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Caspase-3 cleaved p65 fragment dampens NF- κ B-mediated anti-apoptotic transcription by interfering with the p65/RPS3 interaction

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Abstract

Caspase-3-mediated p65 cleavage is believed to suppress nuclear factor-kappa B (NF- κ B)-mediated anti-apoptotic transactivation in cells undergoing apoptosis. However, only a small percentage of p65 is cleaved during apoptosis, not in proportion to the dramatic reduction in NF- κ B transactivation. Here we show that the p65¹⁻⁹⁷ fragment generated by Caspase-3 cleavage interferes with ribosomal protein S3 (RPS3), an NF- κ B “specifier” subunit, and selectively retards the nuclear translocation of RPS3, thus dampening the RPS3/NF- κ B-dependent anti-apoptotic gene expression. Our findings reveal a novel cell fate determination mechanism to ensure cells undergo programmed cell death through interfering with the RPS3/NF- κ B-conferred anti-apoptotic transcription by the fragment from partial p65 cleavage by activated Caspase-3.

Keywords

Fate determination; Apoptosis; NF- κ B; RPS3; Gene transcription; Caspase-3 cleavage

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Author contributions

FW conceived and supervised the study; EMW, KF and FW designed experiments; EMW, KF, AH and XS performed the experiments; EMW, KF and FW analyzed data; EMW and FW wrote the manuscript; EMW, KF and FW made manuscript revisions.

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1. Introduction

The nuclear factor-kappa B (NF- κ B) signaling pathway is pivotal for a wide array of cellular processes [1–5]. Albeit the signaling cascade that leads to NF- κ B activation has been extensively studied [6–11], it remains elusive how NF- κ B specifically activates its target genes [12–15]. Ribosomal protein S3 (RPS3) was identified as an essential component in NF- κ B complexes where RPS3 can interact with the NF- κ B p65 subunit in the cytoplasm and the nucleus [16]. The subcellular localization of RPS3 is precisely regulated by the NF- κ B activation signaling cascade, in particular the Inhibitor of κ B (I κ B) kinase beta (IKK β)-mediated phosphorylation of RPS3 at serine 209 (Ser209) plays an important role for the nuclear translocation and function of RPS3 [17]. The synergistic RPS3-p65 interaction in the nucleus facilitates NF- κ B to achieve optimal binding capacity to target κ B sites, thus conferring the promoter selectivity and transcriptional specificity of NF- κ B [13,16]. An increasing number of studies have highlighted the pathophysiological relevance of the RPS3/NF- κ B signaling pathway. Specifically, RPS3/NF- κ B signaling plays a critical role in immune gene expression in lymphocytes [16], B cell development [18], and islet cell survival [19], and in host defense against enteric pathogen infections [17,20–22]. Blocking the nuclear translocation of RPS3 by small interfering RNA (siRNA) or several bacterial virulence proteins attenuates RPS3/NF- κ B target gene transcription, without affecting NF- κ B stimuli-triggered p65 nuclear accumulation [16,21,22]. Therefore interfering with RPS3-p65 interaction selectively inhibits the transcription of RPS3-dependent, but not all p65-required, NF- κ B target genes, representing a novel strategy to selectively, rather than globally, inhibit NF- κ B transactivation.

Aberrant NF- κ B activation has been acknowledged in various cancers; therefore inhibition of NF- κ B has been explored as a promising intervention in cancer therapy [23,24]. That said, blocking NF- κ B activation by targeting the regulators, in particular upstream ones, of the NF- κ B signaling pathway may introduce unwanted global NF- κ B inhibition in normal and tumor cells [12,25]. It is still a significant challenge to selectively attenuate NF- κ B transactivation in tumor cells without affecting adjacent normal tissue. The essential role of RPS3 in directing NF- κ B to a subset of genes makes it a potential target for selective NF- κ B inhibition in cancer cells. Moreover, RPS3 was recently revealed as a physiologic determinant of NF- κ B-mediated transcription of anti-apoptotic genes in macrophages, including *Birc3* (encoding cellular inhibitor of apoptosis protein-2, cIAP2), *Bcl2l1* (encoding B-cell lymphoma-extra large, Bcl-XL), and *Xiap* (encoding X-linked inhibitor of apoptosis protein, XIAP) [26]. We recently showed that N-terminal fragments of p65, generated by ectopic expression or pathogen protease cleavage, selectively retard RPS3 nuclear translocation and RPS3-conferred NF- κ B gene transcription, without affecting p65 [22,27]. Intriguingly, another N-terminal fragment (amino acids 1-97) of p65 was previously reported by Caspase-3 cleavage during apoptosis [28–30]; however, the relevance of such a p65 fragment in cell fate determination, especially the balance between NF- κ B-mediated anti-apoptotic transcription and programmed cell death, remains unknown. Here we report that Caspase-3 cleaves only a small percentage of p65, thus generating a limited amount of the N-terminal p65¹⁻⁹⁷ fragment during apoptosis. The generated p65¹⁻⁹⁷ fragment interferes with the endogenous RPS3-p65 interaction and selectively retards the nuclear translocation

of RPS3, but not p65, which dampens the transactivation of RPS3-dependent anti-apoptotic NF- κ B genes. Hence our results reveal a novel cell fate determination mechanism during apoptosis by which the Caspase-3 cleavage-generated p65 N-terminal fragment interferes with the RPS3/NF- κ B-conferred anti-apoptotic transcription, thus ensuring cells undergo programmed cell death.

2. Material and Methods

2.1 Cell line, antibodies, and plasmids

HEK293T (ATCC, Manassas, VA) and WT and Bax^{-/-} mouse embryonic fibroblasts (kindly provided by Dr. Richard Youle) were cultured in DMEM medium containing 10% fetal calf serum, 2 M glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. Antibodies used were: p65 (C-terminus, C-20, sc-372), p65 (N-terminus, F-6, sc-8008x), and I κ B α (c-21, sc-371) from Santa Cruz Biotechnology (Dallas, TX); β -actin (AC-15, A5441) from Sigma-Aldrich (St. Louis, MO); PARP-1 (46D11, 9532) and cleaved Caspase-3 (D175, 9661) from Cell Signaling Technology (Danvers, MA); GFP (7.1 and 13.1, 11814460001) from Roche Applied Science (Indianapolis, IN); Hsp90 (610418) and XIAP (610762) from BD Biosciences (San Jose, CA); Bcl-XL (N1C3, GTX105661) from GeneTex (Irvine, CA); RPS3 and phosphorylated RPS3 as previously described [16,17]. Tumor necrosis factor was purchased from R&D System (Minneapolis, MN). The GFP-tagged p65 plasmid was previously described [27]. The GFP-p65¹⁻⁹⁷ plasmid was created by inserting the appropriate fragments into the pEGFP-N1 vector (Clontech Laboratories, Mountain View, CA) using the InFusion Cloning System (Clontech Laboratories).

2.2 RNA interference and transfection

RPS3 siRNA was described previously [17]. Transient transfection of siRNA or plasmids into HEK293T cells or MEFs was performed using Lipofectamine RNAiMAX or Lipofectamine 2000 (Life Technologies, Grand Island, NY) according to the manufacturer's instructions.

2.3 Immunofluorescence microscopy

Immunofluorescence microscopy was performed as previously described [31]. Briefly, cells were seeded on Poly-L-Lysine-coated coverslips and transfected with the appropriate plasmids. Following stimulation, the cells were fixed with 4% paraformaldehyde in PBS, and permeabilized with 0.05% Triton X-100. The nuclei were stained with 1 μ g/ml of Hoechst 33342 (Sigma-Aldrich) and coverslips were mounted onto slides using Fluoro-gel with Tris Buffer (Electron Microscopy Sciences, Hatfield, PA). Cells were examined using an Axio Observer fluorescence microscope (Zeiss, Oberkochen, Germany).

2.4 Semi-quantitative RT-PCR

Total RNA was isolated from MEFs using Trizol reagent (Life Technologies) and treated with the TURBO DNA-free Kit (Life Technologies) to remove genomic DNA. cDNA was synthesized using qScript cDNA SuperMix Kit (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer's instructions. Specific gene products were amplified using MyTaq Red Mix (Bioline, Boston, MA) with the following primers: *Xiap*-f, 5'-

CCATGTGTAGTGAAGAAGCCAGAT-3'; and *Xiap-r*, 5'-TGATCATCAGCCCCTGTGTAGTAG -3'; *Bcl2l1-f*, 5'-AATGAACTCTTTCGGGATGGAG-3'; and *Bcl2l1-r*, 5'-CCAACCTTGCAATCCGACTCA-3'; *Actb-f*, 5'-CACATCAAGAAGGTGGTG-3'; and *Actb-r*, 5'-TGTCATACCAGGAAATGA-3'.

2.5 Subcellular fractionation

Subcellular fractionation was performed by differential centrifugation as previously described [31].

2.6 Immunoprecipitation and immunoblot

Following harvest, cells were lysed on ice with 0.4 ml of lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40 and 0.5% sodium deoxycholate, 1 × complete protease inhibitor cocktail) for 10 min. Lysates were centrifuged at $10,000 \times g$ at 4°C for 10 min. The protein-normalized lysates were subjected to immunoprecipitation by adding 10 mg ml⁻¹ of the appropriate antibody, 30 µl of protein G-agarose (Roche Applied Science), and rotating for more than 2 h at 4°C. The precipitates were washed four times with cold lysis buffer, before separation on SDS-PAGE under reduced and denaturing conditions. The resolved protein bands were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA), and probed as described previously [22]. Blots were developed by the Super Signaling system (Thermo Scientific) according to the manufacturer's instructions, and visualized using a FluorChem E System (Protein Simple, Santa Clara, CA).

2.7 Luciferase reporter gene assays

Luciferase reporter gene assays were performed as previously described [27]. Briefly, cells were cotransfected with 5 × Ig κB site-driven firefly luciferase constructs and the Renilla luciferase pTKRL plasmid (ratio 10:1), together with appropriate plasmids. Cells were cultured for 18 hours, stimulated in triplicate, and analyzed using the Dual-Luciferase Kit (Promega, Madison, WI).

2.8 Recombinant Caspase-3 cleavage assays

The recombinant Caspase-3 cleavage assays were conducted as previously described [28,29]. Briefly, cells expressing indicated GFP-tagged p65 were collected and lysed on ice with 0.4 ml of lysis buffer for 30 min. After centrifuge at $10,000 \times g$ at 4°C for 10 min, 200 µl of supernatant was removed to a separate tube and incubated with 1 µg of recombinant Caspase-3 (kindly provided by Dr. J. Marie Hardwick) at 37°C for 1 h.

2.9 Statistical analysis

All statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA). The difference between treated and control groups were examined by unpaired Student's *t*-tests. Standard errors of means (s.e.m.) were plotted in graphs. n.s. means non-significant difference and significant differences were considered * at $p < 0.05$; ** at $p < 0.01$; and *** at $p < 0.001$.

3. Results and Discussion

To examine the role of Caspase-3-mediated p65 cleavage in cellular fate determination between survival versus death, we treated mouse embryonic fibroblasts (MEFs) with staurosporine (STS), a bacterial alkaloid known to induce apoptosis [32]. As expected, STS treatment caused substantial cell death, in a dose-dependent manner, as measured by morphology and cell counting in wild-type MEFs, whereas STS-induced cell death was abolished in Bax^{-/-} MEFs that are unable to activate executor Caspases (Fig. 1A–C), suggesting that STS triggers the intrinsic cell death pathway. Consistently, we observed cleaved Caspase-3 and cleaved PARP1, that are known biochemical markers of apoptosis, in the STS-stimulated wild-type MEFs, in a time-dependent manner (Fig. 1D). Moreover, an N-terminal fragment of p65 that migrates ~15 kDa by SDS-PAGE separation coincided with the cleaved PARP1 or cleaved Caspase-3 in wild-type MEFs (Fig. 1D), in line with the previous reports that Caspase-3 cleaves p65 during apoptosis [28,29]. In contrast, neither the N-terminal p65 fragment nor cleaved PARP1 and cleaved Caspase-3 were present in STS-treated Bax^{-/-} MEFs, which suggests that the activated Caspase-3 cleaves p65 during apoptosis.

Previous studies proposed that activated Caspase-3 cleaves p65 at Aspartic acid 97/Glycine 98 (D97/G98) [28]. When examining the crystal structure of p65 [33], the D97/G98 residues are located on the exposed surface of the protein, aiding in protease access (Fig. 2A). We first conducted recombinant Caspase-3 cleavage assays using lysates containing wild-type or D97A mutant p65. The GFP-tagged wild-type p65 was reduced in the presence of recombinant Caspase-3, whereas the Caspase-3-mediated p65 cleavage was substantially diminished by an alanine substitution to D97 (Fig. 2B). These results thus suggest that p65 is cleaved by Caspase-3 following apoptotic stimuli, resulting in the production of an N-terminal p65¹⁻⁹⁷ fragment. We then examined the subcellular localization of a GFP-p65¹⁻⁹⁷ fusion protein in resting and tumor necrosis factor (TNF) stimulated cells. As anticipated, GFP-p65¹⁻⁹⁷ was largely excluded from the nucleus, consistent with the fact that it lacks a nuclear localization site, while free GFP localized throughout the cytoplasm and the nucleus, while GFP-p65 mainly resided in the cytoplasm but with a small portion accumulated in the nucleus (Fig. 2C). We confirmed these results biochemically via subcellular fractionation, detecting substantially more GFP-p65¹⁻⁹⁷ in the cytoplasm than the nucleus (Fig. 2D). Hence the p65¹⁻⁹⁷ truncated protein preferentially resides in the cytoplasm in both resting and stimulated cells.

The cleavage of p65 by activated Caspase-3 was believed to be used as a strategy to inhibit NF-κB-mediated anti-apoptotic gene transcription in cells undergoing apoptosis, thus ensuring that they succumb to cell death [28,29]. However, it is noteworthy that the activated Caspase-3 cleaved only a small percentage of p65 during apoptosis (Fig. 1D). The large amount of full-length p65 resistant to Caspase-3 cleavage makes it difficult to explain the remarkable impact of Caspase-3 cleavage on dampening NF-κB activation and anti-apoptotic gene expression. Our recent studies [22,27] showing that N-terminal fragments of p65 interrupt the p65-RPS3 interaction and prevent RPS3 from NF-κB stimuli-induced nuclear translocation and subsequent gene transcription, led us to examine whether the Caspase-3-cleaved p65¹⁻⁹⁷ fragment executes a similar function during apoptosis. Indeed,

when ectopically expressed, full-length and 1-97 truncated p65 interacted with RPS3 (Fig. 3A). However, the p65¹⁻⁹⁷ fragment appeared to have a higher affinity for RPS3 than full-length p65, as evidenced by a substantially higher amount if the p65¹⁻⁹⁷ truncation relative to the full-length in the RPS3 immunoprecipitants (Fig. 3A), indicating that the p65¹⁻⁹⁷ fragment may impair the RPS3-p65 interaction. First we ruled out the possibility that p65¹⁻⁹⁷ influenced the degradation of I κ B α , a protein critical for retaining NF- κ B in the cytoplasm until the proper stimuli. As expected, TNF stimulation induced rapid degradation of I κ B α in GFP transfected cells (Fig. 3B). The presence of GFP-p65¹⁻⁹⁷ protein did not impair I κ B α degradation, suggesting that the p65 truncation product does not interfere with signaling upstream of NF- κ B activation (Fig. 3B). We further examined whether the p65¹⁻⁹⁷ fragment is capable of impairing the translocation of RPS3 from the cytoplasm to the nucleus, a prerequisite for RPS3 to confer selective NF- κ B target gene transcription [17,20,26]. In particular we recently showed that IKK β -mediated phosphorylation of RPS3 on Serine 209 is critical for its interaction with importin- α and subsequent nuclear translocation [17]. TNF stimulation caused a robust increase in phosphorylated RPS3 in the GFP-transfected cells, whereas the phosphorylated RPS3 level stayed constantly low in the cells expressing the p65¹⁻⁹⁷ fragment (Fig. 3C). Moreover, TNF-induced RPS3 nuclear translocation was pronounced in GFP-transfected cells, whereas those transfected with the GFP-p65¹⁻⁹⁷ fragment experienced no increased in nuclear RPS3 levels (Fig. 3D–E). In contrast, the presence of p65¹⁻⁹⁷ fragment did not affect TNF-induced p65 nuclear accumulation (Fig. 3D), suggesting that the p65¹⁻⁹⁷ fragment selectively interferes with RPS3-dependent NF- κ B signaling. To further assess the impact of the p65¹⁻⁹⁷ fragment on NF- κ B gene expression, we employed an Ig- κ B-driven luciferase reporter, whose expression was shown to be RPS3-dependent [16,27]. As expected, TNF treatment robustly induced NF- κ B reporter luciferase expression in GFP-expressing cells (Fig. 3F). In striking contrast, ectopic expression of the GFP-p65¹⁻⁹⁷ fragment dramatically reduced luciferase reporter activity (Fig. 3F). Together these results demonstrate that the p65¹⁻⁹⁷ fragment selectively interferes with the RPS3-dependent NF- κ B signaling and gene expression, without affecting p65 accumulation in the nucleus.

The transactivation of anti-apoptotic genes *Xiap* and *Bcl2l1* were recently revealed to rely on RPS3 [26]. Consistently, the mRNA levels of *Xiap* and *Bcl2l1* decreased in the RPS3-specific siRNA transfected wild-type MEFs, in comparison to those transfected with the non-specific scramble siRNA (Fig. 4A), which suggests that *Xiap* and *Bcl2l1* are RPS3 target genes. As expected, TNF treatment slightly stimulated the transcription of *Xiap* and *Bcl2l1* genes in the GFP vehicle control-transfected MEFs (Fig. 4B). In contrast, the TNF-induced *Xiap* and *Bcl2l1* gene transcription was diminished in the cells expressing GFP-p65¹⁻⁹⁷ fragment (Fig. 4B), demonstrating that ectopic expression of the Caspase-3-cleaved p65¹⁻⁹⁷ fragment is sufficient to block the RPS3-dependent gene transcription in MEFs. Moreover, in parallel to Caspase-3 activation and p65 cleavage in STS-stimulated wild-type MEFs (Fig. 1D), the transcription of anti-apoptotic genes such as *Xiap* and *Bcl2l1* was substantially attenuated (Fig. 4C). Consistently, the protein levels of XIAP and Bcl-XL were also reduced in a time-dependent manner in wild-type MEFs following STS treatment (Fig. 4D). In striking contrast, the STS-induced retardation in *Xiap* and *Bcl2l1* gene transcription was less profound in Bax^{-/-} MEFs (Fig. 4C). Moreover, along with the defective Caspase-3

activation and cell death (Fig. 1C–D), STS treatment elevated XIAP and Bcl-XL protein levels in Bax^{-/-} MEFs (Fig. 4D), which indicates that the Caspase-3 cleavage impacts the anti-apoptotic gene expression during apoptosis.

TNF treatment is well known to induce vigorous NF-κB activation, which leads to expression of a host of anti-apoptotic molecules, while triggering mild programmed cell death, in MEFs. The overwhelming anti-apoptotic signaling supersedes the mild programmed cell death, however, ensuring cell survival. In stark contrast, a combined treatment with TNF and cycloheximide (CHX), which inhibits protein synthesis of NF-κB-transactivated anti-apoptotic molecules, caused significantly more apoptosis in MEFs by switching the death-survival balance (Fig. 4E). Likewise, ectopic expression of the p65¹⁻⁹⁷ fragment substantially sensitized the TNF-treated MEFs to undergo apoptosis, as indicated by cleaved PARP1 and cleaved Caspase-3 (Fig. 4F), which is in line with the finding that p65¹⁻⁹⁷ overexpression diminished TNF-induced anti-apoptotic gene transcription (Fig. 4B) and suggests that the Caspase-3-cleaved p65 fragment plays a crucial role in suppressing NF-κB-mediated anti-apoptotic gene transactivation.

Apoptosis is a primary way cells die in a programmed and controlled fashion, therefore it occurs under various pathophysiological conditions. Among the Caspase superfamily, Caspase-3 is a critical downstream effector/executor protease during apoptosis, which can be activated via both the intrinsic mitochondrial pathway and the extrinsic death receptor pathway [34]. While triggering programmed cell death, most death stimuli activate the NF-κB signaling pathway that leads to the transactivation of an array of anti-apoptotic genes, which provides a balancing mechanism for cells to reevaluate their death versus survival fate. Caspase-3 cleavage of p65 was proposed as a mechanism to suppress NF-κB-mediated anti-apoptotic gene transcription, allowing cells to undergo apoptosis [28,29]. Perplexingly, the portion of Caspase-3 cleaved p65 compared to full-length form is very low, even in cells undergoing apoptosis, despite a dramatic effect on cell viability and reduction in NF-κB anti-apoptotic gene transcription (Fig. 1D) [28,29]. RPS3, the non-Rel subunit that confers the promoter selectivity and transcriptional specificity of NF-κB [12], was recently revealed to play an indispensable role in the transactivation of anti-apoptotic genes *Birc3*, *Bcl2l1*, and *Xiap* [26]. Moreover, the fact that RPS3 occurs in a subset of, rather than all, NF-κB DNA binding complexes in cells [31] suggests that inhibition of anti-apoptotic transcription by interfering with RPS3, rather than p65, would be more efficient to shut down anti-apoptotic NF-κB signaling. Indeed, interference with the nuclear translocation and NF-κB facilitating function of RPS3 dampens RPS3-dependent NF-κB target gene transcription, without affecting p65 [22,27]. Hence our results suggest that the Caspase-3-cleaved N-terminal 1-97 fragment of p65, albeit only a small percentage of total p65, interferes with the activation-induced RPS3 nuclear translocation and subsequent NF-κB promoter selectivity function, thus dampening the transactivation of a host of anti-apoptotic molecules in apoptotic cells (Fig. 5). More importantly, emerging evidence demonstrates that both Caspase-3 activation and late-stage apoptosis are reversible in primary cells [35,36]. This transient and reversible apoptotic response causes accumulation of DNA damage and introduces oncogenic transformation [35]. Therefore, it is extremely pivotal for apoptotic cells to fully and efficiently shut down NF-κB-mediated anti-apoptotic transcription and ensure that they

undergo well-controlled programmed death. Our results suggest a novel cell fate determination mechanism during apoptosis that relies on manipulating RPS3-dependent NF- κ B anti-apoptotic gene transcription via a Caspase-3 produced p65 fragment.

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Abbreviations

Bcl-XL	B-cell lymphoma-extra large
CHX	cycloheximide
cIAP2	cellular inhibitor of apoptosis protein-2
IκB	Inhibitor of κ B
IKKβ	I κ B kinase beta
MEFs	mouse embryonic fibroblasts
NF-κB	Nuclear factor-kappa B
NLS	nuclear localization site
RPS3	Ribosomal protein S3
siRNA	small interfering RNA
STS	staurosporine
TNF	tumor necrosis factor
XIAP	X-linked inhibitor of apoptosis protein

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Highlights

- We examined the cell fate determination mechanism by Caspase-3-mediated p65 cleavage.
- A small percentage of p65 is cleaved by Caspase-3 during apoptosis.
- The generated p65¹⁻⁹⁷ fragment interferes with ribosomal protein S3 (RPS3).
- The p65¹⁻⁹⁷-RPS3 interaction dampens the NF-κB-mediated anti-apoptotic transcription.

Fig. 1.

NF-κB p65 is cleaved by Caspase-3 during apoptosis. (A) Whole cell lysates derived from wild-type (WT) and Bax^{-/-} mouse embryonic fibroblasts (MEFs) were SDS/PAGE separated and immunoblotted (IB) for indicated proteins, with β-actin as a loading control. (B) Representative micrographs of WT and Bax^{-/-} MEFs stimulated with indicated doses of Staurosporine (STS) for 6 h. Scale bars, 200 μm. (C) Relative cell numbers (normalized to untreated samples) of WT and Bax^{-/-} MEFs as stimulated in B. *, $p < 0.05$; **, $p < 0.01$ by unpaired Student's *t*-tests. (D) WT and Bax^{-/-} MEFs stimulated with 100 nM of STS for indicated periods. Whole cell lysates were derived and IB for indicated proteins, with β-actin as a loading control. c-PARP1, cleaved PARP1; c-Casp3, cleaved Caspase-3.

Fig. 2.

The Caspase-3-cleaved N-terminal p65 fragment residues in the cytoplasm. (A) The N-terminal structure of p65, with dimerization domain (DimD), N-terminal domain (NTD), Asp97, and Gly98 highlighted in yellow, orange, blue, and red, respectively. Image was created from PDB file 1VKX [33] using the Pymol software. (B) Whole cell lysates derived from HEK293T cells expressing GFP-tagged wild-type or D97A mutant p65 were incubated with recombinant Caspase-3 (r-Caspase-3) for 30 min and immunoblotted (IB) with GFP antibody. The densitometry of GFP-tagged p65 and p65 (D97A) mutant was quantified and normalized to those in the absence of r-Caspase-3. (C) Diagram of GFP-fused full-length and truncated p65. NTD, N-terminal domain; DimD, dimerization domain; TAD, transcriptional activation domain; NLS, nuclear localization signals (upper). Immunofluorescence micrographs of HEK293T cells transfected with GFP, GFP-tagged p65¹⁻⁹⁷ fragment, and GFP-tagged p65. Nuclei were counterstained with DAPI. Scale bars, 10 μ m. (D) HEK293T cells expressing GFP-tagged p65¹⁻⁹⁷ or full-length p65 were stimulated with 50 ng/ml of TNF for indicated periods. The cytosolic and nuclear fractions

were derived and immunoblotted (IB) for indicated proteins. Caspase-3 and PARP1 served as loading controls and cytosolic and nuclear markers, respectively.

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Fig. 3.

The cleaved p65¹⁻⁹⁷ fragment interferes with the RPS3-dependent NF-κB signaling. (A) HEK293T cells were transfected with GFP-tagged p65 or p65¹⁻⁹⁷ plasmids. 24 h later, whole cell lysates were gathered and immunoblotted (IB) directly, or following immunoprecipitation (IP) with RPS3 antibody, for indicated proteins. (B–C) HEK293T cells expressing GFP-tagged p65¹⁻⁹⁷ or GFP alone were stimulated with 50 ng/ml of TNF for indicated periods. Whole cell lysates were derived and IB for IκBα (B) or phospho-RPS3 (p-RPS3) (C), using β-actin as a loading control. (D) HEK293T cells were transfected and stimulated as in B, and nuclear fractions were collected and IB for indicated proteins. Hsp90 and PARP1 served as loading controls, as well as cytosolic and nuclear markers, respectively. (E) HEK293T cells, transfected and stimulated as in B, were left untreated (Unstim.) or stimulated with 50 ng/ml of TNF for 30 min. Shown are micrographs of transfected GFP or GFP-fused protein, endogenous RPS3, and DAPI-counterstained nuclei. Scale bars, 10 μm. (F) HEK293T cells were transfected with GFP or GFP-p65¹⁻⁹⁷, together with 5 × κB-Luc reporter and pTKRL plasmids. After 28 h, the cells were stimulated in the presence or absence of TNF (50ng/ml) and analyzed for luciferase activity.

Fig. 4.
The p65¹⁻⁹⁷ fragment suppresses RPS3-dependent anti-apoptotic gene expression, overcoming intrinsic NF-κB anti-apoptotic activity. (A) Wild-type (WT) mouse embryonic fibroblasts (MEFs) were transfected with non-specific small interference RNA (si-NC) or siRNA targeting RPS3 (si-RPS3). 48 h later, total RNA was extracted and the mRNA levels of indicated genes were analyzed by semiquantitative RT-PCR. (B) WT MEFs expressing GFP vehicle or GFP-p65¹⁻⁹⁷ fragment were stimulated with TNF (50 ng/ml) for indicated periods. Total RNA was extracted and analyzed as in A. (C) WT and Bax^{-/-} MEFs were treated with STS (100 nM) for indicated periods. Total RNA was extracted and analyzed as in A. Densitometry, normalized to the unstimulated samples (set as 1.0), are shown. (D) WT or Bax^{-/-} MEFs were treated as in C. Whole cell lysates were derived and immunoblotted (IB) for indicated proteins, using β-actin as a loading control. Densitometry, normalized to the unstimulated samples (set as 1.0), are shown. (E–F) WT MEFs were stimulated with TNF (50 ng/ml) alone or TNF plus cycloheximide (CHX, 10 μg/ml) for 4 h (E) and WT MEFs expressing GFP vehicle or GFP-p65¹⁻⁹⁷ fragment were stimulated with TNF (50 ng/ml) for indicated periods (F). Whole cell lysates were derived and IB for indicated proteins, with β-actin as a loading control. c-PARP1, cleaved PARP1; c-Casp3, cleaved Caspase-3.

Fig. 5. Schematic model of how selective inhibition of NF-κB anti-apoptotic gene expression by the Caspase-3-cleaved p65¹⁻⁹⁷ fragment affects cellular balance between survival and apoptosis. The expression of a host of anti-apoptotic NF-κB target genes that require the presence of RPS3 tunes cells in a pro-survival state. During apoptosis, however, activated Caspase-3 cleaves a small percentage of p65 in the N-terminus, generating a p65¹⁻⁹⁷ fragment. This fragment interacts with RPS3 and prevents the nuclear translocation of RPS3, thus selectively inhibiting the RPS3-dependent NF-κB anti-apoptotic transcription and shifting the balance towards apoptosis.