

STING-Dependent Interferon- λ 1 Induction in HT29 Cells, a Human Colorectal Cancer Cell Line, After Gamma-Radiation

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Purpose

To investigate the induction of [type III interferons](#) (IFNs) in human [cancer cells](#) by gamma-rays.

Methods and Materials

Type III IFN expression in human cancer cell lines after gamma-ray irradiation [in vitro](#) was assessed by reverse transcription–quantitative polymerase chain reaction and [enzyme-linked immunosorbent assay](#). [Signaling pathways](#) mediating type III IFN induction were examined by a variety of means, including [immunoblotting](#), [flow cytometry](#), confocal imaging, and reverse transcription–quantitative polymerase chain reaction. Key mediators in these pathways were further explored and validated using gene [CRISPR](#) knockout or [short hairpin RNA](#) knockdown.

Results

Exposure to gamma-rays directly induced type III IFNs (mainly IFNL1) in human cancer cell lines in dose- and time-dependent fashions. The induction of IFNL1 was primarily mediated by the [cytosolic](#) DNA sensors–STING–TBK1–IRF1 signaling axis, with a lesser contribution from the nuclear factor kappa b signaling in HT29 cells. In addition, type III IFN signaling through its receptors serves as a positive feedback loop, further enhancing IFN expression via up-regulation of the [kinases](#) in the STING–TBK1 signaling axis.

Conclusions

Our results suggest that IFNL1 can be up-regulated in human cancer cell lines after gamma-ray treatment. In HT29 cells this induction occurs via the STING pathway, adding another layer of complexity to the understanding of radiation-induced [antitumor](#) immunity, and may provide novel insights into IFN-based cancer treatment.

Summary

Induction of [type I and II interferons](#) (IFNs) in irradiated tumors has been extensively documented, whereas the role of type III IFNs in tumors remains unknown. We found that gamma-rays directly induced IFNL1 in human [cancer cells](#). In HT29 cells this induction occurs via the STING–TBK1–IRF1 signaling axis and, to a lesser extent, [NF-κB](#) signaling. These findings add another layer of complexity to the understanding of irradiation-induced [antitumor](#) immunity and may provide novel insights into IFN-based cancer treatment.

Introduction

It has been increasingly acknowledged that host immunity plays an important role in the therapeutic outcome of [radiation therapy](#) [1](#), [2](#), [3](#), [4](#), [5](#). Irradiated tumor cells release a variety of immunomodulatory factors, including damage associated molecular patterns and [cytokines](#), resulting in an inflammatory [microenvironment](#) with the potential to facilitate tumor-specific immunity.

One of the most important mediators in the [antitumor](#) immune response is [interferons](#) (IFNs), a group of cytokines with the hallmark of [antiviral](#) function. Interferons have multiple antitumor effects and have been used to treat a number of malignancies for decades [\(6\)](#). New concepts have emerged suggesting that IFN [signaling pathways](#) are involved in enhancing the therapeutic efficacy of radiation treatment through immuno-stimulation. For example, localized radiation treatment not only induced lethal damage in [cancer cells](#) but also promoted intratumoral production of type II IFN, [IFN-γ](#), which led to enhanced [T-cell](#) infiltration and tumor target recognition [\(7\)](#). Another study found that the efficacy of ablative radiation treatment relied on type I IFN-dependent innate and adaptive immune responses [\(8\)](#). Irradiation of tumors deficient in STING, a component of the pathway mediating type I IFN induction, results in reduced antitumor immunity in the context of [immune checkpoint blockade](#) [\(9\)](#). These studies suggest that IFNs could help improve therapeutic efficacy by enhancing tumor-specific immune response.

There are 3 types of IFNs, defined by different receptors, all of which consist of a pair of heterologous subunits. Type I IFNs bind [IFN alpha](#) receptor subunit 1&2 and are subdivided into IFN-α (IFNA), -β (IFNB), -ω, -ε, -κ, -δ, -τ, and -ξ. Of them, only IFNA and IFNB are ubiquitously expressed. There is only 1 type II IFN, IFN-γ, which binds to a different receptor complex, [IFNGR](#) 1&2. [Interferon-γ](#) is expressed by activated [immune cells](#). Type III IFN was first discovered in 2003, and 4 [homologous proteins](#) have since been found: IFN-λ 1-4 (IFNL1-4) [10](#), [11](#), [12](#). Type III IFNs utilize a receptor complex of IFNLR1, also called [interleukin](#) (IL)-28R, together with IL-10R2, a receptor subunit shared with [IL-10](#), IL-22, and IL-26. All cell types are capable of expressing type III IFN.

Induction of type I and II IFNs in irradiated tumors has been extensively documented, whereas the role of type III IFNs in tumors remains unknown [8](#), [13](#), [14](#), [15](#). In this study we found that gamma-rays directly induced type III IFNs in human cancer cell lines in a dose- and time-dependent manner.

Induction of IFNL1 in cancer cells was mediated by the [cytosolic](#) DNA sensor–STING–TBK1–IRF1 signaling axis and, to a lesser extent, the nuclear factor kappa b (NF-κB) pathway in HT29 cells. In addition, type III IFN signaling through its receptors serves as a positive feedback loop, enhancing IFNL1 expression via up-regulation of the [kinases](#) in the STING–TBK1 signaling axis.

Methods and Materials

Cells

Human colorectal [cancer cell lines](#) HT29, LOVO, and HCT116 and human cervical cancer cell line HeLa were obtained from American Type Culture Collection. OE21 and OE33 (both human esophageal cancer cell line) were gifts from Dr Ester Hammond (University of Oxford). HT29 cells were grown in Roswell Park Memorial Institute 1640 medium. LOVO, HCT116, OE21, and OE33 were cultured in [Dulbecco's Modified Eagle Medium](#). HeLa cells were maintained in Eagle's Minimum Essential Medium. All mediums were supplemented with 10% (vol/vol) [fetal bovine serum](#) and 1% penicillin–streptomycin (all from Sigma-Aldrich, St. Louis, MO). Cells were used with passage number <8. [Mycoplasma](#) was routinely tested.

Reagents

ML120B (Bio-technie, Minneapolis, MN) was dissolved in [dimethyl sulfoxide](#) at 20 mM and used at 20 μM. Human IFNL1 (PBL, Piscataway, NJ) was used at 1000 U/mL. Human [tumor necrosis factor alphas](#) (TNFα, from Bio-technie) were used at 40 ng/mL. [Lipofectamine](#)3000 (Thermo Fisher, Waltham, MA) was used to deliver the following reagents to cells: DNA salt from herring [testes](#) (from Sigma-Aldrich), 2',3'-cyclic [guanosine monophosphate-adenosine](#) monophosphate (cGAMP), VACV-70, and Poly I:C HMW (InvivoGen, San Diego, CA). Antibodies used in this study are listed in [Table E1](#) (available online at www.redjournal.org).

Radiation treatment

Cells were seeded in 6-well plates with a density of 0.5 to 5 × 10⁴ cells/cm² and maintained at 37°C with 5% CO₂. Twenty-four hours later, cells were treated with gamma-rays at indicated time points. Gamma-ray treatment was delivered using a ¹³⁷Cs-laboratory irradiator (IBL 637; CIS bio International, Saclay, France), which produces gamma-rays (0.662 MeV) at a [dose rate](#) of 0.81 Gy/min. Fractionated radiation was delivered at 1 fraction per day with a time interval of 24 hours.

Reverse transcription–quantitative polymerase chain reaction

[Ribonucleic acid](#) (RNA) was extracted from cells using [Trizol](#) (Invitrogen, Carlsbad, CA). Their quality and quantity were measured using a NanoDrop 2000 microvolume spectrophotometer (Thermo Fisher). All RNAs were treated with [DNase](#) (Turbo DNA-free Kit, Thermo Fisher) before the [complementary DNA](#) (cDNA) synthesis. Approximately 2 μg of RNA for each sample was transcribed into cDNA using the High-Capacity cDNA [Reverse Transcription](#) Kit (Thermo Fisher).

[Quantitative polymerase chain reaction](#) (qPCR) was performed using [SYBR green](#) (Promega, Madison, WI) with specific [primers](#) (listed in [Table E2](#); available online at www.redjournal.org) or hydrolysis probes (Taqman primers/probes from Thermo Fisher), including [IFIT1](#), [IFI44](#), and [beta-actin](#) (B-Actin). B-Actin was used as a reference gene ([Fig. E1](#); available online at www.redjournal.org). Reactions were run on the Mx3005p [QPCR](#) System (Agilent Technologies, Santa Clara, CA) in a final volume of 25 μ L, with cDNA equivalent of 10 ng RNA and 400 nM primers. The cycling conditions were as follows: (1) 50°C 2 minutes, 1 cycle; (2) 95°C 2 minutes, 1 cycle; (3) 95°C 15 seconds \rightarrow 58°C 30 seconds \rightarrow 72°C 30 seconds, 40 cycles; (4) 72°C 10 minutes, 1 cycle. After the amplification, the threshold cycle (C_t) values for target genes and B-Actin were recorded. The difference of C_t (ΔC_t) between the target gene and B-Actin was calculated. The relative gene expression level of the target gene was equal to $2^{(-\Delta C_t)}$. The difference of ΔC_t ($\Delta \Delta C_t$) between the experimental sample and the control sample was calculated as well. The [messenger RNA](#) (mRNA) fold induction of the target gene in the experimental samples was then equal to $2^{(-\Delta \Delta C_t)}$. Additional data for quality control of reverse transcription–qPCR (RT–qPCR) can be found in the [Supplementary Methods](#) (available online at www.redjournal.org).

Immunoblotting

[Immunoblotting](#) was performed as described in the [Supplementary Methods](#) (available online at www.redjournal.org).

NF- κ B reporter assay

Tumor cells were [transfected](#) with Cignal lenti [nuclear factor kappa B](#) (NF- κ B) hMGFP reporter (10 transducing units per cell) using SureENTRY (Qiagen, Hilden, Germany, final concentration 8 μ g/mL) and selected with [puromycin](#) (1.5 μ g/mL) for 10 to 14 days. Human TNF α was used as a positive control to activate the [NF- \$\kappa\$ B](#) pathway in cells. NF- κ B activity of the transfected cells was monitored and quantified using an Attune [flow cytometer](#) (Thermo Fisher). The flow cytometry data were analyzed using FlowJo software (FlowJo, Ashland, OR). NF- κ B activity was calculated as the percentage of hMGFP-positive cells on a histogram plot of [Fluorescein Isothiocyanate](#) filter. Fold change of NF- κ B activity was calculated as the ratio of NF- κ B activity between experimental sample and control sample.

Gene knockdown using short hairpin RNA

Gene knockdown using [short hairpin RNA](#) (shRNA) is described in the [Supplementary Methods](#) (available online at www.redjournal.org).

Generation of knockout cell lines using CRISPR

[Gene knockout cell lines](#) were generated using the [CRISPR](#) technique. [Nickase Cas9 plasmids](#), including pSpCas9n (BB)-2A-GFP (PX461) and pSpCas9n (BB)-2A-Puro (PX462), were obtained from [Addgene](#) (Cambridge, MA, plasmids 48140 and 62987). A pair of guide [RNA primers](#) for each gene was designed using Feng Zhang laboratory's CRISPR Design (<http://crispr.mit.edu/>) and cloned

into plasmid PX461 and PX462, respectively ([Table E3](#); available online at www.redjournal.org). Plasmid [cloning](#) was verified by [sequencing](#). Cells were then transfected with PX461 and PX462 plasmids simultaneously using Lipofectamine 3000. After transfection, cells were selected with puromycin for 24 to 48 hours and processed into single-cell clones by [serial dilution](#). Cells with biallelic gene knockout were selected and validated using immunoblotting or functional assays.

Fluorescent staining and confocal imaging

Fluorescent staining and imaging were performed as described in the [Supplementary Methods](#) (available online at www.redjournal.org).

Enzyme-linked immunosorbent assay

The IFNL1 protein level in cell culture supernatants was measured using the human [IL-29ELISA](#) Ready-SET-Go kit (Thermo Fisher).

Results

Induction of type III IFNs in human cancer cell lines after exposure to gamma-rays

We asked which types of IFNs were induced after irradiation of human colorectal cancer HT29 cells in tissue culture. As shown in [Figure 1A](#), 6 Gy given as a single dose led to robust up-regulation of type I IFN (IFNB) and type III IFN (IFNL1 and IFNL2/3). We also asked whether fractionated administration of gamma-rays at the biologically [equivalent dose](#) (the treatment scheme and biologically equivalent dose calculation can be found in the [Supplementary Methods](#), available online at www.redjournal.org) led to a similar pattern of IFN induction. Despite the differences in timing, the patterns of induction were similar between single-dose and fractionated treatments. Enhanced IFNB expression in [cancer cells](#) was consistent with previous studies [9](#), [15](#), whereas induction of type III IFN in cancer cells after [gamma-irradiation](#) has not previously been reported. Induction of IFNL1 and IFIT2, one of the IFN-stimulated genes (ISGs), in HT29 showed dose- and time-dependent patterns ([Figs. 1B](#) and [1C](#)). Gamma-ray treatment of several other human cancer [cell lines](#) led to significant up-regulation of type III IFN, suggesting that induction of type III IFN by gamma-rays is a more general phenomenon ([Fig. 1D](#)).

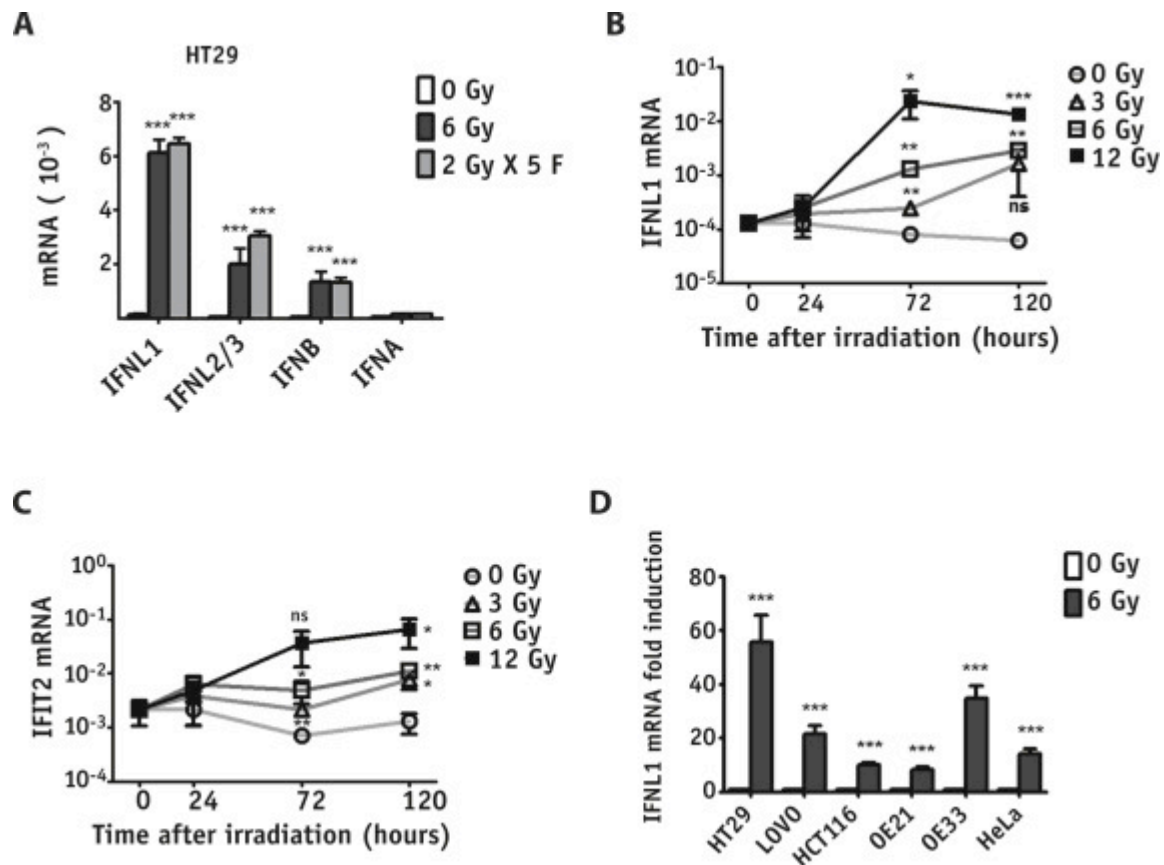


Fig. 1. Induction of [type III interferons](#) (IFNs) in human [cancer cell lines](#) after exposure to gamma-rays. (A). [Interferons messenger RNA](#) (mRNA) expressions in HT29 cancer cells at 120 hours after mock or 6 Gy or 24 hours after 2 Gy \times 5 fractions (F) of gamma-rays. HT29 cells were exposed to 0 to 12 Gy of gamma-rays. Cells were harvested at indicated time points for the assessment of IFNL1 (B) and IFIT2 (C) expression. (D) IFNL1 mRNA expression in a variety of cancer cell lines at 120 hours after mock or 6 Gy gamma-ray treatment. All mRNA expression levels were normalized to [beta-actin](#). Gene mRNA fold induction after gamma-ray treatment was normalized to expression level of control. All data in this study represents means and standard deviations of at least 3 values in more than 1 independent experiment. Comparisons were performed using Student *t* test (ns > .05, **P* < .05, ***P* < .01, ****P* < .001).

TBK1 and NF- κ B pathways contribute to IFNL1 induction by gamma-rays

The [promoters](#) of type III IFN genes have [binding sites](#) for both [NF- \$\kappa\$ B transcription](#) factors and IFN regulatory factors (IRFs) [16](#), [17](#). The [kinases](#) TANK-binding kinase 1 (TBK1) and inhibitor of NF- κ B kinase subunit beta (IKKB) mediate activation of these 2 pathways [\(18\)](#). [Ionizing](#) radiation is known to induce NF- κ B activity [\(19\)](#). We [transfected](#) HT29 cells with an NF- κ B activity hMGFP reporter [plasmid](#), allowing cellular NF- κ B activity to be monitored using [flow cytometry](#) (validated in [Fig. E2A](#); available online at [www.redjournal.org](#)). After a single dose of 6 Gy gamma-rays, HT29 exhibited gradually increased NF- κ B activity ([Fig. E2B](#); available online at [www.redjournal.org](#)). The enhanced NF- κ B activity in HT29 cells generated by either single-dose or fractionated irradiation with gamma-rays was significantly inhibited using ML120B, an IKKB-specific inhibitor ([Fig. 2A](#)).

Gamma-ray treatment also resulted in oscillating activation of TBK1 ([Fig. 2B](#)). To distinguish the contributions of these 2 pathways to IFN induction by gamma-rays, we down-regulated [IKK \$\beta\$](#) or TBK1 in HT29 with shRNA ([Figs. 2C](#) and [2D](#)). Both reduced IFNL1 induction but to different extents. TBK1 knockdown (KD) significantly down-regulated the expression of IFNL1 at both 72 and 120 hours after gamma-ray treatment, whereas IKK β KD only reduced gamma-ray-induced IFNL1 at the later 120-hour time point ([Figs. 2E](#) and [2F](#)). Inhibiting both TBK1 and NF- κ B pathways simultaneously completely abolished the induction of IFNL1. Similar results were found in LOVO cells. IFNL1 was mainly mediated by TBK1 and, to lesser extent, the NF- κ B pathway ([Fig. E3](#); available online at www.redjournal.org). Thus both pathways play a role in mediating IFN induction, with TBK1 having overall a greater effect.

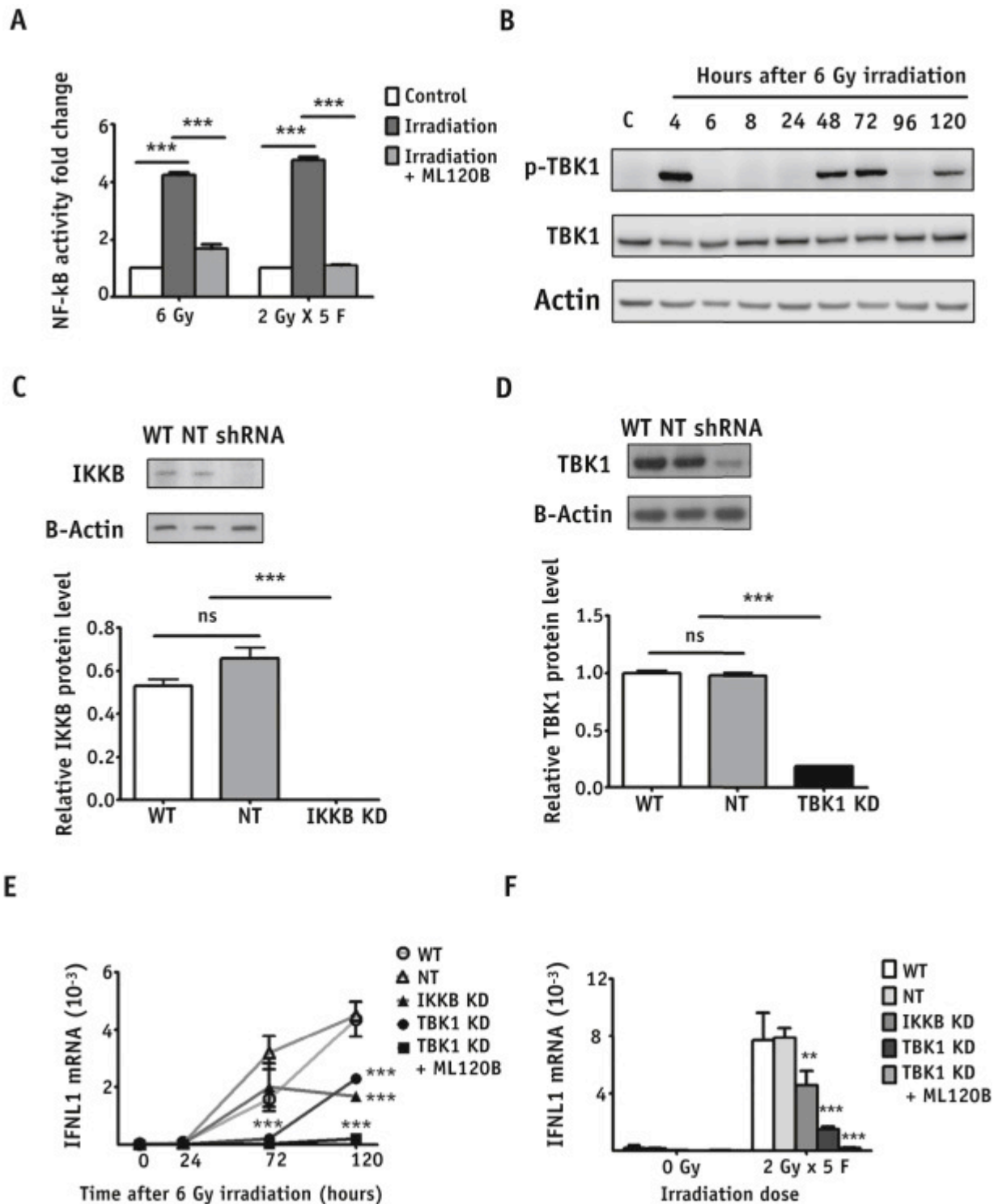


Fig. 2. TANK-binding kinase 1 (TBK1) and nuclear factor kappa B (NF-κB) pathways contribute to IFNL1 induction by gamma-rays. (A) NF-κB activity in HT29 cells at 120 hours after mock or 6 Gy or 24 hours after 2 Gy × 5 F of gamma-rays, with or without ML120B (90 minutes of preincubation and remaining in the medium through the experiment; final concentration 20 μM). Number of replicates (N) for each value = 3. (B) Phosphorylation of TBK1 in HT29 cells after 6 Gy gamma-rays. The knockdown (KD) efficacies of inhibitor of NF-κB kinase subunit beta (IKKB) (C) and TBK1 (D) in HT29 cells were assessed by immunoblotting. N = 3 for (C) and (D). Expression of IFNL1 in a panel of

HT29 cells (wild-type [WT] vs nontarget control [NT] vs [IKKB](#) KD vs TBK1 KD) after gamma-ray treatment with or without ML120B (E, F). N = 4 for (E) and (F).

Cytosolic DNA sensor–STING is the predominant signaling axis in gamma-ray induction of IFNL1

TBK1 can be activated by a variety of sensors (eg, [toll-like receptors](#) [TLRs], [RIG-I-like receptors](#), and [cytosolic](#) DNA receptors [CDRs]) in cooperation with their relevant adaptors. Of

them, [TLR3](#) and [RIG-I](#) have been found to mediate type I IFN expression in cancer cells [13, 15, 20](#).

Interestingly, in contrast to previous reports, we found that TLR3 down-regulation actually resulted in more IFNL1 expression in HT29 after gamma-ray treatment ([Fig. E4](#); available online

at www.redjournal.org). In the cell lines that we used we could barely detect RIG-I by [immunoblot](#),

although its mRNA level increased after a single dose of gamma-rays (data not shown). Thus it is less likely that RIG-I initiates the induction of IFNs after gamma-rays, though its role cannot be totally excluded.

To further delineate the pathways leading to activation of TBK1, we turned to consideration of CDRs, because recently published studies indicated that STING-dependent cytosolic DNA sensing mediated the induction of [type I interferon](#) [9, 21, 22](#). To assess the roles of STING in gamma-ray induction of

type III IFN, we eliminated expression of this gene using [CRISPR](#) (validated in [Fig. E5A](#); available

online at www.redjournal.org). Strikingly, STING KO completely abrogated IFNL1 expression in HT29

after fractionated or single-dose irradiation with gamma-rays ([Figs. 3A and 3B](#)). Consistent with our

mRNA results, gamma-ray [exposure led](#) to substantial induction of IFNL1 protein in the supernatant

of irradiated [wild-type](#) (WT) HT29. This induction was completely abrogated by knockout of STING

([Fig. 3C](#)). Thus, our evidence places STING as an adaptor in gamma-ray–mediated induction of type

III IFN. Activation of STING pathway by cGAMP treatment did not enhance NF-κB activity in

HT29 cells, indicating that NF-κB pathway is upstream of STING ([Fig. E6](#); available online

at www.redjournal.org).

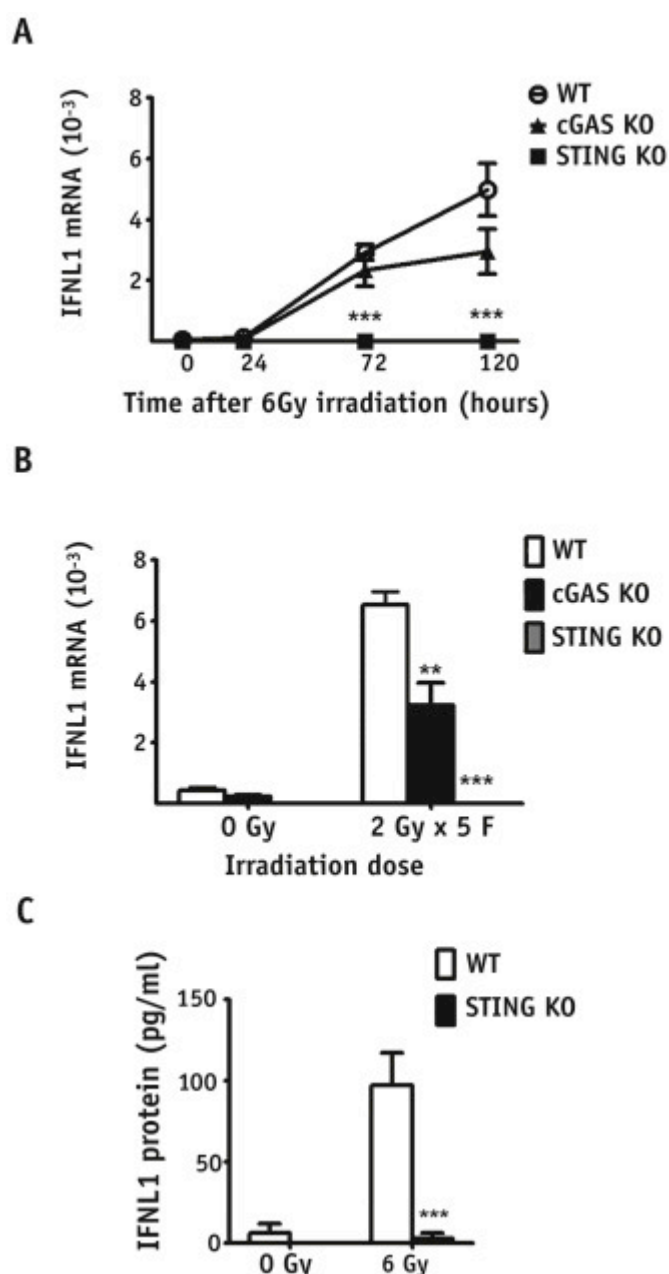


Fig. 3. [Cytosolic](#) DNA sensor–STING is the predominant signaling axis in gamma-ray induction of IFNL1. IFNL1 expression in HT29 cells (WT vs cGAS knockout [KO] vs STING KO) at 24, 72, and 120 hours after mock or 6 Gy (A) or 24 hours after 2 Gy \times 5 F (B) gamma-rays was assessed by reverse transcription–quantitative polymerase chain reaction (RT-qPCR). N = 3. (C) IFNL1 protein level in tissue culture supernatants of HT29 cells (WT vs STING KO) was measured by [enzyme-linked immunosorbent assay](#) (ELISA) at 72 hours after mock or 6 Gy gamma-rays. N = 4.

To further explore this [signaling pathway](#), we evaluated the effects of genetic elimination of Cyclic GMP-AMP [synthase](#) (cGAS) (validated in [Fig. E5B](#); available online at [www.redjournal.org](#)), one of the upstream sensors for STING, which has been implicated in recognition of cytosolic DNA or [micronuclei](#) generated as a consequence of radiation [9](#), [23](#), [24](#), [25](#). In HT29 cGAS KO decreased IFNB by 50% or less, indicating that multiple CDRs may be involved ([Figs. 3A](#) and [3B](#)).

Gamma-ray induction of IFNL1 in HT29 is mediated by IRF1

We next asked which IRFs are acting in response to gamma-rays to induce IFNL1 transcription. IRFs are mainly localized in the [cytoplasm](#) as latent factors and translocate to the nucleus after activation. Of the 9 members of IRFs, [IRF1](#), [IRF3](#), [IRF5](#), and [IRF7](#) positively regulate IFN expression, with only IRF3 and IRF7 shown to be essential [\(26\)](#). To determine the subtypes of IRFs mediating gamma-ray-induction of IFNs, we examined the translocation of IRF1, -3, and -7 from [cytoplasm to nucleus](#) in HT29 cells. As shown in [Figure 4A](#), IRF1, but neither IRF3 nor IRF7, translocated to the nucleus after gamma-radiation. Genetic deletion of IRF1 abrogated induction of type III IFN by gamma-rays ([Figs. 4B and 4C](#)).

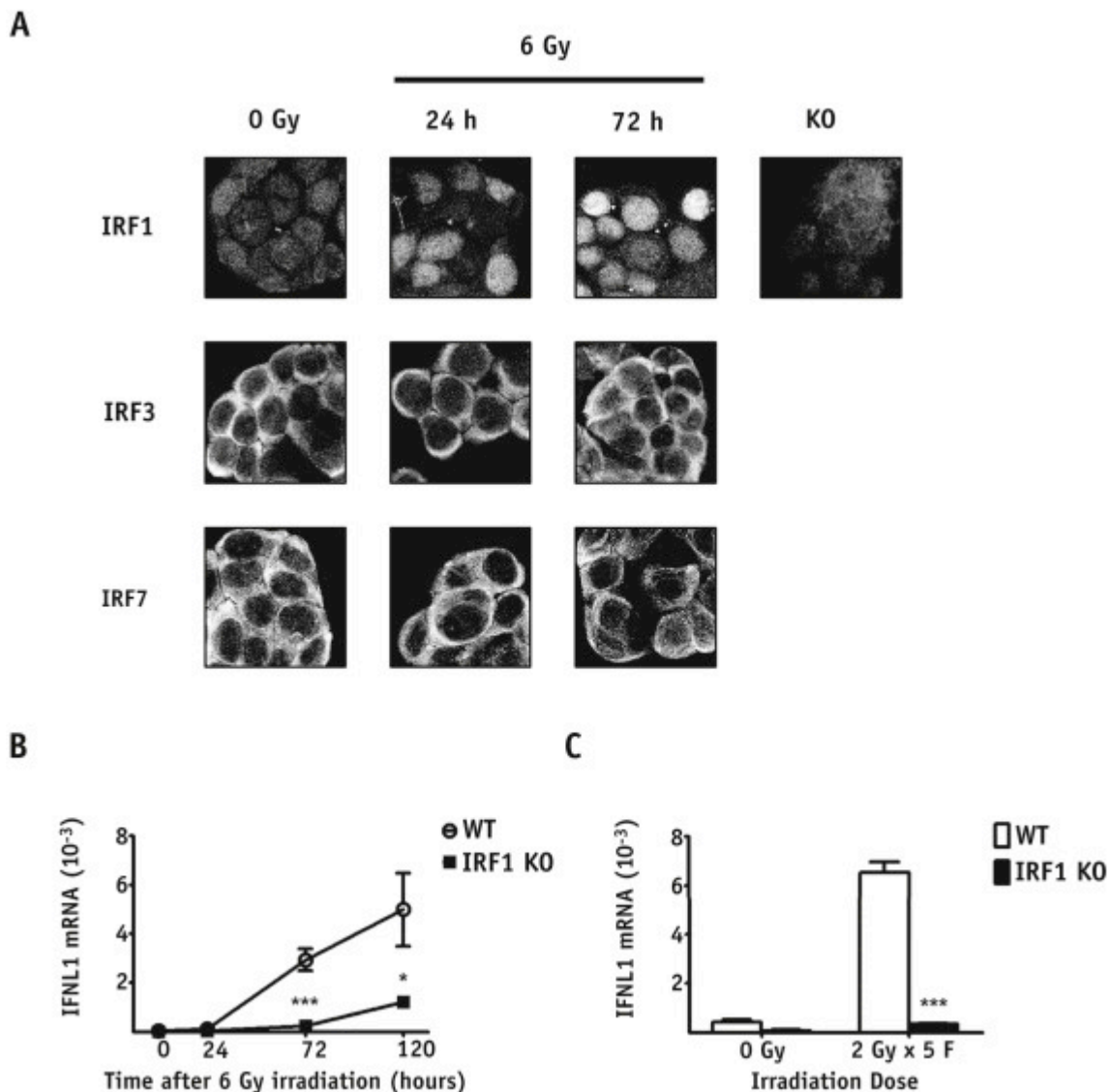
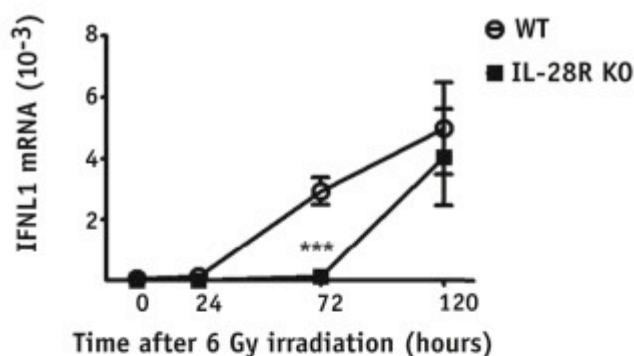


Fig. 4. Gamma-rays induction of IFNL1 is mediated by IFN regulatory factor (IRF)1 in HT29. (A) Distribution of IRF1, [IRF3](#), and [IRF7](#) within HT29 cells after mock or 6 Gy gamma-rays. IRF1 KO was also validated on the right panel. IFNL1 expression in HT29 cells (WT vs IRF1 KO) at 24, 72, and 120 hours after mock or 6 Gy (B) or 24 hours after 2 Gy \times 5 F (C) gamma-rays was assessed by [RT-qPCR](#). N = 3.

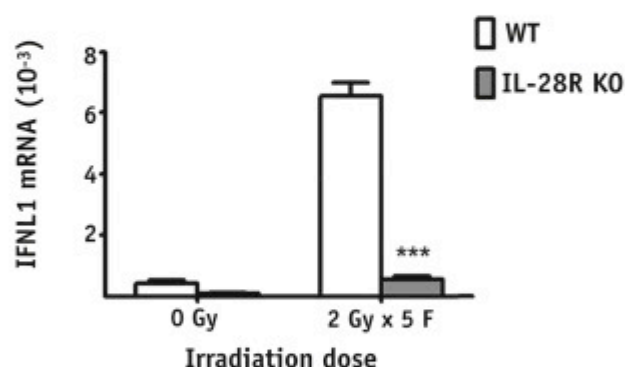
IL-28R signaling pathways act in a positive feedback loop to enhance induction of IFNL1 after radiation

Binding of IFNs to their receptors in an [autocrine](#) or [paracrine](#) fashion leads to up-regulation of ISGs. We found robust induction of IFIT2 after gamma-radiation in HT29 cells ([Fig. 1C](#)). Activation of type III IFN signaling may further enhance IFN expression by a positive feedback loop. To test this possibility, we genetically eliminated IL-28R (IFNLR) to abrogate type III IFN signaling pathways in HT29 cells (validated in [Fig. E5C](#); available online at www.redjournal.org). Remarkably, we found that IL-28R KO significantly reduced IFNL1 induction at the mRNA and protein levels in HT29 ([Fig. 5A-C](#)).

A



B



C

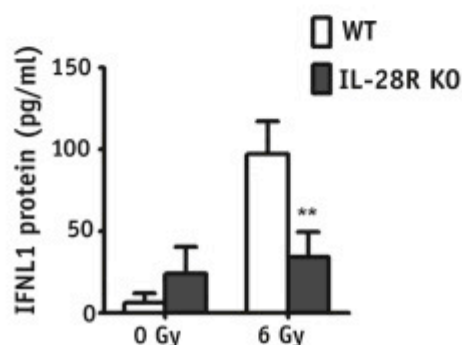


Fig. 5. [Interleukin](#) (IL)-28R [signaling pathways](#) act in a positive feedback loop to enhance induction of IFNL1 after radiation. IFNL1 expression in HT29 cells (WT vs IL-28R KO) at 24, 72, and 120 hours after mock or 6 Gy (A) or 24 hours after 2 Gy x 5 F (B) gamma-rays was assessed by [RT-qPCR](#). N = 3. (C)

IFNL1 protein level in tissue culture supernatants of HT29 cells (WT vs IL-28R KO) was measured by [ELISA](#) at 72 hours after mock or 6 Gy gamma-rays. N = 4.

In further experiments, we found that abrogation of the type III IFN signaling pathway significantly reduced the expression of key factors involved in induction of IFNL1, including cGAS, STING, TBK1, and IRFs ([Fig. E7](#); available online at www.redjournal.org). Taken together, these findings document a critical role for positive feedback loops by type III IFN signaling pathways in induction of IFNL1 by gamma-rays.

Model for induction of IFNL1 in human cancer cells by gamma-rays

On the basis of our findings, we propose a model for induction of type III IFNs in cancer cells by gamma-rays. As shown in [Figure 6, DNA damage](#) induced by gamma-rays activates STING–TBK1–IRF signaling and the NF- κ B pathway as well, leading to induction of type III IFNs. These induced type III IFNs then bind to their relevant receptors. Signaling through type III receptor acts as a feedback loop to further enhance IFNs expression via up-regulation of kinases in the cytosolic DNA signaling pathways.

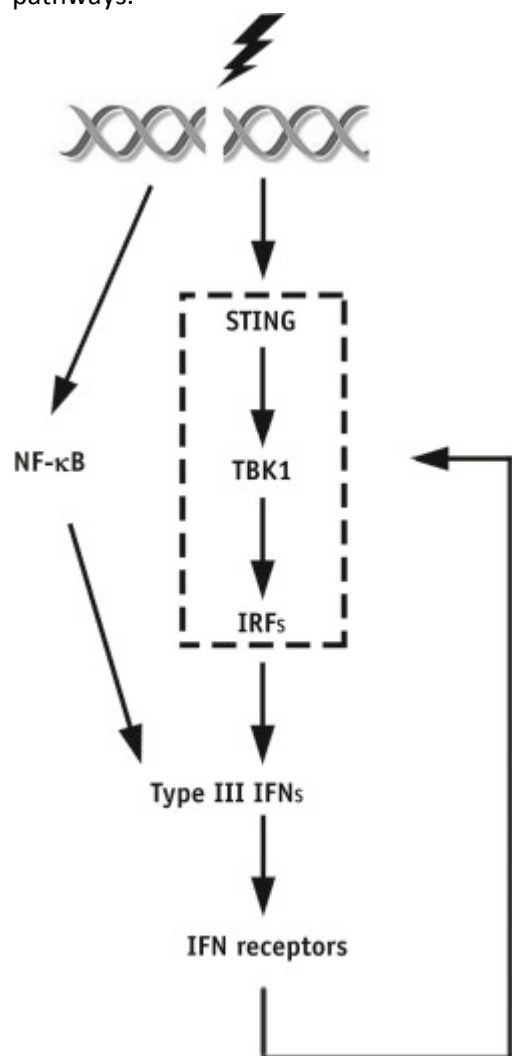


Fig. 6. Model for induction of IFNL in human [cancer cells](#) by gamma-rays.

Discussion

[Ionizing](#) radiation-induced type I or II IFNs have been implicated in [antitumor](#) immune responses after [radiation therapy](#). Here, for the first time, we demonstrate that gamma-rays, a type of ionizing radiation, also led to significant induction of type III IFNs in human [cancer cell lines](#). Moreover, we show that STING–TBK1 signaling and, to a lesser extent, [NF-κB](#) mediate type III IFN induction. The induction occurs over days, with maximum levels of mRNA at 3 to 5 days after a single dose of gamma-rays, consistent with recent findings showing a requirement of [mitosis](#) after a G2 delay for the formation of [micronuclei](#), an important source of [cytosolic](#) DNA [23](#), [24](#), [25](#).

Although both STING–TBK1 and NF-κB [signaling pathways](#) can trigger type III IFNs, NF-κB proved to have a lesser contribution in the cancer [cell lines](#) we tested and at most times. Consistent with our results, Vanpouille-Box Claire et al [\(9\)](#) recently showed that knockdown of cGAS or STING completely abrogated type I IFN induction in several cell lines. Notably, despite our data suggesting that STING is indispensable in induction of type III IFNs after gamma-rays, reduced IFN induction was still found upon inhibition of the NF-κB pathway. No cross-talk was apparent between the NF-κB and the STING pathway in our model, in agreement with a previous study showing that the NF-κB pathway is required for maximum levels of [IFNL1 gene](#) expression and could up-regulate type III in an IRF-independent manner [\(27\)](#). Nevertheless, our results suggest that induction of type III IFN in general is mainly driven by STING–TBK1 after radiation, but this is not universal, and redundancy with NF-κB exists.

The STING pathway is triggered by a variety of cytosolic sensors after detection of DNA fragments [14](#), [28](#), [29](#). Potential sources of these DNA fragments include the [nuclear genome](#), [mitochondrial DNA](#), exosomes released by neighboring cells, micronuclei, and uptake of debris after cell death [22](#), [23](#), [24](#), [25](#), [28](#), [30](#), [31](#). Many cytosolic DNA sensors have been identified, including cGAS, [IFI16](#), [DDX41](#), DNA-PK, and [Ku70](#) [32](#), [33](#), [34](#), [35](#), [36](#). Although we have not comprehensively sought to identify the cytosolic sensors, cGAS elimination only partially reduced IFN up-regulation in HT29. Clearly multiple redundant mechanisms are present.

Many human colorectal carcinoma cell lines have defective STING signaling [\(37\)](#). These cell lines may rely solely on the NF-κB pathway to express type III IFN in response to gamma-rays and may have relatively less induction. These defects in STING may also lead to reduced antitumor immune responses. Cells with deficiency in key [kinases](#) of the [DNA damage](#) machinery elicited dramatically increased presence of cytosolic DNA, resulting in type I IFN induction via the STING pathway [14](#), [38](#). Under basal conditions, cytosolic DNA can be quickly degraded by Trex1, a master DNA [exonuclease](#) in the [cytoplasm](#), and hence would be cleared before activation of the STING pathway [\(39\)](#). Patients with mutated Trex1 exhibit high levels of type I IFN, presumably resulting from the accumulation of cytosolic DNA [40](#), [41](#). In the context of inflammation, it has been shown that oxidative modification of DNA by [reactive oxygen species](#) confers resistance to Trex1 degradation, resulting in STING-dependent IFN production [\(42\)](#). Further, micronuclei induced by ionizing radiation also trigger cGAS–STING–TBK1. Whether Trex1 has any impact on micronuclei is unknown. These factors will clearly add additional heterogeneity to the induction of IFNs after radiation in cancer cells.

Type III IFN expression could be mediated by a variety of IRFs, depending on the cell type and the activated upstream signaling. Induction via [RIG-I](#) or TLR signaling has been suggested to be mediated by both [IRF3](#) and [IRF7](#), whereas [IRF1](#) was also found to play an important role in IFNL up-regulation mediated by [peroxisomal](#) MAVS or in human airway [epithelial cells](#) in response to [RNA virus](#) infection [17](#), [43](#), [44](#), [45](#). Increased type III IFN expression in murine fibroblast cell line after treatment with etoposide, a chemotherapeutic agent that, like gamma-rays, induces DNA [double-](#)

[strand breaks](#), requires the presence of IRF1 and IRF7 as well [\(46\)](#). In our hands, gamma-rays induced type III IFN in the human cancer cell lines we tested. This induction in HT29 cells was solely mediated by IRF1, and clearly IRF1 is an important downstream mediator of the STING–TBK1 pathway. Consistent with our results, a recently published study indicated that STING-mediated type III IFN production in [HEK 293 cells](#), in response to exogenous DNA, required IRF1, IRF3, and IRF7 [\(36\)](#). Collectively, these results suggest that IRF1 is an important transcriptional factor for cytosolic DNA sensing–mediated type III IFN production.

Type III and I IFNs induce a similar profile of ISGs; however, the induction magnitudes and temporal patterns differ [47, 48](#). More importantly, whereas almost all cell types respond to type I IFN, response to type III IFN is restricted to epithelial tissues and a subset of [immune cells 49, 50](#).

Because our results suggest that type III IFN signaling plays a pivotal role in enhancing induction of type III IFN in a positive feedback loop, the impacts of type III IFN on gamma-ray–induced immune response may be more profound in cancer of epithelial origins. The consequences of gamma-rays–induced type III IFN in cancer cells remain to be determined.

In summary, our results suggest that gamma-rays could significantly up-regulate type III IFNs in human cancer cell lines via the STING pathway, which has added another layer of complexity to the understanding of gamma-rays–induced antitumor immunity and may provide novel insights into IFN-based cancer treatment.