Uncoupling p53 functions in radiation-induced intestinal damage via PUMA and p21

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Abbreviations: GI, gastrointestinal; HP, hematopoietic; WBI, whole body irradiation; IR, irradiation; ISC, intestinal stem cells. TUNEL, terminal deoxynucleotidyl transferase mediated deoxyuridinetriphosphate nick end labeling.

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Leibowitz et al. Last updated on 2011 0314

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Abstract

The role of p53 in tissue protection is not well understood. Loss of p53 blocks apoptosis in the intestinal crypts following irradiation, but paradoxically accelerates gastrointestinal (GI) damage and death. PUMA and p21 are the major mediators of p53-dependent apoptosis and cell cycle checkpoints, respectively. To better understand these two arms of p53 response in radiation-induced GI damage, we compared animal survival, as well as apoptosis, proliferation, cell cycle progression, DNA damage, and regeneration in the crypts of WT, p53 KO, PUMA KO, p21 KO, and p21/PUMA double KO (DKO) mice in a whole body irradiation model. Deficiency in p53 or p21 led to shortened survival but accelerated crypt regeneration associated with massive non-apoptotic cell death. Non-apoptotic cell death is characterized by aberrant cell cycle progression, persistent DNA damage, rampant replication stress and genome instability. PUMA deficiency alone enhanced survival and crypt regeneration by blocking apoptosis, but failed to rescue delayed non-apoptotic crypt death or shortened survival in p21 KO mice. These studies help better understand p53 functions in tissue injury and regeneration, and potentially improve strategies to protect or mitigate intestinal damage induced by radiation.
Introduction

Exposure to high doses of radiation causes acute gastrointestinal (GI) injury, which is also a significant dose-limiting factor in abdominal and pelvic radiotherapy (1, 2). In contrast to the lethal hematopoietic (HP) injury developed from lower doses of irradiation, which can be rescued by bone marrow transplantation, there is no approved treatment or preventive measure for GI damage (3). Radiation models have been used extensively to understand the DNA damage response and stem cell biology (1, 4, 5). Rapidly renewing tissues such as the GI epithelium, bone marrow and hair follicles undergo extensive apoptosis in response genotoxic stresses including radiation. Loss of p53 protects the hematopoietic system and skin against DNA damage-induced injuries (6, 7), but unexpectedly exacerbates GI damage despite blocked apoptosis (8-10).

Following genotoxic stress, p53 is stabilized and activates transcriptional programs to restrict inappropriate cell proliferation and maintain genome integrity (11, 12). p53-dependent induction of p21 or PUMA is required for p53-dependent cell cycle arrest or apoptosis following ionizing radiation (IR) in most cell types (12, 13). p21 inhibits several cyclin-dependent kinases (CDKs) to initiate the G1 cell cycle checkpoint, and maintain the G2/M checkpoint in some cells (14). PUMA, a pro-apoptotic BH3-only Bcl-2 family protein, promotes Bax/Bak and mitochondria-dependent apoptosis in various cell types (15-19). Selective expression of apoptotic or cell cycle regulators has been proposed as a mechanism in determining cell fate following DNA damage (12, 13). We and others have shown recently that PUMA deficiency improves survival and tissue regeneration following lethal doses of IR by blocking apoptosis in stem and progenitor cells of the small intestine and bone marrow, which is associated with elevated p21 levels (20-23).
We hypothesized that the paradoxical role of p53 in radiation-induced intestinal damage may be explained by its major downstream targets that regulate apoptosis and cell cycle arrest independently. To test this hypothesis, we compared animal survival, as well as apoptosis, proliferation, cell cycle progression, DNA damage, and regeneration in the crypts of WT, \( p53 \) KO, \( PUMA \) KO, \( p21 \) KO, and \( PUMA/p21 \) DKO mice in a whole body irradiation model. We found that deficiency in \( p53 \) or \( p21 \) leads to accelerated crypt regeneration but shortened survival, which is associated with massive non-apoptotic cell death resulting from aberrant proliferation of clonogenic cells with persistent DNA damage and genome instability. \( PUMA \) deficiency did not rescue delayed non-apoptotic crypt death or shortened survival in \( p21 \) KO mice, indicating that that a \( p21 \)-dependent mechanism is required for the productive regeneration of crypts and improved survival in mice resistant to radiation-induced apoptosis.

**Materials & Methods**

**Mice and treatment**

The procedures for all animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. 7- to 10-week-old \( PUMA^{+/+}/p21^{+/+} \) (WT), \( PUMA^{-/-} \) (\( PUMA \) KO) (24), \( p21^{-/-} \) (\( p21 \) KO) (The Jackson laboratory, Bar Harbor, ME), \( PUMA^{-/-}/p21^{-/-} \) (DKO) and \( p53^{-/-} \) (\( p53 \) KO) (The Jackson laboratory) mice were generated from heterozygote breeding. All strains are in or have been back crossed to the C57BL/6 background for more than 10 generations (F10), and litter mates were used for \( PUMA \) KO, \( p21 \) KO and \( PUMA/p21 \) DKO. Mice were housed in micro-isolator cages in a room illuminated from 0700 to 1900 hours (12:12-hr light–dark cycle), with access to water and chow *ad libitum.*
Leibowitz et al. Last updated on 2011 0314

Genotyping of WT, PUMA KO and p53 KO (25) and p21 KO (26) alleles were performed as described. Mice were irradiated at a rate of 76 cGy/min in a $^{137}$Cs irradiator (Mark I, JL Shepherd and Associates, San Fernando, CA, USA).

**Western blotting**

Antibodies used include those against PUMA (ab9643; Abcam, Cambridge, MA), p53 and p21 (sc-6243 and sc-397; Santa Cruz Biotechnology, Santa Cruz, CA), and actin (A5541; Sigma-Aldrich, St. Louis, MO). Tissue lysates were collected from freshly scraped intestinal mucosa as previously described (25).

**Tissue processing, histological analysis, TUNEL and BrdU staining and crypt microcolony assay**

All mice were injected with 100 mg/kg BrdU (Sigma-Aldrich, St. Louis, MO) 2 hr prior to sacrifice. The intestinal tissues were harvested and processed in bundles as described (20, 27) (Supplemental Material). Histological analysis was performed by hematoxylin and eosin (H&E) staining. Mitoses were scored by visual inspections of H&E sections under microscope at magnification of 600X (28). Normal mitoses contain condensed chromosomes that show even and symmetrical separation and alignment. Aberrant mitoses contain condensed chromosomes with multi-polar spindles, lagging or misaligned chromosomes, anaphase bridges, or micronuclei.

TUNEL and BrdU staining were performed as described (20) (Supplemental Material). In brief, TUNEL staining was performed with the ApopTag Peroxidase Kit or ApopTag Fluorescein In
Situ Apoptosis Detection Kit (Chemicon International, Temecula, CA). Complete crypts extending to neighboring villi and containing at least 17 cells along either side with several Paneth cells at the bottom were used for counting in samples collected up to 48 h after irradiation unless indicated otherwise. TUNEL-positive or BrdU-positive cells were scored in 100 crypts/mouse, with a minimum of three mice per group. Data were reported as means ± SEM.

The crypt microcolony assay was used to quantify stem cell survival by counting regenerated crypts in H&E stained cross sections 3 and 4 days post-irradiation (20). More details are found in the Supplemental Material. At least three mice were used in each group and the data are reported as means ± SEM.

Immunohistochemistry (IHC) and immunofluorescence (IF)


Statistical analysis

Comparisons of the responses were analyzed by one-way ANOVA and Dunnett’s post-hoc test using GraphPad Prism 4 software. The survival data were analyzed by log-rank test using GraphPad Prism 4 software. Differences were considered significant if the probability of the difference occurring by chance was less than 5 in100 (p < 0.05).
Results

Loss of p53 or p21 leads to shortened survival but early crypt regeneration

Deficiencies in p53, PUMA and p21 have been reported to alter survival following irradiation at doses causing GI syndrome and death in mice (9, 20, 28). These experiments were not always conducted in the same genetic background and used varying doses and delivery rates. To better understand the role of p53 and a possible genetic interaction of PUMA and p21 in radiation-induced GI damage, we generated cohorts of mice that were WT or deficient in p53, PUMA, p21, or PUMA and p21 (PUMA/p21 DKO) in the C57/B6 background, and compared their survival following 15 Gy whole body irradiation (WBI) (Figure 1 A). WT mice survived an average of 7.4 days, while p53 KO mice survived an average of 4.5 days, PUMA KO mice survived an average of 10.6 days, p21 KO mice survived an average of 6.5 days, and PUMA/p21 DKO survived an average of 6.3 days (Figure 1B) (20).

Surprisingly, PUMA deficiency failed to extend the survival of p21 KO mice. We have shown earlier that PUMA, but not p53, deficiency improved survival and crypt regeneration 96 hr after IR, while both blocked apoptosis by 24 hr (20). We therefore determined the timing and extent of crypt regeneration using a microcolony assay that monitors the regeneration of single clonogenic cells following IR (1 ). We found that p53 KO, p21 KO, and PUMA/p21 DKO mice all displayed many regenerated crypts 72 hr after IR, while WT and PUMA KO mice had few or
none (Figures 1C, 1D and S1A). No regenerated crypt was found at 48 hr in any genotype (data not shown) (20). By 96 hr, all groups of mice had regenerated crypts, with PUMA KO mice displaying the greatest amount (Figure S1B and S1C). Our results indicate that early crypt regeneration is associated with shortened survival following irradiation, suggesting that cell loss might occur in regenerated crypts via a PUMA-independent or non-apoptotic mechanism.

*p53 or p21 deficiency leads to delayed non-apoptotic cell death in the intestinal crypts*

We first looked for evidence of cell death by TUNEL in the intestinal crypts. The levels of TUNEL-positive cells remained elevated in *p53* KO, *p21* KO, and PUMA/p21 DKO mice while sharply decreased in WT and PUMA KO mice 72 hr after IR (Figures 2A, 2B, and S2A). However, the levels of active caspase-3 or caspase-8, markers for apoptosis, were very low and not significantly different among all five groups 72 hr after IR (Figures S3A and 2B). More TUNEL-positive cells were also observed in the crypts of *p53* KO, *p21* KO, and PUMA/p21 DKO mice 48 hr after IR (Figure S2B and S2C), suggesting extensive non-apoptotic cell death in these otherwise “normal” appearing crypts. Using an epithelial marker pan-cytokeratin, we confirmed that this caspase-independent cell death is largely confined in the intestinal epithelium (Figures 2C and S3B). To better understand the nature of this cell death, we compared TUNEL and active caspase-3 signals in *p21* KO and WT mice at 0, 4, 24, 48, 72, and 96 hr after IR (Figure S4A and S4B). TUNEL signals stayed elevated in *p21* KO mice at 48 hr and later, but diminished in WT mice while active caspase-3 signals peaked at 4 hr and gradually decreased to a minimum at 72 hr similarly in both groups (Figures 2D and S4A and B). Caspase-8 was also activated by radiation, but declined sharply in both WT and *p21* KO crypts from 48 to 72 hr.
Leibowitz et al. Last updated on 2011 0314

(Figure S4C). Activate caspase-8 signals were less than 0.15 cell/crypt in unirradiated WT or p21 KO mice (data not shown). Therefore, loss of p21 led to elevated non-apoptotic cell death in the crypts 48 hr or later after IR during the regeneration phase (Figure 2D).

Cell death in the crypts within 24 hr of IR is largely attributed to p53-dependent apoptosis through PUMA induction (1, 20). To rule out a potential impact of p21 deficiency on apoptosis, we compared TUNEL and active caspase-3 staining in the crypts of all five groups of mice. p53 KO, PUMA KO, and PUMA/p21 DKO mice were largely protected from apoptosis as expected (Figure 3). Contrary to a recent study (29), our data suggest that accelerated crypt regeneration in p21 KO mice does not indicate enhanced survival of the stem cells and nor is correlated with better survival (3). The failure of PUMA deficiency to rescue early crypt regeneration and shortened survival of p21 KO mice (Figure 1), suggesting that a p21-dependent mechanism, independent of apoptosis, must explain the differences between p53 KO and PUMA KO mice.

The p21-dependent checkpoint and DNA repair in the intestinal crypts after IR

Given the established role of p21 in DNA damage-induced checkpoints, we performed a 2 hr BrdU pulse experiment to monitor S phase entry in the intestinal crypts after IR. DNA synthesis decreased significantly 4 hr after 15 Gy in the WT and PUMA KO (p21-competent) groups, but not in the p21- or p53-deficient groups, indicating a failure of G1 checkpoint activation (Figure 4A). Basal crypt proliferation was similar in all groups (Figure 4A). By 24 hr after IR, the rate of DNA synthesis decreased in all groups, with the p53 KO group maintaining the highest level.
In contrast, only a slightly higher rate of DNA synthesis was found in *PUMA* KO crypt, compared to WT crypts, despite blocked apoptosis (Figures 3 and 4A).

We reasoned that elevated p21 (Figure 1A) (20) might facilitate DNA repair and subsequent crypt regeneration by restricting DNA synthesis immediately after IR in *PUMA* KO mice (Figure 4A), and measured DNA double strand breaks by γ-H2AX staining in the crypts (Figure 4B). In the *PUMA* KO group, γ-H2AX foci peaked within 1 hr, sharply decreased by 2-4 hr. In comparison, γ-H2AX foci peaked around 4 hr in the WT group, coincident with extensive apoptosis (Figure 4C). From 4 to 24 hr, γ-H2AX foci decreased 22% (1.16 to 0.9/crypt) in the WT group with significant apoptosis, but decreased by over 62% (0.8 to 0.5/crypt) in the *PUMA* KO group with blocked apoptosis (Figures 4C and 3). Elevated p21 expression in the *PUMA* KO crypts was detected in the stem (CBC) and progenitor (+4-9) cells as early as 4 hr after IR (Figures 4D and S6). These findings suggest that p21 is required for radiation-induced checkpoint activation and DNA repair in the crypts.

*p53 or p21 deficiency leads to persistent DNA damage following proliferation*

Defective checkpoints and DNA repair are predicted to lead to accumulation of DNA damage and non-apoptotic death in regenerated crypts resulting from unusually rapid rounds of cell division and DNA replication (1). Concurrent with the transition from apoptotic to non-apoptotic cell death between 48 and 72 hr after IR, significantly more double strand breaks were found in p21 KO, *PUMA/p21* DKO, and *p53* KO crypts. Approximately 30-fold more cells inappropriately progressed through S phase and mitosis in these three groups, compared to the
WT or PUMA KO group (Figures 5A and 5B, and S7). Unrepaired double strand breaks in S phase are known to cause stalled DNA-replication forks and cell death if not resolved (4). Consistent with this prediction, the number of cells labeled with phospho-RPA32, a marker for stalled replication forks, increased by over 6-fold in p21- and p53-deficient mice, with many co-expressing Ki67. Fewer phospho-RPA32-positive cells were observed in the PUMA KO group than other groups (Figure 5C and 5D), consistent with enhanced DNA repair (Figure 4C). These results indicate that p53-dependent p21 induction prevents accumulation of DNA damage in the intestinal crypts following IR.

p53 or p21 deficiency leads to aberrant mitoses and severe genome instability

Loss of p53 was previously shown to increase “mitotic death” in the crypts 24 hr or later after IR (30). We reasoned that this “delayed” cell death can manifest as aberrant mitoses due to persistent DNA lesions coupled with cell division. Mitosis was suppressed by 60-70% in WT crypts 24 hr after IR, and over two thirds of those seen were abnormal (Figure 6A and 6B). Substantially more mitoses, including 10-fold more abnormal mitoses, were observed in p21- or p53-deficient crypts, consistent with loss of the G2/M checkpoint (Figure 6A and 6B). Common abnormalities included lagging or misaligned chromosomes, anaphase bridges, multi-polar spindles and micronuclei (Figure S8A). Mitosis was completely suppressed 48 hr after IR in all five groups of mice prior to crypt regeneration (data not shown). At 72 hr, significantly more aberrant mitoses were found in the regenerated crypts of p53 or p21- deficient groups compared to the WT or PUMA KO groups, which were dominated by numerous micro-nuclei indicative of severe genome instability (Figures 6C and S8B). These results suggest that “mitotic
catastrophe”, characteristic of the $p21$- or $p53$-deficient crypt cells following IR, is a form of non-apoptotic cell death, attributable to proliferation with persistent DNA damage.

**Discussion**

The role of $p53$ in tissue protection is complex, but not well understood. Using mice deficient in $p53$, \textit{PUMA}, $p21$, or \textit{PUMA} and $p21$ (\textit{PUMA}/$p21$ DKO), we showed that a paradoxical role of $p53$ in radiation-induced GI damage can be uncoupled largely through its transcriptional targets \textit{PUMA} and $p21$. Induction of \textit{PUMA} leads to rapid loss of intestinal stem cells and progenitors through apoptosis (20), and induction of $p21$ suppresses catastrophic regeneration of these cells by facilitating cell cycle arrest and DNA repair. As a result, \textit{PUMA}/$p21$ DKO mice phenocopy $p53$ KO mice, with blocked apoptosis but exacerbated GI damage (Figure 6D). In contrast, $p53$ protects against chronic intestinal degeneration caused by telomere dysfunction by removing chromosomal-unstable stem cells perhaps through apoptosis, independent of $p21$ (31, 32). It appears that a dual, and sometimes paradoxical, role of $p53$ in tissue protection is selectively mediated by its targets, which is influenced by the proliferation demand in a given tissue, stem or progenitor cell reserves, and the extent and nature of the damage. A better understanding of these mechanisms can help protect or heal ailing tissues.

Epithelial damage appears to be the major culprit in acute GI radiation toxicity that involves both apoptotic and less understood non-apoptotic cell death (1, 9, 20, 28). Our data indicate that a failure of both the G1 and G2/M checkpoints in $p21$- and $p53$-deficient mice contributes to
persistent DNA damage, massive replication stress, rampant mitotic defects, and culminates in early crypt regeneration but widespread non-apoptotic cell death. Earlier studies also reported that loss of p21 increases sensitivity to radiation or DNA damage through induction of cell death or senescence depending on the context (17, 26, 33, 34). Cell death induced by radiation appears to be more important in the intestinal epithelium, which is associated with increased proliferation or aberrant mitosis (17, 34, 35). Similarly, loss of the cdk inhibitor p27 leads to a defective G2/M checkpoint and increases genetic instability in the intestinal crypts following DNA damage (36). Deregulation of additional p53 targets might explain the more severe intestinal damage in p53 KO mice than p21 KO mice. Moreover, deficiency in DNA repair proteins ATM (37), p53BP1 (38), or Poly (ADP-ribose) polymerase-1 (PARP-1) (39) exacerbates radiation-induced crypt damage in mice, supporting the notion that DNA repair is critical for productive intestinal regeneration by suppressing genomic instability and non-apoptotic cell death.

Sub-pools of stem cells were recently proposed to be responsible for normal maintenance or regeneration following injury in rapidly renewing tissues including the GI tract (40). The apoptosis-dependent and -independent mechanisms of p53 have been shown to be differentially regulated in hematopoietic stem and progenitor cells following IR (41, 42). Our earlier work demonstrated that PUMA deficiency provided a better protection against IR-induced apoptosis in the CBCs (43), compared to p53 deficiency (27), consistent with the rapidly-renewing CBCs contributing to crypt regeneration (data not shown). It is possible that intestinal stem cells and progenitors might utilize different components of the DNA damage response machinery in response to irradiation or other cytotoxic agents. Well-defined intestinal stem cell markers and
mouse models will help provide a deeper understanding of p53-mediated DNA damage responses important in GI diseases including cancer.

There is currently no effective countermeasure against acute GI damage induced by radiation, and targeting p53 carries a high risk for cancer and is unlikely to be useful in this system (10, 44). Our studies suggest a potential strategy, blocking PUMA-dependent apoptosis helps preserve the stem and progenitor compartments immediately after irradiation, while enhanced DNA repair and genome stability, at least in part via p21, can further aid their productive regeneration (Figure 6D). A number of recent studies support this notion. Blocking PUMA-dependent apoptosis profoundly protects against IR-induced GI and HP damage (20-23) or adult stem cell depletion upon p53 activation (45), but carries little risk for spontaneous tumorigenesis and even suppresses IR-induced lymphoma (24, 46-48). Small molecule PUMA inhibitors are being developed for radiation and mitigation (49), while small molecule CDK4/6 inhibitors suppressed radiation toxicity in the HP system (50). It would be interesting to see whether these molecules can work together to protect normal tissues against radiation or chemotherapy-induced injury. In addition, activation of non-apoptotic cell death pathways might be exploited in p53 negative tumors to improve the efficacy of radiation or chemotherapy through selective killing of tumor cells (51). Therefore, manipulating PUMA and p21, as opposed to p53, might hold a greater promise in clinical settings by balancing short-term tissue reconstitution and long-term cancer risk (Figure 6D).

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Leibowitz et al. Last updated on 2011 0314

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**Author contributions**

BJL, designed and performed experiments, analyzed data and wrote the paper.

WQ and HTL, performed experiments and analyzed data.

TC and LZ, provided key reagents and analyzed data.

JY, designed experiments, analyzed data and wrote the paper.

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Leibowitz et al. Last updated on 2011 0314


Leibowitz et al. Last updated on 2011 0314


**Figure Legends**

**Figure 1.** *p53* or *p21* deficiency led to accelerated death and crypt regeneration following WBI. A) Validation of mice genotypes by PCR (left) and Western blotting using intestinal mucosal extracts 24 h after IR (right). B) The survival of WT, *PUMA* KO, *p21* KO, *PUMA/p21* DKO, and *p53* KO mice following 15 Gy WBI. *PUMA* KO vs. WT, *p* < 0.0001; DKO vs. WT, *p* = 0.0174; *p21* KO vs. WT, *p* = 0.0405; and *p53* KO vs. WT, *p* < 0.0001. C) Mice with indicated genotypes were treated with 15 Gy WBI and received 100 mg/kg BrdU by IP injection 2 h prior to sacrifice. Representative images of regenerated crypts identified by BrdU staining (brown) after 72 hr (magnification 100X). D) Quantitation of regenerated crypts 72 h after IR in mice with the indicated genotypes from 6-8 complete circumferences. Values are means ± SEM; *n* = 3 or more in each group. * indicates *p* < 0.05, and ** indicates *p* < 0.01 compared to WT.

**Figure 2.** *p53* or *p21* deficiency led to non-apoptotic cell death in the crypts following IR. A) Mice with the indicated genotypes were treated with 15 Gy WBI and sacrificed at 72 h. Cell death in the crypts was assessed by TUNEL and active caspase-3 staining. Representative images are shown (magnification 400X). B) TUNEL- or active caspase-3-positive cells were counted in 10 400X fields. When crypt structure was largely absent, the area below the villi was considered
Leibowitz et al. Last updated on 2011 0314

to be the crypt region. Values are means ± SEM; n = 3 in each group. ** indicates p < 0.01 compared to WT. C) Representative images and quantification of double IF staining for TUNEL (green) and pan-cytokeratin (red) 72 h after 15 Gy (magnification 400X). Values are means ± SEM; n = 3 in each group. ** indicates p < 0.01 compared to WT. D) Quantitation (positive cells/400X field) of active caspase-3 (Cas3*, left) and TUNEL (right) in the crypts of WT and p21 KO mice at 0, 4, 24, 48, 72, and 96 h after 15 Gy. Values are means; n = 3 in each group.

Figure 3. p21 deficiency did not affect PUMA-dependent crypt apoptosis induced by IR. Mice with the indicated genotypes were treated with 15 Gy WBI and sacrificed after 4 and 24 h. Apoptosis was analyzed by TUNEL and active caspase-3 staining. A) Representative images of TUNEL IF staining in the crypts (magnification 600X). B) Quantitation of TUNEL-positive cells by counting at least 100 crypts. Values are means ± SEM; n = 3 in each group. * indicates p < 0.05, and ** indicates p < 0.01 compared to WT. C) Representative images of active caspase-3 staining in the crypts (magnification 600X). D) Quantitation of caspase-3-positive cells by counting at least 100 crypts. Values are means ± SEM; n = 3 in each group. ** indicates p < 0.01 compared to WT.

Figure 4. p21 deficiency compromised IR-induced G1/S checkpoint and DNA repair in the intestinal crypts. Mice with the indicated genotypes were treated with 15 Gy WBI and sacrificed at indicated times. A) Proliferation in the intestinal crypts 0, 4, or 24 h after irradiation was assessed by BrdU staining. Upper, representative images are shown (magnification 400X). Lower, Quantitation of BrdU-positive cells from at least 100 crypts. Values are means ± SEM; n = 3 in each group. * indicates p < 0.05, and ** indicates p < 0.01 compared to WT at the same time point. # indicates p < 0.01 compared to WT 0 h. B) Representative images of γ-H2AX foci.
in the crypts of WT and PUMA KO mice 4 h after IR (magnification 400X).  C) Quantitation of γ-H2AX foci in the crypts of WT and PUMA KO mice at 0, 0.5, 1, 2, 4, and 24 h post-IR, time points not drawn in scale.  Values are means; n = 3 in each group.  D) Quantitation of nuclear p21 expressing cells in 100 intestinal crypts from WT and PUMA KO mice at 0, 4, 24, and 48 h after IR.  Values are means ± SEM; n = 3 in each group.  *** indicates p < 0.001 compared to WT.

**Figure 5.** *p53* or *p21* deficiency led to compromised G2/M checkpoint, replication stress and persistent DNA damage in the intestinal crypts following IR. Mice with the indicated genotypes were treated with 15 Gy WBI and sacrificed at 48 and 72 h. Cell proliferation and mitosis were analyzed by Ki67 and phospho-H3 (pH3) staining, respectively. The presence of DNA double strand breaks and single stranded DNA at the replication forks were analyzed by phospho-γ-H2AX (γ-H2AX) and phospho-RPA32 (p-RPA32), respectively.  A) Quantitation of γ-H2AX and Ki67 double positive cells at 48 and 72 h after IR from 10 400X fields in the area below the villus. B) Quantitation of double γ-H2AX and pH3 positive cells at 48 and 72 h after IR from 10 400X fields in the area below the villus. C) Representative pictures of double IF staining for p-RPA32 and Ki67 (magnification 400X). Dashed circles indicate cells stained positive for both markers.  D) Quantification of p-RPA32 (*upper*), or p-RPA32 /Ki67 double positive cells (*lower*) in (C). Values are means ± SEM; n = 3 in each group.  ** indicates p < 0.01 compared to WT.

**Figure 6.** *p53* or *p21* deficiency increased aberrant mitoses and genome instability in the intestinal crypts after IR. Mice with the indicated genotypes were treated with 15 Gy WBI and sacrificed at the indicated times. Mitoses were analyzed on H&E stained sections. A) Examples
of normal and aberrant mitoses before or 24 h after IR (magnification 600X) are indicated by an arrow and asterisks, respectively. B) Quantitation of normal and aberrant mitoses by counting at least 100 crypts before or 24 h after IR. Values are means ± SEM; n = 3 in each group. * indicates p < 0.05, ** indicates p < 0.01 compared to WT. C) Quantitation of aberrant mitoses in 20 regenerated crypts 72 h after IR. Values are means ± SEM; n = 3 in each group. ** indicates p < 0.01 compared to WT. D) A model of p53-mediated responses in IR-induced intestinal damage and protection. PUMA deficiency coupled with p21 induction, but not p53 deficiency, facilitates intestinal stem cell survival and regeneration. The p21-dependent mechanisms suppress genome instability and non-apoptotic cell death following radiation.
Leibowitz et al., Figure 1

A

![Blot images of p21 and PUMA expression in WT and KO samples](image1)

B

![Survival curve after 15 Gy irradiation](image2)

C

![Images of BrdU incorporation](image3)

D

![Bar graph of BrdU incorporation](image4)
Leibowitz et al., Figure 2

A

TUNEL

CaS3

WT  PUMA KO  p21 KO  DKO  p53 KO

72 h

B

Graph 1

WT  PUMA KO  p21 KO  DKO  p53 KO

72 h Post-IR

Graph 2

72 h TUNEL/Cytokeratin

WT  PUMA KO  p21 KO  DKO  p53 KO

Graph 3

TUNEL/Cytokeratin

WT  p21 KO  DKO  p53 KO

Graph 4
Leibowitz et al., Figure 4

A

B

C

D
Leibowitz et al., Figure 5

A

B

C

D

48 h

72 h

48 h

72 h

48 h

48 h
Leibowitz et al., Figure 6

A

24 h

WT
WT
PUMA KO
p21 KO
DKO
p53 KO

0 Gy
15 Gy

B

Untreated

C

72 h

D

p53

Apoptosis
Arrest/repair
Cell loss
Genome Stability
×

PUMA, NOXA
p21, Gadd45

ISC survival & regeneration

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Aberrant
Uncoupling p53 functions in radiation-induced intestinal damage via PUMA and p21

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