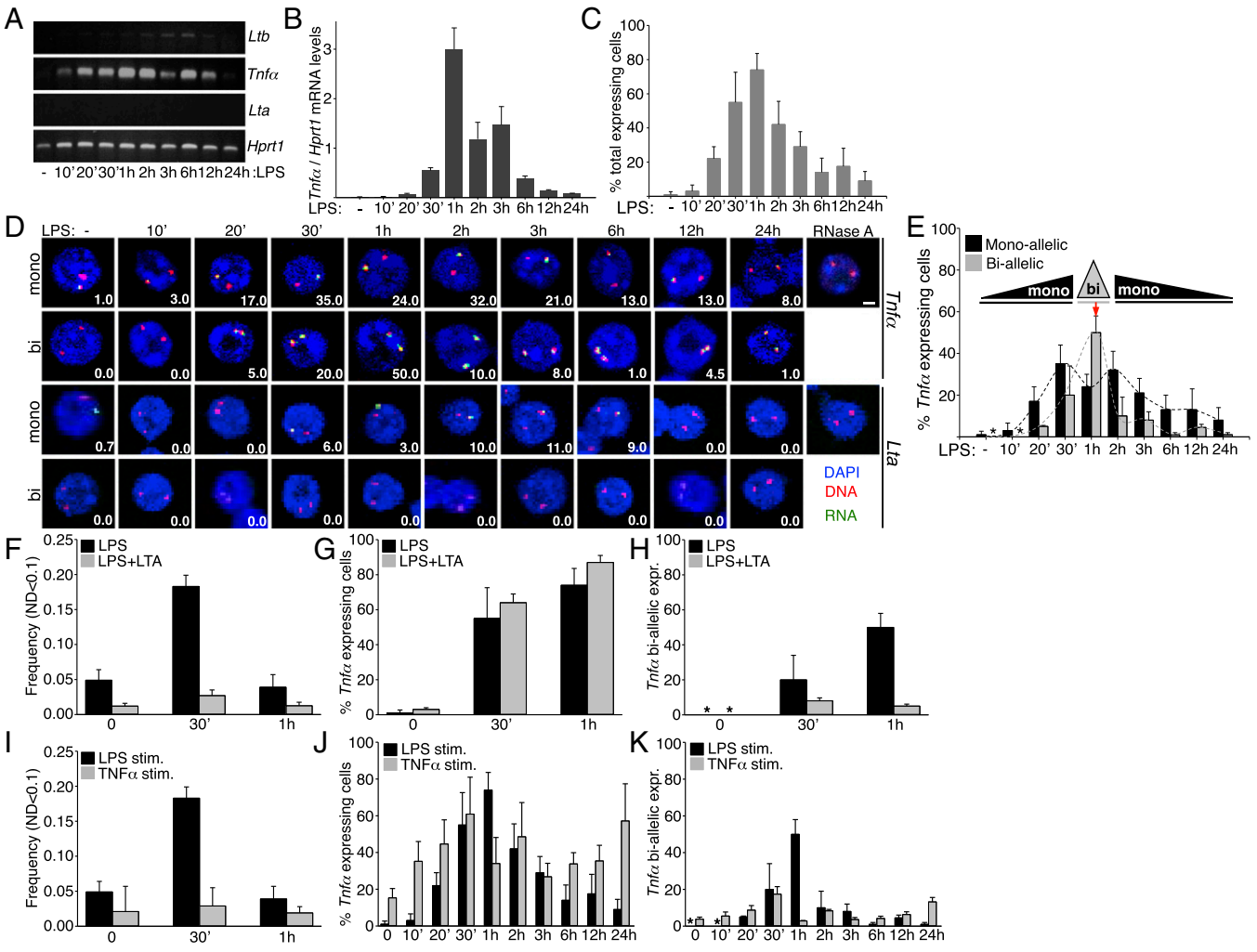

Fig. 3. *Tnfα* biallelic expression depends on LPS-stimulated and actin-mediated homologous spatial proximity. (*A*) mRNA expression analysis for the *Tnfα*, *Ltb*, and *Lta* genes. *Hprt1* was used as a loading control. (*B*) qRT-PCR analysis of the total *Tnfα* mRNA levels in LPS-stimulated macrophages. (*C*) Percentage of *Tnfα* expressing macrophages upon LPS stimulation. (*D*) RNA-DNA FISH analysis of the LT/TNF locus, along with the nascent *Tnfα* or *Lta* mRNA transcripts. (Scale bar, 2 μm.) (*E*) The switch to *Tnfα* biallelic expression is detected after 1 h of LPS stimulation. RNA-DNA FISH experiments were analyzed to plot the allelic pattern of *Tnfα* expression. Bars represent the percentage of *Tnfα* expressing cells with SDs of three independent experiments. Sample sizes/time point (*n*) = 205–352 cells. *Value not detected. (*F*) Frequency of cells with paired *Tnfα* alleles (ND < 0.1) in LPS-stimulated or LTA-pretreated macrophages, with SDs of three independent experiments. Sample size (*n*) = 3,760 cells. (*G*) Frequencies of *Tnfα* expressing cells in LTA-pretreated compared with untreated LPS-stimulated macrophages with SDs of three independent experiments. Sample size (*n*) = 3,334 cells. (*H*) Frequencies of *Tnfα* biallelically expressing cells. (*I*) Frequencies of cells with *Tnfα* allele ND < 0.1 with SDs of three independent experiments. Sample size (*n*) = 3,779 cells. (*J*). Frequencies of *Tnfα* expressing macrophages with SDs of three independent experiments. Sample size (*n*) = 4,288 cells. (*K*) Frequencies of *Tnfα* biallelically expressing macrophages. *Value not detected.

Tnfα from either one or both alleles, unlike the *Lta* gene, which was moderately activated by LPS and was only expressed in a monoallelic manner (Fig. 3*D*). Strikingly, the examination of *Tnfα* transcription at the single-cell level uncovered a unique allelic pattern of expression. Early on in LPS stimulation, during the first 30 min, *Tnfα* was mainly expressed from one allele. After 1 h of stimulation, however, about 70% of the expressing cells displayed a pattern of transcription from both alleles. After the 1 h LPS biallelic switch, the frequency of expressing cells steadily declined until it reached basal levels after 24 h of stimulation (Fig. 3*E*). The switch from mono- to biallelic gene expression coincided with the qRT-PCR results of maximal mRNA levels at the 1h LPS time point, portrayed both by total *Tnfα* transcript levels and expressing cell frequency (Fig. 3 *B* and *C*). In short, *Tnfα* gene expression follows a distinct monoallelic pattern succeeded by a switch to biallelic expression early on in LPS stimulation of macrophages.

The LPS-Induced and Actin-Mediated Homologous Spatial Proximity of the LT/TNF Locus Is Necessary for the Biallelic Switch in *Tnfα* Gene Expression. To further explore whether the homologous spatial proximity of the *Tnfα* alleles played a role in mediating the subsequent biallelic switch in *Tnfα* gene expression, we blocked actin polymerization using latrunculin A (LTA) and found that the movement of the LT/TNF locus was impeded and spatial proximity was reduced (Fig. 3*F*). Although the frequency of cells that expressed *Tnfα* was not affected by the treatment with LTA (Fig. 3*G*), we found that the switch to biallelic gene expression, after 1 h of LPS stimulation, was not observed (Fig. 3*H*). We next investigated whether the TNFα cytokine, produced by macrophages upon LPS stimulation, was responsible for inducing the homologous spatial proximity of the *Tnfα* alleles and the subsequent switch from mono- to biallelic gene expression. We treated RAW 264.7 macrophages with mouse recombinant TNFα and found that it failed to induce homologous spatial proximity (Fig. 3*I*). RNA-DNA FISH experiments on TNFα-treated macrophages revealed that although *Tnfα* gene transcription was detected (Fig. 3*J*), biallelic expression was greatly impaired (Fig. 3*K*). We conclude that the homologous association of the *Tnfα* alleles is LPS-induced, actin-mediated, and indispensable for the biallelic switch in *Tnfα* gene expression.

Identification of Two Complementary Long Noncoding RNAs Transcribed from the LT/TNF Locus. Long noncoding RNAs have been implicated in the regulation of many diverse physiological processes maintaining cell homeostasis (33–36). For example, to compensate for gene dosage differences between sexes, the mechanism of mammalian X inactivation involves the transient pairing of the two X chromosomes, and subsequently the expression of the *Xist* lncRNA results in the down-regulation of gene expression in the inactive X chromosome (13, 35, 37, 38). The involvement of lncRNAs in mammalian X inactivation, as well as in other processes entailing trans-allelic proximity (39), directed us to examine whether there was evidence of such long transcripts in the LT/TNF locus.

Multiple intergenic regions in the LT/TNF locus are highly conserved among mammals, and the locus itself encompasses a genomic region of 17 kb on murine chromosome 17 (Fig. 4*A*). To test for the presence of intergenic transcripts in the LT/TNF locus, we isolated total RNA from LPS-stimulated RAW 264.7 cells in a time course of 6 h; RNA samples were treated with DNase I and then subjected to reverse transcription primed by random sequence hexanucleotides. The produced cDNA was used in PCR reactions, using primer pairs that covered the entire LT/TNF locus and mapped to either the coding regions of the genes (such as the primer pairs for the PCR product 6 mapping on the coding region of the *Lta* gene, PCR product 8 mapping on the coding region of the *Tnfα* gene, and PCR product 10 mapping on the coding region of the *Ltb* gene), encompassing the locus, or to intergenic regions (such as PCR products 1–4, 7, 9, and 11) (Fig. 4*B*). On the basis of this analysis, using random sequence hexanucleotides for the generation of cDNA and the subsequent PCR analysis for the PCR products 1–11, we concluded there was extensive transcription throughout the LT/TNF locus; moreover, these transcripts were up-regulated upon LPS stimulation of the cells. Intergenic transcripts have been detected for PCR reactions 1–9, but not for the PCR reactions 10 and 11, proximal to the *Ltb* gene.

We then asked whether the transcripts we have detected were individual short RNA molecules or were part of longer RNA transcripts. To answer this question, we performed reverse transcription experiments on total RNA isolated from either untreated or 1-h LPS-stimulated macrophages, using single primers mapped on the ends of the transcribed region (genomic regions mapped on either PCR product 1 or PCR product 9) of the LT/TNF locus. For the detection of a sense long RNA transcript, we have used primer 9F (Fig. 4*A* and *SI Appendix*, Fig. S4) to produce cDNA in a reverse transcription reaction with total RNA as template and then used this single primed cDNA as template for PCR reactions 1–9 (Fig. 4 *C* and *D*). To detect an antisense long transcript in the locus, we used primer 1R (Fig. 4*A* and *SI Appendix*, Fig. S4) to produce cDNA in a reverse transcription reaction with total RNA as template and then used this single primed cDNA as template for the PCR reactions 1–11 (Fig. 4 *C* and *D*). On the basis of the PCR analysis of these single-oligonucleotide-primed cDNAs, we concluded that there are two complementary long RNA molecules, with a length of more than 12.0 kb each, that encompassed the *Lta* and *Tnfα* gene loci, and their expression was up-regulated upon LPS stimulation of the cells (Fig. 4*C*, *Upper*, for untreated macrophages and Fig. 4*C*, *Lower*, for 1-h LPS–treated macrophages). We named these transcripts lncRNA *SeT* (for LT/TNF locus lncRNA sense transcript) and lncRNA *AseT* (for LT/TNF locus lncRNA antisense transcript) (Fig. 4 *A* and *C*). We then used the rapid amplification of cDNA ends (RACE) approach to characterize the potential 5′- and 3′-ends of each individual transcript and identified both ends of each transcript (Fig. 4*D* and *SI Appendix*, Fig. S4). LncRNA *SeT* had a size of 12,340 bp, and its transcription start site mapped 8,258 bp upstream from the transcription start site of the *Tnfα* gene, whereas its 3′-end mapped between the *Tnfα* and *Ltb* genes. LncRNA *AseT* had a size of 12,033 bp, its transcription start site mapped 3,835 bp downstream from the transcription start site of the *Tnfα* gene, and its 3′-end mapped 60 bp downstream from the transcription start site of the *lncRNA SeT* (Fig. 4*A*). On the basis of additional RACE reactions performed, we have also identified alternative 5′- and 3′-ends for each transcript, which are presented diagrammatically in *SI Appendix*, Fig. S4. It is noteworthy that we were able to detect the lncRNA *AseT* in differentiated human monocytes (THP1 cell line), but not the lncRNA SeT (*SI Appendix*, Fig. S5). Although informative, a more thorough analysis should be performed for the presence and the transcription start sites of these lncRNAs in human cell types. Moreover, using biotinylated strand-specific riboprobes coupled to extensive signal amplification, we were able to detect each individual *SeT* and *AseT* transcript in LPS-activated macrophages (Fig. 4*E*). On the basis of our results, we conclude there are two long cRNA molecules transcribed from the LT/TNF locus.

LncRNAs *SeT* and *AseT* Regulate the Homologous Spatial Proximity and Biallelic Expression of *Tnfα*. To further characterize the long transcripts *SeT* and *AseT*, we examined the genomic regions upstream from the transcription start site of each transcript, using the Evolutionary Conserved Regions browser, and found them to be conserved (>70% homology) between mammalian species (Fig. 5*A*). To study the chromatin conformation of these conserved genomic regions, we performed DNase I hypersensitivity assays

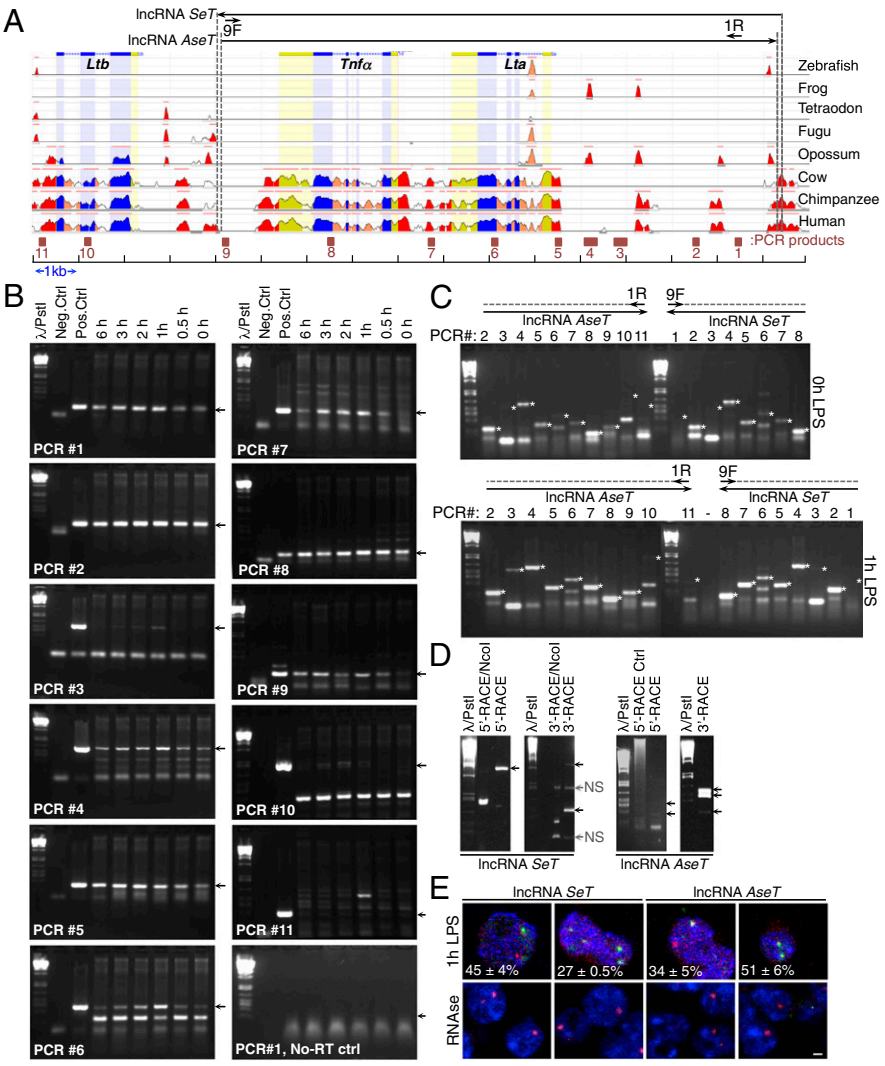

Fig. 4. Two complementary long noncoding RNAs are expressed from the LT/TNF locus. (*A*) Cross-species conservation of the LT/TNF locus. Conservation more than 70% is indicated for gene exons (blue), untranslated regions (yellow), and intergenic regions (red), based on the Evolutionary Conserved Regions browser. Numbered squares: PCR products used to detect transcripts mapped on the locus. (*B*) Intergenic transcripts are detected on the LT/TNF locus. PCR products spanning the locus in 1–2-kb intervals (sequences 1–11), on random-hexamers primed reverse transcription of total RNA isolated from macrophages stimulated with LPS. Arrows indicate expected PCR products. Additional bands of lower molecular weight for PCR reactions 6 and 10: *Lta* and *Ltb* spliced transcripts. Bacterial artificial chromosome DNA was used as a positive control, and no-DNA reactions as a negative control. (*C*) Long complementary transcripts are detected on the LT/TNF locus. PCR reactions harboring the 11 indicated sequences in *A* were performed on cDNA templates created with single specific-primer reverse transcription of RNA from macrophages (9F: RT primer for lncRNA *SeT*; 1R: RT primer for lncRNA *AseT*). (*D*) Rapid amplification of cDNA ends for the 5′- and 3′- ends of the lncRNAs *SeT* and *AseT*. From left to right: lncRNA *SeT* 5′-RACE performed with the primer 1F and nested primers (control digestion with NcoI restriction enzyme), lncRNA *SeT* 3′-RACE performed with primer 9R and nested primers, lncRNA *AseT* 5′-RACE performed with primer 9R and nested primers, and lncRNA *AseT* 3′-RACE performed with primer 1F and nested primers. NS = nonspecific. (*E*) RNA-FISH using strand specific biotinylated riboprobes for the nascent *SeT* and *AseT* lncRNAs. (Scale bar, 2 μm.)

and found them to be hypersensitive in untreated RAW 264.7 murine macrophages, whereas the DNase I hypersensitivity increased upon LPS stimulation of the cells (Fig. 5*B*). We used mammalian expression vectors and cloned these genomic regions upstream of a luciferase reporter gene (*SI Appendix*, Fig. S6). Transient transfection of the two individual constructs in RAW 264.7 cells and subsequent LPS stimulation of the cells revealed that the DNase I hypersensitive site (HSS) 1 upstream from the transcription start site of the lncRNA *SeT* showed increased promoter-reporter activity rapidly (30 min) upon LPS stimulation of the cells, which remained active 3 h after the initial stimulation. In contrast, HSS9, upstream from the transcription start site of the lncRNA *AseT*, showed rapid and transient promoter-reporter activity after 30 min of LPS stimulation of the cells (Fig. 5*C*).

To determine the relative RNA expression levels of the two lncRNAs, we performed qRT-PCR upon specific primer reverse transcription and found that the lncRNAs were both expressed in untreated mouse macrophages and are rapidly up-regulated upon LPS stimulation of the cells (Fig. 5*D*). Interestingly, the two lncRNAs had distinctly different roles in the *Tnfα* homologous spatial proximity and allelic expression profile. Silencing of the lncRNA *SeT* (*SI Appendix*, Fig. S7*A*), using locked nucleic acid (LNA) technology, had no effect on the LT/TNF locus homologous spatial proximity (Fig. 5*E*) and rendered *Tnfα* transcription mainly biallelic (Fig. 5*F*) upon LPS stimulation of macrophages, with no apparent effect on *Tnfα* monoallelic expression (*SI Appendix*, Fig. S8). In contrast, LNA-mediated silencing of lncRNA *AseT* (*SI Appendix*, Fig. S7*B*) impaired both LT/TNF locus homologous spatial proximity and its biallelic expression (Fig. 5 *E* and *F*). Therefore, the specific silencing of each individual of the two complementary lncRNAs had opposite effects on the homologous spatial proximity and allelic *Tnfα* expression profile.

Identification of the Pyruvate Kinase Muscle Isoform 2 Protein with GA Binding Activity. To unravel the functional mechanism behind the regulation of homologous spatial proximity of *Tnfα* alleles and its effect on the regulation of allelic expression profile of the *Tnfα* gene, we have chosen to purify and characterize protein complexes that mediate such a phenomenon. We thus resorted to the protein factors that play a major role in transvection. GAF, a protein encoded by the Trithorax-like (*Trl*) gene in *Drosophila*, is a transcription factor with DNA binding and transactivation properties that binds to GAGAG motifs and locally remodels chromatin to enable enhancer–promoter interactions (40–42). Because there is no biochemically or functionally characterized mammalian homolog of the *Drosophila* GAF, we performed a series of biochemical assays to identify a protein factor with similar activity.

We selected $(GA)_n$ repetitive DNA stretches from the LT/TNF locus or an oligonucleotide harboring a single GAGAG motif mapping on the *Tnfα* gene promoter and performed electrophoretic mobility shift assays, using nuclear protein extracts prepared from LPS-stimulated mouse macrophages. We identified

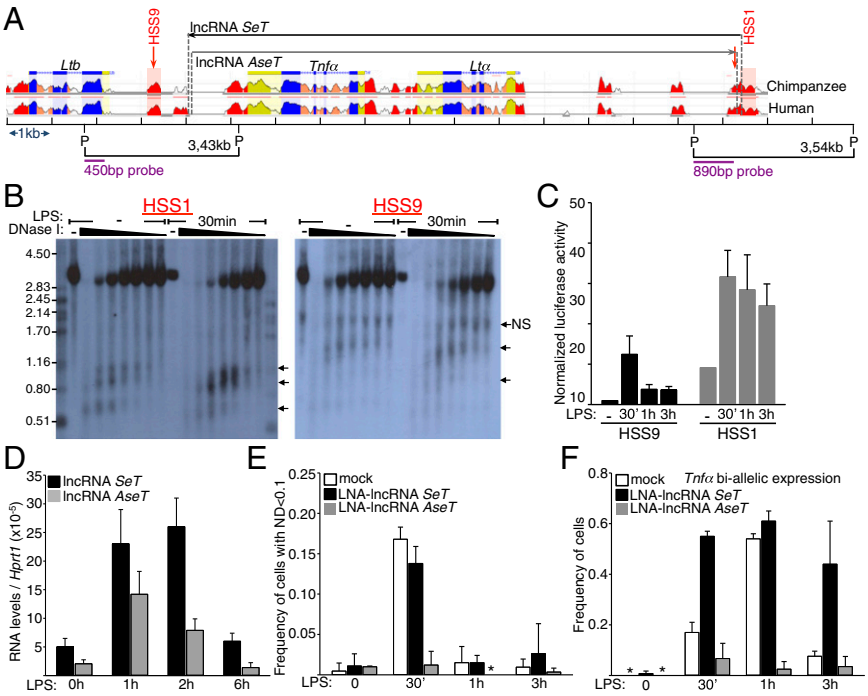

Fig. 5. The complementary lncRNAs *SeT* and *AseT* are LPS-regulated and mediate opposite effects on homologous spatial proximity and *Tnfα* allelic expression. (*A*) Mapping of the lncRNAs *SeT* and *AseT*. Radiolabeled DNA probes were used to hybridize to the *PstI* (P) fragments spanning the 5′ ends of each long transcript. (*B*) DNase I hypersensitivity mapping at the transcription start site of each long transcript. Arrows indicate the DNase I HSSs. NS, non specific. (*C*) Luciferase reporter assays in either untreated or LPS-stimulated macrophages, transiently transfected with promoter-reporter plasmids harboring either the HSS1 or HSS9 region. SDs are from three independent experiments. (*D*) qRT-PCR results of the relative RNA levels of the lncRNAs *SeT* and *AseT*. cDNA was reverse transcribed, using single oligonucleotide primers specific for *SeT* or *AseT*. (*E*) LNA-mediated silencing of the lncRNA *SeT* has no effect on the LT/TNF homologous spatial proximity, whereas silencing of the lncRNA *AseT* abolishes homologous spatial proximity. Frequency of cells expressing *Tnfα* in untreated cells (white) and cells treated with LNA for lncRNA *SeT* (black) or *AseT* (gray). (*n*) = 3,329 cells. (*F*) LNA-mediated silencing of the lncRNA *SeT* increases the biallelic *Tnfα* gene expression, and silencing of *AseT* abolishes biallelic *Tnfα* gene expression. (*n*) = 2,173 cells.

a specific DNA binding activity, which was dependent on LPS stimulation of the cells, and reached maximal binding on the *Tnfα* promoter oligonucleotide after 30 min of LPS stimulation (Fig. 6*A*). In accordance with these experiments, we performed Southwestern blotting experiments and were also able to detect maximal $(GA)_n$ oligonucleotide binding of several proteins present in nuclear protein extracts prepared from 30-min LPS-stimulated macrophages (Fig. 6*B*).

To isolate and characterize the protein or proteins with $(GA)_n$ binding activity, we have undertaken a series of approaches. Nuclear polyadenylated mRNA from LPS-stimulated macrophages (for 30 min) was used for the construction of a cDNA library in a yeast one-hybrid screening, using a sequence of 25 GA nucleotides as bait (*SI Appendix*, Fig. S9). In parallel, fractionated nuclear protein extracts from LPS-stimulated RAW 264.7 macrophages (Fig. 6 *C* and *D*) were used for DNA affinity chromatography coupled to mass spectrometry. Mouse pyruvate kinase muscle isozyme 2 (PKM2) was identified by both approaches as a protein with the ability to bind specifically on GA-repetitive elements (Fig. 6 *E* and *F*). On the basis of our results, we suggest that the PKM2 protein binds GA-containing sequences from the *Tnfα* locus either directly or indirectly, via its interaction with other protein factors.

Homologous Spatial Proximity Depends on PKM2 and T-Helper–Inducing POZ-Krüppel-Like Factor Proteins. PKM2 is a cytoplasmic protein with a role in glycolysis and was also recently shown to translocate into the nucleus and directly regulate transcription as a protein kinase (43–45). Using specific antibodies, we performed immunostaining experiments and found that PKM2 protein was mainly localized in the cytoplasm, but also in the nucleus of macrophages, with a distinct speckled pattern after 30 min of LPS stimulation (Fig. 7*A* and *SI Appendix*, Fig. S10*A*). T-helper–inducing POZ-Krüppel–like factor (ThPOK), in silico predicted to be the GAF mammalian homolog because of its structural and sequence similarity (46), was also tested for its involvement in the *Tnfα* homologous spatial proximity. ThPOK protein also displayed a distinct speckled pattern in the nucleus of macrophages (Fig. 7*B*). SiRNA-mediated knock-down of PKM2 (*SI Appendix*, Fig. S10*B*) or ThPOK in LPS-stimulated RAW 264.7 murine macrophages resulted in reduced *Tnfα* mRNA levels (Fig. 7 *C* and *D*) and, more importantly, disrupted the homologous spatial proximity of the *Tnfα* alleles, as depicted by DNA FISH analysis, based on the reduced frequency of cells with a *Tnfα* allele ND less than 0.1 (Fig. 7*E*). The reduced *Tnfα* mRNA levels could be explained by RNA-DNA FISH experiments, which showed that although the frequency of cells depicting *Tnfα* monoallelic expression was not remarkably affected by the knockdown of PKM2 or ThPOK, the frequency of biallelically expressing cells was greatly reduced (Fig. 7*F*). Taken together, these data show that PKM2 and ThPOK proteins mediate *Tnfα* homologous spatial proximity, and silencing either of them disrupts the association and consequently blocks the switch from mono- to biallelic *Tnfα* expression.

Discussion

Taken together, our data revealed the LPS-induced, transient and rapidly established homologous spatial proximity of *Tnfα* alleles that did not occur to maintain allelic exclusion, as in X chromosome inactivation or the Ig loci, but regulates mRNA dose control through a mono- to biallelic switch in *Tnfα* gene expression (Fig. 7*G*). Used as a way of information exchange *in trans*, *Tnfα* homologous spatial proximity ensures the production of maximal *Tnfα* mRNA levels necessary for macrophage immune responses.

Although evidence for regional pairing of homologous chromosomes has increased over recent years, it still remains unclear how the two alleles find each other and what mediates and/or sustains these associations. It has been speculated to be either a result of the properties of a larger region of the chromosome or a result of the specific settings provided by distinct genomic elements. Homologous pairing has been documented in several studies, the most prominent of which is the establishment of monoallelic silencing of the X chromosome. In X inactivation, the two chromosomes pair and the lncRNA *Tsix* is transiently down-regulated to allow the monoallelic expression of the lncRNA *Xist* to reach levels sufficient for the coating and silencing of the inactive X chromosome (12). Pairing was also shown to occur between the Ig loci. In this case, one of the two alleles undergoes recombination-dependent cleavage, and the other is heterochromatinized (23). In imprinted loci, it is easier to distinguish

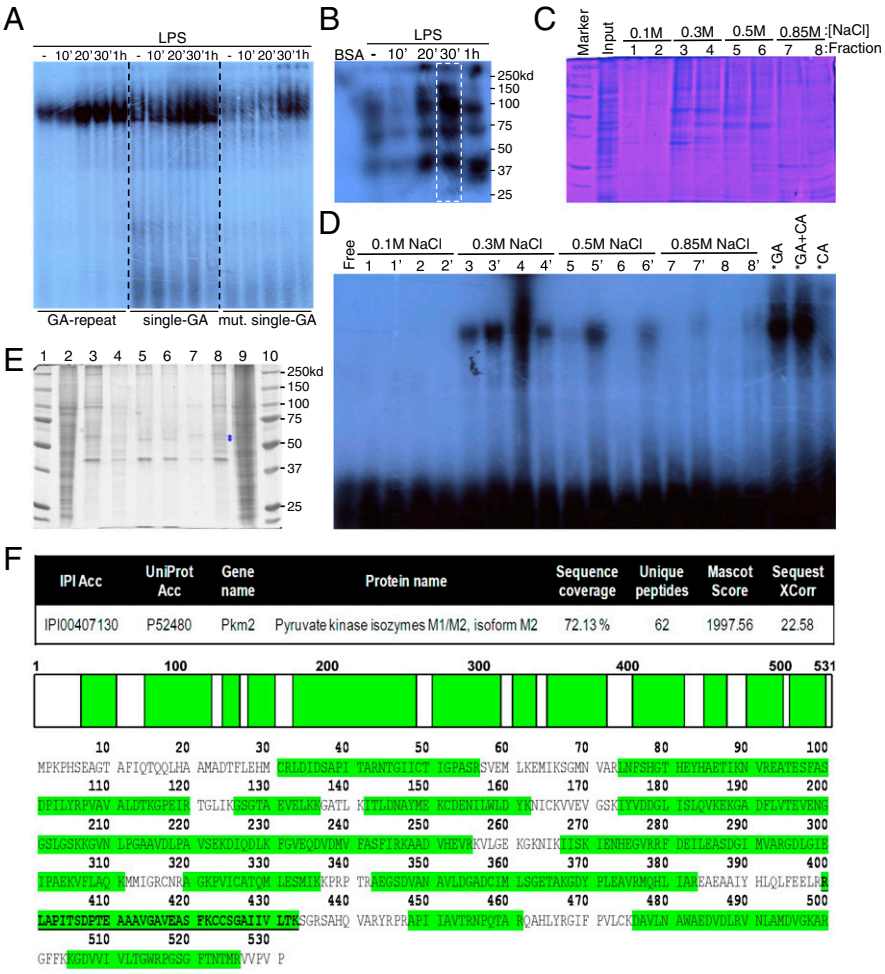

Fig. 6. Identification of the PKM2 protein from murine macrophages with $(GA)_n$ binding activity. (*A*) electrophoretic mobility shift assay with nuclear extracts from LPS-stimulated macrophages shows specific $(GA)_n$ binding activity upon LPS stimulation (GA-repeat: 25-bp oligonucleotide of repeated GA nucleotides, single-GA: specific sequence from the *Tnfα* promoter with a single GAGAG element; mut.single GA: similar to the latter with mutated GAGAG sequence). (*B*) Southwestern blot indicating DNA binding activity on a GA 25-mer labeled oligonucleotide from LPS-stimulated macrophage nuclear extracts. (*C*) SDS polyacrylamide gel electrophoresis of nuclear extracts from 30-min LPS-stimulated murine macrophages fractionated on a P11 phosphocellulose column and eluted with increasing NaCl concentration buffer. (*D*) Electrophoretic mobility shift assay indicating the presence of the desired DNA binding activity of proteins in specific eluted fractions from *C*. (*E*) DNA affinity chromatography. Nuclear protein extracts from mouse macrophages, precleared and fractionated (fractions 3 and 4), were incubated with concatamerized biotinylated oligonucleotides immobilized on streptavidin magnetic beads. Lanes 1/10: molecular weight marker, 2: first flow-through, 3: GA-bound proteins, 4: wash, 5/6: CA-bound, 7: second flow-through, 8: GA-bound, 9: input. Asterisks: bands analyzed. (*F*) PKM2 has been identified by mass spectrometry. Shown is the amino acid sequence of pyruvate kinase muscle isozyme protein. Green indicates the peptides identified by MS analysis (underlined: PKM2 specific).

the two loci, as they are differentially premarked through DNA methylation. Homologous pairing in the cases of Prader-Willi/Angelman region in humans or the Kcnq1 cluster has been extensively studied, and in these cases too, the result of the association was monoallelic expression (24, 25). These examples are associated with allelic exclusion, and it has been suggested that homologous pairing is a feature of regions in which one allele is silenced and monoallelic expression is maintained.

Moreover, homologous pairing has also been associated with DNA repair of double-strand breaks. Although nonhomologous end joining is more commonly used in mammals for the repair of such DNA damage, homologous recombination, which is predominantly used in yeast, is also found in mammals in the case of replication-induced breaks (47). It was recently shown that homologous pairing is not dependent on imprinting or allelic exclusion; in fact, loss of imprinting did not change pairing frequency. Instead, it was shown that somatic homologous pairing, although rare, depends on chromosomal position and transcriptional activity (48). Our data are in line with these findings, as we describe allelic spatial proximity, which is transient and rapidly established, and thus independent of imprinting status, and does not occur to maintain allelic exclusion but, instead, activates the expression of the *Tnfα* gene from the second allele as well.

We have also identified two protein factors mediating the homologous spatial proximity of the two *Tnfα* alleles: a protein kinase with transactivation potential and a transcription factor considered to be the mammalian homolog of the *Drosophila* GAGA factor. PKM2, a kinase involved in glycolysis and in cancer metabolism, is surprisingly capable of functioning as a protein kinase in the nucleus. It has been involved in several phosphorylation and transactivation events and has been found to bind DNA either directly or indirectly. It is intriguing, however, how a glycolytic enzyme is able to simultaneously function as a protein kinase. Studies of PKM2 in tumor cells, where it is predominantly found in its dimeric form, unable to convert phosphoenolpyruvic acid to pyruvate (49), give a possible answer to this question. The tetrameric form of PKM2 in the cytoplasm interacts with several glycolytic enzymes or oncoproteins, which are able to promote the conversion of PKM2 to a dimer (50). In addition, the binding of phosphorylated tyrosine peptides to PKM2 decreases its enzymatic activity (43). This was possibly caused by the exposure of a hydrophobic part of the dimeric protein, able to bind a protein substrate, in contrast to the tetrameric form, where this site would be inaccessible (51, 52). Thus, even without a prominent DNA binding domain, it is possible that LPS stimulation induces the switch to the dimeric form of PKM2 via an interaction in the cytoplasm, facilitating PKM2 to bind the LT/TNF locus, maybe through an interaction with ThPOK. Although a phosphorylation event may be occurring during the binding of PKM2 to ThPOK, altering the function of the latter (ThPOK contains tyrosine residues predicted to be phosphorylated by protein kinases), we have shown that both proteins are functionally necessary for the establishment of *Tnfα* homologous spatial proximity, as well as the switch in allelic expression.

Furthermore, we have implicated two long complementary transcripts, expressed by the LT/TNF locus, in the control of the switch in allelic expression of the *Tnfα* gene. The two lncRNAs involved seem to counteract one another by means of transcript quantity and localization. In correspondence to the function of

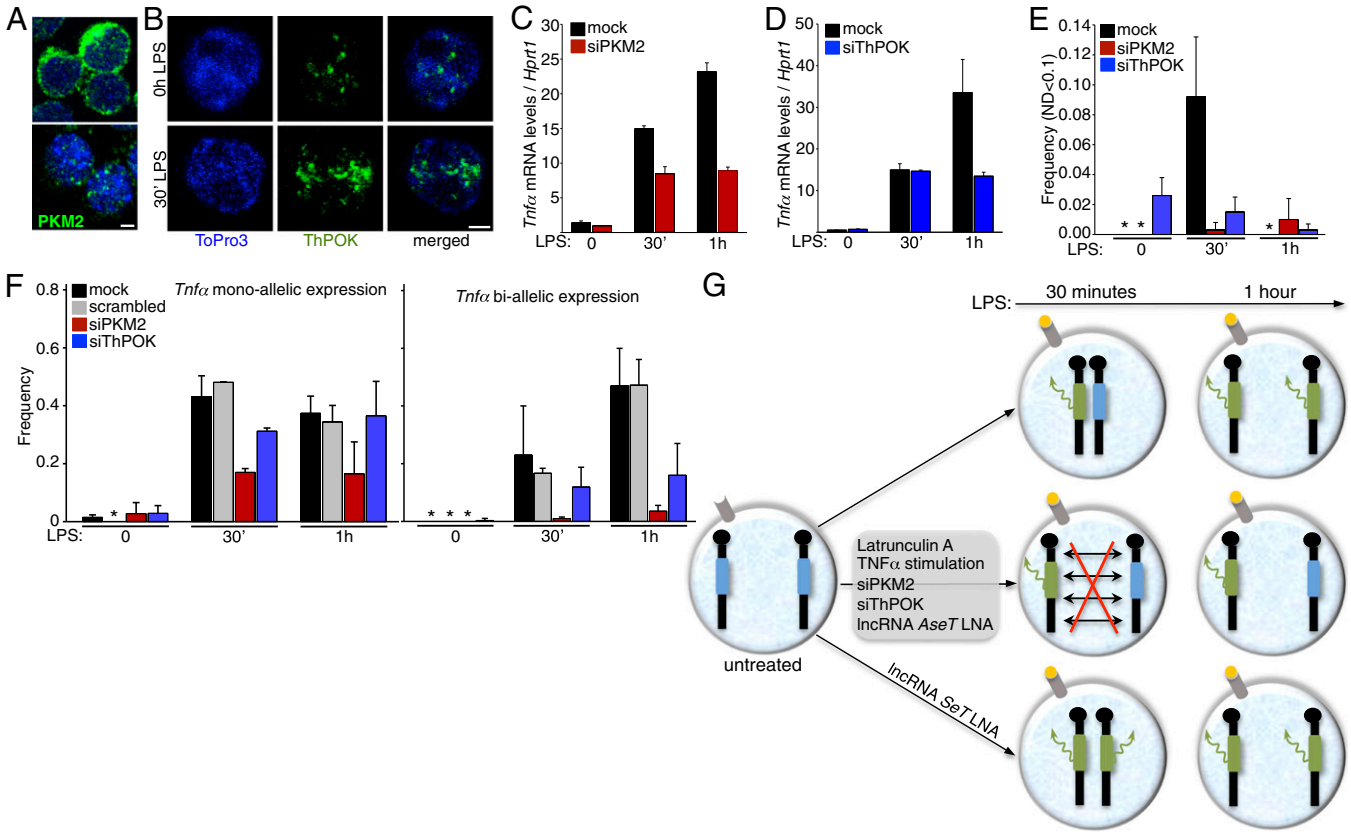

Fig. 7. PKM2 and ThPOK mediate *Tnfα* homologous spatial proximity and subsequent biallelic gene expression. (*A*) PKM2 protein subnuclear localization. (*B*) ThPOK protein nuclear localization. (Scale bar, 2 μm.) (*C*) *Tnfα* mRNA levels in LPS-stimulated macrophages either before or upon siRNA-mediated knockdown of PKM2. (*D*) *Tnfα* mRNA levels in LPS-stimulated macrophages either before or upon siRNA-mediated knockdown of ThPOK. (*E*) Effect of PKM2 or ThPOK siRNA treatment on *Tnfα* homologous spatial proximity. Frequencies of cells with allele ND < 0.1, for untreated cells (black), cells treated with siRNA for PKM2 (red), and siRNA for ThPOK (blue). (*n*) = 1,958. *Value not detected. (*F*) Frequencies of cells with monoallelic *Tnfα* expression for untreated (black), cells treated with siRNA for PKM2 (red), ThPOK (blue), or scrambled siRNA (gray). (*n*) = 5,683 cells. *Not detected. (*G*) Spatial proximity of the *Tnfα* alleles is LPS-induced, actin-mediated, and necessary for the biallelic switch in *Tnfα* expression. The complementary lncRNAs *SeT* and *AseT* transcribed from the LT/TNF locus have diverse effects on the PKM2- and ThPOK-mediated spatial proximity and biallelic *Tnfα* expression.

the lncRNA *Xist* in X inactivation, we may suggest that lncRNA *SeT* blocks *Tnfα* transcription from the second allele by coating the locus after 30 min of LPS stimulation, supported by the fact that LNA-mediated knock-down of this transcript allowed both alleles to express *Tnfα*. In parallel, lncRNA *AseT* functioned oppositely by displacing its complementary lncRNA *SeT* after 1 h of LPS stimulation (RNA-DNA FISH experiments show lncRNA *SeT* to be dispersed around the locus after 30 min of LPS stimulation), allowing the switch to biallelic expression. LNA-mediated knockdown of the lncRNA *AseT* could both disrupt homologous spatial proximity as well as render *Tnfα* expression monoallelic, possibly by allowing lncRNA *SeT* to coat the locus.

Further investigation in the mechanisms involved in the induction and maintenance of TNFα maximal levels is of great importance for both basic research and clinical practice. First, because a mechanism controlling somatic homologous pairing and allelic expression may be occurring in a wide range of inducible systems, and second, because the identification of ways to exploit such a mechanism could be used in the future to study and possibly resolve the deregulation of gene expression in disease models.

Materials and Methods

Detailed experimental procedures are available in the *SI Appendix*.

Cell Treatments. Murine monocyte-derived macrophage RAW264.7 cells were stimulated with 50 ng/mL LPS (InvivoGen) or 10 ng/mL TNFα (R&D Systems) or pretreated with 10 μM Latrunculin A (Sigma) where stated. Knockdown experiments for PKM2 and ThPOK were performed with the use of 5 nM Silencer Select siRNAs incubated with siPORT NeoFX Transfection Agent (Ambion, Applied Biosystems) in OPTI-MEM media (GIBCO) according to the manufacturer's instructions. The LT/TNF locus long transcripts were knocked down using Locked Nucleic Acid oligonucleotides (Exiqon). Thioglycollate-elicited (Brewer's medium, LAB064, Lab M) peritoneal macrophages were harvested from C57BL/6, B6.129P2-Ltb/Tnf/Lta^{tm1Dvk}/J (Delta3$^{-/-}$), or Myd88$^{-/-}$ mice, plated overnight, and stimulated with LPS as described earlier.

RNA-DNA FISH. For DNA FISH experiments, cells were fixed in 4% paraformaldehyde/1× PBS and permeabilized in 0.5% Triton X-100/1× PBS. For RNA-DNA FISH, cells were treated with cytoskeletal buffer prior to fixation. Hybridizations of genomic loci and nascent RNA were performed with bacterial artificial chromosome or cDNA probes labeled with spectrum orange/green dUTP. Nuclear DNA was stained with ToPro3 (ToPro3 Iodide 642/661) and pseudocolored blue.

Imaging Analysis and Statistics. The analyses and measurements of allele distances and nuclear volumes were performed with the use of the Volocity software (Improvision, Perkin-Elmer). Statistical analysis for the randomness of the distance distributions was performed in a pairwise manner, using the Kolmogorov-Smirnov test.

RACE. The FirstChoice RLM-RACE kit (Invitrogen, AM1700M) was used to identify the 5′- and 3′- ends of specific capped mRNA molecules from RAW 264.7 macrophages treated with 50 ng/mL LPS for 1 h.

Protein Identification and Mass Spectrometry. For the isolation of DNA binding proteins, the Yaneva and Tempst protocol was followed (53). Coomassie-stained polyacrylamide gel bands were destained, reduced, alkylated, and digested with trypsin (proteomics grade, Sigma, T6567). The subsequent mass spectrometric analysis involved nano-liquid chromatography–MS/MS analysis (54).

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Supplemental Information

Inventory of Supplemental Information

Supplemental Figures and Legends

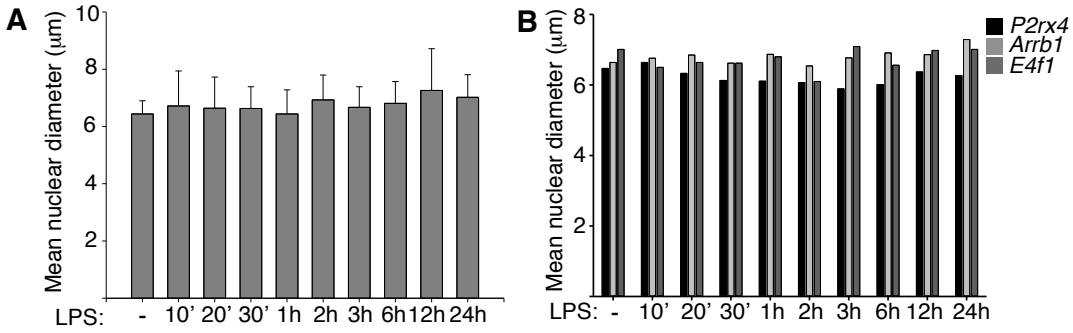

Fig. S1. Steady nuclear diameter of macrophages during the course of LPS stimulation.

(*A*) Homologous association of *Tnfα* alleles is not artificially detected due to changes of the nuclear cell volume upon LPS activation of macrophages. Bars represent the mean nuclear diameter (in μm) of untreated and LPS-stimulated macrophages used in DNA FISH experiments to measure *Tnfα* allele distances with standard deviations of 11 independent experiments. Sample sizes from (*n*) = 441 to 1104 cells for each time point.

(*B*) Bars represent the mean nuclear diameter of cells that were used to measure the *P2rx4* (black bars), *Arrb1* (light grey bars) and *E4f1* (dark grey bars) allele distances. Sample sizes (*n*) = 2022 (for *P2rx4*), 1176 (for *Arrb1*) and 1574 (for *E4f1*) cells.

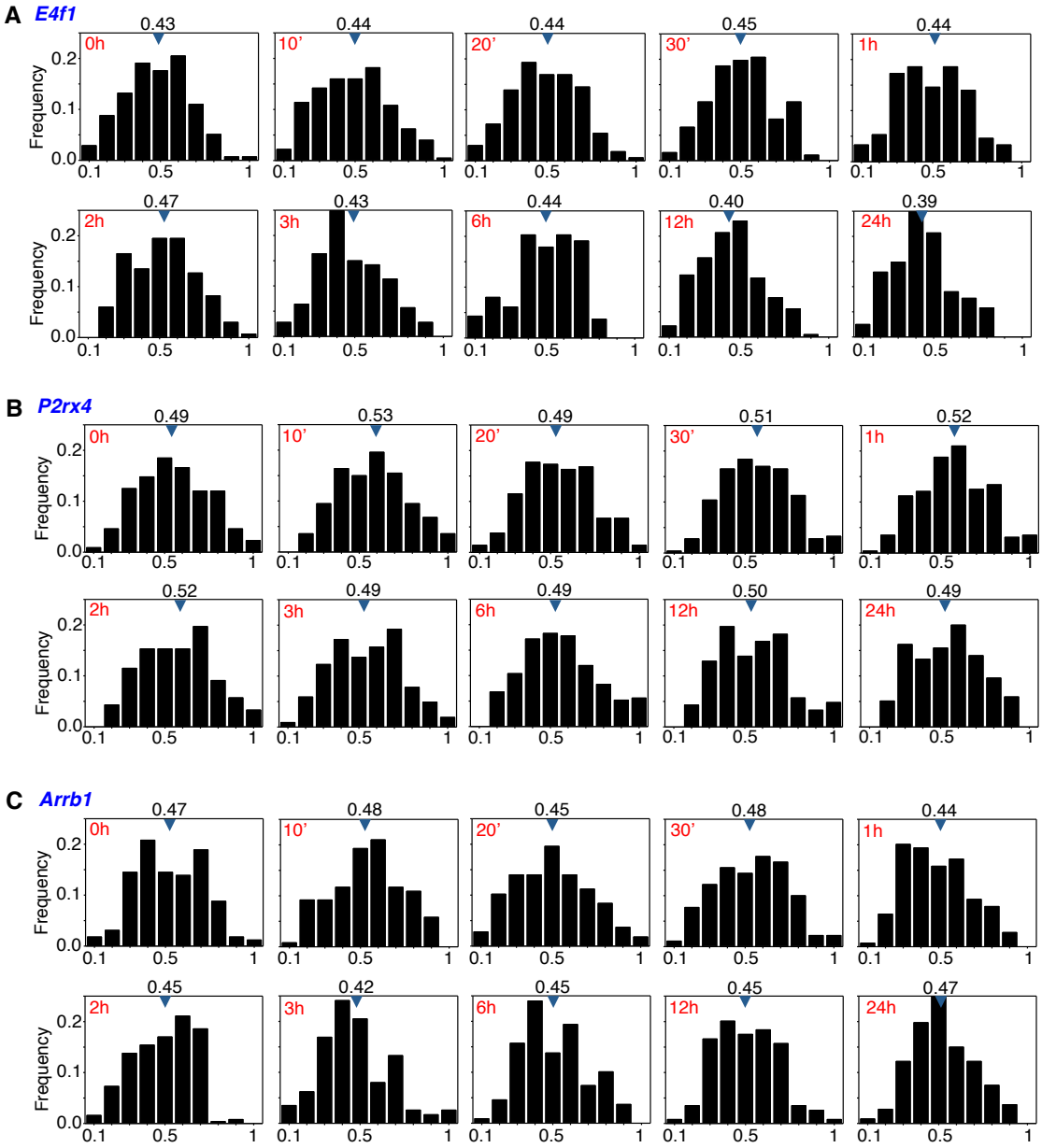

Fig. S2. Spatial proximity of homologous alleles is specific to the LT/TNF locus.

Spatial proximity of homologous alleles upon LPS stimulation of macrophages is not detected for loci located upstream the *Tnfα* gene on the same mouse chromosome (*E4f1*-chr. 17) or other chromosomes (*P2rx4* - chr.5, *Arrb1* - chr.7). Allele distance distributions are plotted in 10 clusters from 0 to 1, for each time point of LPS stimulated macrophages. Distances are normalized for the cell volume of each individual cell, and ND=allele distance/d, where

d=2x(nuclear area/π)$^{0.5}$. ND ranges from 0 to 1 and mean ND is indicated by a blue triangle.

(*A*) *E4f1* alleles distance distributions. Sample size (*n*) = 1574 ranging from 133 to 181 cells/timepoint.

(*B*) *P2rx4* alleles distance distributions. Sample size (*n*) = 2022 ranging from 135 to 223 cells/timepoint.

(*C*) *Arrb1* alleles distance distributions. Sample size (*n*) = 1176 ranging from 90 to 158 cells/timepoint.

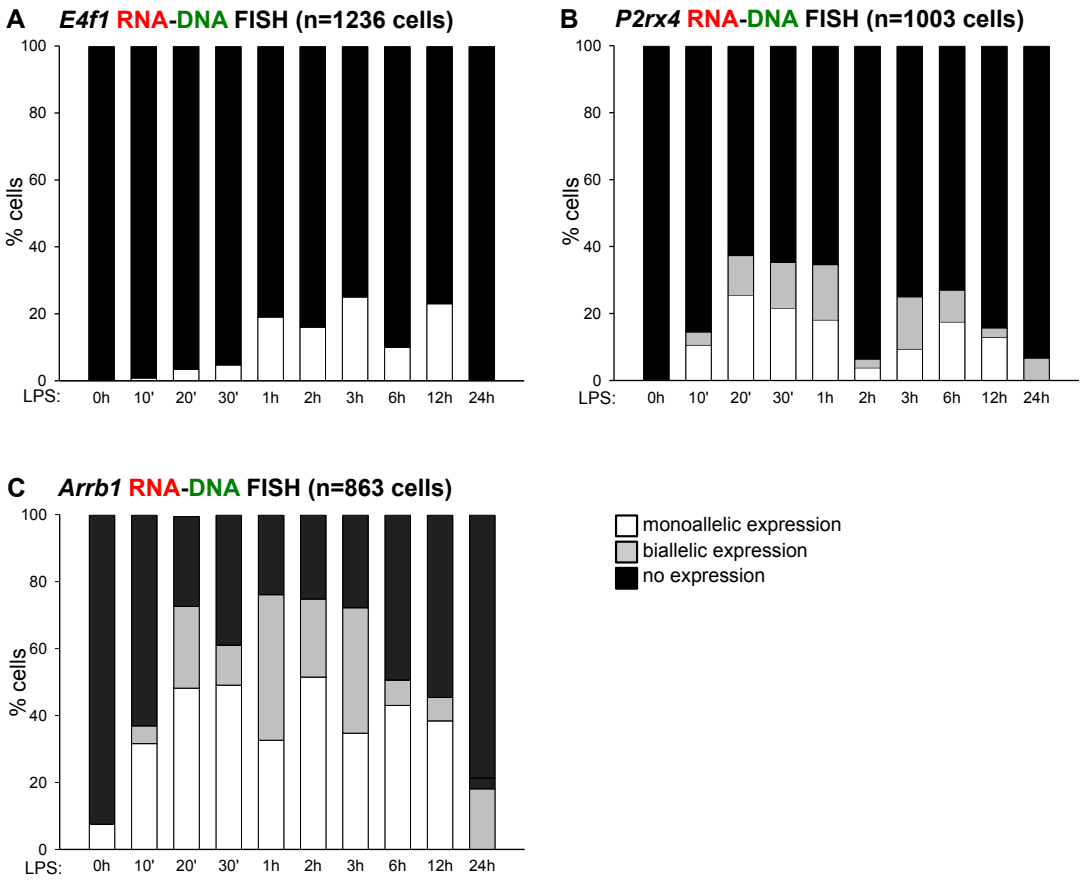

Fig. S3. Allelic expression profile of *E4f1*, *P2rx4* and *Arrb1* genes.

The percentage of expressing cells was plotted for each time point over a time course of LPS stimulation of RAW 264.7 mouse macrophages – monoallelic expression (white bars), biallelic expression (grey bars) and no expression (black bars) is depicted in the graphs.

(*A*) RNA-DNA FISH analysis of *E4f1* expression.

(*B*) RNA-DNA FISH analysis of *P2rx4* expression.

(*C*) RNA-DNA FISH analysis of *Arrb1* expression.

Nucleotide #31= mm9/35.329.096 (mouse chromosome 17)

primer 11F

PCR product #11

primer 11R

```
1     GGACTCTATT TTTTTTTTTT TTTTTTTAAA GATTTATTTA TTTATTTATT TATTTATTAT
      CCTGAGATAA AAAAAAAAAA AAAAAAATTT CTAAATAAAT AAATAAATAA ATAAATAATA

61    ATGGAAGTAC ATTGTAGCTG TCTTCAGACA CTCCAGAAGA GGGAGTCAGA TCTCGTTACG
      TACCTTCATG TAACATCGAC AGAAGTCTGT GAGGTCTTCT CCCTCAGTCT AGAGCAATGC

121   GATGGTTGTG AGCCACCATG TGGTTTGCTG GGATTTGAAC TCTGGACCTT CGGAAGAGCA
      CTACCAACAC TCGGTGGTAC ACCAAACGAC CCTAAACTTG AGACCTGGAA GCCTTCTCGT

181   GTCGGGTGCT CTTACCCACT GAGCCATCTC ACCAGCCCCG AGGGACTCTA TTAAAGCGTT
      CAGCCCACGA GAATGGGTGA CTCGGTAGAG TGGTCGGGGC TCCCTGAGAT AATTTCGCAA

241   GCAGCATCAG GAAGTTTGAG AACCACTGAC CTAGATCTAT AAGCAACACT TTGCTGAGTC
      CGTCGTAGTC CTTCAAACTC TTGGTGACTG GATCTAGATA TTCGTTGTGA AACGACTCAG

301   ACATGCCTCC CTCTCCTTTG TTTGTTCAGT TTTGTTTAAG ATTTATTTAT TATTATATCT
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361   AAGTACACTG AAGCTGTCTT CAGACACACT AGAAGAGGGT GTCAGATCTC ATTACAAATG
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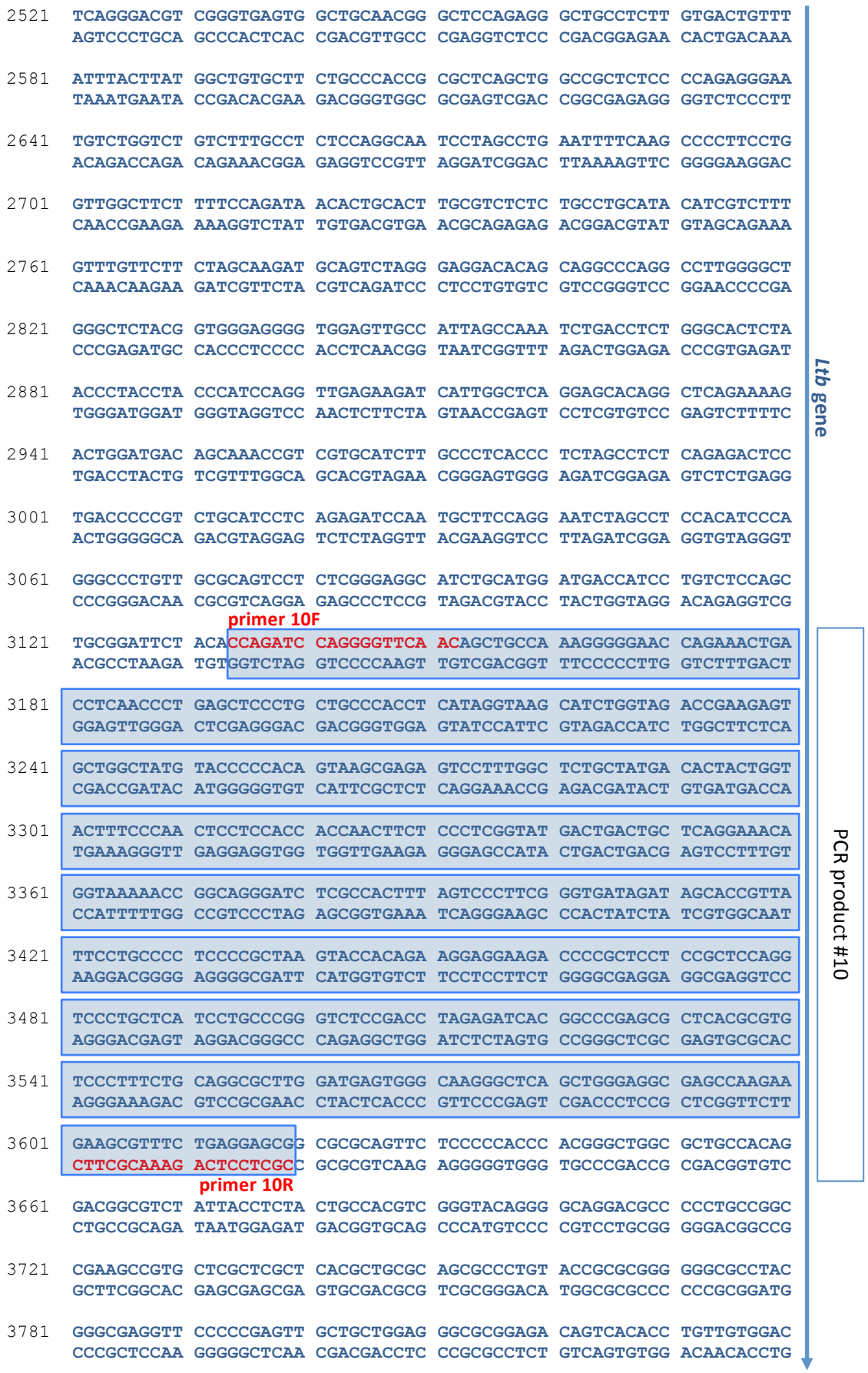
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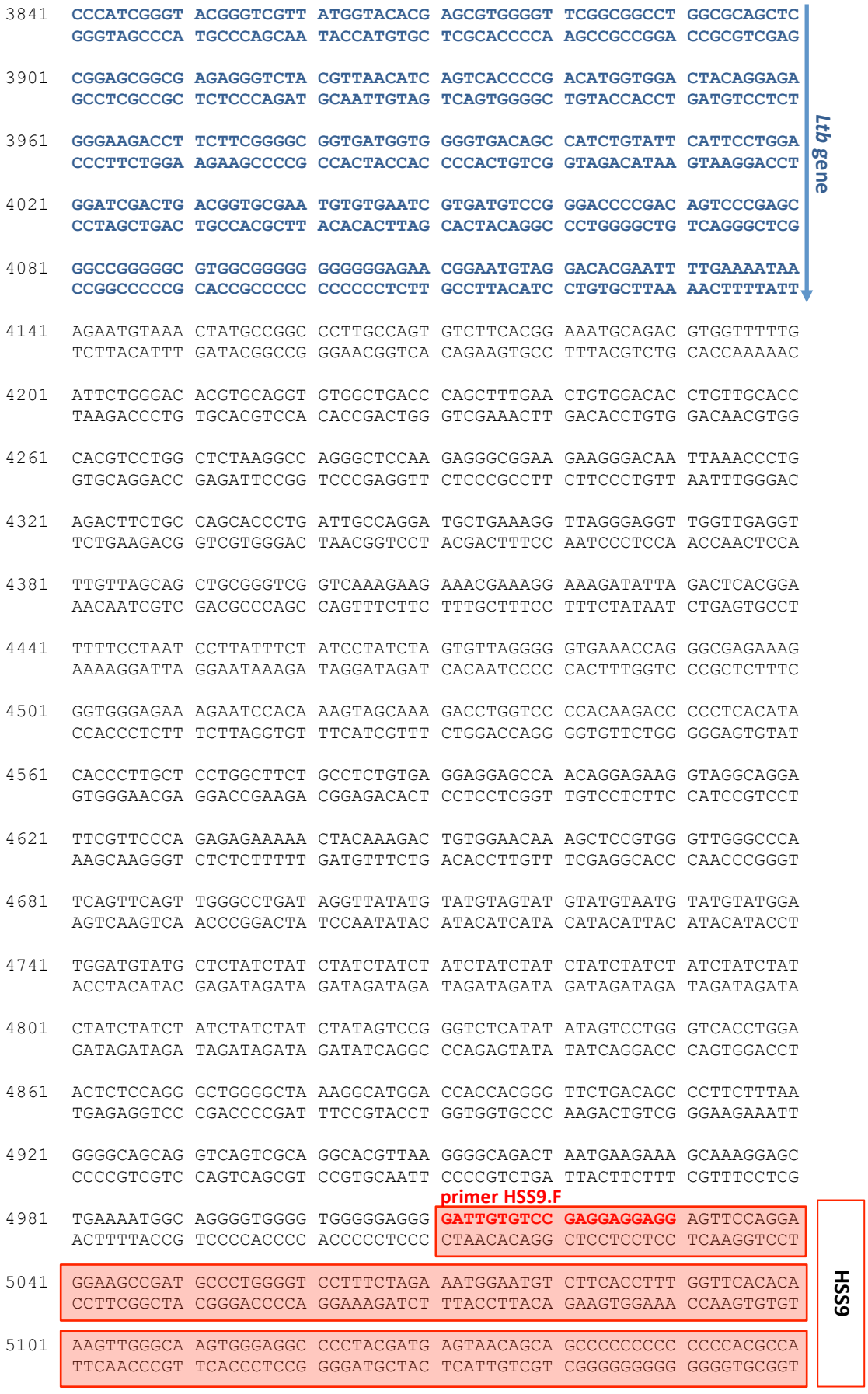
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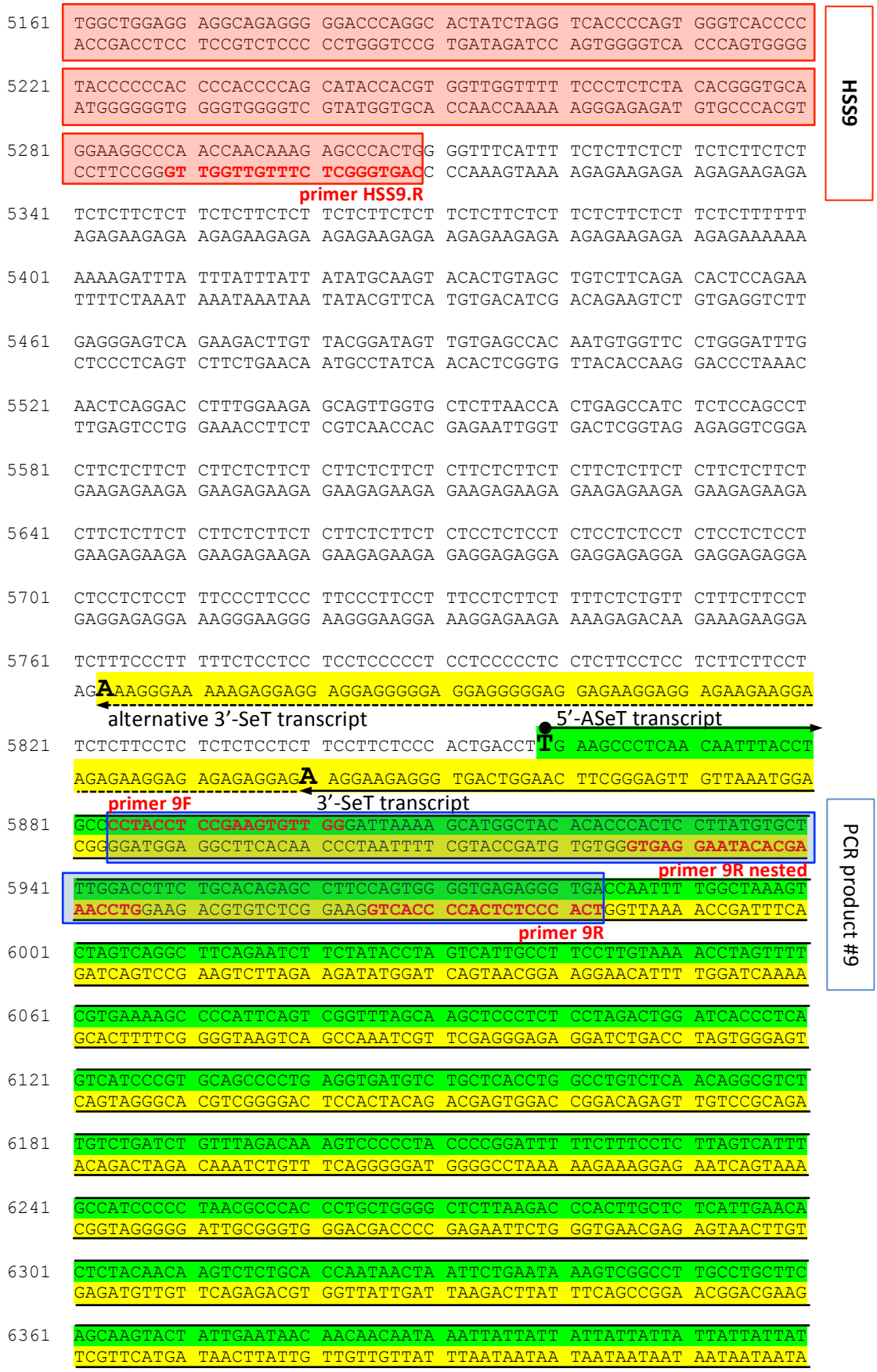
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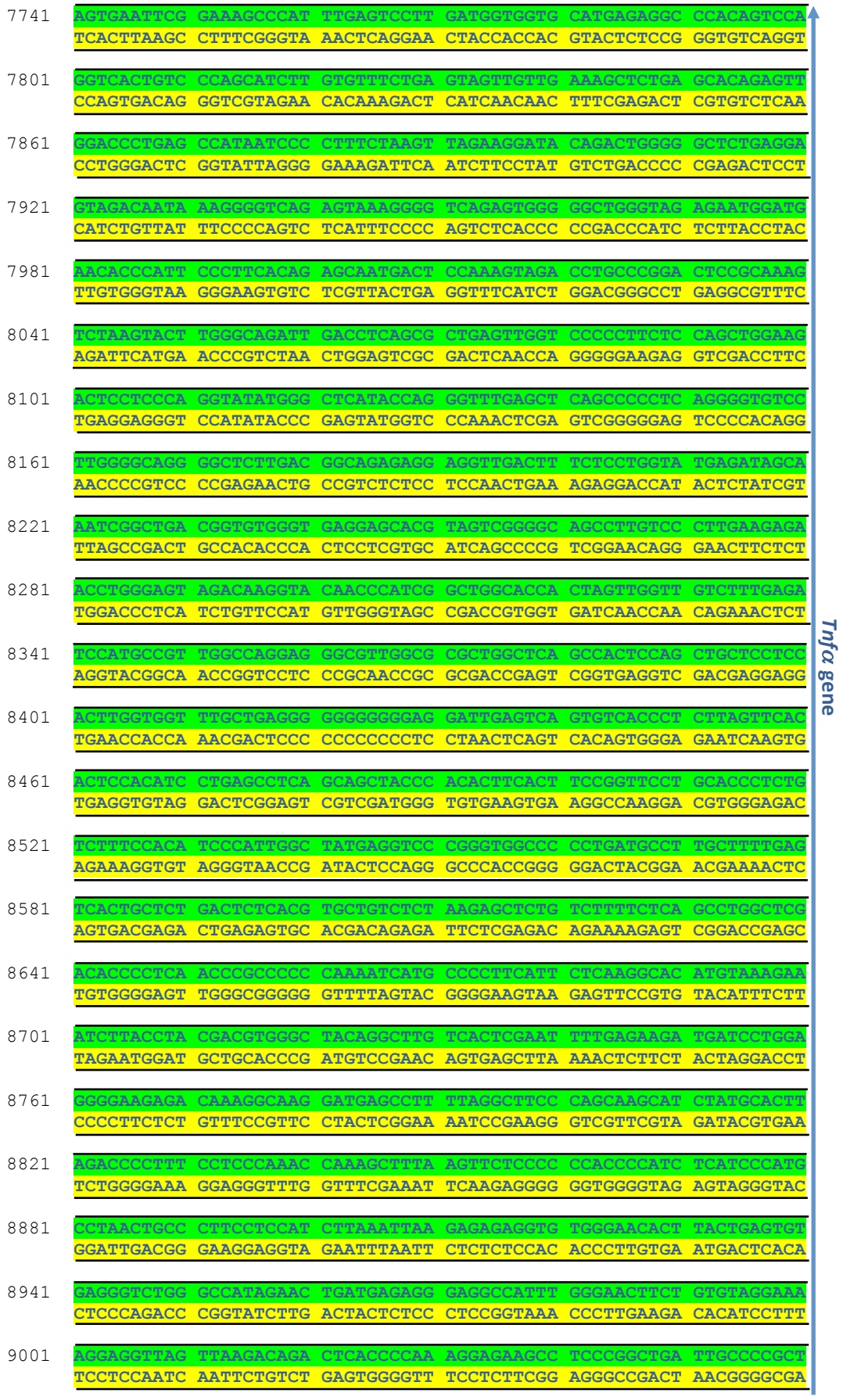
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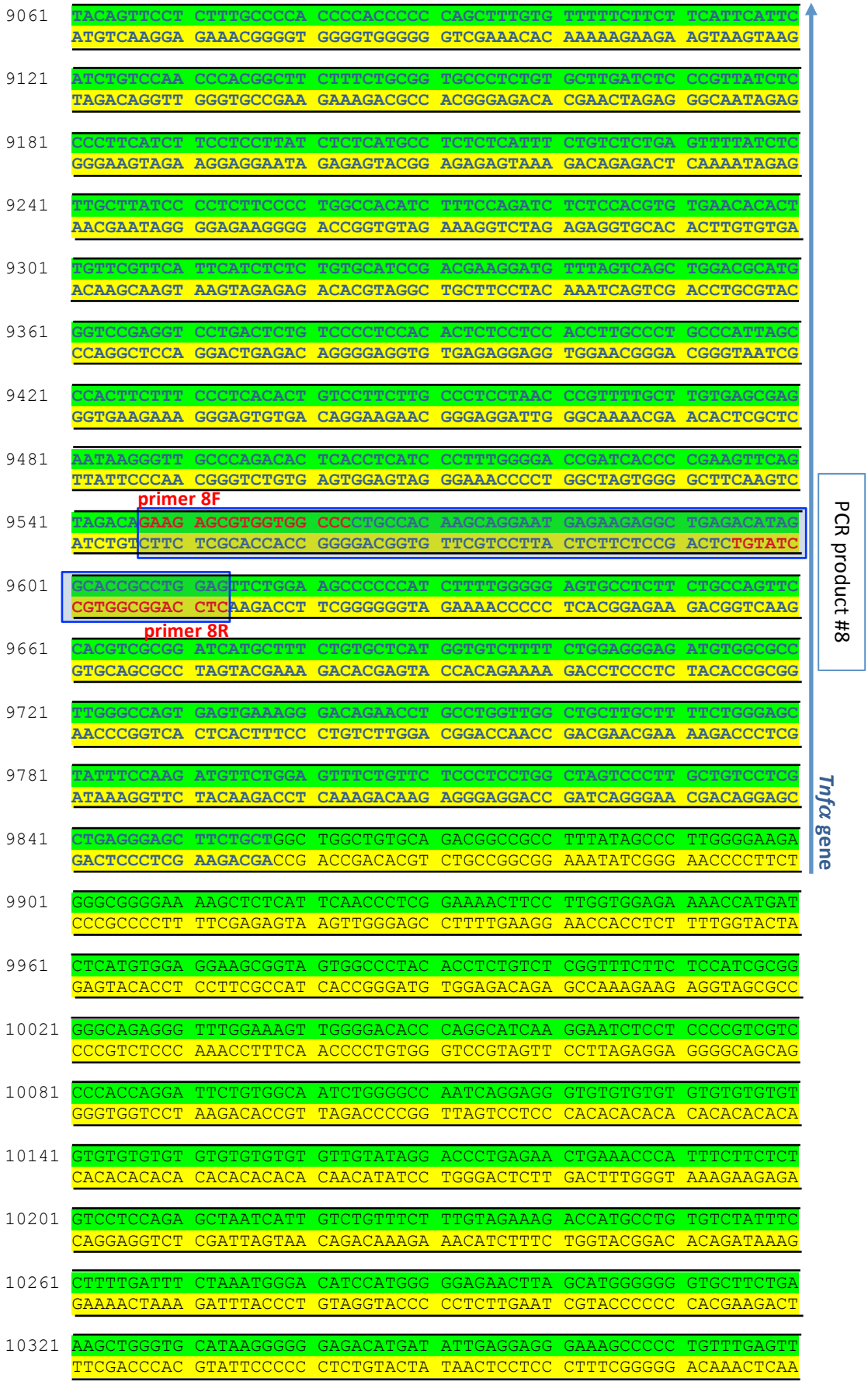
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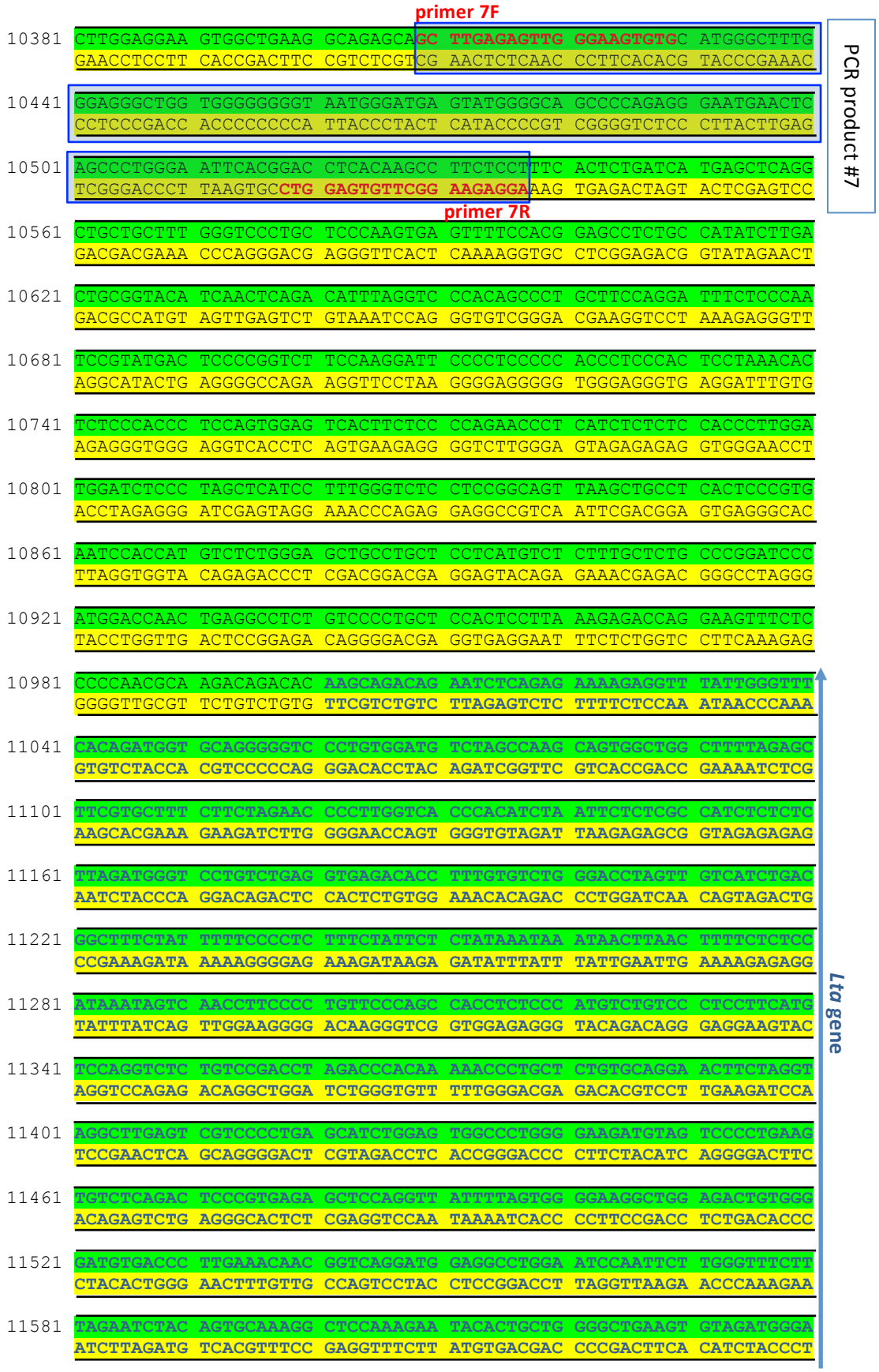
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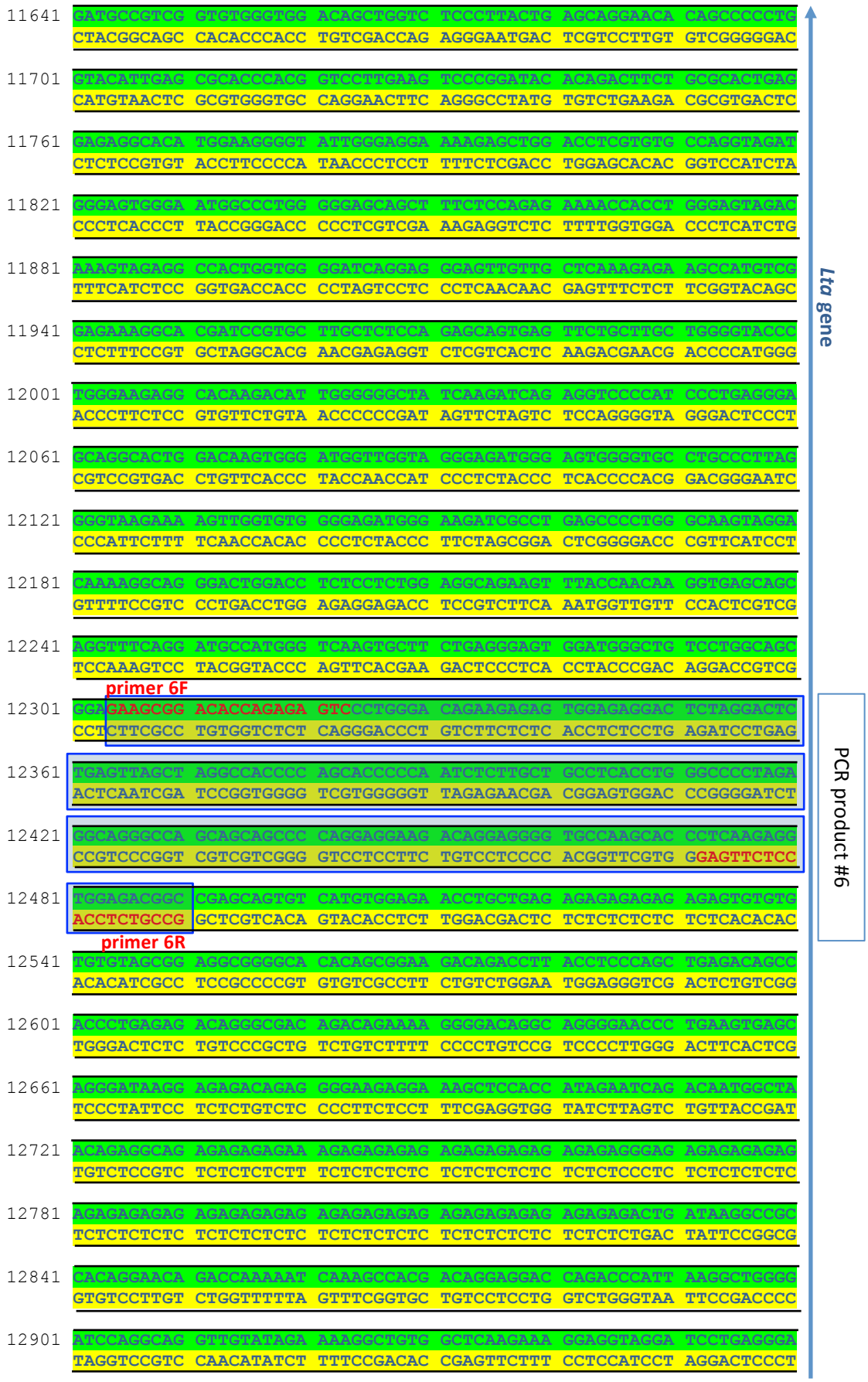
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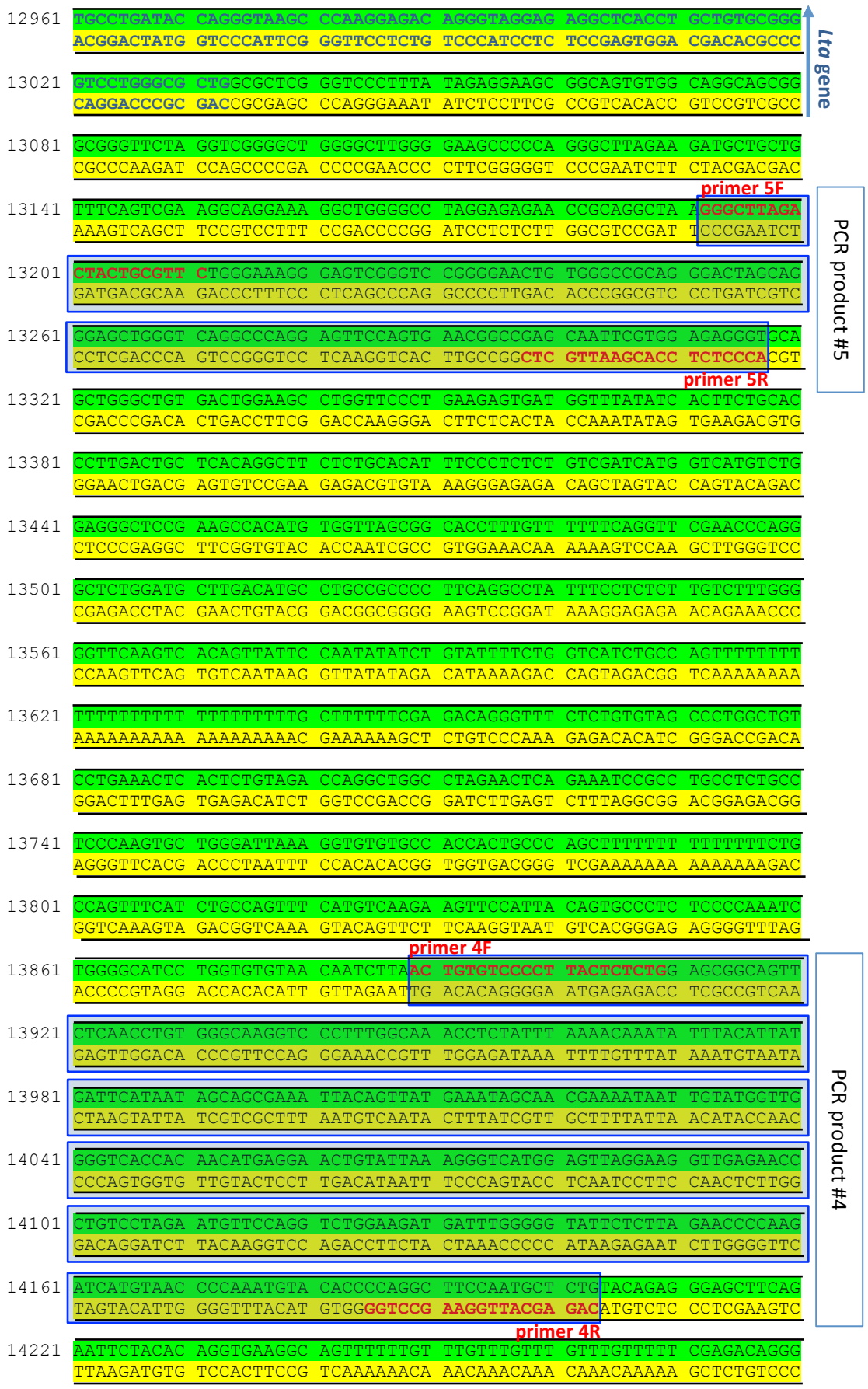
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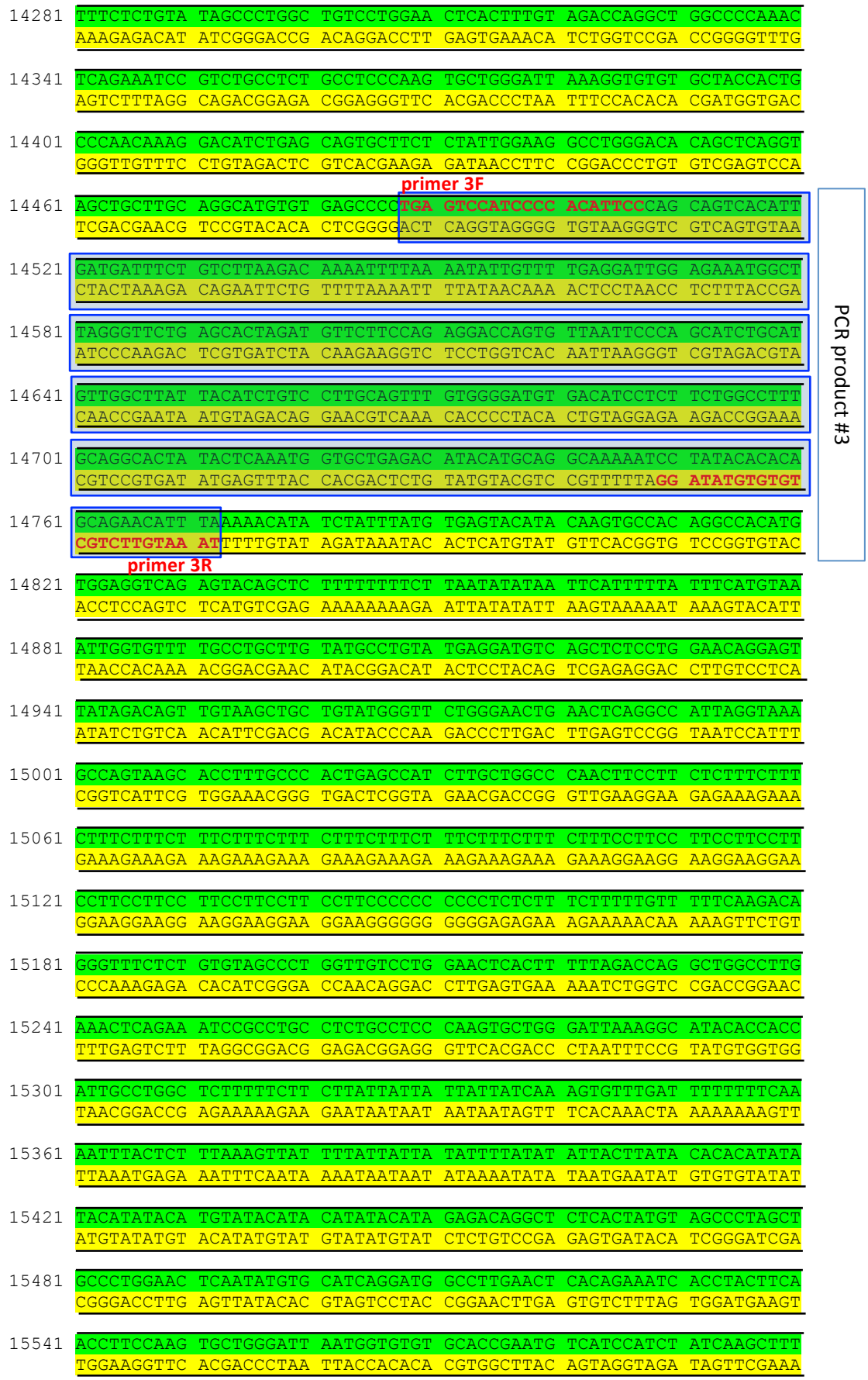
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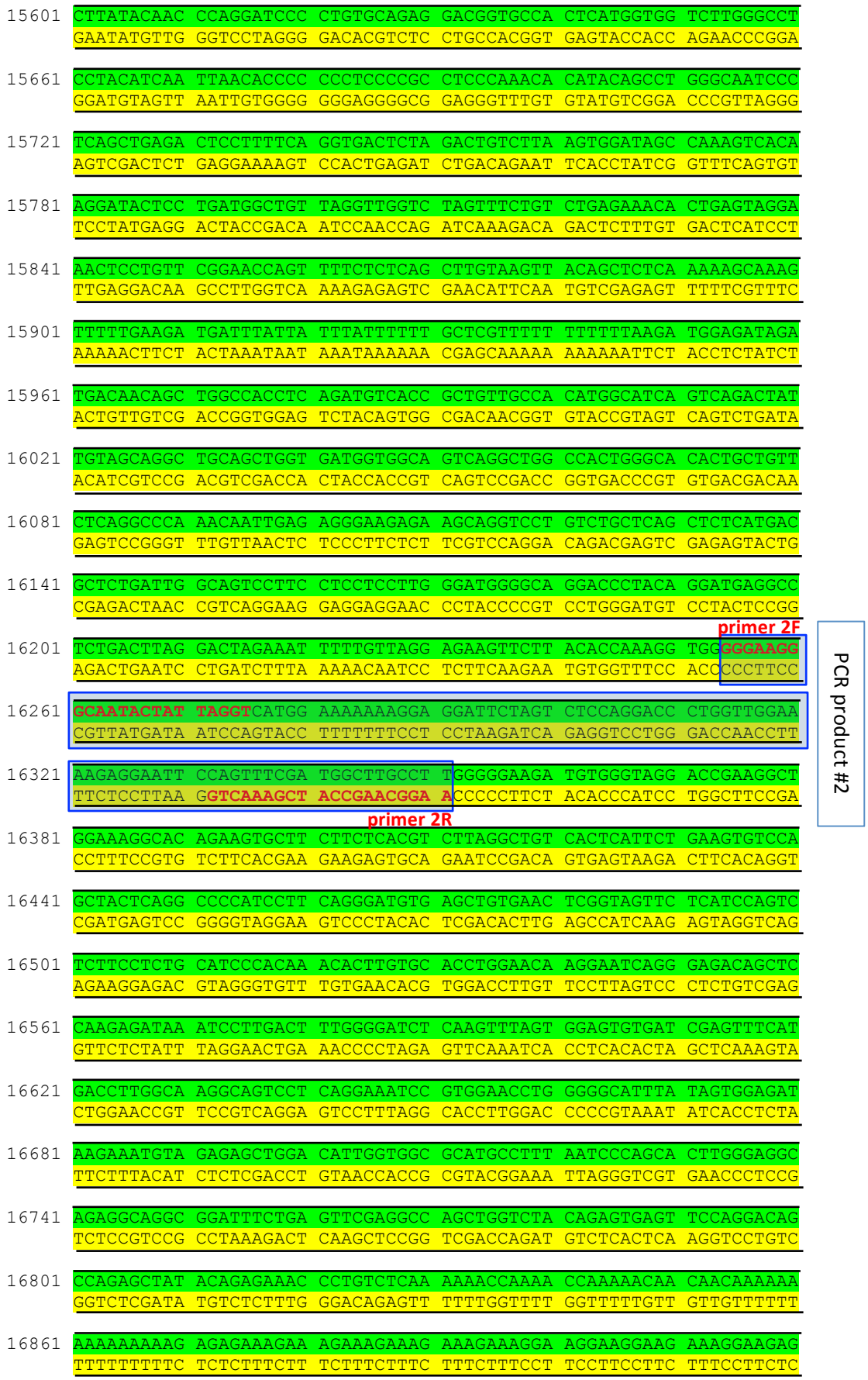
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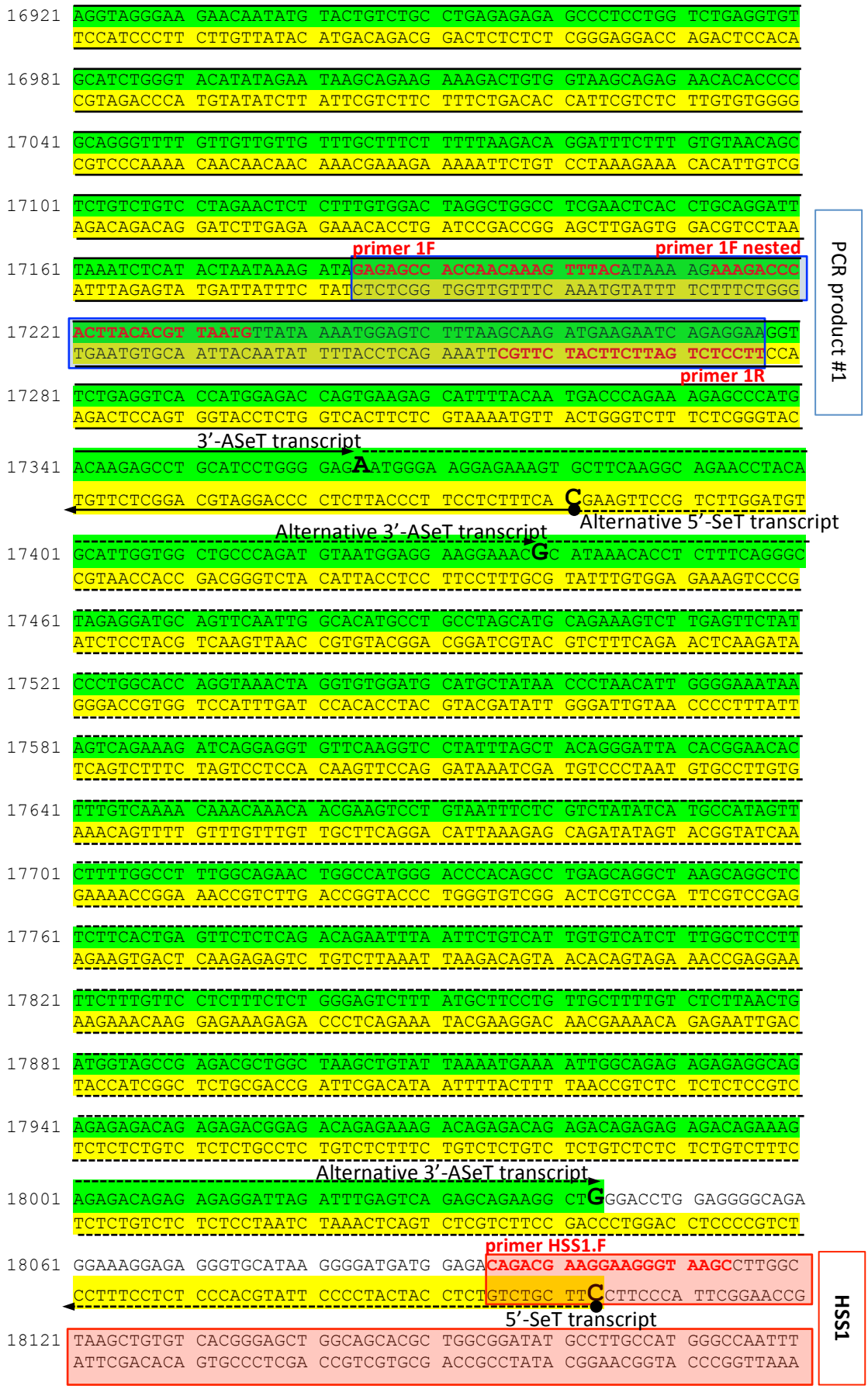
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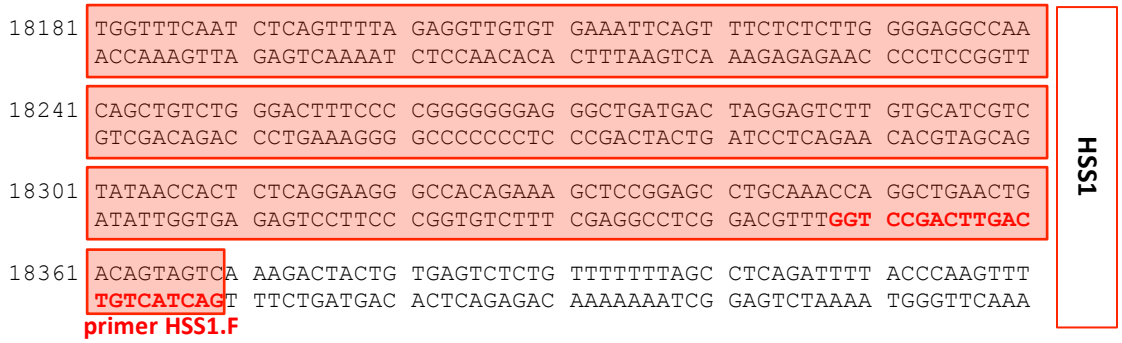


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Nucleotide #20000= mm9/35.349.095 (mouse chromosome 17)

Fig. S4. The LT/TNF locus sequence.

The double-stranded DNA sequence harboring the *Tnfα* gene is presented (mm9, chr. 17: 35.329.096 - 35.349.095). The sequence of the primers used for the identification of the two lncRNA transcripts (in red), the anticipated PCR products (blue frames), the *Ltb*, *Tnfα* and *Lta* genes – from the transcription start site to the last nucleotide of the 3' UTR – (blue, arrows show the direction of gene transcription), DNAse I hypersensitive sites HSS9 and HSS1 (red frames), the sequence of lncRNA *Set* (yellow) and lncRNA *ASet* (green), as well as their transcription start sites and alternative 5' and 3' ends are indicated on the sequence.

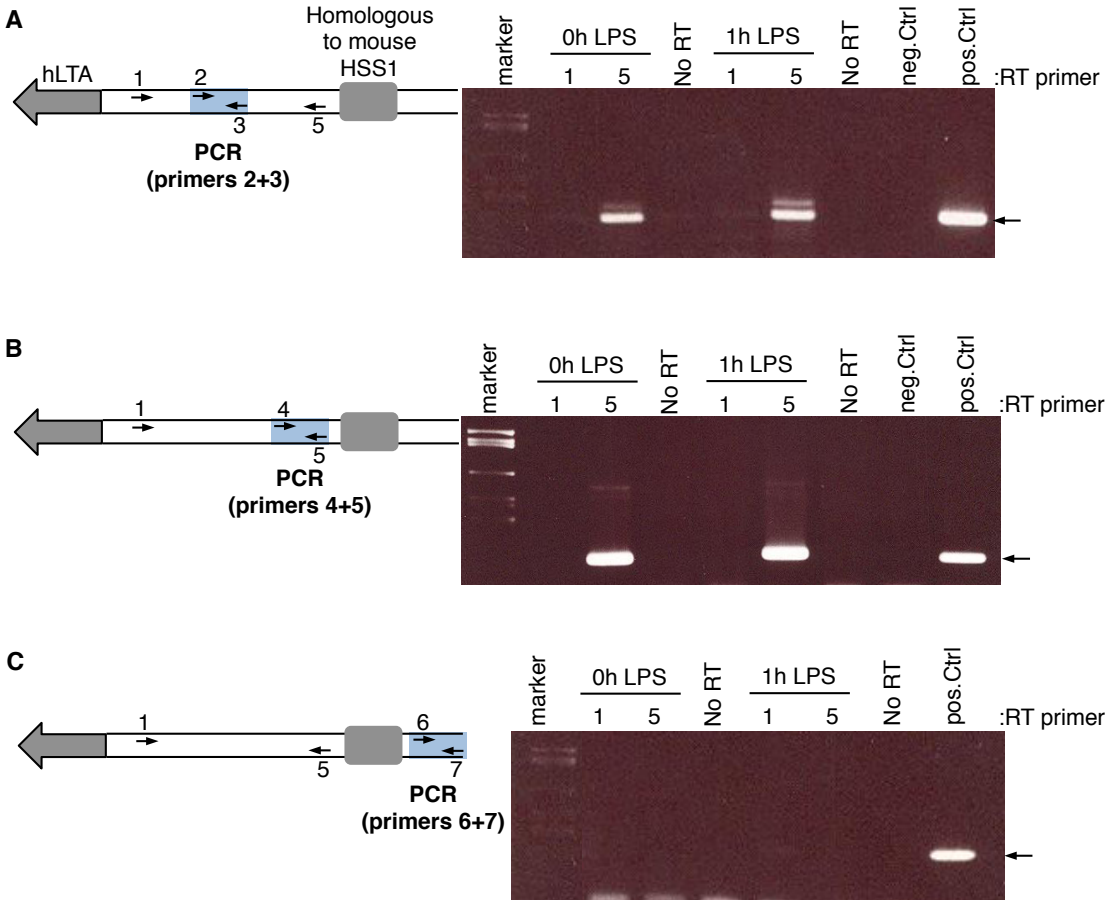

Fig. S5. Expression of *SeT/ASeT* in human macrophages.

Human THP-1 monocytes, differentiated into macrophages (50ng/ml PMA for 24h), stimulated with LPS were used for the detection of the lncRNAs *SeT* and *ASeT*. Specific primer – reverse transcription (primers shown with arrows 1 and 5) was performed and PCR products 2-3 (*A*), 4-5 (*B*), 6-7 (*C*) and no RT, negative PCR and positive (human BAC) controls are shown. Black arrows point to the PCR bands of the expected size.

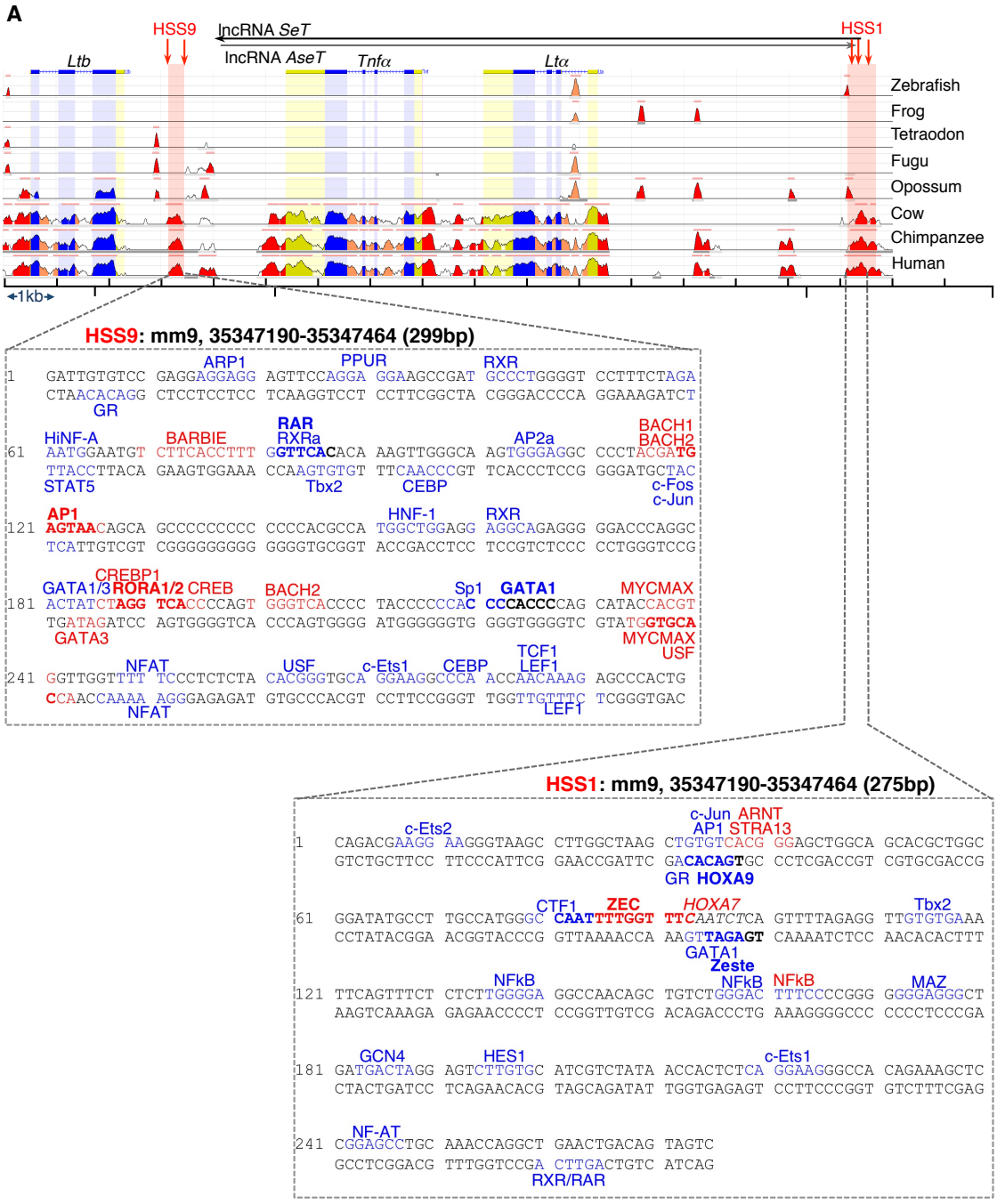

Fig. S6. DNAse I hypersensitive sites.

The DNA sequence conservation of the LT/TNF locus is presented (ECR browser). The conserved regions with DNAse I hypersensitivity HSS9 (299bp) and HSS1 (275bp) were selected and cloned for luciferase assays. Specific DNA binding motifs of transcription factors are shown on the sequence, as provided by the rVista (red) and PATCH (blue) tools.

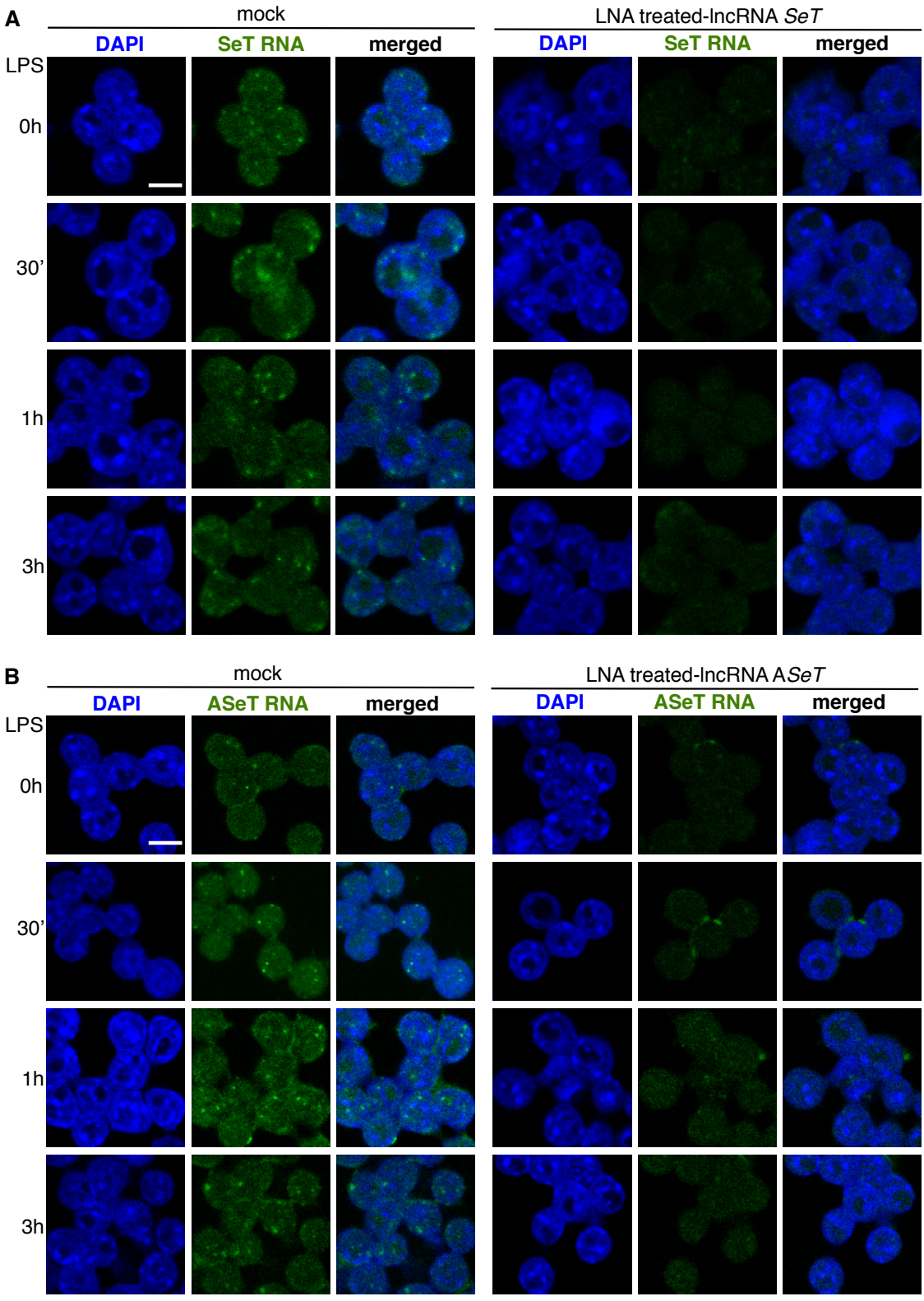

Fig. S7. LNA-mediated knock-down of lncRNAs *SeT* and *ASeT*.

RNA FISH experiments utilizing strand-specific biotinylated riboprobes coupled to tyramide signal amplification were performed in Raw264.7 macrophages

stimulated with LPS (0h, 30min, 1h and 3h), upon the transient transfection of specific LNA probes for 24 hours. Representative images of single z-sections of RNA FISH analysis using confocal microscopy are shown. Scale bar 5 μm. DNA was counterstained with ToPro3.

(*A*) RNA FISH analysis of lncRNA *SeT* expression upon LNA-mediated knock-down of *SeT* transcript.

(*B*) RNA FISH analysis of lncRNA *ASeT* expression upon LNA-mediated knock-down of *ASeT* transcript.

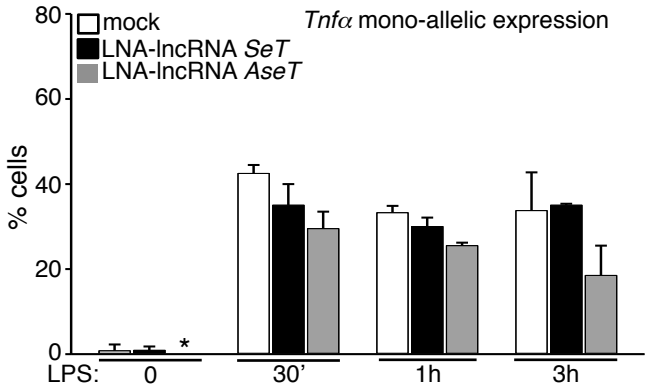

Fig. S8. Effect of the LNA-mediated silencing of lncRNAs *SeT* or *AseT* on *Tnfα* mono-allelic expression.

Percentage of cells with mono-allelic *Tnfα* expression are plotted over time of LPS stimulation of macrophages, for untreated (white bars), cells treated with LNA for lncRNA *SeT* (black bars) and LNA for lncRNA *AseT* (grey bars). *= not detected.

A

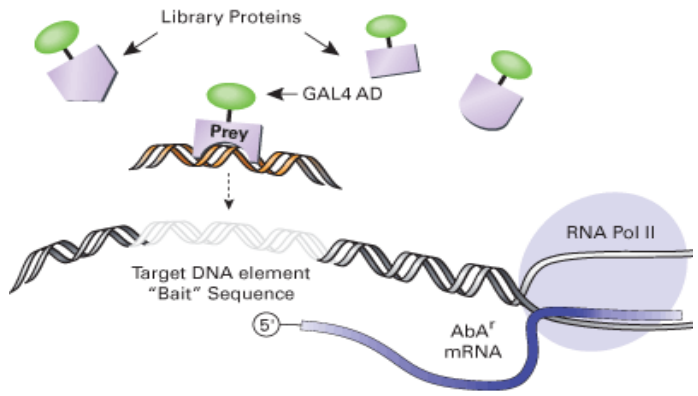

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Fig. S9. Yeast one hybrid library screening.

(*A*) The image presents the co-transformation of competent yeast cells with a high-complexity cDNA library expressing fusions of nuclear proteins from Raw264.7 upon LPS stimulation, with the Gal4 AD, together with the pHIS-GA repeat plasmid. Colonies with the ability to express the His3 reporter were selected for PCR and sequence confirmation.

(*B*) Mus musculus pyruvate kinase muscle isoform 2 (PKM2) mRNA sequence as indicated by BLAST nucleotide alignment of the yeast one hybrid clone. Genbank clone: BC094663.1 (1-2185). Cloned mRNA sequence: polyA transcript (1312-2185, bold letters).

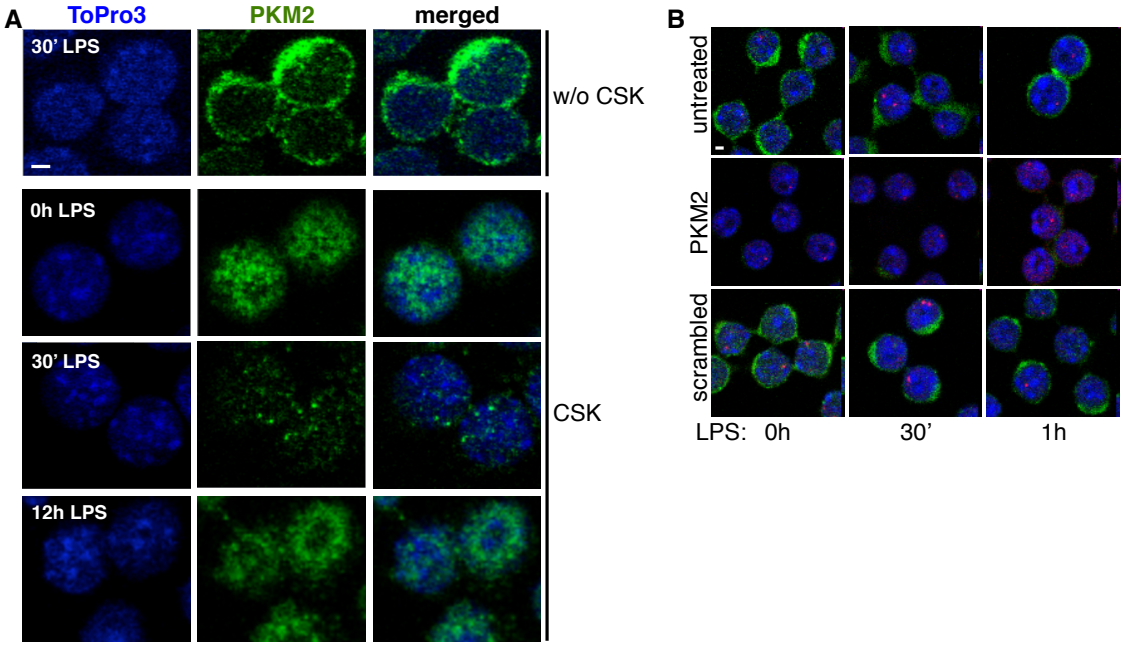

Fig. S10. PKM2 protein is detected both in the cytoplasm and the nucleus of LPS-stimulated mouse macrophages.

(*A*) Detection of PKM2 protein mainly in the cytoplasm [cells w/o cytoskeletal buffer (CSK) treatment] and the nucleus (CSK-treated cells) of untreated and LPS-stimulated macrophages. From left to right, ToPro3 Iodide 642/661 (blue), a-PKM2 (green), merged image.

(*B*) PKM2 protein detection prior and upon siRNA-mediated knock down. Scale bar 2μm.

Supplemental Experimental Procedures

Cell culture and treatments

The murine monocyte-derived macrophage cell line RAW 264.7 and the embryonic stem cell line, CGR8 were cultured under 5% CO_2 at 37°C, in Dulbecco's Modified Eagle's Medium (ATCC, Cat No.30-2002 or GIBCO, Cat.No.41966) supplemented with 10% fetal bovine serum (Biosera, FB1001/500), penicillin and streptomycin (Sigma, P4333 or Biosera LM, A4118) at 5μg/mL each. Macrophages were stimulated with 50ng/ml Lipopolysaccharide (LPS) (EB Ultrapure, Invivogen, O111:B4), or 10ng/ml TNFα (recombinant murine TNFα, R&D Systems, Cat No.410-MT). Actin polymerization was blocked with the pretreatment of cells with 10μM Latrunculin A (LTA, Sigma, L5163) for 1 hour prior to LPS stimulation.

PKM2 and ThPOK mRNAs were knocked-down with the use of 5nM siRNAs (Silencer® Select siRNAs, Ambion, Applied Biosystems), incubated with siPORT NeoFX Transfection Agent (Ambion, Applied Biosystems, AM4510) in OPTI-MEM media (GIBCO Cat No.31985-062) and then introduced to the cells ($3x10^4$ cells/24-well) for 48 hours (for mRNA) or 72 hours (for protein) at 37°C. The siRNAs used were: PKM2 – s71680, ThPOK – s76338, Scrambled – Silencer® Select Negative control #1 Cat. No. 4390843.

Knock-down of the LT/TNF locus long transcripts was achieved with the use of two Locked Nucleic Acid (LNA) oligonucleotides (HPLC, Exiqon, Product No. 500150):

LNA oligonucleotide *SeT*: 5'-GTCTTTATGCTTCCTGTTG-3'

LNA oligonucleotide *AseT*: 5'-ATGTATTGAGGTGGGTGGA-3'

Negative control: 5'-GTGTAACACGTCTATACGCCCA-3' (5-FAM/ miRCURY LNA Inhibitor Control, Exiqon, Product No. 199004-04).

Antibodies

Rabbit anti-mouse ThPOK: Abcam, ab20985

Rabbit anti-mouse PKM2 (D78A4) XP: Cell Signaling, Cat.No.4053

Goat anti-rabbit IgG, 488: Alexa Fluor, Molecular probes, A11008

cDNA synthesis

For quantitative mRNA expression analysis, 10% of the synthesized cDNA was used (2μg total RNA), using the SYBR Green PCR Master mix (Applied Biosystems, Cat.No.4309155) according to the manufacturer's instructions. QPCR was performed in an Opticon 2 DNA Engine (MJ Research) and the results were normalized over *Hprt1* mRNA levels. The primers used for mRNA quantitation were:

Tnfα.F: 5'-GAAGAGCGTGGTGGCCC-3'

Tnfα.R: 5'-CTCCAGGCGGTGCCTATGT-3'

Hprt1.F: 5'-GTCCCAGCGTCGTGATTAGC-3'

Hprt1.R: 5'-TTCCAAATCCTCGGCATAATG-3'

DNA Fluorescence *in situ* hybridization (DNA-FISH)

Probe preparation: DNA FISH probes were constructed with the use of the Nick Translation kit (Roche, Applied Science, Cat.No.11 745 808 910) supplemented with Spectrum Orange/Green dUTP (Abbott Molecular, 02N33-050/02N32-050). The reaction was prepared with 2μg BAC according to the kit's manual. The

probe was purified through a QIAquick PCR purification column (QIAGEN, Cat.No.28104 or Invitrogen, Purelink PCR purification kit, K31001) and stored at -20^{o}C until use. The murine BAC clones (BACPAC Resources Centre, CHORI) that were used for the preparation of DNA-FISH probes were the following: LT locus (chr.17): RP23-446-C22, *E4f1* (chr.17): RP24-162018, *P2rx4* (chr.5): RP24-228I1, *Arrb1* (chr.7): RP23-102I6.

Cell preparation: Cells were seeded on sterile glass coverslips, and stimulated with LPS or TNFα for the desired time. Cells were then fixed with 4% PFA (paraformaldehyde 16% aqueous solution, EM Grade, Electron Microscopy Sciences, Cat.No.30525-89-4) in 1xPBS, permeabilized with 0.5% Triton X-100 in 1xPBS and incubated in 20% glycerol in 1xPBS. After three freeze-thaw cycles in liquid nitrogen, the cells were incubated in 0,1N HCl for 5 minutes. The cells were finally rinsed in 2xSSC (for 20xSSC: 3M NaCl, 0.3M Sodium citrate) and stored in 70% ethanol at 4^{o}C.

Hybridization: 100ng from each DNA probe and 1μg mouse COT-1 DNA (Invitrogen, Cat.No.18440-016) were lyophilized. The pellet was resuspended in 5μl de-ionized formamide (Ambion, AM9342). DNA was then denatured and 5μl 2x hybridization buffer (4xSSC, 20% Dextran sulfate, 50mM Sodium Phosphate) were added. The cells were dehydrated with 4 washes of increasing ethanol concentrations (70%, 80%, 95%, 100%). The coverslips were air-dried and then incubated with denaturation buffer (70% de-ionized formamide, 2xSSC, pH7, pre-warmed at 73^{o}C) at 73^{o}C for 5 minutes. The cells were then dehydrated in increasing concentrations of ice-cold ethanol. The probe was then placed on a glass microscope slide and the coverslip with the cells was flipped on top, sealed and incubated at 37^{o}C for 16 hours in a

humidified hybridization chamber. The coverslips were washed three times with 2xSSC and nuclear DNA was counterstained with ToPro3 in 1:8000 dilution in 2xSSC (ToPro3 Iodide 642/661, Molecular Probes, T3605) for 1 minute at room temperature. The coverslips were then mounted in ProLong Gold anti-fade reagent supplemented with DAPI (Invitrogen, Molecular Probes, P-36931).

RNA-DNA FISH

Probe preparation: RNA FISH probes were constructed as for DNA FISH probes with the use of *Tnfα* cDNA cloned in a pCR® 2.1 plasmid vector.

Cell preparation: For RNA-DNA FISH, after the cells were washed with ice-cold 1xPBS, they were incubated in Cytoskeletal buffer (CSK buffer: 100mM NaCl, 300mM Sucrose, 3mM $MgCl_2$, 10mM PIPES, 0.5% Triton X-100, 1mM EGTA, 2mM Vanadyl Ribonucleoside Complex) for 5 minutes on ice. Then, they were fixed with 4% PFA in 1xPBS, washed three times with 70% ethanol and stored at -20^{o}C until use. For RNase A treatment, the cells were incubated with 100μg/ml RNase A for 30 minutes.

Hybridization: 100ng from each probe and 1μg mouse COT-1 DNA along with 20μg yeast tRNA (Ribonucleic acid, transfer from baker's yeast, SIGMA, R5636) were used as above.

Riboprobes – RNA FISH with indirectly labeled probes

RNA probes were used for the allele-specific hybridization experiments of the LT/TNF locus long RNA transcripts. Probes were prepared with *in vitro* transcription of PCR products spanning the T7 promoter on the 5'end. The PCR

products specific for each long RNA transcript were amplified with the following primers:

lncRNA#1.F: 5'-GAGAGCCACCAACAAAGTTTAC-3'

lncRNA#1.R: 5'-TCTCCATCATCCCCTTATGCACC'3'

lncRNA#9.F: 5'-TATTGGTGTTGGGATCAAATC-3'

lncRNA#9.R: 5'-GCTCTGCCTTTCGGTCAC-3'

The lncRNA#1 and #9 riboprobes (920 and 340bp respectively) were prepared with the Biotin RNA labeling mix (Roche, Cat. No. 11685597910) and were then precipitated with LiCl. The cells were prepared as for the direct RNA-DNA FISH experiments and hybridized with the riboprobes. The Tyramide Signal Amplification System (TSA Biotin System, Perkin Elmer, NEL700A001KT) was used to amplify the signals. Briefly, after hybridization, the coverslips were washed with 2xSSC in 50% formamide, 2xSSC, 1xSSC, 0.5xSSC, 0.2xSSC and 0.1xSSC, the blocked with TNB buffer for 30min and incubated with Streptavidin-HRP (1/200 in TNB) for 30min at RT. After 3 washes with TNT buffer, the coverslips were incubated with biotinyl-tyramide (1/50 in amplification buffer) for 8min, washed with TNT and stained with Streptavidin-488 (1/400 in TNB) for 30min. The cells were then counter-stained with ToPro3 diluted in TNT and mounted in ProLong Gold antifade reagent with DAPI.

Confocal microscopy

Fluorescently-labeled probes and proteins were visualized with the use of a Zeiss-Biorad confocal microscope (Axioskop2 Plus) equipped with a Laser Scanning System (Radiance 2100, BioRad) with 3 lasers (Argon, He-Ne, Red Iode) through the Lasersharp-2000 software.

Nuclear protein extracts

Nuclear extracts were prepared from macrophage cells washed twice in ice-cold 1xPBS. The pellet was resuspended in 5 cell pellet volumes of Buffer A (10mM Hepes pH7.9, 1.5mM $MgCl_2$, 10mM KCl, 5mM NaF, 1mM DTT, 1mM PMSF) and incubated on ice for 10 minutes. 0.1% NP-40 was added and the cells were vortexed and centrifuged at 4000rpm, for 1.5 minutes at 4^{o}C. The pellet (nuclei) was washed with Buffer A and upon resuspension, Buffer C (20mM Hepes pH7.9, 25% Glycerol, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 1mM DTT, 1mM PMSF and 5mM NaF) was added. The extracted nuclei were left on ice for 30 minutes and then centrifuged at 14000rpm for 15 minutes at 4^{o}C. For DNA affinity chromatography, the extracts were also dialyzed twice against 50 volumes of the DNA binding buffer.

Electrophoretic Mobility Shift Assay (EMSA)

The oligonucleotide sequences that were used to create double stranded oligonucleotides were as follows:

GA repeat: 5'- GAGAGAGAGAGAGAGAGAGAGAG -3'

GA single: 5'- GGTGGTGCATGAGAGGCCCACAGTC -3'

GA mutant: 5'- GGTGGTGCATACACAGCCCACAGTC -3'

200ng of double-stranded oligonucleotides (Microchemistry laboratory, IMBB, FORTH) were end-labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P] ATP and then purified with a G-50 column. The binding reactions were carried out in a binding buffer (80mM KCl, 10mM Hepes pH7.9, 5mM $MgCl_2$, 10% Glycerol, 0.1% NP-40, 50μM $ZnCl_2$, 1mM DTT, 1mM PMSF)

containing 3μg macrophage nuclear protein extracts and end-labeled oligonucleotide (60-100x10^3cpm). After a 20 minute incubation on ice the reactions were analyzed in a 6% (39:1) polyacrylamide:bis-acrylamide gel at 120-150V. The gel was dried and exposed on a film.

SouthWestern blotting

Protein samples were separated on a 10% SDS-polyacrylamide gel and transferred on a nitrocellulose membrane (Protran, Whatman) for 16 hours at 50mA (21-25V) at 4°C. The membrane was incubated with 25ml Blocking/Renaturation buffer (25mM Hepes pH7.5, 50mM KCl, 6.25mM $MgCl_2$, 10% Glycerol, 0.1% NP-40, 1mM DTT and 1mM PMSF) for 10 minutes and then another 25ml Blocking/Renaturation buffer were added, this time with 3% non-fat milk for 8 hours. The membrane was then washed with 25ml Binding/Washing buffer (12.5mM Hepes pH7.5, 50mM KCl, 6.25mM $MgCl_2$, 10% Glycerol, 0.05% NP-40, 1mM DTT and 1mM PMSF) for 5 minutes. The membrane was sealed in a plastic bag with hybridization buffer containing ~20x10^6 cpm end-labeled oligonucleotide and 60μg Salmon Sperm DNA in 3ml Binding/Washing buffer for 16 hours at room temperature. Subsequently the membrane was washed 3 times with Binding/Washing buffer for 15 minutes each and exposed on a film.

DNA affinity Chromatography

Binding of biotinylated oligonucleotide on beads: Oligonucleotides were bound on washed beads (200ng biotinylated oligonucleotide with 20μl beads in 5mM Tris-HCl pH7.5, 0.5mM EDTA, 1M NaCl) for >10 minutes according to the

manufacturer's protocol (Dynabeads M-280 Streptavidin, Invitrogen, Cat.No.112.05D).

Pre-clearing of Nuclear Extracts (NEs) with beads: Per 100μg of nuclear extracts 100ng/μl poly(dI:dC) was used in 1xBiotin-Binding buffer (80mM KCl, 10mM Hepes pH7.9, 5mM $MgCl_2$, 10% Glycerol, 50μM $ZnCl_2$, 0.05% NP-40, 1mM DTT, 1mM PMSF, 5mM NaF). 20μl beads per sample were added and left rotating for 1 hour at 4^{o}C.

Binding of NEs to biotinylated-oligonucleotides: Either beads bound to oligonucleotide or beads alone were incubated with the protein extracts for 20 minutes at room temperature. The beads were washed (Washing buffer: 80mM KCl, 20mM Hepes pH7.9, 5mM $MgCl_2$, 50μM $ZnCl_2$, 0.05% NP-40, 1mM DTT, 1mM PMSF, 5mM NaF) three times, with the first wash containing 100ng/μl poly(dI:dC).

Untreated and stimulated (50ng/ml LPS for 30min) RAW 264.7 murine macrophages ($2x10^9$ cells/condition) were lysed in 10mM Tris-HCl pH8.0, 10mM NaCl, 8mM $MgCl_2$, homogenized with a Dounce pestle B in hypotonic buffer (20mM Tris-HCl pH7.5, 20mM KCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 25% glycerol), nuclear extracts were prepared in a high salt buffer (20mM Tris-HCl pH7.5, 1.2M KCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 25% glycerol) and dialyzed against 20mM HEPES-KOH pH7.9, 0.2mM EDTA, 0.5mM DTT, 0.01% NP-40, 10% glycerol and 0.75-1M NaCl. The extracts were fractionated in a P11 phosphocellulose column with 0.1M, 0.3M, 0.5M and 0.85M NaCl stepwise elution and the presence of the desired DNA binding activity was analyzed by EMSA as described.

Concatamers of DNA binding sites were generated by a self-priming PCR method using two direct repeats of complementary single-stranded oligonucleotides. The oligonucleotides used were the biotinylated 30nt GA-repeat and its complementary 30nt CT-repeat. 460ng of each oligonucleotide were used in a PCR reaction, which produced double-stranded GA/CT repeat concatamers of 5-10kb in length. The nuclear extracts were then incubated with 1mg concatamerized DNA bound to magnetic M280 streptavidin beads, in the presence of the competitor DNA oligo(dI:dC) and poly(dI:dC) at 0.1 mg/mL each.

Protein detection and Mass Spectrometry

Protein samples were separated on 8-10% polyacrylamide gels and either stained by Coomassie G250 Colloidal blue (Fluka, Cat No.27815), or silver stained. For protein identification and characterization, Coomassie-stained gel bands were destained, reduced, alkylated and digested with trypsin (Proteomics grade, Sigma, T6567).

nanoLC- MS/MS analysis

Briefly, the analysis performed on an EASY-nLC system (Proxeon, software version 2.7.6 #1) coupled on-line with an LTQ-Orbitrap XL ETD (Thermo Scientific, Bremen Germany) through a nanoES ion source (Proxeon). Data were acquired with the Xcalibur software (LTQ Tune 2.5.5 sp1, Thermo Scientific). The mass spectrometer was calibrated with a standard ESI positive ion calibration solution of caffeine (Sigma), L-methionyl-arginyol-phenylalanylalanine acetate H_2O (MRFA, Research Plus) and perfluoroalkyl

triazine (Ultramark 1621, Alfa Aesar). Samples were reconstituted in 0.5% formic acid (FA) and the tryptic peptide mixture was separated on a reversed phase column [Reprosil Pur C18 AQ, particle size 3μm, pore size 120Å (Dr. Maisch), fused silica emitters, 100mm long, 75μm internal diameter, (Proxeon)] packed in-house using a pressurized (35-40bars of Helium) packing bomb (Loader kit SP035, Proxeon). The nanoLC flow rate was 300nl min^{-1}. The LC mobile phase consisted of 0.5% FA in water (A) and 0.5% FA in acetonitrile (B). A multi-step gradient was employed, from 5% to 16% B in 7 minutes, to 35% B in 33 minutes, and to 85% B in 10 minutes. After holding the gradient at 85% B for 5 minutes, the mobile phase was re-equilibrated at initial gradient conditions. The MS was operated with a spray voltage of 2300V, a capillary voltage of 35V, a tube lens voltage of 140V and a capillary temperature of 180^{o}C. A survey scan was acquired in the range of m/z 400-2000 with an AGC MS target value of 10^6 (resolving power 60,000 at m/z 400). The ten most intense precursor ions from each MS scan were subjected to collision-induced dissociation (CID, isolation width 3Da, normalized collision energy 35%, activation q 0.25, and activation time 30ms) in the ion trap. Each scan included one microscan with a maximum injection time of 200 ms and an AGC MSn target value of 2×10^4.

Data analysis of MS/MS derived data

The MS raw data were loaded in Proteome Discoverer 1.3.0.339 (Thermo Scientific) and run using Mascot 2.3.01 (Matrix Science) and Sequest (Thermo Scientific) search algorithms against the Mouse theoretical proteome [UniProt Knowledge Database, June 2013] containing 73,921 entries (1). A list of common contaminants was included in the database (2). For protein

identification, the following search parameters were used: precursor error tolerance 10 ppm, fragment ion tolerance 0.8 Da, trypsin full specificity, maximum number of missed cleavages 3, methionine oxidation, aspartic and glutamic acid methylation, asparagine's deamidation as variable modifications, and cysteine alkylation as a fixed modification. Peptides were assigned correct if they matched the following criteria: Mascot ion score >25, Sequest XCorr >2.5.

Yeast One Hybrid Screening

The Matchmaker One-Hybrid System (Clontech laboratories, Inc, Cat.No.630304) was used according to the manufacturer's protocol. Briefly, a GA-repeat DNA sequence was cloned upstream of the *His3* reporter in pHIS2.1 and a high-complexity cDNA library, which expressed fusions of nuclear proteins from RAW 264.7 upon LPS-induction, with the Gal4 AD, was generated. After co-transformation of competent yeast cells with the cDNA library, as well as the pHIS-GA plasmid, expression from the *His3* reporter was detected only in colonies that were able to grow on minimal medium that lacked histidine and contained 3-AT. PCR and sequence analysis were used to identify and confirm the positive clones. The library was constructed twice and the results were repeated and verified by PCR and DNA sequencing.

DNase I Hypersensitivity mapping

Murine macrophages ($100x10^6$ cells) stimulated with 50ng/ml LPS for 30min, were incubated in Lysis buffer (50mM Tris pH7.9, 50% glycerol, 100mM KCL, 5mM $MgCl_2$, 0.05% saponin, 200mM β-mercaptoethanol) for 10 minutes on ice.

After a 15 minute-centrifugation at 1300g at 4°C, the resulting nuclei were washed once with 1.5ml buffer A and finally resuspended in 4ml Buffer A (50mM Tris pH7.9, 100mM NaCl, 3mM $MgCl_2$, 1mM DTT and 0.2mM PMSF). Aliquots of 180μl nuclei were dispensed in a series of DNase I two-fold dilutions (initial concentration 1 kunitz unit/μl, SIGMA D5052), incubated for 20 minutes at 37°C and the reactions were terminated by adding 16.6μl 0,5M EDTA and vortexing for three cycles. Samples were then treated with 12μl RNase A (QIAGEN Cat.No.1007885, 10mg/ml in TE) for 30 minutes at 37°C and 40μl Proteinase K (MERCK Cat.No.1.24568.0100, 0.2mg/ml in 50mM Tris/100mM NaCl) were added, as well as 100μl SDS buffer (20mM Tris pH7.9, 70mM EDTA pH8, 100mM NaCl, 2% SDS) and incubated for 16 hours at 50°C.

DNA was extracted and precipitated. Samples were then resuspended in 200μl TE at 55°C for 16 hours, were digested with *PstI* and then separated on a 0.8% agarose gel in 0.5xTBE. DNase I hypersensitive sites were distinguished on Southern blots (Amersham Hybond N nylon membrane, GE Healthcare, Life Sciences) by radio-labeled DNA probes, prepared using random priming-PCR amplification of the template with [α-^{32}P] dCTP (Stratagene, Prime-it II Random Primer Labeling kit, Agilent Technologies, Cat.No.300385). Probe design is indicated in Figure 5.

Luciferase assays

RAW 264.7 cells were co-transfected with the pCMV-LacZ vector as well as with either the pGL3-basic vector, pGL3-basic/HSS-1 or pGL3-basic/HSS-9 constructs, using Lipofectamine 2000 (Invitrogen, Cat.No.11668-019), according to the manufacturer's instructions. The cells were stimulated with LPS

24 hours upon transfection and resuspended in 200μl 0.25M Tris pH7.8. After 3 sonication pulses, 20μl of the cell suspension was supplemented with 20μl luciferin (PROMEGA, E1601) and luciferase activity was measured in a Luminometer (TD-20/20, Turner Designs). The transfection efficiency was calculated by using the *β-galactosidase* gene activity as an internal control. The cell suspension was supplemented with o-nitrophenyl-β-D-galactopyranoside (ONPG, SIGMA, N1127) in lacZ buffer and β-galactosidase activity was measured at 420nm in a Photometer (DigiScan 400, ASYS HITECH GMBH). The cloned promoter sequences were generated using the following primers:

HSS1.F: 5'-CAGACGAAGGAAGGGTAAGC-3'

HSS1.R: 5'-GACTACTGTCAGTTCAGCCTGG-3'

HSS9.F: 5'-GATTGTGTCCGAGGAGGAGG-3'

HSS9.R: 5'-CAGTGGGCTCTTTGTTGGTTG-3'

Rapid Amplification of cDNA Ends (RACE)

The FirstChoice RLM-RACE kit (Invitrogen, AM1700M) was used to identify the 5'- and 3'-ends of specific capped mRNA molecules from RAW 264.7 macrophages treated with 50ng/ml LPS for 1 hour. Briefly, total RNA was treated with Calf Intestine Alkaline Phosphatase to remove the free 5'-phosphates from non-capped molecules. The sample was then treated with Tobacco Acid Pyrophosphatase to uncap the mRNA molecules, which were then ligated to a 45nt RNA adapter with the use of T4 RNA ligase. A random-primed reverse transcription reaction followed and nested PCR amplified the 5'-end of the specific transcript, using an adapter-specific and a gene-specific nested primer. For the 3'-RACE, an oligo(dT)-adapter was used to synthesize

the first cDNA strand from total RNA and then the gene of interest was amplified by PCR using an adapter-specific and a gene-specific primer.

5'-RACE was performed using the following primers:

lncRNA *SeT*: #1.F: 5'-GAGAGCCACCAACAAAGTTTAC-3'

#1Nested.F: 5'-AAAGACCCACTTACACGTTAATG-3'

lncRNA *AseT*: #9.R: 5'-TCACCCTCTCACCCCACTG-3'

#9Nested.R: 5'-GTCCAAAGCACATAAGGAGTG

3'-RACE was performed using the following primers:

lncRNA *SeT*: #9.R: 5'-TCACCCTCTCACCCCACTG-3'

#9Nested.R: 5'-GTCCAAAGCACATAAGGAGTG-3'

lncRNA *AseT*: #1.F: 5'-GAGAGCCACCAACAAAGTTTAC-3'

#1Nested.F: 5'-AAAGACCCACTTACACGTTAATG-3'

The sequences of the primers used for PCR amplification of regions 1 to 11 (as indicated in Figure 4A) are the following:

1.F: 5'- GAGAGCCACCAACAAAGTTTAC - 3'

1.R: 5'- TTCCTCTGATTCTTCATCTTGC - 3'

2.F: 5'- GGGAAGGGCAATACTATTAGGT - 3'

2.R: 5'- AAGGCAAGCCATCGAAACTG - 3'

3.F: 5'- TGAGTCCATCCCCACATTCC - 3'

3.R: 5'- TAAATGTTCTGCTGTGTGTATAGG - 3'

4.F: 5'- ACTGTGTCCCCTTACTCTCTG - 3'

4.R: 5'- CAGAGCATTGGAAGCCTGG - 3'

5.F: 5'- GGGCTTAGACTACTGCGTTC - 3'

5.R: 5’- ACCCTCTCCACGAATTGCTC - 3’

6.F: 5’- GAAGCGGACACCAGAGAGTC - 3’

6.R: 5’- GCCGTCTCCACCTCTTGAG - 3’

7.F: 5’- GCTTGAGAGTTGGGAAGTGTG - 3’

7.R: 5’- AGGAGAAGGCTTGTGAGGTC - 3’

8.F: 5’- GAAGAGCGTGGTGGCCC - 3’

8.R: 5’- CTCCAGGCGGTGCCTATGT - 3’

9.F: 5’- CCTACCTCCGAAGTGTTGG - 3’

9.R: 5’- TCACCCTCTCACCCCACTG - 3’

10.F: 5’- CCAGATCCAGGGGTTCAAC - 3’

10.R: 5’- CGCTCCTCAGAAACGCTTC - 3’

11.F: 5’- AGAAGAGGGAGTCAGATCTCG - 3’

11.R: 5’- CTGCAACGCTTTAATAGAGTCC - 3’

Supplemental References

1. Rappsilber J, Ryder U, Lamond AI, Mann M (2002) Large-scale proteomic analysis of the human spliceosome. *Genome Res.* 12:1231-1245.
2. UniProt Consortium (2010) The Universal Protein Resource (UniProt) in 2010. *Nucleic Acids Res.* 38:D142-D148.