**ARTICLE OPEN**

**IκBα is required for full transcriptional induction of some NFκB-regulated genes in response to TNF in MCF-7 cells**

Minami Ando, Shigeyuki Magi, Masahide Seki, Yutaka Suzuki, Takeya Kasukawa, Diane Lefaudeux, Alexander Hoffmann, and Mariko Okada

Inflammatory stimuli triggers the degradation of three inhibitory κB (IκB) proteins, allowing for nuclear translocation of nuclear factor-κB (NFκB) for transcriptional induction of its target genes. Of these three, IκBα is a well-known negative feedback regulator that limits the duration of NFκB activity. We sought to determine whether IκBα’s role in enabling or limiting NFκB activation is important for tumor necrosis factor (TNF)-induced gene expression in human breast cancer cells (MCF-7). Contrary to our expectations, many more TNF-response genes showed reduced induction than enhanced induction in IκBα knockout cells. Mathematical modeling was used to investigate the underlying mechanism. We found that the reduced activation of some NFκB target genes in IκBα-deficient cells could be explained by the incoherent feedforward loop (IFFL) model. In addition, for a subset of genes, prolonged NFκB activity due to loss of negative feedback control did not prolong their transient activation; this implied a multi-state transcription cycle control of gene induction. Genes encoding key inflammation-related transcription factors, such as JUNB and KLF10, were found to be best represented by a model that contained both the IFFL and the transcription cycle motif. Our analysis sheds light on the regulatory strategies that safeguard inflammatory gene expression from overproduction and repositions the function of IκBα not only as a negative feedback regulator of NFκB but also as an enabler of NFκB-regulated stimulus-responsive inflammatory gene expression. This study indicates the complex involvement of IκBα in the inflammatory response to TNF that is induced by radiation therapy in breast cancer.

npj Systems Biology and Applications (2021) 7:42 ; https://doi.org/10.1038/s41540-021-00204-7

---

**INTRODUCTION**

The phenotype of the cell is determined by the precise expression of its genes, which are regulated by transcription factors (TFs). Nuclear factor-κB (NFκB), a TF, is essential for regulating the transcription of immune response genes and cell death pathway-related genes. The canonical NFκB signaling pathway is triggered by many inflammatory stimuli, including tumor necrosis factor (TNF), an important cytokine involved in chronic and acute inflammation. Radiation therapy induces DNA damage and the release of NFκB, which induces apoptosis in cancer cells. Binding of NFκB to its receptor activates NFκB and its target genes.

NFκB consists of heterodimeric and homodimeric complexes of p65 (RelA), p50 (NFκB1), p52 (NFκB2), RelB, and c-Rel proteins and binds to the κB site, which is a 10 base pair consensus sequence. There are three inhibitory κB (IκB) proteins (IκBα, IκBβ, and IκBε) that bind and retain NFκB in the cytoplasm. Upon TNF stimulation, they are phosphorylated and degraded, allowing NFκB to enter the nucleus, bind DNA, and activate transcription. When all three IκB proteins are removed by mRNA knockdown or gene knockout, NFκB is unresponsive to TNF stimulation. Thus, IκB proteins are responsible for NFκB activation. The three IκB proteins have distinct degradation and synthesis characteristics and therefore have distinct roles in shaping the dynamics of NFκB activity. IκBα not only responds most rapidly to stimuli but also mediates a powerful negative feedback loop that may remove NFκB from DNA and result in oscillatory NFκB activity. IκBβ and IκBε degrade more slowly upon TNF stimulation, and IκBε mediates a second negative feedback mechanism that functions in anti-phase to IκBα. Thus, the precise balance of these IκB proteins determines the dynamics of nuclear NFκB activity in response to stimulation.

Previous studies of IκBα's effect on gene expression have focused on its role as a negative feedback regulator that limits the duration of NFκB activity when stimulated transiently, or that result in oscillations when stimulated with TNF for an extended period of time. In a transient stimulation protocol IκBα-deficient cells show enhanced expression of some genes, as NFκB duration may be discriminated by a slow mRNA decay step or a slow chromatin step. In the persistent stimulation protocol IκBα-deficient cells show NFκB-mediated eviction of many more nucleosomes and establishment of de novo enhancers. However, whether the slowed and diminished NFκB activation observed in IκBα-deficient cells affects gene expression has not been examined.

Here we will address whether rapid NFκB activation by TNF, which is enabled by the degradation of IκBα within the IκBα-NFκB complex, is critical for activation of NFκB target genes in MCF7 breast cancer cells. Time-course NFκB activation and RNA-seq transcriptomic profiling data reveals a cohort of genes that are diminished in an siIκBα knockdown condition. By fitting mathematical models of alternative gene regulatory mechanisms (GRM) models to the data, we characterize the regulatory mechanism that renders some genes sensitive to the presence of IκBα while others are not. Our study repositions the function of IκBα not only...
as a negative feedback regulator that may limit gene expression in some conditions but also as an enabler of NFκB-responsive inflammatory gene expression.

RESULTS
NFκB target gene activation in the presence or absence of IκBα

To observe the difference in NFκB dynamics in the presence or absence of IκBα in MCF-7 cells, we conducted imaging experiments using fixed immunofluorescence to measure the time course of nuclear NFκB activity after stimulation with TNF (Supplementary Table 1). When IκBα was present, NFκB transiently translocated into the nucleus at earlier time points, followed by a dampened peak at later time points, whereas when IκBα was knocked down using small interfering RNA (siRNA), nuclear translocation of NFκB was prolonged (Fig. 1a). Furthermore, the basal level of nuclear NFκB abundance was 13% higher when IκBα was knocked down (Supplementary Fig. 1), and this difference in the basal level produced a significantly higher fold change in expression in the presence of IκBα (Fig. 1b).

To investigate how IκBα-regulated NFκB localization affects the expression of its target genes, we performed bulk RNA-sequencing at early time points (0–180 min) after stimulation with TNF in the presence (control) or absence of IκBα (silkBα). We measured RNA levels every 15 min after stimulation to produce detailed expression patterns and identified 371 and 922 differentially expressed genes (DEGs) in control and silkBα cells, respectively. We then compared the DEGs in each condition and identified 321 overlapping DEGs in both conditions (Fig. 1c). To observe the expression dynamics of DEGs in control cells, we performed Fuzzy c-means clustering and classified the 371 DEGs in control cells into five gene clusters: early response genes (ERGs), intermediate response genes (IRGs), delayed response genes (DRGs), downregulated, and others (Fig. 1d). To investigate genes that were differentially expressed in silkBα but not in control, 601 DEGs that were unique to silkBα were selected (Fig. 1c) and clustered into three groups: upregulated, downregulated, and others (Supplementary Fig. 2).

As the fold change in the expression level of a target gene is proportional to the fold change in the activity of the TF when they participate in an incoherent feedforward loop (IFFL)\textsuperscript{29,30} we statistically compared the fold change in expression between control and silkBα for genes in each TNF-induced cluster. For the ERGs, IRGs, and DRGs, the fold change in gene expression at some time points were significantly higher in control cells (Fig. 1d). In contrast, the mean fold change in the expression of upregulated genes that were unique to silkBα cells was consistently lower in control cells after stimulation (Supplementary Fig. 2). Furthermore, we investigated whether this gene cluster includes genes that are regulated by NFκB by performing motif analysis. This was performed at the promoter regions (±500 bps transcription start site (TSS)) of 193 protein-coding genes among 214 upregulated genes, but none of the NFκB subunit-binding motifs were significantly enriched (Supplementary Fig. 3). Results indicated that most TNF-induced DEGs that were upregulated only in silkBα were unlikely to be controlled by NFκB.

To analyze the ERGs, IRGs, and DRGs in more detail, we further classified each cluster into two subclusters (Fig. 1e). For each gene cluster, genes in subcluster 1 showed a similar fold change in expression between control and silkBα, and genes in subcluster 2 showed a statistically higher fold change in expression in the control than in silkBα. We performed motif analysis at the promoter regions of each subcluster from the ERGs, IRGs, and DRGs and found that a subcluster 2 from all three showed a significantly enriched NFκB binding motif, while only subcluster 1 from the ERGs showed a significant enrichment (Fig. 1f). These data show that NFκB is involved in the induction of many TNF-responsive genes and indicate that IκBα functions not only as a negative feedback regulator\textsuperscript{15,31} of NFκB, but also as an enabler of NFκB-regulated stimulus-responsive gene expression.

NFκB control of chromatin accessibility in the presence or absence of IκBα

To examine how NFκB-binding sites (κB sites) are affected by NFκB activation in the presence or absence of IκBα in individual cells, we performed a single-cell assay for transposable-accessible chromatin using sequencing (ATAC-seq) for early time points (0, 30, 75, and 120 min) after stimulation with TNF for control and silkBα in MCF-7 cells. These time points were selected based on those that showed the maximum and minimum early time course (0–180 min) nuclear NFκB abundance (Fig. 1a). After sample curation, the remaining 989 and 953 single-cell samples in control and silkBα, respectively, were aligned to the genome and their chromatin accessibility was quantified. Furthermore, we quantified the chromatin accessibility of the aggregated samples for each condition and identified the peak regions for each time point.

Using these peak regions in the aggregated data, we identified peak regions of the ATAC-seq signal that were significantly induced at each time point after TNF stimulation. Within these 3643 regions in total, we filtered out regions that did not show higher chromatin accessibility after TNF stimulation than before. When the TMM normalization was applied to normalize the chromatin accessibility, we found that 833, 704, and 1889 regions were induced at 30, 75, and 120 min, respectively, in control cells (Fig. 2a). We then clustered all the 3182 regions based on their time-course chromatin accessibility. These regions were clustered into 13 groups based on their time-course patterns (Fig. 2b). Next, we performed motif analysis at these TNF-induced ATAC peak regions in control cells to capture the trend of the time-course chromatin accessibility pattern of TNF-induced κB sites and to identify transcription regulators other than NFκB. There were five clusters that showed significant enrichment of κB sites (Fig. 2c), including binding sites for the interferon regulatory factor 4 (IRF4)\textsuperscript{42} and inflammatory cytokine regulating AP-1 subunit (Supplementary Fig. 4). We further extracted only the regions that were significantly enriched with κB sites from each of these clusters (Fig. 2c). The patterns of these extracted regions in each cluster reflected the nuclear NFκB abundance in control, showing a high chromatin accessibility at 30 and 120 min, which corresponds to the first and second peaks, respectively, of nuclear NFκB activity (Fig. 2d).

Similarly, we identified 3571 out of 4079 TNF-induced regions that were upregulated in silkBα. These regions were clustered into 13 groups (Supplementary Fig. 5), and motif analysis was performed to capture the trend of time-course chromatin accessibility. We obtained five clusters that showed significant enrichment of κB sites, including IRF4- and AP-1-binding sites (Supplementary Fig. 5). Then we extracted only the regions that were significantly enriched with κB sites from each of these clusters (Supplementary Fig. 6). The time-course patterns of these extracted regions in each cluster reflected the nuclear NFκB abundance in silkBα, showing high and prolonged chromatin accessibility at late time points (Supplementary Fig. 7). To confirm the statistical difference between chromatin accessibility at the TNF-induced clusters in the presence and absence of IκBα, we calculated the aggregated time-course chromatin accessibility for each cluster in control and silkBα cells and performed a one-tailed Wilcoxon rank sum test between the two conditions for each cluster at each time point (Fig. 2d, e and Supplementary Fig. 7). Furthermore, to investigate whether the individual cells in control and silkBα can be distinguished by chromatin accessibility at these TNF-induced regions between the
Fig. 1 TNF (tumor necrosis factor)-induced RelA nuclear translocation and expression of NFκB target gene clusters. Time-course nuclear NFκB abundance (a) and fold change (b) from fixed-cell immunofluorescence of MCF-7 cells treated with 1 ng mL⁻¹ TNF in Ctrl (Control) and silkBα (IκBα knockdown). Line graphs represent the means of two biological replicates. Number of cells used to calculate the mean for each time point ranged from 1336 to 2374 cells (Supplementary Table 1). Gray lines indicate the time points where RNA-seq data was measured, and arrows indicate the time points where single-cell ATAC-seq data was measured. Statistical significance was observed for interpolated (same method used in mathematical modeling) time-course nuclear NFκB abundance (p value: 1.039e⁻⁴) and fold change (p value < 2.2e⁻¹⁶) between Ctrl and silkBα from before stimulation and 75 min (the first peak time point of early response genes) after stimulation by one-tailed Wilcoxon rank-sum test.

c Venn diagram of the TNF-induced DEGs in Ctrl and silkBα.

d Mean of fold change in the expression of five TNF-induced clusters (ERG, IRG, DRG, downregulated, and others) of DEGs in Ctrl. For these 5 clusters, statistical tests were performed for fold change in expression between Ctrl and silkBα (* p value < 0.01 by one-tailed Wilcoxon rank-sum test).

e Two subclusters for each TNF-induced cluster (ERG, IRG, and DRG) in Ctrl (outlier PCSK5 excluded in line graph). For these 6 subclusters, statistical tests were performed for fold change in expression between Ctrl and silkBα (* p value < 0.01 by one-tailed Wilcoxon rank-sum test).

f κB motifs that were enriched at promoter regions (±50 bps TSS) of ERG subclusters 1 and 2, IRG subcluster 2, and DRG subcluster 2.

---

Published in partnership with the Systems Biology Institute
presence and absence of IkBα, we performed 10-fold cross-validation using Bayesian generalized linear model.

As a result, substantial statistical significance (p value < 0.0001) from aggregated cells and classification accuracy of >60% from single cells were observed at 30 and 120 min of TNF-induced regions in the presence of IkBα, which corresponded to the peaks of nuclear NFκB abundance in control (Fig. 2d, e, cluster 2 at 30 min and cluster 3 at 120 min). However, TNF-induced regions in
the absence of IκBα showed these statistical significance and classification accuracy at only late time points (Supplementary Fig. 7, cluster 1 and 5 at 75 min and cluster 4 at 120 min). These results suggest that IκBα is responsible for the rapid NFκB activation dynamics that allows NFκB to mediate early chromatin activation.

A simple mathematical model accounts for many DRGs in subcluster 2

The expression patterns of ERGs, IRGs, and DRGs in subcluster 2 in control cells followed a similar pattern with the time-course nuclear NFκB dynamics. However, whereas nuclear NFκB activity time course showed a prolonged pattern in silkBα, many ERGs showed post-induction repression. This implied that there is a post-induction repression mechanism of transcriptional control that is independent of IκBα.

To unravel this mechanism, we first applied a simple mathematical model to the data on the nuclear NFκB activity and its target genes to identify the regulatory mechanisms (detailed description in "Methods"). To recapitulate the time-course fold change in expression observed in the RNA-seq data, we calculated the fold change of input nuclear NFκB activity and scaled it to avoid assay-specific reductions in the dynamic range for two biological replicates of NFκB translocation data (Supplementary Fig. 8). We optimized the parameters of the model for each biological replicate and identified parameter sets that were the most concordant among the 10,000 pairs of two biological replicates according to the Pearson correlation coefficients (Supplementary Fig. 9) to justify each parameter value. We simulated fold change in expression using these concordant parameter sets and examined whether the model was acceptable for each gene. To examine this, we set a definition referring to the results of the RNA-seq data analysis (Fig. 1e). First, to be a good fit, the nRMSD (normalized root mean square deviation) values in replicates 1 and 2 of the control set should be <0.3, and the nRMSD values in replicates 1 and 2 of the silkBα should be <0.39. In addition, since all ERGs, IRGs, and DRGs in subcluster 2 showed a larger value in the control than in silkBα for at least either their max-fold induction (MFI) or the area under the curve (AUC), we defined that at least either the simulated MFI or AUC should also show the same relationship (control > silkBα) to be a good fit (Supplementary Fig. 10).

In subcluster 2, there were 5 out of 34 ERGs, 19 out of 48 IRGs, and 50 out of 60 DRGs that showed a good fit (Supplementary Fig. 10). Many DRGs showed a good fit with this model, and the fold change in expression was similar between control and silkBα, showing a monotonically increasing expression pattern. Representative good-fit DRGs included LTB, which encodes a membrane protein that promotes inflammation through the activation of the NFκB signaling pathway36, and RELB, which encodes a protein that is a subunit of NFκB complex that is involved in immune tolerance to inflammation35. However, this model failed to recapitulate the patterns of many other in subcluster 2, including ERGs in subcluster 2 which showed post-induction repression. These results implied additional GRMs.

The IFFL model accounts for reduced expression in silkBα

Since the simple model was insufficient to describe the post-induction repression mechanism, we turned to a previously introduced IFFL model29. The IFFL model detects the fold change in the nuclear NFκB abundance and reflects this change on gene expression. This model demonstrates a NFκB-regulated competitor TF that competitively binds to the NFκB target gene promoter region. From among the ERGs, IRGs, and DRGs, we searched for TNF-induced DEGs, which are also NFκB target genes, for possible NFκB competitors29. We identified the NFκB1 encoded protein, which is processed into p50 as the only competitor. We defined the processing time of p50 from information gathered from previous studies and fixed the time between nuclear p50 translocation and mature poly A+ mRNA production as 2 h36,37 (detailed description in "Methods"). After fixing the processing time, we applied this model to the ERGs, IRGs, and DRGs in subcluster 2 by using the same parameter optimization flow, identification process of concordant parameter sets (Supplementary Fig. 11), and the definition of the good fit as those used for the simple model analysis.

In subcluster 2, there were 5 out of 34 ERGs, 5 out of 48 IRGs, and 23 out of 60 DRGs that showed a good fit (Supplementary Fig. 12). The good fit genes in each cluster showed a reduced fold change in expression in silkBα, similar to the nuclear NFκB activity fold change in silkBα, which showed a lower fold change level than in control. The IFFL model detects the nuclear NFκB activity fold change, and thus the fold change in gene expression closely follows this reduction in silkBα. However, the transcription regulatory mechanisms for most ERGs and IRGs in subcluster 2 were not described by this model (Supplementary Table 2).

A 3-state cycle model accounts for post-induction repression

The IFFL model was able to describe the reduced fold change in expression in silkBα, but since many ERGs showed post-induction repression, we hypothesized a mechanism that physically inhibits the binding of NFκB after transcription inhibition. This encouraged us to construct a model that describes the transition of the promoter state. This model consists of nuclear NFκB, closed promoter state that is driven to an open promoter state, and the active promoter state that is driven by the open promoter state. The active promoter state is refractory and is driven to a closed promoter state (note that the rate constant of the regulation from the active state to the closed state may result from a combination of several transcriptional mechanisms, including the dissociation of NFκB). The active state also drives transcription, which includes the synthesis and degradation of mRNA. There are backward reactions between this closed chromatin state and open chromatin state, as well as between the open chromatin state and active chromatin state (detailed description in "Methods"). We hypothesized that post-induction repression occurs when the promoter state of a target gene changes from active to closed after nuclear NFκB translocation.

We applied this model to the ERGs, IRGs, and DRGs in subcluster 2 by using the same parameter optimization flow and the definition of a good fit as the simple model analysis. When fold change in expression was simulated with the concordant parameter set (Supplementary Fig. 13) for each gene in subcluster 2, there were 7 out of 34 ERGs, 24 out of 48 IRGs, and 55 out of 60 DRGs that showed a good fit (Supplementary Fig. 14). A representative IRG, BCL2L11, which encodes a protein that functions as a tumor suppressor by inducing apoptosis38, showed a good fit with this model. However, despite the presence of many ERGs in subcluster 2 that showed post-induction repression similar to the good fit genes in other clusters, there were only a few ERGs that showed a good fit with this model (Supplementary Fig. 14). This was because many of the ERGs in subcluster 2 that showed post-induction repression also showed reduced expression in silkBα.

A combined model v4 accounts for both post-induction repression and reduced expression

Finally, since many ERGs showed both post-induction repression and reduced expression in silkBα, we constructed a model that combines the 3-state cycle model and the IFFL model by applying the previous three models. In this model, the NFκB competitor suppresses the NFκB target gene promoter in the active state, which suppresses transcription of the target gene (detailed description in "Methods"). We hypothesized that by adding the IFFL model to the 3-state cycle model, both post-induction
We applied four mathematical models to each ERG, IRG, and DRG in subcluster 2 to identify the transcription mechanism of TNF-induced gene expression. We first applied a simple model that considers only the relationship between the fold change in nuclear NFκB activity and the transcription of its target gene. This model recapitulated many DRGs in subcluster 2, which showed a similar level of fold change in expression between control and silkBa, and a monotonically increasing expression pattern. However, most of the ERGs in subcluster 2 did not fit well with this model. Next, we applied the IFFL model, which is a detectable fold-change model. Since the fold change in nuclear NFκB is higher than that in silkBa, reduced expression in silkBa was recapitulated by this model. A few ERGs in subcluster 2 showed a good fit with this model, but many genes did not. To recapitulate post-induction repression, we constructed a 3-state cycle model and identified good fit genes. This model was able to recapitulate post-induction repression but not reduced expression in silkBa. Therefore, we constructed model v4, which combines the 3-state cycle and IFFL model. This model was able to recapitulate both post-induction repression and reduced expression in silkBa for many DRGs in subcluster 2.

Since there were genes that showed a good fit with multiple mathematical models, we identified the best-fit model for each gene (Fig. 3A). For each, we calculated the total nRMSD in replicate 1, nRMSD of silkBa in replicate 1, nRMSD in replicate 2, and nRMSD of silkBa in replicate 2 for each model, which showed a good fit. The total nRMSDs were then compared between the good-fit models, and the model with the smallest total nRMSD was identified. This model was defined as the “best-fit” model for each good-fit gene. We found that the best-fit model of many ERGs was model v4 (Fig. 3B, C), showing a transient expression pattern (Supplementary Table 3). Interestingly, among the ERGs recapitulated with model v4, there were well-known NFκB pathway regulators (e.g., A20) and TFs (e.g., JUNB and KLF10), which are key inflammation and breast cancer regulators. For the IRGs, BCL2L11 encoded protein, known as a tumor suppressor, was recapitulated by the 3-state cycle model, showing a monotonically increasing expression pattern (Supplementary Table 4). Among the DRGs, the LTB encoded membrane protein, known as TNF C, which promotes inflammation through the activation of the NFκB signaling pathway, and the RELB, known to be involved in immune tolerance to inflammation and to repress proinflammatory genes were recapitulated by the simple model (Supplementary Table 5), showing a monotonically increasing expression pattern.

Identification of the best-fit model for each ERG, IRG, and DRG in subcluster 2

Chromatin remodeling at kB sites in promoter regions of post-induction repressed genes

DISCUSSION

Previous studies have focused on IkBa’s role as a negative feedback regulator of NFκB activity, which limits the duration when stimulated transiently. Thus, in IkBa-deficient cells, transient stimulation results in prolonged NFκB activation which in turn induces enhanced expression of some genes. It was shown that prolonged NFκB activity allows genes to be
Model v4 recapitulated fold change in expression of many early response genes in subcluster 2. a Schematic of the four mathematical models. The color code corresponds with the background color of each model. b Fraction of the good-fit genes from their best-fit mathematical models. c Heatmaps of the time-course gene expression from experimental results and data fit from the best-fit model. Each color bar indicates the best-fit model (blue: simple model, magenta: IFFL model, green: 3-state cycle model, and purple: model v4), which shows the smallest total nRMSD (nRMSD in Ctrl from rep1 + nRMSD in siIκBα from rep1 + nRMSD in Ctrl from rep2 + nRMSD in siIκBα from rep2). Gray colored bars indicate genes that did not show a good fit with any of the four mathematical models, and the results from the simple model are shown.
Fig. 4  Chromatin remodeling observed for many post-induction repressed ERGs in subcluster 2. a Heatmaps of time-course fold change in expression from data of all post-induction repressed genes that were demonstrated by the 3-state cycle model or model v4 (highlighted in orange). Genes shown in the heatmaps all belong to the ERGs in subcluster 2. b Heatmaps of time-course chromatin accessibility at κB sites in promoter regions of ERGs, IRGs, and DRGs in subcluster 2, which showed the start of decrease in chromatin accessibility at least at 30 (light brown box) or 75 (brown box) min in both Ctrl and silκBα. Genes highlighted with orange are post-induction repressed ERGs in subcluster 2 that were best demonstrated by 3-state cycle model or model v4. Genes highlighted with purple are genes that were demonstrated by any of the 4 models but did not show post-induction repression in data.
regulated by a slow chromatin step or by slow mRNA decay to be activated more fully. Persistent stimulation may result in NFκB oscillations mediated by the IkBa feedback loop; in their absence, NFκB was shown in macrophages to generate hundreds of de novo enhancers by triggering nucleosome eviction, suggesting that IkBα’s role is to preserve the enhancer landscape. However, the absence of IkBα also results in slowed and diminished activation of NFκB, as other IkB family members have slower IKK-responsive degradation kinetics. Whether IkBα’s role to provide rapid NFκB activation is important for gene activation has not been examined.

Physiologically, radiation is known to induce apoptosis through the expression of TNF, which is also known to inhibit cell proliferation. TNF activates the canonical NFκB signaling pathway, which induces IkB degradation and releases NFκB to the nucleus for transcriptional regulation. We confirmed the expression of a gene that is involved in immune tolerance to inflammation (e.g., RELB) and apoptosis-inducing tumor-suppressor genes (e.g., BCL2L11 and KLF10). However, the expression of inflammation and cancer-progressing genes (e.g., A20, JUNB, and LTB) were also observed when IkBα was present, indicating that IkBα enables the full induction of not only NFκB-regulated genes that promote apoptosis, but also NFκB-regulated genes that promote inflammation and cancer progression. Here, our findings provided by studying gene expression in cells that contain IkBα and those that do not suggest that IkBα not only functions as a negative feedback regulator, but also as an enabler of some NFκB-regulated stimulus-responsive inflammatory gene expression and NFκB-regulated early chromatin activation. This indicates the complex involvement of IkBα in NFκB transcription regulation, activated by TNF.

Secondly, we explored how the altered dynamics in IkBα-deficient cells are interpreted by transcription regulatory mechanisms of TNF-induced NFκB target genes. In particular, expression of some ERGs was repression in the absence of IkBα after induction. Given that the time course of nuclear NFκB activity in the absence of IkBα was prolonged, these results indicated the existence of GRMs. We investigated these mechanisms by fitting GRM models to the RNA-seq transcriptomic profiling data.

While the NFκB activation mechanism is common between cells, basal nuclear NFκB activity varies from cell to cell because of heterogeneity in protein expression and kinase activity. A previous study revealed that fold change of NFκB activity rather than absolute NFκB abundance in HeLa cells provided a more statistically robust explanation for the observed variability in expression between cells. The fold-change detection mechanism provides an analog of Weber’s law, which discriminates the signal relative to the background signal. In signaling systems, it may be mediated by an IFFL, in which a TF regulates its target gene and a repressor of the target gene. Since the fold change detection mechanism in the transcription of target genes is introduced by an IFFL of human cells and is also found in the NFκB signaling pathway, we applied the IFFL model. Consequently, we found that a combined model that inserts the IFFL model into the 3-state cycle model recapitulated many ERGs, indicating that transcription is regulated by a post-induction repression mechanism that drives the promoter state from active to closed and a detectable fold change mechanism that renders these genes sensitive to the presence of IkBα. As a whole, the complexity of the transcription regulatory mechanism increased as the response time to TNF stimulation decreased, indicating that inflammation and cancer-related ERGs require a strict and precise transcriptional regulation to avoid overproduction (Fig. 5).

The post-induction repression can be explained by molecular mechanisms that involve chromatin remodeling complexes that have been revealed in previous studies. For example, the nucleosome remodeling and deacetylation (NuRD) complex is recruited to its target sites by transcriptional repressors and/or methylated DNA, and nucleosome remodeling facilitates histone deacetylation by HDAC1/2 subunits of the NuRD complex. The enzymatic reaction promotes the folding of chromatin into a repressed, higher-order structure, which in turn leads to the loss of RNA polymerase II and repression of transcription of some genes. At the same time, resetting the local nucleosome landscape and initializing the TSS of RNA polymerase II establishes a new transcriptional state, where some genes show an overall increase in expression levels. In addition, NuRD complex is known to promote the activity of another chromatin remodeling complex called the polycomb repressive complex 2 (PRC2), which initially targets the genomic region for chromatin remodeling by methylating H3K27. H3K27me3 produced by PRC2 acts as a docking site for the PRC1 to induce chromatin compaction, which reduces not only the accessibility of TFs but also the ATP-dependent chromatin-remodeling machineries, such as the SWI/SNF complex. The SWI/SNF complex also catalyzes ATP-dependent chromatin remodeling by coupling ATP hydrolysis with directional movement over DNA, which represses transcription of some genes. Chromatin remodeling mechanisms including these mechanisms may cooperatively or independently induce the post-induction repression observed for many ERGs found in this study.

This study provides insights that may be used to reveal the transcription regulatory mechanisms in many biological systems, such as systems that require both post-induction repression and fold change detection. TNF-induced NFκB target genes that were recapitulated by either of the four models are likely robust to noise, because the models applied here were all deterministic. In addition, genes that did not show a good fit when either of the four models were applied may be regulated by an additional combination of a transcription regulatory module, such as the existence of multiple repressors and activators. Other factors may also affect the transcription of target genes. For example, the dose of the TNF stimulus changes the NFκB dynamics, which in turn alters the transcription pattern of the target gene, or the abundance of the different NFκB dimers affects the transcription state of the target gene. Transcription regulation is affected by various factors and is controlled by a sensitive balance in each cell. In summary, we found that rapid IkBα-mediated NFκB activation is required for full induction of some NFκB-regulated target genes and for NFκB-regulated early chromatin activation. Among the TNF-induced target genes that showed reduced fold change in expression in the absence of IkBα, we characterized GRMs that render some genes sensitive to the presence of IkBα by fitting GRM models to data. Our study repositions the function of IkBα not only as a negative feedback regulator but also as an enabler of NFκB-regulated stimulus-responsive inflammatory gene expression in the signaling pathway and proposes GRMs that safeguard inflammatory gene expression from overproduction.

METHODS
MCF-7 cell culture and TNF treatment
The human breast adenocarcinoma MCF-7 cell line was purchased (American Type Culture Collection, Manassas, VA, USA) and propagated in Dulbecco’s modified Eagle’s medium (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units mL⁻¹ penicillin and 100 μg mL⁻¹ streptomycin, Nacalai Tesque, Kyoto, Japan). TNFa (Thermo Fisher Scientific) was dissolved in a 0.1% bovine serum albumin/phosphate-buffered saline (PBS) solution at a concentration of 100 ng mL⁻¹ and added to the cells at a final concentration of 1 ng mL⁻¹.

siRNA transfection
Reverse transfection was performed using Hiperfect reagent (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Trypsinized MCF-7 cells were resuspended in an antibiotic-free medium and then
mixed with a suspension of Opti-MEM (Thermo Fisher Scientific) containing 50 nM siRNA and Hiperfect reagent in 60-mm dishes (for ATAC-seq), 6-well plates (for RNA-seq), or 96-well plates (for immunostaining). SMARTpool ON-TARGETplus siRNA targeting I\(\kappa\)B\(\alpha\) (L-004765-00, a mixture of four sequences: AGUCAGAGUUCACGGAGUU, GCUGAUGUCAACAGAGUUA, AGGACGAGCUGCCCUAU, AGUGCUGAUGUCAAUGCUCA) and ON-TARGETplus Non-targeting siRNA (D-001810-02, UGGUUUACUGUUGUGA) were purchased from Dharmacon (GE Healthcare, now Horizon Life Sciences).

**Fig. 5** Distinct regulatory mechanisms of NF\(\kappa\)B target genes depending on their response time. Diagram of the distinct transcription regulatory mechanisms that characterizes each TNF-induced NF\(\kappa\)B target gene cluster. Mean fold change in nuclear NF\(\kappa\)B abundance of two biological replicates in Ctrl and silk\(\kappa\)B\(\alpha\) were interpolated to every second (same method used in mathematical modeling) and were max-normalized from 0 to 1 together, shown as line graphs in the top panel. The dots indicate the data from fixed-cell immunofluorescence experiments. The bottom panel shows the schematic curves of the expression patterns of ERGs, IRGs, and DRGs in subcluster 2. Despite the persistent pattern of nuclear NF\(\kappa\)B abundance in silk\(\kappa\)B\(\alpha\), post-induction repression was observed for the ERGs, and model v4 was the most effective model to recapitulate post-induction repression and reduced expression. For the IRGs, the 3-state cycle model was the most effective model, and for the DRGs, the simple model was the most effective model. The complexity of the transcription regulatory mechanisms increases as the response time of transcription decreases. Inflammation and cancer-related JUNB and KLF10, respectively, showed a transient fold change in expression, indicating that post-induction repression mechanism safeguards gene expression from overproduction, protecting from inflammatory diseases and tumor progression.
Discovery, UK). siRNA transfections were carried out 3 days before TNFα stimulation.

**Immunostaining**

The method of immunostaining and quantification of signal intensity at every single cell was slightly modified from the previously reported method. The control and IkBa-knockdown MCF-7 cells were seeded at a density of $1 \times 10^4$ cells well$^{-1}$ in 96-well plates. Cells were exposed to 1 ng mL$^{-1}$ of TNFα for 0–3 h at 15 min intervals, fixed with 4% paraformaldehyde (Electron Microscopy Science, Hatfield, PA, USA) in PBS for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 5 min, and washed with PBS. After incubating with blocking solution (10% FBS in Blocking One, Nacalai Tesque) for 1 h at room temperature, the cells were exposed to anti-RelA antibody (#8242, Cell Signaling Technology, Danvers, MA, USA) diluted 1:200 with blocking solution at 4 °C. The next day, the cells were stained with Dylight550 anti-rabbit- IgG antibody (#84541, Thermo Fisher Scientific) diluted 1:500 with blocking solution for 1 h at room temperature, and thereafter stained with 0.2 mg mL$^{-1}$ 4,6-diamidino-2-phenylindole (DAPI) in PBS for nuclei detection. Fluorescence images were obtained by using InCell Analyzer 2000 (GE Healthcare, now Cytiva, Marlborough, MA, USA). The Developer Toolbox software (Cytiva) was used to segment area with no salt). Then polymerase chain reaction (PCR) was performed with Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA) using the 2 (Illumina), 1× C1 Loading Reagent with low salt, and 1.1× NEBnext High-Fidelity PCR Master Mix (New England Biolabs) with the following PCR conditions: 72 °C for 5 min; 98 °C for 30 s; and thermocycling at 98 °C for 10 s, 72 °C for 30 s, and 72 °C for 1 min. The PCR products from 96 single cells were collected in a single tube and purified using a single MinElute PCR Purification Kit column (Qiagen). The purified library was then eluted with 20 μL of pure H₂O. To remove primer dimers, pooled libraries were purified twice or thrice using the same volume of AMPure XP beads (Beckman Coulter, Brea, CA, USA). After quantifying the library using the Bioanalyzer 2100, multiplex sequencing (36 bp single-read) was performed using HiSeq2500 System (Illumina).

**RNA extraction and mRNA sequencing**

The control and IkBa-knockdown MCF-7 cells were exposed to 1 ng mL$^{-1}$ of TNFα for 0–3 h at 15 min intervals. Duplicate samples were used for total RNA extraction with NucleoSpin RNA Plus (Takara Bio, Shiga, Japan) according to the manufacturer’s instructions. The concentration and integrity of RNA were evaluated using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Thereafter, library preparation was performed using 50 ng of mRNA with the Sureselect Strand RNA Kit (Agilent Technologies) according to the manufacturer’s protocol. A 36 bp single-read was performed using the HiSeq2500 System (Illumina, San Diego, CA, USA) with distinct samples from the immunostaining experiments.

**Single-cell ATAC-seq**

To investigate whether the expression of NF-κB target genes was affected by chromatin accessibility, single-cell ATAC-seq was performed using the control and IkBa-knockdown MCF-7 cells for three biological replicates (distinct samples from immunostaining experiments and bulk RNA-sequencing experiments were used). The cells were stimulated with 1 ng mL$^{-1}$ TNFα for 0, 30, 75, and 120 min and then collected using TrypLE™ Select (Thermo Fisher Scientific). The cells were washed twice with ice-cold PBS. The cell aggregates were removed by the plurinStrainer 20 μm (plurSelect Life Science, Leipzig, Germany) and resuspended in PBS (250 cells μL$^{-1}$). The preparation of single-cell ATAC-seq libraries using the C1 system (Fluidigm, South San Francisco, CA, USA) and Nextera DNA Library Preparation Kit (Illumina) was performed with reference to the previously reported method in the paper and the deposited protocol in the manufacturer’s platform (Fluidigm script Hub https://www.fluidigm.com/c1openapp/scriptpub/script/2015-06/single-cell-chromatin-accessibility-1433443631246-1, Revision C), with some modifications: The cell suspension and the enzyme mixture were mixed in a 7:3 ratio and loaded into a C1 Single-Cell Open App IFC 17–25 μm (Fluidigm). Thereafter, a phase-contrast microscope was used to check whether any single cell without cell debris was captured. The cells were lysed and exposed to ATAC reaction by Tn5 transposition mix (1.5x TD buffer, 1.5x Tn5 transposase (Nextera DNA Sample Prep Kit, Illumina), 1.5x C1 Loading Regent with no salt (Fluidigm), 0.15x NP-40) at 37 °C for 30 min. Tn5 DNA complexes were dissociated from chromatin via the addition of the EDTA buffer (50 mM EDTA, 8.5 mM Tris-HCl pH 8, 1x C1 Loading Regent with no salt) for 30 min at 50 °C, and thereafter free EDTA was quenched by the MgCl₂ buffer (45 mM MgCl₂, 0.5 mM Tris-HCl pH 8, 1x C1 Loading Regent with no salt). Then polymerase chain reaction (PCR) was performed with ATAC Seq PCR Mix (14 μM non-indexed custom Nextera PCR primers 1 and 2 (Illumina) and 1x C1 Loading Regent with low salt, and 1.1x NEBnext High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA)) using the following conditions: 72°C for 5 min; 98°C for 30 s; and thermocycling at 98°C for 10 s, 72°C for 30 s, and 72°C for 1 min. The amplified transposed DNA in every single cell was collected in approximately 3.5 μL each of C1 Harvest Regent each (Fluidigm). The single-cell DNA library was collected from C1 Single-Cell Open App IFC (Fluidigm) and mixed with 10 μL of C1 DNA Dilution Regent (Fluidigm). To dual-index the harvested libraries, 10 μL of harvested libraries were amplified for an additional 14 cycles in 50 μL of PCR reagent (1.25 μM custom Nextera dual-index PCR primers (Illumina) and 1× NEBnext High-Fidelity PCR Master Mix (New England Biolabs)) with the following PCR conditions: 72°C for 5 min; 98°C for 30 s; and thermocycling at 98°C for 10 s, 72°C for 30 s, and 72°C for 1 min. The PCR products from 96 single cells were collected in a single tube and purified using a single MinElute PCR Purification Kit column (Qiagen). The purified library was then eluted with 20 μL of pure H₂O. To remove primer dimers, pooled libraries were purified twice or thrice using the same volume of AMPure XP beads (Beckman Coulter, Brea, CA, USA). After quantifying the library using the Bioanalyzer 2100, multiplex sequencing (36 bp single-read) was performed using HiSeq2500 System (Illumina).

**Quantification of the expression level**

The size factor and library size were calculated using the “calcNormFactors” function in the R package “edgeR” from the read counts that were normalized to the read counts of each transcript to be at the length of 1000, where $X_i$ is the read counts per 1000 bps, $R_i$ is the read counts that were mapped, $L_i$ is the length of the transcript length, $S_i$ is the library size, $N_i$ is the normalization factor of RLE normalization, and $TPM_i$ is the transcripts per million (TPM).

$$X_i = R_i \frac{10^3}{L_i}$$

(1)

$$TPM_i = X_i \frac{1}{S_i N_i}$$

(2)

The expression level for each protein-coding gene was normalized using RLE normalization, where the raw read counts were normalized by the number of the total read counts to be 1,000,000 after the read counts were divided by the product of the library size and size factor. The mean value of the two replicates was used for further analysis.

**Identification of DEGs**

DEGs were identified using the “DESeq” function in the R package “DESeq2” by performing a Wald significance test between the gene expression levels before and after TNF stimulation for each time point to calculate the adjusted $p$ values and fold change in expression for each gene in control and silkBa. The expression levels of the genes were normalized using the RLE normalization method. Genes with adjusted $p$ values $<0.05$ and log2 fold change $>0$ were classified as upregulated DEGs, and adjusted $p$ values $<0.05$, and log2 fold change $<0$ as downregulated DEGs.

**Clustering and subclustering of DEGs**

The DEGs in control and silkBa were clustered into groups by their z-score normalized expression level using Fuzzy $c$-means clustering from the “mcearns” function in the R package “e1071.” Used parameters are: center = 5 for control and 3 for silkBa (number of clusters), iter.max = 2 (maximum number of iterations), method = “mcearns” (clustering method), $m = 1.3$ (fuzzy partition matrix), and dist = “Euclidean” (similarity measure). Furthermore, for each TNF-induced cluster (ERGs, IRGs, and DRGs), we calculated the z-score normalized fold change for both conditions and identified the mean time point of the maximum expression level. The z-score normalized fold change was extracted at the corresponding time.
points until it reached the time point in data just before the calculated mean time point of the maximum expression level of all genes in each cluster. The extracted z-score normalized fold change in the control and silkw8a were combined for each gene. The genes were subclustered into two groups in each cluster based on these values using Fuzzy c-means clustering. Used parameters are: centers = 2 (number of clusters), itermax = 2 (maximum number of iterations), method = cmeans (clustering method), m = 1.3 (fuzzy partition matrix), and dist = Euclidean (similarity measurement). Motif analysis was performed using "findMotifsGenome" command in HOMER77 version 4.10.4 for each cluster in control and silkw8a.

**Sequence mapping of single-cell ATAC-seq data**

Single-cell ATAC-seq datasets containing two or more cells and samples that were contaminated were filtered out. A total of 989 cells remained in the control and 953 cells in the silkw8a, which were used for further analysis. In addition, a dataset was prepared in which all cells of the three replicates were aggregated into one dataset for each time point in each condition. The single-cell and aggregated datasets were aligned using BOWTIE78 version 2.3.4.1, to build version UCSC26/hg38 of the human genome. The signal of peak regions was calculated using peakRegionIdentifier function in deepTools80, using the option "–binSize 1 -e 200 -ignoreForNormalization chrX -p max" indicates the format of the input BAM file. BAM indicates the format of the input.

**Peak region identification**

Peak calling was performed with MACS2 (https://pypi.org/project/MACS2/) version 2.1.2.1 using the option "-f BAM -q h -s 0.05 -mfold 6 50" where -f BAM indicates the format of the input file; -g h indicates the mappable human genome size; -q 0.05 indicates the cutoff to call significant regions is 0.05; and --mfold 6 50 indicates the lower and upper threshold of the height of the ATAC-seq peaks.

**Quantification of chromatin accessibility**

The signal of peak regions was calculated using "multiBlightwigSummary" function in deepTools80, using the option "–BED" where a BED file with promoter regions defined as ±500 bps TSS of protein-coding genes from version GRCh38/hg38 of the human genome was provided. To normalize each signal at each time point, reads per million (RPM) were calculated, where the raw signal was normalized by the number of the total signal to be 1,000,000 after the read counts were divided by the product of the library size and size factor. The size factor and library size to normalize the remaining regions were clustered based on their time-course chromatin accessibility was more than 0 (|z-score| ≥ 0.05). Then the remaining regions were clustered based on their time-course chromatin accessibility. The chromatin accessibility at each time point was classified into a group in which its z-score normalized chromatin accessibility was smaller than 0 (z-score < 0) and a group whose z-score normalized chromatin accessibility was more than 0 (z-score ≥ 0). For each cluster, motif analysis was performed using "findMotifsGenome" command in HOMER77 and identified clusters which showed enrichment of x8 sites. From this result, we extracted only the regions that included x8 sites in each cluster. The aggregated single-cell chromatin accessibility at those regions in each condition was calculated and a paired t-test from "ttest" function in the R package "stats" was performed to determine whether they show statistical significance. In addition, we calculated the single-cell chromatin accessibility at those regions in each condition and performed a 10-fold cross validation using a Bayesian generalized linear model from the "train" function in the R package "caret" to calculate the accuracy of the classification between the single cells of control and silkw8a in those regions.

**Mathematical modeling**

Basal nuclear NFkB activity from the two independent immunostaining results was subtracted from all time points in control and silkw8a. For replicate 1, the fold change of nuclear NFkB activity in control and silkw8a were calculated. Then, the fold change in control and silkw8a were converted together using the "rescale" function in the R package "scaler" to span a range of 2--100 to avoid assay-specific reductions of the dynamic range. The fold change in nuclear NFkB activity of replicate 2 from the immunostaining result in control was converted to span a range of 2--100 to avoid assay-specific reductions in the dynamic range. Then we converted the immunostaining result in the silkw8a of replicate 2 using the same scale used for the control. Scaling methods were different between the two replicates because the time-course nuclear NFkB activity of replicate 2 in both conditions was consistently lower than the activity of replicate 1 in both conditions. While the maximum activity in control which appeared at the first peak was also the highest among both conditions in replicate 1, the maximum activity among both conditions in replicate 2 appeared at late time points in silkw8a. Thus, to standardize the maximum activity of replicates 1 and 2, activity of replicate 2 in control was scaled individually to avoid the scaled maximum activity (which is 100 in replicate 1) to appear at late time points in silkw8a but to appear at the first peak in control, similar to the scaled maximum activity of replicate 1. Each of the scaled nuclear NFkB activities was interpolated to every second using the "pchipfun" function in the R package "pracma." These interpolated data for each replicate were used as the input of all ODE models, which were numerically solved using the "ode" function in the R package "deSolve."

**The simple model**

A simple model was used to reproduce the transcription regulating mechanism that considers only the nuclear NFkB activity and its target gene. Where, \( k_{syn} \) is the synthesis rate constant for the target gene, \( k_{deg} \) is the mRNA degradation rate constant for the target gene, \( k_t \) is the NFkB-regulation strength constant for the target gene, \( h \) is the Hill function exponent for the target gene, and \( t \) is the time between nuclear NFkB and mature poly A+ mRNA production.

\[
\text{d}[\text{mRNA}(t)]/\text{dt} = k_{syn} \left( \frac{NFKB(t-t_\tau)}{[NFKB(t-t_\tau)]_{\tau}^h + k_{deg}\text{[mRNA}(t)]} \right)
\]

The free parameters \( k_{deg}, k_{syn}, h \), and \( \tau \) were optimized with bound constraints (2 \( e - 5 \leq k_{deg} \leq 2e - 3, 0.001 < k_{syn} < 1000, \) and \( 0 < \tau < 7200). \) For simplicity, \( h \) and \( k_{syn} \) were fixed at 1.

**The IFFL model**

The IFFL model79 was used to enable a transcription-regulating mechanism that is fold change detectable. This model considered the competitor that binds to the target gene promoter to interfere with the transcriptional regulation by NFkB. Where, \( k_{deg,\text{IF}} \) is the mRNA degradation rate constant for the competitor TF gene, \( k_t \) is the NFkB-regulation strength constant for the competitor TF gene, \( k_{syn} \) is the NFkB-regulation strength constant for the target gene, \( k_{deg} \) is the competitor TF-regulation strength constant for the target gene, \( h_{TF} \) is the Hill function exponent for the competitor TF gene, and \( t_{TF} \) is the time between nuclear NFkB and mature poly A+ mRNA production.

\[
\text{d}[\text{mRNA}(t)]/\text{dt} = k_{syn} \left( \frac{[NFKB(t-t_\tau)]_{\tau}^h}{[NFKB(t-t_\tau)]_{\tau}^h + k_{deg}\text{[mRNA}(t)]} \right) - \left( k_{deg,\text{IF}} + k_{deg} \right) \text{[mRNA}(t)]
\]

The free parameters \( k_{deg,\text{IF}}, k_{syn}, h \), and \( \tau \) were optimized with bound constraints (2 \( e - 5 \leq k_{deg,\text{IF}} \leq 2e - 3, 0.001 < k_{syn} < 1000, \) and \( 0 < \tau < 7200). \) For simplicity, \( h \) was fixed at 1, \( h_{TF} \) was fixed at 2, \( k_{deg,\text{IF}} \) was 0.001, and \( k_{syn} \) was fixed at 1.
was fixed at 8.022537e-6, K_{DFF} was fixed at 100, τ_{TF} was fixed at 7200^{36,44}, and K_{syn} was fixed at 1.

The 3-state cycle model

The 3-state cycle model was constructed to recapitulate the promoter state transition from the active state (state A) to the closed state (state C), which induces transcriptional repression of the target gene. Where K_{DD} is the cooperativity of the active chromatin state at the promoter region of the target gene, and k_f, k_r, k_2, k_3, and k_4 are the reaction rate constants.

\[
\frac{dC}{dt} = -k_f \left( \frac{K_{DD}[\text{NFkB}(t)]}{K_{DD}[\text{NFkB}(t)] + 1} \right) \cdot C + k_r \cdot O + k_3 \cdot A
\]

\[
\frac{dO}{dt} = k_f \left( \frac{K_{DD}[\text{NFkB}(t)]}{K_{DD}[\text{NFkB}(t)] + 1} \right) \cdot C - k_r \cdot O + k_2 \cdot A
\]

\[
\frac{dA}{dt} = k_f \left( \frac{K_{DD}[\text{NFkB}(t)]}{K_{DD}[\text{NFkB}(t)] + 1} \right) \cdot C - k_r \cdot O + k_3 \cdot A
\]

The parameters for each model were optimized using the subplex algorithm to identify concordant parameter sets for IRGs and DRGs from 3-state cycle model and model v4 are the same as simple model and IFFL model. The model v4 is a combination of the 3-state cycle and the IFFL models, where the model v4 was constructed to recapitulate the promoter state transition from the active state (state A) to the closed state (state C), which induces transcriptional repression of the target gene. Where K_{DD} is the cooperativity of the active chromatin state at the promoter region of the target gene, and k_f, k_r, k_2, k_3, and k_4 are the reaction rate constants.

\[
\frac{dC}{dt} = -k_f \left( \frac{K_{DD}[\text{NFkB}(t)]}{K_{DD}[\text{NFkB}(t)] + 1} \right) \cdot C + k_r \cdot O + k_3 \cdot A
\]

Parameter optimization process and identification of concordant parameter sets

To identify the best-fit parameter, a two-step parameter optimization process was applied. First, using the scaled input nuclear NFkB activity, we simulated the expression and calculated the time-course fold change in the expression in control and silkB. The time-course fold change in expression from the entire dataset was also calculated, and these were max-normalized to span a range of 0 to 1 for both simulation and data. Next, we minimized the nRMSD between the max-normalized fold change between the simulation and data in the control for 100 initial parameter sets. Within these optimized parameter sets, we selected parameter sets with nRMSD < 0.5, some of which were set to 0.6 or 0.7, and identified the minimum and maximum values for each parameter. Using these two values as the lower and upper bound constraints, we further produced 100 initial parameter sets and optimized them by minimizing the nRMSD of the max-normalized fold change between the simulation and data in silkB.

Chromatin remodeling at promoter regions of post-induction repressed ERGs

There were 11 post-induction repressed genes that were best demonstrated by the 3-state cycle model or model v4. These genes all belonged to the ERGs in subcluster 2, and all the other genes that were best demonstrated by any of the four models were 89 in total. The peak regions at each time point in control and silkB were that were included in the promoter regions (±500 bps TSS) of these 100 genes were identified. For these peak regions, motif analysis was performed using a "findMotifsGenome" command in HOMER to identify peak regions which showed enrichment of kB sites. From this result, only the peak regions that included kB sites were further extracted. When kB site enriched regions were identified for multiple time points, regions were merged using the "mergePeaks" command in HOMER using the default setting.

The signal of these peak regions was calculated using "multBigwigSummary" function in deepTools, using the option "-BED" where a BED file with promoter regions defined as ±500 bps TSS of protein-coding genes from version GRCh38/hg38 of the human genome was provided. To normalize each signal at each time point, RPM were calculated, where the raw signal was normalized by the number of the total signal to be 1,000,000 after the read counts were divided by the product of the library size and size factor. The size factor and library size to normalize the ATAC-seq signal for each protein-coding gene were calculated using the TMM normalization method from the calcNormFactors function in the R package "edgeR". The kB site-enriched regions that showed a start of decrease in chromatin accessibility at least at 30 or 75 min in both Ctrl and silkB were selected and z-score normalized for each condition for heatmaps. The heatmaps of the time-course fold change in expression of
数据分析
全部数据集报告在本论文中已存放在DNA数据银行，使用accession numbers DRA011742和DRA011743，这些数据集已公开可访问，可在 https://www.ddbj.nig.ac.jp/index-e.html获取。

代码可用性
此代码可由 GitHub 提供，网址：https://github.com/okadalabipr/Ando2021.git。

收到：2023年2月21日；已接受：2021年11月；在线出版：2021年12月1日

参考文献
1. Behar, M. & Hoffmann, A. Understanding the temporal codes of intra-cellular signals. Curr. Opin. Genet. Dev. 20, 684–693 (2010).
2. Hoffmann, A. & Baltimore, D. Circuity of nuclear factor Xb signaling. Immunol. Rev. 210, 171–186 (2006).
3. Mischeu, O., Lens, S., Gaide, O., Alevizopoulos, K. & Schopp, J. NF-kB signaling induces the expression of c-FLIP. Mol. Cell. Biol. 21, 5299–5305 (2001).
4. Pham, C. G. et al. Ferritin heavy chain upregulation by NF-kB inhibits TNF-induced apoptosis by suppressing reactive oxygen species. Cell 119, 529–542 (2004).
5. De Smaele, E. et al. Induction of gadd45b by NF-kB downregulates pro-apoptotic JNK signaling. Nature 414, 308–313 (2001).
6. Bradley, J. R. TNF-mediated inflammatory disease. J. Pathol. 214, 149–160 (2008).
7. Baskar, R., Lee, K. A., Yeo, R. & Yeoh, K. W. Cancer and radiation therapy: current advances and future directions. Int. J. Med. Sci. 9, 193–199 (2012).
8. Rube, C. E. et al. Modulation of radiation-induced tumour necrosis factor α (TNF-α) expression in the lung tissue by pentoxifylline. Radiother. Oncol. 64, 177–182 (2007).
9. Rath, P. C. & Aggarwal, B. TNF-induced signaling in apoptosis. J. Clin. Immunol. 19, 350–364 (1999).
10. Blank, V., Kourilsky, P. & Israel, A. NF-kB and related proteins: Rel/dorsal homologies meetakyrin-like repeats. Trends Biochem. Sci. 17, 135–140 (1992).
11. Goldberg, A. L. Functions of the proteasome: the lysis at the end of the tunnel. Science 268, 522–523 (1995).
12. Liou, H. C. & Baltimore, D. Regulation of the NF-kappa B/Rel transcription factor and I kappa B inhibitor system. Curr. Opin. Cell Biol. 5, 47–487 (1993).
13. Siebenlist, U., Franzeno, G. & Brown, K. Structure, regulation and function of NF-kB. Annu. Rev. Cell Biol. 10, 405–455 (1994).
14. Hoffmann, A. & Lesang, T. H. Genetic analysis of NF-kB/Rel transcription factors defines functional specificities. EMBO J 22, 5530–5533 (2003).
15. Hoffmann, A., Levenkoch, A., Scott, M. L. & Baltimore, D. The Idκ-NF-kB signaling module: temporal control and selective gene activation. Science 298, 1241–1245 (2002).
16. Tergaonkar, V., Correa, R. G., Ikawa, M. & Verma, I. M. Distinct roles of Idκ proteins in regulating constitutive NF-kB activity. Nat. Cell. Biol. 7, 921–923 (2005).
17. Basak, S. et al. A fourth Idκ protein within the NF-kB signaling module. Cell 128, 369–381 (2007).
18. Bauerle, P. A. & Baltimore, D. Idκ: a specific inhibitor of the NF-kB transcription factor. Science 242, 540–546 (1988).
19. Basak, S., Behar, M. & Hoffmann, A. Lessons from mathematically modeling the NF-kB pathway. Immunol. Rev. 246, 221–238 (2012).
20. O’Neill, L. A. & Hoffmann, A. NF-kB signaling. Wiley Interdiscip. Rev. Syst. Biol. Med. 1, 107–119 (2009).
21. Mitchell, S., Vargas, J. & Hoffmann, A. Signaling via the NF-kB system. Wiley Interdiscip. Rev. Syst. Biol. Med. 8, 227–241 (2016).
22. Beg, A. A., Finco, T. S., Nantermet, P. V. & Baldwin, A. S. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NF-kappa B activation. Mol. Cell. Biol. 13, 3301–3310 (1993).
23. Henkel, T. et al. Rapid proteinase of Idκ-a is necessary for activation of transcription factor NF-kB. Nature 365, 182–185 (1993).
24. Kearn, J. D., Basak, S., Werner, S. L., Huang, C. S. & Hoffmann, A. Idκα Provides negative feedback to control NF-kB oscillations, signaling dynamics, and inflammatory gene expression. J. Cell Biol. 173, 659–664 (2006).
25. Dembinski, H. E. et al. Functional importance of stripping in NF-kB signaling revealed by a stripping-impared Idκ mutant. Proc. Natl. Acad. Sci. USA 114, 1916–1921 (2017).
57. Tyler, J. K. & Kadonaga, J. T. The "dark side" of chromatin remodeling. Cell 99, 443–446 (1999).

58. Di Croce, L. & Helin, K. Transcriptional regulation by Polycomb group proteins. Nat. Struct. Mol. Biol. 20, 1147–1155 (2013).

59. Bornólov, S. et al. The nucleosome remodeling and deacetylation complex modulates chromatin structure at sites of active transcription to fine-tune gene expression. Mol. Cell 71, 56–72 (2018).

60. Morey, L. et al. MBDD3, a component of the NuRD complex, facilitates chromatin alteration and deposition of epigenetic marks. Mol. Cell Biol. 28, 5912–5923 (2008).

61. Reynolds, N. et al. NuRD-mediated deacetylation of H3K27 facilitates recruitment of polycomb repressive complex 2 to direct gene repression. EMBO J. 31, 593–605 (2012).

62. Bantignies, F. & Cavalli, G. Polycomb group proteins: repression in 3D. Trends Genet. 27, 454–464 (2011).

63. Ribeiro-Silva, C., Vermeulen, W. & Lans, H. SWI/SNF: complex complexes in genome stability and cancer. DNA Repair 77, 87–95 (2019).

64. Galardi, S., Mercatelli, N., Farace, M. G. & Ciafrè, S. A. NF-kB and c-Jun induce the expression of the oncogenic miR-221 and miR-222 in prostate carcinoma and glioblastoma cells. Nucleic Acids Res. 39, 3892–3902 (2011).

65. Messner, B., Stütz, A. M., Albrecht, B., Peitrisch, S. & Woisetschläger, M. Cooperation of binding sites for STAT6 and NF kappa B/rel in the IL-4-induced up-regulation of the human IgE germine promoter. J. Immunol. 159, 3330–3337 (1997).

66. Planeta, C. S., Lepsch, L. B., Alves, R. & Scavone, C. Influence of the dopaminergic system, CREB, and transcription factor-kB on cocaine neurotoxicity. Braz. J. Med. Biol. Res. 46, 909–915 (2013).

67. Toleadino, M. B. & Leonard, W. J. Modulation of transcription factor NF-kB binding activity by oxidation-reduction in vitro. Proc. Natl Acad. Sci. USA 88, 4328–4332 (1991).

68. DeFelice, M. M. et al. NF-B signaling dynamics is controlled by a dose-sensing autoregulatory loop. Sci. Signal. 12, eaau3568 (2019).

69. Lewin, S. R., Lambert, P., Deacon, N. J., Mills, J. & Crowe, S. M. Constitutive expression of p50 homodimer in freshly isolated human monocytes decreases with in vitro and in vivo differentiation: a possible mechanism influencing human immunodeficiency virus replication in monocytes and mature macrophages. J. Virol. 71, 2114–2119 (1997).

70. Magi, S. et al. Transcriptionally inducible pleckstrin homology-like domain, family a, member 1, attenuates ERBB receptor activity by inhibiting receptor oligomerization. J. Biol. Chem. 293, 2206–2218 (2018).

71. Buenrostro, J. D. et al. Single-cell chromatin accessibility reveals principles of regulatory variation. Nature 523, 486–490 (2015).

72. Kim, D., Langmead, B. & Salzberg, S. L. Hisat2. Nat. Methods 12, 357–360 (2015).

73. Krueger, F. Trim galore!: a wrapper tool around Cutadapt and FastQC to consistently apply quality and adapter trimming to FastQ files. Babraham Institute. https://www.bioinformatics.babraham.ac.uk/projects/ (2015).

74. Liao, Y., Smyth, G. K. & Shi, W. FeatureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30, 923–930 (2014).

75. Robinson, M., McCarthy, D. & Smyth, G. K. edgeR. Most 26, 139–140 (2010).

76. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550 (2014).

77. Benner, C., Heinz, S. & Glass, C. K. HOMER - software for motif discovery and next generation sequencing analysis. http://homer.ucsd.edu/homer/ (2017).

78. Langmead, B., Trapnell, C., Pop, M. & Salzberg, S. L. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 10, R25 (2009).

79. Li, H. et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25, 2078–2079 (2009).

80. Ramírez, F., Dündar, F., Diehl, S., Grünig, B. A. & Manke, T. DeepTools: a flexible platform for exploring deep-sequencing data. Nucleic Acids Res. 42, W187–W191 (2014).

81. Grant, B. J., Rodrigues, A. P. C., ElSawy, K. M., McCammon, J. A. & Caves, L. S. D. Bio3d: an R package for the comparative analysis of protein structures. Bioinformatics 22, 2695–2696 (2006).

ACKNOWLEDGEMENTS

The study was supported by JSPS KAKENHI Grant Nos. 17K18359 and 19H04963 to S. M.; 17H06306 and 17H06299 to Y.S.; 17H06302, 17H06302, and 18H04031 to M.O.; JST-Mirai program No. JPMJMI19G7, JST Crest program No. JPMJCR21N3, JST Moonshot R&D Grant No. JPMJMS2021, and the Uehara Memorial Foundation to M. O.; and NIH/NIAID AI127864 to A.H.

AUTHOR CONTRIBUTIONS

M.A., T.K., and D.L. performed bioinformatics analysis of sequence data. M.S. and Y.S. performed the sequencing analysis. S.M. performed experiments. M.A., S.M., A.H., and M.O. interpreted the results and wrote the manuscript. A.H. and M.O. conceived the study and were in charge of overall direction.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41592-021-00204-7.

Correspondence and requests for materials should be addressed to Alexander Hoffmann or Maniko Okada.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021