Cellular and molecular characterization of Blimp1 activation in response to ionizing radiation and its relevance to radiogenic breast cancer

Amy Gill

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Committee on Cancer Biology

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ABSTRACT

Radiogenic cancer is a common consequence of ionizing radiation (IR) exposure from radiation therapy (RT). Genetic variants in the Blimp1 (PRDM1) locus modulate the risk of second cancers following RT. Preliminary data suggest Blimp1 is activated by IR and protects against radiogenic cancer. I intend to determine the cellular and molecular mechanisms by which Blimp1 protects against radiogenic cancer. Blimp1 is a pleiotropic transcriptional repressor with diverse roles in regulating stress responses in the immune system and epithelial cells. I hypothesize that IR-mediated Blimp1 activation protects against breast tumorigenesis by modulating stress response pathways in breast cells and/or immune cells. First, I will determine the mechanism of Blimp1 activation by IR, both by determining the cell types in which Blimp1 is activated by IR and by testing whether IR activates Blimp1 via NFκB. Then, I will determine the cellular and molecular consequences of IR-mediated Blimp1 activation. In response to stress, Blimp1 often reduces proliferation and survival in inflammatory and epithelial cells. Consequently, I will test whether IR-mediated Blimp1 activation modulates cell proliferation and survival. I will determine whether IR-activated Blimp1 represses a set of candidate Blimp1 target genes that regulate proliferation, survival, and inflammation. Last, I will establish whether Blimp1 protects against radiogenic cancers *in vivo* in two mouse models of radiogenic cancer. I will knock down Blimp1 in $p53^{-/-}$ primary mouse mammary epithelial cells, xenograft these cells into cleared mammary fat pads, then irradiate the mice and determine whether Blimp1 knockdown reduces breast tumor latency and therefore increases radiogenic cancer risk. In addition, I will test whether Blimp1 more generally protects against a range of radiogenic cancers by irradiating $p53^{+/-}$ mice in a Blimp1 wild-type or heterozygous background to determine whether Blimp1 deficiency reduces the latency of radiogenic cancers in a variety of tissues. If successful, I will elucidate the mechanisms by which IR activates Blimp1, the cellular and molecular consequences of IR-mediated Blimp1 activation, and whether Blimp1 indeed protects against radiogenic cancers *in vivo*. This proposal is significant because Blimp1 activity could be modulated to protect individuals undergoing RT from radiogenic cancers, and because Blimp1 is a common component of stress response and inflammatory processes and these findings could have wider implications for normal tissue biology and protection against other stress-induced cancers.

SPECIFIC AIMS

Radiation therapy (RT)-induced second malignant neoplasms (SMNs) are a late complication of exposure to ionizing radiation (IR) and the second leading cause of death for survivors of pediatric Hodgkin's lymphoma (HL). Breast cancer is the most common radiogenic solid tumor and has a worse prognosis than sporadic breast cancer. We discovered genetic variants in the Blimp1 (PRDM1) locus that modulate the risk of second cancers following RT for HL. The risk variants are associated with lower basal expression of Blimp1 and impaired Blimp1 induction after IR. This suggests Blimp1 activation by IR protects against radiogenic cancer. I intend to elucidate the cellular and molecular mechanisms by which IR-driven Blimp1 induction protects against radiogenic cancer. This proposal is *significant* because Blimp1 activity could be modulated to protect individuals from second cancers during RT. As Blimp1 activation is a common component of stress response and inflammatory processes, these findings may have wider implications for other stress response processes and protection against other cancers.

IR promotes cancer both by acting directly on epithelial cells and by modifying the microenvironment. Blimp1 is a key stress response factor in both epithelial and inflammatory cells, and could act in either cell type to protect against radiogenic cancer. IR induces inflammation, and Blimp1 plays a key role in limiting inflammation by reducing cytokine production and inflammatory cell proliferation and survival. Blimp1 also protects epithelial cells from stress, including DNA damage, and might prevent radiation injury by promoting breast cell cycle arrest or apoptosis of damaged cells. Known Blimp1 response pathways controlling proliferation, survival, and inflammation likely regulate these tissue-level responses and contribute to radiogenic cancer protection. **I hypothesize that IR-mediated Blimp1 activation protects against breast tumorigenesis by modulating stress response pathways in breast cells and/or immune cells.** To test this hypothesis, I propose three specific aims:

Aim 1: To determine whether IR activates Blimp1 in breast and immune cells in an NFκB-dependent manner. As we have shown that IR-mediated Blimp1 induction is dependent upon NFκB signaling in lymphoblastoid cell lines (LCLs), in this aim I will test the hypothesis that Blimp1 activation by IR involves an NFκB-dependent stress response process. First, I will determine whether IR activates Blimp1 in breast epithelial cells, T cells, and monocytes. I will explore the kinetics and dose-dependence of IR-mediated Blimp1 induction. Second, I will determine whether NFκB mediates IR-dependent Blimp1 activation. By using inhibitors of various NFκB activation pathways, I will determine the molecular mechanism by which IR activates Blimp1 via NFκB. I will also determine whether NFκB directly regulates the Blimp1 locus after IR exposure by chromatin immunoprecipitation (ChIP). If successful, I will show that IR-induced NFκB activates Blimp1 transcription in particular tissues.

Aim 2: To determine the cellular and molecular consequences of IR-mediated Blimp1 activation. In this aim, I will investigate the cellular and molecular effects of Blimp1 after IR exposure and characterize the role of Blimp1 in radiation-induced injury using Blimp1 knockdown and inducible overexpression. I hypothesize that IR-mediated Blimp1 activation reduces proliferation and survival of epithelial and/or immune cells. First, I will study the effects of IR-activated Blimp1 on proliferation and survival by flow cytometry using knockdown and overexpressing cell lines. Second, I will elucidate the molecular network regulated by Blimp1 in response to IR. I will analyze the effects of IR-induced Blimp1 on target genes known to be involved in other stress response processes by ChIP, RT-PCR, and Western blot using Blimp1 knockdown and overexpressing cell lines. I will study a variety of Blimp1 targets including inflammatory cytokines involved in IR response (IL-2, IL-6, TNFα, IFN-γ) and key regulators of proliferation and survival (c-Myc, Fos, PI3K). I expect Blimp1 promotes cell cycle arrest and apoptosis of breast or immune cells and inhibits inflammation. If successful, I will determine the mechanisms by which Blimp1 activation protects cells from IR.

Aim 3: To determine whether Blimp1 protects against radiogenic cancers *in vivo* in mice. In this aim, I will test the hypothesis that Blimp1 is a general protective factor against radiogenic cancer in a variety of tissues. First, I will determine whether Blimp1 dosage affects the risk of radiogenic cancer in breast epithelial cells. I will knock down Blimp1 in primary $p53^{-/-}$ mouse mammary epithelial cells and transplant these cells into cleared mammary fat pads, and then irradiate the chimeric mice. I expect Blimp1 dosage in breast epithelial cells affects the risk of radiogenic breast cancer. Second, I will test the more general hypothesis that Blimp1 protects against all radiogenic cancers. I will breed $Blimp1^{+/-}$ mice onto a $p53^{+/-}$ background which develops a wide variety of cancers following IR exposure, then irradiate these mice and determine whether Blimp1 deficiency makes mice more susceptible to all types of radiogenic cancer. If I am successful, I will show that Blimp1 protects against radiogenic cancer in the breast and in various other tissues.

BACKGROUND AND SIGNIFICANCE

Radiogenic cancer is a late complication of radiation therapy for pediatric and young adult cancer

The successful treatment of up to 80% of primary tumors in children and young adults is a clear milestone in modern cancer therapeutics. However, the need to use genotoxic and pro-inflammatory treatment modalities such as ionizing radiation (IR) has resulted in a significant risk of second malignant neoplasms (SMNs) in these survivors.[1-3] 16% of newly diagnosed cancers in the United States are SMNs.[4] At least 10% of individuals treated with ionizing radiation (IR) developed a SMN within 30 years.[3,5] Survivors of Hodgkin's lymphoma (HL), which is classically treated with thoracic radiation, have the highest risk of SMNs, with 18% of men and 26% of women developing a malignancy within 30 years of completing treatment.[6] SMNs are the second leading cause of death among HL patients.[7,8] These cancers affect a variety of tissues in the field of radiation. In particular, the developing and premenopausal female breast is highly susceptible to radiation carcinogenesis, and breast cancer is the most common radiogenic solid tumor. Female HL survivors have an 18.3% cumulative risk of breast cancer 30 years after treatment,[8] and up to a 40% risk at IR doses above 40 Gy.[2] Interestingly, women of childbearing age treated with IR for breast cancer have a dose-dependent elevated risk of developing contralateral breast cancer.[9] This suggests radiation exposure causes tissue-wide changes that are sufficient to transform cells that have not been directly damaged by radiation. Radiogenic breast cancers are more likely to be ER negative and have a worse prognosis than sporadic breast cancers.[10,11] If the risk factors for radiogenic cancer were better understood, methods could be developed to prevent some second cancers by protecting individuals from tumorigenesis as a result of RT. A greater understanding of the modulators of radiation carcinogenesis not only has important implications for cancer prevention in the growing population of pediatric and young adult cancer survivors at risk for radiogenic cancers, but could also provide significant insights into carcinogenesis in the context of genotoxic and pro-inflammatory stress and have broader implications for cancer prevention.

Radiogenic cancer risk can be modulated by effects on IR-damaged cells and on the microenvironment

Radiation can cause cancer both by acting directly on epithelial cells and by altering the microenvironment. Consequently, radioprotective agents can act in the epithelium or the stroma. At early time points after IR exposure (up to 4 hours), the most drastic morphological responses to IR are seen in the breast epithelium rather than the stroma.[12] IR induces DNA damage and oxidative stress that can cause mutations, induce genomic instability, and activate stress response processes. While stromal cells have very few γ-H2AX foci indicating sites of DNA damage and repair, γ-H2AX foci are abundant in the irradiated breast epithelium.[12] IR induces p53 expression, stabilization, activation, and nuclear localization in a dose-dependent manner exclusively in the epithelium, at least up to 4 hours after IR. IR-damaged cells are susceptible to mutations, and these cells can be protected from transformation by minimizing mutation accumulation or promoting apoptosis of irreparably damaged cells. Cell cycle inhibition protects against mutation by allowing more time for DNA repair before mutations are passed on to daughter cells. Therefore, attenuation of proliferation and survival are two important mechanisms that protect against transformation of IR-damaged breast epithelial cells.

Radiation-induced tissue damage also causes permanent changes in the irradiated microenvironment.[13,14] As radiogenic cancers affect a variety of tissues in the field of radiation, there are likely shared microenvironment effects that modulate radiation carcinogenesis in many tissues. IR promotes transformation of cells that have not been directly irradiated.[15,16] This is clearly demonstrated in the 'radiation chimera' model of radiogenic breast cancer, in which mice with cleared mammary fat pads are irradiated and then transplanted with non-irradiated $p53^{-/-}$ mammary epithelium.[17] IR significantly increases breast cancer formation in these mice, although the direct effects of IR are confined to the microenvironment.[17] The mechanism of this bystander effect is complex but clearly involves tissue-wide effects mediated by a variety of cell types. The breast microenvironment is a complex community that includes breast epithelial cells, fibroblasts, adipose tissue, and immune cells. All of these cell types could contribute to IR response. However, the most well understood effect of IR on the microenvironment is induction of inflammation. IR drives inflammation in irradiated tissue both by increasing cytokine production and by promoting inflammatory cell infiltration and activation.[18-22] Macrophages are the first cells recruited to the irradiated microenvironment and are the major component of the radiogenic inflammatory infiltrate for at least the first 24 hours after IR exposure.[19] IR promotes macrophage activation in

damaged tissues,[19] and these macrophages are both directly cytotoxic and recruit other inflammatory cells to sites of radiation damage. After a few days, T cells take over the inflammatory response. Despite the inherent radiosensitivity of lymphocytes, IR increases the number of activated T cells in tissues.[23] In particular, low dose IR increases activation and proliferation of T cells.[24] Proliferation and survival control macrophage and T cell numbers, and activation controls the cytotoxic effects of these cells. Therefore, agents could control IR-induced inflammation by affecting inflammatory cell proliferation, survival, or activation. Radiogenic inflammation is observed in all irradiated tissues and is both acute and chronic, with waves of inflammatory responses persisting in irradiated tissues weeks, months, and years after exposure.[25] Inflammation plays a key role in the bystander effect, as drugs that reduce inflammation decrease genomic instability, mutation, and transformation of cells in an irradiated microenvironment.[20,26] In addition, chronic inflammation promotes cancer in many contexts. It is important to determine the factors that regulate radiogenic cancer sensitivity in the microenvironment because these factors could act in a wide range of tissues to protect against transformation following radiation. In summary, both direct genotoxic damage and inflammation induced by IR contribute to the development of radiogenic cancer.

Genetic variants at the PRDM1 (Blimp1) locus are associated with radiogenic cancer risk and altered induction of Blimp1 expression by IR

Genetic factors affect radiogenic cancer risk. By performing a genome-wide association study (GWAS) using survivors of pediatric HL, our group identified two genetic variants at chromosome 6q21 that dramatically affect the risk of SMNs after RT for HL[27] (Table 1). These variants form a common haplotype near the PRDM1 locus. Nearly 30% of HL survivors homozygous for the risk version of the haplotype developed a SMN within 30 years, but only 3% of HL survivors homozygous for the protective haplotype did. PRDM1 encodes Blimp1, a pleiotropic protein with roles in differentiation, development, stress response, and immune system function. Blimp1 is a transcriptional repressor that acts on target genes from a variety of pathways, including cytokine signaling, proliferation, and survival. The haplotype associated with increased risk of radiogenic cancer confers lower basal expression of Blimp1 and, importantly, impaired induction of Blimp1 expression after IR exposure relative to the risk haplotype (Figure 1). These data suggest Blimp1 expression and induction by IR protects against radiogenic cancer and implicate Blimp1 as a radiation-responsive tumor suppressor.

SNP	Risk/protective allele	Risk allele frequency	SMN case genotypes	Control genotypes	Odds Ratio (95% CI)	p value
rs4946728	C/A	.712	3/40/114	18/75/60	3.32 (2.25-4.90)	5.99×10^{-10}
rs1040411	A/G	.509	16/77/64	46/78/28	2.39 (1.73-3.30)	1.18×10^{-7}

Table 1. Two SNPs near the PRDM1 locus are strongly associated with development of second cancers after radiation therapy for pediatric HL. GWAS data are from a combined discovery and replication set of 158 cases and 153 controls who were treated with 25-44 Gy RT for pediatric HL and who did (case) or did not (control) develop an SMN. Genotypes are given as protective/heterozygous/risk. p values were calculated using a two-tailed Chi squared test. Allele frequencies are from the HapMap CEU population. Adapted from Best et al 2011 (27).

NFκB activates Blimp1 transcription in response to a variety of stresses

Blimp1 has been implicated in stress responses in both immune and epithelial tissues. Noxious stimuli induce Blimp1 transcription by activating the stress response factor NFκB. NFκB is an inducible transcription factor activated by a variety of stimuli including cytokines, bacterial and viral antigens, oxidative stress, and ER stress.[28] Its most important roles are in the immune system, but it is also a critical element of stress responses in epithelial tissues. The NFκB family consists of 5 members (p65, p105/p50, p100/p52, RelB, and RelC) that act as homo- or heterodimers to regulate transcription.[28] The most common NFκB dimer is p65:p50. In the basal state, most NFκB dimers are sequestered in the cytosol by binding to IκB. Dimers containing p100 do not bind IκB but are kept inactive by the p100 autoinhibitory domain, which is cleaved upon activation to generate p52.[28] There are three main pathways of NFκB activation.[28] Most known cytokines activate NFκB through the canonical pathway by activating IKK, leading to IκB degradation and release of NFκB dimers (primarily p50/p65), which then translocate to the nucleus and act as transcription factors.[28] In the non-canonical pathway, different cytokines promote cleavage of the p100 NFκB precursor into active p52, allowing p52:RelB

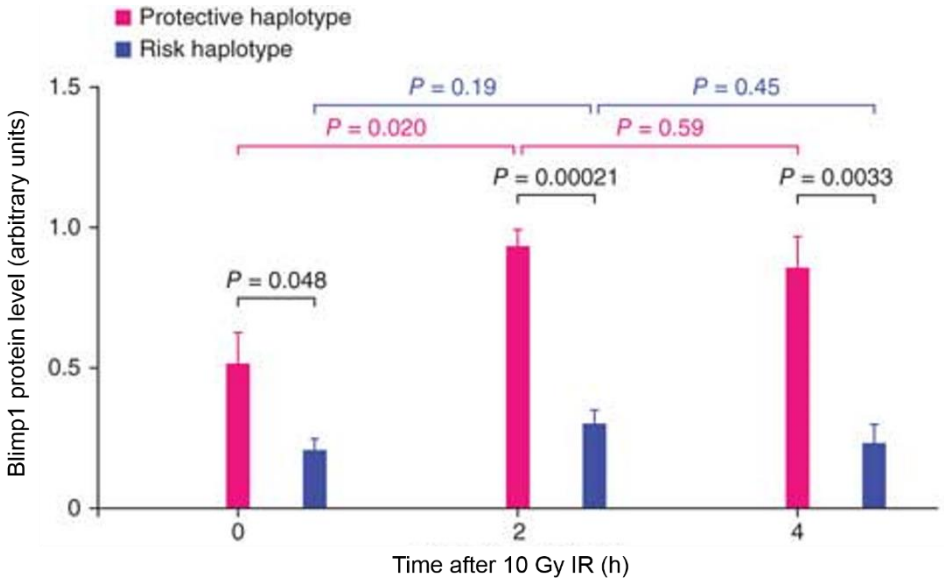

Figure 1. Blimp1 expression is induced by IR in LCLs with the protective haplotype, but not in those with the risk haplotype. In addition, Blimp1 basal expression is higher in LCLs with the protective haplotype than in those with the risk haplotype. Adapted from Best et al 2011 (27).

dimers to translocate to the nucleus and initiate transcription.[28] The alternative pathway is driven by DNA damage as active ATM promotes the activation of IKK and subsequent NFκB dimer release.[28] NFκB activates Blimp1 by binding to several promoter and enhancer elements in the PRDM1 locus.[29,30] NFκB promotes Blimp1 expression in response to diverse stresses, including viral infection, bacterial antigens, cytokines, and ER stress.[29,30] NFκB inhibition blocks induction of Blimp1 by IR in LCLs (Figure 2),[31] suggesting IR also induces Blimp1 via NFκB. If Blimp1 protects against radiogenic cancer, then NFκB could play a novel role as a radiation-responsive tumor suppressor by activating Blimp1.

Blimp1 acts in the microenvironment to repress inflammation by reducing inflammatory cell activation, proliferation, and survival

The most well characterized roles of Blimp1 are in the immune system. Blimp1 is involved in differentiation and activation of a wide variety of immune cell types.[32-38] Critically, Blimp1 plays a role in terminating immune responses. Notably, the protective haplotype is also protective against several autoimmune diseases, including rheumatoid arthritis,[39] lupus,[40] and Crohn's disease.[41] This is intriguing because the observed *in* vivo effects of the Blimp1 haplotype are consistent with known cellular effects of Blimp1.These data also suggest Blimp1 could regulate inflammatory diseases and radiogenic cancer risk by a common mechanism. As IR induces an inflammatory response, Blimp1 could affect radiogenic cancer risk by modulating inflammation. Blimp1 plays known roles in both key inflammatory cell types induced by IR, macrophages and T cells. Blimp1 is induced during monocyte maturation and macrophage activation. Blimp1 regulates activation-induced cell death of T cells by attenuating their proliferation and survival.[42] A T cell specific knockout of Blimp1 leads to lethal autoimmune disease characterized by greatly increased numbers of activated T cells and extensive inflammatory infiltrates in epithelial tissues.[43,44] Blimp1 also reduces proliferation and survival of other immune cell types[36,37] suggesting a common role in limiting inflammatory cell number and curbing inflammation. Blimp1 directly represses expression of several pro-inflammatory cytokines.[45-48] By repressing these cytokines, Blimp1 generally blocks inflammatory processes by inhibiting activation of NFκB. Blimp1 also reduces inflammatory cell number by inhibiting target genes involved in survival and proliferation, and by promoting cytokine deprivation. If Blimp1 protects against radiogenic cancers by acting on the immune

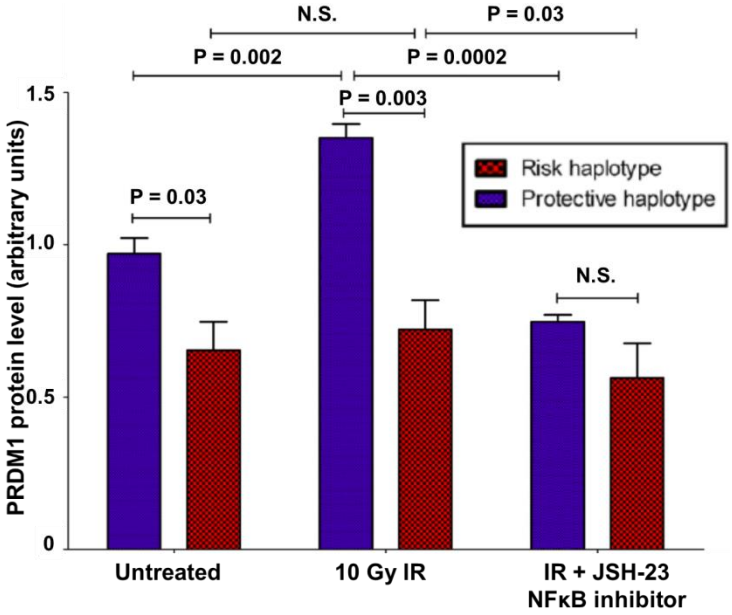

Figure 2. NFκB inhibition blocks Blimp1 induction after IR in LCLs. Cells were treated with or without 20μM JSH-23 for 2 hours before IR and samples were collected 1 hour after IR. 4 LCLs of each haplotype were treated. Adapted from Best 2011. (31)

system, then Blimp1 could be a general radioprotective agent in a variety of tissues.

Blimp1 promotes stress responses in the epithelium by several cellular and molecular mechanisms

Growing evidence suggests Blimp1 is a stress response factor in epithelial cells. RelB NFκB activates Blimp1 in breast epithelial cells.[49] In addition, mouse colon epithelial cells that overexpress constitutively active IKKβ, the upstream activator of NFκB, have increased Blimp1 expression compared to wild-type cells (M. Karin, personal communication). ER stress induces Blimp1 via NFκB in HeLa cells. These results suggest that other processes that induce NFκB, such as inflammation, could activate Blimp1 in epithelial cells. Genotoxic stress also induces Blimp1, though the role of NFκB in this process has not been studied.[50] In response to the DNA damaging agent 5-FU, Blimp1 promotes cell cycle arrest of colon cancer cells.[50] Several Blimp1 target genes affect intracellular proliferation and survival pathways, including c-Myc,[51] Fos,[42] and PI3K.[52] Although little is known about Blimp1 target genes in epithelial cells, Blimp1 could protect the breast epithelium against radiation damage by promoting cell cycle arrest and repair or by promoting apoptosis in damaged cells.

Summary

I hypothesize that IR-mediated Blimp1 activation protects against breast tumorigenesis by modulating stress response pathways in breast epithelial cells and/or immune cells. (Figure 3) I believe NFκB mediates Blimp1 activation by IR in breast epithelium, macrophages, and/or T cells. Blimp1 induction after IR likely modulates target pathways that regulate proliferation, survival, and inflammatory cell activation. The protective effect of Blimp1 against radiation carcinogenesis should be recapitulated in mice, and we can use these mice to determine whether Blimp1 acts in the epithelium or microenvironment to protect against radiogenic cancer. Blimp1 may also have a more general effect in protecting all tissues from radiation carcinogenesis. This proposal is *significant* because Blimp1 signaling could be manipulated to protect individuals undergoing RT against second cancers. The study of stress-induced Blimp1 induction also has important implications for all cancers initiated by DNA damaging or inflammatory processes. If Blimp1 plays a wider role in reducing cancer risk, then Blimp1 could be targeted for cancer prevention in many contexts. Blimp1 also has key roles in response to infection and autoimmunity, so these studies have wider implications in immunology and could benefit a variety of patients. This proposal is *innovative* because the role of Blimp1 in IR response has never been studied. In addition, few studies of radiation carcinogenesis investigate contributions of both the epithelium and the microenvironment, and our agnostic approach increases the probability we uncover the mechanism by which Blimp1 affects radiogenic cancer risk.

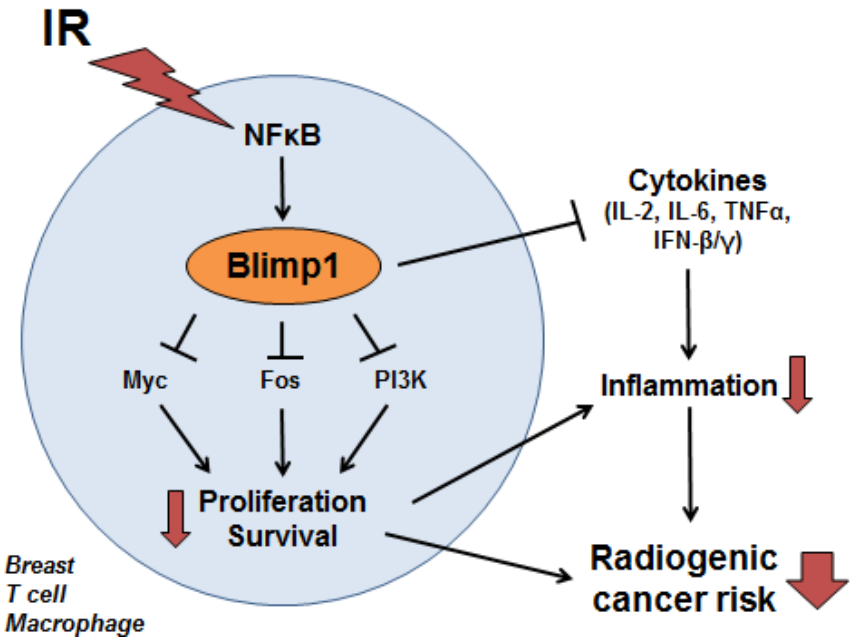

Figure 3. Model for Blimp1-mediated protection against radiogenic cancer. I will test the hypothesis that IR-activated Blimp1 activates stress response pathways in breast epithelial cells, T cells, and macrophages, and that repression of known Blimp1 target genes has cellular effects that reduce the risk of radiogenic cancer.

EXPERIMENTAL DESIGN

Specific Aim 1: To determine whether IR activates Blimp1 in breast and immune cells in an NFκB-dependent manner.

Rationale: Blimp1 is an important stress response factor in both epithelial and inflammatory cells. In this aim, I will determine which of the three main cell types involved in the inflammatory response to IR (breast epithelial cells, T cells, and monocytes/macrophages) activate Blimp1 in response to IR by establishing a cell culture model of IR-mediated Blimp1 activation. IR causes direct genotoxic damage to breast epithelial cells which can cause mutations and also activates stress response pathways. IR also initiates an inflammatory response that leads to massive macrophage infiltration followed by T lymphocyte invasion. These two main IR-responsive immune cell types help clear IR-damaged tissue of debris, but also propagate acute and chronic inflammation that causes additional tissue damage and can promote development of cancer. In all of these cell types, stress typically activates Blimp1 by an NFκB-dependent process, and IR appears to also induce Blimp1 through NFκB. I will determine the molecular mechanism by which NFκB mediates Blimp1 activation in response to IR. I will inhibit the three main NFκB activation pathways to determine which of these pathways contribute to IR-mediated Blimp1 activation. I will also determine whether NFκB directly binds and regulates the PRDM1 gene by ChIP. If I am successful, I will determine whether IR activates Blimp1 in breast cells, T cells, and macrophages in an NFκB-dependent manner.

Aim 1a: Determine whether IR activates Blimp1 in breast epithelial cells, T cells, and monocytes.

Methods: I will determine whether IR induces Blimp1 in breast, T, and/or monocytic cell lines. For all experiments in this proposal, I will preferentially choose cell lines homozygous for the protective haplotype,[53] as LCLs with this haplotype induce Blimp1 after IR. To begin, I will use HCC1500 breast cells, MOLT16 T cells, and AML-193 monocytes. However, most common cell lines do not have the protective haplotype. If homozygous protective cell lines are not available, I will use cell lines heterozygous for the protective haplotype, which have induce Blimp1 at an intermediate level between protective and risk. We will expand our findings in cells homozygous for the risk haplotype to determine whether Blimp1 is induced in a haplotype-specific manner in these cell types. I will perform a time course of Blimp1 induction in our cells of interest to determine when Blimp1 is maximally induced after IR. Cells will be sham treated or treated with 5 Gy IR, a dose known to induce Blimp1 in LCLs. Cells will be collected at 0, 0.5, 1, 2, 4, 8, 12, 24, 48, and 72 hours after IR. These time points span the range of known maximal induction times of Blimp1 in response to a variety of stimuli. RNA will be isolated by the Trizol method and used for RT-PCR against Blimp1α and Blimp1β, the two main isoforms of Blimp1. All primers used in our studies will be designed by standard criteria (T_m = 60-65°C, GC content 40-60%, no off-target matching sequences found by BLAST, fragments 60-150 bp). We will also perform Western blots on cell lysates using a Blimp1 antibody (Cell Signaling). Last, I will perform a dose-response curve of Blimp1 induction in order to define the optimal radiation doses to use in later experiments. I will sham treat or treat cells with varying doses of IR from 10 cGy to 10 Gy. I will collect cells at the time point of maximal Blimp1 induction, as determined above. I will then measure Blimp1 RNA by RT-PCR and protein by Western blot.

Expected results: I expect IR will induce Blimp1 in one or more cell types. If Blimp1 expression increases after IR in breast cells, T cells or monocytes, I will conclude that Blimp1 is induced by IR in these cells. In future experiments, cells will be collected at the time point of maximal Blimp1 induction. I also expect that all doses of IR will induce Blimp1 and that Blimp1 induction will increase with dose. In future experiments, I will treat cells with two doses of IR: the minimum dose required for Blimp1 induction (low dose) and the dose at which maximal Blimp1 induction occurs (high dose). IR may induce Blimp1 in multiple lineages. If so, in subsequent experiments we will prioritize the cell type with the largest change in Blimp1 expression after IR. The results of these experiments will also inform the design of our mouse experiments in aim 3, as they will suggest whether Blimp1 is more likely to act in the breast epithelium or in the microenvironment to modulate radiation carcinogenesis

Pitfalls and alternatives: Blimp1 may not be induced by IR in these cell lines. This may be an artifact of the particular cell lines chosen. When possible, I will investigate multiple cell lines with the protective or heterozygous haplotype of each lineage to minimize this risk. Alternatively, this result may suggest the investigated cell types do not induce Blimp1 after IR and are not important mediators of Blimp1's protective effect against radiogenic cancer. I will test for IR-mediated Blimp1 induction in other cell types with known roles for Blimp1, including B cells, NK cells, and granulocytes. Alternatively, these results may be an artifact of cell culture and these cell types may induce Blimp1 after IR *in vivo* in the context of the tissue microenvironment.

To evaluate this possibility, I will examine Blimp1 expression after IR in various mouse tissues using Blimp1-YFP reporter mice to determine which endogenous cell types respond to IR by activating Blimp1. To ensure our results are not particular to immortalized cell lines, I can investigate the IR-mediated Blimp1 response in primary cells. I can also explore the relationships between the irradiated cell types tested by co-culture or treatment with conditioned medium.

Aim 1b: Determine whether NFκB mediates Blimp1 induction by IR.

Methods: First, I will determine whether NFκB inhibition blocks IR-mediated Blimp1 induction. I will pre-treat cells with pharmacological or RNAi inhibitors of various NFκB activation pathways. I will inhibit global NFκB activity with bortezomib (10 nM, 1 hour pre-treatment),[54] canonical NFκB signaling with JSH-23 (20 μM, 2 hour pre-treatment),[31] non-canonical activation with NIK siRNA (designed by standard methods, transfected 24 hours before IR treatment), and atypical NFκB activation with caffeine (4 mM, 30 minute pre-treatment).[55,56] Cells will be sham treated or treated with IR at the low and high doses determined in aim 1a and then collected at the time point of maximal Blimp1 induction determined in aim 1a. I will measure Blimp1 mRNA by RT-PCR and protein by Western blot. Second, I will determine whether NFκB directly binds the Blimp1 locus after IR. I will sham treat or IR treat cells of the protective and risk haplotype. As NFκB binds target genes within the first hour after stimulation, 1 hour after treatment I will cross-link protein and DNA with formaldehyde and pellet cells. Cell lysates will be made and chromatin will be sheared by sonication. I will immunoprecipitate NFκB using p65 antibody (Cell Signaling) conjugated to protein A beads, reverse protein-DNA crosslinks, and purify DNA. I will then perform RT-PCR for NFκB binding sites in the PRDM1 locus, as determined by ENCODE ChIP-Seq data, NFκB consensus sites, and previous reports. I will test the 10 known NFκB sites in the 100 kb region centered on the PRDM1 gene.

Expected results: If NFκB binds a target site in response to IR but not in the absence of IR, I will conclude NFκB regulates PRDM1 at this site in response to IR. If NFκB binds a site after IR in cells with the protective haplotype but not in cells with the risk haplotype, I will conclude that NFκB binds that site after IR in a haplotype-specific manner. I expect one NFκB site in the PRDM1 locus to be regulated in a haplotype-specific manner. Our top candidate is an NFκB site 170 bp 5' of the SNP rs11152966, which we believe is the functional variant responsible for the phenotype of the PRDM1 haplotype. I expect that NFκB binds this site after IR in cells with the protective haplotype but not in cells with the risk haplotype.

Pitfalls and alternatives: NFκB dimers that do not include p65 may modulate Blimp1 transcription. This experiment could be repeated performing IP for other NFκB subunits such as p52 or RelB to test this possibility. NFκB may not mediate IR-driven Blimp1 induction. If not, we will test the role of several other pathways known to induce Blimp1 using pharmacological inhibitors, including p38, MAPK, JNK, and STAT. If this also fails, we will take an unbiased approach to identify inhibitors of IR-driven Blimp1 induction using a drug screen of compounds with known mechanisms of action. In addition, this line of inquiry does not fully address the mechanism of the protective/risk haplotype as the functional variant does not lie in an NFκB site. We believe a second transcription factor binds the functional variant and modulates NFκB binding to a nearby enhancer. As rs11152966 modifies a consensus binding site for SP3, we can also investigate whether SP3 binds the PRDM1 locus in a haplotype-specific manner and whether SP3 interferes with NFκB binding.

Specific Aim 2: To determine the cellular and molecular consequences of IR-mediated Blimp1 activation.

Rationale: In this aim, I want to determine how Blimp1 modulates oncogenesis. Based on existing knowledge about Blimp1 and an understanding of the cellular effects of IR, the most rational mechanisms by which Blimp1 could protect against breast cell transformation are by affecting proliferation and survival of the breast epithelial cells or the inflammatory cells activated by IR. Blimp1 often regulates proliferation, survival, and inflammatory cell activation. By promoting cell cycle arrest of breast epithelial cells in response to IR, Blimp1 could minimize DNA damage by allowing extra time for cells to repair lesions before replication. Alternatively, Blimp1 could promote apoptosis of damaged epithelial cells and reduce the odds that a badly damaged cell survives and becomes transformed. Blimp1 induction also plays important anti-inflammatory roles in several contexts by reducing immune cell proliferation and survival, decreasing the number of active inflammatory cells. I will investigate the molecular targets of IR-activated Blimp1 using a candidate approach. I chose these target genes because they have demonstrated roles in Blimp1 regulation of proliferation, survival, and inflammatory cell activation. Blimp1 often attenuates proliferation and survival by repressing c-Myc[51,57,58] and Fos.[42] Recent ChIP-seq data show that Blimp1 also binds the PIK3CA gene encoding PI3K, an important regulator of survival via the Akt pathway, and reduces PI3K transcription.[52] Blimp1 induction also prevents inflammatory cell

activation, proliferation, and survival by inhibiting production of effector cytokines, including IL-2,[46] IL-6,[45] TNFα,[36] IFN-β,[47] and IFN-γ.[48] These cytokines are required for recruiting and activating additional immune cells in an inflammatory response and are important survival factors and mitogens for activated inflammatory cells. In addition, all of these cytokines are induced in irradiated tissues.[59] I hypothesize that IR-mediated Blimp1 induction represses proliferation and promotes apoptosis of breast and/or immune cells. I will test this hypothesis by measuring breast and immune cell proliferation and survival after IR by flow cytometry in Blimp1 wild-type, knockdown, and overexpressing cells. Furthermore, I will determine the molecular effectors of these changes by investigating how IR-mediated Blimp1 induction regulates the candidate target genes described above using ChIP, RT-PCR, and Western blot in Blimp1 wild-type, knockdown, and overexpressing cells. Importantly, if this candidate approach fails to explain the protective effects of Blimp1 in response to IR, I will use unbiased approaches including ChIP-seq and gene expression profiling to determine the effectors downstream of IR-activated Blimp1. This will allow us to investigate whether Blimp1 regulates IR response by known mechanisms or through previously uncharacterized pathways. If I am successful, these experiments will reveal that IR-mediated Blimp1 reduces proliferation and survival of breast and immune cells by repressing known Blimp1 target genes and will allow further functional characterization of the pathways downstream of Blimp1 in the context of IR.

Aim 2a: Establish Blimp1 knockdown and overexpression systems.

Methods: Knockdown and overexpression methods allow me to take a reductionist approach to study the effects of Blimp1. By establishing cell lines that only differ in Blimp1 status, I can specifically identify the effects of Blimp1 after IR. In particular, Blimp1 knockdown cells will block the effects of Blimp1 and allow us to focus on the processes regulated by Blimp1 in this context. Furthermore, our Blimp1 knockdown cell lines will approximate the effect of Blimp1 depletion in mice in aim 3 and can inform future mouse experiments. Once an appropriate cell culture model of IR-mediated Blimp1 induction has been developed in aim 1a, I will knock down Blimp1 using lentiviral shRNAs. I have cloned 5 shRNAs into lentiviral constructs. Constructs will be transfected into HEK293T cells to generate lentivirus, and then target cell lines will be transduced and positive clones will be isolated by puromycin selection. Knockdown will be confirmed by RT-PCR and Western blot. I am also developing a lentiviral Tet-inducible Blimp1 overexpression system. Blimp1 has 2 main isoforms (Blimp1α and Blimp1β) with distinct expression kinetics and biological activities. As it is unclear which isoforms are involved in IR response, I am developing overexpression constructs of both isoforms. These constructs will be introduced into cells as described for lentiviral shRNAs, and inducible overexpression will be confirmed by RT-PCR and Western blot. I chose an inducible system because I believe Blimp1 signaling has important temporal aspects that would not be accurately recapitulated in a constitutive overexpression system. In order to more faithfully mimic an IR-induced burst of Blimp1 expression in experiments, I will treat cells with doxycycline and IR such that Blimp1 expression is turned on by doxycycline at the time when IR would normally induce Blimp1. In order to determine the appropriate time to treat with doxycycline relative to IR, I will perform a time course of Blimp1 induction following doxycycline treatment.

Expected results: I expect to establish stable Blimp1 knockdown and overexpression in appropriate cell types of both the protective and risk haplotype.

Pitfalls and alternatives: Our shRNAs may not successfully knock down Blimp1. If that occurs, we can use commercially available shRNAs against Blimp1 that were successful in other studies. It may not be practical to time our inducible overexpression to coincide with the normal IR-mediated Blimp1 induction. We can ignore the temporal aspect of signaling and pre-treat the cells with doxycycline to induce overexpression before IR, though our responses may not reflect the endogenous behavior of Blimp1 after IR. In these studies, we will also create and test the lentiviral constructs to be used on primary mouse mammary epithelial cells in aim 3.

Aim 2b: Analyze the effects of IR-mediated Blimp1 expression on breast and inflammatory cell proliferation and survival by flow cytometry.

Methods: First, I will perform cell cycle analysis by propidium iodide (PI) staining. Blimp1 wild-type, knockdown, and overexpressing cells will be sham treated or treated with IR. Two IR doses will be used: the minimal dose required to induce Blimp1 (low dose) and the dose at which maximal Blimp1 induction occurs (high dose). 4 hours after maximal activation of Blimp1, as determined in aim 1a, cells will be fixed in ethanol and stained with PI, then analyzed by flow cytometry on an LSR-II flow cytometer in the Cunningham lab or at the flow cytometry core facility. The percentage of cells in S, G1, G2, and sub-G1 phase will be determined. Second, we will analyze the effects of IR-mediated Blimp1 on proliferation by measuring DNA synthesis by BrdU incorporation. Blimp1 wild-type, knockdown, and overexpressing cells will be sham treated or treated with

low or high dose IR as described above. 4 hours after maximal induction of Blimp1, cells will be incubated in BrdU-containing medium at 37°C for 30 minutes and then pelleted and fixed in ethanol. DNA will be denatured by HCl treatment. Cells will be stained with mouse monoclonal BrdU antibody (Cell Signaling) and FITC-conjugated goat anti-mouse IgG antibody (Abcam) and analyzed by flow cytometry.
Expected results: If IR decreases the S phase fraction and decreases BrdU incorporation to a greater extent in Blimp1 wild-type cells than in Blimp1 knockdown cells, I will conclude that IR-mediated Blimp1 promotes cell cycle arrest and reduces proliferation. This conclusion will be strengthened if Blimp1 overexpression further decreases the S phase fraction and BrdU incorporation. Similarly, if IR increases the sub-G1 fraction and increases annexin V staining to a greater extent in Blimp1 wild-type cells than in Blimp1 knockdown cells, I will conclude that IR-mediated Blimp1 reduces cell survival by promoting apoptosis.
Pitfalls and alternatives: All methods are standard in the Cunningham and Onel labs. We anticipate no difficulties. Blimp1 overexpression with IR may not significantly change proliferation or survival relative to IR alone, potentially because endogenous IR-mediated Blimp1 already induces maximal biological response. In this case, we will base our conclusions solely on the wild-type and knockdown data. We can also test our overexpression constructs in cells with the risk haplotype that normally express low levels of Blimp1 after IR. We expect the overexpression data to be informative in the context of the risk haplotype. However, overexpression can lead to non-specific effects; therefore, we will interpret the results of all overexpression experiments with caution and ensure they are consistent with observed effects in Blimp1 wild-type and knockdown cells.

Aim 2c: Analyze the effects of IR-mediated Blimp1 induction on candidate Blimp1 target genes by RT-PCR and ChIP.
Methods: First, I will determine whether Blimp1 represses primary transcript levels of candidate genes (c-Myc, Fos, PI3K, IL-2, IL-6, TNFα, IFN-β, IFN-γ). Wild-type, knockdown, and overexpressing cells will be sham treated or treated with low or high dose IR as described above. I will collect RNA by the Trizol method and perform a time course of primary transcript levels of candidate genes by RT-PCR. We will analyze primary transcripts rather than mature transcripts to avoid confounding issues of mRNA stability and focus only on transcription levels. Primers will be designed by standard criteria (fragments 60-150 bp, T_m = 60-65°C, GC content 40-60%, no off-target matching sequences found by BLAST). If Blimp1 represses expression of a given primary transcript, I will test whether Blimp1 directly binds that locus by ChIP to confirm whether the gene is indeed a Blimp1 target. At the time of maximal Blimp1 expression as determined in aim 1a, I will cross-link protein and DNA with formaldehyde and pellet cells. Cell lysates will be made and chromatin will be sheared by sonication. I will immunoprecipitate Blimp1 using Blimp1 antibody (Cell Signaling) conjugated to protein A beads, reverse protein-DNA crosslinks, and purify DNA. I will then perform RT-PCR for Blimp1 binding sites in candidate target genes as determined by ENCODE ChIP-seq footprints, existing Blimp1 ChIP-seq data, and Blimp1 consensus sequence.
Expected results: I expect that Blimp1 represses one or more candidate target genes. If IR reduces levels of a given primary transcript in wild-type but not in Blimp1 knockdown cells, I will conclude that Blimp1 regulates expression of that gene. This conclusion will be strengthened if primary transcript levels are further repressed in Blimp1 overexpressing cells. I expect that Blimp1 reduces expression of that target gene by direct binding of its locus and subsequent transcriptional repression. If Blimp1 ChIP pulls down the Blimp1 binding site in a given gene at a higher frequency in IR-treated cells than in sham treated cells, I will conclude that Blimp1 binds the target gene in response to IR. Decreased Blimp1 binding to these promoters in Blimp1 knockdown cells and increased binding in Blimp1 overexpressing cells will demonstrate that differences in Blimp1 expression affect regulation of these target genes and will increase our confidence that Blimp1 specifically binds and regulates these genes.
Pitfalls and alternatives: The candidate genes investigated may not be the effectors of Blimp1 after IR. If these target genes do not bind Blimp1 and there is no change in gene expression after IR when comparing wild-type and Blimp1 knockdown or overexpressing cells, I will take an unbiased genome-wide approach to identify the Blimp1 target genes involved in IR response. I will perform expression microarrays of sham treated and IR treated Blimp1 wild-type and knockdown cells. If expression of a gene is repressed by IR in wild-type cells but de-repressed in Blimp1 knockdown cells, I will infer that gene is a target of Blimp1 after IR and follow up with ChIP, RT-PCR, and Western blots to determine whether the gene is a direct or indirect target of Blimp1. I will combine the expression data set with ChIP-seq data from ENCODE and other groups and with existing data on radiation response signatures and inflammatory signatures to perform network analysis and

identify key networks that likely mediate the Blimp1 response to IR. These networks can be further explored in order to identify the effectors of IR-activated Blimp1.

Specific Aim 3: To determine whether Blimp1 protects against radiogenic cancers *in vivo* in mice.
Rationale: In this aim, I will test the hypothesis that Blimp1 protects against radiogenic cancers *in vivo* in a variety of tissues. I will test this hypothesis both in radiogenic breast cancer and more generally in all radiogenic cancers. I intend to replicate our observation in humans that decreased Blimp1 dosage is associated with increased radiation carcinogenesis. Therefore, I would like to study the effects of Blimp1 depletion on radiogenic cancer risk. However, Blimp1$^{-/-}$ mice are embryonic lethal. To get around this, in aim 3a I will knock down Blimp1 in mammary epithelial cells *ex vivo* and then will xenograft these cells into mice, irradiate the mice, and determine how Blimp1 dosage in the mammary epithelium modifies radiation carcinogenesis. This will allow me to determine whether Blimp1 acts in a cell autonomous manner to protect against breast cell transformation after IR exposure. In aim 3b, to test whether Blimp1 depletion in other tissues and in the microenvironment increases radiogenic cancer risk, I will irradiate Blimp1$^{+/-}$ mice in a p53$^{+/-}$ background known to be susceptible to radiogenic cancers. Results from other systems demonstrate that Blimp1 effects are highly dependent on gene dosage, and Blimp1$^{+/-}$ cells consistently have phenotypes intermediate between Blimp1$^{+/+}$ and Blimp1$^{-/-}$ cells. Therefore, I hypothesize that Blimp1$^{+/-}$ status decreases Blimp1 protein level and increases radiogenic cancer risk. Because PRDM1 haplotype is associated with increased risk of a variety of cancers following radiation therapy, I hypothesize Blimp1 plays a general role in radiation carcinogenesis and that Blimp1 deficiency will increase the risk of all radiogenic cancers.

Aim 3a: Determine whether Blimp1 knockdown or overexpression in the irradiated mammary gland modulates radiogenic breast cancer development
Methods: This aim will be performed in collaboration with the Barcellos-Hoff lab at NYU, the creators of the radiation chimera mouse model. We will transplant Blimp1-modified p53$^{-/-}$ breast epithelial cells into cleared mammary fat pads of mice, then irradiate these mice and compare the latency of breast cancer development. I will use lentiviral Blimp1 shRNA and scrambled shRNA based on those used in aim 2. I will generate virus by transfecting HEK293T cells. I will confirm that these lentiviruses successfully knock down Blimp1 in primary BALB/c mouse mammary epithelial cells. To establish Blimp1 knockdown and overexpressing primary mouse mammary epithelium for experimental use, I will remove mammary epithelium from 8-10 week old female p53$^{-/-}$ BALB/c mice (Barcellos-Hoff lab). The mammary gland will be dissociated into single cells and enriched for mammary epithelial cells as previously described,[60] then plated and allowed to attach overnight. I will then transduce cells with the lentiviruses described above lentivirus to knock down or overexpress Blimp1, or a lentivirus containing a scrambled shRNA as a negative control.[61] Then, I will surgically clear the fourth inguinal mammary fat pads of 3 week old BALB/c mice as previously described.[17] All surgical procedures will be learned from the Barcellos-Hoff lab at NYU. During this surgery, 0.5*10^{6} transduced mammary epithelial cells will be injected into the cleared mammary fat pad in a

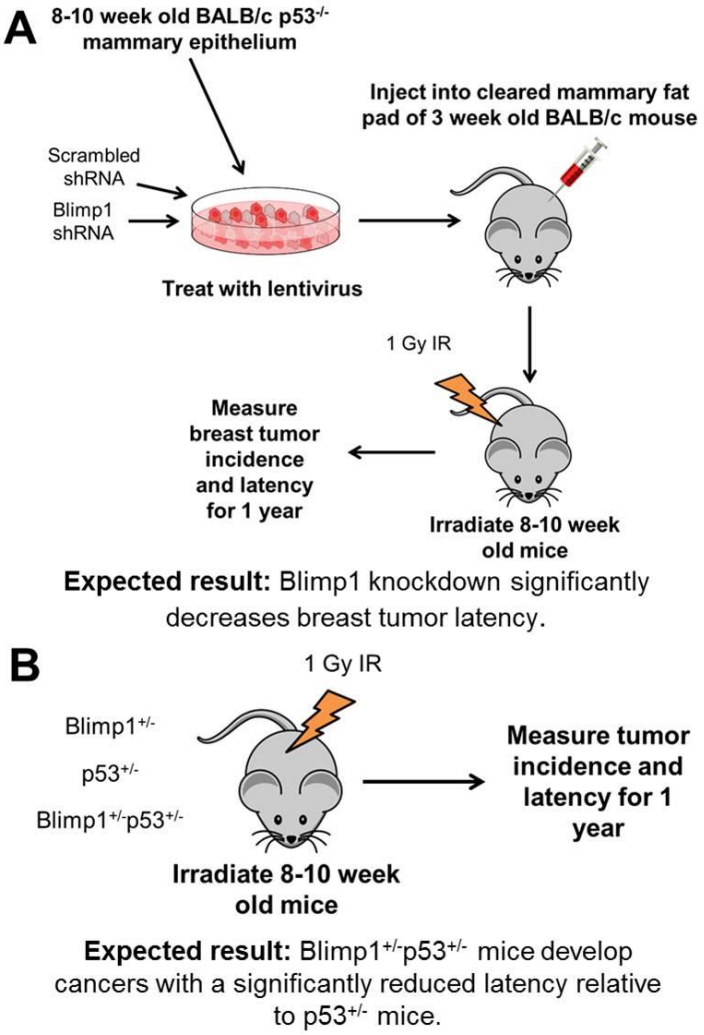

Figure 4. Experimental design for aim 3. **A)** p53$^{-/-}$ mammary epithelial cells will be treated with Blimp1 shRNA, then xenografted into cleared mammary fat pads. Mice will be irradiated and monitored for breast tumors for 1 year. **B)** Blimp1$^{+/-}$, p53$^{+/-}$, and Blimp1$^{+/-}$p53$^{+/-}$ mice will be irradiated and monitored for tumors for 1 year.

volume of 10 µL as previously described.[61] When these mice are 8-10 weeks old, I will treat them with 1 Gy whole body irradiation, a dose shown to induce mammary tumors in $p53^{-/-}$ breast cells.[17] (Figure 4a) Mice will be monitored for breast tumor formation by palpation for 1 year after IR treatment. Tumors will be measured with calipers. Mice will be sacrificed when tumors reach > 1 cm or the mice reach human endpoints as detailed in University of Chicago IACUC guidelines. Our primary endpoints will be tumor incidence at 9 months and tumor latency. Secondary outcomes will be tumor incidence at 6 and 12 months, tumor growth rate, and tumor pathology, including ER status. Based on data comparing breast cancer latency in $p53^{-/-}$ epithelium in the presence and absence of the tumor suppressor BRCA1, I estimate that deficiency of the tumor suppressor Blimp1 will reduce breast cancer latency by 2 months. In order to detect a 2 month decreased latency of breast cancer with 80% power, we will use 10 mice per group, or 20 mice total. I will consult with a statistician to obtain better estimates of the required number of mice for our study.

Expected results: Based on existing literature, I expect the median latency of 1 Gy irradiated $p53^{-/-}$ tumors in the negative control group (no Blimp1 modification) to be 9 months.[17] If Blimp1 knockdown significantly decreases tumor latency, I will conclude that Blimp1 loss in the mammary epithelium increases the risk of radiogenic breast cancer.

Pitfalls and alternatives: If we see a significant decrease in breast cancer latency in Blimp1 knockdown epithelium, we will repeat the experiment with and without IR treatment to determine whether Blimp1 status has an effect on breast cancer incidence in the absence of radiation. In addition, we will transduce $p53^{-/-}$ breast epithelial cells with a Blimp1 overexpression construct to determine whether increased Blimp1 dosage is protective against radiogenic breast cancer. Lentiviral transduction will have variable efficiency on the $p53^{-/-}$ mammary epithelial cells. Therefore, what we transplant will be a mix of transduced and non-transduced cells with varying levels of Blimp1. This may obscure our results. Blimp1 levels in the mammary epithelium may have less impact on breast cancer development than Blimp1 levels in the microenvironment. If so, we expect that our experiment in aim 3b will show that global Blimp1 levels impact radiogenic cancer risk. If our cell culture and mouse results suggest Blimp1 acts in the microenvironment to protect against radiogenic cancer, we can test this hypothesis by performing radiation chimera experiments in Blimp1 wild-type and $Blimp1^{+/-}$ recipient mice and would expect that irradiated $Blimp1^{+/-}$ mice are more likely than Blimp1 wild-type mice to develop cancer in transplanted non-irradiated $p53^{-/-}$ epithelium.

Aim 3b: Determine whether Blimp1 deficiency increases the risk of radiogenic cancer.

Methods: C57BL/6 $Blimp1^{flox/flox}$ mice (Cunningham lab) will be crossed onto the BALB/c background using speed congenics. We chose the BALB/c background because it is the most radiation-sensitive mouse strain and is the most likely to develop radiogenic cancers.[62,63] Briefly, $Blimp1^{flox/flox}$ mice will be crossed with BALB/c mice and progeny will be genotyped at a panel of markers designed to discriminate between C57BL/6 and BALB/c background. The progeny with the highest percentage of BALB/c markers will be backcrossed with BALB/c mice and the process will be repeated. After 3-4 generations, we expect to obtain $Blimp1^{+/flox}$ mice with >99% BALB/c background. BALB/c $Blimp1^{+/-}$ mice will be generated by crossing BALB/c $Blimp1^{+/flox}$ mice with BALB/c CMV-Cre mice (Jackson). CMV-Cre is expressed in all cells from early embryonic development, including the germline, so the $Blimp1^{+/-}$ genotype is heritable. $Blimp1^{+/-}$ mice will be crossed with BALB/c $p53^{+/-}$ mice (Barcellos-Hoff lab) to generate $p53^{+/-}$, $Blimp1^{+/-}$, and $Blimp1^{+/-}p53^{+/-}$ mice. 8-10 week old mice of each genotype will be treated with 1 Gy IR, a dose known to generate radiogenic cancers in $p53^{+/-}$ mice.[64] (Figure 4b) Mice will be monitored for breast tumor formation by palpation for 1 year after IR treatment, the time at which all irradiated $p53^{+/-}$ mice are expected to have developed tumors.[65] However, our primary endpoints will be median tumor latency in irradiated mice and tumor incidence at 40 weeks, the time point at which 50% of irradiated $p53^{+/-}$ mice are expected to develop tumors,[40] and it may not be necessary to follow mice for the full 12 months. Tumors will be measured with calipers. Mice will be sacrificed when tumors reach > 1 cm or the mice reach human endpoints as detailed in University of Chicago IACUC guidelines. Secondary outcomes will be tumor tissue of origin and tumor growth rate. As described above, I estimate that Blimp1 will decrease tumor latency by 2 months. Based on data for $p53^{+/-}$ tumor latency after IR,[64] I estimate we need 10 mice per group to have 80% power to detect a 2 month decrease in tumor latency, or 30 mice for the whole study. I will consult with a statistician to obtain better estimates of the required number of mice for our study.

Expected results: Irradiated 129/Ola $p53^{+/-}$ mice develop a variety of cancers (primarily lymphomas and sarcomas) with a mean latency of 40 weeks.[40] I expect our irradiated BALB/c $p53^{+/-}$ mice to develop cancers with a similar latency, although we are treating a strain of mice more sensitive to radiation carcinogenesis[62] at a lower dose (1 Gy vs 4 Gy). I expect IR treated $Blimp1^{+/-}$ mice will not develop cancers in the absence of the $p53^{+/-}$ genotype that sensitizes mice to radiogenic cancers. However, it would be interesting if irradiated

Blimp1$^{+/-}$ mice developed cancers because it would suggest Blimp1 deficiency alone permits development of radiogenic cancers. Importantly, I expect irradiated Blimp1$^{+/-}$p53$^{+/-}$ to have decreased tumor latency and increased tumor incidence relative to irradiated p53$^{+/-}$ mice. If this occurs, I will conclude that Blimp1 deficiency increases the risk of radiogenic cancers.

Pitfalls and alternatives: If Blimp1 deficiency has a clear effect on radiation carcinogenesis in p53$^{+/-}$ mice, then in subsequent experiments we will compare tumor formation in IR treated and sham treated mice to determine whether Blimp1 has any effect on tumorigenesis in the absence of radiation. Blimp1 deficiency may change the spectrum of tumors that arise in irradiated p53$^{+/-}$ mice. We will analyze tumor histology to determine whether there are significant differences in either tissue of origin or tumor biology in Blimp1 wild-type and heterozygous mice. We will follow up with mouse experiments to elucidate the mechanism by which Blimp1 protects against radiation carcinogenesis, guided by our findings in aims 1 and 2.

REFERENCES

1. Inskip, P. D. et al. Radiation Dose and Breast Cancer Risk in the Childhood Cancer Survivor Study. JCO 27, 3901–3907 (2009).
2. Travis, L. B. et al. Cumulative Absolute Breast Cancer Risk for Young Women Treated for Hodgkin Lymphoma. J Natl Cancer Inst 97, 1428–1437 (2005).
3. Travis, L. B. et al. Second Malignant Neoplasms and Cardiovascular Disease Following Radiotherapy. J Natl Cancer Inst 104, 357–370 (2012).
4. Altekruse SF, Kosary CL, Krapcho M, et al (eds): SEER Cancer Statistics Review, 1975-2007. National Cancer Institute, Bethesda, MD, 2010. http://seer.cancer.gov/csr/1975_2007/)
5. Friedman, D. L. et al. Subsequent Neoplasms in 5-Year Survivors of Childhood Cancer: The Childhood Cancer Survivor Study. J Natl Cancer Inst 102, 1083–1095 (2010).
6. Hodgson, D. C. et al. Long-Term Solid Cancer Risk Among 5-Year Survivors of Hodgkin's Lymphoma. JCO 25, 1489–1497 (2007).
7. Aleman, B. M. P. et al. Long-Term Cause-Specific Mortality of Patients Treated for Hodgkin's Disease. JCO 21, 3431–3439 (2003).
8. Castellino, S. M. et al. Morbidity and mortality in long-term survivors of Hodgkin lymphoma: a report from the Childhood Cancer Survivor Study. Blood 117, 1806–1816 (2011).
9. Stovall, M. et al. Dose to the Contralateral Breast From Radiotherapy and Risk of Second Primary Breast Cancer in the WECARE Study. International Journal of Radiation Oncology*Biology*Physics 72, 1021–1030 (2008).
10. Castiglioni, F. et al. Radiation Effects on Development of HER2-Positive Breast Carcinomas. Clin Cancer Res 13, 46–51 (2007).
11. Milano, M. T., Li, H., Gail, M. H., Constine, L. S. & Travis, L. B. Long-Term Survival Among Patients With Hodgkin's Lymphoma Who Developed Breast Cancer: A Population-Based Study. JCO 28, 5088–5096 (2010).
12. Coates, P. J. et al. Differential contextual responses of normal human breast epithelium to ionizing radiation in a mouse xenograft model. Cancer Res. 70, 9808–9815 (2010).
13. Barcellos-Hoff, M. H. Stromal Mediation of Radiation Carcinogenesis. J Mammary Gland Biol Neoplasia 15, 381–387 (2010).
14. Seymour, C. B. & Mothersill, C. Radiation-induced bystander effects — implications for cancer. Nat Rev Cancer 4, 158–164 (2004).
15. Lorimore, S. A., McIlrath, J. M., Coates, P. J. & Wright, E. G. Chromosomal Instability in Unirradiated Hemopoietic Cells Resulting from a Delayed In vivo Bystander Effect of γ Radiation. Cancer Res 65, 5668–5673 (2005).
16. Barcellos-Hoff, M. H. & Ravani, S. A. Irradiated Mammary Gland Stroma Promotes the Expression of Tumorigenic Potential by Unirradiated Epithelial Cells. Cancer Res 60, 1254–1260 (2000).
17. Nguyen, D. H. et al. Radiation Acts on the Microenvironment to Affect Breast Carcinogenesis by Distinct Mechanisms that Decrease Cancer Latency and Affect Tumor Type. Cancer Cell 19, 640–651 (2011).
18. Schaue, D., Kachikwu, E. L. & McBride, W. H. Cytokines in Radiobiological Responses: A Review. Radiation Research 178, 505–523 (2012).
19. Lorimore, S., Coates, P. & Scobie, G. Inflammatory-type responses after exposure to ionizing radiation in vivo: a mechanism for radiation-induced bystander effects? , Published online: 26 October 2001; | doi:10.1038/sj.onc.1204903 20, (2001).
20. Mukherjee, D., Coates, P. J., Lorimore, S. A. & Wright, E. G. The in vivo expression of radiation-induced chromosomal instability has an inflammatory mechanism. Radiat. Res. 177, 18–24 (2012).
21. Rodel, F. et al. Modulation of Inflammatory Immune Reactions by Low-Dose Ionizing Radiation: Molecular Mechanisms and Clinical Application. Current Medicinal Chemistry 19, 1741–1750 (2012).
22. Lorimore, S. A., Rastogi, S., Mukherjee, D., Coates, P. J. & Wright, E. G. The Influence of p53 Functions on Radiation-Induced Inflammatory Bystander-Type Signaling in Murine Bone Marrow. Radiation Research 179, 406–415 (2013).
23. Demaria, S. & Formenti, S. C. Role of T lymphocytes in tumor response to radiotherapy. Front. Oncol 2, 95 (2012).

24. Pandey, R., Shankar, B. S., Sharma, D. & Sainis, K. B. Low dose radiation induced immunomodulation: Effect on macrophages and CD8 + T cells. International Journal of Radiation Biology 81, 801–812 (2005).
25. McBride, W. H. et al. A Sense of Danger from Radiation. Radiation Research 162, 1–19 (2004).
26. Zhou, H. et al. Mechanism of radiation-induced bystander effect: Role of the cyclooxygenase-2 signaling pathway. PNAS 102, 14641–14646 (2005).
27. Best, T. et al. Variants at 6q21 implicate PRDM1 in the etiology of therapy-induced second malignancies after Hodgkin's lymphoma. Nat Med 17, 941–943 (2011).
28. Hayden, M. S. & Ghosh, S. Shared Principles in NF-κB Signaling. Cell 132, 344–362 (2008).
29. Morgan, M. A. J. et al. Blimp-1/Prdm1 Alternative Promoter Usage during Mouse Development and Plasma Cell Differentiation. Mol. Cell. Biol. 29, 5813–5827 (2009).
30. Doody, G. M., Stephenson, S. & Tooze, R. M. BLIMP-1 is a target of cellular stress and downstream of the unfolded protein response. European Journal of Immunology 36, 1572–1582 (2006).
31. Best, T. Cancer risk at the interface of genomics and the environment. PhD dissertation, University of Chicago (2012).
32. Kallies, A., Xin, A., Belz, G. T. & Nutt, S. L. Blimp-1 Transcription Factor Is Required for the Differentiation of Effector CD8+ T Cells and Memory Responses. Immunity 31, 283–295 (2009).
33. Rutishauser, R. L. et al. Transcriptional Repressor Blimp-1 Promotes CD8+ T Cell Terminal Differentiation and Represses the Acquisition of Central Memory T Cell Properties. Immunity 31, 296–308 (2009).
34. Wang, L. et al. Blimp-1 Induced by IL-4 Plays a Critical Role in Suppressing IL-2 Production in Activated CD4 T Cells. J Immunol 181, 5249–5256 (2008).
35. Shapiro-Shelef, M. et al. Blimp-1 Is Required for the Formation of Immunoglobulin Secreting Plasma Cells and Pre-Plasma Memory B Cells. Immunity 19, 607–620 (2003).
36. Smith, M. A. et al. PRDM1/Blimp-1 Controls Effector Cytokine Production in Human NK Cells. J Immunol 185, 6058–6067 (2010).
37. Kim, S. J., Zou, Y. R., Goldstein, J., Reizis, B. & Diamond, B. Tolerogenic function of Blimp-1 in dendritic cells. J Exp Med 208, 2193–2199 (2011).
38. Chang, D. H., Angelin-Duclos, C. & Calame, K. BLIMP-1: trigger for differentiation of myeloid lineage. Nat Immunol 1, 169–176 (2000).
39. Raychaudhuri, S. et al. Genetic variants at CD28, PRDM1 and CD2/CD58 are associated with rheumatoid arthritis risk. Nat Genet 41, 1313–1318 (2009).
40. Gateva, V. et al. A large-scale replication study identifies TNIP1, PRDM1, JAZF1, UHRF1BP1 and IL10 as risk loci for systemic lupus erythematosus. Nat Genet 41, 1228–1233 (2009).
41. Ellinghaus, D. et al. Association Between Variants of PRDM1 and NDP52 and Crohn's Disease, Based on Exome Sequencing and Functional Studies. Gastroenterology 145, 339–347 (2013).
42. Martins, G. A., Cimmino, L., Liao, J., Magnusdottir, E. & Calame, K. Blimp-1 directly represses Il2 and the Il2 activator Fos, attenuating T cell proliferation and survival. J Exp Med 205, 1959–1965 (2008).
43. Martins, G. A. et al. Transcriptional repressor Blimp-1 regulates T cell homeostasis and function. Nat Immunol 7, 457–465 (2006).
44. Kallies, A. et al. Transcriptional repressor Blimp-1 is essential for T cell homeostasis and self-tolerance. Nat Immunol 7, 466–474 (2006).
45. Chan, Y.-H. et al. Absence of the Transcriptional Repressor Blimp-1 in Hematopoietic Lineages Reveals Its Role in Dendritic Cell Homeostatic Development and Function. J Immunol 183, 7039–7046 (2009).
46. Gong, D. & Malek, T. R. Cytokine-dependent Blimp-1 expression in activated T cells inhibits IL-2 production. J. Immunol. 178, 242–252 (2007).
47. Keller, A. D. & Maniatis, T. Identification and characterization of a novel repressor of beta-interferon gene expression. Genes Dev. 5, 868–879 (1991).
48. Cimmino, L. et al. Blimp-1 Attenuates Th1 Differentiation by Repression of ifng, tbx21, and bcl6 Gene Expression. J Immunol 181, 2338–2347 (2008).
49. Wang, X. et al. RelB NF-κB Represses Estrogen Receptor α Expression via Induction of the Zinc Finger Protein Blimp1. Mol. Cell. Biol. 29, 3832–3844 (2009).
50. Yan, J. et al. BLIMP1 regulates cell growth through repression of p53 transcription. PNAS 104, 1841–1846 (2007).

51. Lin, Y., Wong, K. & Calame, K. Repression of c-myc Transcription by Blimp-1, an Inducer of Terminal B Cell Differentiation. Science 276, 596–599 (1997).
52. Doody, G. M. et al. An extended set of PRDM1/BLIMP1 target genes links binding motif type to dynamic repression. Nucl. Acids Res. 38, 5336–5350 (2010).
53. Barretina, J. et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. Nature 483, 603–607 (2012).
54. Codony-Servat, J. et al. Differential cellular and molecular effects of bortezomib, a proteasome inhibitor, in human breast cancer cells. Mol. Cancer Ther. 5, 665–675 (2006).
55. Sarkaria, J. N. et al. Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. Cancer Res. 59, 4375–4382 (1999).
56. Cortez, D. Caffeine inhibits checkpoint responses without inhibiting the ataxia-telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR) protein kinases. J. Biol. Chem. 278, 37139–37145 (2003).
57. Horsley, V. et al. Blimp1 Defines a Progenitor Population that Governs Cellular Input to the Sebaceous Gland. Cell 126, 597–609 (2006).
58. Magnúsdóttir, E. et al. Epidermal terminal differentiation depends on B lymphocyte-induced maturation protein-1. PNAS 104, 14988–14993 (2007).
59. Schaue, D., Kachikwu, E. L. & McBride, W. H. Cytokines in Radiobiological Responses: A Review. Radiation Research 178, 505–523 (2012).
60. Stingl et al., Methods in Molecular Biology 290, 249-263 (2005).
61. Welm, B. E., Dijkgraaf, G. J. P., Bledau, A. S., Welm, A. L. & Werb, Z. Lentiviral Transduction of Mammary Stem Cells for Analysis of Gene Function during Development and Cancer. Cell Stem Cell 2, 90–102 (2008).
62. Storer, J. B., Mitchell, T. J. & Fry, R. J. Extrapolation of the relative risk of radiogenic neoplasms across mouse strains and to man. Radiat. Res. 114, 331–353 (1988).
63. Ullrich, R. L., Bowles, N. D., Satterfield, L. C. & Davis, C. M. Strain-Dependent Susceptibility to Radiation-Induced Mammary Cancer Is a Result of Differences in Epithelial Cell Sensitivity to Transformation. Radiation Research 146, 353–355 (1996).
64. Kemp, C. J., Wheldon, T. & Balmain, A. p53-deficient mice are extremely susceptible to radiation-induced tumorigenesis. Nature Genetics 8, 66–69 (1994).
65. Kuperwasser, C. et al. Development of Spontaneous Mammary Tumors in BALB/c p53 Heterozygous Mice. Am J Pathol 157, 2151–2159 (2000).