Transcriptional repression of NFKBIA triggers constitutive IKK- and proteasome-independent p65/RelA activation in senescence

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Abstract

The IκB kinase (IKK)-NF-κB pathway is activated as part of the DNA damage response and controls both inflammation and resistance to apoptosis. How these distinct functions are achieved remained unknown. We demonstrate here that DNA double-strand breaks elicit two subsequent phases of NF-κB activation in vivo and in vitro, which are mechanistically and functionally distinct. RNA-sequencing reveals that the first phase controls anti-apoptotic gene expression, while the second drives expression of senescence-associated secretory phenotype (SASP) genes. The rapidly activated first phase is driven by the ATM-PARP-TrAF6-IKK cascade, which triggers proteasomal destruction of inhibitory IκBα, and is terminated through IκBα re-expression from the NFKBIA gene. The second phase, which is activated days later in senescent cells, is on the other hand independent of IKK and the proteasome. An altered phosphorylation status of NF-κB family member p65/RelA, in part mediated by GSK3β, results in transcriptional silencing of NFKBIA and IKK-independent, constitutive activation of NF-κB in senescence. Collectively, our study reveals a novel physiological mechanism of NF-κB activation with important implications for genotoxic cancer treatment.

Keywords  DNA damage response; IκBα; NF-κB; SASP; senescence
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Introduction

Chemo- and radiotherapies activated oncogenes and shortened telomeres trigger via the DNA damage response (DDR) a terminal proliferative arrest called cellular senescence (Blagosklonny, 2014; Salama et al., 2014; Lasry & Ben-Neriah, 2015; Lee & Schmitt, 2019). The associated alterations include formation of senescence-associated heterochromatin foci (SAHF), increased synthesis of cell cycle inhibitors, including p21 (CDKN1A) and p16 (CDKN2A) and of inflammatory cytokines and chemokines that constitute the senescence-associated secretory phenotype (SASP) and a related, low-grade inflammation termed senescence inflammatory response (SIR) that affects surrounding tissues in a paracrine manner (Shelton et al., 1999; Lasry & Ben-Neriah, 2015). The epigenetically controlled cell cycle cessation serves as a cell-autonomous barrier to tumor formation (Braig et al., 2005; Collado et al., 2005; Reimann et al., 2010). Therefore, induction of senescence was considered as important in treating cancer and other pathologies. The inflammatory response, however, comprises factors that may instigate oncogenic transformation, cell migration, and cancer stemness (Acosta et al., 2008; Reimann et al., 2010; Chien et al., 2011; Freund et al., 2011; Jing et al., 2011; Acosta et al., 2013; Salama et al., 2014; Hoare et al., 2016; Milanovic et al., 2018).

The majority of SASP factors are transcriptional targets of NF-κB (Acosta et al., 2008; Kuillman et al., 2008; Chien et al., 2011; Freund et al., 2011; Jing et al., 2011; Lasry & Ben-Neriah, 2015). Although it is well established that NF-κB drives inflammatory gene expression in senescence, whether it also contributes to cell cycle arrest remained unclear. DNA double-strand breaks lead to rapid activation of NF-κB RelA/p65-p50, the most prevalent heterodimer (Smale, 2012). A signaling cascade that is activated by ataxia

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**Results**

DNA damage activates NF-κB in two distinct phases: a transient anti-apoptotic first and a persistent inflammatory second phase

To investigate the kinetics of activation of NF-κB, we first examined the establishment of senescence and SASP over time in human diploid fibroblasts (HDFs) and cancer cell lines that experienced DNA damage. Onset of senescence was marked by senescence-associated β-galactosidase (SA-β-gal) activity and elevated CDKN1A (p21) expression (Fig 1A and Appendix Fig S1A). Proliferation ceased 1–2 days following irradiation (IR), as expected, and cells entered a lasting senescent state (Appendix Fig S1B and C). Unresolved DNA damage, evidenced by γH2AX foci and phosphorylation of p53 at serine 15, was seen within minutes and hours and persisted through all time points. However, acute activation of upstream ATM and Checkpoint kinase 2 (Chk2) was observed predominantly only within minutes and hours after the IR treatment (Appendix Fig S1D and E). Strikingly, a single dose IR generated a biphasic NF-κB activation, with two temporally separate phases of its nuclear translocation and DNA binding, first, within hours and then days later (Fig 1B, and Appendix Fig S1D, F and G). An RNA-seq analysis revealed distinct transcriptomes in both NF-κB phases (Fig 1C and Tables EV1A–C). During the first phase, 326 transcripts showed significant upregulation (Dataset EV1A), which included direct targets of NF-κB, such as early response genes and negative feedback inhibitors of the pathway (NFKBIA and TNFAIP3; see Dataset EV1B). This transcript group was enriched for GO terms “cell cycle arrest” and “regulation of apoptotic process” (Table EV1). In contrast, the 2,980 transcripts upregulated during the second phase were enriched for GO terms “inflammatory response”, “immune response”, and “response to wounding” (Table EV1). The biphasic NF-κB response thus coincided with two distinct transcriptomes, an anti-apoptotic first phase and a pro-inflammatory second phase.

We next analyzed murine tissues for IR-induced expression of the representative first- and second-phase target genes of NF-κB, Nfkbia and Il6, respectively. IR strongly activates NF-κB in various tissues (Li et al., 2001). Indeed, IR significantly induced Nfkbia mRNA in skin and kidney tissues only at early time points and of Il6 mRNA only at late ones, representing the first and second NF-κB phases (Fig 1D). Other examples for induced phase-restricted gene expression in skin are Tnfaip3 (only first phase) and Cx3b or Cxcl3 (only second phase; Appendix Fig S1H).

To ensure that the expression of SASP during the second phase depended on NF-κB, we analyzed primary kidney cells from irradiated mice, which ubiquitously express the NF-κB super-repressor IkBzxAN (Krappmann et al., 1996; Schmidt-Ullrich et al., 2001). Compared to littermate controls, expression of Il6 in the second phase was abolished, whereas Cdkn1a and Cdkn2a upregulation was unaffected (Appendix Fig S1I). These results further reveal that DNA damage activates NF-κB that drives SASP, but NF-κB is not essential for the proliferative arrest observed in senescence.

A strong response was seen in hair follicles (HF), which require NF-κB activation for development and morphogenesis (Schmidt-Ullrich et al., 2001). Upregulation of Nfkbia mRNA and IkBβ protein was restricted to the first phase of NF-κB activation in HF following whole-body IR (Fig 1E and F). At Day 7 post-IR, IkBβ expression in the proximal HF was strongly reduced, concomitant with an increase in IL-6 expression in the same region (Fig 1G and H). These results demonstrate that two distinct, subsequent NF-κB transcriptional programs also occur in vivo. Because IkBβ is required to terminate NF-κB signaling, we postulated that loss of IkBβ in senescence could trigger SASP.

Loss of IkBβ expression in senescence triggers the second phase of NF-κB activation and generates SASP

IkBβ expression was either undetectable or strongly reduced in senescence in the different cancer- and non-transformed cell lines tested (Fig 2A and Appendix Fig S2A and B). Likewise, NFKBIA mRNA was upregulated only in the first phase (Fig 2A, right panel; Appendix Fig S2C), despite robust activation of NF-κB in both phases (Fig 1B). Repeat IR treatment, with the aim to re-stimulate first-phase-like transcriptional induction of NFKBIA in senescent cells, restored neither NFKBIA mRNA nor IkBβ protein expression (Fig 2A left, lane 5, and right panel).

Activated oncocenes cause DNA strand breaks and induce DDR signaling, thereby promoting cellular senescence similar to cells exposed to DNA-damaging agents (Acosta et al., 2008; Coppe et al., 2008; Kuijlman et al., 2008). Inducible activation of oncogenic RASV12 led to activation of NF-κB and expression of the representative SASP factor IL-8 (encoded by CXCL8) that negatively correlated with IkBβ expression (Appendix Fig S2D and E). In summary, these data show that different pro-senescent triggers lead to loss of NFKBIA mRNA expression together with the onset of an NF-κB-driven SASP.
To investigate if experimental NFKBIA depletion mimics the second-phase NF-κB activation and SASP type gene expression, we knocked down NFKBIA (Fig 2B and Appendix Fig S2F). We confirmed that DNA damage signaling was not altered due to NFKBIA knockdown (Appendix Fig S2F), and importantly, that constitutive NF-κB activation in untreated cells with NFKBIA

Figure 1.
knockdown (Fig 2B, lane 5) did not stem from a DDR (Appendix Fig S2F). These data indicate that knockdown of NFKBIA, irrespective of DNA damage, is sufficient for constitutive NF-κB activation.

We next asked which target genes would be regulated by constitutive NF-κB activation resulting from NFKBIA knockdown. Remarkably, the transcriptome of non-irradiated NFKBIA-depleted cells shared a strong overlap and the same GO terms with the senescent cells of the second phase (Fig 2C and D, and Table EV1). Thus, loss of IkBα in the absence of DNA damage was sufficient to activate transcription of SASP factors. IkBα rescue through ectopic overexpression of an NFKBIA mutant encoding the protease-insensitive IkBαS32A/S36A blocked the induction of the bona fide SASP factors (Appendix Fig S2G), consistent with the conclusion that loss of IkBα drives expression of SASP. Secretion of a set of the identified cytokines and chemokines was confirmed by an antibody array (Appendix Fig S2H). Furthermore, supernatants from either senescent or IkBα-depleted cells induced migration of macrophages (Appendix Fig S2I).

Since we showed that suppression of NFKBIA was sufficient for SASP, we next asked whether it was also sufficient for senescence-associated proliferative arrest. At least in the case of Eμ-myc lymphomas, it has been suggested that NF-κB also positively regulates cell cycle arrest (Chien et al., 2011). We therefore analyzed proliferation of cells with NFKBIA knockdown. The duplication rate of cells with NFKBIA knockdown was comparable to that of control cells (Appendix Fig S3A). Similarly, BrdU incorporation in senescent cells with NFKBIA knockdown was not affected, despite constitutive activation of NF-κB (Appendix Fig S3B).

We next asked whether in vivo, constitutive NF-κB resulting from a knockdown of Nfkbia would also trigger SASP, but not senescence-associated proliferative arrest. Since ubiquitous loss of IkBα causes early postnatal lethality (Beg et al., 1995; Klement et al., 1996), we generated intestinal epithelium-restricted (villin-Cre × floxed Nfkbia) knockout mice (Mikuda et al., 2020). Importantly, we observed hyperproliferation of cells, leading to crypt hyperplasia and enrichment of genes responsible for cell cycle progression. Increased proliferation (evidenced by increase in Ki67+ cells) was observed both in vivo and in organoids derived from these mice (Mikuda et al., 2020). Thus, constitutive NF-κB activation in vivo regulates proliferation and cell cycle progression in a positive manner. To determine if SASP factors could be detected in vivo, we performed GSEA analysis on epithelium from (villin-Cre × floxed) Nfkbia knockout mice and matched sibling controls (Fig 2E). Importantly, only enrichment of SASP-coding transcripts was detected, but not of those transcripts belonging to the broader category of cellular senescence, which in addition comprises factors involved in cell cycle regulation (Fig 2E and Appendix Fig S3C). We confirmed our findings by RT-qPCR by analyzing expression of selected SASP factors that are also targets of NF-κB, including Cc20, Icam1, Ile6 and Tnfa (Fig 2F). These were significantly upregulated in the absence of DNA damage, evidenced by lack of p53 pSer15 nuclear foci (Appendix Fig S3D and E), confirming our conclusion in an in vivo setting.

p65 regulates SASP in senescence but not proliferative arrest

Since we observed distinct NF-κB targets upregulated during the two phases (Dataset EV1B), we next asked which family member(s) were responsible for NF-κB activity in senescence. NF-κB showed robust DNA binding during both the first and the second phase (Fig 3A, lanes 7 and 13), consisting in each case of p65-containing heterodimers (lanes 10 and 16). We therefore knocked down RELA/p65 to analyze how it regulates the first and the second-phase transcriptomes. All NF-κB targets, including SASP-encoding genes, showed decrease in expression (Fig 3B) in cells bearing shRNA against RELA/p65 (Appendix Fig S4A). Nevertheless, knockdown of RELA did not rescue cells from senescence (Fig 3C and Appendix Fig S4A–C). These data confirm that p65 containing dimers regulate SASP but not the senescence-associated proliferative arrest.

Suppression of NFKBIA in senescence through p65 pSer468

Since loss of IkBαs in the second phase resulted from a decline of mRNA expression, we investigated its regulation by p65. The phosphorylation status of this subunit differentially determines...
transcription of its target genes (Schmitz et al., 2004; Wietek & O’Neill, 2007). We therefore analyzed the phosphorylation status of p65 during the two phases (Fig 4A). Phosphorylation on p65 Ser536, the substrate site of IKK, peaked during the first phase and declined in the second. Unlike phosphorylation of p65 at S536 and S276, which enhance the p65 transactivation potential, phosphorylation at S468 is inhibitory and is mediated in part by GSK3β (Buss et al., 2004; Christian et al., 2016). Of note, GSK3β exhibits increased kinase activity in senescence to activate formation of SAHF, through downregulation of Wnt signaling (Ye et al., 2007). Indeed, nuclear phosphorylation on S468 increased in senescence (Fig 4A left and right panels). To determine if phosphorylation on S468 repressed expression of IkBα, we overexpressed p65 bearing a S468A mutation in cells where endogenous p65 was knocked down. Overexpression of p65 S468A rescued IkBα expression (Fig 4B). Similar results were observed with ectopic expression of wild-type p65, likely due to the abundance of the substrate in relation to the S468 kinase(s). As a negative control, we transfected the S276A mutant. Since phosphorylation at S276 is required for p65 activity (Christian et al., 2016), its overexpression did not rescue IkBα in senescence (Fig 4B).

This model strongly predicts that the NFκBIA locus is bound during the second but not the first phase by inhibitory p65, which is phosphorylated at serine 468. To explore this possibility, we performed chromatin immunoprecipitation (ChIP) with both p65 (total) and p65 pSer468 antibodies. Strikingly, unlike total p65,
showed increased phosphorylation following irradiation, albeit with factors associated with DNA damage, including p53 and Chk2, showed a significant change in response to IR (Fig 4D). As expected, proteome kinase array. Of 45 selected kinases and proteins, 12 showed equivalent binding affinity during both phases, only p65 Ser468 showed strongly induced binding during the second phase (Fig 4C). Together, these data show that translational modifications on p65 lead to repression of its inhibitor-encoding gene, NFKBIA, in senescence.

To determine which kinases could contribute to the phosphorylation of S468, we analyzed the nuclear fraction of cells with a proteome kinase array. Of 45 selected kinases and proteins, 12 showed a significant change in response to IR (Fig 4D). As expected, factors associated with DNA damage, including p53 and Chk2, showed increased phosphorylation following irradiation, albeit with differing kinetics (Fig 4D). Phosphorylation of GSK3α and β at the inhibitory sites, serine 21 and 9, respectively, was present in untreated cells and at 1.5 h post-IR, but was decreased in senescence, indicating that nuclear GSK was more active. This correlated with decreased abundance of β-catenin (Fig 4D), whose phosphorylation by GSK leads to its degradation (Kitagawa et al., 1999). Due to the previously described role of GSK in phosphorylation of p65 on serine 468, we knocked down GSK3β or inhibited GSK3β by lithium chloride and CHIR-99021 (Fig 4E–G). Both modes of interference diminished serine 468 phosphorylation and led to a partial restoration of IκBα expression (protein and mRNA) in senescence, with

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**Figure 3. RelA regulates SASP but not cell cycle arrest.**

A Nuclear fractions from either untreated or irradiated (20 Gy) U2-OS cells, 1.5 h or 7 days prior to harvest, were analyzed by EMSA with supershift analysis. For supershifting, lysates were incubated with antibodies or water, as indicated. Arrows point to shift location of a given subunit, due to antibody binding. ns, non-specific band. All antibodies were previously tested for supershift compatibility. Representative gel shown from untreated cells and at 1.5 h post-IR, but was decreased in senescence, indicating that nuclear GSK was more active. This correlated with decreased abundance of β-catenin (Fig 4D), whose phosphorylation by GSK leads to its degradation (Kitagawa et al., 1999). Due to the previously described role of GSK in phosphorylation of p65 on serine 468, we knocked down GSK3β or inhibited GSK3β by lithium chloride and CHIR-99021 (Fig 4E–G). Both modes of interference diminished serine 468 phosphorylation and led to a partial restoration of IκBα expression (protein and mRNA) in senescence, with
concomitant decrease in expression of \textit{IL6} (Fig 4G). These data show that changes in p65 phosphorylation contribute to an attenuation of NFKBIA expression in senescence.

**Second-phase NK-κB activation in senescence is independent of IKK signaling and the proteasome**

We next investigated the contribution of the known regulators of the genotoxic stress-induced IKK pathway (Fig 5A) in the two NK-κB phases. The activation of ATM and IKK correlated only with the first NK-κB phase and peaked in the first hours after IR followed by a decline afterward (Fig 5B). We also found that TRAF6 depletion only abrogated activation of the first, but not the second NK-κB phase (Fig 5C). Expression of target genes of NF-κB, \textit{IL6} and \textit{IL1A}, in the second phase was completely unaffected by depletion of ATM (Appendix Fig S5A and B). Furthermore, proteasomal inhibition abrogated IR-induced IκBα destruction in the first NK-κB phase, but not loss of IκBα in the second phase (Fig 5D). In line with this, upregulation of the \textit{bona fide} SASP component IL-1α was not affected by proteasome inhibition (Fig 5D).
We eliminated IKKβ using CRISPR/Cas9 (Fig 5E). Loss of IKKβ impeded only first-phase NF-kB activation (Fig 5E, lanes 2 and 6). Remarkably, IKKβ was not required for the second phase of NF-kB activation (Fig 5E, lanes 4 and 8).

Our conclusion was further corroborated by identification of genes that depended on p65 expression (Fig 3B), yet showed unaltered expression in senescent cells with CRISPR mediated knockout of IKKB (Fig 5F). These IKK-independent genes encoded many bona fide SASP factors, such as IL8 and metalloproteinases MMP1 and 3 (Fig 5F, lower half of the panel). Likewise, siRNA-mediated IKKγ depletion only diminished p65 nuclear translocation at 1.5 h, but not 5 days following IR (Appendix Fig S5C). To determine if CHUK/IKKα that regulates non-canonical signaling might regulate SASP, we checked expression of select NF-kB genes in senescence inhibits NFKBIA expression. We and others have previously shown that irreparable DNA damage sequentially activates two temporally and functionally distinct transcriptomes of NF-kB, separated by a span of several days. An anti-apoptotic first phase is driven by an ATM-, PARP-1-, and TRAF6-dependent IKK signaling cascade (Silimann et al, 2009; Hinz et al, 2010), resulting in proteasomal destruction of IκBz. A pro-inflammatory second phase occurs in senescence and comprises SASP (Fig 6). Importantly, we demonstrate that the second phase of NF-kB and expression of the majority of the SASP genes are both IKK- and proteasome-independent. A fraction of transcripts that were IKK-dependent in senescence could be regulated in an alternative manner that does not require phosphorylation in the activation loop of IKKβ. Indeed, we have recently shown that basal activity of IKKβ suffices for its interaction with EDC4 (Enhancer of Decapping 4) and for post-transcriptional stabilization and destabilization of scores of transcripts, including of CXCL8 and TNFA (Mikuda et al, 2018). It is therefore possible that IKK-dependent regulation of some SASP genes occurs at the level of their RNA stability. It is also possible that additional phosphorylation sites on IKK, not analyzed in this paper, contribute to its activation and to regulation of IKK-dependent genes expressed in senescence.

Interestingly, absence of IKK at the instant of DNA damage abolishes only the first phase of NF-kB activation, but does not affect the second phase. This suggests that the first phase is not required for the second, and that the changes accumulated over time activate distinct signaling pathways that enable the second phase of NF-kB. In accordance with this, we show that post-translational modifications on p65 contribute to the two distinct transcriptomes. IKK phosphorylates p65 on serine 536 within minutes following DNA damage, however in senescence a switch in phosphorylation from serine 536 to 468, and to recruitment of p65-Ser468 to the NFKBIA locus, leads to repression of NFKBIA. GSK3β, which is hyperactive in senescence (Ye et al, 2007), phosphorylates p65 at Ser 468.

### Discussion

Most NF-kB activation pathways depend on IKKs (Hoffmann & Baltimore, 2006; Hinz & Scheidereit, 2014). Here we provide a physiologically relevant context for IKK-independent activation of NF-kB both in human cells lines and in murine models in vivo. We found that in DNA damage-induced senescence of epithelial cells, two interconnected events comprising a decline in IKK phosphorylation and a drop in transcription of the inhibitor of the pathway, NFKBIA (IκBz), initiate a persistent, IKK-independent activation of NF-kB (Fig 6).

We and others have previously shown that irreparable DNA damage leads to cellular senescence and to SASP driven by NF-kB (Rodier et al, 2009; Chien et al, 2011; Jing et al, 2011). However, since DNA damage triggers prompt activation of NF-kB, whose immediate transcriptional targets do not feature SASP, it was not clear how NF-kB could be responsible for two such drastically different transcriptomes arising from the same initial stimulus. Here we demonstrated both in vitro and ex vivo that a single dose of DNA damage sequentially activates two temporally and functionally distinct transcriptomes of NF-kB, separated by a span of several days. An anti-apoptotic first phase is driven by an ATM-, PARP-1-, and TRAF6-dependent IKK signaling cascade (Silimann et al, 2009; Hinz et al, 2010), resulting in proteasomal destruction of IκBz. A pro-inflammatory second phase occurs in senescence and comprises SASP (Fig 6). Importantly, we demonstrate that the second phase of NF-kB and expression of the majority of the SASP genes are both IKK- and proteasome-independent. A fraction of transcripts that were IKK-dependent in senescence could be regulated in an alternative manner that does not require phosphorylation in the activation loop of IKKβ. Indeed, we have recently shown that basal activity of IKKβ suffices for its interaction with EDC4 (Enhancer of Decapping 4) and for post-transcriptional stabilization and destabilization of scores of transcripts, including of CXCL8 and TNFA (Mikuda et al, 2018). It is therefore possible that IKK-dependent regulation of some SASP genes occurs at the level of their RNA stability. It is also possible that additional phosphorylation sites on IKK, not analyzed in this paper, contribute to its activation and to regulation of IKK-dependent genes expressed in senescence.
However, inhibition of GSK3β did not completely reinstate ΙκBα expression, indicating that additional kinases and/or epigenetic changes may contribute to downmodulation of NFκBIA expression in senescence.

Silencing of NFκBIA in senescence results in IKK-independent and persistent activation of NF-κB (Fig 6). Consequently, the second phase can be mimicked without induction of DNA damage by inactivation of the NFκBIA gene, as we have shown in human cell.
Kolesnichenko et al. firmly establish proliferative arrest (Narita et al.) through additional regulators including TORC1, MAPK, and Toll-like receptors, likely because full-featured senescence relies on activation of the IKK-signalosome or the proteasome being ineffective in suppressing SASP in tumor therapies. On a positive note, direct inhibition of NF-κB would inhibit detrimental SASP, while leaving the cell cycle arrest intact.

**Materials and Methods**

**Transfection/transduction**

pTRIPz clones IκBα, p65, IKKβ, and IKKy and Scrambled or pGIPZ-IκBα and pGIPZ-scrambled (Dharmacon, Lafayette, USA) were transfected into HEK293T cells and supernatant used for transduction as described in manufacturer’s protocol (http://dharmacon.genelifesciences.com/uploadedfiles/resources/ptripz-inducible-lentiviral-manual.pdf). Clonal selection was performed using puromycin. Doxycycline hydrochloride was added daily (2 μg/ml, Sigma). Unless specified otherwise, Dox treatment was done for 5–6 days prior to harvest. As controls, cells inducibly expressing scrambled shRNAs were treated with Dox.

CRISPR knockout cells were generated as described previously (Mikuda et al., 2018).

**Preparation of murine tissues from in vivo experiments**

All mouse protocols in this study followed the regulatory standards of the governmental review board (Landesamt Berlin). All mouse protocols in this study followed the regulatory standards of the governmental review board (Landesamt Berlin), Reg. G007/08, G-0029/15, G-0082/13, G0358/13, and X9013/11). B6;129P2-NKBia<sup>tm1(R2Sp)</sup> and Tg(Vil-cre)20Syr mice were sacrificed at 5 or 8 weeks of age. Additionally, 12- to 16-week-old C57Bl6/N female mice were sacrificed either 1.5 h or 7 days post-whole-body IR (5Gy).

**Nuclear cytoplasmic fractionation, Western Blot analysis, and EMSA**

Nuclear cytoplasmic fractionation, Western Blot analysis, and EMSA performed as described previously (Mikuda et al., 2018).

**In situ hybridization**

In situ hybridization was performed as described previously (Schmidt-Ullrich et al., 2001).

**Antibody array**

Proteome profiler (R&D Systems) antibody array was performed on 1 ml of culture medium according to manufacturer’s protocol. Quantitation was performed with FusionCapt Advanced software.

**Kinase array**

(R&D Systems) was performed on nuclear lysates according to manufacturer’s protocol. Quantitation was performed with Fusion-Capt Advanced software.

**Quantitative RT–PCR**

Quantitative RT–PCR (RT-qPCR) was performed using the CFX96 Real Time System (Bio-Rad) and GoTaq® qPCR Master Mix (Promega), using a minimum of two reference genes (TBP, Rpl13a, HRP1).
**RNA-seq**
RNA samples were prepared in quintuplicates and extracted using Trizol reagent according to manufacturer’s instructions (Thermo Fisher). Stranded mRNA sequencing libraries were prepared with 500 ng total RNA according to manufacturer’s protocol (Illumina). The libraries were sequenced in 1 × 100 + 7 manner on HiSeq 2000 platform (Illumina).

**Antibodies**
For a list of antibodies, See Table EV2.

**Data availability**
The RNA-seq data from this publication have been deposited to the GEO database (www.ncbi.nlm.nih.gov/geo) and assigned the identifier GSE158743 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE158743).

**Expanded View** for this article is available online.

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**Author contributions**
Conceptualization: MK, CS; Data curation: WS; Formal analysis: MK, BU, EK, UEH; Funding acquisition: CS; Investigation: MK, NM, EK, MH, ABT, MM; Project administration: MK; Resources: CAS, CS, RS-U, SL, AA; Supervision: CS; Validation: IK, MW, SJ, LH, KS; Visualization: MK, NM, EK, CS; Writing original draft: MK; Writing—review & editing: CS, MK, CAS, RSU, UEH, EK.

**Conflict of interest**
The authors declare that they have no conflict of interest.

**References**


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