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Inhibition of Notch signaling reduces the number of surviving Dclk1+ reserve crypt epithelial stem cells following radiation injury

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Abstract

We have previously reported that doublecortin-like kinase 1 (Dclk1) is a putative intestinal stem cell (ISC) marker. In this report, we evaluated the use of Dclk1 as a marker of surviving ISCs in response to treatment with high-dose total body irradiation (TBI). Both apoptotic and mitotic Dclk1+ cells were observed 24 h post TBI associated with a corresponding loss of intestinal crypts observed at 84 h post TBI. Although the Notch signaling pathway plays an important role in regulating proliferation and lineage commitment within the intestine, its role in ISC function in response to severe genotoxic injury is not yet fully understood. We employed the microcolony assay to functionally assess the effects of Notch inhibition with DAPT on intestinal crypt stem cell survival following severe (> 8 Gy) radiation injury. Following treatment with DAPT, we observed a nearly 50% reduction in the number of surviving Dclk1+ crypt epithelial cells at 24 h after TBI and similar reduction in the number of surviving small intestinal crypts at 84 h. These data indicate that inhibition of Notch signaling decreases ISC survival following radiation injury, suggesting that the Notch signaling pathway plays an important role in ISC mediated crypt regeneration. These results also suggest that crypt epithelial cell Dclk1 expression can be used as one potential marker to evaluate the early survival of ISCs following severe radiation injury.

Keywords: DCLK1, Notch, stem cells, radiation, crypt survival

Introduction

The adult intestinal epithelium is continuously and rapidly replaced by cell replication within the crypts of Lieberku□hn and subsequent migration of their progeny onto the villus epithelium in the small intestine, or onto the surface epithelium in the colon (9). Intestinal epithelial cells are ultimately derived from multipotent stem cell(s) located near the base of each intestinal crypt (3, 4, 32, 37). In the adult mouse small intestine, these crypt stem cells divide to produce a daughter stem cell (self-renewal) as well as a more rapidly replicating transit cell. Their progeny subsequently differentiate into the mature epithelial cell types found in the small intestine as the epithelial cells migrate away from the proliferative zone in each intestinal crypt (3, 26, 27).

Crypt stem cells also play a central role in mucosal regeneration following injury (22). Intestinal injury induced by genotoxic/cytotoxic agents can disrupt the epithelial barrier resulting in the loss of crypts. Restoration of normal epithelial architecture and barrier function is a multistep process: (*a*) stem cells proliferate to increase their numbers and to give rise to the more rapidly proliferating transit cell population; (*b*) the transit cell population expands rapidly to form a regenerative crypt; and (*c*) normal patterns of epithelial differentiation are reestablished by migration and differentiation of cells produced in these regenerative crypts (9). Moreover, if the injury has completely destroyed some crypts, the surviving crypt stem cells can divide to replete the number of viable crypts (22).

A functional assay for quantifying stem cell survival following acute radiation injury to the replicating cell population has been developed based on the capacity of the surviving stem cells to regenerate crypt-like foci of cells (termed microcolonies) (22, 38). The actively proliferating transit cells are the most sensitive to ionizing radiation-induced injury; the

slowly proliferating stem cells are less sensitive to radiation. In this assay, 3 or 4 d after irradiation, the number of crypt-like foci of surviving epithelial cells is scored on histologic sections of intestine. Each epithelial foci is thought to represent the survival of one or more clonogenic stem cells able to give rise to the regenerative crypt. This assay allows for quantitative assessment of the role of exogenous agents and or molecular signal transduction pathways on crypt survival and indirectly stem cell survival following lethal dose total body irradiation.

We have reported that doublecortin-like kinase 1 (Dclk1), previously know as DCAMKL-1, is a putative ISC marker (14, 15). Dclk1+ cells were primarily found at the +4 position of the normal uninjured mouse intestinal crypt. Dclk1+ cells were also found in other positions along the crypt villus axis including crypt based columnar cells (15). Some Dclk1+ cells, particularly on the villus, appear to be cells with the morphologic appearance of Tuft/brush cells (8). The precise function of this morphologically distinct cell remains unknown. Yet in the crypt, Dclk1 also appears to mark a subpopulation of non-Tuft cells and occasionally Lgr5 expressing cells (15). In a modified label retention (following 10 Gy IR) assay, Dclk1 label-retaining cells were functionally quiescent and appeared to be functionally “anchored” at or near the +4 position (15). These data suggest that post radiation injury, all of the label retaining Dclk1+ cells are not sloughed off at the villus tip. Furthermore, Dclk1+ cells sorted from the mouse small intestine using anti-Dclk1 antibody form spheroids in suspension culture and develop into nodules when injected into the flanks of athymic nude mice. These nodules contain cells demonstrating markers of early intestinal epithelial lineage commitment (15). These studies demonstrate that Dclk1 cells isolated from intestinal crypts have self-renewal capacity.

The Notch signaling pathway plays an important role in the regulation of critical biological processes, including cellular proliferation and lineage commitment within the intestine. Inhibition of Notch signaling accelerates epithelial differentiation, while over-activation results in inhibition of all secretory lineage commitment and amplification of the intestinal progenitor pool (6). The Notch signaling pathway is considered as an intestinal stem and progenitor cell gatekeeper (13). Whether the Notch signaling pathway plays a role in ISC functions in response to radiation is not clear.

In this report we investigated the effects of inhibiting the Notch signaling pathway using DAPT on the number of surviving crypts 3.5 days after IR. We also evaluated the effects of Notch inhibition on the number of Dclk1+ crypt epithelial cells at 24 h after IR. Our results demonstrate that the effect of Notch inhibitor on Dclk1+ crypt epithelial cells was proportional to the effect on surviving crypts, which suggests that Dclk1+ cells may provide a useful surrogate for evaluating the survival of ISCs following radiation injury. This study also demonstrates that inhibition of Notch signaling decreases ISC survival with an associated loss of surviving Dclk1+ stem cells following radiation injury, suggesting that the Notch signaling pathway plays an important role in ISC mediated crypt regeneration.

Materials and Methods

Experimental animals. Six to eight-wk-old female C57Bl/6 mice (The Jackson laboratory, Bar Harbor, ME) were used in the experiments. Mice were housed under controlled conditions, including a 12-h light-dark cycle, with ad libitum access to food and water. All animal experiments were performed with the approval and authorization from the Institutional Review Board and the Institutional Animal Care and Use Committee, University of Oklahoma Health Sciences Center.

Irradiation procedure. Adult mice were exposed to TBI with air being pumped into the chamber during exposure. A Gamma-cell 40 ^{137}Cs gamma irradiator was used with a dose rate of 1 Gy IR per minute. For Difluorophenacetyl-L-alanyl-S-phenylglycine t-butyl ester (DAPT, Sigma Aldrich, St. Louis, MO) treatment, mice (n=5) were injected with DAPT (100 mg/kg in corn oil, *i.p*.) 24 h prior to IR exposure. Mice in the control group (TBI without DAPT, n=5) were injected with corn oil only. Two hours before the 84 h time interval, each mouse was injected with 5-bromo-2'-deoxyuridine (BrdU, Sigma Aldrich, St. Louis, MO, 200 µl of 5 mg/ml BrdU solution in PBS). Mice were euthanized at 6, 24, or 84 h post IR exposure.

Immunohistochemistry. Heat-induced epitope retrieval was performed on 4-µm formalin-fixed paraffin-embedded sections by utilizing a pressurized Decloaking Chamber (Biocare Medical, Concord, CA) in citrate buffer (pH 6.0) at 99°C for 18 min. For brightfield microscopy, slides exposed to peroxidase blocking solution prior to the addition of primary antibodies (anti-Dclk1 ab31704, 1:8000 dilution [Abcam, Cambridge, MA], anti-p-β-cat-Ser552, 1:500 dilution, anti-Ki67, 1:1000). After incubation with primary antibody overnight at 4^{o}C, the slides were incubated in peroxidase-conjugated polymer (Promark Series-

144 Biocare Medical, CA). Slides were developed with either Betazoid DAB or Bajoran Purple
145 HRP chromogens (Biocare Medical). To detect apoptotic cells, ApopTag Peroxidase in Situ
146 Apoptosis Detection Kit was used following the manufacturer's instructions (Millipore,
147 Billerica, MA). The apoptotic cells were detected with anti-digoxigenin conjugated with
148 FITC. To detect Dclk1+ apoptotic cells, following the incubation with anti-Dclk1 polyclonal
149 antibody, anti-rabbit secondary antibody conjugated with Alexa 547 was used.
150 *Microscopic examination.* Slides were examined with a Nikon 80i microscope and
151 DXM1200C camera for brightfield microscopy. Fluorescent images were taken with
152 PlanFluoro objectives, utilizing a CoolSnap ES2 camera (Photometrics, Tucson, AZ).
153 Images were processed using NIS-Elements software (Nikon Instruments, Melville, NY).
154 *Crypt survival study.* The number of surviving crypts was scored across each intestinal
155 cross-section circumference (a surviving crypt was defined as containing five or more
156 adjacent, BrdU-positive nuclei (7)). Twenty cross sections were measured for each mouse
157 and four mice per experimental group.
158 *Real-time RT-PCR Analyses.* Total RNA isolated from small intestine was subjected to
159 reverse transcription using Superscript™ II RNase H-Reverse Transcriptase and random
160 hexanucleotide primers (Invitrogen, Carlsbad, CA). The complementary DNA (cDNA) was
161 subsequently used to perform real-time polymerase chain reaction (PCR) by SYBR™
162 chemistry (SYBR Green I, Molecular Probes, Eugene, OR) for specific transcripts using
163 gene-specific primers and JumpStart™ Taq DNA polymerase (Sigma-Aldrich, St. Louis,
164 MO). The crossing threshold value assessed by real-time PCR was noted for the
165 transcripts and normalized with β-actin messenger RNA (mRNA). The quantitative changes
166 in mRNA were expressed as fold-change relative to control ± SEM value.

The following primers were used:

β-actin: forward: 5'-GGTGATCCACATCTGCTGGAA-3',

reverse: 5'-ATCATTGCTCCTCCTCAGGG-3';

Dclk1: forward: 5'- CAGCAACCAGGAATGTATTGGA -3',

reverse: 5'- CTCAACTCGGAATCGGAAGACT-3';

Notch1: forward: 5'-CGGGTCCACCAGTTTGAATG-3',

reverse: 5'-GTTGTATTGGTTCGGCACCAT-3'.

Hes1: forward: 5'-TCTGACCACAGAAAGTCATCA -3',

reverse: 5'-AGCTATCTTTCTTAAGTGCATC -3';

Statistical analysis. All experiments were performed in triplicate. Results were reported as average+/- SEM. Data was analyzed using the Student's t test for comparison of mean values between groups. A *P value* <0.05 was considered statistically significant.

Results

Intestinal crypt response to total body irradiation.

Adult C57Bl/6 mice were subjected to 12 Gy TBI to study the response of the small intestinal crypt to genotoxic injury. The small intestines were isolated at 6, 24, 84 and 168 h post TBI, fixed and stained with H&E (Figure 1). Morphologically apoptotic cells appeared at 6 h post radiation, suggesting the initiation of cell death induced by radiation damage. Morphologically mitotic cells appeared at 24 h post radiation, suggesting the release of stem/progenitor cells from radiation-induced cell cycle arrest allowing surviving stem/progenitor cells to divide following radiation injury. Regenerative crypts appeared at 3.5 days post radiation, and the return of normal crypt/villus axis architecture appeared at 7 days post radiation even though the crypt was still hyperplastic.

Response of Dclk1+ crypt cells and small intestinal crypts to total body irradiation.

To investigate the response of Dclk1+ crypt epithelial cells and intestinal crypts to genotoxic injury, C57Bl/6 mice were subjected to 12 Gy TBI. Apoptotic cells were identified by TUNEL assay at both 6 h and 24 h post TBI (Figure 2, center panel, green staining). Dclk1 in the crypt cells was identified by specific antibody immunostaining (Figure 2, left panel, red staining). Dclk1 staining was observed occasionally in apoptotic cells at both 6 h and 24 h post TBI (Figure 2, right panel for overlay), indicating apoptosis of potential resident stem cells after high dose radiation injury. Approximately 15% of Dclk1 positive cells were detected undergoing apoptosis at both 6 h and 24 h post TBI. In our previous report, apoptotic Dclk1 positive cells were only found at 24 h after low dose radiation (6 Gy), but not found at 6 h post 6 Gy radiation (14). These data suggested that Dclk1 cells were

resistant to low dose IR at 6 h. Moreover, these studies suggested that the 24 h mitotic activity of Dclk1+ cells could compensate for any stem cell loss as there is essentially no substantial crypt loss at doses below 8 Gy. He et al. have reported that ISCs that undergo crypt fission and crypt budding contain nuclear phosphorylated β-catenin-Ser552 (p-β-cat-Ser552) (10). In order to assess this following TBI, crypt cells were immunostained with anti-p-β-cat-Ser552 antibody 24 h post TBI. We found nuclear p-β-cat-Ser552 staining in the crypts, indicating the presence of mitotic stem cells (Figure 3, black arrows in all three sections). Dclk1 in the crypt cells was identified by specific antibody immunostaining (Figure 3, purple arrows in all three sections). Moreover, Dclk1 positive cells were found adjacent to or co-localized with p-β-cat-Ser552 positive cells with approximately 40% incidence (Figure 3, black arrowheads). Furthermore, Dclk1 staining was also found in some Ki67 positive cells 24 h post 12 Gy IR (Figure 4). These data suggest that the potential descendants of Dclk1+ cells are able to divide and proliferate 24 h after high dose TBI. We also observed the loss of intestinal crypts 84 h post 12 Gy again demonstrating the high dose requirement for crypt loss following TBI (Figure 6A). These results provide additional support for the long-standing Potten hypothesis that lethal dose (12 Gy) IR induces crypt stem cell sterilization in a majority of intestinal crypts and the descendants of the few surviving cells are able to divide and ultimately repopulate the entire intestinal epithelium (25, 28, 30).

Inhibition of the Notch signaling pathway reduces surviving Dclk1+ cells in response to TBI.

In uninjured adult mice, there are approximately 20-30 Dclk1+ cells per cross section (~150 crypts) (Data not shown). 24 h post TBI, there are approximately 11 Dclk1+ cells per cross section counted (Figure 5A), a greater than 50% reduction. This reduction is presumably the result of apoptotic cell death induced by radiation injury. Apoptotic Dclk1+ cells were detected post TBI (Figure 2). To investigate the role of the Notch signaling pathway on crypt epithelial Dclk1+ cell fate after TBI, mice were treated with the γ-secretase inhibitor, DAPT, 24 h prior to IR exposure. DAPT treatment alone for 48 h didn't affect the number of Dclk1+ cells per cross section (data not shown). Following DAPT pre-treatment and 24 h post IR exposure, there were approximately 6 Dclk1+ cells per cross section (Figure 5B, 5C), an approximately 50% reduction in the number of Dclk1+ crypt epithelial cells after DAPT treatment. These data suggest that Notch signaling pathway plays an important role in the survival of Dclk1 expressing cells measured *in situ* 24 h after TBI, and inhibition of Notch pathway may sensitize Dclk1 cells to the effects of high dose TBI.

Inhibition of Notch signaling pathway reduces the number of regenerative crypts in response to TBI.

Lethal TBI injury induces crypt stem cell sterilization in a majority of intestinal crypts (11). The appearance of regenerative crypts 84 h post radiation injury is thought to represent the survival of at least one progenitor/stem cell per crypt (22). There were several BrdU incorporated crypts (regenerative crypts) detected 84 h post TBI (Figure 6A, brown stain). After DAPT pre-treatment and 12 Gy IR exposure, the number of BrdU incorporated crypts were dramatically decreased (Figure 6B). The number of BrdU+ cells per cross section were quantified and an approximately 50% reduction in crypt survival was observed (Figure

6C). These data taken together suggest that the Notch signaling pathway plays an important role in crypt stem cell survival and/or crypt regeneration following severe radiation injury.

Inhibition of Notch signaling pathway reduces Dclk1 expression levels in the small intestine in response to high-dose radiation injury.

In our previous report, immunoreactive Dclk1 staining was not observed in regenerative crypt epithelial cells 84 h post TBI (14). Restoration of Dclk1 expression within the crypts was only observed 7 days post radiation injury when the morphologic features of the intestine are returning to normal. In this study, we measured Dclk1 mRNA levels by quantitative real time RT-PCR, 24 and 84 h post-IR with or without DAPT pre-treatment. In the DAPT alone groups, Dclk1 mRNA levels were decreased in intestine approximately 20% and 30% 48 h and 108 h post DAPT pre-treatment, respectively (Figure 7A, dotted bars of 24 h and 84 h. Note: 24 h and 84 h in this figure indicate for post TBI time, DAPT pre-treatment started 24 h prior to TBI). This result suggests that the Notch signaling pathway regulates Dclk1 expression in the small intestine. In the 24 h post TBI groups, Dclk1 mRNA levels decreased by 60% in the absence of DAPT, and approximately 50% in the presence of DAPT (Figure 7A). Thus there was no further reduction of Dclk1 mRNA levels in the presence of DAPT. In the 84 h post TBI groups, Dclk1 mRNA levels decreased about 45% in the absence of DAPT, and approximately 70% in the presence of DAPT. Since regenerative crypts at 84 h post IR were reduced 50% in the presence of DAPT (Figure 6), these data taken together support the hypothesis that inhibition of Notch signaling results in a reduction in the number of Dclk1 expressing cells.

We also measured mRNA levels of Notch1, and Hes1, one of the down-stream effectors of Notch signaling pathway, in the small intestine after DAPT and TBI treatment. In the DAPT alone groups, Notch1 mRNA levels decreased approximately 40% 24 h after treatment, and more than 50% 48 and 108 h after treatment (Figure 7B, dotted bars of 0 h, 24 h, and 84 h groups. Note: 24 h and 84 h indicate for post TBI time, DAPT pre-treatment started 24 h prior to TBI). In the 24 h post TBI groups, Notch1 mRNA levels decreased dramatically in the absence of DAPT, and DAPT treatment furthered the reduction (Figure 7B). In the 84 h post TBI groups, Notch1 mRNA levels recovered more than 30% in the absence of DAPT, but remained decreased (70%) in the presence of DAPT. In the DAPT alone groups, Hes1 mRNA levels remained unchanged 24 h after DAPT treatment, but decreased significantly (30%) after 48 h, and recovered back to baseline levels 108 h after DAPT (Figure 7C, dotted bars). In the 24 h post TBI groups, Hes1 mRNA levels decreased 60% in the absence of DAPT, and 50% in the presence of DAPT (Figure 7C). In the 84 h post TBI groups, Hes1 mRNA levels increased about 30% in the absence of DAPT, and remained decreased (60% reduction) in the presence of DAPT. These results suggest that the Notch signaling pathway is very active during crypt regeneration, supporting its importance in crypt stem epithelial cell dynamics after radiation injury.

Discussion

In this study we detected apoptotic and mitotic Dclk1+ cells at 24 h, and loss of crypts at 84 h post 12 Gy TBI. Using Notch pathway inhibitor DAPT, we demonstrated the effect of Notch inhibitor on Dclk1+ crypt epithelial cells was proportional to the effect on surviving crypts, suggesting that Dclk1+ cells may provide a useful surrogate for evaluating the survival of ISCs following radiation injury. A recent report by Hua et al. has shown that following 12 Gy TBI, the Lgr5 labeled crypt based columnar stem cells undergo apoptosis starting at 6 h and peaking at 24 h, followed by mitotic death at 24-48 h post TBI (12). Their results suggest that examining at 24 h time point may overestimate the number of surviving stem cells, and the surviving stem cells at 48 h may better predict crypt regeneration at 3.5 d post TBI (12).

Notch signaling has been implicated in stem cell maintenance in many systems through its downstream target Hes1 (5). Notch signaling drives progenitor cells towards an enterocyte lineage, while inhibition of Notch signaling drives progenitor cells towards a secretory lineage resulting in an abundance of Goblet cells (17). Despite this seemingly protective phenotype, Notch inhibitors were not protective against colitis induced by the chemical irritant DSS. Moreover, Notch inhibition appears to exacerbate colitis in other models of inflammatory injury (20). In addition, gut toxicity has been a major limiting factor in many clinical trials of Notch inhibitors for a variety of liquid tumors. This toxicity can be attenuated by limiting the dosing schedule to every 4 days allowing for recovery of the intestinal epithelium which turns over every 3-4 days. This may represent a plausible explanation for the improved tolerability of this regimen. These data led us to investigate

whether stem cells were actually deleted or at least functionally inhibited by Notch inhibition. Our results demonstrate that systemic Notch inhibition beginning 24 h prior to IR results in a 50% reduction in crypt survival after IR compared to vehicle treated controls, suggesting that inhibition of Notch can functionally reduce ISC's activity. This result seems to confirm the well-known role of the Notch pathway in stem cell biology, and these studies demonstrate a direct effect of Notch inhibition on intestinal stem cells in vivo.

To further analyze this result mechanistically, we sought to determine the effect of Notch inhibition on the expression of the putative ISC marker Dclk1 in response to radiation injury. In response to DAPT treatment alone without radiation injury, we found an approximately 20% decrease in Dclk1 mRNA levels, but no change in the number of Dclk1+ cells per cross section. Since the Dclk1+ cells were detected by immunostaining, it may not be sensitive enough to detect small changes in Dclk1 protein levels. We have reported that treating HCT116 colon cancer xenografts with DAPT results in both Dclk1 mRNA and protein downregulation in the tumor, suggesting a Notch regulatory mechanism (33). We evaluated the effects of radiation injury on Dclk1 expression 24 h post IR, the postulated time point at which p53 independent stem cell apoptosis occurs following lethal dose IR (14, 16). We chose Dclk1 for several reasons: 1) its limited expression pattern within the crypt allowed for quantitative assessment; 2) its distinct subcellular expression pattern in conjunction with labeling with nuclear markers (such as p-β-cat-Ser552) within a particular crypt could be used to determine the identity of DNA damaged cells; and 3) other putative stem cell markers are expressed in crypt based columnar and progenitor populations making it impossible to quantitatively assess the effects of Notch inhibition on crypt dynamics in two dimensional histologic sections (24). Although skepticism still exists

regarding the identity of Dclk1 expressing cells (8), there is increasing data in other models suggesting that Dclk1 plays a functional role in many cancers and is unlikely to be a marker of wholly differentiated cells. Studies are currently underway to determine the function of Dclk1 in the gut in normal homeostasis and in cancer. The possibility that Dclk1 and other markers that are observed at the +4 position (i.e. Msi1, Bmi1, Lrig1, and mTert) (18, 23, 29, 31, 34, 39) may serve to mark "rescue stem cells" in response to severe genotoxic/cytotoxic injury must also be considered.

The studies presented here attempt to assess the role of Notch signaling on epithelial crypt stem cell fate in response to genotoxic/cytotoxic injury using Dclk1 as an *in situ* marker of reserve or rescue stem cells. Notch signaling may play a key role as a regulatory mechanism for protection of the stem cell under severe DNA damage. Recent studies using Notch deficient mice have demonstrated that Notch deletion results in a reduction in Wnt target genes including the novel stem cell marker Lgr5 (21). These findings suggest in a correlative manner that Notch may be upstream of Wnt with respect to stem cell dynamics and that Notch inhibition may indeed result in reduced Wnt signaling. This combined reduction in stem cell pathway signaling may be a welcome approach in attenuating the stem cell phenotype in cancer by reducing the cell of origin as well as the proliferative potential of the stem cell offspring. This hypothesis would certainly be in play if a hierarchical or interconversion model of the stem cell niche were the reality. Because the identity and validation of novel stem cell markers continues to be debated, functional assays of stem cell survival such as clonogenic microcolony assays remain necessary. Evaluation of Dclk1 expression in radiation and related injury models may serve as an effective model to test the effects of therapeutic agents on gut stem cells directly. This use

of Dclk1 is currently feasible as commercial antibodies that are easy to use and yield reproducible results in many tissues are available. Furthermore, the expression in a minority of cells allow for quantitative assessment of the response to genotoxic/cytotoxic injury particularly 24 h after radiation injury. We are aware of reports that Dclk1 represents a fully differentiated Tuft cell or enteroendocrine cell based on immunohistochemistry (8). We contend the functional properties of Dclk1 and previous reported sorting studies identify a subset of Dclk1 cells that are not fully differentiated. In fact, a recent study by Bjerknes *et al.* confirmed the presence of an early Tuft cell in the crypt with a different marker expression profile (1). Finally, there is a possibility that a subset of tuft cells has stem cell like properties. In fact, several recent reports suggest that other cell types can revert to stem cells upon crypt damage (2, 35, 36). Using a transgenic mouse model (Cyp1A1-H2B-YFP), Buczacki et al reported that label-retaining cells (Histone 2B positive) at the crypt base of the small intestine have a combined secretory and stem-cell signature, and demonstrate clonogenicity after injury (2). Additionally, Van Es et al. showed that Dll1+ secretory progenitor cells can revert to stem cells during intestinal crypt regeneration following injury (35). Using the Sox9-EGFP transgenic mouse model, Van Landeghem et al. found that Sox9-EGFP high cells have both an enteroendocrine and a +4 ISC signature, and express Dclk1 protein. Moreover, isolated Sox9-EGFP high cells form organoids in vitro after radiation injury, suggesting that Sox9-EGFP high cells contain radiation-activated cells with ISC characteristics that likely participate in crypt regeneration post injury (36). A recent report by Nakanishi et al. shown that there was no detectable BrdU incorporation in Dclk1+ cells in the normal intestine, and extremely small number of blue stripes comprised LacZ-labeled Dclk1+ cell lineages 14 days after 8 Gy IR in the Dclk1-Cre-ERT2 lineage

tracing mouse (19). It is hypothesized that the +4 position quiescent stem cell is a potential rescue stem cell, and may play a minor role in normal intestinal homeostasis where Lgr5+ rapidly cycling stem/progenitor cells may have a predominant role. In this report, they used 8 Gy IR to activate lineage tracing. However, it may be necessary to use a > 8 Gy high dose IR to functionally achieve lineage tracing from a “rescue stem cell”.

The results reported here lend additional support to the hypothesis that the Notch signaling pathway is a valid target for anti-stem cell based therapies. Furthermore, these studies provide a reasonable explanation for the side effect profile observed with GSIs and establish Dclk1 expression as an effective marker for testing the effects of therapeutic agents in radiation and related injury models. Additional approaches to the inhibition of the Notch signaling pathway may be beneficial for anti-cancer drug development.

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Figure Legends

Figure 1. The intestinal crypts response to radiation injury. Wild type C57Bl/6 mice were subjected to 12 Gy IR. The small intestinal crypts isolated 6, 24, 84, and 168 h post IR were stained with H&E and presented. The magnification is 400x.

Figure 2. Identification of Dclk1 positive apoptotic cells in small intestinal crypts after TBI. The small intestine isolated 6 and 24 h post TBI were fixed with formalin and embedded in paraffin. Paraffin-embedded sections were immunostained with anti-Dclk1 antibody (red in the left panel) and ApopTag Peroxidase for apoptotic cells (green in the middle panel). Co-localization of Dclk1 (red) in apoptotic cells (green) is indicated by arrows in the overlay pictures (right panel). Upper panels are 6 h post TBI and lower panels are 24 h post TBI. The magnification is 200x, and the magnification for the inset is ~600x.

Figure 3. Identification of Dclk1 positive mitotic cells in small intestinal crypts after TBI. The small intestine isolated 24 h post TBI were fixed with formalin and embedded in paraffin. Paraffin-embedded sections were immunostained with anti-Dclk1 antibody (purple, purple arrow) and anti-phospho β-catenin Ser552 antibody (brown, black arrow). Co-localization of Dclk1 with phospho β-catenin was indicated by arrowhead. The magnification is 400x.

Figure 4. Identification of Dclk1 positive proliferative cells in small intestinal crypts after TBI. The small intestine isolated 24 h post TBI were fixed with formalin and embedded in paraffin. Paraffin-embedded sections were immunostained with anti-Dclk1

antibody (purple) and anti-Ki67 antibody (brown) (**A**). Co-localization of Dclk1 with Ki67 was indicated by arrow. The magnification for the inset is ~600x (**B**).

Figure 5. Inhibition of Notch1 signaling pathway reduced surviving Dclk1 positive cells after radiation injury. C57Bl/6 mice in control group (**A**) or DAPT treated group (**B)** are subjected to TBI. 24 h post IR, the small intestines were isolated, fixed, processed, and stained with anti-Dclk1 antibody. Dclk1 positive cells in one cross section were indicated by arrows in panels A and B. The magnification is 40x for the cross section pictures, and ~600x for the inset. The average number of Dclk1 positive cells per cross section was counted for total of 80 cross sections and presented in panel **C**. Values in the bar graph are given as average ± SEM and * denote statistically significant difference ($^{*}p<0.01$) compared to control.

Figure 6. Inhibition of Notch1 signaling pathway reduced the number of surviving crypts after radiation injury. C57Bl/6 mice in control group (**A**) or DAPT treated group (**B**) were subjected to TBI. These mice were injected with BrdU solution 82 h post IR, and killed 84 h post IR. The small intestines were isolated, fixed, and processed. BrdU positive cells are the indication for surviving crypts in panels **A** and **B**. The magnification is 40x for the cross section pictures, and ~600x for the inset. The average number of surviving crypts per cross section was counted for total of 80 cross sections and presented in panel **C.** Values in the bar graph are given as average ± SEM and * denote statistically significant difference ($^{*}p<0.01$) compared to control.

Figure 7. Inhibition of Notch1 signaling pathway decreased Dclk1 mRNA expression after TBI. Total RNAs were isolated from the small intestine after each treatment. mRNA expression levels were measured by quantitative real-time RT-PCR, and normalized against β-actin level. **A.** Dclk1 mRNA after DAPT and radiation injury. **B**. Notch1 mRNA after DAPT and radiation injury. **C**. Hes1 mRNA after DAPT and radiation injury. Values in the bar graphs are given as average ± SEM and * denote statistically significant difference ($^{*}p<0.05$) compared to control.

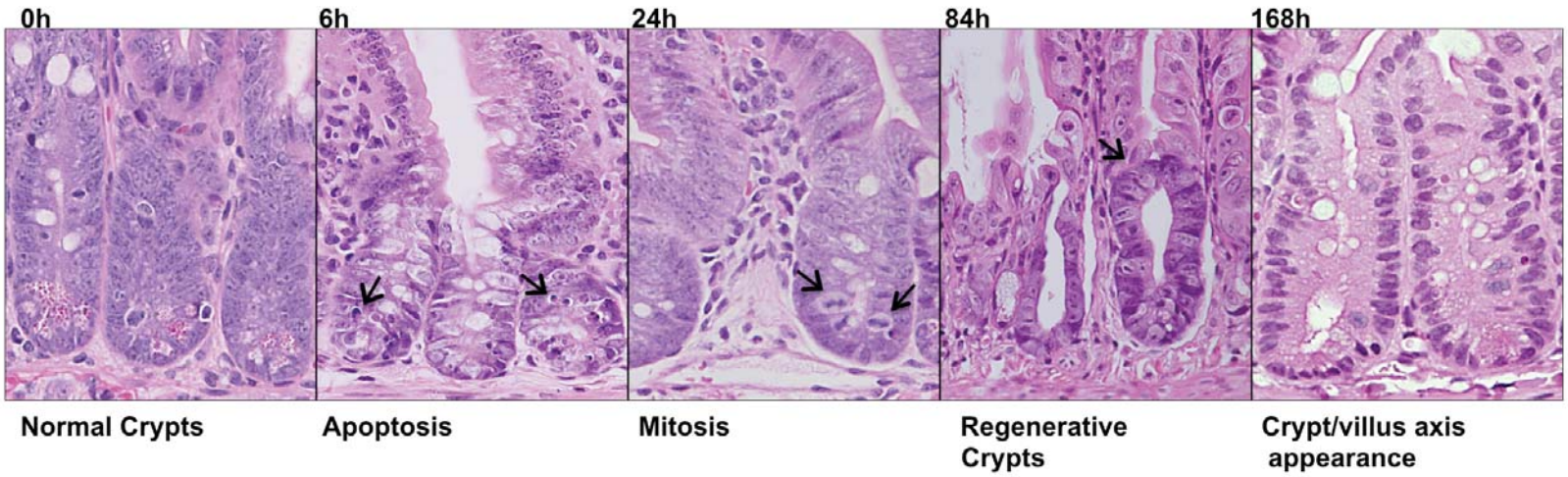

0h
6h
24h
84h
168h
Normal Crypts
Apoptosis
Mitosis
Regenerative Crypts
Crypt/villus axis appearance

Figure 1.

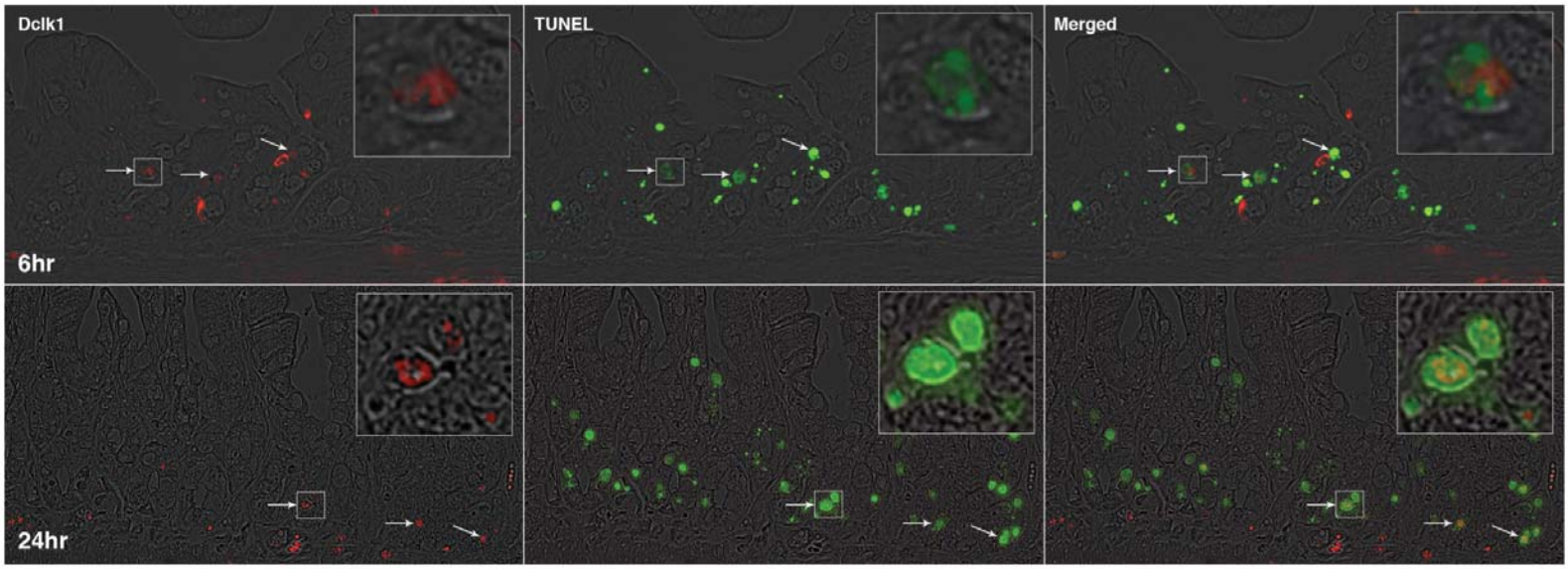


Figure 2.

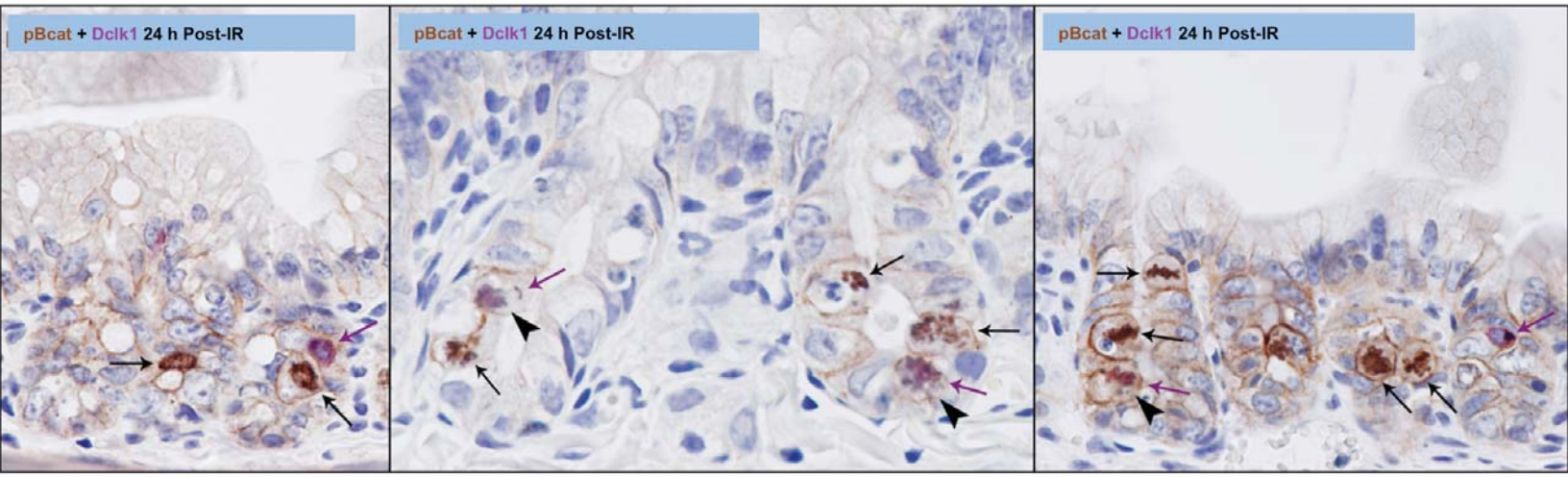

pBcat + Dclk1 24 h Post-IR
pBcat + Dclk1 24 h Post-IR
pBcat + Dclk1 24 h Post-IR

Figure 3.

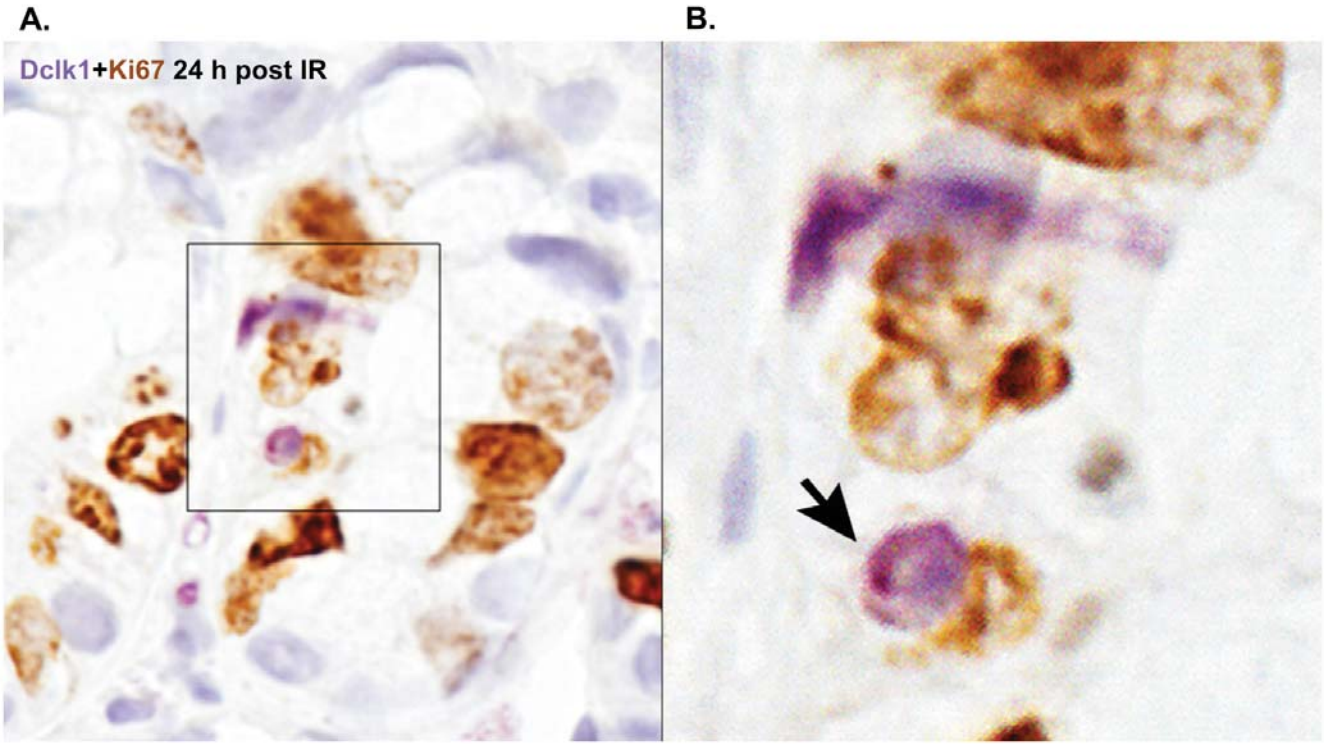
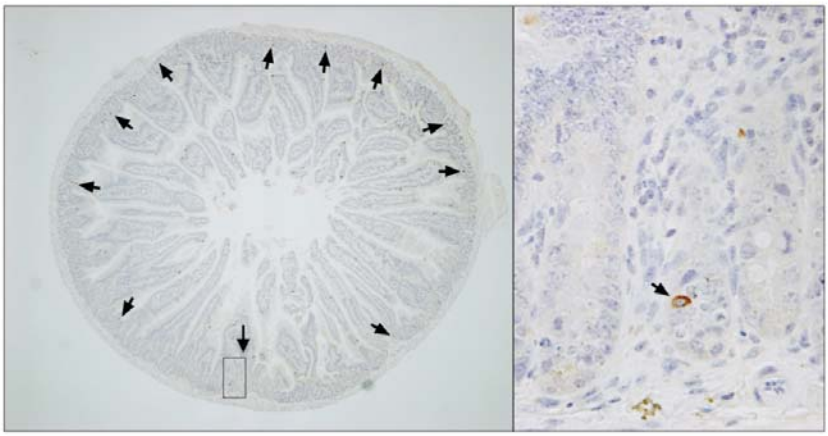
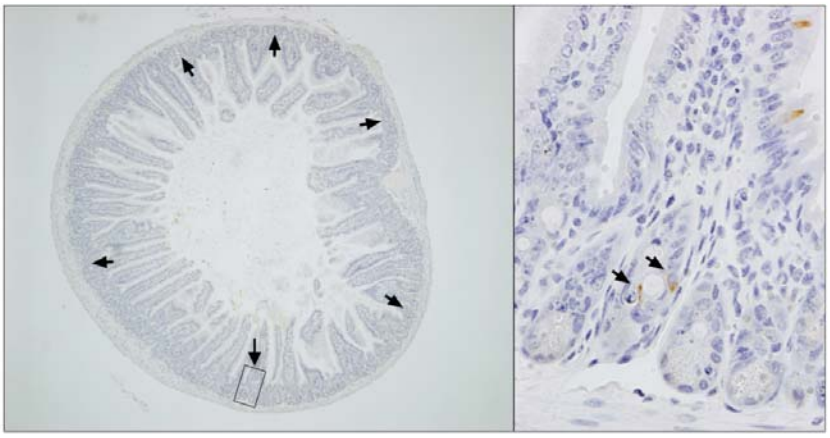


Figure 4

A. Control



B. +DAPT



C.

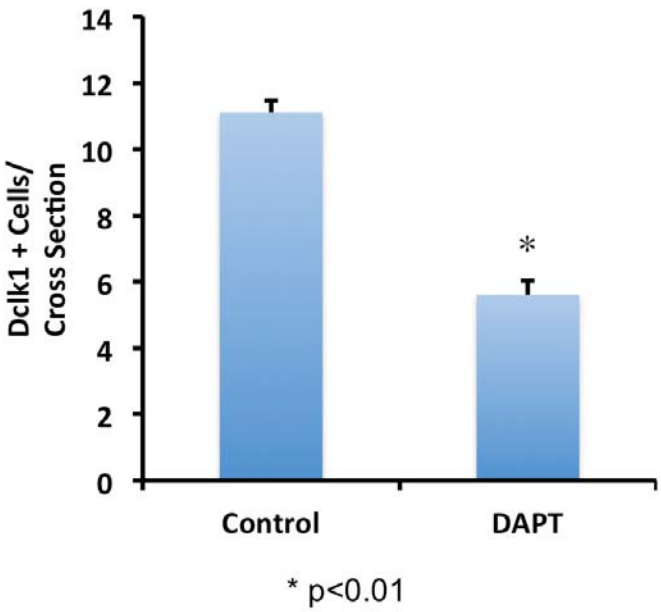


Figure 5.

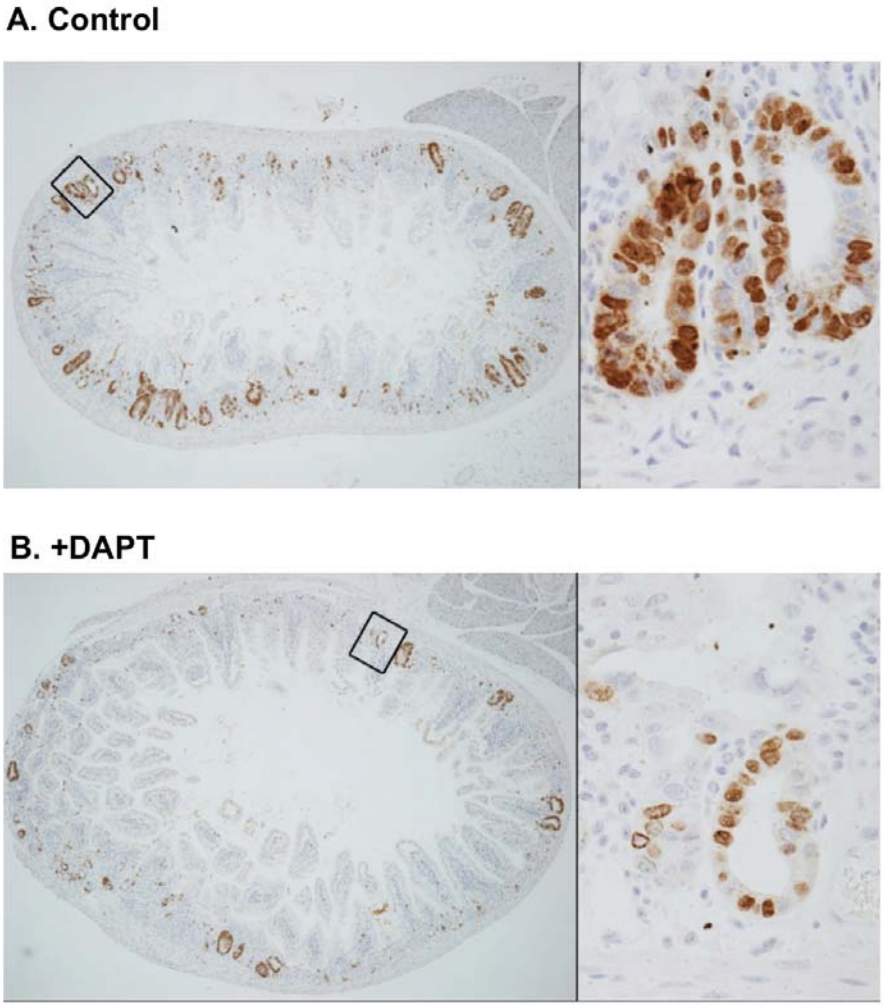

A. Control
B. +DAPT
C.
Surviving Crypts/ Cross Section
40
35
30
25
20
15
10
5
0
*
Control
DAPT

Figure 6.

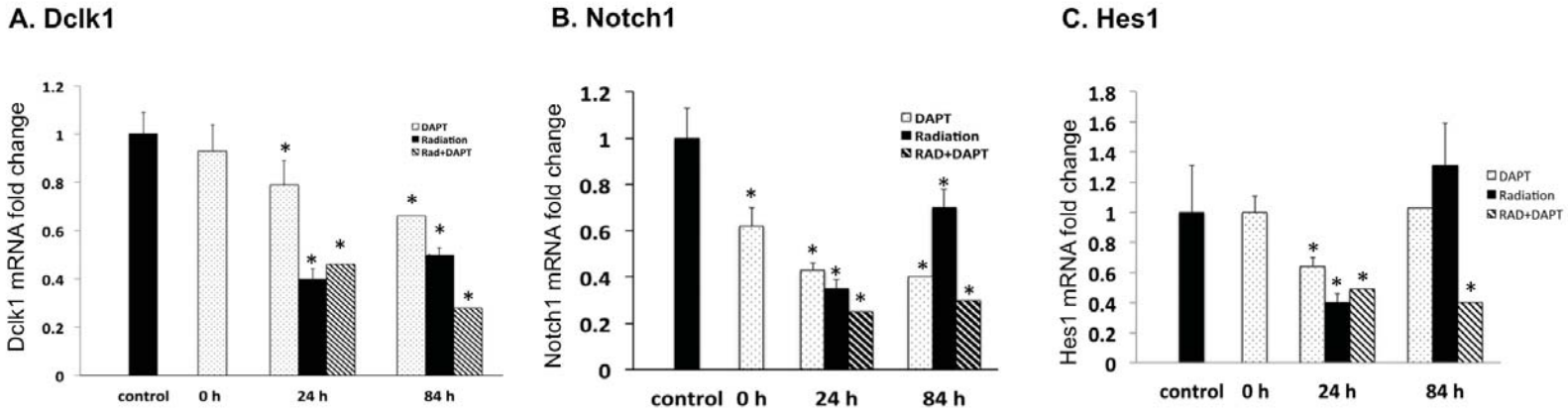

A. Dclk1
Dclk1 mRNA fold change
1.2
1
0.8
0.6
0.4
0.2
0
DAPT
Radiation
Rad+DAPT
control
0 h
24 h
84 h
B. Notch1
Notch1 mRNA fold change
1.2
1
0.8
0.6
0.4
0.2
0
DAPT
Radiation
RAD+DAPT
control
0 h
24 h
84 h
C. Hes1
Hes1 mRNA fold change
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
DAPT
Radiation
RAD+DAPT
control
0 h
24 h
84 h

Figure 7.