Title: **Enhancer hijacking reveals a multimodal role of NF-κB during the immediate-early inflammatory response**

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Authors: Petros Kolovos1,\*, Theodore Georgomanolis2, Milos Nikolic2, Anna Koeferle3,§, Joshua D. Larkin3,¤, Alexander Feuerborn3, Wilfred F. van Ijcken4, Eduardo G. Gusmao5, Ivan G. Costa5, Peter R. Cook3, Frank G. Grosveld1, Argyris Papantonis2,\*

Affiliation: *1Department of Cell Biology, Erasmus Medical Centre, 3015 CN Rotterdam, The Netherlands; 2Center for Molecular Medicine, University of Cologne, 50931 Cologne, Germany; 3Sir William Dunn School of Pathology, University of Oxford, OX1 3RE Oxford, United Kingdom; 4Center for Biomics, Erasmus Medical Centre, 3015GE Rotterdam, The Netherlands; 5IZKF Computational Biology Research Group, RWTH Aachen University Medical School, 52062 Aachen, Germany.*

Present address: §Department of Cancer Biology, University College London, WC1E 6BT London, United Kingdom; ¤Department of Electrical and Biomedical Engineering, University of Nevada, NV 89557 Reno, USA.

Correspondence: \*A.P.; Email: [argyris.papantonis@uni-koeln.de](mailto:argyris.papantonis@uni-koeln.de)

\*P.K.; Email: [p.kolovos@erasmusmc.nl](mailto:p.kolovos@erasmusmc.nl)

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**ABSTRACT**

**Mammalian cells have developed intricate mechanisms to interpret, integrate, and ultimately respond to signaling. Tumor necrosis factor alpha acutely remodels the cell’s transcriptional program, but our understanding of how the activation of proinflammatory genes is achieved at the expense of the ongoing transcriptional program is far from complete. We use human endothelial cells to monitor the immediate-early phase of this cascade at high spatiotemporal resolution. We find that NF-κB, the main driver of the response, predominantly hijacks the regulatory machinery of the cell by binding already-active enhancers, more than half of which do not carry NF-κB recognition motifs. We apply targeted chromatin capture, in combination with genome editing and inhibitors, to understand the function exerted by hijacked enhancers in a 3-Mbp locus. We present evidence for a multimodal role of NF-κΒ that satisfies the need for both transcriptional activation and suppression, and is linked with changes in the higher-order structure of regulated loci. Our work reveals a hitherto unappreciated complexity in enhancer functionality in the context of acute transcriptional responses.**

*Key words*: tumor necrosis factor alpha; chromosome conformation capture; T2C; hijacked enhancers; nuclear factor kappa B; activator; repressor

**INTRODUCTION**

Mammalian cells have developed intricate mechanisms to interpret, integrate, and ultimately respond to extra-cellular stimuli. Inflammatory signaling (Natoli and Smale, 2014) constitutes a prime example: TNFα triggers acute remodeling of the cell’s gene expression program and this is achieved via its main downstream effector, transcription factor NF-κB (Hayden and Ghosh, 2008). Stimulation by TNFα leads to the induction of the same genes across different cell types (Moynagh, 2005); but, in order for the cascade to unfold, the diverse ongoing transcriptional programs of each cell need to be suppressed. To date this was shown to be achieved directly via repressive NF-κB heterodimers (Marienfeld *et al*., 2033) or indirectly via redistribution of cofactors (Schmidt *et al*., 2015), and is tightly linked to the choice of NF-κB binding positions *in vivo*. However, the cell-type specific principles that guide this choice are still not fully understood, despite the wealth of data on the different phases of the inflammatory response and its NF-κB–centered regulatory landscape (Ghisletti *et al*., 2010; Rao *et al*., 2010; Bhatt *et al*., 2012; Danko *et al*., 2013; Ostuni *et al*., 2013; Brown *et al*., 2014; Zhao *et al*., 2014; Schmidt *et al*., 2015). In addition, our spatiotemporal understanding of how NF-κB contributes to such diverse regulatory output is far from complete.

We now understand that non-genic *cis*-regulatory elements, such as enhancers and insulators, play an important role in the implementation of cell type-specific gene expression programs. Enhancers loop onto the promoters they regulate and via the combinatorial recruitment of transcription factors affect their transcriptional status (Heinz *et al*., 2015); in fact, many enhancers regulating TNFα-inducible genes were found to be pre-looped prior to stimulation (Jin *et al*., 2013). Insulators, like the abundant CCCTC-binding factor (CTCF), have been shown to confine chromatin interactions between genomic elements within specific “topological” domains (Ong and Corces, 2014), the disruption of which leads to gene expression dysregulation (Dowen *et al*., 2014; Lupianez *et al*., 2015; Tsujimura *et al*., 2015). Hence, the three-dimensional organization of chromatin influences (and is influenced) by the spatial interplay of enhancers and insulators (Kolovos *et al*., 2012; Pombo and Dillon, 2015), and is bound to be involved in the regulation of inflammatory signaling.

Previously we characterized NF-κB–driven spatial networks of coregulated genes in endothelial cells (Papantonis *et al*., 2012), as well as global “priming” of the chromatin landscape for the ensuing transcriptional events in both a NF-κB–dependent and -independent manner (Diermeier *et al*., 2014). In this cell type inflammatory stimulation is linked to a number of acute or chronic disorders (Levy *et al*., 2007; Libby *et al*., 2011; Medzhitov *et al*., 2012; Brown *et al*., 2014) and we use these cells as a model to study the inflammatory response by focusing on the following. First, on how the inflammatory gene expression program imposes on the ongoing one in human endothelial cells. Second, on how the three-dimensional organization of responsive loci refines this. We stimulate human umbilical vein endothelial cells with TNFα for 0-90 min and follow changes in 30-min intervals. We analyze chromatin marks and transcription factor binding by ChIP-seq, nascent RNA production by total RNA sequencing and the iRNA-seq pipeline (Madsen *et al*., 2015), as well as 3D chromatin topology by a combination of chromosome conformation capture-based assays (3C; de Wit and de Laat, 2012) and genome editing technology (Gaj *et al*., 2013). We find that NF-κB predominantly “hijacks” thousands of already-active enhancers, >50% of which do not carry canonical NF-κB binding motifs; a considerable fraction of these enhancers is also linked to transcriptional repression. Then, we apply 3C-seq (Stadhouders *et al*., 2013) to compare and contrast the spatio-temporal deployment of interactions along two Mbp-long genomic loci, one that is induced upon stimulation and one already active. Finally, we revisit the TNFα-responsive locus global conformation at sub-kbp resolution (using T2C; Kolovos *et al*., 2014), disrupt the interaction between the *SAMD4A* promoter and its intronic hijacked enhancer cluster using zinc-finger nucleases, and dissect its responsiveness to stimulation. Taken together, our results suggest a multimodal role for NF-κΒ during the immediate-early phase of the inflammatory cascade. This role satisfies the need for proinflammatory gene activation and repression, and is tightly interlinked with changes in the three-dimensional topology of the regulated loci. It also reveals a hitherto unappreciated complexity in enhancer functionality in the context of acute transcriptional responses.

**RESULTS**

***TNFα stimulation rapidly remodels the nascent transcriptome of HUVECs***

The inflammatory response is characterized by acute changes in gene expression that manifest within <30 min of TNFα stimulation (Hao and Baltimore, 2009; Diermeier *et al*., 2014; Schmidt *et al*., 2015). As a result, changes in mRNA levels can be misleading since many genes responding to the stimulus will not produce mature mRNA within such a short time frame. To circumvent this, we isolated and sequenced total (and depleted of rRNA species) RNA at 0, 30, 60, and 90 min post-stimulation. Each sample was sequenced to ~150 million read pairs to obtain robust coverage at intronic regions, and data were analyzed via the iRNA-seq pipeline (Madsen *et al*., 2015) to assess changes at the nascent RNA level. This analysis revealed >1000 genes as up- or down-regulated at any given time (**Figure 1A,C**), twice as many as the equivalent analysis on poly(A)+-selected RNA sequenced to the same depth (**Supplementary Fig. 1A,B**). We also examined the distribution of fold-change values for up- and down-regulated genes at each time point. Our data show comparable fold-change responses throughout the time course (**Figure 1B**), with a more pronounced effect for 60-min down-regulated genes (similar to data from adipocytes; Schmidt *et al*., 2015). Gene ontology (GO) term analysis of differentially-regulated genes at each time revealed that up-regulated genes were highly associated with processes like transcription factor (TF) regulation, stimulus/immune responses, or regulation by RNA polymerase II (RNAPII) transcription, while down-regulated ones were mostly involved in primary and nucleic acid metabolism, and angiogenesis (**Figure 1D**). Finally, the sensitive approach for detecting transcriptional changes used here (i.e., changes in intronic RNA) reveals a dynamic network of differentially-regulated genes along the time course; the 30-, 60-, and 90-min time-points only share 569 and 269 up- and down-regulated genes, respectively (±0.6 log2 fold-change compared to 0 min; **Figure 1E**). Notably, this dynamic remodeling of the cell’s transcriptome relies mainly on the diverse outputs of NF-κB activity.

Genes are not randomly strung along the DNA fiber; we have come to appreciate that genomic architecture contributes significantly to the regulation of gene expression (Pombo and Dillon, 2015), also in response to extra-cellular stimuli (Jin *et al*., 2013; Le Dily *et al*., 2014). To begin addressing this we performed whole-genome 3C (Hi-C; Belton *et al*., 2012) using HUVECs stimulated with TNFα for 30 min, sequenced the resulting library to 200 million read pairs, and analyzed data (see **Methods**) to obtain interaction maps at 100-kbp resolution (**Supplementary Fig. 1C**). Using these maps we identified “topologically associating domains” (TADs; Dixon *et al*., 2012; Levy-Leduc *et al*., 2014) and used them to examine if TNFα-responsive genes cluster within particular TADs. We found that 688 up-regulated genes reside singly in a TAD, whereas 218 and 110 genes reside in a given TAD in pairs or groups of ≥3 genes, respectively. Interestingly, highly-clustered TNFα-responsive genes display a more confined breadth of fold-change values, perhaps indicative of co-regulation (**Figure 1F**); this also applies to down-regulated genes (**Supplementary Fig. 1E**).

We now also understand that a fraction of genes are poised for prompt activation in response to developmental or signaling cues (Danko *et al*., 2013; Jonkers *et al*., 2014). We took advantage of the iRNA-seq analysis features and devised a “poising index” (PI; see **Methods** for details) to identify genes at 0 min that might be poised for prompt response to the TNFα stimulus. Focusing on TNFα-responsive genes, we found 261 with a log2PI >0.6; these included known immediate-early TNF-responders (e.g., *NFKBIA*, *NFKBIE*, *TNFAIP2*, *CXCL1*), as well as previously-identified poised genes (e.g., *MYC*, *IL1A, SELE*). Poised genes displayed significantly higher fold-change values at 30 min compared to non-poised up-regulated genes, and their vast majority resided as singletons in TADs (**Figure 1G**).

***NF-κB hijacks already-active enhancers to both stimulate and suppress gene expression***

The NF-κB factor is mainly represented by the p65::p50 heterodimer, which strongly binds the canonical 5’-GGRRNNYYCC-3’ motif (Wong *et al*., 2011; Zhao *et al*., 2015). There exist >575000 such motifs in the human genome (hg18), yet ChIP-seq data on p65 global binding (Papantonis *et al*., 2012; Brown *et al*., 2014) reveal ~8500 high confidence binding events at 30 or 60 min post-stimulation. Re-analysis of these datasets (for a typical example see **Figure 2A**) showed that NF-κB mostly binds enhancer regions which do not associate exclusively with TNFα-induced genes [assessed using HUVEC HMM motifs (Hoffman *et al*., 2012) and an algorithm for assigning peaks to genes (Sikora-Wohlfeld *et al*., 2013); **Supplementary Fig. 2A**]. Intriguingly, ~70% of the 30-min p65 peaks do not overlap the expected “canonical” p65::p50 motif. This prompted us to perform motif analysis on the sequence underlying each peak. At 30 min, peaks overlapping the canonical motif (“with”) carried NF-κB and AP-1 motifs, as expected; peaks that lacked the motif (“without”) carried motifs for REL, AP-1, and STAT3 binding. This remained so for 60-min peaks, with STAT3 now also present in “with” peaks (**Supplementary Fig. 2B**). Next, we examined how ΝF-κB redistributes genome-wide between the 30- and 60-min time points. About 55% of 60-min peaks are “without” canonical motifs, while half also seen bound at 30 min (**Supplementary Fig. 2C**). Interestingly, p65 peaks are positioned progressively more distant to the nearest transcription start site (TSS; defined as that also positioned within the same TAD), and “without” peaks are consistently more distant to TSSs than peaks “with” motif (**Supplementary Fig. 2D**).

NF-κB binding predominantly occurs at enhancers already-active before stimulation (**Figure 2B** and **Supplementary Fig. 2A**); >60% of 30-min NF-κB peaks overlap sites marked by monomethylation of lysine 4 (H3K4me1) and acetylation of lysine 27 (H3K27ac) of the histone H3 tails at 0 min (**Figure 2B** and **Supplementary Fig. 2E**). Reassuringly, we also observe binding at “latent” enhancers as previously reported (Ostuni *et al*., 2013). Of these 5366 sites, >3/4 do not overlap TSSs (**Figure 2B**), and hereafter constitute the “hijacked” enhancers. These are mostly intragenic, display little overlap with CTCF-bound sites, and half carry the canonical motif or remain bound by NF-κB at 60 min post-stimulation (**Figure 2B**; *bottom*). To obtain a more precise view of NF-κB binding choices, we combined p65 ChIP-seq data with genome-wide DNase I-hypersensitivity footprinting data from the ENCODE project (0 min; ENCODE, 2012). We performed motif analysis at the footprints indicative of TF binding, thus alleviating much of the noise from intervening unbound sequences at each p65 peak (**Supplementary Fig. 2E**). Looking for differentially-enriched factors in “with” and “without” peaks, we find p65 (RELA) and p50 (NFKB1) motifs markedly enriched at peaks carrying the canonical motif, whereas those lacking the motif predominantly harbored JUN and FOS motifs (the monomers forming the AP-1 factor; Zenz *et al*., 2008; **Figure 2C**). This prediction was verified by ENCODE ChIP-seq data from HUVECs, where co-binding of NF-κB and JUN/FOS (and to a lesser extent GATA2) was most prominent at “without” sites (**Figure 2D**). Notably, both “with” and “without” hijacked enhancers are marked by divergent eRNAs (assessed using factory RNA-seq; Caudron-Herger *et al*., 2015; **Figure 2E**; **Supplementary Fig. 2F**) indicating they are active (Kaikkonnen *et al*., 2013). Last, we assigned the closest gene to each p65 peak (again, ensuring both lie within the same TAD) and performed GO term analysis. Genes proximal to “with” peaks associated with processes like regulation of RNAPII, cell death, metabolism, or stimulus response, unlike those proximal to “without” peaks that associated with cell communication, adhesion, or angiogenesis (**Figure 2F**).

The above GO terms differed from those assigned to up-regulated genes (see **Figure 1D**); hence, we speculated that NF-κB, via its p65 subunit, might also contribute to transcriptional repression. We examined RNAPII ChIP-seq levels (Papantonis *et al*., 2012) at p65-bound sites. This revealed a general increase in RNAPII signal around p65 peaks with the exception of a subset of ~770 “without” peaks that showed marked decrease in polymerase binding (**Figure 2G**). As before, “without” clusters harbor FOS/JUN-bound regions, with repressive cluster “4-without” specifically encompassing binding sites for the AP-1 repressor, JDP2 (Aronheim *et al*., 1997; **Supplementary Fig. 2G-J**). Strikingly, “without” peaks appear more conserved (amongst 17 vertebrates) that “with” peaks (**Figure 2I**). Next, we used H3K27ac ChIP-seq generated at 30 and 60 min to ask whether these clusters retain this “active” histone mark post-stimulation; most did, but the repressive cluster lost >60% of its H3K27ac peaks (**Figure 2H**). We also plotted nucleosome occupancy (using MNase-seq data; Diermeier *et al*., 2014) along these clusters, and—again—only the repressive one showed increased occupancy post-stimulation, which points to transcriptional deactivation (**Figure 2J**). Finally, looking at eRNA production and changes in expression levels before and after stimulation per cluster, “4-without” showed an overall decrease in eRNA levels and contained most repressed genes than any other cluster (**Supplementary Fig. 2K,L**).

***Comparative analysis of two genomic loci reveals different modes of regulation by NF-κB***

Our global analysis on p65 binding and enhancer activity suggests different modes of regulation by NF-κB. To further dissect this, and to incorporate the aspect of 3D chromatin conformation, we performed 3C-seq from multiple viewpoints along two 2.5-Mbp genomic loci. One, on chromosome 14, harbors the inducible *SAMD4A* and *GCH1* (both also identified as poised; **Supplementary Table 1**) and the repressed *BMP4* gene, amongst a total of 15 genes. The other, on chromosome 6, harbors 6 already-active genes that respond variably. We monitored the interactions of the *SAMD4A*, *BMP4*, *CDKN3*, and *EDN1*, *NEDD9* TSSs at 0, 30, and 60 min, focusing on contacts with hijacked enhancers (**Figure 3A** and **Supplementary Fig. 3A**). On chromosome 14, the TSS of induced *SAMD4A* develops new contacts by 30 min, although it is contacting a large cluster of enhancers in its first intron already at 0 min. These contacts persist, unlike those with its proximal upstream enhancer which essentially disappear at 60 min (**Figure 3A**) when the gene is “locked” into elongation (Wada *et al*., 2009; Larkin *et al*., 2012). *SAMD4A* also forms contacts with other active genes in the same TAD, like *GMFB*, *CGRRF1*, and *SOCS4*, with which it shares similar response profiles (assessed by fold-changes in intronic RNA levels at 30 and 60 min; **Figure 3B**). *CDKN3* displays an interaction profile mostly confined by its two flanking H3K27me3 heterochromatic islands, as does down-regulated *BMP4* that resides in the same TAD. However, *BMP4* 3C-seq profiles stretch over longer (gene poor) genomic space and involve no other genes (**Figure 3A**). Intriguingly, the majority of hijacked enhancers contacted by inducible *SAMD4A* are “with” motif, whereas the strongest *BMP4* contact (upstream the gene) is “without” motif and belongs to repressive cluster 4 leading to a change in the genes interaction profile, and ultimately to its repression (**Supplementary Fig. 4A,B**).

In contrast, on chromosome 6, *EDN1* displays a rather constant 3C-seq profile by contacting two enhancer clusters up- and downstream its TSS, plus the *HIVEP1* promoter. Its contacts span three >200 kbp-long TADs, involve hijacked enhancers that are predominantly “without” motif (**Supplementary Fig. 3A**). *NEDD9* contacts are mostly confined within its own TAD, mutually exclusive to those formed by *EDN1*, and involve both “with” and “without” enhancers, plus the *SMIM13* TSS with which intronic fold-change profiles match (**Supplementary Fig. 3B**). In fact, the correlation of the changes in intronic RNA levels proved an adequate predictor of spatial interactions (**Supplementary Fig. 3C**).

In order to understand how NF-κB acts to differentially-regulate gene expression within spatial compartments, we compared the *SAMD4A* and *EDN1* interactomes. We only used contacted fragments with >100 rpm (to focus on “strong” interactions; see **Methods**) and correlated them to own and public ChIP-seq data. The outcome of these correlations is in line with our initial observations; i.e., before TNFα stimulation *EDN1* contacts other active (as it is itself active) and AP-1–bound regions (as its contacted enhancers are “without” motif). *SAMD4A* contacts fewer AP-1–bound regions (as most of its contacted enhancers are “with” motif), associates with a strong intragenic CTCF site (see Larkin *et al*., 2012) and a large intronic enhancer cluster, presumably to ensure prompt activation (**Supplementary Fig. 3D**). On stimulation, *SAMD4A* become strongly associates with NF-κΒ–bound and actively-transcribed regions (**Figure 3C**; in line with previous observations; Papantonis *et al*., 2012).

***NF-κB hijacks large clusters of active enhancers***

NF-κB peaks redistribute along chromosomes between 30 and 60 min post-stimulation (**Supplementary Fig. 2B,C**). While examining this, we observed large, dense, clusters of hijacked enhancers forming at 30 min, many of which are dismantled at 60 min (**Supplementary Fig. 5A**). Our 3C-seq data show that the most prominent contacts for both *EDN1* and *SAMD4A* are with dense enhancer clusters that resemble “super-enhancers” (SEs; Hnisz *et al*., 2013), but are of even larger breadth. As SEs have been implicated in the inflammatory response (Brown *et al*., 2014; Schmidt *et al*., 2015), we examined if they can also be discriminated according to their underlying motifs.

The original definition of SEs requires the “stitching” of adjacent enhancers separated by ≤12.5 kbp; we used the list of “stitched” HUVEC enhancers generated previously (Hnisz *et al*., 2013), assigned the 0-min H3K27ac ChIP-seq signal to each, and sorted all 12999 clusters according to it. This allowed identification of 608 SEs prior to stimulation, of which 77% are hijacked by NF-κB, and only few associate with TNFα-responsive genes (**Supplementary Fig. 5B**). We repeated the analysis starting with 3849 “stitched” peaks carrying both H3K27ac (0 min) and NF-κB (30 min), sorted according to p65 ChIP-seq signal, and identified 212 that associated with increasing eRNA levels and TNFα-inducible genes (also seen by Brown *et al*., 2013; **Supplementary Fig. 5C-F**). NF-κB–hijacked SEs associate with a spectrum of GO terms similar to that of standalone enhancers (**Figure 2F** and **Supplementary Fig. 5G**), but >80% are also bound by NF-κB at 60 min, >75% are intragenic, and ≥33% lie within >50 kbp-long genes and classify as “without” canonical NF-κB motifs (**Supplementary Fig. 5H**).

***TNFα-induced changes in the SAMD4A locus 3D architecture at high resolution***

3C-seq does not allow analysis of “all-to-all” interactions, and Hi-C that does is not cost-effective if high resolution is required; hence, we applied “targeted chromatin capture” (T2C; Kolovos *et al*., 2014) to understand interactions involving a large NF-κB–hijacked enhancer cluster. We focused on the ~3-Mbp locus that harbors the *SAMD4A* gene (**Figure 4A**). T2C was performed at 0 and 30 min post-stimulation (**Figure 4B**), it was highly reproducible (**Supplementary Fig. 6A,B**), and the overall architecture at <2-kbp resolution matched that obtained in high-resolution Hi-C studies (Rao *et al*., 2014; **Supplementary Fig. 6C**). At this resolution we were able to record a finer definition of TADs within the locus, which markedly changes upon stimulation (e.g., to now-include coregulated *SAMD4A* and *GCH1* in a single TAD; **Figure 4B**). Analysis of contacts forming by CTCF-bound regions showed a strong contact between the *SAMD4A* TSS and a CTCF peak downstream the enhancer cluster is lost on stimulation (**Figure 4C**), thus mediating the shift in TADs (**Figure 4B**). Reassuringly, the *SAMD4A* CTCF-mediated gene loop observed at 0 min is also lost on stimulation (**Figure 4C**) as we previously reported (Larkin *et al*., 2012). A similar analysis of contacts involving NF-κB–hijacked enhancers showed that the *SAMD4A* intronic enhancer cluster is prelooped onto the gene’s promoter already at 0 min, and that this interaction is weakened at 30 min; notably the individual enhancers in the >50 kbp-long hijacked cluster interact strongly with one another (**Figure 4D**). Again, reassuringly, T2C data verify the previously-documented NF-κB–mediated interaction network connecting TNFα-responsive genes and *cis*-regulatory regions on chromosome 14 (Papantonis *et al*., 2012; **Supplementary Fig. 6D,E**).

The *SAMD4A* intragenic enhancer cluster may be seen as an extended super-enhancer (SE), and in part of it does classify as such (**Supplementary Fig. 6B,C**). It has been demonstrated that SEs can be selectively inhibited using the JQ1 inhibitor (Filippakopoulos *et al*., 2010; Brown *et al*., 2014). Similarly, since the *SAMD4A* enhancer cluster is already active before NF-κB enters HUVEC nuclei, we reasoned that inhibiting H3K27ac acetylation via the p300 inhibitor, C646 (Bowers *et al*., 2010), might also affect its function. RT-qPCR showed that JQ1 does not affect transcription at the *SAMD4A* TSS, but represses mRNA production; C646 has a negligible effect, and DRB reduces both nascent and steady-state RNA levels (**Figure 4D** and **Supplementary Fig. 6F**). Note that inducible genes relying on hijacked SEs for their activation, like *TNFAIP3*, are affected by JQ1 but not C646, while *CXCL3* that is not dependent on an SE, but requires *de novo* enhancer acetylation is only sensitive to C646 (**Figure 4D** and **Supplementary Fig. 6F-H**). Using 3C-seq, we also asked how the *SAMD4A* spatial interactions are affected. Pretreatment of HUVECs with C646 abrogates pre-looping, but on TNFα stimulation much of the TSS-enhancer cluster interaction is restored; DRB treatment completely abolishes interactions (**Figure 4E**). In contrast, *EDN1*, which is already active before stimulation and contacts an intergenic SE consisting of “without” peaks, does not respond to C646, but is sensitive to JQ1 treatment (**Figure 4E** and **Supplementary Fig. 6I,J**).

***Altering the SAMD4A locus topology affects local regulatory crosstalk***

The different behavior, as regards looping and sensitivity to inhibitors, of the *SAMD4A* enhancer cluster hinted that it might be disconnected from the direct regulation of transcriptional initiation. To examine this we used targeted genome editing, via custom zinc-finger nucleases, to insert a strong RNAPIII-driven promoter between the *SAMD4A* TSS and enhancer cluster (**Supplementary Fig. 7A**). Active RNAPIII genes are often positioned at TAD boundaries (Dixon *et al*., 2012; Rao *et al*., 2014), and we previously speculated that they contribute to spatial gene regulation (Kolovos *et al*., 2012; Zirkel and Papantonis, 2014). We chose the *7SK* gene promoter that is not responsive to TNFα (**Supplementary Fig. 7B**) and has been shown to drive formation of specialized spatial networks (Xu and Cook, 2008). We inserted this 128-bp element ~13.5 kbp downstream the *SAMD4A* TSS so as not to interfere with its characteristic transcriptional initiation (Wada *et al*., 2009; Larkin et al., 2012; **Supplementary Fig. 7C**) and selected a single-cell–derived HUVEC clone (“7SKi”) with a homozygous insertion. 7SKi cells responded normally to TNFα (assessed by RNA-seq; **Supplementary Fig. 7D**). We used these and wild-type (wt) cells to perform T2C with *Apo*I, and achieved ~0.5-kbp resolution (**Figure 5A** and **Supplementary Fig. 7E**). Comparison of locus-wide interactions profiles revealed that the overall 3D topology was not affected by the insertion (**Supplementary Fig. 7F**), but distinct changes occurred around *SAMD4A* already at 0 min (**Figure 5A**). We highlight the loss of most prelooping, also verified using 3C-seq (**Figure 5B** and **Supplementary Fig. 7G**), despite NF-κB and p300 still binding the enhancer cluster (assessed by ChIP-seq in 7SKi cells; **Figure 5B**), and transcriptional initiation at the TSS and eRNA production at the enhancer cluster remaining essentially unaffected (**Figure 5C**). Looking at changes in the induction or suppression of neighboring genes (at the intronic RNA level to assess engagement in transcription) in the T2C-studied locus, we noticed a general suppression of genes upstream the 7SK insertion and variable behavior downstream (**Figure 5D**). This is explained by the redirection of the spatial contacts formed by the enhancer cluster (**Figure 5E**). In wt-HUVECs contacts mainly involve genes upstream, but in 7SKi cells they seem mostly redirected to downstream genes (**Figure 5F**), which are variably affected. Notably, as observed with JQ1 treatment, the *SAMD4A* response to stimulation is not significantly affected, but *GCH1* is markedly over-induced and *DLGAP5* over-suppressed compared to wild-type levels (**Figure 5D**).

**DISCUSSION**

The transcriptome of human endothelial cells is rapidly remodeled during the immediate-early inflammatory phase that follows TNFα stimulation (**Figure 1**). This is essentially the result of the nuclear translocation and chromatin binding of the NF-κB (p65::p50) heterodimer, which, despite the presence of hundreds of thousands canonical recognition motifs throughout the genome, binds sites not carrying the expected motif in >50% of cases (also observed for 30% of cases in LPS-stimulated B-cells and *in vitro*; Zhao *et al*., 2014; Wong *et al*., 2011). Similarly, >60% of all early NF-κB binding events involve “hijacking” of already-active *cis*-regulatory elements, mostly AP-1–bound enhancers (**Figure 2**). Such NF-κB cooperativity with master lineage regulators has been proposed for LPS-stimulated macrophages (Ghisletti *et al*., 2010; Saliba *et al*., 2014); however, here we find that binding site selection is not only marked by the absence of a canonical p65::p50 recognition motif, but notably by increased conservation of the sequence underlying the hijacked enhancer. We suggest that this essentially marks conserved regulatory elements for this cell type that control its core transcriptional program and, thus, need to be hijacked immediately after stimulation to establish the inflammatory cascade.

Repression of the ongoing cell type-specific gene expression program is an integral part of the deployment of any given cascade. NF-κB has been known to achieve this either by repression involving non-p65 containing complexes (p50::p52, p52::p52), which we are not detecting here (Hayden and Ghosh, 2008; Zhao *et al*., 2014), or by redistribution of cofactors (Schmidt *et al*., 2015). Here, we show a mode of direct p65-mediated repression and dissect how it is deployed in 3D space and over time using the *BMP4* gene as an example (**Supplementary Fig. 4**). This highlights an underappreciated function of NF-κB, and hints towards such features being carried by other drivers of signaling responses.

NF-κB–driven enhancer clusters, also known as “super-enhancers” (Hnisz *et al*., 2013), have been implicated in the proinflammatory response (Brown *et al*., 2014; Schmidt *et al*., 2015). Here, we observe an immediate-early specific arrangement of NF-κB–hijacked enhancers along chromosomes that produces clusters of even larger breadth than SEs (**Supplementary Fig. 5**). As the SE definition is still debated (Pott and Lieb, 2015), we comparatively interrogated one such large cluster for its function. The large *SAMD4A* intragenic hijacked enhancer cluster, mostly consisting of “with” peaks, appears to act as a unity. It is prelooped onto the *SAMD4A* TSS before stimulation, yet its pharmacological inhibition does not affect transcriptional initiation, but rather mRNA production (**Figure 4**). Similarly, even though we achieved physical insulation of the *SAMD4A* TSS from this enhancer cluster via the 7SK insertion, the *SAMD4A* gene was not deactivated (**Figure 5**), but its mRNA levels dropped (from 1.22 in wt cells to 0.67 log2 fold-change in 7SKi ones). These results suggest a different role for this enhancer cluster than what perhaps expected; it more in line with the “anti-pause release” model (Liu *et al*., 2013), where *SAMD4A* TSS-bound RNA polymerases are driven into productive elongation and with the model we previously proposed (Diermeier et al., 2014), whereby large intronic enhancer clusters might function to “open up” chromatin and facilitate elongation rather transcriptional activation *per se*. Besides, such an atypical role for this enhancer cluster is best reconciled with our previous observation on the transcription cycle of this long gene (Papantonis *et al*., 2010; Larkin *et al*., 2012; 2013).

Finally, in 7SKi-modified HUVECs the enhancer cluster in question develops a different spatial interactome, which results in the variable regulation of neighboring genes (**Figure 5**). This diverse output (e.g., *GCH1* is now most contacted and up-regulated; *DLGAP5* is also contacted, but repressed) is in line with a bimodal role for NF-κB as both an activator and repressor (**Figure 2**), but also with the formation of NF-κB–driven specialized networks of particular hierarchy (Papantonis *et al*., 2012; Fanucchi *et al*., 2013). Obviously, although our observations establish a multi-modal role for NF-κB during the acute phase of inflammatory signaling, they raise a number of questions that need to be followed up. How we can identify more cell-type determinants of TF binding choices or a quantitative understanding of the extent and strength of these TF-centered networks are just two of them.

**MATERIALS AND METHODS**

***Cell culture***

HUVECs from pooled donors were grown to 85-90% confluence in Endothelial Basal Medium 2-MV with supplements (EBM; Lonza) and 5% FBS, “starved” for 16-18 h in EBM+0.5% FBS, treated with TNFα (10 ng/ml; Peprotech), and harvested at 0, 30, 60 or 90 min post-stimulation; in some cases, 50 μM C646 (a p300 inhibitor; Bowers *et al*., 2010), 10 μM JQ1 (a BRD4 inhibitor; Filippakopoulos *et al*., 2010) or 50 μM DRB (5,6-dichloro-1-*β*-D-ribo-furanosyl-benzimidazole) was added for 1 h before harvesting.

***High throughput RNA sequencing (RNA-seq) and data analysis***

Total RNA was extracted from HUVECs at different times post-stimulation using Trizol (Ambion), DNase-treated as above, rRNA-depleted using a kit (RiboMinus; Epicentre), chemically fragmented into pieces of ~350 nt, and cDNA generated using random hexamers according to the True-seq protocol (Illumina) before sequencing on an Illumina HiSeq 2000. Poly(A)+-enriched samples were obtained using the same procedure with the addition of a selection on an oligo-dT column (see Kelly *et al*., 2015). Approximately 180 and 120 million read pairs/sample of total and poly(A)+-selected RNA were generated, respectively. Reads were then aligned using to the human genome (NCBI build 36; hg18) using Tophat (Trapnell *et al*., 2009) and default parameters (“no-coverage-search”, “segment-length 18” as input options) while reads that did not map uniquely were discarded. Counts of uniquely mapped reads from total, ribodepleted, RNA-seq were used as input for the “iRNA-seq” pipeline (Madsen *et al*., 2015) using the “intron” option for analysis of differential gene expression. For poly(A)+ RNA-seq analysis, uniquely mapped reads were counted per RefSeq gene exon using HTseq (<http://www‐huber.embl.de/users/anders/HTSeq/>), and statistical analysis of differentially-expressed genes was performed via DESeq (Anders and Huber, 2010). In all cases up- and down-regulated genes were selected to have more than ±0.6 log2 fold change in RNA levels compared to 0-min levels, and ≥1 FPKM at the stimulated and unstimulated state, respectively. Resulting gene subsets were used for generating box plots using BoxPlotR (<http://boxplot.tyerslab.com/>) and for Gene Ontology analysis using the Panther database (<http://www.pantherdb.org/>). Finally, for generating the “poising index” we calculated expression levels of TNFα-responsive genes using all three iRNA-seq options (“intron”, “gene”, and “polII”) to produce the ratio of RNA-seq signal between the 5’ end and the rest of each gene using the following formula:

PI = log2((gene0min *x* gene length)-(polII0min *x* polII length)/(gene length-polII length)/intron0min)

Genes with a PI >0.6 were considered poised (for a full list see **Supplementary Table 1**).

***Chromatin immunoprecipitation (ChIP-seq) and data analysis***

Typically, 15-30 million HUVECs were cross-linked (10 min, 20°C) in 1% paraformaldehyde (Electron Microscopy Science) at the appropriate times after TNFα induction, nuclei were isolated and chromatin was prepared, fragmented, and collected using the ChIP-It High Sensitivity kit (Active motif); immuno-precipitations were performed on aliquots of ~25 μg chromatin using antisera targeting acetylated lysine 27 of histone H3 (Abcam; ab4729), the phospho-CTD of RNA polymerase II (Papantonis *et al*., 2012) or IgG controls (Active motif; 53017). DNA was purified using a DNA clean-up kit (Zymo Research) and used either for qPCR analysis on a Rotor-Gene 3000 cycler (Qiagen) or as template for high throughput DNA sequencing on an Illumina HiSeq2000 platform. ChIP-seq data for NF-κB (targeting its p65 subunit) at 0, 30, and 60 min, as well as MNase-seq data at 0 and 30 min post-stimulation were generated previously (Papantonis *et al*., 2012; Brown *et al*., 2014; Diermeier *et al*., 2014). All other data on CTCF binding, DNase I footprinting, and histone modifications in unstimulated HUVECs come from the ENCODE project (ENCODE, 2012). In all cases, raw read files were aligned to the human reference genome (NCBI build 36; hg18) using Bowtie (Langmead *et al*., 2009) and discarding reads that mapped to multiple genomic locations. Uniquely-mapped reads were given a fragment size of 200 bp in MACS, which was used to identify binding peaks (Zhang *et al*., 2008), and the ShortRead package (Morgan *et al*., 2009) was used to to convert these into genome-wide coverage files. Using negative binomial distributions, we assigned *P*-values and false discovery rates (FDR) to each binding region (Rozowsky *et al*., 2009), and the final peak lists were compiled according with the following criteria: ≥20 read counts at each peak summit and FDR ≤0.001. Overlaps between binding peaks in the different datasets were identified using the “findOverlaps” function from the GenomicRanges suite, while the iRanges package (Lawrence *et al*., 2013) was used in custom R scripts for annotating the genomic location of peaks and defining the closest gene to each. For the latter, both the ChIP-seq peak and the gene’s TSS were required to reside in the same topological domain (TAD; see below). Finally, heat maps and line plots were generated using seqMINER (Ye *et al*., 2011) and NGSplot (Shen *et al*., 2014), respectively.

***Chromosome conformation (3C) and Targeted Chromatin (T2C) capture***

3C-seq and T2C were both performed in duplicates as described (Stadhouders *et al*., 2013; Kolovos *et al*., 2014). In brief, ~10 million HUVEC nuclei were isolated at 0, 30, and 60 min post-stimulation, following cross-linking in 1% paraformaldehyde (10 min, 20°C; Electron Microscopy Science), chromatin was cut using *Apo*I or *Hind*III (New England Biolabs), and ligated in dilution under conditions that allowed most nuclei to remain intact. For 3C-seq, ligated DNA was recut with *Dpn*II and ligated, and PCR was performed for each viewpoint (primer list in **Supplementary Table 2**) before the amplified material was sequenced to # million reads/sample on average (details in **Supplementary Table 3**). For T2C, ligated DNA was reduced to a size of ~### bp by a combination of *Dpn*II digestion and sonication, and sequenced to ## million reads/sample (details in **Supplementary Table 4**) on a HiSeq2000 platform (Illumina). Raw data were mapped to the reference genome (hg18) and analyzed using the r3Cseq (Thongjuea *et al*., 2013) or a custom pipeline (Kolovos *et al*., 2014) for 3C-seq and T2C, respectively. 3C-seq data were visualized on the UCSC browser (<http://genome-euro.ucsc.edu/>) using “bedGraph” files and the 6-pixel smoothing option; T2C data were visualized in 2D interaction plots as described (Kolovos *et al*., 2014). The RCircos package (Zhang *et al*., 2013) was used to depict interacting segments containing NFκB and/or H3K27ac peaks based on T2C profiles. NGSplot (Shen *et al*., 2014) was used to generate coverage plots along 3C-seq fragments carrying >100 reads per million of signal (a cutoff defined based on the average 3C-seq read distribution, so as to avoid analysis of poorly-contacted fragments by a given viewpoint).

***Whole-genome chromosome conformation capture (Hi-C) and analysis***

Hi-C was performed using 35 million HUVECs stimulated for 30 min with TNFα as described (Belton *et al*., 2012). In brief, nuclei were isolated, treated overnight with 800 units *Hind*III (New England Biolabs), overhangs were filled-in with biotin and ligated under dilute conditions, before being sonicated to ~800 bp, and biotinylated 3C junction being selected on streptavidin beads. After washing non-captured DNA away, end-repair and ligation of Illumina linkers was performed, and the library was sequenced (in two replicates) on a HiSeq2000 platform to generate 200 million read pairs (100 bp in length). The resulting reads were then mapped to the reference genome (hg18) iteratively (to ensure maximum recovery of uniquely mapped pairs) using BWA (Li and Durbin, 2009). The .BAM files containing mapped forward and reverse reads were merged, duplicates were removed using Picard (<http://picard.sourceforge.net/>) and the output is converted into “BEDPE” format using BEDTools (Quinlan and Hall, 2010). Then, custom R scripts were used to bin the genome into equally-sized bins (25-250 kbp), assigne reads to bins, and normalize read counts to library size. Next, the HiTC package (Servant *et al*., 2012) was used to annotate and correct matrices for biases in genomic features (Yaffe and Tanay, 2011), and visualize 2D heat maps. Finally, topologically-associating domain (TAD) boundaries were identified at a resolution of 100-kbp using “HiCseg” and default parameters (Lévy-Leduc *et al*., 2014).

***Differential motif and co-binding analysis in DHS footprints***

DNase-seq raw reads from HUVECs were obtained (GEO accession: GSM816646) and aligned to the human genome (hg18). Footprints were predicted using the HINT method (Gusmao *et al*., 2014) and extended by 5 bp up- and downstream. Then, motif-predicted binding sites (MPBSs) were determined by performing motif search inside footprints overlapping “with” or “without” NF-κB peaks against position frequency matrices obtained from public databases (Robasky and Bulyk, 2011; Mathelier *et al*., 2014). MPBSs were used to assess the enrichment of transcription factor binding sites using an approach based on Fisher’s exact test as described (Lin *et al*., 2015). All tools used here are available as part of the Regulatory Genomics toolbox (<http://www.regulatory-genomics.org>).

***Generation of genome-edited HUVECs using zinc-finger nucleases (ZFNs)***

A pair of ZFNs designed to specifically target an intronic region 13.5 kbp downstream the *SAMD4A* TSS (chr14: 54,117,868-54,117,908; hg18) was custom-produced (Sigma-Aldrich)*.* Plasmids carrying the ZFN open reading frames were *in vitro* transcribed to obtain the ZFN mRNAs according to the manufacturer’s instructions. In brief, plasmids were linearized by digestion with *Xba*I (New England Biolabs), purified and *in vitro* transcribed using the Message MAX T7 ARCA-Capped Message Transcription Kit (Epicentre). Poly-adenylation was carried out using the Poly(A) Polymerase Tailing Kit (Epicentre) and ZFN mRNAs were purified using the MEGAclear Kit (Ambion). ZFN mRNA quality was assessed on denaturing 1.2% agarose gels containing formaldehyde. The cutting and targeting efficiency of the ZFNs was verified via *Cel*I enzymatic assays (Transgenomic SURVEYOR Kit) as per manufacturer’s instructions in HUVECs transfected with 2.5 μg of each ZFN plasmid or 2 μg of each ZFN mRNA. To achieve insertions a repair vector was generated by inserting the pUC19 polylinker between the upstream and downstream *SAMD4A* homology regions (each ~750 bp-long), and then subcloning the 128-bp 7SK promoter (Xu and Cook, 2008) between *Xba*I and *Kpn*I sites. The constructs were validated by sequencing, amplified in One Shot TOP10 Chemically Competent cells (Invitrogen) and purified using a maxiprep kit (QIAGEN). The inserted promoter sequence is as follows:

1 AAGCTTAGATCAGCTTGCATGCCTGCAGGTCGACTCTAGATAGCGCTACCGGACTCAGATCTCGA

66 GCTCAAGCTTCGAATTCTGCAGTATTTAGCATGCCCCACCCATCTGCAAGGCATTCTGGATAG

As HUVECs are difficult to transfect we optimized conditions using the Amaxa HUVEC Nucleofector Kit (Lonza) and the pmax-GFP plasmid (Lonza), and assessed efficiency by fluorescence microscopy. Finally, HUVECs from pooled donors (Lonza), grown to 80-90% confluence in 10 cm dishes, were transfected with 2 μg of the 7SK-repair template plus 1.5 μg of each ZFN plasmid or 2 μg of each ZFN mRNA, and harvested at 80% confluence to isolate genomic DNA using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) for an initial PCR-based screen. In our hands ZFN plasmid transfections gave best results; hence, these cells were then grown in 96-well plates at approximately single-cell dilution and maintained in quadruplets for downstream work. Single-cell–derived HUVEC populations were PCR-screened for the insertion using primers that (i) anneal to the homology regions and should amplify both remaining plasmid and the genomic locus, (ii) anneal in the upstream homology region and a region only found on the repair template to control for the amount of non-degraded plasmid, (iii) anneal to the upstream homology region or insert respectively and just downstream of the homology region within *SAMD4A* to amplify only the genomic locus. PCRs were conducted using the GoTaq DNA polymerase (Promega) and amplimers were resolved by electrophoresis in ready-made 1% agarose gel containing ethidium bromide (E-Gel 96, Invitrogen). Clones that were positive in these screens were grown into increasingly larger tissue culture containers (24-well, 12-well, 10-com, and 15-cm plates) to obtain sufficient cell numbers for RNA-seq, 3C-seq, and ChIP-seq for p65 and p300 (in collaboration with Active Motif) at 0 and/or 30 min post-stimulation, making sure cells did not enter senescence.

***Oligonucleotides***

Oligonucleotides used in qPCR were designed using Primer 3.0 Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) using the default “qPCR” settings; optimal length was set to 20-22 nt, melting temperature to 62°C, and amplimer length to 100-250 bp. Primer sequences are available on request.

***Statistical analysis***

*P*-values (two-tailed) from unpaired Student’s *t*-tests and Fisher’s exact tests were calculated using GraphPad (<http://www.graphpad.com>); they were considered significant when <0.01. Pearson’s correlation coefficients were calculated using the build-in function of the Excel suite (Microsoft).

***Data availability***

All high throughput sequencing data generated in this study are available at ###.

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**Author contributions.** P.K., P.R.C., and A.P. conceived experiments; P.K., T.G., A.K., J.D.L., A.F., and A.P. performed experiments; P.K., T.G., and M.N. performed bioinformatic analysis; E.G.G. and I.G.C. analyzed NF-κB binding data; W.F.I. sequenced 3C and T2C libraries; all authors interpreted data and prepared figures; P.K., P.R.C, F.G.G., and A.P. wrote the manuscript.

**Competing interests.** The authors declare no competing interests.

**Supplementary Online Data** include Supplementary Figures 1-6 and Tables 1-#.

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**FIGURE LEGENDS**

**Figure 1. High temporal resolution analysis of gene expression at the level of intronic RNA.**

HUVECs were stimulated with TNFα for 0, 30, 60 or 90 min, and harvested to isolate total RNA for massively parallel sequencing. Changes in intronic RNA levels were assessed using the iRNA-seq pipeline. (**A**) Scatter plots illustrating log2 normalized mean counts of RefSeq introns in TNFα-stimulated vs. 0-min HUVECs. Only up- (*orange*) and down-regulated (*blue*) genes are shown per each time point. (**B**) Box plots display fold changes in intronic RNA levels for up- (log2FC ≥ 0.6) and down-regulated genes (log2FC ≤ -0.6) at each time. (**C**) Browser views of RNA-seq coverage along typical induced and repressed genes (*HIVEP2* and *ARHGAP18*, respectively) at the different times post-stimulation. (**D**) Heat map displaying *P*-values for the top five most and least enriched biological processes associated with genes up- and down-regulated by TNFα at different times. (**E**) Venn diagrams illustrating the overlap of genes up- and down-regulated by TNFα at 30, 60, and 90 min. (**F**) Box plots display log2 fold changes in intronic RNA levels after 30 min for TNFα-induced genes that lie alone (*yellow*), in pairs (*orange*), or in groups of ≥3 (*brown*) in a given topological domain (TAD). Numbers per group are shown on top of each box plot. (**G**) *Top left*: example of the “poising index” for the *NFKBIE* gene. Using iRNA-seq we calculate the log2 ratio of the signal at 5’ ends of genes (*grey*) over that along gene bodies (*orange*). *Bottom left*: pie charts show that most poised genes lie unpaired in a TAD. *Right*: box plots display log2 fold changes in intronic RNA levels after 30 min for poised (*brown*) or all up-regulated genes (*yellow*). \*: significantly different; *P*<0.01, two-tailed Student’s unpaired *t*-test.

**Figure 2. NF-κB mostly hijacks already-active enhancers in HUVECs.**

HUVECs were stimulated with TNFα for 0, 30 or 60 min, and NF-κB binding was assessed by analyzing ChIP-seq of its p65 subunit. (**A**) Browser view of data coverage along a typical TNFα-stimulated gene, *NFKBIA*, on chromosome 14. Data include topological domain breadth (*dark* and *light* *grey* boxes), genome-wide binding patterns for p65 (*orange*) and CTCF (*grey*), positioning of the H3K27ac (*blue*), H3K4me1 (*green*) and H3K4me3 marks (*black*), levels of total RNA (*magenta*) displayed using reads per million for the time points indicated. The closed and open arrows below the image denote a p65 peak overlapping a canonical and non-canonical recognition motif, respectively. (**B**) *Top*: The Venn diagram shows the overlap between 0-min H3K4me1 (*green*), H3K27ac (*blue*), and 30-min p65 (*orange*) ChIP-seq peaks; the 5366 common peaks signify sites hijacked by NF-κB on stimulation. *Bottom*: bar graphs show the fraction of hijacked peaks that are also bound by NF-κB at 60 min, carry (or not) the canonical motif, are intragenic, overlap the TSS or a CTCF 0-min peak. (**C**) Heat map showing the enrichment of *bona fide* binding motifs for transcription factors (TFs) in the DNase-hypersensitive footprints encompassing p65 binding sites “with” (*blue*) or “without” motif (*red*). TFs induced or repressed by TNFα are demarcated orange and blue, respectively. (**D**) Line plots showing occupancy (in reads per million) by the MAX, FOS, JUN, and GATA2 TFs at p65 peaks “with” (*orange*) or “without” (*black*) a canonical motif. (**E**) Line plots showing 0-min levels of divergently transcribed eRNAs at hijacked enhancers (5’ to 3’ end), ±1.5 kbp up-/downstream. (**F**) Heat map displaying *P*-values for the most enriched biological processes involving genes associated with peaks carrying the canonical NF-κB motif (“with”) or not (“without”) at 30 min; *P*-values of all up-regulated genes serve as a control. (**G**) Heat maps illustrating signal from 0- and 30-min RNAPII ChIP-seq for ±4 kbp around “with” or “without” peaks clustered hierarchically into five groups. (**H**) Heat maps illustrating the fraction of NF-κB peaks per cluster (from panel G) that are marked by H3K27ac at different times post-stimulation. (**I**) Plots showing conservation for ±2.5 kbp around p65 (“with”, *orange*; “without”, *black*) or CTCF peaks (*grey*; data from 17 ENCODE vertebrates). (**J**) Line plots showing nucleosome occupancy (in reads per million) for ±2 kbp around p65 peaks from “with” and “without” clusters 2, and 4 (from panel G) at 0 (*blue*) and 30 min (*orange*) post-stimulation.

**Figure 3. Analysis of spatial interactions along a TNFα-responsive locus.**

HUVECs were stimulated with TNFα for 0, 30 or 60 min, and 3C-seq was performed using the TSSs of the *BMP4*, *CDKN3*, and *SAMD4A* genes as viewpoints. (**A**) A browser view of data coverage along a 2.5-Mbp locus on chromosome 14 (ideogram on top). 3C-seq viewpoints are demarcated by triangles, topological domains by rectangles, and ChIP-seq data for CTCF (*light grey*), H3K27me3 (*green*), H3K27ac (*brown*), and NF-κB (p65; *black*) are also shown. Arrows denote hijacked enhancers contacted by *BMP4* (*yellow*), *CDKN3* (*light* *blue*) and/or *SAMD4A* (*purple*). (**B**) Plots showing log2 fold changes in intronic RNA levels of all genes in panel A at 30 and 60 min post-stimulation. (**C**) Line plots illustrating raw read coverage of nascent RNA-seq, and NF-κB (p65) or H3K27ac ChIP-seq along *Apo*I fragments contacted by *SAMD4A* at 0 (*grey*) and 30 min (*orange*) post-stimulation. Only contacted fragments with >100 rpm were used.

**Figure 4. High resolution spatiotemporal analysis of interactions in the *SAMD4A* locus.**

HUVECs stimulated with TNFα for 0, 30 or 60 min and analyzed. (**A**). *Top*: The cumulative separation of 30-min NF-κΒ–hijacked (*orange*) and 0-min CTCF peaks (*grey*) along a 3-Mbp region of chromosome 14 (52,902,464-55,702,766; hg18) is plotted. *Bottom*: Browser view of RNA- and ChIP-seq data (in reads per million) along *SAMD4A* at different times post-stimulation. The cluster of NF-κΒ–hijacked enhancers (*magenta*) and peaks “with” (*black arrows*) or “without” NF-κΒ motif (*white arrows*) are demarcated. (**B**) Heat maps illustrating interaction frequency of *Hind*III fragments in the SAMD4A locus on chromosome 14 (ideogram on top) as captured by T2C at 0 and 30 min post-stimulation. Between the two heat maps the location of TADs identified at sub-2 kbp resolution for each time and of RefSeq genes (up-regulated, *orange*; down-regulated, *blue*) is shown. (**C**) Heat map illustrating interactions captured by T2C between 29 CTCF-bounds sites in the locus at 0 and 30 min post-stimulation. The rectangle (*magenta*) demarcates the region of the SAMD4A intronic enhancer cluster. (**D**) As in panel C, but for all 13 NF-κΒ–hijacked enhancers in the locus. (**E**) RT-qPCR analysis of nascent (intronic) RNA generated at the TSS-proximal intron of *SAMD4A*, *EDN1*, and *TNFAIP3*. The fold change in RNA levels ±S.D. between 30 and 0 min post-stimulation is plotted for untreated cells (*grey*) or treated with C646 (*orange*), JQ1 (*black*) or DRB (*blue*). \*: significantly different; *P*<0.01, two-tailed Student’s unpaired *t*-test. (**F**) Browser view of 3C-seq data (in reads per million) using the *SAMD4A* TSS as a viewpoint (*yellow* *triangle*) at 0 and 30 min post-stimulation with or without C646 and DRB treatment.

**Figure 5. HUVECs carrying a 7SK insertion in *SAMD4A* show altered local regulatory crosstalk.**

Single-cell–derived HUVECs carrying the 7SK insertion were stimulated with TNFα for 0 or 30 min and analyzed. (**A**) Heat maps illustrating the interaction profiles captured by T2C along the 3-Mbp *SAMD4A* locus on chromosome 14 obtained at 0 min using wild-type (wt) or modified (7SKi) HUVECs. White circles denote two major interactions of the *SAMD4A* TSS that change between the two cell populations. The first (*left*) involves contacts to the intronic cluster of hijacked enhancers, the second (*right*) contact to the CTCF site immediately downstream the enhancer cluster. (**B**) Browser view showing 3C- and ChIP-seq profiles along the *SAMD4A* locus obtained at 0 and 30 min post-stimulation using wild-type (wt) or ZFN-modified (7SKi) HUVECs. The 3C-seq viewpoints (TSS, *yellow*; 7SK insertion, *orange*) and the intronic cluster of hijacked enhancers (*magenta*) are also indicated. (**C**) Browser view illustrating RNA-seq data (in reads per million) along 221-kbp *SAMD4A* obtained at 0 and 30 min post-stimulation using wild-type (wt) or ZFN-modified (7SKi) HUVECs. p65 ChIP-seq profiles obtained at 30 min are also shown. (**D**) Plot showing 30- vs. 0-min log2 fold changes in intronic RNA levels of genes in the extended *SAMD4A* locus obtained using wild-type (wt; *black*) or ZFN-modified (7SKi; *white*) HUVECs; *TNFAIP3* and *CXCL3* serve as controls. (**E**) Heat maps illustrating interactions captured by T2C between the 13 hijacked enhancers at 0 and 30 min post-stimulation in wild-type (wt) or modified (7SKi) HUVECs. The *SAMD4A* intronic cluster of hijacked enhancers (*magenta*) and 7SKi insertion (*orange arrowhead*) are indicated, and p65 ChIP-seq levels for each peak are shown (*right*). (**F**) Heat maps show interactions captured by T2C that connect the SAMD4A intronic hijacked enhancer cluster with TSS up- and downstream (within 3 Mbp) obtained at 0 and 30 min post-stimulation using wild-type (wt; *top*) or modified (7SKi; *bottom*) HUVECs; non-contacted *TNFAIP3* and *CXCL3* serve as controls. Spider plots show interactions with ≥0.13 rpm.

**SUPPLEMENTARY ONLINE DATA**

**Enhancer hijacking reveals the multimodal role of NF-κB**

**during the immediate-early inflammatory response**

Petros Kolovos1,\*, Theodore Georgomanolis2, Milos Nikolic2, Anna Koeferle3,§, Joshua D. Larkin3,¤, Alexander Feuerborn3, Wilfred F. van Ijcken4, Eduardo G. Gusmao5, Ivan G. Costa5, Peter R. Cook3, Frank G. Grosveld1, Argyris Papantonis2,\*

*1Department of Cell Biology, Erasmus Medical Centre, 3015 CN Rotterdam, The Netherlands*

*2Center for Molecular Medicine, University of Cologne, 50931 Cologne, Germany*

*3Sir William Dunn School of Pathology, University of Oxford, OX1 3RE Oxford, United Kingdom*

*4Center for Biomics, Erasmus Medical Centre, 3015GE Rotterdam, The Netherlands*

*5IZKF Computational Biology Research Group, RWTH Aachen University Medical School, 52062 Aachen, Germany.*

*Present address: §Department of Cancer Biology, University College London, WC1E 6BT London, United Kingdom; ¤Department of Electrical and Biomedical Engineering, University of Nevada, NV 89557 Reno, USA.*

*Correspondence: \*A.P.; Email:* [*argyris.papantonis@uni-koeln.de*](mailto:argyris.papantonis@uni-koeln.de) */ \*P.K.; Email:* [*p.kolovos@erasmusmc.nl*](mailto:p.kolovos@erasmusmc.nl)

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—**Supplementary Tables 1-#**

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**Supplementary Figure 1. Analysis of HUVEC gene expression on TNFα stimulation.**

HUVECs were stimulated with TNFα for 0, 30, 60 or 90 min, and harvested to isolate total RNA for massively parallel sequencing. Changes in RNA levels were assessed using DEseq or iRNA-seq. (**A**) Scatter plots illustrating log2 normalized mean counts of RefSeq exons in TNFα-stimulated vs. 0-min HUVECs. Only up- (*orange*) and down-regulated (*blue*) genes are shown per each time point. (**B**) Plots comparing changes in gene expression profiles assessed using exonic (*black*) or intronic RNA levels (*magenta*). The profiles of *SAMD4A* and *EDN1* are highlighted. (**C**) Two-dimensional Hi-C interaction maps at a 100-kbp resolution along the short arm of chromosome 6 and the long arm of chromosome 14. The zoom-in compares a 20-Mbp segment of chromosome 14 to 25-kbp Hi-C data from HUVECs published previously (Rao *et al*., 2014). (**D**) Enrichment of different marks at TAD boundaries. The per cent of TAD boundaries overlapping histone-tail marks (H3K4me3, *purple*; H3K27me3, *blue*), CTCF (*grey*) or hijacked enhancers (*orange*) is plotted for increasingly larger distances up to 10 kbp up- and down-stream the boundary. TAD mid-points are used as a control. (**E**) Box plots display log2 fold changes in intronic RNA levels after 30 min for TNFα-repressed genes that lie alone (*light blue*), in pairs (*green*), or in groups of ≥3 (*dark green*) in a given topological domain (TAD). The number of genes per group is shown on top of each box plot. \*: significantly different; *P*<0.01, two-tailed Student’s unpaired *t*-test. (**F**) Scatter plot showing changes in poising index at 30 vs. 0 min are anti-correlated (Pearson’s coefficient correlation; ρ=-0.37) to changes in gene expression (whole gene RNA levels; log2) at those times.

**Supplementary Figure 2. Analysis of NF-κB (p65) genome-wide binding features in HUVECs.**

HUVECs were stimulated with TNFα for 0, 30 or 60 min, and NF-κB binding was assessed by analyzing ChIP-seq of its p65 subunit. (**A**) *Top*: Comparison of 30- and 60-min p65 peaks with HUVEC HMM motifs; most peaks overlap enhancer regions. *Bottom*: Plot showing the distribution of *q*-values derived from the statistical assignment of p65 peaks to genes (Sikora-Wohlfeld *et al*., 2013) for 21,006 RefSeq genes. Genes with a q-value <0.2 were used to plot the Venn diagram (*inset*). (**B**) Known motif analysis at p65 peaks. Line plots show the distribution of the most enriched motifs for ±500 bp around “with” and “without” p65 peaks, at 30 and 60 min post-stimulation. Motif logos and its recovery *P*-values (in parentheses) are shown next to each plot. (**C**) *Top*: the Venn diagram shows the overlap between 0- (*white*), 30- (*yellow*), and 60-min (*orange*) p65 ChIP-seq peaks “with” motif. *Bottom*: as above, but for p65 peaks “without” motif. (**D**) *Top*: box plots illustrating the increase in distance (in Mbp) of p65 peaks from their closest TSS (within the same TAD) over time. For both “with” and “without” peaks 30-min unique peaks are shown in yellow, 60-min ones in orange, and those shared between the two time points in grey. *Bottom*: Bar plots showing the genomic location of “with” and “without” peaks over time. (**E**) Heat maps illustrating the fraction of NF-κB peaks per cluster (from panel G) that are marked by H3K4me1, -K4me3, and -K27ac at different times post-stimulation at 30 (*left*) or 60 min (*right*) post-stimulation. (**F**) Line plots showing 0-min levels of divergently-transcribed eRNAs at inter- (*top*) and intragenic (*bottom*) hijacked enhancers ±1.5 kbp up-/downstream. (**G**) DNase I hypersensitivity profiles for ±250 bp around “with” or “without” p65 peaks per cluster (as defined in **Figure 2G**; cluster 1: black; 2: *orange*; 3: *magenta*; 4: *blue*; 5: *grey*). (**H**) Heat map showing the enrichment of known TF binding motifs in the DNase-hypersensitive footprints encompassing with” (*blue*) or “without” (*red*) p65 binding sites clustered as in **Figure 2G**. TFs induced or repressed by TNFα are demarcated orange and blue, respectively. (**I**) Line plots showing occupancy (in reads per million) by the MAX, JUN, and FOS TFs at p65 “with” (*orange*) or “without” (*black*) peaks clustered as in **Figure 2G**. (**J**) Table showing statistical predictions (*P*<0.05) of TFs expected to be co-bound (TF2) with JUN or FOS (TF1) at “without” clusters 1-5 based on motif analysis. TFs induced by TNFα are demarcated orange. (**K**) Line plots showing 0- and 30-min eRNA levels (in reads per million; assessed using data from Caudron-Herger *et al*., 2015) at “with” or “without” p65 peaks from cluster 4 ±1.5 kbp up-/downstream. AS: antisense strand signal; S: sense strand signal. (**L**) Scatter plot showing the fold change in intronic RNA levels (30- or 60- vs. 0-min levels; only changes of at least 0.6-log2 fold are shown) of genes associated (within the same TAD) with p65 peaks belonging to clusters 1, 3, and 4. Genes associated with “with” and “without” p65 peaks are coloured orange and black, respectively.

**Supplementary Figure 3. Analysis of spatial interactions along a genomic locus on chromosome 6.**

HUVECs were stimulated with TNFα for 0, 30 or 60 min, and 3C-seq was performed using the TSSs of the *NEDD9* and *EDN1* genes as viewpoints. (**A**) A browser view of data coverage along a 2.8-Mbp locus on chromosome 6 (ideogram on top). 3C-seq viewpoints are demarcated by triangles, topological domains by rectangles, and ChIP-seq data for CTCF (*light grey*), H3K27me3 (*green*), H3K27ac (*brown*), and NF-κB (p65; *black*) are also shown. Arrows denote hijacked enhancers contacted by *NEDD9* (*yellow*) and *EDN1* (*light blue*). (**B**) Plots showing log2 fold changes in intronic RNA levels of the genes in panel A at 30 and 60 min post-stimulation. (**C**) Heat maps show pairwise correlations of changes in expression profiles (from panel B and **Figure 3B**) for the genes that reside in the two loci on chromosomes 14 (*left*) and 6 (*right*) investigated by 3C-seq. Correlated profiles are shown in shades of orange, anti-correlated ones in shades of blue (using Pearson’s correlation coefficient). (**D**) Line plots illustrating raw read coverage of DHS- and ChIP-seq along *Apo*I fragments (±1 kbp) contacted by *SAMD4A* (*orange*) or *EDN1* (*blue*) before stimulation. Only contacted fragments with >100 rpm were used.

**Supplementary Figure 4. Analysis of spatial interactions along the *BMP4* locus.**

HUVECs were stimulated with TNFα for 0 and 30, and 3C-seq was performed using the *BMP4* TSS as a viewpoint. (**A**) A browser view of data coverage along a 200-kbp region on chromosome 14 (ideogram on top). The 3C-seq viewpoint is demarcated by a triangle, and RNA-seq (*magenta*) and ChIP-seq data for CTCF (*light grey*), H3K4me3 (*green*) H3K4me1 (*dark green*), H3K27ac (*brown*), and NF-κB (p65; *black*) at 0 or 30 min are also shown. The hijacked enhancer that belongs to the “4/without” cluster (see **Figure 2G**) and is contacted by *BMP4* is highlighted. (**B**) Line plots illustrating raw read coverage of DHS-, ChIP-, and nascent RNA-seq along *Apo*I fragments (±1 kbp) contacted by *BMP4* at 0 (*grey*) or 30 min (*orange*) post-stimulation. Only contacted fragments with >100 rpm were used. (**C**) ##JDP2 ChIP-qPCR##.

**Supplementary Figure 5. NF-κB–hijacked super-enhancers in HUVECs.**

HUVECs were stimulated with TNFα for 0, 30 or 60 min, and NF-κB binding was assessed by analyzing ChIP-seq of its p65 subunit. (**A**) NF-κB forms large, clusters on chromosomes. The cumulative separation of p65 “with” or “without” peaks along chromosomes 1 (*purple*), 6 (*green*), 14 (*orange*), and 17 (*blue*) is plotted at 30 and 60 min post-stimulation. (**B**) Diagrams illustrating the distribution of H3K27ac ChIP-seq signal (0 min; ENCODE) for “stitched” peaks that lie <12.5 kbp apart (Hnisz *et al*., 2013). Super-enhancers are those contained within the part of the curve that has a slope of >1 (highlighted grey). SEs include those associated with *CLEC9A*, *SOX17*, *FOS*, and *SAMD4A*. *Inset*: The Venn diagram shows that 468 out of 608 SEs defined using H3K27ac are hijacked by NF-κB at 30 min. (**C**) Diagrams illustrating the distribution of 30-min p65 ChIP-seq signal for “stitched” peaks that lie <12.5 kbp apart (as in panel B). Super-enhancers are those contained within the part of the curve that is highlighted grey, and include those associated with *CXCL2,* *HIVEP2*, *IRF1, FOS*, and *SAMD4A* (but not *CLEC9A* and *SOX17*). *Inset*: The Venn diagram shows that 98 out of 212 NF-κB–SEs are also identified when using H3K27ac signal. (**D**) Browser view illustrating a typical SE associated with the *FOS* gene on chromosome 14. ChIP- and RNA-seq data are displayed in reads per million. (**E**) Line plots showing 0- (*blue*) and 30-min (*orange*) levels of divergently-transcribed eRNAs at NFκΒ- (*top*), H3K27ac- (*middle*) and hijacked SEs (*bottom*) ±3 kbp up-/downstream. (**F**) Box plots representing log2 fold change in intronic RNA levels of genes associated (within the same TAD) with NFκΒ- (*orange*), H3K27ac- (*blue*) or hijacked SEs (*grey*). \*: significantly different; *P*<0.01, two-tailed Student’s unpaired *t*-test. (**G**) Heat map displaying *P*-values for the most enriched biological processes involving genes associated with NFκΒ-, all or hijacked SEs. (**H**) Bar graphs showing the fraction of hijacked SEs that are also bound by NF-κB at 60 min, carry (or not) the canonical motif, associated (within the same TAD) with TNFα-responsive genes, are intragenic, and contained with genes of >50 (*light* *grey*), 10-50 (*grey*) or <10 kbp (*dark* *grey*) in length.

**Supplementary Figure 6. High-resolution analysis of the *SAMD4A* locus using T2C.**

HUVECs stimulated with TNFα for 0, 30 or 60 min and analyzed. (**A**) Plot showing pairwise correlations of interaction profiles obtained using the different T2C replicas, or their merge, at 0 and 30 min post-stimulation. (**B**) Violin plots showing the number and distribution of mapped reads for which 0, 1, or 2 probes were present on the array used in T2C experiments. (**C**) Heat maps illustrating interaction frequencies detected using T2C (and *Apo*I) or Hi-C (and *Mbo*I; Rao et al., 2014) along the *SAMD4A* locus on chromosome 14 (coordinated below) at 0 min. (**D**) Heat maps illustrating interactions captured by T2C between CTCF- (*left*) or NF-κB–bound sites (*right*) in the *SAMD4A* locus at 0, 30 or 60 min post-stimulation. Between each heat map pair the location and directionality of CTCF peaks (*left*) of the position RefSeq genes (*right*; up-regulated, *orange* – down-regulated, *blue*) is shown. (**E**) Circos diagram demonstrating NF-κB–driven contacts in chromosome 14 (*circular* *ideogram*) at 30 (*red*) and 60 min (*blue*) post-stimulation. NF-κB and H3K27ac ChIP-seq coverage (in rpm) is shown for two typical examples (*below*). (**F**) Browser views of the TNFα-induced *TNFAIP3* and *CXCL3* loci on chromosomes 6 and 4, respectively. ChIP-seq coverage for NF-κB (*orange*), H3K27ac (*blue*), and CTCF (*grey*) in reads per million is shown. (**G**) RT-qPCR analysis of *SAMD4A*, *EDN1*, *CXCL3*, and *TNFAIP3* mRNA. Fold changes in mRNA levels ±S.D. between 30 and 0 min post-stimulation are plotted for untreated cells (*grey*) or cells treated with C646 (*orange*), JQ1 (*black*) or DRB (*blue*). \*: significantly different; *P*<0.01, two-tailed Student’s unpaired *t*-test. (**H**) ChIP-qPCR analysis of H3K27ac enrichment at *SAMD4A*, *CXCL3*, and *TNFAIP3* NF-κB–hijacked peaks. H3K27ac levels ±S.D. obtained at 0 min using untreated cells (*grey*) or cells treated with C646 (*orange*) are shown; inactive OR1 serves as a negative control. \*: significantly different to untreated; *P*<0.01, two-tailed Student’s unpaired *t*-test. (**I**) Browser view of RNA- and ChIP-seq data (in reads per million) in the *EDN1* locus at 0 and 30 min post-stimulation. Black and white arrows demarcate “with” and “without” NF-κB peaks, respectively. (**J**) Browser view of 3C-seq (in reads per million) using the *EDN1* TSS as a viewpoint (*yellow* *triangle*) at 0 and 30 min post-stimulation with or without C646 and DRB treatment. The rectangle (*magenta*) demarcates the region of the downstream hijacked enhancer cluster.

**Supplementary Figure 7. Analysis of ZFN-modified HUVECs carrying the 7SK promoter insertion.**

ZFN-modified HUVECs were stimulated with TNFα for 0 or 30 min and analyzed. (**A**) The strategy for the targeted genome editing of HUVECs (*left*) involves co-transfection of ZFN-carrying plasmids plus a vector carrying the 7SK promoter insert between two 750-bp regions homologous to the *SAMD4A* target. After homologous recombination, a PCR screen is performed on a fraction of the whole population to assess modification efficiency. Amplimers are resolved by gel electrophoresis and results confirm that the use of ZFN plasmids works more efficiently than the transfection of ZFN mRNA for 7SK insertions. The rest of the HUVEC population was diluted to single-cells, which were grown in 96-well plates. These were also screened by PCR and resolved by electrophoresis in 96-well E-Gels (Invitrogen; *middle*); red ovals denote positive PCR controls, light purple ovals selected single-cell–clones that were trypsinized and regrown in higher titers. Clones G4 and G5 were subjected to a final selection PCR-screen; results indicated that G5 was heterozygous and G4 homozygous for the 7SK insertion. Hence, all downstream experiments were conducted using G4. (**B**) Browser view showing total RNA-seq profiles along the endogenous *7SK* gene at 0 and 30 min post-stimulation in both wild-type (wt) and modified (7SKi) HUVECs. ChIP-seq for NF-κB (p65), CTCF, H3K27ac, and H3K4me3 are also shown. (**C**) Heat maps illustrating the interaction profiles captured by T2C along the 3-Mbp *SAMD4A* locus on chromosome 14 at 0 and 30 min post-stimulation in both wild-type (wt) and modified (7SKi) HUVECs. (**D**) Heat maps illustrating the interaction profiles captured by T2C along the 3-Mbp *SAMD4A* locus obtained at 30 min post-stimulation in the presence of transcriptional inhibitor, DRB, using replicates of 7SKi HUVECs. (**E**) Scatter plots illustrating log2 mean counts of RefSeq introns from 30- vs. 0-min modified (7SKi) HUVECs. Up- (*orange*) and down-regulated (*blue*) genes are shown, and typical TNFα-responsive genes indicated. (**F**) ChIP-qPCR along 221-kbp *SAMD4A* using antibodies targeting RNA polymerase II isoforms phosphorylated at either the Ser5 (*white data points*) or Ser2 (*magenta data points*) residues of their C-terminal domain. Results are displayed in per cent enrichment over input for 0, 30, and 60 min post-stimulation, in the presence or absence of DRB. The position of the 7SKi insertion is also indicated (*dashed line*). (**G**) A browser view illustrating coverage along the SAMD4A locus of 3C-seq and p65 ChIP-seq data obtained at 0 and 30 min post-stimulation using either wild-type (wt; *grey background*) or modified (*white background*) HUVECs. The intronic cluster of hijacked enhancers in *SAMD4A* is indicated by a rectangle (*magenta*).