Supplementary data

Papantonis *et al* (2012) TNFα signals through specialized factories where responsive coding and miRNA genes are transcribed

This section contains:

—Supplementary Methods and References

—Supplementary Figures S1-S8 and their respective legends

—Legends to Supplementary Tables S1-S7 (Tables provided separately as an .xls file)

Supplementary Methods

Oligonucleotides

Oligonucleotides were designed to be 20-22 nt-long, have a T*m* of 62°C, and yield amplimers of 75-225 bp using Primer 3.0 Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus /primer3plus.cgi). For 4C libraries prepared using *Sac*I, primer sequences used for nested inverse PCR on fragments spanning the TSS of our reference genes were (ExF—external forward primer; InF—internal forward primer; ExR—external reverse primer; InR—internal reverse primer):

(i) For *SAMD4A*:

(Sac-ExF) ACATTGAGGGAGATTCCATTGAG,

(Sac-ExR) TGAAGACGAAGCTCTAAAACCAGA,

(Sac-InF) TTCCTCCTCCCTAGTATGGTGTG,

(Sac-InR) AAGTAACCCACTTCATGCCTGTC.

(ii) For *EXT1* (semi-nested PCR):

(Sac-ExF/InF) CTAGAGGCTGGGGACAGAGAGTT,

(Sac-InR) CAAAGTTGGGTCGGAAGTTTTC,

(Sac-ExR) TGGGATGATCCTTAGAAAAGAGG.

For libraries prepared using *Hind*III, they were:

(i) For *SAMD4A*:

(Hind-ExF) ATATCCGGAAACTAGCCAAGAAC,

(Hind-ExR) ACGCTAGCAAATAGGAAACTCGT,

(Hind-InF) GAGAATATTTCAGGCCCTCTCTCA,

(Hind-InR) AAGTAACCCACTTCATGCCTGTC.

(ii) For *EXT1*:

(Hind-ExF) CCACCAAGAGAATAACATCACTTTG,

(Hind-ExR) CCAACTGTCCCAGCTATAGAAG,

(Hind-InF) ATCTTTAACACCACCACCACCAC,

(Hind-InR) AAGGACATATGACTGGTAGAATTGC.

(iii) For *ETS2*:

(ExF) ATACAATGGAAGCGCCTGTG,

(ExR) TCTCCAAAGGGGACTGCTC,

(InF) GTTATCTGCCTGCCCACAC,

(InR) TAGCGCGTCAACTACTGTTTTAG.

All other primer sequences are available on request.

Chromatin immunoprecipitation (ChIP)

For p65 ChIP followed by quantitative PCR, ~10^7 HUVECs were cross-linked (10 min; 20°C) in 1% paraformaldehyde and chromatin was prepared using the ChIP-IT-Express kit (Active motif). Immunoprecipitations were performed using a polyclonal antibody against the p65 subunit of NFκB (4 µg/reaction; sc-372X, Santa Cruz Biotechnology) or against the N-terminus of the largest subunit of RNA polymerase II (2 µg/reaction; sc-889X, Santa Cruz Biotechnology). DNA was purified using a MicroElute Cycle-Pure kit prior to quantitative real-time PCR (Platinum SYBR Green qPCR Mix-UDG, Invitrogen). Reactions were 50°C/2 min, 95°C/5 min, and 40 cycles at 95°C/15 sec, and 60°C/50 sec. Data was analyzed (Nelson *et al,* 2006) using the *TNFAIP3* promoter (for p65 binding) and *GAPDH* TATA box (for RNAPII binding) as positive, and the *AFP* 3' UTR as a negative control.

For p65 ChIP followed by next-generation sequencing, cells were crosslinked as for ChIA-PET, sonicated (Branson sonicator 250, 10 min), and immunoprecipitation performed using an anti-p65 polyclonal antibody (Abcam, ab7970) and Protein A-coated magnetic beads (Dynal). DNA bound to beads was isolated, and enrichment evaluated by qPCR using primers targeting promoters of genes known to bind p65 (*TNFAIP3* and *CXCL1*). Primer sequences were: TNFAIP3F—CTGGGAGTTTGTT GGACGTT, TNFAIP3R—AACCTCTGCAGCAGTGACCT; CXCL1F—AGGGAATTCACCCCAAGAAC, CXCL1R—GGCGGGACTTACATGACTTC. From ~13 x 10^6 36-bp reads (for each of the 0 and 30 min datasets), ~8 x 10^6 uniquely mapped t the genome (hg18). These were clustered into >12,000 p65-binding sites (consistent with results of Kasowski *et al*, 2009), and it is now known that NFκB binds to many sites scattered around the genome, including *Alu* repeats (Antonaki *et al*, 2011).

ChIP for RNA polymerase II and histone modifications was carried out as described (Wada *et al,* 2009), and coupled to next-generation sequencing. Briefly, 2x10^6 HUVEC cells were grown, serum-starved, stimulated with TNFα for 30 min, cross-linked using 1% paraformaldehyde (10 min; 20°C), neutralized in 0.2 M glycine/PBS, lyzed in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% SDS, plus 1mM EDTA, and DNA sonicated to ~300 bp. RNA polymerase II-bound chromatin was pulled down using the mouse monoclonal antibody Pd75C9 (as above). After purification, DNA was sequenced, reads mapped as for 4C-seq and extended to 200 bp. Clusters containing significantly more reads were identified by comparison to a Poissonian background model (P <10^{-9}). A gene was considered able to bind polymerase if a peak >5-fold higher than background was detected between -3 to +1 kbp of the transcription start site. From ~20 x 10^6 36-bp reads (for each of the 0 and 30 min datasets), ~14 x 10^6 were uniquely mapped against the genome (hg18). ChIP-seq data are available at the GEO database (NCBI) under accession number GSE34500. Views shown in **Supplementary Figures S2E**, **S3C**, and **S8** were obtained using the Integrated Genome Browser (IGB) v 6.4.

Quantitative reverse transcriptase PCR (qRT-PCR)

Total RNA was isolated using TRIzol (Invitrogen) from 10^7 cells stimulated with TNFα (0-60 min), treated with RQ1 DNase (1 unit of DNase/μg of total RNA; 37°C for 45 min; Promega), and nascent RNA amplified (54°C/10 min followed by 1 cycle at 95°C/5 min, 40 cycles of 95°C/15 sec, 60°C/50 sec, and a single cycle at 40°C/2 min; Rotor-Gene 3000 cycler, Corbett) using the One-Step qRT-PCR kit (Invitrogen) with primers targeting introns. The presence of single amplimers was confirmed by melting curve analysis. Reactions in which Platinum *Taq* polymerase (Invitrogen) replaced the RTase/*Taq* polymerase mix were performed to ensure amplimers did not result from residual genomic DNA. Precursor and mature miRNAs were detected using miScript assays (Qiagen) with levels normalized relative to mature RNU6.

Immunofluorescence (IF)

HUVECs grown on coverslips etched with hydrofluoric acid were fixed with 4% paraformaldehyde (Electron Microscopy Science) in 250 mM HEPES (pH 7.6; 20 min; 20°C), washed 3x in PBS (5 min; 20°C), permeabilized using 0.5% Triton X-100/0.5% saponin in PBS (20 min; 20°C), washed with 0.05% Tween 20 in PBS (10 min; 20°C), and blocked with 3% BSA/0.2% gelatin in PBS (Sigma; 20 min; 20°C). Phosphorylated (at Ser536) p65 was detected using a rabbit monoclonal antibody (1:1,000 dilution; #3033, Cell Signalling Technology) and Alexa488- or Cy3-conjugated donkey anti-rabbit IgG (0.5 μg/ml; Invitrogen). After DAPI counter-staining, images were collected as for RNA FISH and analysed using ImageJ (Abramoff *et al,* 2004); an area of 1292x1292 pixels was arbitrarily selected in the nucleus, the mean intensity calculated, and nuclear fluorescence (arbitrary units, au) calculated by subtracting the background (measured as the minimum intensity in the area).

Immunofluorescence and fluorescence in situ hybridization (Immuno-FISH)

HUVECs on coverslips were fixed and washed as above, stored in 70% ethanol (4°C; 48 h), transferred to PBS (5 min; 20°C), permeabilized with 0.5% Triton X-100/0.5% saponin in PBS (20 min; 20°C), rinsed in water, post-fixed with 4% paraformaldehyde in PBS (5 min; 20°C), washed in PBS (10 min; 20°C), gradually dehydrated in ethanol (70%, 90% and 100%), and allowed to hybridize (16 h; 37°C) with 25 ng (for *SAMD4A* and *EXT1*) or 10 ng (for *EDN1*) of the relevant probes in hybridization mix (25% deionized formamide, 2x SSC, 200 ng/μl sheared salmon sperm DNA, 5x Denhardt's, 50 mM phosphate buffer, 1 mM EDTA, 0.5 μl murine RNase inhibitor). RNA FISH probes were the sets of five 50-mers described above. Next day, coverslips were washed 3x in 2xSSC (10 min; 37°C), processed for immunofluorescence, and imaged as described above. For the panel in **Supplementary Figure S5D** illustrating non-colocalization of nascent *EDN1* RNA and $p65^P$ (detected using Alexa 488 and Cy3, respectively), the red channel is pseudo-colored green, and the green

channel pseudo-colored red (to facilitate comparison with the other panels). A (red) FISH focus (defined as above) was deemed to colocalize with a (green) p65 focus if ≥33% of red pixels overlapped green pixels.

BrUTP labeling, immunofluorescence, and nearest-neighbour analysis

HUVECs on coverslips were induced with TNFα, washed in ice-cold PB+ (100 mM CH_3COOK, 30 mM KCl, 10 mM Na_2HPO_4, 1 mM $MgCl_2$, 10 mM NaF, 10 mM β-glycerophosphate, 200 μM Na_3VO_4, 1 mM Na_2ATP, 1 mM DTT, 1:1000 PIC, 1:2000 RNaseOUT; pH 7.6) for 1 min, permeabilized in 170 μg/ml saponin (Sigma)/PB+ for 5 min on ice, washed 3x in ice-cold PB+, incubated (5 min; 33˚C) in transcription buffer (PB+ with 100 μM each of ATP, GTP and CTP, 100 μM $MgCl_2$, 1:100 RNaseOUT), 100 μM BrUTP (Sigma) added and a "run-on" carried out (15 min; 33˚C), and stopped by adding 2.5 mM EDTA (pH 8.0). Then, cells were washed 2x in ice-cold PB+, fixed in 4% PFA/250 mM HEPES (20 min; room temperature), washed 3x in PBS (5 min; room temperature), and prepared for immunofluorescence, imaged, and images aligned as for high-resolution RNA FISH. Primary antibodies were: (i) rabbit monoclonal against phosphorylated (Ser 536) p65 (1:1000 dilution; Cell Signaling Technology), and (ii) mouse monoclonal against BrUTP (1:1000 dilution; Phoenix Flow Systems). Secondary antibodies were: (i) Alexa488-conjugated donkey anti-rabbit IgG (0.5 μg/ml; Invitrogen), and (ii) DyLight649-conjugated donkey anti-mouse IgG (7 μg/ml; Jackson ImmunoResearch). For "nearest-neighbour" (NN) analysis, foci were identified automatically as follows. The nuclear region was selected (using a threshold in the DAPI image), foci with Gaussian shape selected independently of intensity, features too small to represent true foci removed (*i.e.*, ≤4 pixels in a 2x2 array), and foci selected in which the brightest pixel had an intensity greater than both the average global background (*i.e.*, the mean nuclear intensity plus half a SD) and the average local background (measured in the 43 outermost pixels in the 9x9 array around the brightest pixel); >90% foci seen by eye survived this selection. Then, peak intensities within the selected foci were localized with 22-nm precision using the "tuned" version of the JD algorithm (Larkin and Cook, 2012), and the distance from the peak of each red (or green) focus to its closest (in 2D space) green (or red) focus was determined. A total of 2865 foci from 8 nuclei were analyzed. As a control, 8243 randomly-distributed foci with the same density were computer-generated. All calculations were performed in MATLAB (MathWorks) using custom software routines available on request.

Association of $p65^P$ with large fragments of transcription factories

Here, a method for isolating large fragments of factories of >8 MDa was followed exactly (Melnik *et al,* 2011). HUVECs were treated with TNFα for 0 or 15 min, nuclei isolated in PB+ buffer (see above),

and most chromatin detached from the nuclear sub-structure using DNase I; after spinning, the supernatant (*i.e.*, fraction "4super") contains chromatin. The pellet was resuspended, treated with caspases, and respun; the supernatant (*i.e.*, fraction "5super") contains large fragments of factories. The two supernatants containing either "chromatin" or large fragments of "factories" were then resolved by SDS-PAGE electrophoresis in 10% acrylamide gels (Bio-Rad), proteins transferred onto nitrocellulose using the iBlot Transfer System (Invitrogen), and phospho-p65 ($p65^{P}$), RCC1 (a negative control), and RNA polymerase II detected by immuno-blotting using the rabbit monoclonal antibody (1:3,000 dilution; #3033, Cell Signalling Technology), a mouse monoclonal anti-RCC1 (1:3,000 dilution; R35420, Transduction Laboratories), and a mouse monoclonal anti-RPB1 (7C2; 1:10,000 dilution; a gift of Marc Vigneron), respectively.

DNA fluorescence in situ hybridization (DNA FISH)

DNA FISH was performed as described (Li *et al*, 2012). In brief, HUVECs grown on coverslips were stimulated with TNFα for 30 min, fixed in 3:1 methanol/acetic acid (15 min; room temperature), washed 3x in PBS, and stored in 70% ethanol at -20°C. Then, cells were washed once in PBS, once in 100 mM Tris-HCl (pH 7.5) plus 150 mM NaCl, digested with pepsin/HCl (5 min; 37°C; as for RNA FISH), rinsed in water, post-fixed in 1% PFA/PBS (5 min; room temperature), washed 2x in PBS, dehydrated via a 70%-90%-100% ethanol series, and chromatin denatured in 2x SSC/60% formamide (40 min; 80°C). Meanwhile, probes were denatured (10 min; 90°C) in hybridization buffer (as for RNA FISH, but with 50% formamide), quenched on ice, added to cells, coverslips sealed on to slides using rubber cement (Fixogum, Marabu), and hybridized in a moist chamber (37°C; 24 h). Next day, slides were washed twice in 2x SSC (5 min; 37°C) to remove coverslips, which were in turn washed 3x in 2x SSC (10 min; 37°C), mounted on to new slides, and imaged as for RNA FISH. Probes were produced from BAC clones (CTD-2589I5 for *SAMD4A*, RP11-720F6 for *SLC6A5*, and RP11-194M2 for *EDN1*; all from CHORI) nick-translated and conjugated to Alexa 488 or 555 using the FISH-Tag kit (Invitrogen). Genomic loci were considered as colocalizing when >10% of pixels in the respective FISH foci overlapped; ~100 cells per probe pair were analyzed.

Estimating numbers of "NFκB" factories

After stimulation our reference genes share few contacts (**Supplementary Figure S7**, and **Tables S1** and **S2**), and RNA FISH using multiplexed probes reveals that most nascent targets do not colocalize with each other. We estimate that ~8 "NFκB" factories are accessible to *SAMD4A* and to each of the 7 other responsive genes analyzed in **Figure 4A** as follows: the probability, *P*, that at least one of the 7 nascent transcripts colocalizes with a nascent *SAMD4A* transcript is given by $1-[(n-1)/n]^{7}$, where *n* is the number of "NFκB" factories that all 8 genes can access at a high frequency; as $P = 0.6$ (**Figure**

4A), *n* = 8. That a gene like *SAMD4A* can access ~8 "NFκB" factories is consistent with the fact that 30 min after stimulation (in nuclei containing at least one green and one red focus) ~8% nascent *SAMD4A* transcripts colocalize with nascent RNA copied from one gene in the multiplexed set, *TNFAIP2* (Papantonis *et al*, 2010). If both can access the same 8 "NFκB" factories with equal chance, the probability that a nascent *TNFAIP2* transcript is found in a factory already containing a *SAMD4A* transcript is 1/8 or 12% (comparable to the 8% seen experimentally). The total number of polymerase II factories in the (G0) HUVEC nucleoplasm is ~2,200 assuming: (i) the density of factories in the HUVEC nucleoplasm is the same as in several other mammalian cells—~9.3 factories/μm^3 (Faro-Trindade and Cook, 2006; densities corrected for a revised factory diameter of 87 nm; Eskiw *et al*, 2008), (ii) the nuclear volume of HUVECs is 300 ± 87 μm^3 (*n* = 15; measured by confocal microscopy of fixed, DAPI-stained nuclei), and (iii) non-nucleolar volume occupies 80% nuclear volume (*i.e*., in the middle of the range seen in other mammalian cells; Faro-Trindade and Cook, 2006). The number of "NFκB" factories will be a fraction of the above, and we can place an upper bound on it in two ways. First, using RNA FISH data; 31% yellow foci (*i.e*., 60%-29%=31%; see **Supplementary Figure S5A**) are then expected using probes targeting *SAMD4A* and 3 multiplexed targets encoded by the same chromosome as *SAMD4A*. Using the same equation as above, *P* is then 0.31, thus *n*=8. If all responsive genes on chromosome 14 (which represents 2.5% of the genome) can access the same ~8 "NFκB" factories, and if the number of "NFκB" factories scales with amount of DNA, there will be 320 "NFκB" factories. This is an upper estimate, as responsive genes on different chromosomes clearly share "NFκB" factories. Second, using ChIA-PET data; we select 606 genes that are up-regulated >3-fold between 0 and 30 min (using contact frequencies, as these accurately reflect transcriptional activity; Li *et al*, 2012). 496 genes in this set contact at least one other member in the set (6,266 interactions in total). If these 496 genes access essentially all "NFκB" factories, successively removing the gene making the fewest contacts from the set, then the next fewest, and so on, should initially have little effect on the total number of interactions; this is the case (**Supplementary Figure S6D**; data for other sets of genes are included for comparison). Then, when too few up-regulated genes in the set remain to saturate all "NFκB" factories with contacts, the number of interactions should begin to fall; this number is ~250 and this is also an upper limit of the number of "NFκB" factories. These values are consistent with ~150 $p65^P$ foci per nucleus (**Supplementary Figure S5D**); if such foci mark most "NFκB" factories, there might be ~150-250 such factories. Note that the estimates given here are inevitably coarse ones, given the number of assumptions.

Statistical analysis

P values (two-tailed) from Fisher's exact test, Chi-square test with Yates' correction, and unpaired Student's *t*-test were calculated using GraphPad (http://www.graphpad.com); they were considered significant when <0.05. Pearson's correlation coefficient (*R*) was calculated using Excel (Microsoft). *P* values from the binomial distribution and Kolmogorov-Smirnov two-sample tests (in **Supplementary Figures S4** and **S5**, respectively) were calculated manually.

Supplementary references

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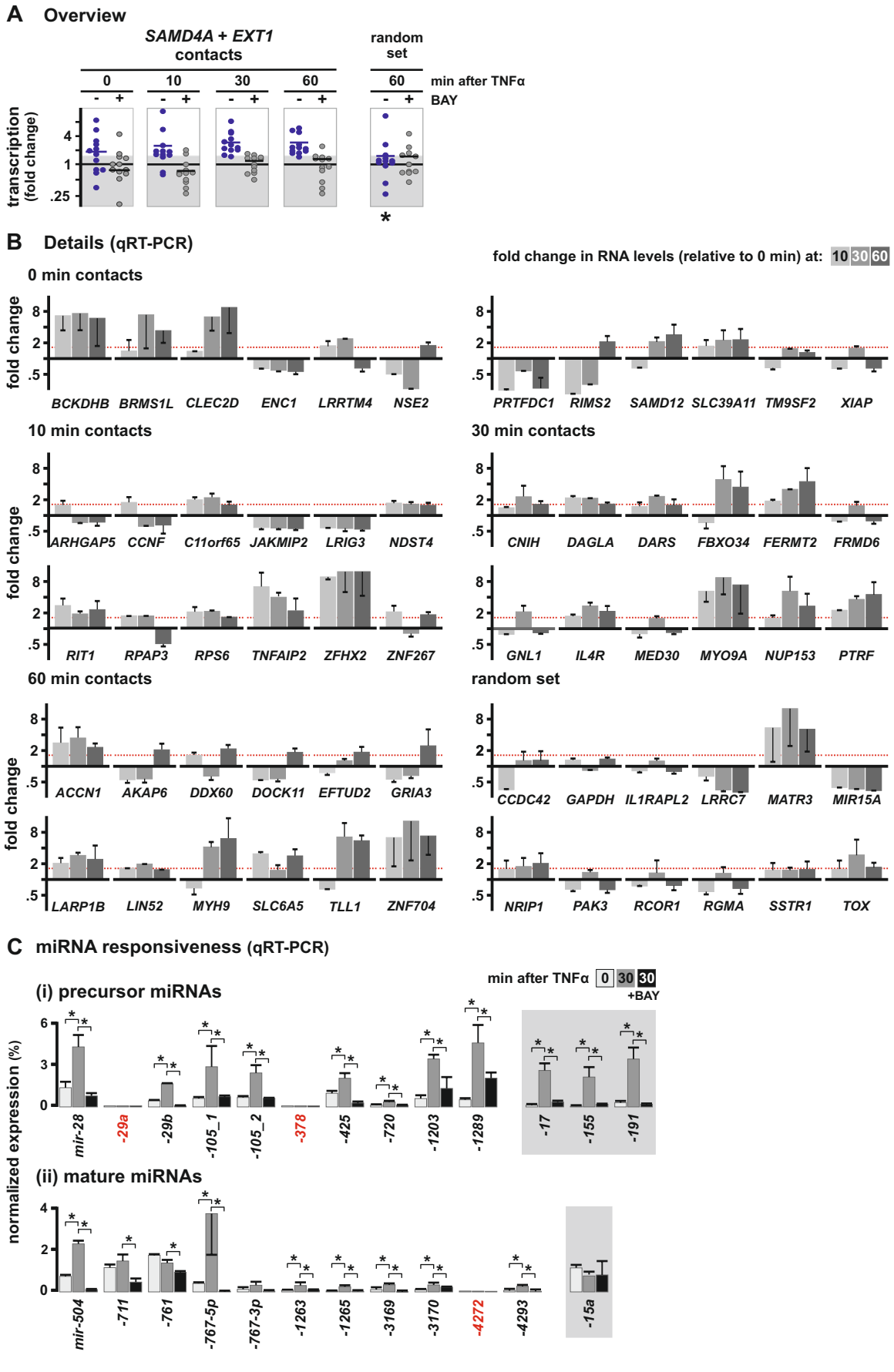
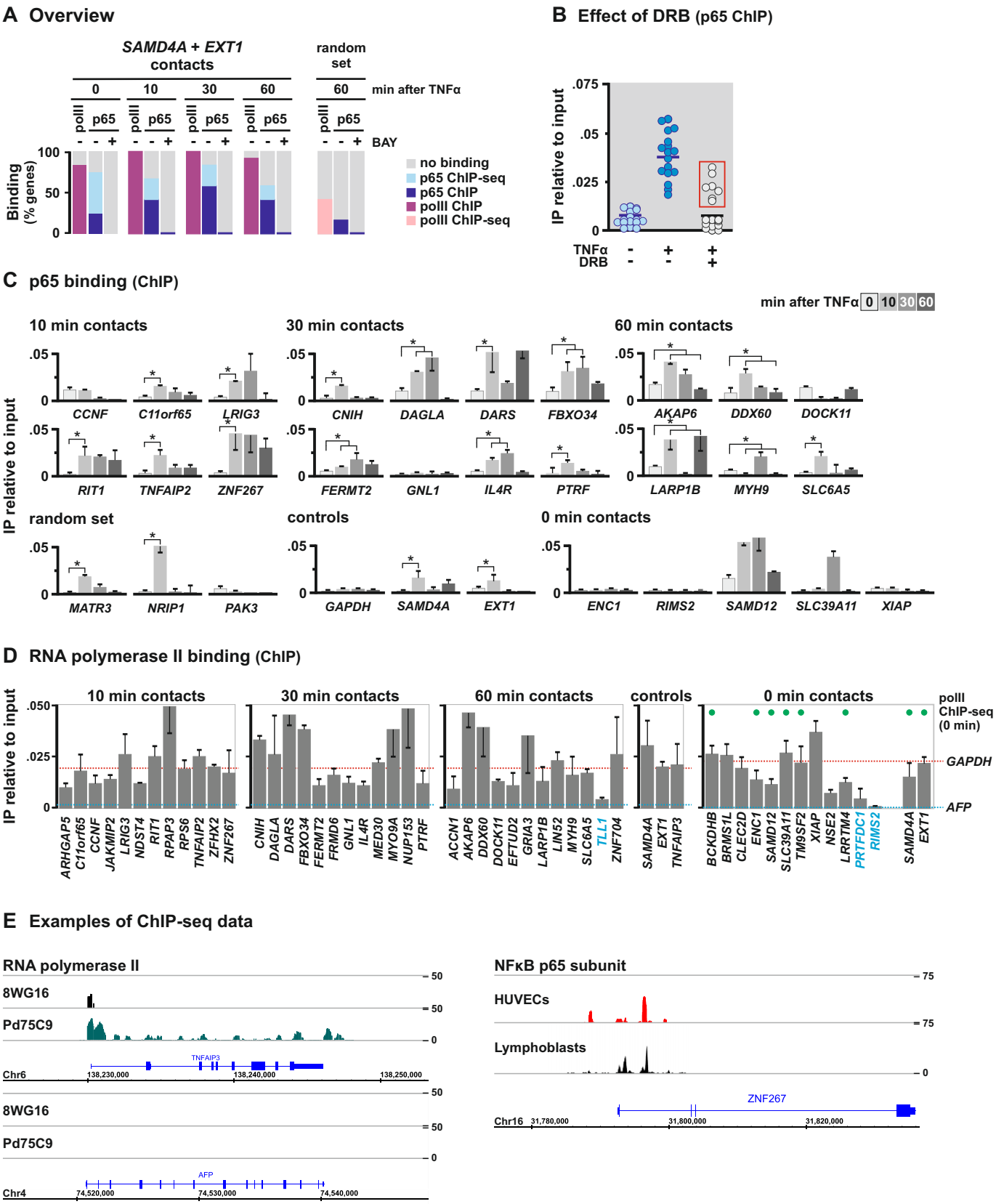
Supplementary Figure S1



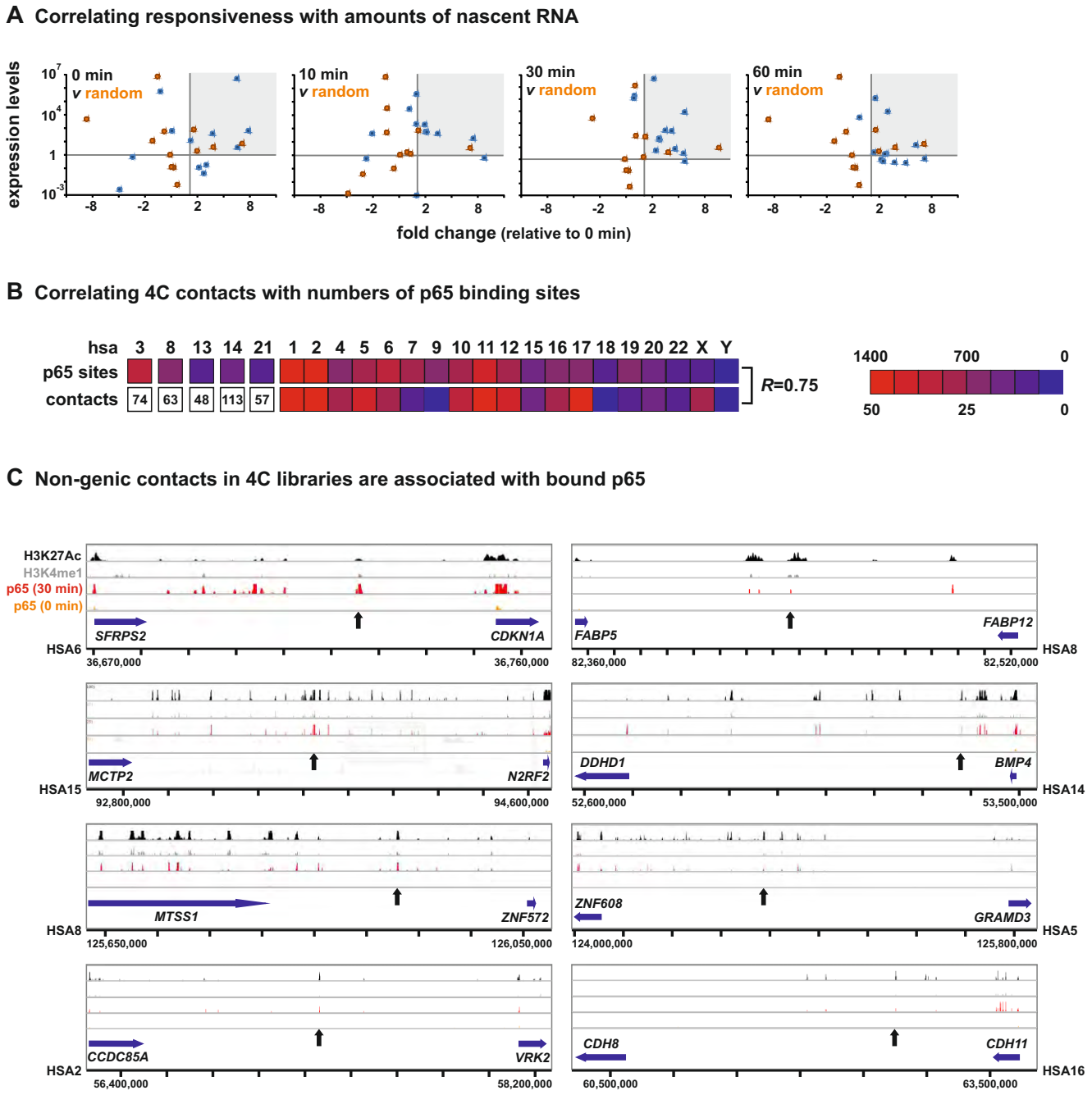
Supplementary Figure S1 Quantitative RT-PCR shows that most contacted coding and non-coding genes respond to TNFα. HUVECs were grown ±BAY for 1 h, treated with TNFα for up to 60 min, and relative levels of nascent transcripts assessed using qRT-PCR with intronic probes. (**A**) Overview of results for a set of 12 genes contacted by *SAMD4A* and *EXT1* at 0-60 min after stimulation (genes selected at random from the set of all those contacted); a set of randomly-selected human genes provides a control. Circles indicate fold-change relative to 0-min value (for 0-min contacts, the respective 60-min values are used) and black lines give mean. Genes in each experimental set were significantly up-regulated, compared to the random set (1.5-fold threshold indicated by grey shading). BAY abolished this up-regulation (grey circles). *: $P<0.01$ ($n=4$; unpaired two-tailed Student's *t*-test). (**B**) Details for the genes analyzed in (A). Changes in nascent RNA levels (relative to 0-min level; ± SD; $n=4$) are shown for each gene. No more than 3 genes in the random set are up-regulated >1.5-fold (red dotted line) at any time-point. In contrast, significantly more genes in each of the experimental sets were up-regulated at the relevant time ($P=0.0375$, 0.003, 0.01, and 0.003 for the 0-, 10-, 30-, and 60-min sets, respectively; two-tailed Fisher's exact test). (**C**) A similar analysis of (**i**) pre-miRNAs and (**ii**) mature miRNAs. Pre- and mature miRNAs were chosen because their host genes were contacted 30 min after stimulation by *MIR17HG*, *MIR155HG*, or *MIR191* (in 4C or ChIA-PET libraries). Levels are expressed as a percentage of those of RNU6 RNA. mir-17, -155, and -191 are presented as positive control, and mir-15a as a negative control (grey boxes). 67% miRNAs tested are significantly up-regulated 30 min after induction, and pretreatment with BAY prevents this (*: $P<0.01$; $n=3$; unpaired two-tailed Student's *t*-test). Pre-miRNAs and miRNAs shown in red could not be detected.

Supplementary Figure S2



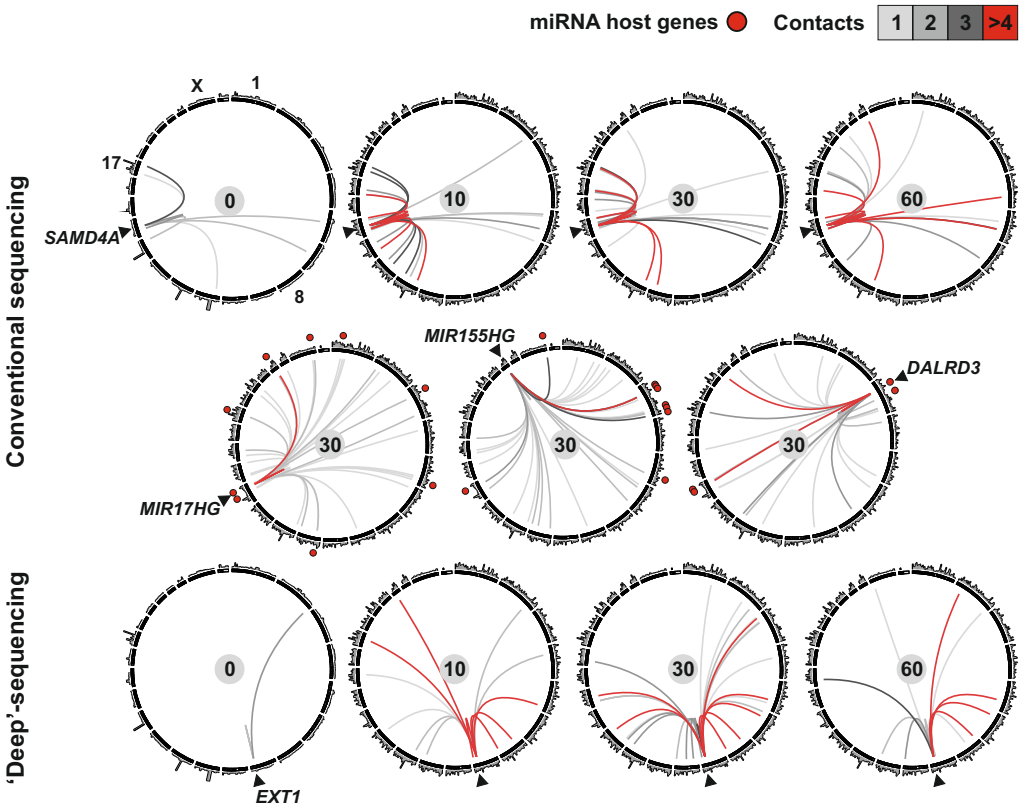
Supplementary Figure S2 ChIP shows that RNA polymerase II and the p65 subunit of NFκB bind to the promoters of most genes contacted by *SAMD4A* and *EXT1*. HUVECs were grown ± DRB for 1 h, treated with TNFα for up to 60 min, and binding of p65 and polymerase II monitored by ChIP at the relevant times (amount bound ± SD is expressed as a fraction of input; *n*=3). (**A**) Overview of results for sets of 12 randomly-selected genes contacted by *SAMD4A* and *EXT1*; a set of randomly-selected human genes provides a control. Contacts tend to bind the polymerase or p65 (compared to the random set), and BAY prevents this binding. Polymerase II binding to promoters (*i.e*., ±1 kbp of the TSS) was assessed by ChIP (ChIP-seq for the random set). Binding of p65 to genes possessing at least one binding site (5'-GGGRNNYCC-3') within 3 kbp upstream of the TSS was assessed by ChIP; binding to genes lacking such p65 binding motifs (as qPCR primers could not be designed) was assessed by ChIP-seq. (**B**) The transcriptional inhibitor, DRB, partially prevents p65 binding to the 17 promoters from the 10-, 30-, and 60-min sets in (A) and (C) that bound significantly more p65 after TNFα stimulation. Stimulation with TNFα increases binding to all promoters (line gives mean); DRB does not affect this increase for 7 out of the 17 genes tested (red box). (**C**) Details for the p65 ChIP presented in (A). *GAPDH* (plus *SAMD4A* and *EXT1*) provide additional negative (and positive) controls. Of the three genes in the random set that possessed at least 1 binding site, binding to only two increased significantly on stimulation. In contrast, binding to 5, 7, and 5 genes in the 10-, 30-, and 60-min sets, respectively, was significantly higher (*: $P<0.01$; unpaired two-tailed Student's *t*-test). (**D**) Details for the RNA polymerase II ChIP presented in (A). The *GAPDH* TATA box and the *AFP* 3' untranslated region are included as controls, as well as three responding promoters (*SAMD4A*, *EXT1*, *TNFAIP3*). Most promoters at each of the four times were associated with polymerase levels comparable to those of constitutively-expressed *GAPDH* (dotted red line). Only the *TLL1, PRTFDC1,* and *RIMS2* promoters (light blue) were associated with low levels seen with *AFP* (dotted blue line). (**E**) Typical genome browser views (IGB v. 6.4) of genes showing RNA polymerase II and p65 binding determined by ChIP-seq. *Left*: Results obtained using two different antibodies against RNA polymerase II (commercially-available 8WG16, and Pd75C9); a transcribed (*TNFAIP3*) and non-transcribed gene (*AFP*) which were tested in (D) are shown. *Right*: Results obtained using different antibodies targeting p65 using HUVECs or lymphoblasts (data for the latter from Kasowski *et al,* 2010); patterns are similar.

Supplementary Figure S3



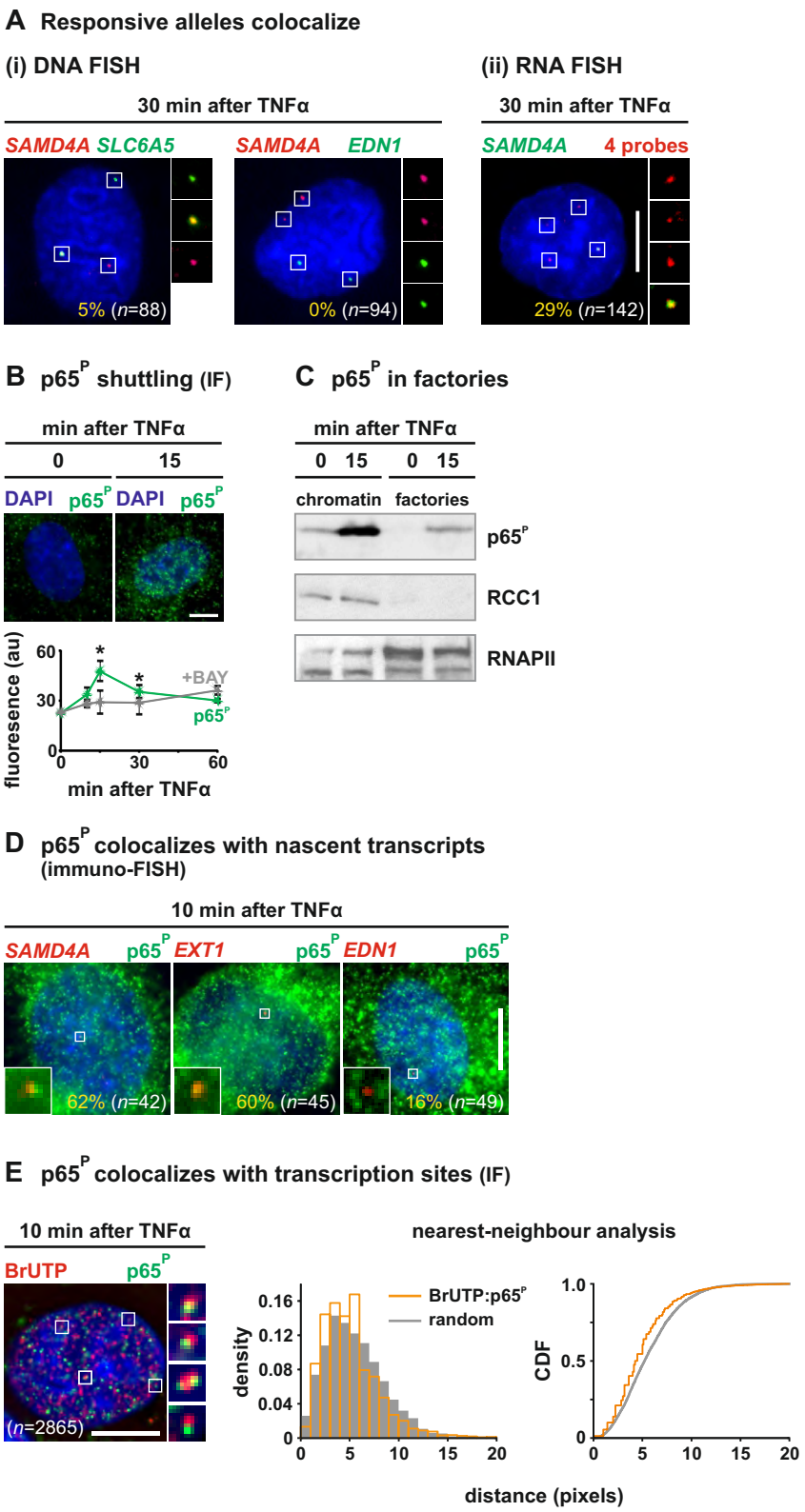
Supplementary Figure S3 Characteristics of contacts seen by 4C. Data is derived from 4C libraries prepared without BAY between 0-60 min after stimulation with TNFα and analyzed by conventional sequencing. (**A**) Correlation between TNFα responsiveness and the expression level of a gene. The four sets of 12 genes contacted by *SAMD4A* or *EXT1* and seen in 4C libraries 0, 10, 30, and 60 min after stimulation—plus the random control set—were analyzed (these sets were also analyzed in **Supplementary Figure S2A**). Fold change is the nascent RNA level at the time indicated relative to the 0-min level; expression level is the amount of nascent RNA normalized relative to the level of mature *RNU6* RNA. The horizontal grey line indicates the level given by *GAPDH*, and the vertical grey line marks 1.5-fold up-regulation. Many contacted genes (blue circles), but few in the random set (brown circles), are up-regulated more than 1.5-fold (and so lie in the two quadrants on the right); this confirms that contacted genes tend to be up-regulated. Of the minority of contacted genes that are not up-regulated, many tend to be transcribed more than *GAPDH* (and so lie in the top left quadrant). (**B**) The numbers of contacts made by *SAMD4A*, *EXT1*, *MIR17*, *MIR155*, and *MIR191* with different chromosomes (assessed using 4C) is compared with the number of p65 binding sites on each chromosome (determined using published ChIP-seq data; Kasowski *et al,* 2010). *Open boxes*: numbers of intra-chromosomal contacts made by each reference gene (*SAMD4A*, *EXT1*, *MIR17HG*, *MIR155HG*, and *MIR191* reside on chromosomes 14, 8, 13, 21, and 3, respectively) which were not included in the analysis. Pearson's correlation coefficient (*R*) for the two sets was 0.75; correlation coefficients for contact number and chromosomal length or site density/Mbp were significantly lower (*i.e.*, *R*=0.58 and 0.33, respectively). No contacts map to the Y chromosome, which is not present in (female) HUVECs. (**C**) Browser views (IGB v. 6.4) of eight of the 10 most-frequently seen non-genic contacts (the other two lacked any bound p65 and are not shown) seen in the 4C libraries. Vertical arrows indicate contact points; y-axis for each track in reads per million (from 0 to 50). In most cases, the contact point exhibits a peak in two "active" chromatin marks (*i.e.*, H3K27Ac and H3K4me1), as well as increased binding of p65 30 min after stimulation.

Supplementary Figure S4



Supplementary Figure S4 Contacts evolve over time and contacting regions are rich in bound p65. Circos software (Krzywinski *et al,* 2009) was used to depict *SAMD4A, EXT1,* and miRNA contacts detected by 4C coupled to conventional or "deep"-sequencing. Chromosome ideograms are drawn to scale and presented clockwise from 1 to Y; positions of chromosomes 1, 8, X, and 17 are indicated, and no contacts are with the Y chromosome—which is not present in (female) HUVECs. Contacts are color-coded according to their frequency of occurrence (from light grey for singletons to red for >4 contacts). p65 ChIP-seq data are shown on the outer track (peak height reflects amount bound). *Arrowheads*: positions of reference genes. *Red* circles: gene hosting a micro-RNA. For simplicity, non-genic hits seen by 4C coupled to "deep"-sequencing are omitted. Results show that many new contacts appear after 10 min, and then contacts evolve thereafter. Note that reference genes contact other chromosomes more often than their own (*e.g*., ~60 of *SAMD4A* and ~84% of *EXT1* contacts are inter-chromosomal); globin genes frequently make such inter-chromosomal contacts (Brown *et al*, 2008; Schoenfelder *et al,* 2010). 47% *SAMD4A* contacts and 41% *EXT1* contacts are with chromosomes not contacted by the other—a percentage significantly higher than that expected by chance ($P < 10^{-100}$, calculated using the binomial distribution assuming chromosomes contact each other with equal probability); this is consistent with the respective territories lying in different parts of the nucleus. This differential location was confirmed by analyzing images like those in **Figure 4A**; 71% foci containing nascent RNA encoded by *SAMD4A*, compared to 43% *EXT1* foci, lay in the peripheral half of the nuclear area.

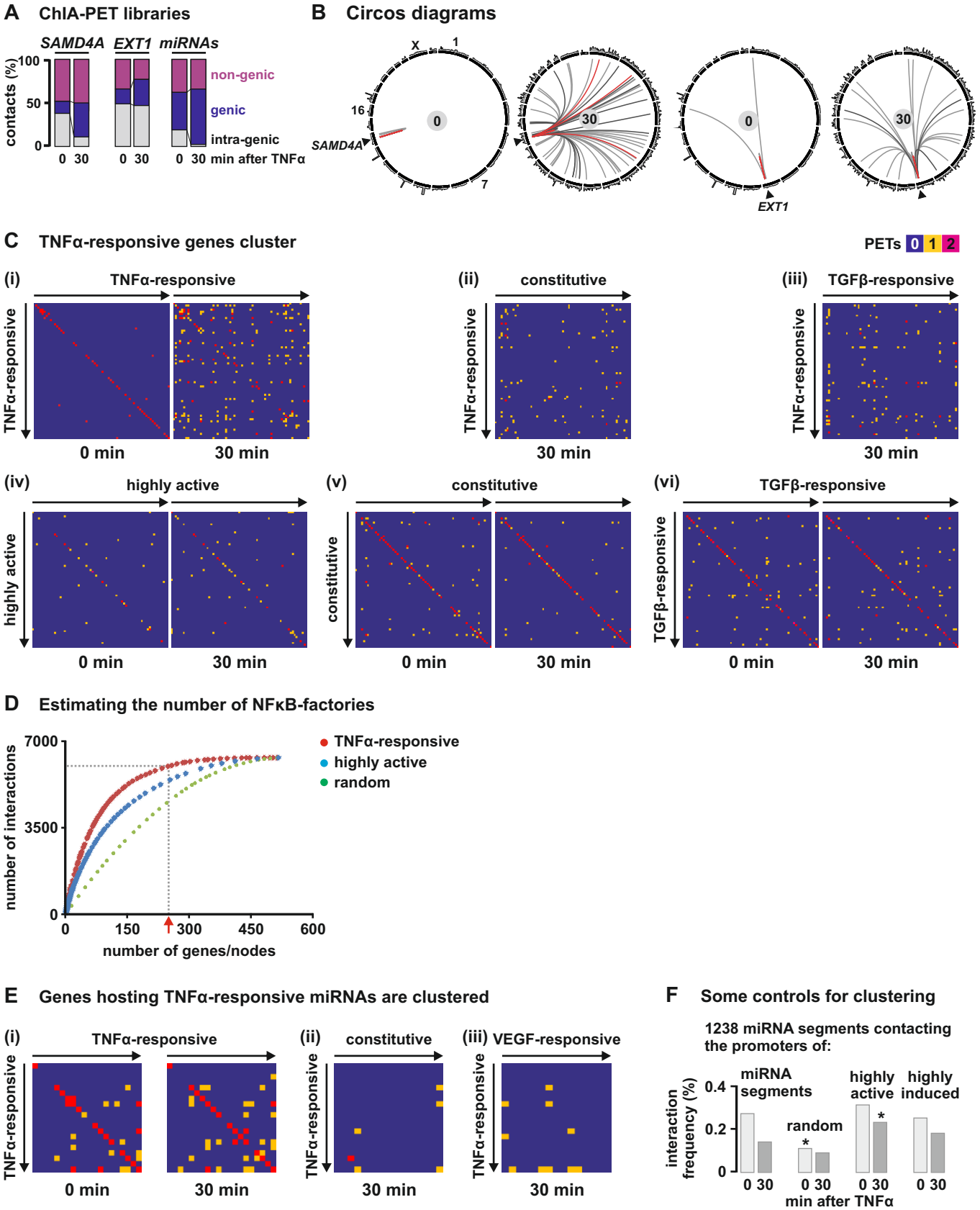
Supplementary Figure S5



Supplementary Figure S5 *SAMD4A* and *EXT1* alleles/transcripts colocalize in NFκB-factories. HUVECs were stimulated using TNFα for 0-60 min in the presence or absence of the BAY inhibitor. *Bars*: 5 μm. (**A**) Responsive alleles colocalize 30 min after stimulation. (**i**) DNA FISH was performed using probes targeting *SAMD4A* (on HSA 14), *SLC6A5* (on 11), and *EDN1* (on 6). 5% nuclei screened (*n*=88) had at least one *SAMD4A* allele colocalizing with a *SLC6A5* allele (*left*); these two genes were shown to contact each other by 4C (**Supplementary Table S1**), and their nascent transcripts also colocalize (RNA FISH showed ~7% active alleles colocalized; Papantonis *et al*, 2010). A control targeting *SAMD4A* and constitutively-active *EDN1* (*right*) yielded no overlapping foci (*n*=94); this difference is statistically significant (*P*=0.0249; two-tailed Fisher's exact test). (ii) Nascent RNA FISH was performed as in Figure 4, using probes targeting *SAMD4A* (green) and a set of 4 multiplexed ones (red) on chromosomes 4, 5, 16, and 22; 29% (*n*=142) of green foci overlapped at least one red focus, significantly more than the 2% yielded by the control in Figure 4 (*P*<0.0001; two-tailed Fisher's exact test). (**B**) Phospho-p65 ($p65^P$; phosphorylated at Ser 536) shuttling. *Left*: the nucleus (DAPI-stained) contains more $p65^P$ 15 min after induction. *Right*: the intensity of nuclear fluorescence (arbitrary units, au) is significantly higher after 15 and 30 min, compared to 0 min or in BAY-treated cells (*: *P*<0.01; *n* = 20; unpaired two-tailed Student's *t*-test). (**C**) $p65^P$ becomes enriched in purified factories after stimulation. Nuclei were isolated from HUVECs, chromatin detached with DNase I, and spun to leave "chromatin" in the supernatant. The pellet was resuspended, treated with caspases, and spun; this supernatant contains large fragments of "factories" released from the substructure. $p65^P$, RNA polymerase II, and RCC1 (used as a negative control, as it is an abundant nuclear protein not present in factories; Melnik *et al,* 2011) in "chromatin" or "factories" were then detected by immuno-blotting. RNA polymerase II serves as a loading control. $p65^P$ becomes enriched in "factories" after 15 min, but not RCC1 is detected within factories at either time. (**D**) Nascent *SAMD4A* and *EXT1* transcripts colocalize with $p65^P$ after induction. Fixed cells were hybridized with intronic probes targeting *SAMD4A* or *EXT1* (or *EDN1*, a constitutively-expressed gene used as a control), and $p65^P$ immunolabeled. A single red focus in each nucleus marks nascent RNA copied from one allele; the insets show nascent *SAMD4A* and *EXT1* transcripts colocalize with $p65^P$ foci (green). 62% *SAMD4A* foci (*n*=42) and 60% *EXT1* foci (*n*=45) colocalize with $p65^P$ foci, significantly more (*P*=0.004 and 0.003, respectively; two-tailed Fisher's exact test) than the 16% found with *EDN1* foci (*n*=49). (**E**) Sites of active transcription colocalize with $p65^P$ after induction. HUVECs were stimulated for 10 min, permeabilized, and engaged polymerases allowed to "run on" by few nucleotides in BrUTP; nascent BrRNA and $p65^P$ were then visualized by immunolabeling. A typical wide-field image is shown (*left*; *insets* show magnified examples of overlapping red/green foci). Many red foci lie near green foci to give yellow in the merge. To assess whether the degree of colocalization was significant, foci were

selected automatically using a computer algorithm, peak intensities within the selected foci localized with 22-nm precision, and the distance from the peak of each red (or green) focus to its closest green (or red) focus determined (see **Supplementary methods**). A total of 2865 foci were analyzed. As a control, 8243 randomly-distributed foci with the same density were computer-generated. Plots (*right*) of probability density (density) and cumulative density function (CDF) versus distance to the nearest neighbour in the other channel (in pixels; 1 pixel=90 nm) show that $p65^{P}$ and BrRNA foci are significantly closer together than random (P<0.001, Kolmogorov-Smirnov two-sample test; compare orange and grey bins/lines; in the plot on the right, error bars ± 99% confidence limit are contained within the grey line). Note that the experimental sample gives a lower density in the first bin, which is consistent with one antibody (length ~9 nm) blocking access of a second to a nearby antigen.

Supplementary Figure S6

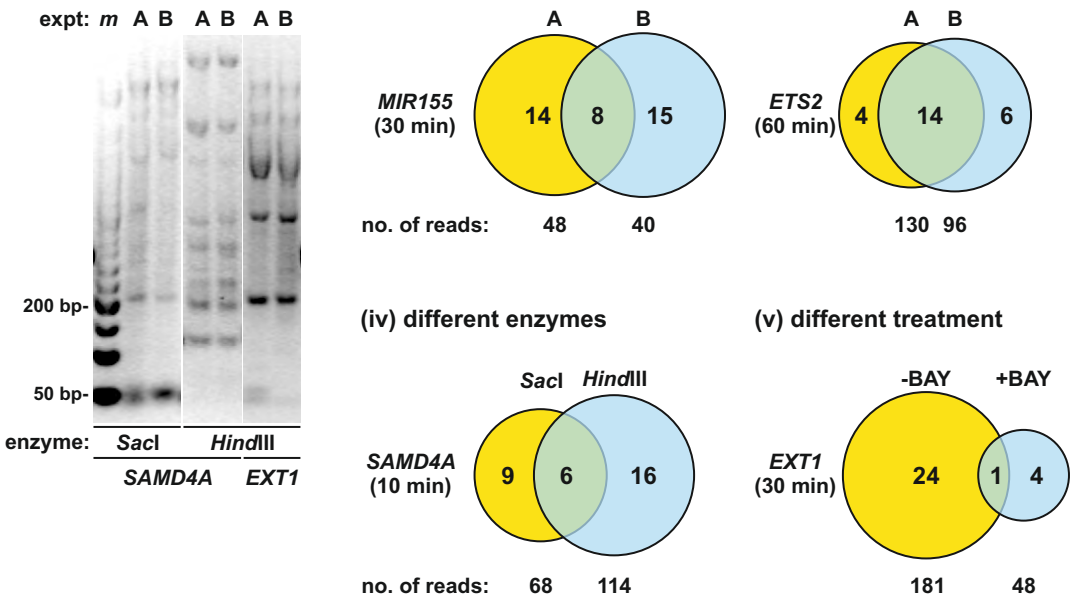


Supplementary Figure S6 ChIA-PET shows that TNFα-responsive genes, and genes encoding miRNAs, co-associate. HUVECs were grown in TNFα for 0 or 30 min, active forms of RNA polymerase II immuno-selected, ChIA-PET performed, and PETs (contacts) between selected genes analyzed. (**A**) Contacts made by *SAMD4A*, *EXT1*, and three miRNA genes (*MIR17*, *MIR155*, and *MIR191*; results pooled) classified as in **Figure 2A**. As for 4C, more genic contacts are detected after stimulation (see also **Supplementary Tables S5** and **S6**). (**B**) Contacts evolve over time. Circos software was used to depict contacts made by *SAMD4A* and *EXT1* (p65 ChIP-seq data is shown on the outer track). *Arrowheads*: positions of reference genes. For the sake of simplicity, non-genic hits are omitted. Results show that many new contacts appear after 30 min. (**C**) Stimulation induces TNFα-responsive genes to associate. Colored boxes within each matrix indicate no PET/contact between two genes (blue), 1 contact (yellow), or at least 2 contacts (red). Significance was assessed using Fisher's two-tailed exact test. (**i**) The 69 genes most up-regulated by TNFα versus the same 69 genes (reproduced from **Figure 5B** for comparison). Genes are ranked from high-to-low up-regulation (from left to right, and top to bottom), determined using microarray data obtained 0 and 30 min after stimulation (all up-regulated at least 1.9-fold). There are significantly more PETs/contacts between responsive genes at 30 min compared to 0 min ($P<0.0001$), and in the 30-min sample here compared to the 30-min samples in sub-panels (ii-vi), for which P values were <0.0001 in all cases. (**ii**) The 69 genes most up-regulated by TNFα versus a set of 69 randomly-chosen constitutively-active genes (of equivalent activity to the 69 genes up-regulated by TNFα). (**iii**) The 69 genes most up-regulated by TNFα versus the 69 genes most up-regulated by TGFβ (determined using microarrays, and ranked as above). (**iv**) The 69 most highly-active genes (but not up-regulated by TNFα) versus each other; there is no significant difference in the number of contacts between 0 and 30 min ($P=0.34$). (**v**) A set of 69 randomly-chosen constitutively-active genes (of equivalent activity to the 69 genes up-regulated by TNFα) versus each other. There was no significant difference in the number of contacts between 0 and 30 min ($P=0.06$). (**vi**) The 69 genes most up-regulated by TGFβ versus each other. There was no significant difference in the number of contacts between 0 and 30 min ($P=0.9$). (**D**) Estimating the number of "NFκB" factories. 496 genes, upregulated >3-fold, make 6,266 ChIA-PET contacts between them. If these 496 genes access essentially all "NFκB" factories in the cell, successively removing the gene making the fewest contacts from the set, and then the next fewest, and so on, should initially have little effect on the total number of interactions; this is the case. When too few up-regulated genes remain to saturate all "NFκB" factories, the number of interactions should begin to fall; ~250 genes recover ~95% interactions (dotted lines), and this provides us with an upper limit of the number of "NFκB" factories. Results for a random network (created by randomizing contacts in the observed network), and for the most-active, but non-responsive genes, are included for comparison.

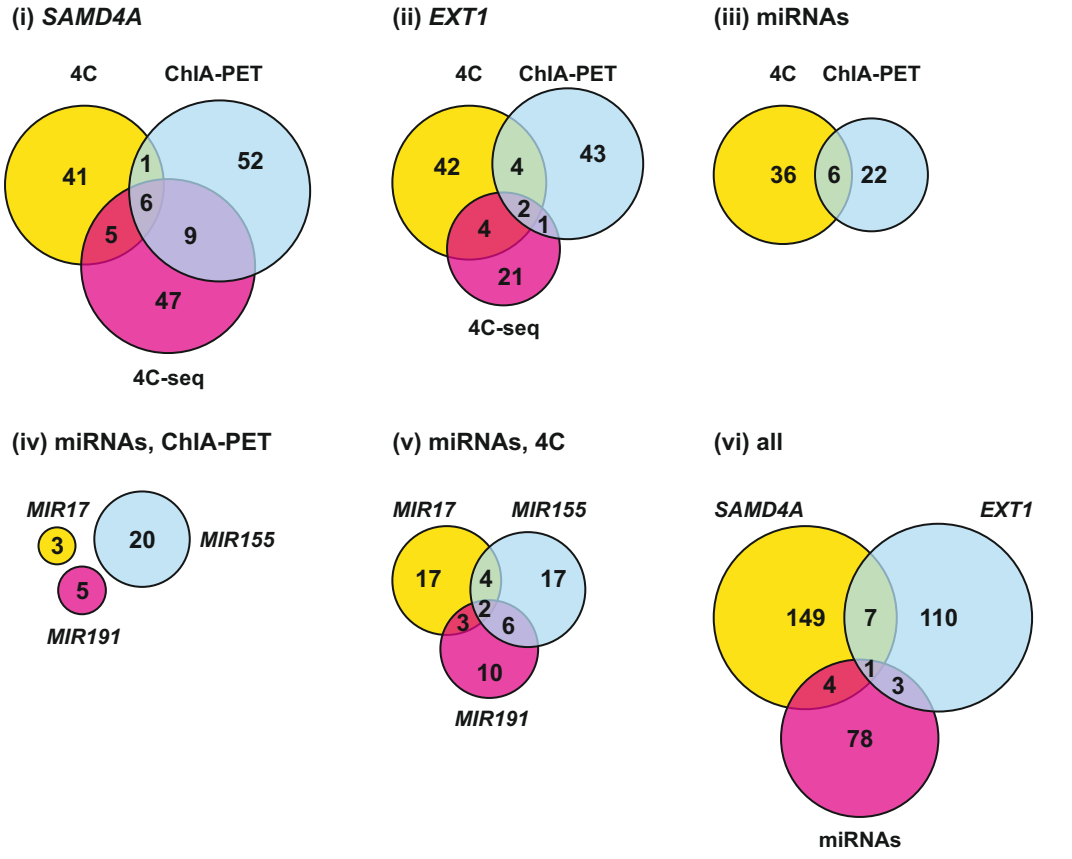
(**E**) Genes hosting TNFα-responsive miRNAs cluster. The 20 genes encoding miRNAs were chosen because data from microarrays (Suárez *et al,* 2010) and qRT-PCR (**Supplementary Figure S1C**) showed they were up-regulated. Colored boxes within each matrix indicate 0 (blue), 1 (yellow), or at least 2 contacts (red) between two genes; genes are ranked (top to bottom, left to right) in order of increasing responsiveness. (**i**) The 20 responsive miRNA genes versus themselves at 0 and 30 min. Although the increase in the number of contacts is small (13 to 16, respectively; *P*=0.92) there is a noticeable rearrangement after stimulation. Most importantly, there are significantly more contacts in the 30-min sample here compared to those in sub-panels (ii) and (iii) (*P*=0.0021, and 0.0034, respectively; two-tailed Fisher's exact test). (**ii**) The 20 responsive miRNA genes versus a set of 20 constitutively-active miRNA genes (Suárez *et al,* 2010) 30 min post-stimulation. (**iii**) The 20 responsive miRNA genes versus a set of 20 VEGF-responsive miRNA genes (Suárez *et al,* 2008) 30 min post-stimulation. (**F**) Some additional controls for miRNA clustering. For the sake of completion, we also analyzed interactions between essentially all genes encoding miRNAs in the genome. We expect many miRNAs not to be expressed (Suárez *et al,* 2010), and thus would not expect members of this complete set of miRNA genes to interact more with themselves, compared to interactions with similarly-sized segments encoding expressed promoters; this proved to be the case. Essentially all segments of the genome encoding miRNAs were selected as follows. First, each of the 1,523 miRNAs in the database were mapped to the genome, each region extended +/- 5 kbp, and combined if they overlapped by >1 bp. Next, PETs/contacts were counted between the resulting 1238 "miRNA segments", these miRNA segments and an equal number of randomly-selected promoters (+/- 5 kbp), these miRNA segments and 886 or 890 promoters (+/- 5 kbp) at 0 or 30 min respectively that are the most highly-active but non-induced, and these miRNA segments and 879 highly-induced promoters (+/- 5 kbp) 30 min after stimulation. Finally, the interaction frequency (*i.e*., the number of PETs divided by the number of possible pairwise combinations expressed as a percentage) was calculated. No significant differences were seen, with the exceptions of randomly-selected promoters at 0 min, and highly-active promoters at 30 min (compared to miRNA segments at 0 and 30 min, respectively). *: *P*=<0.0001 (two-tailed Chi-squared test with Yates' correction). The higher interaction frequency seen after stimulation in panel (D,i) is consistent with the clustering of active miRNA genes—this is obscured by the large fraction of inactive miRNA genes in the sample used here.

Supplementary Figure S7

A Reproducibility in 4C libraries

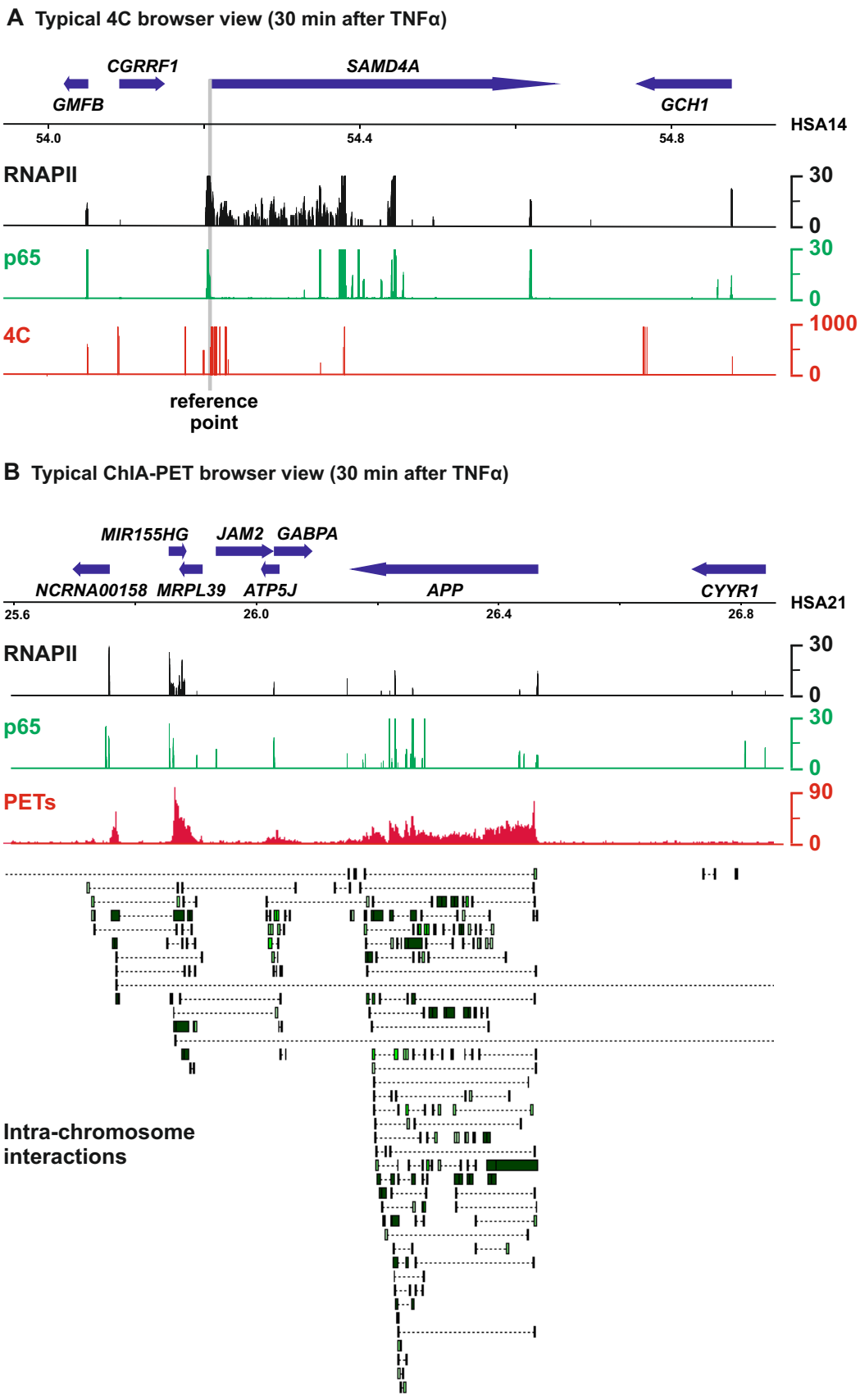


B Shared contacts between the different approaches



Supplementary Figure S7 Reproducibility between different libraries prepared and analyzed by 4C, 4C-seq, and ChIA-PET. Venn diagrams indicate the number of contacts shared between different libraries/approaches. (**A**) Comparison of both genic and non-genic contacts detected in different 4C libraries. (**i**) Representative examples of electrophoresis profiles after 4C and nested inverse PCR amplification between biological replicates (experiments A and B) using *SAMD4A* or *EXT1* as reference points, and *Sac*I or *Hind*III for cutting chromatin. *m*: marker DNA. (**ii**) Comparison between two libraries prepared using the same time after stimulation with TNFα (30 min), enzyme (*Hind*III), and reference gene (*MIR155*); ~36% contacts are shared, indicative of partial coverage. (**iii**) Comparison between two libraries prepared using the same time after stimulation with TGFβ (60 min), enzyme (*Hind*III), and reference gene (*ETS2*); as the number of 3C products screened is greater than in (ii), more contacts are shared (~74%). (**iv**) Comparison between two libraries prepared using the same time after stimulation with TNFα (10 min) and reference gene (*SAMD4A*), but different enzymes (*i.e*., *Hind*III and *Sac*I, yellow and blue, respectively); ~30% contacts in each are shared. (**v**) Comparison between two libraries prepared at the same time after stimulation (30 min) using the same reference point (*EXT1*), but with different treatments (*i.e*., addition or not of BAY); only 1 contact is shared. (**B**) Comparison of genic contacts detected using different 3C variants 0-60 min after TNFα stimulation. (**i**) *SAMD4A* contacts detected by 4C coupled to conventional sequencing ("4C"), 4C coupled to deep-sequencing ("4C-seq"), and ChIA-PET; ~13% 4C contacts are shared between all three approaches, ~22% between 4C and 4C-seq, and ~24% between 4C-seq and ChIA-PET. (**ii**) *EXT1* contacts detected by 4C coupled to conventional sequencing, 4C coupled to deep-sequencing, and ChIA-PET; ~7% 4C-seq contacts are shared between all three approaches. (**iii**) Contacts between genes hosting miRNAs detected by 4C and ChIA-PET; ~21% ChIA-PET contacts are shared. (**iv**) Contacts between genes hosting miRNAs detected using ChIA-PET; no contacts were shared, indicative of low coverage. (**v**) Contacts between genes hosting miRNAs detected using 4C; ~17% of *MIR191* contacts are now shared with both *MIR17* and *MIR155*. (**vi**) Comparison of pooled contacts (detected using all 3C variants) made by *SAMD4A*, *EXT1*, and three miRNA host genes; <1% contacts are shared between all three, indicative of distinct interactomes for each gene.

Supplementary Figure S8



Supplementary Figure S8 Typical browser views of 4C-seq and ChIA-PET data obtained 30 min after stimulation. The number of reads per million for different genomic regions (*y*-axis) are shown for data from ChIP-seq (RNA polymerase II—*black*; p65—*green*), and 4C coupled to next-generation sequencing (*red*); the number of PETs (*y*-axis) is given for ChIA-PET data (*red*). (**A**) Region of ~1 Mbp around *SAMD4A*. *Grey line*: position of the TSS of *SAMD4A* used as the reference point for 4C. (**B**). Region of ~1 Mbp around *MIR155HG*. PETs (rectangles connected by dotted lines) in this part of the genome are shown at the bottom.

Legends to Supplementary Tables

Supplementary Table S1 Properties of genic contacts seen after conventional sequencing in 16 4C libraries prepared using *SAMD4A* and *EXT1* as reference points. Libraries were prepared using *Hind*III or *Sac*I 0-60 min after adding TNFα, and ~80 inserts in each sequenced. Gene name, chromosomal location, time(s) after stimulation when seen, the number of times seen (hits; the number of sequenced clones including identical ones), and TNFα responsiveness plus p65/RNA polymerase II binding (red and blue boxes indicate responsiveness/binding and non-responsiveness/non-binding, respectively, as in **Figure 2B**) are indicated. A random set of genes provides a control. *Grey highlight*: gene seen in at least two different libraries. *: gene randomly selected from those contacted at one time for detailed analysis (see **Supplementary Figures S1** and **S2**). *Orange highlights*: genes hosting miRNAs (*GCH1, PRKCA*, *STRN3*, and *MIR15A* encode mir-4308, -634, -624, and -15a, respectively). Over-representation of red boxes in the *SAMD4A* (43%; *n*=53) and *EXT1* (29%; *n*=52) columns (compared to random sample; *n*=75) indicates that significantly more contacted genes both respond to the cytokine and bind p65 and RNA polymerase II ($P<0.0001$ and 0.0011, respectively; two-tailed Fisher's exact test). Results obtained with each of the two enzymes were broadly similar.

Supplementary Table S2 Properties of genic contacts seen after "deep" sequencing 4C libraries prepared using the TSS of *EXT1* or *SAMD4A* as a reference. All genic contacts detected in libraries prepared using *Hind*III are listed, with chromosomal location, time(s) after stimulation when seen, the number of different sites contacted within the gene (hits; this definition differs from that in **Supplementary Table S1**), and TNFα responsiveness plus p65/RNA polymerase II binding (red and blue boxes indicate responsiveness/binding and non-responsiveness/non-binding, respectively, assessed as in **Figure 2B**). *Grey highlight*: gene also detected by conventional sequencing or ChIA-PET (**Supplementary Tables S1** and **S5**). *Orange boxes*: genes hosting miRNAs. Over-representation of red boxes (compared to the random sample in **Supplementary Table S1**) indicates that significantly more contacted genes are responsive and bind p65/RNA polymerase II. For example, 34% (*n*=50) *EXT1* and 27% *SAMD4A* (*n*=67) contacts are responsive and bind both p65 and the polymerase—significantly more than the 7% (*n*=75) in the random set ($P=0.0002$ and 0.0024, respectively; two-tailed Fisher's exact test).

Supplementary Table S3 Properties of genic contacts seen after conventional sequencing in 4C libraries prepared using genes encoding mir-17, -155, and -191 as reference points. Libraries were prepared using *Hind*III at 30 min after adding TNFα, and ~96 inserts in each sequenced. Gene name, miRNA encoded, chromosomal location, the number of times seen (hits; the number of sequenced

clones including identical ones), and TNFα responsiveness plus p65/RNA polymerase II binding (red and blue boxes indicate responsiveness or binding and non-responsiveness or nonbinding, respectively, assessed as in **Figure 2B**), are indicated. *Grey highlight*: gene hosting one or more miRNAs. Comparisons of the combination of the three sets (*n*=75) with the random sample (*n*=75) or the *SAMD4A* contacts (*n*=53) in **Supplementary Table S1** show significant enrichment in miRNA-hosting genes (*P*<0.0001 and 0.0012, respectively; two-tailed Fisher's exact test).

Supplementary Table S4 Micro-RNAs encoded by three reference genes (*MIR17HG*, *MIR155HG*, and *MIR191/DALRD3*) and their contacts target mRNAs down-regulated by TNFα. The lists depict the 100 genes most down-regulated by TNFα 1 and 4 h after stimulation (selected using microarray data, and listed in order of the most down-regulated to the least); 100 randomly-selected genes serve as a control. Gene name and ratios of expression (compared to 0 min levels) are given. Grey highlights indicate that the 3' UTR of the corresponding mRNA possesses one or more targets for the miRNAs encoded by the three reference host genes and their contacts. Contacted genes encoding miRNAs are listed in **Supplementary Figure S1C**, and the mRNA targets of these miRNAs were detected using the algorithm in the miRWalk database. The down-regulated genes (*n*=200) encode mRNAs with significantly more (*P*=0.0082; two-tailed Fisher's exact test) target sites for the miRNAs encoded by the contacted genes, when compared to the random set (*n*=100).

Supplementary Table S5 Properties of *SAMD4A* and *EXT1* genic contacts detected by ChIA-PET. ChIA-PET libraries were prepared 0 or 30 min after TNFα stimulation and genic contacts formed by *SAMD4A* and *EXT1* mined from the genome-wide interactome. Contacts are listed in rank order of number of PETs seen with gene name, number of PETs seen (only >2 PETs listed), chromosomal location, and TNFα responsiveness plus p65/RNA polymerase II binding (red and blue boxes indicate responsiveness/binding and non-responsiveness/non-binding, respectively, as in **Figure 2B**). *Grey highlight*: contacts also seen by 4C. As in the 4C libraries (**Figure 2A**), more contacts develop after 30 min. *Dots*: contacted genes that are pre-loaded with RNA polymerase at 0 min (from ChIP-seq data). Over-representation of red boxes (compared to the random sample in **Supplementary Table S1**) indicates that significantly more contacted genes are responsive and bind p65/RNA polymerase II. For example, 48.5% (*n*=50) *SAMD4A* and 35.7% (*n*=28) *EXT1* contacts are responsive and bind both p65 and the polymerase—significantly more than the 6.67% (*n*=75) in the random set (*P*<0.0001 and 0.0006, respectively; two-tailed Fisher's exact test).

Supplementary Table S6 Properties of genic contacts made by genes hosting mir-17, -155, and -191 (detected by ChIA-PET). ChIA-PET libraries were prepared 0 or 30 min after TNFα stimulation and contacts formed by *MIR17HG, MIR155HG* and *DALRD3* (which hosts miR-191) mined from the complete genome-wide interactome. Contacts are listed in rank order of number of PETs seen with gene name, number of PETs seen (all PETs detected are listed), chromosomal location, name of hosted miRNAs, and TNFα responsiveness plus p65/RNA polymerase II binding (red and blue boxes indicate responsiveness or binding and non-responsiveness or non-binding, respectively, assessed as in **Figure 2B**). *Grey highlight*: contacts that involve miRNA-hosting genes; genes encoding non-coding RNAs (NCRNA or LOC) are also shown. Comparison of each of the three sets (n=28, 189, and 51, respectively) with the random sample (n=75) in **Supplementary Table S1** shows significant enrichment in miRNA-hosting genes (P=0.0054, 0.0022, and 0.0174, respectively; two-tailed Fisher's exact test).

Supplementary Table S7 Some genic contacts seen in 4C libraries (prepared using *SAMD4A* and *EXT1* as reference points) are TGFβ-responsive. Gene name, chromosomal location, time(s) after stimulation when seen, the number of times seen (hits; the number of sequenced clones including identical ones), and TNFα responsiveness plus p65/RNA polymerase II binding (red and blue boxes indicate responsiveness/binding and non-responsiveness/non-binding, respectively, as in **Figure 2B**) are indicated. Some contacted genes (28% and 40% for *SAMD4A* and *EXT1*, respectively) are responsive to both TNFα and TGFβ, revealing some overlap between the two pathways. However, responsiveness to TNFα and TGFβ appears uncorrelated (R=0.19 and 0.16 for *SAMD4A* and *EXT1*, respectively), consistent with contacts made by these two reference genes being specific for the TNFα cascade.