Cooperative effects of RIG-I-like receptor signaling and IRF1 on DNA damage-induced cell death

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INTRODUCTION

DNA damage is a ubiquitous and existential threat to organisms. Potential causes comprise ionizing radiation (IR), genotoxic chemicals, but also cell-intrinsic mechanisms. Among various possible DNA alterations, the most drastic and impactful are DNA double-strand breaks (DSBs). Complex mechanisms involving detection by ATM, ATR, and downstream processes including the tumor suppressor p53 and checkpoint inhibition, either lead to sufficient repair of the damage or to induction of programmed cell death [1, 2]. The latter mostly comprises apoptosis, but other forms such as necroptosis and pyroptosis have recently been reported as well. Mutations of the central DSB sensors can cause severe diseases such as ataxia telangiectasia, associated with carcinogenesis and serious immunodeficiency [3–5]. Originally discovered and best-studied in the context of the antiviral innate immune response, IRF1 has been implicated in the DNA damage response and tumor suppressor functions [6–9].

Following the IRF1 example, it became apparent that cell-intrinsic antiviral signaling pathways also substantially contribute to DNA damage-induced cell death. Both STING and RIG-I-like receptor (RLR) pathways detect damage-associated molecular patterns (DAMPs), such as endogenous DNA fragments and nuclear RNA, and can trigger cell death [10, 11]. Previously, RIG-I stimulation has been shown to induce death of breast cancer cells, putting forward a potential application in tumor therapy [12]. Typically, the RLRs, RIG-I, and MDA5, are stimulated by non-self RNA in the event of viral infection. Interaction with their adaptor MAVS leads to activation of the transcription factors IRF3, NF-κB and IFNs through expression of pro-apoptotic factors, direct cell death mediating effects have recently been reported for MAVS and IRF3 [13, 14]. Chattopadhyay et al. were first to identify and characterize the RLR-induced IRF3-mediated pathway of apoptosis (RIPA) [15]. Stimulation of RLRs with dsRNA or viral infection induces MAVS-dependent ubiquitination of IRF3 and subsequent activation of pro-apoptotic factors independent of IRF3’s transcriptional activity [16]. Furthermore, MAVS was shown to directly interact with procaspase-8, forming so-called MAVS-death-inducing signaling complexes upon viral infection [17].
Here we show that RLR signaling, IRF1, and canonical DNA damage response pathways, comprising ATM/ATR and p53, are involved in efficient triggering of cell death upon DNA damage. We show that these pathways have independent pro-apoptotic capacities, and we present new insights into IRF1’s complex cellular functions.

METHODS

Cell culture, cell line generation, and stimulation

Cells were grown at 37 °C, 95% humidity, and 5% CO2 in Dulbecco’s modified eagle medium (DMEM high glucose, Life Technologies, Carlsbad, CA, USA), supplemented with final 10% (v/v) fetal calf serum (FCS, Thermo Fisher Scientific, Waltham, MA, USA), 1x non-essential amino acids (Thermo Fisher Scientific), and 100 U/ml penicillin and 100 ng/ml streptomycin (LifeTechnologies). For generation of transgene expressing A549 cell lines by lentiviral transduction, lentiviral particles were produced by transfecting HEK 293T cells with plasmids pCMV-dR8.91, pMD2.G, and the respective retroviral vector (pWPI) using calcium phosphate transfection (CalPhos Mammalian Transfection Kit, Takara Bio Europe, Saint-Germain-en-Laye, France). After 2 days the supernatant was harvested, sterile filtered, and used to transduce target cells two times for 24 h. Transduced cells were selected with antibiotics appropriate for the encoded resistance gene (5 µg/ml blasticidin, MP Biomedicals, Santa Ana, CA, USA; 1 µg/ml puromycin, Sigma Aldrich; 1 mg/ml gentamicin (G418), Santa Cruz, Dallas, TX, USA). Knockout (KO) cell lines were generated by clustered regularly interspersed short palindromic repeats (CRISPR)/Cas9 technology. DNA oligonucleotides coding for guideRNAs against the respective genes (sequences shown in Supplementary Table S1) were cloned into the expression vector LentCRISPRv2 (Feng Zhang, Addgene #52961).

Transduced A549 wild-type cells were selected with puromycin, single-cell clones were isolated, and KO was validated by immunoblotting and functional tests (Fig. S5). We have previously generated, validated and published the following A549-based cell lines: IFNAR (IFNAR1 / IFNLR1) / FGNR (IFNFR TKO) [18], IFI16 / IFI16L1 / IFI16L2 [19], MAVS / MAVS [20], MVD88 / MVD88 [21], RELA / RelA / RELA and RIG-1 / RIG-1 [21]. A549 RIG-I OE cells and cells stably expressing IRF3-eGFP or histone H2B-mCherry were generated by stable lentiviral transduction as described previously [22]. Cells transduced with non-targeting gRNA (sequence taken from the GeCKO CRISPR v2 library) were used as controls. PHSCH non-neoplastic hepatocytes and HepG2 cells were kindly provided by Dr. Volker Lohmann (Heidelberg University, Heidelberg, Germany). HuH7.5 cells were generously provided by Dr. Charles Rice (Rockefeller University, New York). A549, HepG2 and HuH7.5 were authenticated by SNP typing (Multiplexion, Germany). Cell lines are regularly tested to be free of mycoplasma.

Stimulation was performed with doxorubicin (DOX, Hölzel Diagnostica, Cologne, Germany), etoposide (ETO, Cell Signaling Technology, Danvers, MA, USA), or cells were transfected with in vitro transcribed and chromatographically purified 200 bp 5’ppp-dsRNA (23), poly(C) (Sigma-Aldrich), and poly(C) (Sigma-Aldrich) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol. Cells were γ-irradiated with doses of 0–30 Gy using a Gammacell 40 Exactor (Best Theranotrons, Ottawa, Canada).

Real-time imaging of cell death

A549 cells stably expressing histone H2B mCherry [19] were seeded at density of 2 × 10^4 cells per 96-well. The next day, cells were stimulated with 1–2 µM DOX (10 h), 25 µM ETO (10 h), 0.1 ng/ml dsRNA (8 h), or γ-IR. DMSO (Carl Roth, Karlsruhe, Germany), poly(C) [23], and mock irradiation were used as appropriate controls. Post treatment, fresh medium was supplemented with 1:10 000 IncuCyte® Cytotoxic Green Reagent (Sartorius, Göttingen, Germany) to determine dead cells. Total cell number and dead cells were monitored every 2 h using a 10x magnification in an IncuCyte® S3 Live-Cell Analysis System (Sartorius). For IFN pre-stimulation, 200 IU/ml IFN-β (IFN-β1, Bioferon, Laupheim, Germany) or IFN-γ (R&D Systems, Minneapolis, MN, USA) were added at the time of seeding. For inhibitor administration, 40 µM Z-VDAD-FMK (Z-VDAD, R&D Systems) and 10 µM Necrostatin-7 (Sigma Aldrich) were added 2 h prior to stimulation. IncuCyte® Software (2019B Rev.2, Sartorius) was used to mask cells in phase contrast images. Calculations were performed applying the following settings: red fluorescence: segmentation top-hat, radius 100 µM, threshold (GCU) 0.4, edge split sensitivity –35, area 60–1000 µm², integrated intensity ≥60; green fluorescence: segmentation top-hat, radius 100 µM, threshold (GCU) 10, edge split sensitivity –40, area 100–700 µm², eccentricity ≤0.8, mean intensity 7–1000, and integrated intensity ≥2500. Percentage of dead cells was calculated relative to total cell count. Data represent the results of at least three biologically independent experiments. For curve charts, results were normalized to the control cell line of each replicate. Bars represent non-normalized means 36 h post treatment.

Immunofluorescence microscopy and determination of cellular IRF3 distribution

Fluorescence microscopy was performed to visualize phosphorylated histone H2A.X. After 4 h treatment with 2 µM DOX or DMSO, or 1 h post γ-IR with 20 Gy or 0 Gy, cells were permeabilized with −20 °C methanol and fixed with 4% paraformaldehyde. To block non-specific background, cells were incubated with 1 % (w/v) bovine serum albumin (BSA) and 10 % (v/v) FCS for 30 min. Primary antibodies specific for phospho-H2A.X (Cell Signaling Technology, 9718, 1:1000) were applied at 4 °C overnight. Slides were incubated with Alexa Fluor® 488 anti-rabbit (ThermoFisher Scientific, A11008, 1:1000) and DAPI (ThermoFisher Scientific, D1306, 1:5000) for 1 h. For determination of cellular IRF3 distribution, A549 cells stably expressing IRF3-eGFP and histone H2B-mCherry were stimulated either with DOX or poly(C) for 12 h. Fluorescence was visualized using a Primoview microscope (Carl Zeiss, Jena, Germany).

Immunoblotting

Stimulated cells were lysed in Laemmli sample buffer, and digested with Benzonase® Nuclease (Merck Millipore, Burlington, MA, USA). For inhibitor administration, 20 µM KU-55933 (Sigma-Aldrich), 25 µM Rabusertib (Hölzel Diagnostika), 25 µM TP-CA-1 (Sigma Aldrich), or 10 µM VE-822 (Hölzel Diagnostika) were added 2 h prior treatment. For stimulation with IFNs, 200 IU/ml IFN-α (PBL Assay Science, Piscataway, NJ, USA), IFN-β, or IFN-γ were applied over-night. Lysed samples were further denatured at 95 °C for 5 min and cleared from detritus. Resulting protein extracts were subjected to 10 % (w/v) SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA, 0.2 µm pore size). Upon incubation with 5% (w/v) BSA for 2 h to block non-specific background, membranes were probed using antibodies specific for β-actin (Sigma-Aldrich, A5441, 1:5000), calnexin (Enzo Biochem, Farmingdale, NY, USA), GAPDH (Fisher Scientific, A-1962), IL-16 (Fisher Scientific, A11008, 1:1000), or STAT1 (BD Biosciences, Franklin Lakes, NJ, USA, sc-126, 1:1000), or p53 (Santa Cruz Biotechnology, Dallas, TX, USA), p53, 1:1000, or p53 at 120 °C, 1:1000, or STAT1 (BD Biosciences, Franklin Lakes, NJ, USA, 611151, 1:1000) at 4 °C over-night. For detection, anti-rabbit horseradish peroxidase conjugate (HRP) (Sigma-Aldrich, A6154-5X1ML, 1:20 000) or anti-mouse conjugate (HRP) (Cell Signaling Technology, 7074S, 1:10 000). For detection, anti-mouse horseradish conjugate (HRP) (Sigma-Aldrich, A4416-5X1ML, 1:10 000) were applied for 1 h, membranes were covered with Amersham ECL Prime Western Blotting Detection Reagent (ThermoScientific, Westborough, MA, USA) for 5 min, and exposed to a phosphorimager (Bio-Rad). Sequences of specific antibodies are listed in Supplementary Table S2. 

Quantitative PCR with reverse transcription

Upon stimulation, cells were lysed and total RNA was isolated with the Monarch RNA isolation kit (New England Biolabs, Ipswich, MA, USA), following the manufacturer’s protocol. After extraction, complementary DNA (cDNA) was generated using the High Capacity cDNA Reverse Transcription kit (ThermoFisher Scientific). Determination of messenger RNA (mRNA) expression was performed using Taqman UNIJET® Green Supermix (Bio-Rad) on a CFX96 real-time-system (Bio-Rad). Sequences of specific oligo-primers are shown in Supplementary Table S2. GAPDH mRNA was used as a housekeeping gene control and relative expression determined by 2^-ΔΔCt (thus, not normalizing to reference gene).

Cell viability

A549 cells were seeded at a density of 6 × 10^3 cells per well. Upon treatment with 2 µM DOX or DMSO for 24 h, cell viability was determined by

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using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI, USA) following the manufacturer’s protocol. Luciferase activity was measured using a Mithras LB 943 multimode reader (Berthold Technologies, Bad Wildbad, Germany).

**Caspase activity**

A549 cells were seeded at density of 6 x 10^3 cells per 96-well. In all, 48 h post treatment with 0–2 μM DOX for 10 h, caspase-3/7 activity was determined using the Apo-ONE – PUMA assay (Promega) following the manufacturer’s instructions. Resulting fluorescence was measured using the Mithras LB 943 multimode reader (Berthold Technologies).

**Statistical analysis and reproducibility**

Sample size was chosen in accordance with the general standards in the field; no statistical method was used to predetermine the sample size. If not stated otherwise, all experiments were done in three biologically independent repetitions (n = 3), with multiple technical replicates (≥3) per experiment; experiments were not randomized, researchers were not blinded. Individual experiments were excluded only in case of technical failure. Shown are mean and standard deviation (error bars) of the replicate means. For all datasets that underwent statistical comparison, normality of the data could be assumed and variance was similar between the tested samples. Hence, a paired, two-tailed Student’s t-test was employed; *p ≤ 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

**RESULTS**

**Apoptosis induction via DNA damage response pathway in A549 cells**

To investigate the molecular links between DNA damage-induced cell death and innate immune signaling, we used A549 human lung carcinoma cells, as they are immunocompetent with intact virus sensing and IFN pathways [18]. Furthermore, we previously generated numerous functional knockouts (KOs) of components of the antiviral system (see methods) and could successfully generate KOs of further components of the antiviral and DNA damage pathways. Cells were treated with DNA DSB inducers, specifically γ-IR or the topoisomerase II inhibitors doxorubicin (DOX) and etoposide (ETO), and the resulting cell death was monitored on single-cell level by real-time imaging.

Treatment of A549 cells with DOX resulted in pronounced cell death (Fig. 1A) and a corresponding reduction of bulk cell viability (Fig. 1B), accompanied by the detection of the DNA damage marker phospho-histone H2A.X by immunofluorescence (Fig. 1C). As in DMSO control conditions no cell death was observed (Fig. 1A), for the clarity of presentation we omitted this control in the following figures but provide the control data in the supplements. In order to characterize the type of cell death predominant upon DOX-induced DNA damage, we first evaluated activation of caspase-3 and -7 being pivotal markers of apoptosis. DOX treatment activated caspase-3 and -7 in a dose-dependent manner (Fig. 1D). Conversely, we treated cells with the pan-caspase inhibitor Z-VAD in combination with Nec-7 to prevent spill-over to necroptosis [24], or depleted caspase-3 or -9. Both approaches resulted in a significant reduction of cell death upon DOX treatment (Fig. 1E, F, H). These findings confirmed prior reports that cell death driven by DOX is mainly due to apoptosis [25]. Next, we investigated typical components of the DNA damage response upstream of caspase activation. In line with p53’s (TP53) essential role in inducing apoptosis, depletion of p53 showed a significant reduction of cell death (Fig. 1G, H). Interestingly, *TP53* had the opposite effects at late time points, elevating cell death for time points >54 h (Fig. 1G). Amongst others, p53 induces apoptosis via activation of PUMA and NOXA. Accordingly, we found PUMA and NOXA transcript levels to be increased in DOX-treated cells (Fig. 1I), supporting a canonical DNA damage response through p53 in DOX-treated A549 cells.

Relevance of innate antiviral immunity pathways in DNA damage-induced cell death

In order to investigate the contribution of antiviral signaling cascades to the induction of DSB-induced cell death, we compared the impact of the major antiviral pathways using KOs of their respective signaling adapters. We observed DOX-induced cell death to be significantly reduced only by MAVS depletion (RLR signaling), but not so in the absence of STING (cGAS signaling), TRIF (TLR3 signaling), or MYD88 (general TLR signaling) (Fig. 2A–C). Despite RLR signaling appeared to play a major role, neither canonical IRF3 phosphorylation nor its nuclear translocation could be detected (Fig. 2D, E). Consistently, there was also no characteristic RLR-mediated induction of ISGs, such as *IFIT1* (Fig. 2F).

Given the observed relevance of MAVS in DOX-induced cell death, we further analysed the effect of specific RLR depletion. Both *RIG1−/−* and *MDA5−/−* reduced cell death upon DOX treatment, however, RIG-I exhibited a considerably stronger effect (Fig. 3A, C). Reciprocally, *RIG1* overexpression (OE) markedly increased cell death upon DOX treatment (but not in untreated conditions, compare Fig. S3B), underlining the decisive role of RLR signaling in this process (Fig. 3B, C). In order to determine the factors responsible for mediating cell death downstream of MAVS, we further examined the influence of transcription factors IRF3 and NF-κB p65/RELA. We observed that depletion of either factor significantly reduced DOX-induced cell death (Fig. 3D, F), what would be in line with a putative role for type I/III IFNs, whose transcription is co-dependent on both. However, using IFN-“blind” A549 *IFNAR1−/−* *IFNLR1−/−* *IFNγR−/−* (IFNR TKO) cells, we demonstrated that this effect was independent of a response mediated by secreted IFNs (Fig. 3E, F), which was further confirmed using *STAT1−/−* cells (Fig. S3E). This was in accordance with the lack of ISG expression observed previously (Fig. 2F). Thus, IRF3, and possibly NF-κB, appear to have death sensitizing effects distinct from their classical transcriptional activity in the antiviral program.

Taken together, we demonstrated that RLR signaling strongly contributes to the induction of cell death after DNA damage and that this function is independent of IFN secretion and the induction of canonical ISGs.

**Role of IRF1 in DNA damage-induced cell death**

Another transcription factor of the IRF family important for antiviral defenses [6, 18], IRF1, has previously also been implicated with the DNA damage response [26]. We hypothesized that upon genotoxic insult, IRF1 might be a downstream target of the RLR/IRF3 pathway, as reported for virus infection [18], and thereby link RLR activity to the DNA damage response. Indeed, upon DOX treatment, we observed *IRF1* upregulation at the mRNA (Fig. 4A) and protein level (Figs. 4B and S4A). Of note, *IRF1* induction occurred independently of the presence of p53 (Figs. 4B and S4A). In order to determine the relevance of IRF1 to cell death, we next tested *IRF1−/−* cells in DOX treatment. Strikingly, IRF1 depletion almost completely abolished DOX-induced cell death (Fig. 4D, G). Conversely, increasing IRF1 abundance, either by OE through stable transduction or by pre-stimulation of cells with IFN-β or IFN-γ, markedly increased cell death upon DOX treatment (Fig. 4D, E, G), and the percentage of dead cells correlated with IRF1 levels in western blot (Figs. 4C and S4B, C). Notably, neither IFN stimulation alone, nor DOX treatment in IFN-primed but IRF1-depleted cells did induce cell death (Fig. S4E–G). Surprisingly, the same phenotype was observed in *RIG1−/−* conditions (Fig. S4H), in which IRF1 was present, suggesting an independent requirement on both, RLR signaling and *IRF1* induction, for proper triggering and/or execution of cell death. Similar observations were also made after ETO treatment (Fig. S5A, C, D), ruling out DOX-specific effects.
The fundamental importance of IRF1 was additionally demonstrated in response to γ-IR. Although irradiation did induce DNA damage in A549 cells (Fig. S5F, G), we could neither observe induction of IRF1 expression nor any cell death upon administration of up to 30 Gy (Fig. 4F, G, I). Strikingly, induction of cell death upon γ-IR was restored under conditions of elevated IRF1 levels, such as stable OE or IFN-γ pre-stimulation (Fig. 4F–H). In line with this, cells in which γ-IR naturally leads to an upregulation of IRF1 expression, such as PH5CH cells, did exhibit a dose-dependent induction of cell death (Fig. S5H, I).

Thus, we showed that besides clear involvement of p53 and RLR signaling, IRF1 is essential for proper triggering of cell death upon DNA damage. IFNs, in particular IFN-γ, sensitize cells for DNA damage-induced cell death through upregulation of IRF1.

Regulation of IRF1 expression upon DNA damage

Above we have shown that RLR/IRF3 signaling as well as expression of IRF1 are important for DNA damage-induced cell death. Furthermore, across all conditions of DNA damage-induced cell death that we tested for IRF1 expression, we found the transcription factor to be upregulated. We now aimed to confirm whether IRF1 is in fact induced as a downstream target of RLR signaling. We first investigated the induction of IRF1 expression after RIG-I stimulation using dsRNA as a canonical, highly specific agonist [23]. Indeed, we observed a fully RLR-dependent (RIG-I, MAVS, IRF3) increase of IRF1 levels, with a partial contribution of p65/RELA and IFN signaling (IFNR TKO) (Fig. 5A), in line with a recent report of our lab [18]. dsRNA-stimulation furthermore also led to the induction of cell death, which was fully abolished upon depletion of the RLR signaling components RIG-I, MAVS, or IRF3 (Fig. 5B, D). Depletion of p65/RELA and the IFN receptors (IFNR TKO) had minor pro-survival effects, suggesting a potential role for transcription-independent RIPA with a possible but limited role for IFN signaling and ISG induction (Fig. 5C, D). Interestingly and in clear contrast to the situation upon DNA damage, dsRNA-induced cell death was independent of IRF1 and, for unknown reasons, rather increased in IRF1−/− conditions (Fig. 5C). Nonetheless, experimentally elevating IRF1 levels markedly increased the percentage of dead cells also in this setting (Fig. S6E–G).

These findings confirmed that, despite not being essential for cell death induction, IRF1 is induced downstream of RLR signaling, at least when stimulated by a strong RIG-I specific agonist. We
next investigated whether this would also be the case in the context of DNA damage. Unexpectedly, upon treatment of cells with DOX, induction of IRF1 expression was neither affected by depletion of RLR nor of IFN signaling components, including JAK1 (Figs. 5E, F and S6H). This suggested IRF1 expression is induced independently of and coincidentally with antiviral RLR signaling upon DNA damage. We therefore hypothesized sensing of DNA damage might directly induce IRF1. To test this, we treated cells with specific inhibitors of the prototypical DSB sensors ATM and ATR, as well as potential downstream pathways. We found IRF1 induction upon DOX-treatment to be completely blocked by the ATM inhibitor KU-55933 [27] and the ATR inhibitor VE-822 [28], suggesting important roles of these sensors in activation of IRF1 (Figs. 5G and S6I).

As IRF1 expression has previously been shown to be NF-κB sensitive [29], we employed the common pan-NF-κB and JAK1 inhibitor TPCA-1 [30, 31]. Remarkably, TPCA-1 treatment strongly diminished IRF1 expression upon DOX treatment, and even virtually completely depleted basal expression levels (Figs. 5G and S6I). This effect could further be confirmed upon RLR-stimulation with dsRNA (Fig. S7A) and even upon IFN-γ treatment, which is a strong and well-studied canonical inducer of IRF1 (Fig. S7B). We could rule out a cell line (A549) specific effect by testing three other human cell lines, PH5CH, HeLa, and Huh7.5 (Fig. S7C). To our knowledge, this striking effect of TPCA-1 on IRF1 expression has not been reported before. Again, corroborating IRF1’s crucial role in DNA damage-induced cell death, suppressing IRF1 induction by TPCA-1 also reduced cell death in DOX-treated A549, PH5CH, HeLa, and Huh7.5 cells (Fig. S7D).

Finally, we aimed to identify which signaling pathway and NF-κB subunit would be responsible for IRF1 expression upon triggering the DNA damage response. As reported in literature, ATR may signal through CHK1 to activate p50/NFKB1, a potential target of TPCA-1 [32, 33]. We therefore inhibited CHK1 by Rabusertib [34] prior to DOX-treatment. However, our experiments did not reveal any effect of CHK1 inhibition or p50/NFKB1 depletion on IRF1 levels (Figs. 5G and S6I, J). We hence conclude that a so far elusive pathway downstream of the ATM/ATR system induces IRF1.

Taken together, we demonstrated that IRF1 expression upon DOX-treatment is induced by the DSB sensors ATM/ATR rather than RLR signaling. This induction is independent of CHK1 signaling. Additionally, we identified a previously unappreciated IRF1-depleting effect of the NF-κB inhibitor TPCA-1.

DISCUSSION

Cells, particularly of multicellular organisms, have elaborate systems in place ensuring the integrity of their genome, as DNA damage poses severe risks of accumulating tumorigenic mutations or alterations. In response to excessive DNA damage beyond the potential of being properly repaired, cells trigger the execution of cell death programs, most commonly apoptosis [35]. This is also exploited for common cancer chemoradiotherapies, in which excessive DNA damage is radiologically (e.g., γ-IR) or pharmacologically (e.g., DOX or ETO) introduced, leading to the induction of cell death programs particularly in dividing tissues such as tumors. Elucidating the underlying mechanisms of how DNA damage molecularly leads to cell death is crucial to a better understanding of the circumstances leading to cancer and the pathways relevant for chemoradiotherapy. While classical DNA damage checkpoint control via p53 has been investigated thoroughly [1], much less is known about the relevance and contribution of non-canonical pathways. For example, a ground-

Fig. 2 Relevance of antiviral signaling adapters and ISG response during DOX-induced DNA damage response. A–C Percentage of dead A549 cells with functional KO of the indicated genes relative to total cells counted over time (A, B) or 36 h (C) post DOX treatment. See Fig. S2 for DMSO controls. D A549 were stimulated with 1 µM DOX or 1 ng/ml dsRNA for 8 h. Phosphorylated IRF3 (S396) was determined by western blot. Two replicate lanes per condition: A549 wild-type cells (left lanes) and A549 cells with a KO of an irrelevant gene (IRF1, right lanes). Uncropped image of western blot shown in Fig. S9A. E A549 cells were stimulated with 1 µM DOX or 2 µg/ml poly(I:C) for 12 h. Cellular distribution of IRF3 eGFP (cyan) and histone H2B (magenta) was visualized by immunofluorescence microscopy. F A549 cells were stimulated with 1 µM DOX or 10 ng/ml dsRNA for 24 h. IFIT1 mRNA transcripts were determined by qRT-PCR. A–C, F Data shown represent the results of at least three biologically independent experiments.
Functional KO or OE of the indicated genes relative to total cells counted over time (essential for certain chemotherapies)

Fig. S3A–D for DMSO controls. Data shown represent the results of at least three biologically independent experiments.

breakingly found the antiviral type I IFN pathway essential for certain chemotherapies’ efficacy [36]. Cytostatic and pro-apoptotic effects of IFNs have long been noticed [37–39]; however, it remained unresolved what triggered the production of IFNs in the studied context in the first place. Recent data also revealed cell-intrinsic triggering of cell death upon activation of antiviral signaling adapters, such as MAVS and STING. Interestingly, this was not only the case for viral infections, but also in response to DNA damage [10, 11, 40].

In the present study, we confirm this interrelationship between DNA damage response and antiviral signaling pathways, and we demonstrate a strong dependence of DOX- and ETO-triggered cell death on the presence of intact RLR/MAVS signaling. In clear contrast to recently published data, other branches of the cell-intrinsic antiviral defense, such as the TLR or the cGAS/STING system [10, 41, 42], did not affect DOX-induced cell death in our experimental setup; MyD88 KO even appeared to increase cell death (Fig. 2B). Instead, the cytosolic RNA sensors RIG-I and, to a lesser extent, MDA5 were triggered and essential for the induction of cell death. This is in line with a study by Ranoa et al. suggesting small nuclear RNAs U1 and U2 translocate into the cytoplasm in irradiated cells and trigger RIG-I activation [11]. In our experimental system, an intact RIG-I/MDA5-MAVS-IRF3 axis was essential for the full extent of cell death observed upon DNA damage; however, we could not observe canonical transcriptional activities of IRF3, such as the induction of IFN genes or ISGs. While the relevance of both IRF3 and p65/RELA suggested the involvement of IFNB expression, KO of the receptors for all three types of IFNs (IFNR TKO) did not impact cell death. A plausible mechanism for this IFN-independent triggering of apoptosis is RIPA, involving LUBAC-dependent ubiquitylation of IRF3 and subsequent activation of pro-apoptotic BH3-only proteins [16]. The clear contribution of p65/RELA in our experiments might be through its transcriptional activation of further pro-apoptotic proteins [43]. To our knowledge, cooperative effects between RIPA and NF-κB have not been described before and may be an interesting subject for future investigations.

Efficient sensing of nuclear DSBs and triggering an appropriate response is critical for cell survival upon DNA damage, or for initiating cell death and preventing potentially cancerous transformation. As expected, we observed an clear role for p53, highlighting its central function in checkpoint control, coordinating DNA damage repair and triggering apoptosis as a last resort [44]. Interestingly, depletion of p53 strongly reduced the number of apoptotic cells at early time points, but increased cell death at later times. Thus, absence of p53 led to a lack of induction of apoptosis in response to DOX-mediated DSBs at first, but likely massive accumulation of unrepaird DNA damage eventually led to increased, putatively necrotic cell death [45]. As a factor potentially linking the DNA damage response and antiviral signaling, we investigated the role of the multifunctional transcription factor IRF1, as it is known to be involved in both the DNA damage response [8, 26] and IFN signaling [6, 18, 46]. Indeed, we found that IRF1 was considerably upregulated upon DOX and ETO treatment as well as γ-IR in different cell lines. Interestingly, only in A549 cells, described to be relatively radioresistant as a common characteristic for non-small cellular lung cancers [47], IRF1 was not appreciably induced upon irradiation. We also observed reduced histone H2AX phosphorylation after γ-IR compared to DOX treatment, but potential underlying mechanisms are only partially understood and may comprise several processes [48, 49]. Nonetheless, we could further corroborate this clear correlation between IRF1 induction and triggering/execution of a cell death program on a functional level. Experimentally increasing IRF1 levels by stable OE or by pre-treatment of cells with IFN-γ, known as a strong inducer of IRF1 [46], radioresistance of A549 cells could be overcome. A similar effect has previously been demonstrated in T cells [26]. In our experiments, increased IRF1 expression also led to a sensitization towards DOX-treatment. Vice versa, IRF1 KO almost completely rescued cell survival upon DOX-, ETO-, and γ-IR-induced DNA damage. These observations clearly establish a fundamentally important role of IRF1 in DNA damage-induced cell death. This is in accordance with literature suggesting IRF1 as a biomarker for
radioresistance in tumor cells [50]. For example, extremely radioresistant osteosarcomas were shown to exhibit significantly reduced IRF1 expression levels [51]. Our data further support establishing IRF1 as a predictive biomarker in chemoradiotherapy in tumor patients.

Our finding strongly suggested IRF1 to be the functional link between the DNA damage response and the antiviral system, with RLR signaling (either directly or via the IFN/JAK/STAT cascade) leading to transcriptional activation of IRF1. However, KO experiments clearly refuted this hypothesis. Neither KO of essential factors of the RLR pathway nor of IFN signaling components abolished IRF1 induction upon DNA damage, suggesting that RLR signaling may activate IRF1 post-translationally. Generally, IRF1 is thought to be only regulated on a transcriptional level [46]. However, one study reports the requirement for “licensing” of IRF1 to become fully active, which required TLR signaling and MYD88 [52]. In preliminary experiments, we did not find any evidence for post-translational modifications in our setting, but this may warrant deeper investigations in the future. Alternatively, IRF1 might enhance the transcriptional response of IRF3, as reported before [53]. While we cannot rule out this possibility, the virtually complete inhibition of cell death in IRF1+/− despite abundant presence of IRF3 makes this unlikely. In another study, we have also not found any indication of a dampening of IRF3 responses in A549 IRF1−/− cells [18], and we see no effect of IRF1 KO on IRF3 phosphorylation (Fig. 2D, last lane). Notably, despite IRF1 being critically important for cell death induction in our system, IRF1 (over-)expression alone did not suffice to elicit cell death programs. We therefore suspect RLR signaling and IRF1 activity to cooperate further downstream, putatively via the transcriptional activation of complementary cell death promoting factors.

It is interesting to note that cell death is also elicited upon RLR stimulation by dsRNA (the canonical way to trigger antiviral signaling). Also in this case, IRF1 is induced, but strictly dependent on RIG-I and to a lesser extent dependent on IFN signaling. Surprisingly, however, depletion of IRF1 did not affect the cell death rate upon dsRNA stimulation, pointing towards transcription-independent mechanisms such as RIPA [15]. Still, KO of NF-κB (RELA) or the IFN receptors (IFNR TKO) affect cell
death, suggesting some transcriptional regulation, which, however, was independent of IRF1. This may suggest that full-fledged RLR signaling upon dsRNA encounter induces a sufficiently broad transcriptional response, which (in contrast to the situation upon DNA damage) itself is capable of triggering apoptosis or other cell death pathways. Strikingly, even in dsRNA stimulation, ectopic OE of IRF1 or pre-treatment of cells with IFN-γ led to a notable increase in the number of dying cells, putatively by the same cooperative pro-apoptotic/cell death promoting effects observed in the case of DNA damage. This observation of a general sensitization for cell death by IRF1 is in line with data showing that IRF1 OE enhances apoptosis in breast or gastric cancer treatment [54–56]. It is further plausible to speculate that reported pro-apoptotic effects of type I IFN [57, 58] would also be mediated by upregulation of IRF1 through homodimeric STAT1 transcription factor complexes (GAF) inadvertently formed early upon IFNAR engagement [59]. This could mechanistically explain how IFN-α-induced chemotherapy response and overall survival in a murine tumor model [36]. Thus, evidence further accumulates suggesting IRF1-inducing agents to be more broadly considered as adjuvants in tumor therapy.

Two central questions remain: firstly, which cell death promoting factors are specifically induced by IRF1 upon DNA damage that so potently sensitize cells to committing suicide upon (slight) RLR triggering. To this end, we are currently investigating IRF1-dependent candidate genes induced upon DOX-treatment at a transcriptomic level. Secondly, how is IRF1 induced upon DNA damage in the first place if not through classical STAT1:STAT1 activity. In our study, we found its transcriptional regulation to be fully independent of RLR signaling and p53 but completely reliant on DNA DSB sensing via ATM and ATR. Still, the downstream pathway leading to IRF1 expression remains elusive. While p65/RELA or p50/NFKB1 depletion did not affect IRF1 induction, it was almost completely abolished by TPCA-1, a commonly known inhibitor of NF-κB. Interestingly, TPCA-1 considerably reduced baseline IRF1 expression independent of the cell line used, and
could even abolish the strong induction upon IFN-γ treatment. Thus, in addition to its inhibitory effects on NF-κB, JAK1, and STAT3 [30, 31, 60], TPCA-1 appears to specifically and very efficiently inhibit the activity of an essential transcription factor for IRF1.

In conclusion, our study highlights the relevance of the antiviral RLR system for the proper and timely induction of cell death upon DNA damage. We provide evidence for independent but cooperative involvement of p53, IRF1, and IRF3 activity upon detection of DNA DSBs by the ATM/ATR machinery. We show that elevating expression levels of IRF1 lead to the sensitization towards cell death across different genotoxic insults, such as chemotherapeutics, γ-IR or cytosolic dsRNA (i.e. virus infection). These data corroborate a fundamental role for IRF1 and considerable involvement of RLR signaling in DNA damage-mediated cell death and suggest future exploration of IRF1 inducers, such as IFN-γ, together with low-dose RIG-I agonists for their potential as highly efficacious adjuvants in chemoradiotherapy. Additionally, our findings support IRF1 as a biomarker predictive for chemo- and radio-sensitivity of tumors.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY
The raw data acquired for this study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

This study was conceived and designed by M.B. and D.Y.Z., critical intellectual input was provided by V.G.M., experiments were performed by D.Y.Z. with assistance and contributions by S.W., S.S.B., and V.G.M., data was evaluated by D.Y.Z. and M.B., the manuscript was written by D.Y.Z. and M.B. and edited and approved of by all authors.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICAL APPROVAL

This research did not involve human or animal material; ethical approval was not required.

ADDITIONAL INFORMATION

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