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NF κ B dynamics-dependent epigenetic changes modulate inflammatory gene expression and induce cellular senescence

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Keywords

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Upregulation of nuclear factor κ B (NF κ B) signaling is a hallmark of aging and a major cause of age-related chronic inflammation. However, its effect on cellular senescence remains unclear. Here, we show that alteration of NF κ B nuclear dynamics from oscillatory to sustained by depleting a negative feedback regulator of NF κ B pathway, NF κ B inhibitor alpha (I κ B α), in the presence of tumor necrosis factor α (TNF α) promotes cellular senescence. Sustained NF κ B activity enhanced inflammatory gene expression through increased NF κ B-DNA binding and slowed the cell cycle. I κ B α protein was decreased under replicative or oxidative stress *in vitro*. Furthermore, a decrease in I κ B α protein and an increase in DNA-NF κ B binding at the transcription start sites of age-associated genes in aged mouse hearts suggested that nuclear NF κ B dynamics may play a critical role in the progression of aging. Our study suggests that nuclear NF κ B dynamics-dependent epigenetic changes regulated over time in a living system, possibly through a decrease in I κ B α , enhance the expression of inflammatory genes to advance the cells to a senescent state.

Introduction

Aging is a complex and irreversible phenomenon that reflects lifelong biochemical responses to the environment and recent research has shown that age-related dysfunction occurs from the genome to organ levels [1,2]. Inflammatory aging, which refers to aging-

associated chronic inflammation, critically impacts the induction of a wide range of age-related human diseases [3], and its regulation is becoming increasingly important in aging societies. Notably, elderly individuals exhibit low-grade and persistent chronic inflammation

Abbreviations

ATAC-seq, assay for transposase-accessible chromatin sequencing; ChIP-seq, chromatin immunoprecipitation sequencing; DEGs, differentially expressed genes; I κ B α , NF κ B inhibitor alpha; I κ B α DN, dominant-negative I κ B α ; KD, knockdown; KEGG, Kyoto Encyclopedia of Genes and Genomes; KO, knockout; NF κ B, nuclear factor κ B; PDL, population doubling levels; SASP, senescence-associated secretory phenotype; SA- β -gal, senescence associated β -galactosidase; TNF α , tumor necrosis factor α ; TSS, transcription start site.

in their tissues along with elevated levels of circulating inflammatory factors, such as interleukin-6 and tumor necrosis factor α (TNF α), that activate NFκB signaling [4,5]. Meanwhile, the accumulation of senescent cells during aging produces various pro-inflammatory cytokines that represent a senescence-associated secretory phenotype (SASP), which increases the risk of developing age-related diseases [6]. As NFκB is the master transcription factor for SASP genes [7,8], NFκB is considered a positive feedback regulator in inflammatory aging and cellular senescence. However, several studies have also shown that NFκB prevents cellular senescence; therefore, it remains unclear which properties of NFκB activity are related to cellular senescence [9,10]. Herein, we speculate that a quantitative rather than qualitative understanding of NFκB activity is necessary to unravel the role of NFκB in cellular senescence and inflammatory aging.

Nuclear factor κB (NFκB) regulates the expression of genes involved in immune responses and cell proliferation, apoptosis, and senescence [11–13] and excessive NFκB activity induces pathological inflammation and contributes to the progression of various human diseases [14]. Interestingly, NFκB also induces transient, sustained, or oscillatory dynamics associated with different biological functions and cell fate [15]. In general, the canonical NFκB pathway undergoes negative feedback regulation in response to environmental factors and exhibits stimulus-specific oscillatory dynamics. In particular, NFκB inhibitor alpha (IκBα), which is rapidly expressed upon NFκB activation, acts as a strong negative feedback regulator by binding to NFκB and inhibiting its nuclear translocation, thereby inducing characteristic NFκB oscillatory patterns [16]. Accordingly, knockout (KO) or knockdown (KD) of the IκBα gene often results in sustained NFκB nuclear activation, altering epigenetic status and expression dynamics of target genes [17–19].

Although there is growing evidence that NFκB is involved in inflammatory aging, the relationship between NFκB dynamics and its global effects on the cell remains poorly understood. In this study, by combining experiments and multi-omics data analysis, we investigated the impact of the changes in NFκB dynamics on cell functions in the presence of pro-inflammatory cytokine TNF α and found that the alteration from oscillatory to sustained NFκB nuclear dynamics promotes cellular senescence. Our results suggested that sustained NFκB modulated by IκBα KD rewired epigenetic states and enhanced NFκB-DNA binding and promoted inflammatory gene expression. Indeed, IκBα level was found to be decreased in aged mouse heart *in vivo* and furthermore IκBα level also decreased in

response to replicative stress or oxidative stress (H₂O₂) *in vitro*. These results indicate that spatiotemporal changes in NFκB dynamics by its negative feedback regulator IκBα play an important role in controlling the expression levels of inflammatory genes, thereby driving cellular senescence.

Results

Sustained NFκB activity induces growth arrest and cellular senescence

In order to analyze the effect of NFκB dynamics on cellular senescence under conditions that eliminate the influence of replication stress, we first performed analyses using human breast cancer MCF-7 cell, which does not senesce by replication. First, we confirmed that IκBα KD alters oscillatory NFκB nuclear activity to sustained activity in MCF-7 cells stimulated with TNF α (Fig. 1A–C). The change in NFκB dynamics suppressed cell proliferation after 24 h of TNF α stimulation (Fig. 1D) and led to a flattened cell shape and significantly increased cell size (Fig. 1E). These features are similar to the well-known phenotype of senescent cells [20]. Therefore, we further examined SA-β-gal activity, one of the representative indicators of cellular senescence [21]. Although TNF α alone did not promote SA-β-gal activity, under IκBα KD/KO conditions, TNF α increased the number of SA-β-gal positive cells after 24 h (Fig. 1F–I). The number of SA-β-gal positive cells was reduced by KD of RELA, a subunit of NFκB, in IκBα KD + TNF α conditions (Fig. 1J), thus confirming that the phenomenon is NFκB-dependent. As senescent cells typically exhibit a SASP [6,21], we next examined the effects under IκBα KD + TNF α conditions. The expression of various SASP genes [6,21], including different types of cytokines (IL6: interleukin, TNF α : tumor necrosis factor, IL8: interleukin and CXC chemokine, CXCL1: CXC chemokine, CCL2: CC chemokine), was drastically elevated (Fig. 2A,B). The amount of Cyclin D1, which is essential for the G1/S transition, was decreased compared to that with TNF α alone (Fig. 2C). Furthermore, a time-course cell cycle analysis using FUCCI live cell imaging revealed that IκBα KD + TNF α increases the number of cells at the G2/M phase after 24 h and prolongs the duration of the G2/M phase (median values: control, 3.8; IκBα KD, 8.3; $P < 0.05$) (Fig. 2D–F). Although the difference in the duration of the G1 phase, which appeared within 24 h after TNF α stimulation, could not be quantitatively assessed in this experimental setting, these results indicate that the sustained NFκB activity mimicked by IκBα

KD + TNF α modulates Cyclin D1 and G2/M checkpoints and inhibits cell proliferation.

Decrease of I κ B α was associated with cellular senescence induced by replicative or oxidative stress

Next, we examined whether the downregulation of I κ B α is indeed associated with cellular senescence in representative cell models. Long-term culturing causes replicative stress and thereby induces cellular senescence in primary human foreskin fibroblast cells (HFF-1) [22]. We prepared HFF-1 cells with different population doubling levels (PDL) and confirmed that the number of SA- β -gal positive cells increases consistently with PDL (Fig. 3A). In our subsequent studies, HFF1 cells with PDL 43 or higher were defined as senescent cells. The expression of I κ B α in senescent HFF-1 cells (PDL46) was significantly lower than that in younger HFF-1 cells (PDL30) (Fig. 3B). As well as in MCF-7 cells, the number of SA- β -gal positive cells was increased by I κ B α KD + TNF α in HFF-1 cells (Fig. 3C). Since oxidative stress (H₂O₂) exposure is known to induce cellular senescence in MCF-7 cells [23], we also investigated the effect of H₂O₂ on I κ B α expression in MCF-7 cells and confirmed that I κ B α expression was indeed downregulated by H₂O₂ in a dose-dependent manner (Fig. 3D). Overexpression of dominant-negative I κ B α (I κ B α DN) [24], which has a mutation in the ubiquitination site and exerts a dominant-negative effect on activation of NF κ B, suppressed the increase of SA- β -gal staining triggered by H₂O₂ (Fig. 3E). Furthermore, nuclear RELA levels were increased by H₂O₂ treatment or by replication (Fig. 3F,G). These results suggest that downregulation of I κ B α expression and increased RELA level may also play an important role in replication or oxidative stress-induced cellular senescence.

Sustained NF κ B activation enhanced DNA-NF κ B binding on senescence-promoting inflammatory genes *in vitro*

Subsequently, we investigated the relationship between NF κ B nuclear dynamics and target gene regulation. First, we extracted differentially expressed genes (DEGs) in MCF-7 cells between I κ B α KD + TNF α (48 h) and TNF α conditions and identified 687 upregulated DEGs, including cellular senescence-promoting and SASP genes (e.g., *CD36*, *BCL3*, *CCL2*, and *SI00A8/A9*) [1,21,25,26] (Fig. 4A). These DEGs were classified into four clusters based on the time-course patterns of gene expression, which were measured

every 15 min under the I κ B α KD + TNF α condition [17] (Fig. 4B). To clarify whether the differences in gene expression patterns were associated with the chromatin status, chromatin accessibility was assessed using an assay for transposase-accessible chromatin sequencing (ATAC-seq) data. We found that the ATAC signal was elevated in the flanking regions of genes in cluster 2, which showed a monotonically increasing pattern, and their signals were stronger in the I κ B α KD + TNF α condition than the TNF α or I κ B α KD alone (Fig. 4C). Considering that the chromatin accessibility enhanced by I κ B α KD + TNF α might be critical for the regulation of genes controlled by NF κ B, we extracted the regions where chromatin accessibility was significantly enhanced by I κ B α KD + TNF α (Fig. 4D) and assessed the affinity of NF κ B for these regions. We analyzed NF κ B (RELA)-chromatin immunoprecipitation sequencing (ChIP-seq) data and found that the RELA signal intensity in chromatin open regions proximal to genes in cluster 2 was markedly increased under I κ B α KD + TNF α conditions; however, this trend was not observed under TNF α alone (Fig. 4E). Furthermore, we investigated whether the number of NF κ B binding sites affects gene expression in each cluster [27]. Notably, the average number of RELA binding sites associated with genes in cluster 2 was significantly higher than that for all NF κ B target genes (Fig. 4F). These results suggest that the expression of a set of genes (genes in cluster2) is controlled by NF κ B dynamics-dependent epigenetic changes. Genes in cluster 2 were enriched with several cytokine signaling pathways such as TNF α and IL17 (Fig. 4G), suggesting that a positive feedback loop mediated by inflammatory cytokines might be epigenetically enhanced by sustained nuclear NF κ B activity.

Reduced I κ B α level, NF κ B activation, and upregulation of senescence-promoting inflammatory genes were associated with aging in mouse hearts

Senescent cells accumulate in aged tissues in a variety of mammals [21]. Given that sustained NF κ B activity may be involved in promoting cellular senescence and chronic inflammation associated with aging *in vivo*, we examined the levels of proteins that regulate NF κ B activation dynamics in young (8 weeks old) and aged (69–73 weeks old) mice (Fig. 5A–E). We found decreased I κ B α and A20, another negative feedback regulator of the NF κ B pathway, and increased TNF α protein expression, but no significant change in RELA levels in the heart tissue homogenates of aged mice

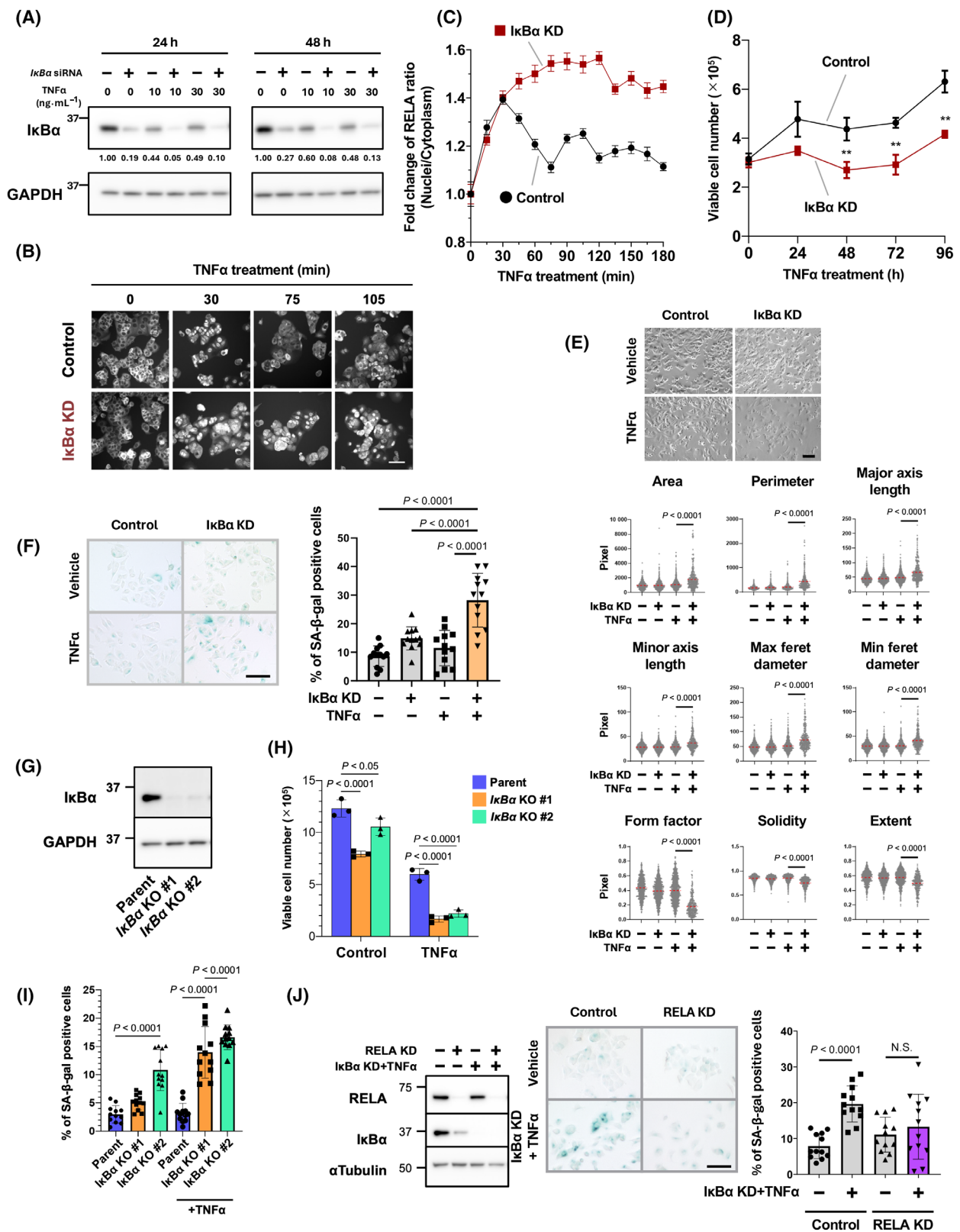


Fig. 1. Effect of IκBα knockdown on NFκB dynamics, cell growth, cell shape, and cellular senescence in MCF-7 cells treated with TNFα. (A) IκBα knockdown in MCF-7 cells by siRNA. MCF-7 cells were treated with TNFα (10 or 30 ng·mL⁻¹) for 24 or 48 h. Representative blot images are shown. The values under each lane indicate the relative density of the band normalized to GAPDH. One of the five replicates is shown as representative. (B) Representative images of RELA immunofluorescence staining in MCF-7 cells transfected with control and IκBα siRNA. The cells were treated with 10 ng·mL⁻¹ TNFα for the indicated times. Scale bars, 25 μm. One of the thirty replicates is shown as representative. (C) Time-course of NFκB (RELA) abundance in the control and IκBα knockdown (KD) MCF-7 cells treated with TNFα (10 ng·mL⁻¹) (refer to the [Materials and methods](#) section). Representative images are shown in (B). *n* = 30. (D) Effect of IκBα KD on cell viability. *n* = 3. (E) Changes in cell morphology in the IκBα KD MCF-7 cells treated with TNFα for 24 h. Representative brightfield images are shown. Scale bars, 100 μm. Each dot on the graph represents the geometric parameters for single cells. Red dashed bars indicate the average. *n* = 713 (control – TNFα), 1014 (IκBα KD – TNFα), 954 (control + TNFα), and 336 (IκBα KD + TNFα). (F) Effect of IκBα KD on SA-β-gal staining in MCF-7 cells treated with TNFα for 24 h. Scale bars, 100 μm. *n* = 12. (G) IκBα knockout (KO) in MCF-7 cells by CRISPR-Cas9. (H) Effect of IκBα KO on cell viability in MCF-7 cells treated with TNFα (10 ng·mL⁻¹) for 48 h. *n* = 3. (I) Effect of IκBα KO on SA-β-gal staining in MCF-7 cells treated with TNFα for 24 h. *n* = 12. (J) Effect of RELA KD on the IκBα KD-induced SA-β-gal staining. *n* = 12. Scale bars, 100 μm. Data represent the mean ± SD (D, F, H, I, J) or ± SE (C). *P* values were determined using unpaired two-tailed Student's *t* tests (D) or one-way ANOVA followed by Tukey's test (E, F, H, I, J). ***P* < 0.01. N.S., not significant.

(Fig. 5A–E). However, we found a marked increase in the NFκB-positive area in the cell nuclei of aged hearts at the single cell level (Fig. 5F) and upregulation of the cellular senescence markers *p16* and *Il6* (Fig. 5G). RNA-seq analysis of heart tissues from young and aged mice and predictions of active transcription factors using DoRothEA [28] suggested that NFκB family members NFKB1 and RELA were activated in aged tissue (Fig. 6A). The genes in cluster 2 had a significant positive correlation between the fold change values of gene expression upon alteration of NFκB activity *in vitro* (IκBα KD + TNFα vs. control MCF-7) and aging *in vivo* (aged vs. young mouse hearts) (*R* = 0.39, *P* < 0.0001), and this significantly-correlated gene set contained cellular senescence-promoting and SASP genes (Fig. 6B). In addition, we examined the relationship between the number of NFκB binding sites and the increase in gene expression with aging in the mouse heart. After predicting putative NFκB targets within ±500 bp from the transcription start site (TSS) using the ChIP-Atlas [29] (see [Materials and methods](#)), we classified the genes into four groups (no, low, medium, and high upregulation) according to the degree of fold change in expression (aged vs. young mice) (Fig. 6C). Counting NFκB binding sites in the flanking regions (within ±10 000 bp from TSS) of the genes using HOMER [30] revealed that the “high” expression group included a significantly higher number of NFκB binding motifs (*P* < 0.0001, high vs. no upregulation) (Fig. 6D). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the high group genes in the aged heart (Fig. 6E) showed a high enrichment of inflammatory signals, which was similar to the KEGG enrichment results for the cluster 2 gene set in MCF-7 cells (Fig. 4F). These results suggested that the increase in frequency of DNA-NFκB binding might be

a common mechanism for the induction of age-related inflammatory genes in aging tissue and senescent cells.

Discussion

The duration of nuclear NFκB activity has significant effects on gene expression by regulating mRNA half-life and epigenetic status [18,19]. In this study, we show that the aging-associated phenotypic changes in inflammatory gene expression and the downregulation of cell cycle could be explained by a core regulatory mechanism stemming from the duration of NFκB activity.

Our *in vitro* study showed that the loss of the negative feedback regulator, IκBα, significantly elevated the nuclear abundance of NFκB and induction of cell senescence. Our *in vivo* data analysis showed that reduced IκBα expression and upregulation of NFκB activity and senescence-promoting inflammatory genes were also observed in aged mouse heart. These results suggest that the nuclear NFκB level is critical for inflammatory aging. Indeed, the decrease in IκBα protein levels under replicative or oxidative stress (Fig. 3) indicates that decreases in IκBα may occur over a lifetime by these stresses. Meanwhile, previous studies have demonstrated that defects in the feedback regulation of IκBα in the NFκB pathway cause Sjögren's syndrome and shortened life span [31] and that mice expressing vascular-specific IκBα overexpression have a prolonged life span [32].

It has been reported that the number of senescent cells in tissues increases with age [33–35]. However, it is also known that the number of senescent cells rarely exceeds a few percent within the cell population of most aged tissues [33–36]. Therefore, the mechanism behind the regulation of the number of senescent cells

during the process of aging is considered to involve a system that generates cell heterogeneity. Previous reports indicate that the increase in the frequency of DNA-NFκB binding can lead to higher fold change and threshold response in target gene expressions [27], which enhances transcriptional heterogeneity [15]. Our data indicate the positive correlation between the frequency of DNA-NFκB interaction and the fold

changes in age-associated NFκB target gene expressions in mouse hearts (Fig. 6), which suggests a possibility that enhanced DNA-NFκB binding in aged tissues contributes to the increase in heterogeneity and thereby regulate the number of senescent cells. Further analysis of heterogeneity in transcriptome and chromatin states in aged tissue at a single-cell level may clarify this point. Furthermore, considering that the threshold

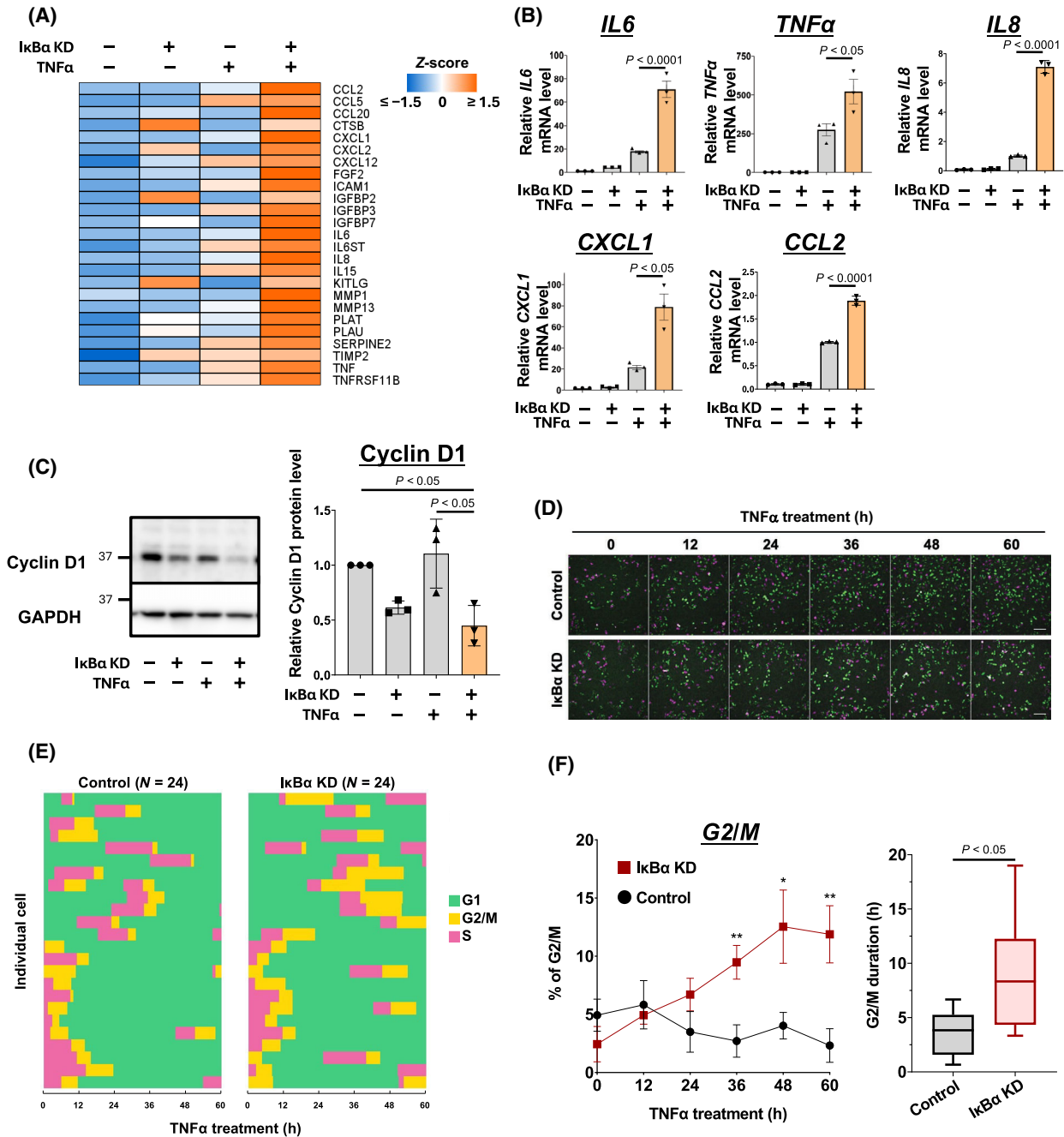


Fig. 2. Effect of I κ B α knockdown on expression of SASP genes and cell cycle in MCF-7 cells treated with TNF α . (A, B) Senescence-associated secretory phenotype (SASP) gene expression in MCF-7 cells under I κ B α KD + TNF α conditions. Cells were treated with TNF α for 24 h. The mRNA level of SASP genes was measured by RNA-seq (A) and qPCR (B). $n = 3$. (C) Effect of I κ B α KD on the protein expression of Cyclin D1 in MCF-7 cells treated with TNF α for 48 h. $n = 3$. (D–F) Cell cycle analysis of MCF-7 cells under I κ B α KD + TNF α conditions. MCF-7 cells were transfected with the control and I κ B α siRNA and treated with 10 ng·mL^{−1} TNF α for the indicated times. Representative images of FUCCI immunofluorescence (D). MCF-7 cells show G1-phase (green color; PIP-tag-mNeonGreen), S-phase (magenta color; mScarlet-hGem), and G2/M-phase (merged color; PIP-tag-mNeonGreen and mScarlet-hGem). Scale bars, 100 μ m. Cell cycle dynamics mapped at an individual cell level ($n = 24$) (E). G2/M ratio (left) and duration (right) in TNF α -treated control and I κ B α KD MCF-7 cells (F). Cell cycle phase data were obtained for cells that could be continuously tracked for 60 h after TNF α treatment. As the experiment was performed under non-synchronized cell cycle conditions, an equal number of cells starting from the G1 or S/G2/M phase ($n = 12$ for each) were selected for analysis. Data represent the mean $\pm$ SD [(B, C, F (left)), or median and interquartile range (25th and 75th percentiles), with error bars showing the minimum to maximum (F, right)]. P values were determined using unpaired two-tailed Student's t tests (F), or one-way ANOVA followed by Tukey's test (B, C). * $P < 0.05$, ** $P < 0.01$.

response of the NF κ B signaling pathway is regulated by both its network structure [37] and epigenetic features [15,27], these regulatory processes can be promising targets for controlling the number of senescent cells.

Our study showed that the cell cycle is prolonged during the G2/M phase under I κ B α KD + TNF α . However, issues regarding the G2/M delay remain unresolved. An earlier study showed that NF κ B activation in response to ionizing radiation or etoposide arrested cells at the G2/M phase for a prolonged time [38]. Identifying a mechanism that can distinguish between cellular senescence and cell death induced by TNF α [39] and its role of I κ B α would be also important in future study. Overall, in this study, we show that the differences in temporal NF κ B dynamics, rather than the presence of NF κ B signaling activity alone, induce senescence through unexpectedly broad and complex interactions in signaling, epigenetics, and inflammatory response feedback. These findings provide unprecedented insights into the molecular basis of the progression of cellular senescence.

Materials and methods

Reagents and cell culture

TNF α was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Methanol, Hydrochloric Acid, Ethanol, Xylene, Mayer's hematoxylin solution, 1% Eosin Y solution, and PathoMount were purchased from Fujifilm Wako Pure Chemical Industries, Ltd., Osaka, Japan. 9-Aminoacridine (9AA) was purchased from Merck (Darmstadt, Germany). Adhesive-coated glass slides for hematoxylin and eosin staining (HE), and coverslips were purchased from Matsunami Glass (Osaka, Japan).

The human breast adenocarcinoma MCF-7 cell line and primary human foreskin fibroblast HFF-1 cells were obtained from the American Type Culture Collection

(ATCC, Manassas, VA, USA). All cell lines used were authenticated by ATCC. Cells were grown in Dulbecco's modified Eagle's medium (Nakalai Tesque, Kyoto, Japan) containing 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO, USA), and antibiotics (100 U·mL^{−1} penicillin, 100 mg·mL^{−1} streptomycin, and 0.25 mg·mL^{−1} amphotericin B; Nakalai Tesque) at 37 °C under a humidified atmosphere with 5% CO₂. All experiments were performed with Mycoplasma-free cells.

Mouse experiments

All mice were purchased from the Japan SLC and maintained under specific pathogen-free conditions at the Graduate School of Frontier Biosciences Osaka University. Female 8-weeks-old (young) and 69- to 73-weeks-old (aged) C57BL/6 N mice were used in experiments. All animals were separately caged with a 12 : 12-h light–dark cycle and had free access to water and chow throughout the study. The excised tissues were immediately frozen in liquid nitrogen and stored at −80 °C until use. All experiments were approved by the Animal Care and Use Committee of Osaka University and were performed following institutional guidelines (approval number: 01-002-059).

Quantitative RT-PCR (qRT-PCR) analysis

Real-time PCR analysis was conducted as described previously [40]. Briefly, RNA was isolated from cells or tissues using NucleoSpin RNA Plus (Cat. No. 740984.250; Macherey-Nagel, Duren, Germany) and subjected to cDNA synthesis using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) and quantitative PCR using a KOD SYBR qPCR kit (TOYOBO) with CFX96 Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. The $\Delta\Delta C_q$ method was used to quantify gene expression, using RPL27 expression as an internal reference [41]. All experiments were performed in triplicate.

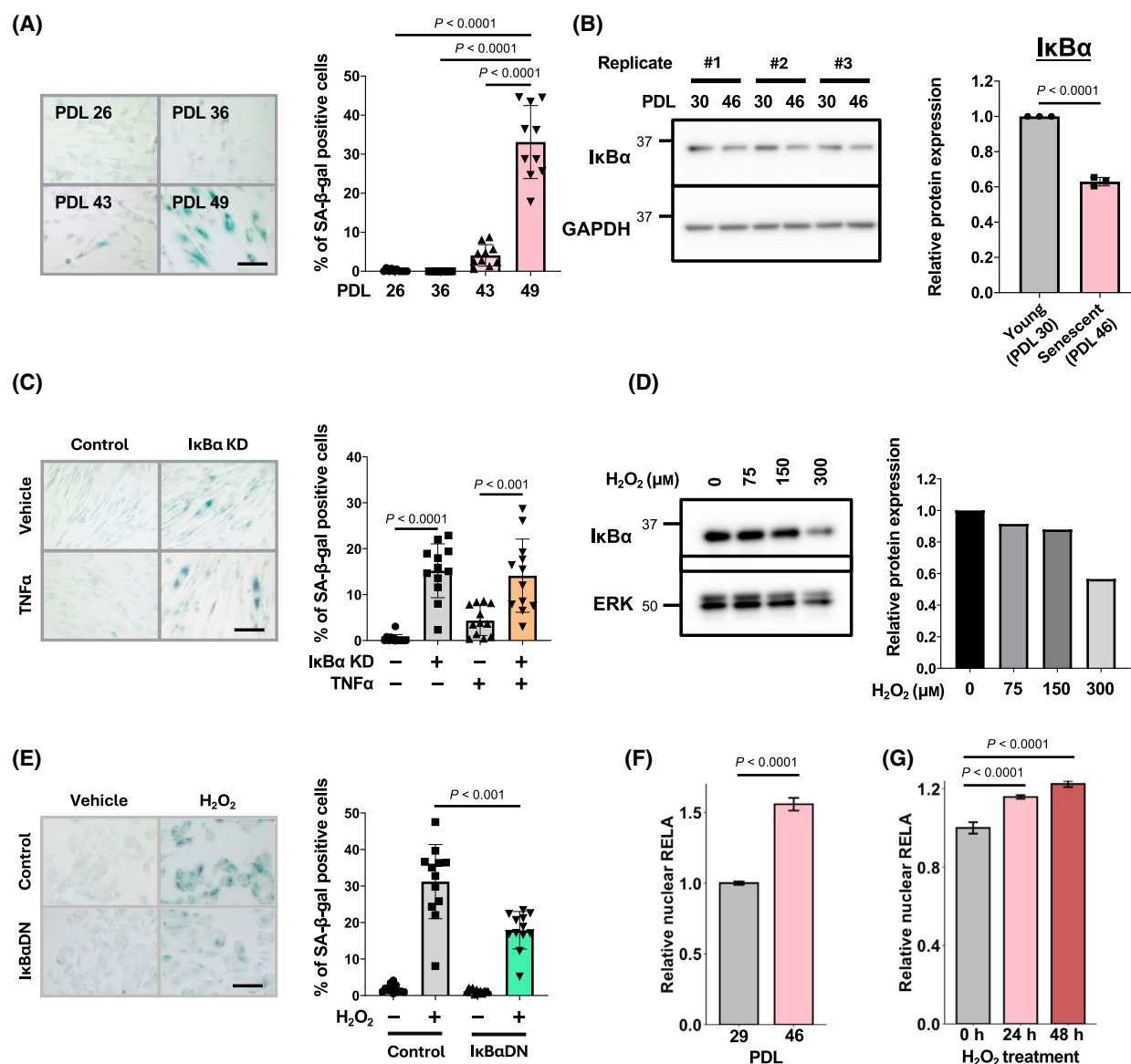


Fig. 3. Downregulation of IκBα by replication or oxidative stress-induced cellular senescence. (A) Induction of SA-β-gal staining by replicative stress in HFF-1 cells. Scale bars, 100 μm. $n = 10$. (B) Expression of IκBα in young (population doubling level, PDL30) and senescent (PDL46) HFF-1 cells. $n = 3$. (C) Effect of IκBα KD on SA-β-gal staining in HFF-1 cells treated with TNFα for 24 h. Scale bars, 100 μm. $n = 12$. (D) Effect of H₂O₂ on IκBα expression in MCF-7 cells. Cells were treated with the indicated concentration of H₂O₂ for 96 h. Representative image and quantification of one of the three replicates are shown. (E) Effect of dominant-negative IκBα (IκBαDN) on SA-β-gal staining in MCF-7 cells treated with 300 μM H₂O₂. $n = 12$. Scale bars, 100 μm. (F, G) Nuclear RELA levels of young (PDL29) and senescent (PDL46) HFF1 cells (F) and H₂O₂ treated MCF7 cells (G) measured by immunostaining. Error bars, standard error of the mean. $n = 30$. P values were determined using unpaired two-tailed Student's t -tests (B, F, G) or one-way ANOVA followed by Tukey's test (A, C, E).

Western blotting

Immunoblot analysis was performed as described previously [40] using primary antibodies against GAPDH (1 : 2000, MAB374; Sigma-Aldrich), GAPDH (1 : 4000, 10494-1-AP; Proteintech, Rosemont, IL, USA), α-tubulin (1 : 1000,

ab15246; Abcam, Cambridge, MA, USA), IκBα (1 : 1000, #4814; Cell Signaling Technology, Danvers, MA, USA), IκBα (1 : 1000, #4812; Cell Signaling Technology), RELA (1 : 1000, #8242; Cell Signaling Technology), A20 (1 : 1000, #5630; Cell Signaling Technology), Cyclin D1 (1 : 1000,

#2978; Cell Signaling Technology), ERK (1 : 1000, #9102; Cell Signaling Technology), and secondary antibodies [HRP-linked anti-mouse IgG (1 : 5000, #7076; Cell Signaling Technology), HRP-linked anti-goat IgG (1 : 5000, sc-2020; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and HRP-linked anti-rabbit IgG (1 : 5000, #7074; Cell Signaling Technology)].

RELA immunocytochemistry

Immunostaining was conducted as described previously [42]. Cells were fixed with 4% paraformaldehyde (Thermo Fisher Scientific) in phosphate-buffered saline (PBS) for 15 min, rinsed with PBS, and permeabilized with 0.2% Triton X-100 (Nacalai Tesque) in PBS for 10 min. Cells were blocked using 1% goat serum (#16210-064; Thermo Fisher Scientific) in PBS for 15 min and incubated with RELA antibodies (1 : 1600, #8242; Cell Signaling Technology) diluted in 1% goat serum/PBS at 4 °C overnight. After rinsing in PBS, cells were incubated with Alexa Fluor 488-conjugated secondary antibodies (1 : 2000, A32731; Thermo Fisher Scientific), 0.125 ng·mL⁻¹ CellMask™ (Thermo Fisher Scientific) as a cell body stain, and 1 µg·mL⁻¹ 4',6-diamidino-2-phenylindole (DAPI; Nacalai Tesque) as a nuclear stain for 1 h at 25 °C in the dark. Fluorescent images were acquired using IN Cell Analyzer 2500HS (Cytiva, Preston, UK) at 20× magnification. Images of 30 fields were taken for each condition. CELLPROFILER (ver. 3.1.9) (Broad Institute, Cambridge, MA, USA) was used to segment cellular regions from CellMask™ images, to segment nuclear regions from DAPI images, and to quantify RELA signal intensities for each cell [43]. The nuclear-to-cytoplasm signal ratio for RELA was calculated based on the integrated signal density.

Analysis of cell morphology

Cell morphology analysis was conducted as described previously [40]. Briefly, Cells were fixed using 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton-X100 for 5 min. Cell membrane and nucleus were stained with 0.125 ng·mL⁻¹ CellMask™ (Thermo Fisher Scientific) and 1 µg·mL⁻¹ DAPI (Nacalai Tesque) for 30 min at 25 °C in the dark, respectively. Fluorescent images were acquired using IN Cell Analyzer 2500HS (Cytiva) at 20× magnification. CELLPROFILER (ver. 3.1.9) was used to extract geometrical features from the cell images [43]. DAPI and CellMask™ staining were used to visualize the segmentation of the nucleus and cell body, respectively. The following morphological parameters of the cells were evaluated: Area, Form Factor, Solidity, Extent, Orientation, Eccentricity, Compactness, major axis length, minor axis length, maximum ferret diameter, minimum ferret diameter, Perimeter, and Mean radius (total of 13 parameters).

Senescence associated β -galactosidase (SA- β -gal) staining

The senescent cells were stained by a senescence β -galactosidase staining Kit (#9860; Cell Signaling Technology), according to the manufacturer's instructions. Briefly, cells in 12-well plates were washed with PBS, fixed for 10 min in a fixative solution, washed twice with PBS, and incubated in SA- β -gal staining solution overnight at 37 °C. Images were acquired with a Keyence BZ-9000 microscope (Keyence, Osaka, Japan) on 20× magnification. Four random views per well were selected to count the number of cells stained positive for SA- β -gal. Each independent experiment was repeated three times and the percentage of SA- β -gal positive cells per field from a total of 12 fields was analyzed with IMAGEJ software (version 1.53t, NIH, Bethesda, MD, USA). DAPI staining was used to identify nuclei and count the number of cells per field. The threshold for identifying senescent cells was set by a positive control (etoposide treatment).

siRNA transfection

siRNA duplexes for I κ B α (ON-TARGET plus SMART pool, L-004765-00), RELA (ON-TARGET plus SMART pool, L-003533-00), and negative control (D-001810-02) were purchased from Dharmacon/Horizon Discovery (Cambridge, UK). Cells were transfected with siRNA oligomer (final concentration 50 nM) mixed with Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific) in serum-reduced Opti-MEM (Thermo Fisher Scientific) according to the manufacturer's instructions.

Cell viability assay

Cell viability was measured using trypan blue exclusion assay as described previously [42]. 2×10^5 cells were seeded in each well of 12-well plates and incubated at 37 °C. After treatment with reagents and/or siRNAs, cells were disaggregated in 400 µL medium, and 10 µL of the suspension was mixed with 10 µL trypan blue (Thermo Fisher Scientific). Viable cells were counted using a Countess Automated Cell Counter (Thermo Fisher Scientific).

Cell cycle analysis using the FUCCI biosensor

MCF-7 cells stably expressing the FUCCI biosensor were obtained by the PiggyBac transposase system [44]. MCF-7 cells were transfected with the pPBbsr2-H2B-iRFP-P2A-mScarlet-I-hGem-P2A-PIP-tag-NLS-mNeonGreen (<https://benchling.com/s/seq-LPZ1tLdpgnpJIYOG2ujR>) and the hyPBase (<https://benchling.com/s/seq-oGkw53b41IZqvzF5y-Q9K>) [45] plasmids using Lipofectamine LTX following manufacturer's protocol (Thermo Fisher Scientific). The plasmids, pPBbsr2-H2B-iRFP-P2A-mScarlet-I-hGem-P2A-PIP-

tag-NLS-mNeonGreen and hyPBase, were a gift from K. Aoki (National Institute for Basic Biology, National Institutes of Natural Sciences). After transfection, cells were

released into a drug-free medium for 48 h followed by blasticidin (10 μg·mL⁻¹) selection until single colonies were formed. Single clones were expanded, and the FUCCI

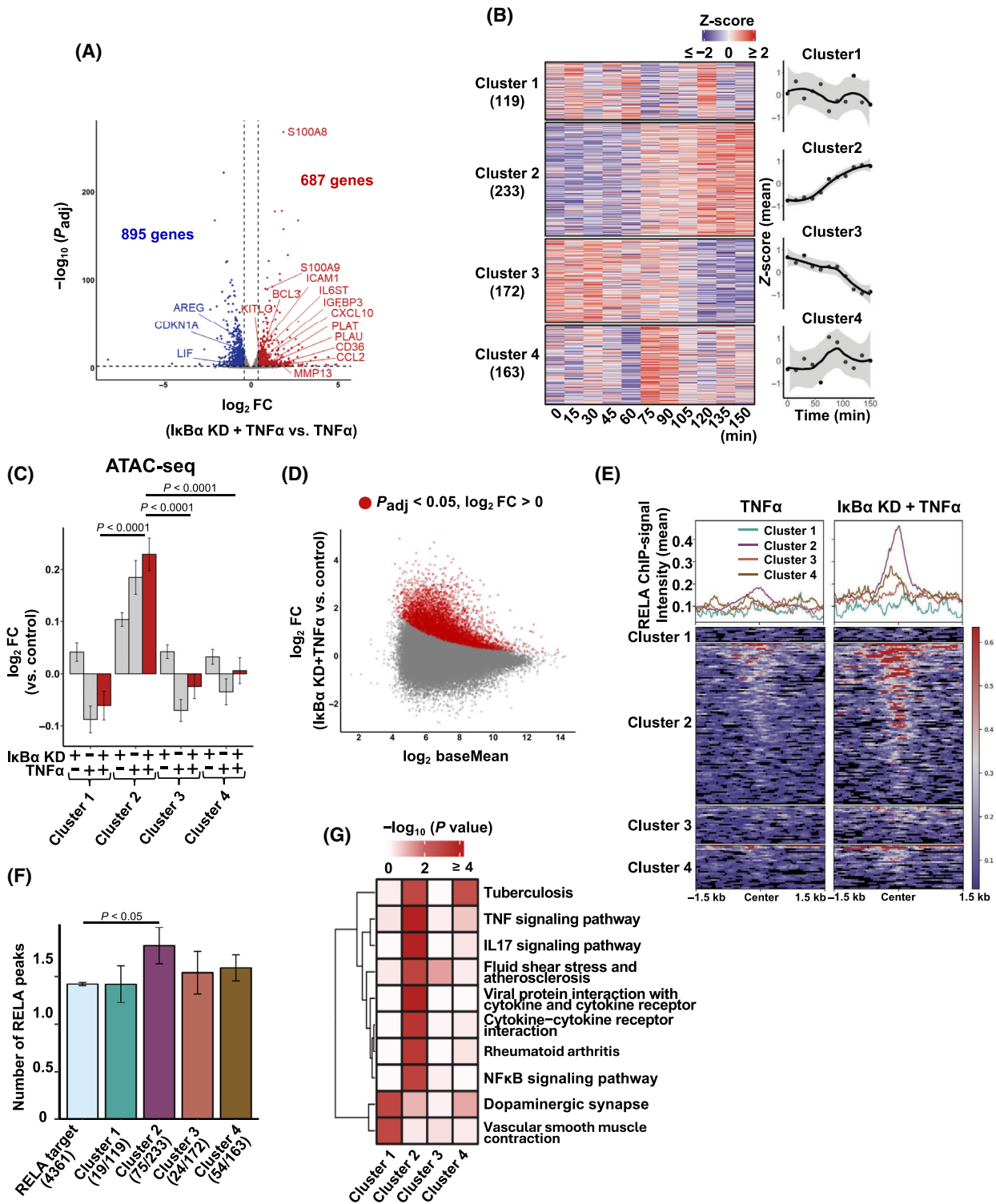


Fig. 4. Transcriptional regulation by sustained NF κ B activation. (A) Volcano plot of differentially expressed genes (DEGs) between TNF α alone and I κ B α KD+ TNF α in MCF-7 cells. Cells were treated with TNF α for 48 h. Red dots indicate DEGs identified by adjusted *P* value (*P*_{adj}) < 0.01 and log₂ fold change (log₂ FC) > 0.4. Blue dots indicate DEGs identified by *P*_{adj} < 0.01 and log₂ FC < −0.4. Among the DEGs, the cellular senescence-promoting and SASP genes are labeled. Horizontal dashed line indicates *P*_{adj} = 0.01, and vertical dashed lines indicate |log₂ FC| = 0.4. *n* = 3. (B) Time-course clustering of mRNA levels (Z-scored log₂TPM) in I κ B α KD MCF-7 cells stimulated with TNF α (10 ng·mL^{−1}) up to 150 min. Lines on the right indicate approximate curves and colored areas indicate 95% confidence intervals. (C) Effect of I κ B α KD on chromatin accessibility for genes in each cluster in MCF-7 cells treated with TNF α (48 h). Data represent the log₂ fold change (FC) vs. control conditions. *n* = 119 (cluster 1), 233 (cluster 2), 172 (cluster 3), and 163 (cluster 4). (D) MA plot of the control vs I κ B α KD + TNF α ATAC-seq signals. Each dot represents each open chromatin region, and red dots indicate open chromatin regions identified by *P*_{adj} < 0.05 and log₂ FC > 0. (E) Effect of I κ B α KD and TNF α (120 min) on RELA ChIP signal intensity in open chromatin regions. Rows in the heatmap indicate the signal intensity in each region. Lines in the line chart show the average RELA signal intensity. (F) The average count of RELA ChIP peaks for NF κ B target genes in each cluster. Genes with RELA peaks in the vicinity of transcriptional start sites (TSS) were defined as NF κ B target genes (4361 genes). NF κ B target genes in each cluster were identified (cluster 1, 19 genes; cluster 2, 75 genes; cluster 3, 24 genes; cluster 4, 54 genes). (G) Heatmap of KEGG enrichment analysis of each cluster genes identified in B. Data represent the mean $\pm$ SE (C, F). *P* values were determined using one-way ANOVA followed by Tukey's test (C, F).

biosensor signal was confirmed by fluorescent microscopy. Time-lapse microscopy images were acquired using an IN Cell Analyzer 2500HS (GE Healthcare Life Science) with a CFI S Plan Fluor ELWD dry objective lens 20 $\times$ (NA: 0.45) using an excitation wavelength of 475 nm and emission wavelength of 511 nm for PIP-tag-mNeonGreen and excitation wavelength of 542 nm and emission wavelength of 597 nm for mScarlet-hGem. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and imaged at 20-min intervals for 60 h. Image processing and quantifications for a percentage of cells in each cell cycle phase were performed using tools in IMAGEJ-FIJI software. Masks of mScarlet-hGem and PIP-tag-mNeonGreen signals were obtained by applying a fixed threshold (160, 65 535). The obtained masks were identified with “Analyze Particles” (size = 10.00–infinity, circularity = 0.00–1.00) and automatically counted as ROIs (region of interest). Cell cycle phases were determined by G1-phase: PIP-tag positive, S-phase: hGem positive, G2/M-phase: PIP-tag and hGem positive. The average fluorescence brightness of mScarlet and mNeonGreen was obtained by cell tracking software (Olympus, Tokyo, Japan). The threshold parameters of mScarlet and mNeonGreen for each cell were manually set. Subsequently, an algorithm for estimating the cell cycle phase assembled with R packages [BRUNNEMUNZEL (v.1.4.1), MULTIMODE (v.1.4), RLST (v.0.4.6.1), TIDYVERSE (v.1.3.0)] was applied to determine each cell cycle phase at the single-cell level. For quantifications of the G2/M phase length from single-cell data, measurements from when the effects of TNF α were observed (24–60 h) were used.

Generation of I κ B α KO MCF-7 cells

I κ B α knockout (KO) MCF-7 cells were generated by CRISPR-Cas9 technology. We designed three independent guide RNAs targeting I κ B α (NFKBIA) with the gRNA design tool (<https://chopchop.cbu.uib.no/>) [46]. Two gRNAs were designed to target exon 1 and one gRNA to target intron 1 of NFKBIA (gRNA_1, TAACCTCGCGG

AAAACACCG, chr14:35403909 (−); gRNA_2, CTGGACGACCGCCACGACAG, chr14:35404547 (−); gRNA_3, ACGGCGGCACGGACTGCTGT, chr14:35404730 (+)). Primers were then designed by adding guanosine base on the 5' and sticky ends corresponding to BbsI site. The pair of primers were then annealed and ligated into BbsI-digested eCas9-P2A-Puro plasmid [47]. MCF-7 cells were transfected with I κ B α gRNA-containing eCas9-P2A-Puro plasmids using Lipofectamine LTX following the protocol of the manufacturer (Thermo Fisher Scientific). After transfection, cells were released into a drug-free medium for 48 h followed by puromycin (1 μ g·mL^{−1}) selection until single colonies were formed. Single clones were expanded, and gene deletion was confirmed by western blotting. eSpCas9(1.1)-T2A-Puro was a gift from Andrea Németh (#101039; Addgene, Watertown, MA, USA).

Generation of I κ B α DN-overexpressing MCF-7 cells

The plasmid used for I κ B α DN overexpression was commercially purchased from Takara (Otsu, Japan; #631923), which contains mutant I κ B α with serine to alanine mutations at residues 32 and 36. MCF-7 cells were transfected with the I κ B α DN plasmid using Lipofectamine LTX following the manufacturer's protocol (Thermo Fisher Scientific). After transfection, cells were released into a drug-free medium for 48 h followed by G418 (100 mg·mL^{−1}) selection until single colonies were formed. Single clones were expanded, and expression of I κ B α was confirmed by western blotting.

Immunohistochemistry of RELA in heart tissue from aged mice

Immunohistochemistry was performed at Genostaff (Tokyo, Japan). Tissue sections were fixed with 4% paraformaldehyde for 10 min. Endogenous peroxidase was blocked with 0.3% H₂O₂ in methanol for 30 min, followed by incubation with G-Block (Genostaff) and avidin/biotin

blocking kit (Vector Laboratories, Peterborough, UK). The sections were incubated with anti-RELA rabbit monoclonal antibody ($0.004 \mu\text{g}\cdot\text{mL}^{-1}$, #8242; Cell Signaling

Technology) at 4°C overnight and then incubated with biotin-conjugated anti-rabbit IgG (Vector Laboratories) for 30 min at room temperature. Subsequently, peroxidase-

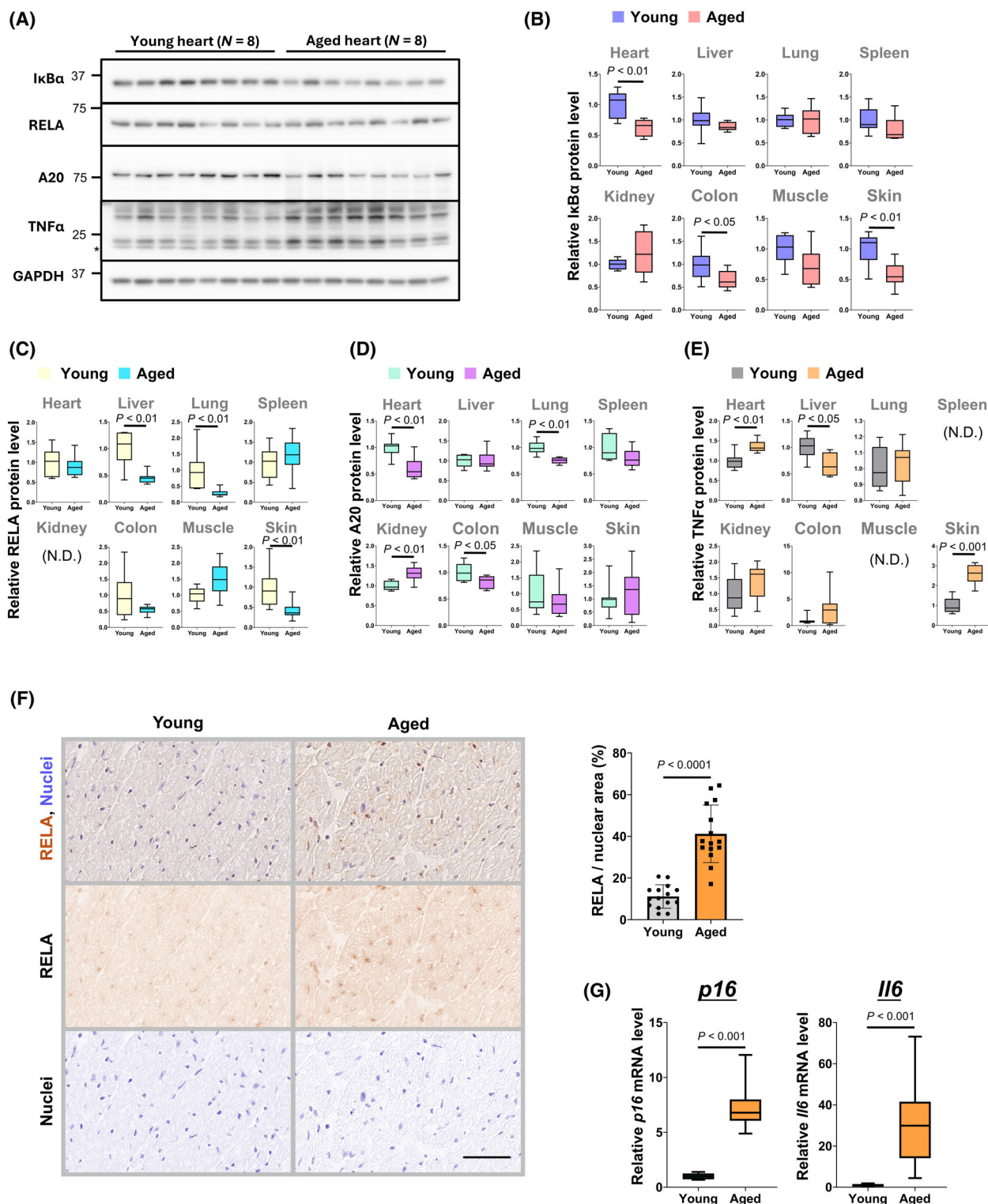


Fig. 5. Expression of I κ B α , A20, and TNF α in young and aged mouse tissues. (A–E) Protein expression of I κ B α , RELA, A20, and TNF α in young and aged mouse tissues. Young mice: 8 weeks old, $n = 8$. Aged mice: 69 to 73 weeks old, $n = 8$. Representative blot images are shown (A). * Signals of interest. Box plots quantifying the relative protein level of I κ B α (B), RELA (C), A20 (D), and TNF α (E). Data represent the median and interquartile range (25th and 75th percentiles) with error bars showing minimum to maximum values. N.D. indicates that the signal was not detected. P values were determined using the Mann–Whitney U test. (F) Immunohistochemistry of RELA in heart tissues from young and aged mice. Young mice: 8 weeks old, $n = 3$. Aged mice: 69–73 weeks old, $n = 3$. Representative images are shown. Scale bar, 50 μ m. Bar plots showing the percentage of RELA-positive staining relative to nuclear areas. Five high-power fields were obtained from each heart tissue. (G) mRNA expression of *Il6* and *p16* in the hearts of young (8 weeks old, $n = 5$) and aged mice (69–73 weeks old, $n = 5$). Data represent the mean $\pm$ SD (F), or median and interquartile range (25th and 75th percentiles), with error bars showing minimum to maximum (B–E, G). P values were determined using Mann–Whitney U test (B–G).

conjugated streptavidin (Nichirei, Tokyo, Japan) was added for 5 min, and peroxidase activity was visualized using diaminobenzidine. The sections were counterstained with Mayer's Hematoxylin (Muto Pure Chemicals Co., Ltd., Tokyo, Japan), dehydrated, and then mounted with Malinol (Muto Pure Chemicals Co., Ltd.).

Stained slides were digitally scanned using a NanozoomerS210 (Hamamatsu Photonics, Hamamatsu, Japan). For histopathological assessment, five random digital images of each mouse heart tissue were captured at 40 $\times$ magnification using NDP VIEW2 software (Hamamatsu Photonics). RELA-positive staining in nuclei was quantified by the IMMUNOTRATIO IMAGEJ plugin [48]. The brown (RELA) and blue (hematoxylin) threshold adjustments were set at 10 and 15, respectively.

RNA-sequencing (RNA-seq) of MCF-7 cells and murine heart tissues

Total RNA was isolated using NucleoSpin RNA Plus (Cat. No. 740984.250; Macherey-Nagel). Library construction and RNA-seq were performed using the Illumina sequencing platform (Rhelixa, Tokyo, Japan). Poly(A) RNA was prepared with the Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Ipswich, MA, USA), the sequencing library was generated with the NEBNext[®] Ultra[™]II Directional RNA Library Prep Kit (New England Biolabs), and paired-end sequencing was processed with the NovaSeq 6000 (Illumina, San Diego, CA, USA).

Acquisition of time course RNA-seq data

Time course bulk RNA-seq data sets (DRA011742 and DRA011743 of DNA Data Bank of Japan) in which gene expression was measured every 15 min after TNF α stimulation in I κ B α KD MCF-7 cells were obtained from our previous study [17].

MCF-7 RNA-seq data analysis

For the analysis of MCF-7 RNA-seq, sequencing reads were preprocessed using nf-core/rnaseq pipeline (v.3.5) [49] (<https://github.com/nf-core/rnaseq/blob/1.4.2/docs/output>).

Read quality was assessed using FASTQC (v.0.11.9). TRIM GALORE (v.0.6.7) was used for adaptor trimming. The resulting reads were mapped onto the GRCh38 genome using STAR (v.2.7.6a) and then quantified by SALMON (v.1.5.2). Protein coding genes registered in GENCODE (https://www.encodegenes.org/human/release_40.html) were used for downstream analysis. Differentially Expressed Genes (DEGs) were identified using the R package DESEQ2 [50] by performing a Wald significance test between gene expression levels in each condition 48 h after TNF α stimulation and calculating adjusted P -values and log₂ fold change (TNF α vs I κ B α KD + TNF α). Genes with adjusted P -value < 0.01 and log₂ fold change > 0.4 were classified as upregulated DEGs, and genes with adjusted P -value < 0.01 and log₂ fold change < -0.4 as downregulated DEGs. The expression pattern of each upregulated DEG in Fig. 4B was obtained using time course TPM data. Time course TPM data were Z-scored after the logarithm of 2 was taken before clustering. Clustering was performed using the partitioning around medoids (PAM) algorithm with the R package cluster. Heat maps were created using the R package COMPLEXHEATMAP [51]. The creation of the regression line and the 95% confidence intervals were calculated by the function “stat_smooth ()” of the R package GGPlot2 [52].

Murine heart RNA-seq data analysis

Mapping of data to perform Gene/transcript quantification was performed using nf-core/rnaseq pipeline (v3.5.) and the default GRCm38 genome. For transcription factor (TF) activity analysis, the DoRothEA [28] Regulon database was used in combination with VIPER [53]. Only TFs with a confidence score of A and interacting with at least 30 genes were included. Genes that were significantly upregulated with aging were identified using the R package DESEQ2 [50]. The criteria were set as adjusted P -values < 0.01 and log₂ fold change > 0.

Assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) and ATAC-seq data analysis

Library preparation, sequencing, mapping, peak calls and annotation, and identification of differentially accessible

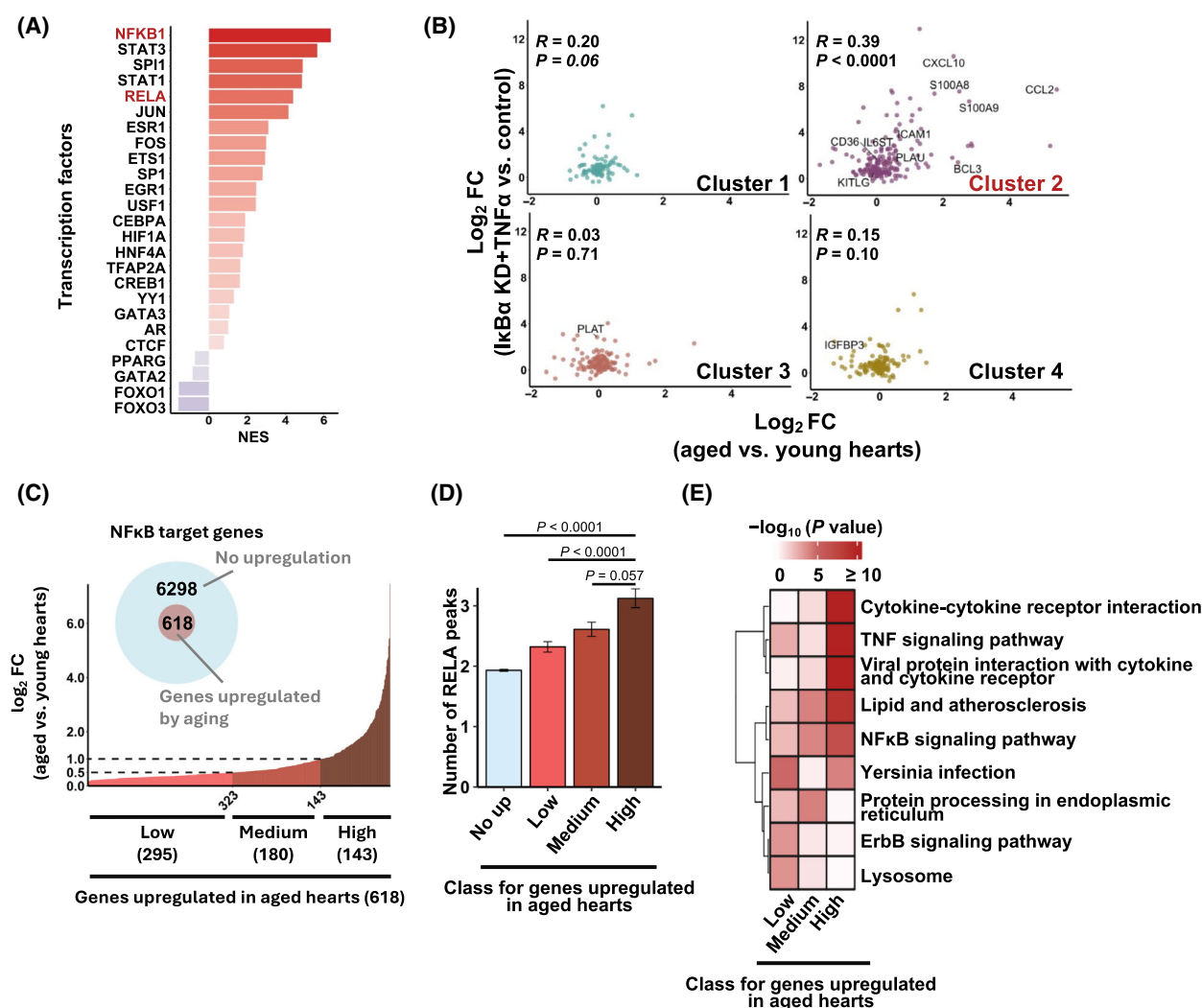


Fig. 6. Enhanced NFκB-DNA binding in the heart tissues of aged mice. (A) Transcription factor (TF) activity predicted by DoRothEA. Only TFs with a confidence score of “A” (highest confidence) and interacting with at least 30 genes were included. The log₂ FC values calculated from the average gene expression levels of the five replicates were analyzed. (B) Correlation between the expression FC (IkBα KD + TNFα vs. Control MCF-7) of genes in cluster 2 and FC of aged vs. young mouse hearts. The log₂ FC values calculated from the average gene expression levels of the five replicates (x axis, mouse heart) and the three replicates (y axis, MCF7) were compared. Cellular senescence-promoting and SASP genes are labeled. The correlation was analyzed using Spearman’s correlation. (C) Classification of the NFκB target genes upregulated by aging in the mouse hearts. The light blue circle indicates NFκB target genes and the pink circle indicates genes whose expression is markedly upregulated in aged mice ($P_{adj} < 0.01$, $FC > 0$). Genes included in the pink circle were classified according to their FC values (Low: $0 < FC \leq 0.5$, Medium: $0.5 < FC \leq 1.0$, High: $1.0 < FC$) calculated from the average gene expression levels of the five replicates. (D) Average count of RELA ChIP peaks annotated with genes in each group classified based on FC expression in aged mouse hearts. Data represent the mean $\pm$ SE. P values were determined using the Kruskal–Wallis test followed by Steel–Dwass test. $n = 6298$ (No up), 295 (Low), 180 (Medium), and 143 (High). (E) KEGG enrichment analysis of the low, medium, and high group genes (618 genes in total) from the analysis in (C).

peaks for ATAC-seq were performed at DNAFORM (Yokohama, Japan). Fragmentation and amplification of ATAC-seq libraries were conducted as described previously [54]. Briefly, approximately 5×10^4 cells were lysed and transposition reactions were carried out using Tn5 Transposase (#FC121-1030; Illumina) at 37 °C for 30 min. The

reaction solution was purified with the Qiagen (Venlo, the Netherlands) MinElute PCR Purification Kit. PCR with custom Nextera PCR primers was then performed for five cycles using NEBNext Q5 Hot Start HiFi PCR Master Mix (New England Biolabs) [55]. The number of additional PCR cycles was determined by qPCR of partially amplified

products [55]. The PCR products were purified using Agencourt AMPure XP beads (A63881; Beckman Coulter, Brea, CA, USA) by double size selection (left ratio: 1.4 $\times$, right ratio: 0.5 $\times$) following the manufacturer's protocol. Paired-end sequencing was performed on the Illumina HiSeq sequencer. Mapping and peak calls were conducted by ENCODE ATAC-seq pipeline (<https://github.com/ENCODE-DCC/atac-seq-pipeline>). Reads were mapped to the GRCh38 reference sequence using BOWTIE2 (v.2.3.4.3), and duplicate reads were removed with PICARD (v.2.20.7) and SAMTOOLS (v.1.9). Peak calling was performed using MACS2 (v.2.2.4) with default parameters. After the removal of the blacklist region, the consistency of peaks was tested by Irreproducible Discovery Rate (IDR) using IDR (v.2.0.4.2). Peak annotation was conducted by HOMER (v.4.9.1) with default settings. Changes in chromatin accessibility near the transcription start site (TSS $\pm$ 5000 bp) per condition in each region and the list of differentially accessible peaks that were differentially accessible under I κ B α KD + TNF α conditions compared to controls were obtained using DESEQ2 (v.1.20.0). The criteria for determining a peak with a difference in accessibility were adjusted P -value < 0.05 and log₂ fold change > 0.

RELA chromatin immunoprecipitation-sequencing (ChIP-seq)

ChIP was carried out using the Simple ChIP Enzymatic Chromatin IP Kit (#9005; Cell Signaling Technology) following the manufacturer's protocol. Protein/DNA was crosslinked using 1% formaldehyde. Nuclei were isolated and sheared using an ultrasonicator (Epishear probe sonicator, Active Motif, Carlsbad, CA, USA). The sheared chromatin was incubated with RELA antibody (ab7970; Abcam) and Dynabeads Protein G beads (10004D; Thermo Fisher Scientific). After elution of ChIP products, DNA was purified using MinElute PCR Purification Kit (28004; QIAGEN), the library was prepared using the TruSeq ChIP Library Preparation Kit (Illumina), and 36-bp single-end sequencing of the libraries was processed with a HiSeq3000 system (Illumina).

RELA ChIP-seq data analysis

Mapping of data to produce BigWig files and call peaks was performed using the nf-core/chipseq pipeline (v.1.2.2) [49] (<https://doi.org/10.5281/zenodo.3240506>) and default GRCh38 genome. BigWig files were quantified by “computeMatrix” and visualized by “plotHeatmap” and “plotProfile” in DEEPTOOLS (v3.5.1) [56]. Each peak was assigned to the TSS of the closest gene by HOMER (v.4.11) [30]. The RELA target genes were defined as the genes whose peak was within $\pm$ 5000 bp from the TSS and were extracted from each cluster. The number of RELA peaks annotated to each target gene was counted using R.

RELA binding site analysis using public databases

The ChIP Atlas peak browser [29] was used to identify RELA binding regions in the mouse genome (mm10). The ChIP-seq peak significance threshold was set to 500 (q value < 1E-50) and ChIP-seq peaks from the groups “TFs and other” and “All cell types” and “RELA” were used. Multiple RELA ChIP peaks in a single region were merged by mergeBed in BEDTOOLS (v.3.5.1) [57]. These RELA ChIP peaks were then assigned to the TSS of the closest gene by HOMER (v.4.11). The final RELA target gene was defined as the gene whose Rela peak was within 500 bp of the TSS. The number of RELA peaks within $\pm$ 10 000 bp from the TSS annotated to each target gene was then counted on R.

KEGG enrichment analysis

KEGG enrichment was compared by the “comparecluster ()” function in CLUSTERPROFILER [58] R package with “fun = ‘enrichKEGG’.” The result was plotted by the R package COMPLEXHEATMAP. Terms with adjusted P -values < 0.05 were displayed.

Quantification and statistical analysis

We performed all experiments at least twice and confirmed similar results as described previously [42]. Statistical analyses were performed using GRAPHPAD PRISM ver. 8.0 software (GraphPad Software, Inc., La Jolla, CA, USA) and R STUDIO ver. 4.2.2 (Rstudio, Boston, MA, USA). For *in vitro* experiments, data from two or more groups were analyzed using Student's t -tests and one-way analysis of variance (ANOVA). For *in vivo* experiments, data from two or more groups were analyzed using the Mann–Whitney U and Kruskal–Wallis tests. Data represented as mean $\pm$ SEM or $\pm$ SD; P values < 0.05 were considered statistically significant.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

ST, K Matsuda, and MO contributed to conceptualization; ST, K Matsuda, and KN contributed to formal analysis; TB and MO contributed to funding acquisition; ST, SS, YI, MT, YM, and AIN contributed to investigation; ST, K Matsuda, KN, YI, MT, YM, AIN, SS, K Moro, TB, and MO contributed to methodology; ST and MO contributed to project administration; K Moro, TB, and MO contributed to resources; K Moro, TB, and MO contributed to supervision; ST, K Matsuda, KN, AIN, and MO contributed to visualization; ST, K Matsuda, SS, KN, AIN, and MO contributed to writing.

Peer review

The peer review history for this article is available at <https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/febs.17227>.

Data availability statement

The sequence data for RNA-seq, ChIP-seq, and ATAC-seq reported in this paper have been deposited in the DNA Data Bank of Japan with accession number DRA015837 (<https://ddbj.nig.ac.jp/resource/sra-submission/DRA015837>). The code is available at <https://github.com/okadalabipr>. All other data will be available upon reasonable request.

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