CELL CYCLE
2024, VOL. 23, NO. 3, 308–327
https://doi.org/10.1080/15384101.2024.2325802

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# Sustained activation of NF-κB through constitutively active IKKβ leads to senescence bypass in murine dermal fibroblasts

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**ABSTRACT**

Although the transcription factor nuclear factor κB (NF-κB) plays a central role in the regulation of senescence-associated secretory phenotype (SASP) acquisition, our understanding of the involvement of NF-κB in the induction of cellular senescence is limited. Here, we show that activation of the canonical NF-κB pathway suppresses senescence in murine dermal fibroblasts. IκB kinase β (IKKβ)-depleted dermal fibroblasts showed ineffective NF-κB activation and underwent senescence more rapidly than control cells when cultured under 20% oxygen conditions, as indicated by senescence-associated β-galactosidase (SA-β-gal) staining and *p16*$^{INK4a}$ mRNA levels. Conversely, the expression of constitutively active IKKβ (IKKβ-CA) was sufficient to drive senescence bypass. Notably, the expression of a degradation-resistant form of inhibitor of κB (IκB), which inhibits NF-κB nuclear translocation, abolished senescence bypass, suggesting that the inhibitory effect of IKKβ-CA on senescence is largely mediated by NF-κB. We also found that IKKβ-CA expression suppressed the derepression of *INK4/Arf* genes and counteracted the senescence-associated loss of *Ezh2*, a catalytic subunit of the Polycomb repressive complex 2 (PRC2). Moreover, pharmacological inhibition of Ezh2 abolished IKKβ-CA-induced senescence bypass. We propose that NF-κB plays a suppressive role in the induction of stress-induced senescence through sustaining *Ezh2* expression.

**ARTICLE HISTORY**
Received 19 September 2023
Revised 16 February 2024
Accepted 26 February 2024

**KEYWORDS**
Senescence bypass; nuclear factor κb; IκB kinase β

## Introduction

Although the acute inflammatory response plays a crucial role in defense against pathogens and tissue repair, chronic inflammation has been linked with aging and age-associated diseases [1]. The nuclear factor κB (NF-κB) transcription factor plays a central role in the inflammatory response through its regulation of genes encoding various cytokines and chemokines [2]. The NF-κB family consists of RelA (p65), c-Rel, RelB, NF-κB1 (p50/p105) and NF-κB2 (p52/p100), which form homo- or heterodimers [3]. Two distinct pathways lead to the activation of NF-κB: the canonical pathway and the noncanonical pathway. The canonical pathway is activated by various stimuli including proinflammatory cytokines and bacterial products, which typically leads to the activation of RelA- or c-Rel containing dimers [3]. The noncanonical pathway is activated by stimuli that are involved in lymphoid tissue organogenesis, and leads to the processing of p100 to yield p52 and the activation of p52/RelB heterodimers [4]. In unstimulated cells, NF-κB is sequestered in the cytoplasm via its interaction with a family of IκBs. After stimulation, the upstream NF-κB regulator IκB kinase (IKK) complex phosphorylates IκBs, which triggers their polyubiquitination and proteasome-dependent degradation, ultimately allowing nuclear translocation of NF-κB [3]. The IKK complex contains two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ [5]. IKKβ is generally considered the dominant kinase in the canonical NF-κB signaling, with the contributions of IKKα and IKKβ varying depending on the cell type and stimulus [6,7].

Numerous studies have revealed the involvement of NF-κB in organismal aging [8]. Several models of accelerated-aging mice and naturally aged mice exhibited increased NF-κB activity and expressed higher levels of inflammatory genes compared to young and healthy mice [9–12]. Importantly, inhibition of NF-κB not only

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/15384101.2024.2325802

suppressed the expression of inflammatory genes but also ameliorated or delayed aging-related phenotypes in these mice [9–13]. Furthermore, mice lacking the gene encoding the inhibitory subunit of NF-κB presented with chronic inflammation and accelerated-aging phenotypes [14,15].

NF-κB is also involved in cellular senescence [16]. Cellular senescence is a state of essentially irreversible cell cycle arrest induced by a potentially oncogenic stressor, and senescent cells undergo distinct morphological and functional changes [17]. These changes include upregulation of cyclin-dependent kinase (CDK) inhibitors such as $p16^{INK4a}$, p21 and, in some cases, $p15^{INK4b}$ and increased activity of lysosomal β-galactosidase, referred to as senescence-associated β-galactosidase (SA-β-gal) [18]. Senescence is also characterized by secretion of various cytokines and matrix modifying enzymes, collectively known as the senescence associated secretory phenotype (SASP) [18]. Although the effects of the SASP can be beneficial depending on its duration and the composition of the secretome [19], a chronic SASP is generally thought to disrupt tissue homeostasis and may contribute to systemic chronic inflammation [1,20]. Recent studies revealed that the accumulation of senescent cells during aging is a contributor to aging-associated pathologies [21–23], and the SASP explains many of the detrimental effects produced by senescent cells [20]. Given that NF-κB plays a pivotal role in the regulation of SASP acquisition [20], NF-κB may be the link between cellular senescence and organismal aging through its regulation of SASP acquisition.

In addition to SASP regulation, NF-κB has also been implicated in the induction or maintenance of cellular senescence [9,14,15,24–33]. Although previous studies mostly point to a pro-senescence and an anti-senescence role for the canonical and the noncanonical NF-κB pathway, respectively, their roles seem highly context-dependent. For example, the ectopic expression of p65, p50 or c-Rel results in cell cycle arrest associated with increased SA-β-gal activity in human keratinocytes [26,27], whereas, when overexpressed, the same subunits exerted no effect on human fibroblasts [34]. Expression of a phospho-mimetic mutant of p65, presumably the active form, induced senescence in the HCT116 human colon cancer cell line, but not in the MCF-7 breast cancer cell line [28]. Furthermore, while RelB and NF-κB2 suppressed the senescence of human fibroblasts and melanoma [30,32], another report showed that *inhibition* of NF-κB activation, including RelB and NF-κB2, abrogated senescence in a different context [24]. Finally, whereas one study showed that p65 depletion promoted escape from Ras-induced senescence [25], suggesting a pro-senescence role of the canonical NF-κB pathway, another study showed that chronic activation of the canonical NF-κB pathway through a constitutively active form of IKKβ delayed Ras-induced senescence [31]. Thus, the precise role of NF-κB in senescence remains unclear.

Previously, we reported that IKKβ-deficient mouse skin fibroblasts promoted the proliferation of melanoma cells when cocultured [35]. We noticed that IKKβ-deficient fibroblasts lost proliferation potential earlier than control cells during serial passages, similar to the pattern observed in cells undergoing premature senescence. This observation prompted us to investigate whether IKKβ deficiency renders cells susceptible to senescence induction. We extended the study by using cells with constitutively active IKKβ and evaluated whether the activation of IKKβ suppresses senescence induction.

## Materials and methods

### *Reagents and antibodies*

Murine recombinant tumor necrosis factor-α (TNF-α) (#575204) and interleukin-1β (IL-1β) (#575102) were purchased from Biolegend. GSK126 was obtained from MedChemExpress. SB 203580 and PD98059 were purchased from AdooQ Bioscience.

The following primary antibodies were used : Lamin A/C (#2032), GAPDH (#2118), β-Actin (#4967), p65 (#6956), phospho-p65 (Ser536) (#3033), IKKβ (#2678), p53 (Rodent Specific) (#32532), phospho-p53 (Ser15) (#9284), IκBα (#4812), phospho-IκBα (#2859), NF-κB1 p105/p50 (#13586), NF-κB2 p100/p52 (#4882), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (#4370), phospho-p38 MAPK (Thr180/Tyr182)

(#4511), phospho-SAPK/JNK (Thr183/Tyr185) (#4668), DYKDDDDK Tag (#2368), phospho-histone H2A.X (Ser139) (#9718), and acetyl-p53 (Lys379) (#2570), CDK6 (#3136), Ezh2 (#5246), RelB (#4922), all purchased from Cell Signaling Technology, and p21 (#sc-6246), which was purchased from Santa Cruz. Phospho-p53 (Ser20) (# PA5–104741), phospho-p53 (Ser37) (#HY-P80843) and phospho-RelA/p65 (Ser276) (#NB100–82086) antibodies were purchased from Invitrogen, MedChemExpress and Novus Biologicals, respectively.

### Mice, preparation of skin fibroblasts and cell culture conditions

Wild-type C57BL/6J mice were obtained from Nihon SLC. Transgenic mice with a constitutively active form of IKKβ (IKKβ-CA) (R26Stop$^{FL}$IKK2CA, #008242) [36], Smooth muscle 22 α (SM22α) Cre mice (Tg (Tagln-cre)1Her/J, #004746) [37], and ROSA$^{mT/mG}$ mice (B6.129(Cg)-Gt(ROSA) 26Sortm4 (ACTB-tdTomato, EGFP) Luo/J, #007676) [38] were obtained from The Jackson Laboratory. IKKβ floxed mice [39] were generously provided by Dr. Michael Karin. IKKβ floxed mice, R26Stop$^{FL}$IKK2CA mice and Sm22αCre mice were crossed into a C57BL/6J background more than 10 times as described previously [40,41]. IKKβ floxed mice were mated with Sm22αCre mice. The resultant IKKβ$^{FL/WT}$ Sm22αCre mice were mated with IKKβ$^{FL/FL}$ mice to generate IKKβ$^{FL/FL}$ Sm22αCre mice [40]. In the following generations, IKKβ$^{FL/FL}$ Sm22αCre mice were mated with IKKβ$^{FL/FL}$ mice. Since this mating protocol generates both Cre-positive (IKKβ$^{FL/FL}$ Sm22αCre) and Cre-negative (IKKβ$^{FL/FL}$) mice, we used Cre-negative (IKKβ$^{FL/FL}$) mice as a control. To generate R26Stop$^{FL}$IKK2CA Sm22αCre mice, R26Stop$^{FL/FL}$IKK2CA were mated with Sm22αCre mice, giving rise to R26Stop$^{FL/WT}$ IKK2CA Sm22αCre and R26Stop$^{FL/WT}$IKK2CA mice. The latter Cre-negative mice were used as a control for IKK2CA. To generate ROSA$^{mT/mG}$ Sm22αCre reporter mice, ROSA$^{mT/mG}$ were mated with Sm22αCre mice.

Dermal fibroblasts were isolated from the dorsal skin of adult female mice (10–20 weeks old). Skin explants were plated on plastic dishes and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Wako, #043–30085) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (Wako, #168–23191) under 3–5% $O_2$ and 5% $CO_2$ conditions (designated passage 0). Cells that had been maintained in 3–5% $O_2$ during early passages (P1-P3) were subsequently passaged according to a 3T3 or 3T2 protocol under 20% $O_2$ conditions to induce their senescence or according to a 3T1 protocol under 3% $O_2$ conditions. In the 3T1, 3T2 and 3T3 protocols, cells are passaged every 3 days, with 1,2 or $3 \times 10^5$cells, respectively, seeded on a 60-mm dish or at the equivalent cell density on a 100-mm dish. Since fibroblasts proliferate faster under 3% $O_2$ conditions than under 20% $O_2$ conditions, we chose the 3T1 protocol for culturing cells under 3% $O_2$ conditions, to prevent cells from reaching confluency before they can be properly analyzed.

### Plasmid construction and lentiviral transduction

To construct a lentiviral vector expressing the non-degradable form of IκBα (IκBαSR), pLVSIN-CMV Pur (Takara, # 6183) and Ubc.IkBSR-Flag Pgk.Cre (Addgene plasmid # 22503) [42] were digested with NotI and EcoRI, and the DNA fragment encoding IκBαSR was inserted into the digested pLVSIN-CMV Pur plasmid using a DNA ligation kit "Mighty Mix" (Takara, # 6023). For the construction of lentiviral vectors expressing wild-type IKKβ and IKKβ-CA, plasmids containing wild-type human IKKβ (Addgene plasmid # 11103) and a constitutively active form of human IKKβ (Addgene plasmid # 11105) were digested with NotI, and the DNA fragments thus formed were inserted into a pLVSIN-EF1α Neo plasmid (Takara, # 6184) or a pLVSIN-CMV Neo plasmid (Takara, # 6181). For p65 and mouse IKKβ-CA overexpression, complementary DNA from IKKβ-CA (Cre-positive) cells was amplified by polymerase chain reaction (PCR) using the following primers: NotI-p65-f, ATATGCGGCCGCATGGACGATCTGT TTCCCCTCA; NotI-p65-r, ATATGCGGCCG CTTAGGAGCTGATCTGACTCAAAAGA; NotI-FLAG-f, ATATGCGGCCGCATGGACTACAAG

GACGACGATGACAAG; and mIKK2-3end-NotI-r, ATATGCGGCCGCTCAGTCACAGGCCTGCTCCA. For CRISPR-Cas9 mediated gene editing, EZH2 sgRNA CRISPR/Cas9 All-in-One Lentivector set (Mouse) (#19601114), Relb sgRNA CRISPR/Cas9 All-in-One Lentivector set (Mouse) (#38803114), Nfkb2 sgRNA CRISPR/Cas9 All-in-One Lentivector set (Mouse) (#31777114) and Scrambled sgRNA CRISPR/Cas9 All-in-One Lentivector (#K010) were purchased from Applied Biological Materials. For small hairpin RNA (shRNA)-mediated knockdown of Ezh2 (ENX-1), ENX-1 shRNA Plasmid (m) (#sc -156,000-SH) and control shRNA Plasmid-A (#sc -108,060) were obtained from Santa Cruz.

Lentivirus particles were produced using Lentiviral High Titer Packaging Mix (TaKaRa) according to the manufacturer's protocol. Briefly, a lentiviral vector plasmid and Lentiviral High Titer Packaging Mix were transfected into 293T cells using TransIT-293 Transfection Reagent (TaKaRa). After 48 hours of incubation, the culture supernatant was harvested, centrifuged, and passed through a 0.45-μm filter. Early-passage (P2-P3) skin fibroblasts and mouse embryonic fibroblasts (MEFs), which had been maintained under 3% $O_2$ conditions, were cultured in DMEM plus 10% FBS containing virus particles with 8 μg/ml polybrene overnight under 3% $O_2$ conditions. The infected cells were treated with 2 μg/ml puromycin for 2–3 days.

### SA-β-gal activity assay

A total of $2 \times 10^4$ cells were seeded on 6-well plates and cultured overnight. The cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde at room temperature for 5 minutes. After washing with PBS, the cells were stained with a solution containing 1 mg/ml X-gal (Takara), 40 mM citric acid/Na phosphate buffer, 5 mM $K_4[Fe(CN)_6]$ 3 $H_2O$, 5 mM $K_3[Fe(CN)_6]$,150 mM sodium chloride and 2 mM magnesium chloride (pH 6.0) overnight. The cells were incubated at 37°C for 1 day and were photographed. At least 80 cells from more than 5 fields were counted.

### Preparation of whole-cell extracts and nuclear/cytoplasmic fraction and Western blotting

Whole cell extracts were prepared from cultured fibroblasts using RIPA buffer (Cell Signaling Technology) with Halt™ Protease Inhibitor Cocktail (Thermo Fischer Scientific).

The extraction of cytoplasmic and nuclear fractions was conducted using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) according to the manufacturer's instructions. Halt™ Protease Inhibitor Cocktail was added to the CER I and the NER.

Extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Bio-Rad Criterion TGX, transferred to Amersham Protran nitrocellulose membranes (GE Healthcare) and analyzed by Western blotting. Immunoreactive bands were visualized by Amersham ECL™ Prime Western Blotting detection reagents and ECL™ Western Blotting detection reagents.

### RNA extraction and RT-qPCR

Total RNA was isolated with TRIzol reagent (Thermo Fisher Scientific) and reverse-transcribed using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO) according to the manufacturer's instructions. The cDNA was analyzed on a StepOnePlus Real-time PCR system (Thermo Fisher Scientific) using Thunderbird SYBR qPCR Mix (TOYOBO).

For measuring the expression of genes associated with senescence, the web tool RefFinder (http://blooge.cn/RefFinder/) [43] was used to determine reference genes for normalization. *GAPDH*, *18S* ribosomal RNA (rRNA), *TMEM199*, *VAMP7* and *WDR55* were chosen as candidate reference genes. The latter three were recently identified as stable reference genes in fibroblast senescence [44]. $IKK\beta^{FL/FL}$Sm22α-Cre (n = 5) and $IKK\beta^{FL/FL}$ (n = 5) fibroblasts which had been maintained under 3% oxygen until P3 were passaged according to the 3T2 protocol under 20% oxygen conditions as described above. Total RNA isolated at P4, P5, P6 and P7 was used to evaluate the stability of candidate gene expression. The comprehensive rankings obtained from

RefFinder showed that *GAPDH* and *TMEM199* showed the highest stability and thus relative mRNA levels of genes of interest were normalized to the geometric mean of *GAPDH* and *TMEM199* expression.

For measuring of expression levels of NF-κB target genes in nonsenescent cells, relative mRNA levels were normalized to the level of *18S* ribosomal RNA.

The primers used in this study are listed in Supplemental material.

### Statistical analysis

The data were analyzed with Microsoft Excel and are presented as the mean ± standard error of the mean. Differences between two groups were determined by two-sided, unpaired Student's t-test. *p* values lower than 0.05 were considered significant.

## Results

### IKKβ protects skin fibroblasts from culture-induced senescence

Dermal fibroblasts were isolated from the dorsal skin of adult IKKβ$^{FL/FL}$Sm22αCre and IKKβ$^{FL/FL}$ mice. As we previously observed [35], IKKβ was depleted in the IKKβ$^{FL/FL}$Sm22αCre dermal fibroblasts under standard culture conditions (DMEM +10% serum) (hereafter, these fibroblasts are called KO cells) (Figure 1(a)). This result suggests that although Sm22α is known as a marker for smooth muscle cells [45], the Sm22α promoter/enhancer was activated in fibroblasts *in vitro* without the addition of inducing agents, and drove the expression of the downstream *Cre* gene. Spontaneous recombination by Sm22αCre in cultured fibroblasts was confirmed by the conversion of the fluorescence in ROSA$^{mT/mG}$ reporter cells from red to green (Figure 1(b)). In the KO cells, IL-1β-induced degradation of IκBα and phosphorylation of p65 at Ser-536 (an IKKβ-phosphorylation site [46] were significantly, although not completely, attenuated compared to their levels in control cells (Figure S1A). Correspondingly, the IL-1β- and lipopolysaccharide-induced upregulation of NF-κB target genes was partially suppressed in KO cells (Figure S1B).

We sought to investigate whether KO cells are susceptible to senescence induction. Mouse fibroblasts undergo senescence after several passages under standard culture conditions (20% oxygen [47] and serum [48]). This outcome is mainly due to accumulated oxidative damage to DNA caused by supraphysiological levels of oxygen (20% $O_2$), and lowering the oxygen level (to 3% $O_2$), which is the physiological oxygen level, can abolish culture-induced senescence in MEFs [47]. Thus, in the present study, primary fibroblasts were maintained at physiological oxygen levels (3–5% oxygen) during early passages, and the cells were subjected to 20% $O_2$ to induce their senescence.

KO and control cells (n = 5) that had been maintained under 3% oxygen conditions until P3 were passaged in parallel under 20% $O_2$ conditions according to a 3T2 protocol (Figure 1(c)) or under 3% $O_2$ conditions according to a 3T1 protocol (Figure 1(d)) as described in Materials and methods. In the 20% $O_2$ group, the KO cells exhibited slower proliferation than the control cells and reached a nondividing state before the control cells (Figure 1(c)). Since the difference between control and KO cells was modest, we repeated the experiment using a different set of primary dermal fibroblasts and obtained a similar result (Figure S2A). We also observed that more KO cells were positively stained for SA-β-gal at every passage analyzed (Figures 1(e,f)), which indicated accelerated senescence. In contrast, when passaged in 3% $O_2$, the proliferation rates of control and KO cells did not decline until the end of the experiment, indicating that both control and KO cells evaded senescence under 3% $O_2$ conditions, although the KO cells exhibited a slightly lower proliferation rate than the control cells (Figure 1(d)). Taken together, we concluded that loss of IKKβ alone is not sufficient to induce senescence, but rather renders cells susceptible to senescence induced by the supraphysiological levels of oxygen.

In contrast to an increase in SA-β-gal activity, no or negligible upregulation was observed in the genes encoding inflammatory cytokines and metalloproteinases analyzed in both control and

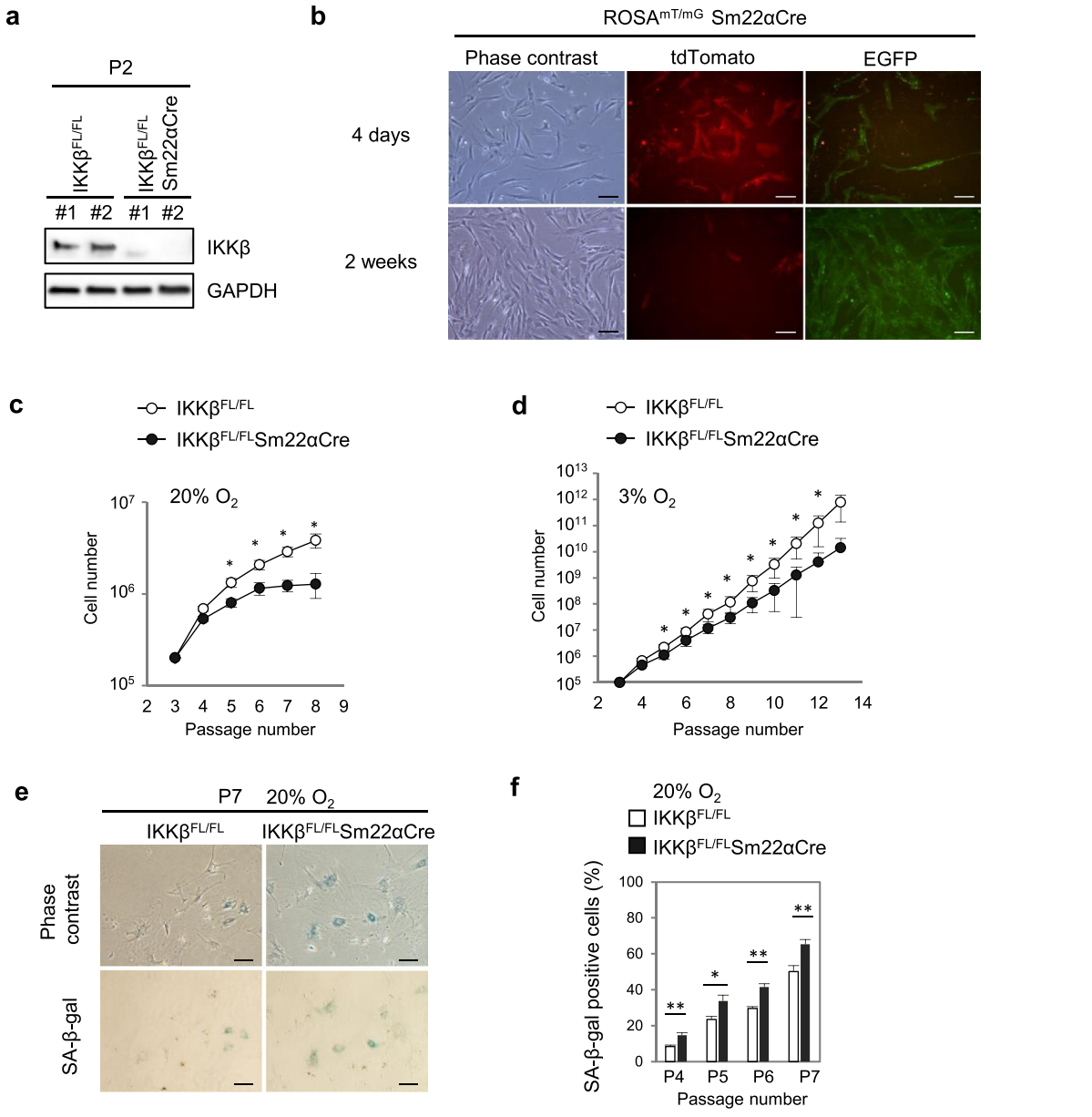


**Figure 1.** IKKβ knockout accelerates senescence in mouse skin fibroblasts.

(a)(b) Sm22αCre-mediated recombination occurred spontaneously under standard culture conditions. Skin explants from adult IKKβ$^{FL/FL}$ and IKKβ$^{FL/FL}$Sm22αCre mice (n = 2) (a) and ROSA$^{mT/mG}$ Sm22αCre reporter mice (n = 2) (b) were plated on plastic dishes and cultured in DMEM supplemented with 10% FBS under 3% $O_2$ and 5% $CO_2$ conditions. The protein levels of IKKβ and GAPDH (the internal control) at passage 2 were measured by Western blotting (a) and the expression of EGFP and tdTomato was examined by fluorescence microscopy (b). Scale bars, 100 µm. (c)(d) Growth curves of skin fibroblasts under 20% and 3% $O_2$ conditions, respectively (n = 5). Primary skin fibroblasts isolated from five adult IKKβ$^{FL/FL}$ and IKKβ$^{FL/FL}$Sm22αCre mice were cultured under physiological oxygen level (3-5% oxygen) conditions until passage 3 (P3), after which time, they were passaged in parallel according to a 3T2 protocol under 20% $O_2$ conditions (c) or maintained under physiological oxygen level (3% oxygen) conditions according to a 3T1 protocol (d). The graph shows the cumulative number of cells in sequential passages. (e) Representative images showing senescence-associated β-galactosidase (SA-β-gal) staining of IKKβ$^{FL/FL}$ and IKKβ$^{FL/FL}$Sm22αCre cells cultured as in (c) (P7: 5 passages under 20% oxygen conditions). Scale bars, 100 µm. (f) Quantification of SA-β-gal staining of skin fibroblasts harvested during the indicated passages. Error bars represent the standard error of the mean. (*$p$ value < 0.05, **$p$ value < 0.01)

KO cells that underwent senescence under 20% $O_2$ conditions (Figure S1C). This outcome was consistent with a previous report showing that mouse fibroblasts exhibited no overt signs of the SASP when induced to senescence via serial passaging under 20% $O_2$ conditions [49].

### *Expression of constitutively active IKKβ induces senescence bypass*

We next evaluated whether activation of IKKβ suppresses culture-induced senescence. To this end, we utilized dermal fibroblasts expressing a constitutively-active form of IKKβ (IKKβ-CA) isolated from R26Stop$^{FL}$IKK2CA mice crossed with Sm22αCre mice. As expected, cells with IKKβ-CA (hereafter, IKKβ-CA cells) showed elevated levels of phosphorylated p65 at Ser-536 and nuclear p65 (Figures 2(a,b)). Accordingly, NF-κB target genes were upregulated in IKKβ-CA cells (Figure S3A), although the upregulation of some of these genes was modest compared to the upregulation induced by IL-1β or TNF-α in control cells (Figure S3B). The expression of certain NF-κB target genes requires IKK-independent post-translational modifications of NF-κB subunits (such as p65 phosphorylation at Ser-276) and changes in chromatin structure, which are partly mediated by mitogen- and stress-activated protein kinases (MSKs) [50], kinases downstream of the ERK and p38 MAPK pathways. Thus, the modest upregulation of NF-κB target genes in IKKβ-CA cells (Figure S3A) was presumably due to the lack of contributions of these pathways. Consistent with this idea, IL-1β or TNF-α treatment, which activates ERK and p38 and induces phosphorylation of p65 at Ser-276 (Figures S3C and S3D), led to further upregulation of some NF-κB target genes (e.g. *IL-6*, *Cxcl2* and *Lif*) in IKKβ-CA cells (Figure S3B), and the IL-1β-induced upregulation of these genes was abrogated by treatment with inhibitors of the ERK and p38 pathways in both control and IKKβ-CA cells (Figure S3E). These results suggest that not all of the NF-κB target genes are fully activated by IKKβ-CA expression alone.

We next measured the proliferation rate of IKKβ-CA cells and control cells during serial passaging. P2 IKKβ-CA cells and control cells were passaged according to a 3T3 protocol under 20% $O_2$ conditions and to a 3T1 protocol under 3% $O_2$ conditions (Figures 2(c,d)). Strikingly, in contrast to control cells, IKKβ-CA cells exhibited no loss of proliferation potential until the end of the culture period (passage 20) under 20% $O_2$ conditions (Figure 2(c)), indicating that senescence was bypassed by IKKβ-CA expression. Consistent with this finding, IKKβ-CA cells exhibited negligible SA-β-gal staining during passage 8 under 20% $O_2$ conditions, while approximately 50% of control cells were SA-β-gal-positive (Figures 2(e,f)).

### *IKKβ-CA-induced senescence bypass depends on NF-κB*

IKKβ phosphorylates various substrates in addition to IκBs [51], and most of the substrates (e.g. p16, p53, and p63 isoform [52–54]) are not directly involved in the NF-κB pathway. We thus wondered whether "senescence bypass" in IKKβ-CA cells depends on NF-κB. To evaluate this possibility, we utilized an undegradable form of IκBα (IκBα super-repressor (SR) [55]), in which serine residues 32 and 36 (IKK-phosphorylation sites) were substituted with alanine, to prevent its degradation and inhibit NF-κB nuclear translocation. After infection with lentivirus carrying a FLAG-tagged IκBαSR plasmid and subsequent selection by puromycin, the expression of IκBαSR was confirmed by Western blotting (Figure 3(a)). As expected, the expression of IκBαSR abrogated the nuclear translocation of p65 and p50 (Figure 3(b)) and the upregulation of NF-κB target genes in IKKβ-CA cells (Figures S4A and S4B). The IKKβ-CA cells expressing IκBαSR were then subjected to serial passaging under 20% $O_2$ conditions, and we found that the senescence bypass by IKKβ-CA cells was abolished by the expression of IκBαSR, as determined by cell proliferation and SA-β-gal staining assays (Figures 3(c-e)). These results suggest that the senescence-inhibiting effect of IKKβ-CA expression was largely dependent on NF-κB activity. To examine whether activation of NF-κB alone can suppress senescence, p65 was overexpressed in wild-type skin fibroblasts through lentiviral transduction (Figures S5A and S5B). Approximately

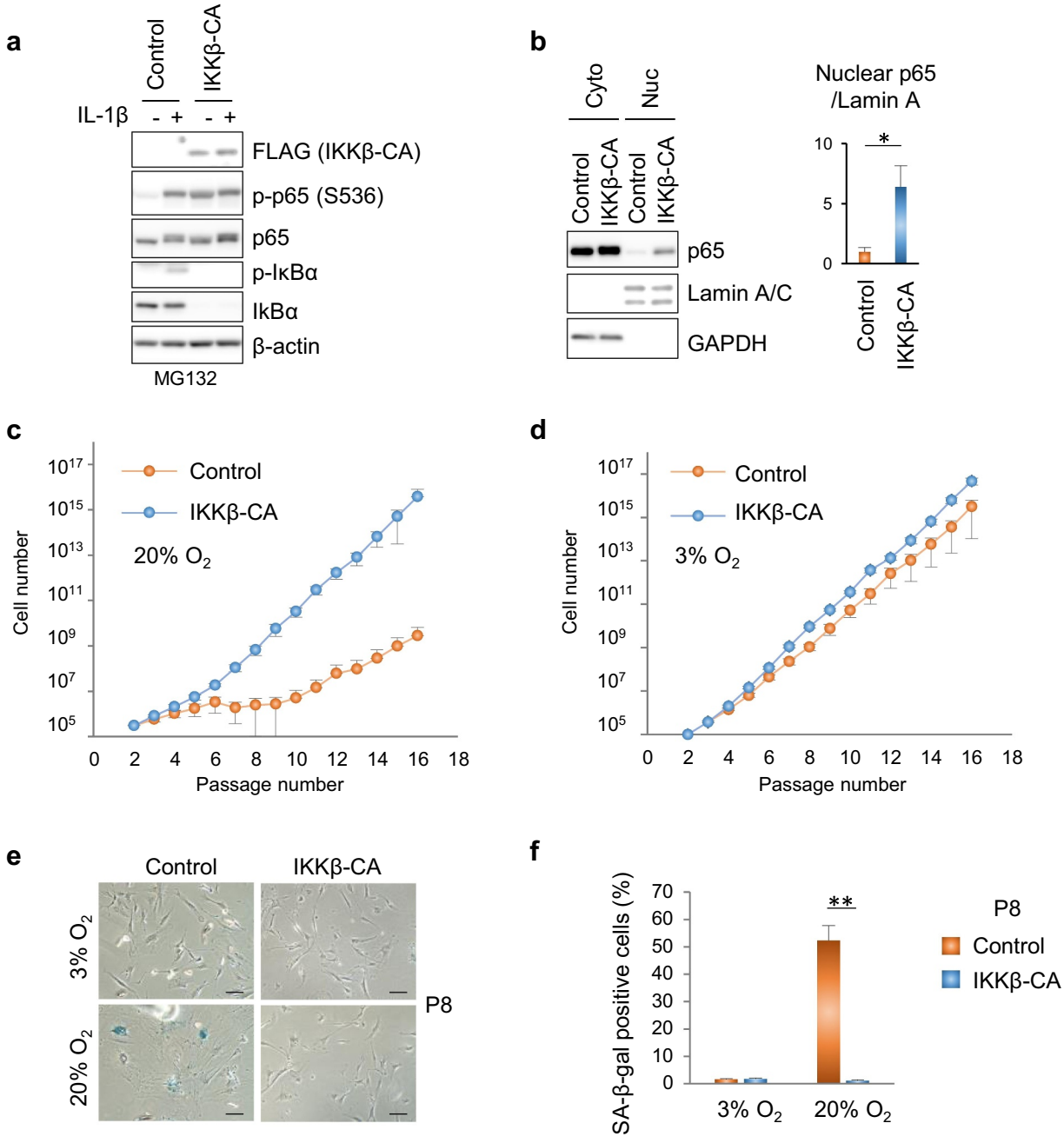


**Figure 2.** Expression of constitutively active IKKβ bypasses cellular senescence.

(a) Control (R26Stop$^{FL}$IKK2CA Cre-negative) and IKKβ-CA (R26Stop$^{FL}$IKK2CA Sm22αCre) skin fibroblasts were treated with MG132 (10 μM), a proteasome inhibitor, for 30 min prior to stimulation with IL-1β (10 ng/ml) for 5 min. Whole-cell lysates were analyzed by Western blotting using the indicated antibodies. Representative images are shown (n = 3). (b) Nuclear and cytoplasmic fractions were prepared from control and IKKβ-CA skin fibroblasts and analyzed by Western blotting. Nuclear p65 levels were quantified by ImageJ and normalized to the level of Lamin A (n = 3). (c)(d) Growth curves of control and IKKβ-CA skin fibroblasts under 20% and 3% oxygen conditions (n = 5). P2 Skin fibroblasts isolated from five adult control and IKKβ-CA mice were passaged in parallel under 20% oxygen conditions according to a 3T3 protocol and under 3% oxygen conditions according to a 3T1 protocol. The graph shows the cumulative number of cells in sequential passages. (e)(f) Representative images and quantification of senescence-associated β-galactosidase (SA-β-gal) staining of control and IKKβ-CA cells in passage 8 cultured under 3% and 20% oxygen conditions (n = 5). Scale bars, 100 μm. Error bars represent the standard error of the mean. (*$p$ value < 0.05, **$p$ value < 0.01)

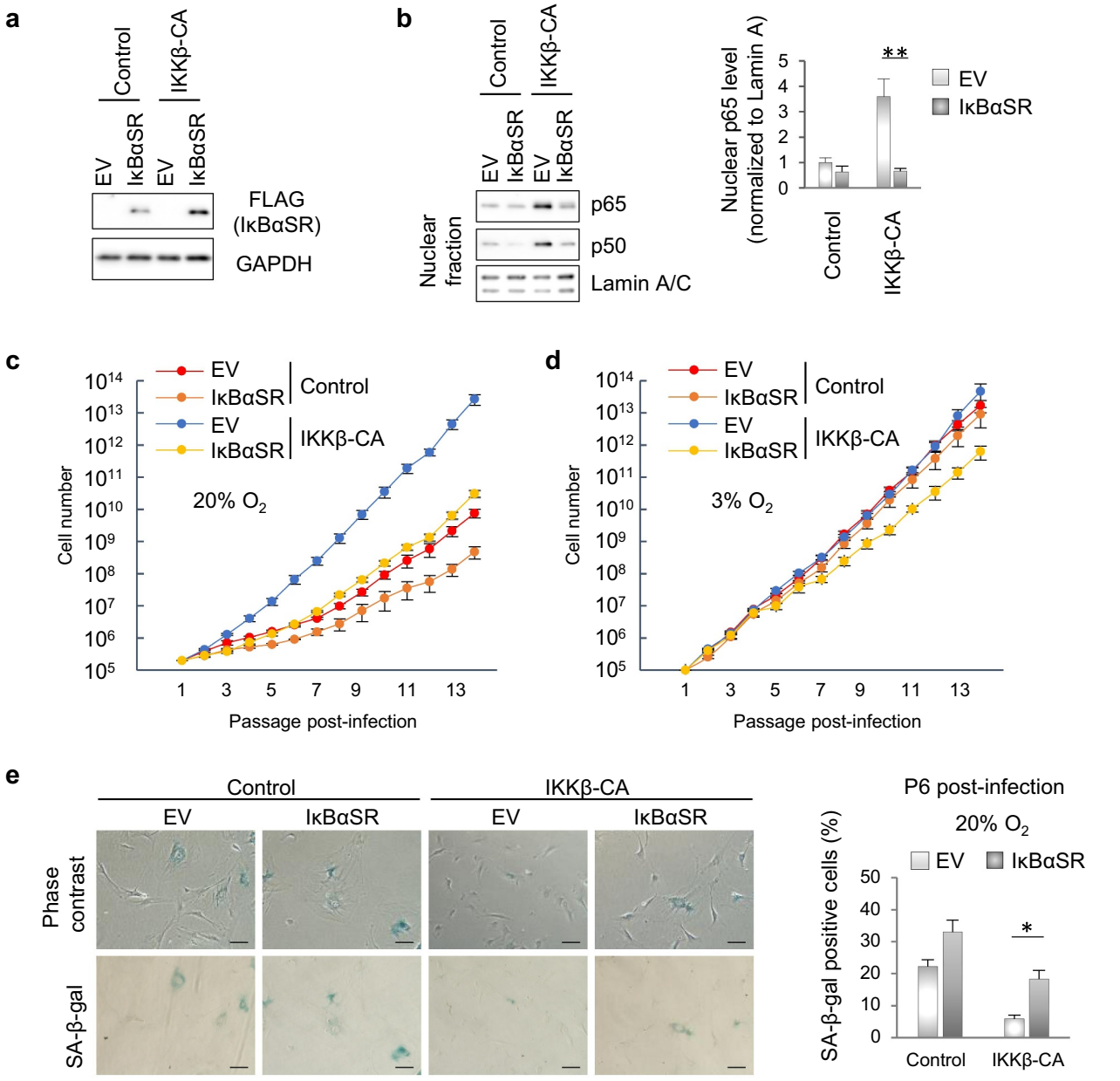


**Figure 3.** Expression of nondegradable IκBα abolishes IKKβ-CA-induced senescence bypass.

(a) Control (R26Stop$^{FL}$IKK2CA Cre-negative) and IKKβ-CA (R26Stop$^{FL}$IKK2CA Sm22αCre) skin fibroblasts were infected with lentivirus carrying an empty vector (EV) or FLAG-tagged IκBαSR. The expression of IκBαSR was confirmed by Western blotting using an antibody against the DYKDDDDK Tag. Representative images are shown (n = 4). (b) Nuclear and cytoplasmic fractions were prepared from control and IKKβ-CA skin fibroblasts harboring EV or IκBαSR. The nuclear p65 and p50 levels were measured by Western blotting, with quantification performed with ImageJ and normalized to the level of Lamin A (n = 4). (c)(d) Growth curves of control and IKKβ-CA skin fibroblasts harboring EV and IκBαSR cultured under 20% and 3% oxygen conditions, respectively. Skin fibroblasts were cultured under physiological oxygen level (3-5% oxygen) conditions until passage 3, at which time, they were infected with lentivirus. The cells were passaged again and treated with 2 μg/ml puromycin for 3 days under 3% oxygen conditions. The selected cells were passaged under 20% oxygen conditions according to a 3T2 protocol (c) or maintained under physiological oxygen level (3% oxygen) conditions according to a 3T1 protocol (d). The graph shows the cumulative number of cells in sequential passages. (e) Representative images and quantification of senescence-associated β-galactosidase (SA-β-gal) staining of control and IKKβ-CA cells harboring EV or IκBaSR cultured under 20% oxygen conditions. Scale bars, 100 μm. Error bars represent the standard error of the mean. (*$p$ value < 0.05, **$p$ value < 0.01)

a 4-fold increase in p65 protein levels was observed at P1 post-infection, although it declined to about 2-fold at P3 post-infection (Figure S5A), supposedly due to transcriptional silencing of the CMV promoter. Despite the decline in the expression level, ectopic expression of p65 significantly reduced the number of SA-β-gal-positive cells in wild-type skin fibroblasts at P5 post-infection (Figure S5B), suggesting a suppressive role of the canonical NF-κB in culture-induced senescence

Senescent cells can promote proliferation or induce senescence in neighboring cells in a paracrine manner through SASP factors (e.g. IL-6, IL-1 and CCL2) [56], which are largely regulated by NF-κB. Furthermore, a proinflammatory cytokine MIF (macrophage migration inhibitory factor), whose expression is regulated by NF-κB [57], was previously shown to delay senescence in MEFs when added to the growth media [58]. Considering that the genes regulated by NF-κB include those encoding various cytokines and chemokines, it is possible that IKKβ-CA cells may affect cell proliferation in a non-cell-autonomous manner, which might help to overcome 20% oxygen-induced cell cycle arrest. To test the hypothesis, we collected conditioned medium using IKKβ-CA cells, and control cells were cultured in the conditioned medium. However, the conditioned medium from IKKβ-CA cells did not significantly affect proliferation of control cells at least during the period tested (Figure S6). Thus, we concluded that IKKβ-CA induces senescence bypass in a cell-autonomous manner.

### *IKKβ-CA-induced senescence bypass is not associated with suppression of p53*

p53 plays a critical role in the induction of senescence, and MEFs from p53-null mice do not undergo senescence [59]. Genetic alterations that allow senescence bypass in mouse cells are frequently associated with the suppression of p53 activity [58,60–62]. Moreover, loss of some p53 target genes, such as *p21* and *pai-1*, facilitated immortalization [63–65].

Thus, we next measured the expression of p53 during serial passaging. If the levels of p53 and its target genes are reduced in IKKβ-CA cells, it may explain the senescence bypass. Surprisingly, however, a significant increase in both total and acetylated p53 protein was observed in IKKβ-CA cells (Figures 4(a), S7A and S7B). Higher levels of nuclear p53 were also found in IKKβ-CA cells than in control cells, although the difference in p53 levels in the nuclear fraction was less profound than that observed in whole-cell extract (Figure 4(b)). A previous report showed that the expression of the p53 gene (*TP53*) is controlled by NF-κB [66]. Consistent with this report, we observed an increase in the p53 mRNA level in IKKβ-CA cells cultured under 20% $O_2$ conditions (Fig. S7C) and the upregulation of p53 at both mRNA and protein levels was almost completely abolished by the expression of IκBαSR (Figures S7D and S7E), suggesting that the increase in p53 expression in IKKβ-CA cells is at least partly mediated by NF-κB-dependent transcription.

Notably, despite the upregulation of p53, the protein levels of p21, a p53 target gene, were comparable in control and IKKβ-CA cells (Figures 4(a) and S7A). In addition, the mRNA levels of other p53 target genes, such as *Mdm2* and *Pai-1*, were also unchanged by IKKβ-CA expression (Figure 4(c)), suggesting that overall p53 activity is comparable in control and IKKβ-CA cells. p53 transcriptional activity is regulated by post-translational modifications, among which phosphorylation at N-terminus transactivation domain such as S15 and S20 (S18 and S23 in mouse, respectively) is induced in response to DNA damage [67]. Since oxygen induces senescence via DNA damage [47], we evaluated the phosphorylation status of p53 at these sites, and no obvious difference was observed between control and IKKβ-CA cells (Figure S7F). Taken together, the above results suggest that IKKβ-CA expression did not reduce the transcriptional activity of p53, and therefore, we concluded that IKKβ-CA-induced senescence bypass is not mediated through the suppression of p53.

We also tested the possibility that senescence bypass in IKKβ-CA cells was due to mutation in the p53 gene. Mouse fibroblasts frequently undergo spontaneous immortalization during long-term cultivation [68], and mutant p53 is commonly observed in these immortalized cells [69]. It is conceivable that IKKβ-CA cells, seemingly resistant to senescence, are in fact derived

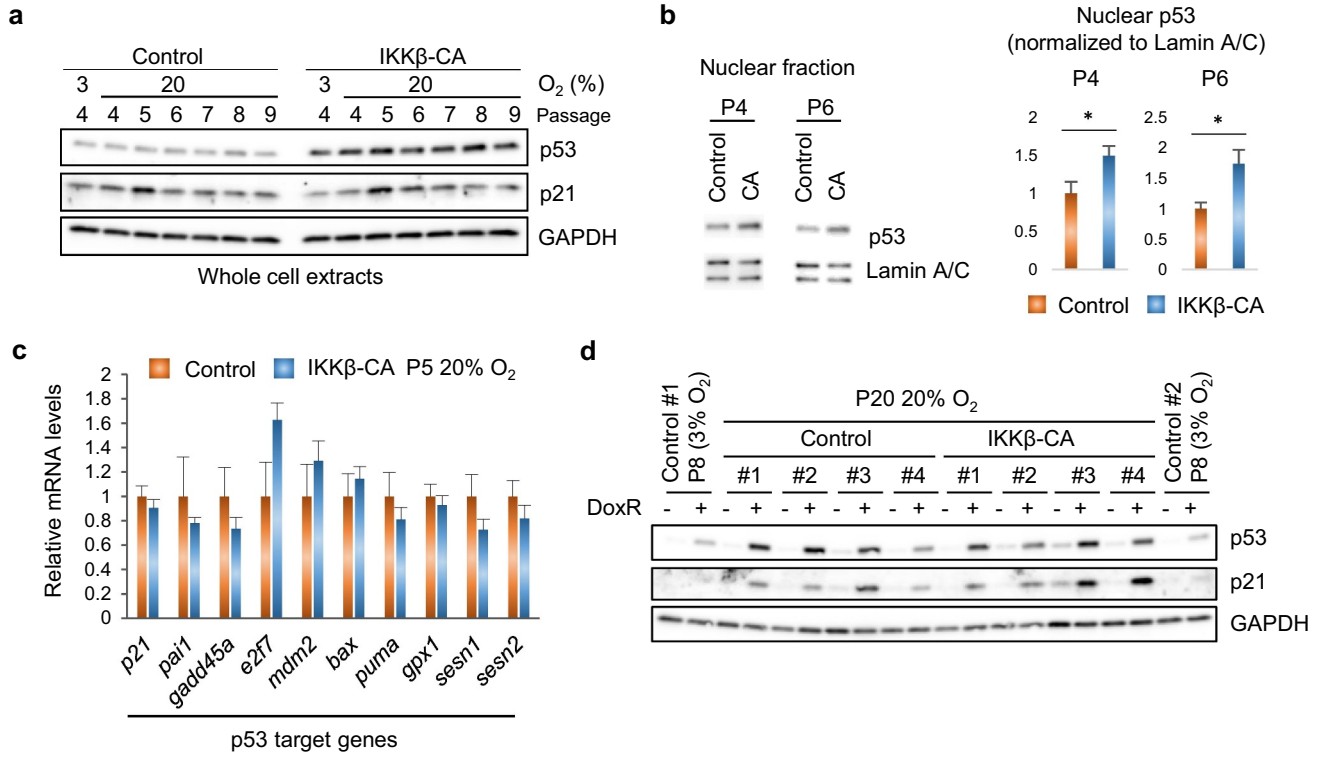


**Figure 4.** IKKβ-CA cells bypass senescence with functional p53 and normal levels of p21.

(a) (b) Control (R26Stop$^{FL}$IKK2CA Cre-negative) and IKKβ-CA (R26Stop$^{FL}$IKK2CA Sm22αCre) skin fibroblasts derived from four mice which had been maintained under 3% oxygen conditions until P3 were passaged under 20% oxygen conditions according to a 3T2 protocol. The cells were harvested during the indicated passages. p53 and p21 levels in whole cell extracts were analyzed by Western blotting (a). The nuclear fraction was extracted during passages 4 and 6, and analyzed by Western blotting (b). Representative images are shown. The nuclear p53 levels were quantified by ImageJ and normalized to the Lamin A/C levels (n = 4). (c) Control and IKKβ-CA skin fibroblasts were cultured as in (A). The mRNA levels of p53 target genes were measured by RT-qPCR and normalized to the level of GAPDH. (d) Control and IKKβ-CA skin fibroblasts derived from four mice were cultured as described in Figure 2(c) until passage 20, at which time they were treated with 500 nM doxorubicin (DoxR) for 4 hours. The p53 and p21 levels were measured by Western blotting. Error bars represent the standard error of the mean. (*$p$ value < 0.05, **$p$ value < 0.01)

from a subset of spontaneously immortalized cells expressing mutant p53 due to a selective advantage during long-term culture. To examine p53 functionality, late-passage cells (P20) cultured under 20% $O_2$ conditions were treated with doxorubicin to induce DNA damage or with Nutlin-3a to inhibit Mdm2 an E3 ligase for p53 to allow p53 accumulation. A Western blot analysis showed that p21 was induced in both control and IKKβ-CA cells following doxorubicin or Nutlin-3a treatment (Figures 4(d) and S7G), suggesting that the p53 gene remained intact in IKKβ-CA cells even after many passages under 20% $O_2$ conditions. Consistent with this finding, the cell cycle of IKKβ-CA cells was arrested in response to ultraviolet light (UV)-induced DNA damage (Figure S7H), whereas the cell cycle in p53- or p21-deleted cells have been reported to fail to undergo arrest in response to genotoxic stress [70].

### *Expression of IKKβ-CA represses the* INK4/Arf *genes and counteracts the senescence-associated downregulation of Ezh2*

The *INK4/Arf* locus, which includes genes encoding $p16^{INK4a}$, *Arf* and $p15^{INK4b}$, is critical to senescence [71]. *Arf*-null MEFs bypass senescence [72] and loss of $p16^{INK4a}$ facilitates escape from senescence in MEFs [73]. The expression of genes encoded at this locus is silenced by the Polycomb repressive complex (PRC) in presenescent cells [74]. Progressive loss of *Ezh2*, the catalytic subunit of PRC2, has been observed in MEFs during serial passaging [75–77], and is considered critical for

the derepression of *INK4/ARF* in cells undergoing senescence [75].

Early-passage IKKβ-CA and control cells (P3), which had been maintained under 3% oxygen conditions, were passaged under 20% oxygen conditions to induce their senescence. Total RNA was isolated and analyzed by RT-qPCR. During the passage under 20% oxygen conditions, $p15^{INK4b}$, $p16^{INK4a}$ and *Arf* were upregulated and *Ezh2* levels declined in control cells (Figure 5(a)), similar to the observation described in MEFs [75]. In contrast, IKKβ-CA cells maintained *Ezh2* mRNA levels and the expression levels of the genes encoded within the *INK4/ARF* remained unchanged during the same period (Figure 5(a)). Sustained expression of Ezh2 in IKKβ-CA cells was further confirmed by Western blot analysis (Figure 5(b)). We also observed that expression of IκBαSR reduced the *Ezh2* mRNA level and increased $p16^{INK4a}$, $p15^{INK4b}$ and *Arf* levels in IKKβ-CA cells (Figure 5(c)). These results suggest that activation of IKKβ counteracts the senescence-associated downregulation of *Ezh2* and prevents the derepression of the *INK4/Arf* genes in an NF-κB-dependent manner. In support of this finding, *Ezh2* was more rapidly lost in KO cells than in control cells during passages under 20% oxygen conditions, and KO cells expressed higher levels of $p16^{INK4a}$ mRNA than control cells (Figure 5(d)). Furthermore, IκBαSR expression alone in control cells reduced *Ezh2* and increased $p16^{INK4a}$ mRNA (Figure 5(c)), and correspondingly reduced their proliferation under 20% oxygen conditions (Figure S2B)

### *IKKβ-CA-induced senescence bypass requires Ezh2 activity*

Sustained PRC activity can suppress the derepression of the *INK4/Arf* genes and is sufficient to induce senescence bypass in MEFs [78,79]. Since IKKβ-CA counteracted the downregulation of *Ezh2*, we evaluated whether IKKβ-CA-induced senescence bypass requires PRC activity by using the Ezh2 inhibitor GSK126. 5 days of treatment with 5 μM GSK126 under 3% $O_2$ conditions successfully induced the expression of $p16^{INK4a}$ and $p15^{INK4b}$ in both control and IKKβ-CA cells (Figure 5(e)). Correspondingly, treatment with GSK126 under 3% $O_2$ conditions blocked proliferation in both control and IKKβ-CA cells (Figure 5(f)) and significantly increased the number of SA-β-gal-positive IKKβ-CA cells (Figure 5(g)). Furthermore, under 20% $O_2$ conditions, IKKβ-CA cells did not bypass senescence in the presence of the Ezh2 inhibitor (Figure 5(f)), suggesting that the maintenance of Ezh2 activity is a prerequisite for IKKβ-CA-induced senescence bypass. Taken together, our results suggest that IKKβ prevents derepression of gene expression in the *INK4/Arf* locus and suppresses senescence, possibly by counteracting the senescence-associated downregulation of *Ezh2*.

## Discussion

### *IKK activation and cell proliferation*

Previous studies reported that ectopic expression of IKKβ-CA mediated through viral transduction inhibited the proliferation of human lung fibroblasts and MEFs [80,81]. Seemingly contradictory to these reports, in the present study, IKKβ-CA expression driven by the endogenous Rosa26 promoter did not inhibit the proliferation of mouse dermal fibroblasts; in contrast, it conferred a growth advantage under 20% $O_2$ conditions. Considering that the reported growth inhibition was transient [80], the long-term culture in our experiments may have allowed the outgrowth of a subset of adapted cells. However, the acute induction of IKKβ-CA expression mediated through lentiviral transduction of the *Cre* gene in $R26Stop^{FL}IKK2CA$ (Cre-negative) cells did not block proliferation (Figures S8A and S8B). Notably, we observed that lentiviral transduction of IKKβ-CA indeed inhibited the proliferation of both MEFs and mouse skin fibroblasts (Figures S8C-F). Furthermore, the proliferation inhibition was abrogated by the coexpression of IκBαSR (Figures S8G and S8H), which was similar to the above-mentioned reports [80,81]. Importantly, proliferation blockade was observed only when the IKKβ-CA gene was highly expressed (driven by the EF1α promoter), but not when it was moderately expressed (driven by the CMV promoter) (Figures S8C and S8D). Thus, the relatively low expression levels of IKKβ-CA driven by the

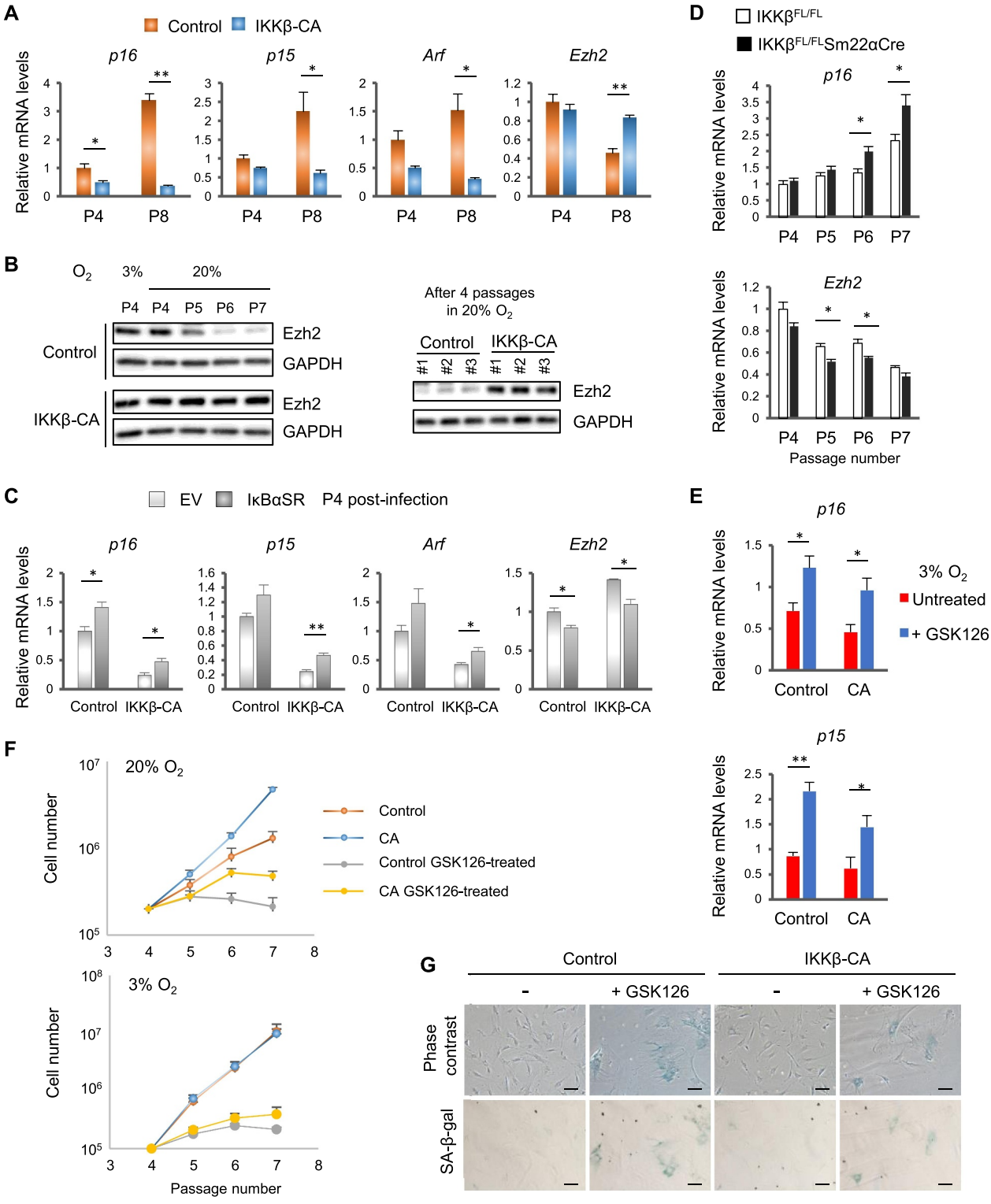


**Figure 5.** IKKβ suppresses the derepression of the genes in the *INK4/Arf* locus and counteracts the senescence-associated down-regulation of Ezh2.

(A)(B)(C) IKKβ-CA and control cells (n = 4) which had been maintained under 3% oxygen conditions until P3 were passaged under 20% oxygen conditions according to a 3T2 protocol (a)(b). IKKβ-CA and control skin fibroblasts harboring an empty vector (EV) or IκBαSR were generated and passaged as described in Figure 3(c). The mRNA levels of $p16^{INK4a}$, $p15^{INK4b}$, *Arf* and *Ezh2* were measured by RT-qPCR and normalized to the geometric mean of *GAPDH* and *TMEM199* expression levels (a)(c). Ezh2 levels in whole-cell extracts were analyzed by Western blotting (b). (d) $IKK\beta^{FL/FL}$Sm22α-Cre and $IKK\beta^{FL/FL}$ cells (n = 5) which had been maintained under 3% oxygen until P3 were passaged according to a 3T2 protocol under 20% oxygen conditions. The mRNA levels of $p16^{INK4a}$ and *Ezh2* were measured by RT-qPCR at the indicated passages and normalized to the geometric mean of *GAPDH* and *TMEM199* expression

Rosa26 endogenous promoter (Figure S8C) may explain the lack of proliferation inhibition in our experiments. The mechanism by which low and high levels of IKKβ-CA differentially affects cell proliferation needs further investigation.

### Chronic activation of IKK induces senescence bypass in an NF-κB-dependent manner

IKKβ-CA cells bypassed culture-induced senescence, and expression of IκBαSR abrogated the senescence bypass-inducing effect of IKKβ-CA. Since IκBα can potentially bind and inhibit CDK4 directly *in vitro* [82], we cannot exclude the possibility that IκBαSR directly blocked cell cycle progression independently of NF-κB. However, considering that p65 overexpression alone suppressed senescence in wild-type skin fibroblasts (Figure S5), we conclude that the effect of IκBαSR is most likely attributable to its inhibition of NF-κB activation. Thus, we propose that chronic activation of NF-κB suppresses senescence caused by culture-induced stress.

Our observations contrast with previous reports suggesting a pro-senescence role of the canonical NF-κB signaling [24,25]. The apparent discrepancy may arise from differences in signaling networks in different cell types and contexts, such as the presence of cooperating factors for inducing senescence. Alternatively, NF-κB may have differential effects on senescence depending on its activity as previously reported for the Ras-Raf-MEK-ERK pathway where only the high intensity, but not moderate level, of the activation in the pathway can induce senescence [83]. NF-κB activity appeared modest in IKKβ-CA cells (Figure S3) and its further activation may shift its effect from growth-promotion to senescence induction. So far, we observed no effect of further NF-κB activation by exogenous IL-1β on the senescence bypass phenotype of IKKβ-CA cells (Figure S9). However, since cytokine treatment only transiently activates NF-κB (Figure S3), presumably due to negative feedback regulation, genetically engineered mutations that constitutively increase NF-κB activity in addition to IKK activation may be appropriate to further test the above hypothesis.

NF-κB target genes include various antioxidant genes, such as manganese superoxide dismutase and ferritin heavy chain [84]. Therefore, it is reasonable to speculate that sustained activation of these genes suppresses intracellular reactive oxygen species accumulation, the main cause of culture-induced senescence in murine cells [47], and thereby allows senescence bypass. However, H2AX phosphorylation levels (γH2AX), a DNA double-strand-break marker, were not lower in IKKβ-CA cells (Figures S7A and 7F), implying that DNA damage accumulation was not suppressed in IKKβ-CA cells and that the senescence bypass phenotype may not be simply explained by increased protection against culture-induced damage.

The senescence bypass in IKKβ-CA depends on NF-κB activation; however, this does not necessarily mean that genes directly targeted by NF-κB mediate senescence bypass. It has been reported that NF-κB can physically interact with other transcription factors and cofactors, thereby affecting the expression of genes not targeted by NF-κB [81,85,86]. For example, p65 and p53 compete for a limited pool of coactivators, namely, p300 and CBP, and therefore, p65 indirectly inhibits p53-dependent transcription [87,88]. These potential indirect effects should also be considered in future studies to identify genes critical for the senescence bypass phenotype.

### Effects of IKKβ-CA on p53 activity

It has been established that there exists a mutually antagonistic relationship between p53 and IKKβ/NF-κB [89], and several mechanisms have been proposed to explain the negative effect of IKKβ/NF-κB on p53 activity, such as the regulation of

levels. (e) After 5 days of treatment with 5 μM GSK126, an Ezh2 inhibitor, under 3% oxygen conditions, total RNA was isolated from IKKβ-CA and control fibroblasts and the mRNA levels of $p16^{INK4a}$ and $p15^{INK4b}$ were measured by RT-qPCR and normalized to the levels of *18S* rRNA. (f) Control and IKKβ-CA cells were serially passaged in the presence or absence of 5 μM GSK126 in 20% and 3% oxygen. (g) Representative images showing senescence-associated β-galactosidase (SA-β-gal) staining in control and IKKβ-CA cells treated with or without 5 μM GSK126 for 5 days (n = 4). Scale bars, 100 μm. Error bars represent the standard error of the mean. (*$p$ value < 0.05; **$p$ value < 0.01)

*Mdm2* [90] and phosphorylation of p53 by IKKβ and its subsequent degradation [52]. Therefore, we hypothesized that constitutive activation of IKKβ may inhibit cellular senescence through the suppression of p53 activity. Unexpectedly, however, p53 protein levels were found to be significantly higher in IKKβ-CA cells than in control cells. Lentiviral introduction of IKKβ-CA also increased p53 protein levels in MEFs (Figure S8C). It should be noted that the level to which the p53 protein is increased by IKKβ-CA expression is much lower than that induced by doxorubicin treatment (Figure 4(d)), which may at least partly explain the lack of increase in the mRNA levels of p53 target genes. Since p53 target gene levels were comparable in control and IKKβ-CA cells, we concluded that IKKβ-CA expression did not have a significant impact on overall p53 activity. These results also imply that IKKβ-CA expression induces senescence bypass likely independently of the p53-p21 pathway.

### Possible mechanism(s) underlying IKK-induced senescence bypass

We observed that *Ezh2* expression was sustained in IKKβ-CA cells during serial passaging, and KO cells lost *Ezh2* expression earlier than control cells, suggesting that IKKβ counteracts the senescence-associated loss of *Ezh2* expression. A previous report showed that the *Ezh2* gene is a direct target of NF-κB [32], and RelB and NF-κB2 (the noncanonical pathway) regulate *Ezh2* expression [30,32]. We observed that *RelB* and *NF-κB2* mRNA levels were increased in IKKβ-CA cells (Figure S10A), which was expected since these genes are known NF-κB targets [91,92]. However, although the level of total p52 protein (the active form of NF-κB2) was increased in IKKβ-CA cells (Figure S10B), that of the nuclear fraction was unchanged (Figure S10C), suggesting that the noncanonical NF-κB pathway was not activated in IKKβ-CA cells. Hence, the sustained expression of *Ezh2* in IKKβ-CA cells was unlikely to be a result of the direct upregulation by the noncanonical NF-κB pathway. Indeed, CRISPR/Cas9-mediated depletion of NF-κB2 or RelB did not reduce Ezh2 protein levels in IKKβ-CA cells (Figure S10D and S10E), and did not abolish the IKKβ-CA-induced senescence bypass (Figure S10F), indicating that the noncanonical NF-κB pathway is dispensable for the senescence bypass phenotype.

IKKβ-CA may prevent upstream events that culminate in the downregulation of *Ezh2* under senescence-inducing conditions. Several factors, including Kat6a (Moz), Snf5, Twist1, c-Myc and Kdm2b (FBXL10), have been proposed to regulate the *Ezh2* gene [93–97], and the latter three were reported to be NF-κB-dependent genes [98–100]. However, none of these genes were upregulated in IKKβ-CA cells (Figure S11A). Notably, *Ezh2* expression is also regulated by E2F transcription factors [101], which play critical roles in controlling the expression of key regulators of cell proliferation. Ezh2 in turn regulates the expression of *INK4/Arf* genes and therefore can indirectly affect the CDK-pRB-E2F pathway. Thus, E2F and Ezh2 constitute a feedback loop (Figure S11B). Hence, it is difficult to determine whether IKKβ regulates the upstream regulator of *Ezh2* expression or whether the sustained expression of *Ezh2* in IKKβ-CA cells is merely an outcome of sustained proliferation (sustained E2F activity). In either case, our experimental results based on the action of an Ezh2 inhibitor GSK126 (Figure 5) suggested that in the absence of Ezh2 activity, IKKβ-CA did not induce senescence bypass. Thus, we hypothesize that the maintenance of *Ezh2* expression may be a mechanism explaining the senescence bypass of IKKβ-CA cells.

Although GSK126 is a highly selective inhibitor for Ezh2 [102], we cannot exclude the possibility that the results obtained using GSK126 may be due to its off-target effects. We thus attempted to deplete Ezh2 using shRNA and CRISPR/Cas9 system targeting *Ezh2*. However, although both lentivirus-mediated shRNA and CRISPR/Cas9 system reduced Ezh2 protein levels to nearly 20% of those in IKKβ-CA cells with control vectors at P1 post-infection, Ezh2 protein levels in these cells recovered at a later passage (P5 or P6 post-infection) to nearly 60% of those in IKKβ-CA cells with control vectors, which are comparable to or more than the levels in early passage (pre-senescent) control cells (Figures S12A, B, D and E). Consistent with the incomplete depletion of Ezh2, proliferation of IKKβ-CA cells was

only partially affected (Figures S12C and S12F). Although we were unable to fully demonstrate the importance of Ezh2 in IKKβ-CA-induced senescence bypass due to the limitations in the knockdown and knockout efficiency, the results suggest that Ezh2 at least contributes to the vigorous proliferation of IKKβ-CA cells under 20% oxygen conditions, and are not inconsistent with the result obtained using the inhibitor. We speculate that the recovery of Ezh2 levels at later passages was due to a selective advantage of the cells in which Ezh2 was poorly depleted and their dominance at later passages. More efficient methods for Ezh2 depletion, such as Cre induction in $Ezh2^{FL/FL}$ cells [103], are required to further test the role of Ezh2 in IKKβ-CA-induced senescence bypass.

Given that immortal IKKβ-CA cells express p21 proteins at levels equivalent to those observed in control cells undergoing senescence (Figures 4(a) and S7A), IKKβ-CA may additionally modulate the expression or function of cell cycle regulators such as CDKs and cyclins, thereby rendering cells insensitive to p21 expressed at the normal level. NF-κB regulates the transcription of several CDKs and cyclins [104], and among the genes evaluated, only *Cdk6* was upregulated in IKKβ-CA cells in an NF-κB-dependent manner (Figure S13). The cyclinD-CDK4/6 complex has been proposed to bind and sequester the p21 protein, thereby freeing CDK2 from inhibition by p21 [105]. The elevated levels of CDK6 in IKKβ-CA cells may indirectly confer resistance to p21 activity through a similar mechanism. Additional experiments are needed to clarify whether the increase in the CDK6 level contributes to senescence bypass of IKKβ-CA cells.

## Acknowledgements

We are grateful to Prof. Michael Karin (University of California, San Diego, CA) for generous permission to use IKKβ flox mice.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

## Funding

This research was supported by AMED under Grant Number DNW-19009 and DNW-20021.

## Author contributions

MH and KSH designed and performed the study; TK, KO and NA supervised the study; MH, KSH and NA analyzed and interpreted the data; MH and KSH wrote the manuscript

## Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

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