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DISSERTATION

OF MICE, GENES, AND RADIATION: THE GENETICS OF NON-HEREDITARY BREAST CANCER EXPLORED USING THE COMMON LABORATORY BALB/C MOUSE

Submitted by

Lila Ramaiah

Department of Microbiology, Immunology, and Pathology

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY LILA RAMAIAH ENTITLED "OF MICE, GENES, AND RADIATION: THE GENETICS OF NON-HEREDITARY BREAST CANCER EXPLORED USING THE COMMON LABORATORY BALB/C MOUSE" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Christine S. Olver

William H. Hanneman

Susan M. Bailey

Michael M. Weil

Adviser: Robert L. Ullrich

Co-Adviser: Stephen A. Benjamin

Department Head/Director : Susan Deines

ABSTRACT OF DISSERTATION

OF MICE, GENES, AND RADIATION:

THE GENETICS OF NON-HEREDITARY BREAST CANCER EXPLORED USING THE COMMON LABORATORY BALB/C MOUSE

In this dissertation we describe the generation and characterization of two novel strains of mice carrying alternate genetic variants of the DNA repair gene *Prkdc* (DNA-PKcs). Strains congenic for the common ($Prkdc^{B6}$) and variant ($Prkdc^{BALB}$) alleles of *Prkdc* are developed, genotypically validated, and used to examine the functional consequences of $Prkdc^{BALB}$ and its linkage with radiation sensitivity. DNA-PKcs (DNA-dependent protein kinase) expression, double strand break (DSB) repair, cell survival, breeding depression, and gene expression are examined. By western blot we demonstrate that $Prkdc^{BALB}$ is required and sufficient to decrease DNA-PKcs expression. Using three DSB repair quantification methods we show that $Prkdc^{BALB}$ is required for reduced radiation-induced DSB rejoining in BALB/c. We also show that $Prkdc^{BALB}$ is both sufficient and required for decreased cell survival after exposure to ionizing radiation. Thus we demonstrate that $Prkdc^{BALB}$ modulates and even diminishes the ability of cells to maintain genomic homeostasis.

Using our newly developed congenic mice, we present the first evidence that $Prkdc^{BALB}$ has a significant effect on gene expression in unirradiated as well as irradiated mice. Microarray analysis of gene expression reveals that $Prkdc^{BALB}$ may have a greater impact on overall gene expression than does radiation, and that *Prkdc* may play a role in constitutive and DNA damage-induced apoptotic and transcriptional responses.

The results presented within this dissertation support the hypothesis that the main role of *Prkdc*^{BALB} in radiation-induced breast cancer is the initiation of mammary epithelial cells. Our data show that *Prkdc*^{BALB} is strongly associated with diminished DNA-PKcs expression and function, diminished survival, and altered transcriptional regulation. The congenic strains developed and characterized here will be instrumental in ongoing studies aiming to clarify the role of *Prkdc* and genomic instability in radiation-induced mammary carcinogenesis in the BALB/c mouse. Future studies should endeavor to quantify DNA-PKcs specific kinase activity and protein metabolism and to evaluate cytogenetic instability, with particular emphasis on telomeres. The congenic strains developed and characterized in this work serve as compelling rodent models of sporadic and radiation-induced human breast cancer, and provide proof of principle for the role of genetic polymorphisms and genomic instability in breast cancer susceptibility.

Lila Ramaiah
Department of Microbiology, Immunology and Pathology
Colorado State University
Fort Collins, CO, 80523
Spring 2008

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1.0 INTRODUCTION

Cancer is a complex and deadly disease that affects more than 25 million people and causes over 6.7 million deaths worldwide.[2] In the United States, cancer is the second leading cause of death, causing one of every four deaths.[3] Although our understanding of carcinogenesis has grown dramatically over the past two decades, cancer survival rates have only increased from 50% to 64%.[3] This is because the majority of cancers are identified at an advanced stage, when tumor heterogeneity allows cancers to persist using alternate molecular pathways to evade cancer therapeutics. Early detection has proven to be one of the best approaches for effective control of cancer mortality rates.[4] Cancer biomarker identification and development is a promising approach for the detection of early stage and pre-malignant lesions that could eventually allow for rapid non-invasive cancer screening.

Incidence can be further improved through prevention activities focused on minimizing risk. Evidence indicates that successful prevention approaches could reduce cancer mortality and incidence rates by 50% and 25% respectively, by the year 2015.[5] Comprehensive Cancer Control (CCC) efforts at the Centers for Disease Control and Prevention's National Comprehensive Cancer Control Program (NCCCP) of the Division of Cancer Prevention and Control (DCPC) and at the American Cancer Society (ACS) include prevention strategies that promote risk reduction. Prevention strategies focus on implementing behavioral changes for improving nutrition, diet and physical activity and reducing obesity, minimizing exposures (tobacco, radiation, infectious agents, occupational and chemical carcinogens), screening for early detection, and expanding the knowledge base of genetic susceptibility factors.[4] These demographic, environmental,

lifestyle, biological and genetic factors act in conjunction to impart risk and affect prognosis. They can be used as multi-factorial predictors to assess individual cancer risk and prognosis and to suggest prevention and control modalities. Lifestyle and environmental risk factors are known and well documented. Effective cancer prevention through lifestyle change requires patient education, compliance, and access to health care, as described in plans proposed by the CDC (Centers for Disease Control) and ACS. Similarly, actions can be taken by individuals and by society to minimize environmental exposures.

Though the risk association between exposures and cancer is well recognized, not all exposed individuals develop cancer. The factors that complicate the relationship between exposure/lifestyle risk factors and cancer are still poorly understood.[6] Current approaches to exploring this black box include identification of genetic variants (polymorphisms) that modify exposure-disease associations. Although at the population level most cancers depend on environmental factors, at the cellular level cancer is characterized by genetic alterations. Understanding how these two processes intersect is of fundamental importance for prevention even if in most instances, hereditary susceptibility genes are different from the somatically mutated genes. Individual genetic susceptibility differences resulting from polymorphisms in genes involved in nutrient, carcinogen and steroid metabolism, DNA repair, cell cycle, immune response and apoptosis can be independent predictors of cancer, or can act as modifiers of exposure-disease relationships. Molecular epidemiological and cancer susceptibility studies identifying such inherited genetic factors have the potential to yield important information regarding cancer etiology. Knowledge about the relationships between inherited factors and cancer

outcomes can be used for reinforcing healthy lifestyles, motivating positive behavior changes, and helping to target medical therapy. Such knowledge would also allow us to elucidate risk relationships more clearly, identify subsets of the population that are most susceptible to certain exposures, and aid in better focusing surveillance activities.

1.1 Inherited Susceptibility to Cancer: Mutations and Polymorphisms

Alterations in the sequence of DNA (mutations) form the foundation for inter-individual phenotypic variability. They can take many forms, from silent single nucleotide substitutions to large chromosomal deletions or translocations, and have varying effects on health (phenotypic expressivity or penetrance) depending on whether and to what extent they modify RNA or protein function.

A version of a gene (allele) that is highly penetrant will express itself almost regardless of the effects of environment whereas an allele with low penetrance will only sometimes produce its associated phenotype. Highly penetrant allelic variants tend to be caused by large or severe gene mutations such as deletions or missense mutations that markedly disturb protein function, whereas low penetrance allelic variants tend to consist of single nucleotide polymorphisms (SNPs) with subtle consequences. SNPs are defined as single base pair variations that occur at a population frequency of at least 1%. More than 10 million SNPs have been identified.[6] As an example of this diversity, two random individuals would differ by 1 in 1,000 single nucleotide polymorphisms or by more than 3 million base pairs. SNPs are likely to have a less pronounced effect on protein function than large deletions, thus they are less penetrant/expressive.

The identification of highly penetrant mutations in cancer susceptibility genes such as BRCA1/2 (hereditary breast and ovarian cancer), MEN1 (Multiple Endocrine Neoplasia), MSH/MLH (hereditary nonpolyposis colorectal cancer) and TP53 (Li Fraumeni syndrome) provides compelling evidence for the inherited basis of cancer. Although high penetrance alleles can be readily discovered using association and pedigree studies, due to their consistent expression in related affected individuals, this class of predisposing mutation can only account for the rare familial cancer syndromes, which comprise only a small fraction of all cancers.[7] Non-familial (sporadic) cancers are more likely to be caused by interactions between multiple genetic variants and the environment, rather than a dominant effect by a specific gene. Emerging research on gene–environment interactions highlights the possibility that individual differences in metabolism and excretion of pollutants and in DNA repair may be responsible for effects in susceptible subgroups. However, these effects are obscured in analyses of the general population. In addition, because cancer involves multiple correlated risk factors, misspecification of models may hide causal effects. For example, variables treated as confounders such as age at menarche and menopause could be on the causal pathway from pollutant exposures to breast cancer. Sample size and diversity of study populations have often been inadequate to explore interactions or effect modification. In some cases, the phenotype in question will occur only when the gene is present; in other cases, it may occur for unrelated reasons.

1.2 Polymorphic Cancer Susceptibility Genes

It is hypothesized that low-penetrance alleles contribute to cancer risk by augmenting the effects of environmental exposures. In such cases it is often difficult to distinguish environmental from genetic factors and to identify low-penetrance alleles using epidemiologic approaches.[8] The existence of low-penetrance alleles is supported by clinical observations noting differences in therapeutic response or side effects from various treatment regimens as well as by association studies in highly inbred species such as laboratory mice. An abundance of recently reported case control studies confirms that although susceptibility alleles may have minimal individual impact on cancer risk, they have high attributable risk and great public health relevance. Some examples are presented in the following discussion.

The pathway leading from environmental exposure to cancer is complex (Fig.1.1). Exogenous agents may be metabolized into different reactive intermediates, which might in turn be excreted or form DNA adducts. Adducts and other types of DNA damage may be repaired, may cause replicative death, or may result in apoptosis. Failure or deficiency in these mechanisms results in mutation, continued cell proliferation, and miscoding, leading to permanent mutations in critical genes that allow cancer progression. The immune system also plays a role by scavenging for mutant cells. Thus, attention has focused on genes involved in carcinogen metabolism, ROS scavenging, apoptosis, DNA repair, cell-cycle control, chromosome segregation, transcriptional regulation, methyl group metabolism, growth promotion and immune status, and their roles as modifiers of exposure to various carcinogenic risks.

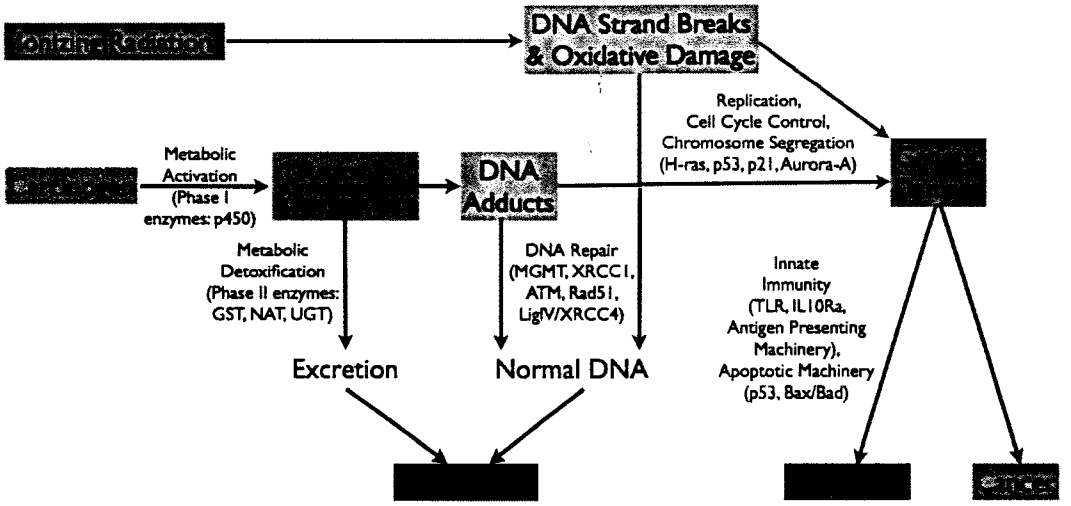

Figure 1.1: Polymorphic genes modify risk in the pathways to cancer. (Adapted from Ambrosone & Furberg, Trends in Mol. Med., 2001.)

1.2.1 Xenobiotic Metabolizing Enzymes

In the area of carcinogen metabolism, research on tobacco-related lung, breast, and bladder cancers has demonstrated that polymorphisms in *CYP1A1* and *CYP1B1* (cytochrome p450 family, phase I enzymes) modify breast cancer risk. In one study, though individuals carrying the *CYP1B1* 432Val allele were not at higher risk for breast cancer, women carrying the allele who had smoked 1-9 cigarettes/day, were.[9] The polymorphic alleles were found in subpopulations of Caucasians and were associated with increased inducibility and heightened activation of tobacco smoke carcinogens.[9,10] In contrast, an association between *CYP1A1* and squamous cell lung cancer is restricted to non-smokers, indicating that tissue-specific factors also modify risk.[11] Polymorphisms in the phase II enzyme *N*-acetyltransferase 2 (*NAT2*) result in a "slow acetylation" phenotype with lesser detoxification of aromatic amines found in tobacco smoke, and are associated with bladder cancer in smokers.[10] "Slow acetylators" are also at increased risk for breast cancer if they carry polymorphic *CYP1B1* alleles, indicating the influence of gene-gene interactions as well as gene-environment interactions.[9]

1.2.2 Steroid Metabolism Enzymes

Genetic variability in genes involved in steroidogenesis and estrogen metabolism such as *CYP17A* and catechol-*O*-methyltransferase (*COMT*) also contributes to cancer risk in a manner that may depend on environmental factors. Direct associations between variant *CYP17A* or *COMT* alleles and breast cancer are inconsistent, thus they are not independent predictors of breast cancer risk. Rather, the variant alleles act as modifiers, where associations vary by menopausal status, body size, dietary intake, and smoking. For example, late menarche is only protective against breast cancer for women homozygous for the variant *CYP17A1* allele, suggesting that its role depends on hormonal environment.[12-16] Similarly, a polymorphism in UDP-glucuronosyltransferase 1A1 (*UGT1A1*) is only predictive of increased breast cancer risk among premenopausal African American women.[10]

1.2.3 Tumor Immunity

Polymorphisms in genes involved in immune regulation and inflammation are suspected to underlie susceptibility to lymphoma. Lymphoma can be observed in association with immune deficiency and certain infectious agents, and is inversely correlated with asthma and allergy.[17] Toll-like receptors mediate host immune responses to infectious agents. They act as a bridge between innate and acquired immunity and regulate the nature of the lymphocyte responses. Polymorphisms in Toll-Like Receptor 2 (TLR2) are overrepresented in lymphoma cases and gastric mucosa-associated lymphoid tissue lymphoma, and are underrepresented in chronic lymphocytic leukemia. This effect is further modified by variant alleles of interleukin 10 receptor a (IL10RA). Exposure to

pathogens including *Helicobacter pylori* and Epstein Barr Virus is suspected in association with these diseases.[17]

Human papillomavirus (HPV) is an oncogenic virus that infects and can transform cells of the uterine cervix, leading to cervical carcinoma. Viral proteins interfere with the antigen presenting machinery of the immune system to help virally infected cells avoid tumor immunity, promoting persistent infection. However, the extent of viral interference with antigen presentation does not always correlate with persisting oncogenic HPV infection and malignant transformation. Epidemiological evidence points toward a genetic predisposition to HPV-induced cervical cancer. Variants of the antigen presenting machinery (TAP2, LMP7, and ERAP1) appear to act in concert to impart risk for cervical carcinoma in high risk HPV exposed patients.[18] Polymorphisms in IL-10 have also been linked to high-risk HPV-induced cervical carcinoma in immunodeficient individuals.[19]

1.2.4 Growth Promoters

Polymorphisms in genes involved in the estrogen receptor signaling pathway, such as ER, PR, MTA3, Snail, E-Cad, and MTA1, that trigger cell growth, chromatin signaling, and tumor invasion, all modify breast cancer risk in obese and nulliparous women. In fact, obesity is only a risk factor for breast cancer in women with a higher number of high-risk genotypes in estrogen and progesterone receptor pathways.[20, 21]

1.2.5 Cell Cycle Control and Apoptosis

Polymorphisms in cell cycle control genes such as *CCND1, TTK, PRRG1, H-ras, p53*, and *p21* also modify cancer risks.[22] Over 100 SNPs have been identified in Cyclin D1 (*CCND1*).[23] The G870A polymorphism is highly prevalent, and both GG and AA alleles have been linked to increased risk for a wide variety of tumors in many conflicting studies.[23] For a single tumor type, some studies show no effect, while some show increased risk with the A/A allele and others with the G/G allele. One study demonstrated increased post-menopausal breast cancer risk associated with hormonal exposure in A/A patients.[24] A different study found that the A/A genotype did not affect breast cancer risk, but that the heterozygous G/A allele significantly reduced breast cancer risk when compared to the G/G allele in women with a high dietary intake of *n*-6 fatty acids, low *n*-3 fatty acid intake, or lack of *GSTM1* or *GSTT1*.[25]

TTK and *PRRG1* regulate the mitotic checkpoint by ensuring chromosome attachment to the spindle and ensuring sister chromatid separation, respectively. Variants in *TTK* and *PRRG1* have been shown to modify breast cancer risk when associated with reproductive risk factors such as number of full-term pregnancies, consistent with the mitogenic effect of estrogen.[22]

Two rare *H-ras1* alleles have been associated with lung and breast cancer.[10] Increased prevalence of prostate cancer, squamous cell carcinoma of the head and neck, and endometrial cancer is also seen in individuals with polymorphisms in *p21*.[26]

1.2.6 Chromosome Segregation

Aurora-A/BTAK/STK15 is a serine/threonine kinase that regulates chromosome segregation and cytokinesis. Its overexpression in breast cancers causes centrosome amplification, chromosomal instability, aneuploidy, and transformation.[27,28] An A91T polymorphism that alters kinase function and causes aneuploidy is strongly associated with elevated breast cancer risk in women with a high body mass index, early menarche, and late first full term pregnancy, factors related to endogenous estrogen exposure.[28,29] Furthermore, the strongest correlation exists in younger patients with estrogen receptor positive tumors.[30] These findings provide evidence for the modifying effect of a mutator phenotype (*Aurora-A* A91T) on breast cancer risk, the contribution of which is enhanced as a result of increased mitosis due to estrogen exposure.

1.2.7 Transcription Factors

Transcription factors are risk modifiers for a variety of tumor types. Aryl hydrocarbon receptor (*ArH*) is a transcription factor that induces expression of *CYP1A1* in response to the tobacco carcinogen PAH (polycyclic aromatic hydrocarbon). Although polymorphisms in *ArH* are not associated with lung cancer risk in the general population, associations are seen in smokers relative to non-smokers, and a synergy between smoking and *ArH* haplotypes is observed for squamous cell carcinoma.[31] Functional polymorphisms in the transcription factor *NF-κB1* modify the risk for oral squamous cell carcinoma in older male areca (betel) chewers.[32]

1.2.8 One-Carbon/Methyl Group Metabolism

The transcription and expression of genes is also regulated by epigenetic DNA methylation, and polymorphisms in methyl group metabolism genes can modify cancer risk. Methyltetrahydrofolate reducatse (MTHFR) is an enzyme that regulates folate-dependent one-carbon metabolism. A C677T polymorphism in MTHFR has high prevalence in the population (15% to 40%) and results in a mild enzyme deficiency but has no effect on cancer risk.[33] However, dietary folate restriction in TT homozygotes results in decreased methionine production and has a protective effect on the risk for acute lymphocytic leukemia and malignant lymphoma. Polymorphisms in MTHFR also appear to influence genomic stability via unknown mechanisms.[34]

1.2.9 DNA Repair

A key feature of malignant neoplasia is the acquisition and persistence of mutations in a proliferating cell population. The impact of DNA damage caused by ionizing radiation (IR) and chemical carcinogens is dependent on its balance with the many mechanisms that serve to repair the damage or halt division of a damaged cell. DNA repair genes are "caretakers" of the genome. They are the tumor suppressors that maintain the integrity of the genome. Deficiencies in DNA repair, even when mild, are suspected to increase susceptibility to cancer, even with low doses of environmental exposures.[35] DNA adducts and oxidative DNA damage resulting from carcinogen or IR exposure and cellular metabolism are primarily repaired via direct damage reversal or base and nucleotide excision repair pathways (BER and NER), whereas strand breaks are repaired via recombination mechanisms or single strand annealing.

Alkyl and methyl DNA adducts induced by cigarette smoking are removed by direct reversal. O^6-methylguanine DNA methyltransferase (MGMT) repairs such damage and also acts as an ER (estrogen receptor) antagonist to inhibit ER-mediated cell proliferation. An Ile143Val polymorphism in this gene is not associated with endometrial or breast cancer risk, however it does appear to modify risk for endometrial cancer in smokers.[36, 37]

Bulky adducts induced by polycyclic aromatic hydrocarbons (PAHs) can be repaired via the NER pathway (Xeroderma Pigmenosum proteins). Polymorphic NER genes such as XPD have been shown to modulate susceptibility to PAH exposure in a vitamin C-dependent manner.[38]

One well-characterized enzyme involved in BER and single strand break (SSB) repair is X-Ray Cross-Complementing group 1 (XRCC1). Many polymorphisms in XRCC1 are described in association with increased risk for oral, pharyngeal, salivary, lung, hepatocellular, bladder, prostate, and breast cancer.[39-48] Aflatoxin exposure has been linked with hepatocellular carcinoma (HCC), but not all exposed individuals get HCC. Polymorphisms in GST, which detoxifies aflatoxin B1 metabolites, are only weakly associated with HCC risk. However, exposed individuals with polymorphic GST and XRCC1 are at significantly greater risk.[35] Conversely, the *Arg399Gln* polymorphism in XRCC1 has a protective effect on sporadic breast cancer risk.[49] XRCC1 polymorphisms associate with malignant but not benign salivary gland tumors, consistent with a requirement for instability, not just proliferation.[44]

Ionizing radiation causes direct DNA damage in the form of frank SSBs and double strand breaks (DSBs), as well as indirect damage in the form of oxidative damage by

reactive oxygen species (ROS). Allelic variants of genes involved in the generation and metabolism of ROS have been linked with increased cancer risk in subpopulations exposed to unique hormonal environments. A polymorphic manganese superoxide dismutate (MnSOD) alanine allele reduces capacity to metabolize hydroxyl radicals and is associated with a fourfold increase in breast cancer risk among premenopausal women, thus linking estrogen exposure with ROS metabolism.[50, 51] A polymorphic inducible nitric oxide synthase (iNOS) (C608T) does not itself increase risk for developing gastric cancer, however, it modifies susceptibility to cigarette/alcohol induced gastric cancer.[52]

1.2.9.1 Double Strand Break Repair

DSBs are induced by intrinsic sources (by-products of cellular metabolism, SSBs in replication, collapsed replication forks, V(D)J recombination, class switch, and meiosis) or by extrinsic sources (IR and radiomimetic chemotherapeutic drugs). The principal pathways for mammalian DSB repair are homologous recombination (HR) and non-homologous end joining (NHEJ). HR is a highly accurate process that utilizes an intact unaffected sister chromatid as a template. However HR can only be employed in the S/G2 phases of the cell cycle in mammalian cells due to its dependency on a homologous DNA template, which is present only after DNA synthesis. In contrast, NHEJ, the predominant DSB repair mechanism in mammalian cells, is an error-prone process of illegitimately linking broken pieces back together without accurate reference to the original sequence or correct base-pairing.[53]

The gene mutated in Ataxia Telangiectasia, *ATM* (Ataxia Telangiectasia Mutated), encodes a 350 kDa protein kinase that plays a key role in the detection of and response to

DNA DSBs. ATM triggers phosphorylation of p53, Mdm2, Chk2, BRCA1, SMC1, NBS1, and FANCD2, resulting in activation of cell cycle checkpoints, DNA repair, and/or apoptosis. Variant ATM alleles have been reported in association with many cancers, including lung cancer, chronic lymphocytic leukemia, acute lymphoblastic leukemia, hereditary nonpolyposis colorectal carcinoma, endometrial carcinoma, and mammary carcinoma.[54-59] Though many studies yield contradictory and false negative or false positive results due to small sample size and poor study design, the cumulative evidence indicates that DNA repair variants can significantly modify cancer risk.[58, 60-62] Some variants modify breast cancer risk by imparting radiation sensitivity. Homozygous carriers of *ATM* polymorphisms T77C and C238G are radiosensitive and at increased risk for breast cancer.[63] Homozygosity for the G5557A variant of *ATM* is over-represented in cells from radiation-sensitive breast cancer cases compared with those from non-radiosensitive breast cancer cases.[63]

In the HR pathway of DSB repair, the MRN (MRE11/RAD50/NBS1) complex initially recognizes breaks. RAD51 and its paralogs (XRCC2/3), BRCA1/2, RPA, RAD52, RAD54, BLM and WRN then initiate nucleolytic processing and sister chromatid pairing and facilitate Holiday junction resolution. A polymerase fills nucleotide gaps and strands are resolved by a ligase/resolvase complex. Polymorphisms affecting genes that repair DSBs via HR are frequently described in association with predisposition to breast/ovarian cancers. The *RAD51* G135C polymorphism increases risk for familial breast cancer.[49] One case control study found that the rare homozygote CC of *WRN* A46729C was significantly associated with a risk for breast cancer.[64] Another case control study examining the association of SNPs with breast cancer risk in US radiologic

technologists found that *WRN* V114I significantly modified the association between cumulative radiation exposure to the breast and risk for breast cancer, and that *BRCA1* significantly altered the occupational exposure-response relationship.[65] One literature review focusing on associations between DNA repair polymorphisms and cancer risk found associations between the *BRCA2* N372H variant and increased risk for breast cancer.[60] The R188H polymorphism in XRCC2 modifies the risk for breast cancer in association with plasma β-carotene level and age of cancer onset.[66, 67]

Non-homologous end-joining (NHEJ), the predominant DSB repair pathway of mammalian cells, involves juxtaposition of DNA ends by the DNA-dependent protein kinase holoenzyme (DNA-PK) followed by ligation by the DNA Ligase-XRCC4 complex.[53] The DNA-PK holoenzyme consists of the Ku70/80 heterodimer and a 470-kD catalytic subunit (DNA-PKcs) with serine/threonine protein kinase activity.[68] NHEJ proceeds as follows: 1) DNA-end binding Ku 70/80 heterodimer initially recognizes and binds the DNA break, 2) the protein kinase DNA-PKcs binds Ku bound DNA, activating its kinase activity, signals the presence of a break and recruits repair proteins to the break, 3) enzymes such as Artemis help process the DNA ends, and 4) the XRCC4–Ligase IV complex re-ligates the broken DNA ends.

As in HR, polymorphisms in NHEJ genes have been found in association with risk for breast cancer. Polymorphisms in the LigaseIV gene have been reported to modify risk for multiple myeloma and breast cancer.[66, 69, 70] Polymorphisms in Ku70 and XRCC4 have also been associated with increased breast cancer risk. Specifically, they appear to modify risk in women who were more susceptible to estrogen (nulliparous).[71] XRCC4 polymorphisms were also linked with early age at diagnosis.[72] In the only case control

study to link polymorphisms in DNA-PKcs with breast cancer risk, investigators examined the association of SNPs with breast cancer risk in US radiologic technologists exposed to known low doses of radiation.[65] *Prkdc IVS15* + T6C (rs11231202), *Prkdc IVS34* + C39T (rs8178097), and *Prkdc IVS31* – A634C (rs10109984) significantly modified the personal diagnostic radiation exposure - response relationship.[65]

A role for DNA-PKcs polymorphisms in carcinogenesis is further supported by studies demonstrating that DNA-PKcs expression is lower cancerous colon tissue when compared to non-cancerous lung and colon tissue,[73] and lower in patients with lung cancer than healthy controls.[74]

The paucity of reports of cancer risk linked with polymorphisms in NHEJ genes is due in part to the fact that NHEJ is a relatively newly characterized cellular process whose players are still being elucidated. In addition, scant genetic evidence has been found linking severe defects in NHEJ genes with cancer in humans. Most likely, the lack of severe defects in NHEJ genes results from the fact that they are so crucial for genomic stability that a serious defect would cause premature death (i.e. embryonic lethality, defective neurogenesis, lymphoma, immunodeficiency) and would preclude the development of late onset tumors. Only subtle defects in low-penetrance alleles such as hypomorphic variants or mild polymorphisms would likely result in repair deficient phenotypes that are viable and permitting sufficient lifespan for the development of other cancers such as breast cancers.

1.3 Challenges in Radiation Carcinogenesis

As described in earlier sections, modifiers of risk are generally identified in relation to quantifiable doses of carcinogen exposure. The study of radiation carcinogenesis poses unique challenges that are not faced in chemical carcinogenesis studies. Data on radiation carcinogenesis comes primarily from epidemiologic studies of populations exposed to unknown quantities of radiation from accidental exposures. Because radiation-induced cancers are histologically indistinguishable from cancers due to other causes it is very difficult to attribute one individual's cancer to radiation, given the normal variation in cancer mortality rates. In addition, risk estimates derived from populations with different genetic, life-style, and environmental backgrounds may not be applicable to all individuals. Cancer risk resulting from background radiation is nearly impossible to quantify in the general population and information on effects at low doses usually relies on extrapolation from data obtained at higher doses.

Nevertheless, some studies have identified genetic modifiers that cause subpopulations of individuals to be more susceptible to the mutagenic and carcinogenic effects of low doses of radiation. Heritability has been found to play a more prominent role than environment (socio-cultural and lifestyle factors) in determining mutagen sensitivity to carcinogens (bleomycin, BPDE, 4NQO, and radiation).[75] Furthermore, gamma-radiation sensitivity is the most heritable (62.5%, compared to bleomycin: 40.7%, BPDE: 48.0%, 4NQO: 53.8%) and has the least contribution from shared environment (0%).[75] Therefore, though modifiers of radiation-induced cancer risk are among the least well characterized and the most difficult to ascertain, their identification is very likely to provide candidate genetic markers for cancer risk assessment and clinical prevention.

1.4 What we know from human exposures to radiation

Humans are exposed to very low doses of IR on a daily basis throughout their lives. Ionizing radiation is emitted from naturally occurring sources such as cosmic rays and radionuclides in the ground and building materials, as well as from radon gas. The average individual effective dose from natural background radiation is 2-3 mSv/year.[76] Geographic factors may increase effective background dose for some populations.

Quantitative data on cancer induction by IR comes from populations exposed to medical diagnostic and therapeutic procedures, nuclear weapons (deliberate and inadvertent), and occupational exposures.[76-78] Cancer risk in each exposed population must be interpreted independently due to differences in radiation type, dose, dose rate, organ dose, and individual susceptibility. Radium dial painters were likely to develop bone tumors because divalent radium/polonium mimics calcium and is deposited in bone and releases radon.[79] Radon gas inhalation leads specifically to lung tumors in miners.[80] Therapeutic exposures of the thoracic cavity in children with enlarged thymuses or tuberculosis lead specifically to thyroid cancer and breast cancer.[81-83] Regional and ethnic differences were observed in children epilated with x-rays to treat *tinea capitis* who developed leukemia and thyroid, skin, salivary gland, and brain tumors.[84-86] Spinal radiation therapy administered to relieve pain due to ankylosing spondylitis in 14,000 British patients resulted in excess risk of leukemia mortality. Unfortunately, these historical human experiences of radiation exposure are of little use in risk assessment due to highly uncertain dosimetry in each instance and lack of appropriate controls.[78, 87] Quantitative data from modern medical and occupational exposures is more useful in determining dose-effect relationships but suffers from two drawbacks. Medical and some

occupational exposures involve very high doses targeted to specific organs rather than low dose whole body exposures such as those the general population experiences. Also, medical patient susceptibility may be skewed since inherited factors causing their illness likely increase susceptibility to carcinogenic radiation effects, as evidenced by the high incidence of secondary tumors in radiation therapy oncology patients.

The Life Span Study of cancer in the Japanese atomic bomb survivors provides the best currently available single group data because of the high number of people exposed to whole body irradiation.[88] Japanese A-bomb survivors represent a wide cross-section of the population in terms of human health status, age and sex, and include a wide range of doses from lethal doses to those nearing or at background levels. Early studies examining cancer development in A-bomb survivors suggested that the radiation-induced tumor spectrum was similar to that of spontaneously occurring cancers in contrast to rare inherited cancers. It follows that radiation exposure may play a causal role in the development of many spontaneous tumors observed in the general population. In addition, radiation-induced cancers looked identical to spontaneously occurring cancers in the general population. This characteristic is central to the problem of determining risk from IR exposure.

In the last two decades, the meticulous A-bomb survivor follow-up as well as new chemical ^{63}Ni extraction/accelerator mass spectrometry methods have permitted precise exposure estimations and consistent determinations of radiation-induced cancer risks.[89] One drawback of these studies was that these exposures were acute rather than chronic or lifelong. Also, these exposures happened relatively recently, and data for late and lifelong effects is still incomplete. Data from the Chernobyl Nuclear Power Plant

accident in 1986 also represents a large and heterogeneous population exposed to a wide range of doses that will yield invaluable information in the coming years.[77]

When interpreted collectively, human exposure data indicates that IR effects are stochastic (risk increases with dose, not severity) and that excess risk exists for leukemia in the short term and for a wide variety of solid cancers in the long term.[76] Myeloid leukemias are short latency (5-7 years) consequences of radiation exposure in adults. Acute lymphoid leukemia and thyroid carcinoma develop rapidly in exposed children. Individuals who do not succumb from leukemia or thyroid cancer are likely to develop solid tumors with increasing risk as they age, in a spectrum following natural age-related cancer risk (0.47 ERR/Sv).[88] The most recent data also reveal that excess risk from IR exposure exists for specific tumor types, with significant influence from gender and age at exposure.[90] The excess relative risk (ERR) for lung cancer in nonsmokers is 0.9/Sv.[88] The ERR in those exposed under the age of 20 is 6.3/Sv for thyroid cancer [91] and 2.41/Sv for breast cancer.[92] Numerous studies have documented increased vulnerability of the breast to radiation exposure. In an examination of the most recent data from atomic bomb survivors and medical exposures that derived site-specific risk coefficients with respect to LET, organ-specific dose, gender and age at exposure, investigators concluded that the female breast, bladder, and esophagus were at greater ERR per gray than a variety of other organs (lung, liver, gall bladder, stomach, rectum, colon, pancreas, ovary, and uterus).[93] Excess risk correlated inversely with organ depth, suggesting that surface organs are more vulnerable. Another study examining female radiologic technologists chronically exposed to low/moderate doses of IR documented increased relative risk (RR) for all cancers (1.28), leukemia (1.64) and breast cancer (2.92).[94, 95]

1.5 Breast Cancer Risk Factors: The Hormone – Radiation Link

Breast cancer accounts for 20% of all female cancers and is the most prevalent cancer worldwide.[2] It is the most frequent cause of death among high-income cancer patients and poses the greatest burden to those societies.[96] Approximately 1-2% of breast cancers are familial and caused by inheritance of high penetrance susceptibility genes.[97] The remaining 98% of cases occur sporadically, and the responsible factors remain largely obscure. Risk factors for sporadic breast cancer include gender, age, race, presence of preneoplastic lesions in the breast, high lifetime exposure to estrogen and progesterone (nulliparity, late age at first pregnancy, early menarche and late menopause, oral contraceptive use, post-menopausal hormone therapy), not breastfeeding, alcohol, obesity, smoking, consumption of well-done meats, and previous chest radiation.[98-100] Radiation is the most thoroughly documented breast carcinogen.[101, 102]

Data from epidemiologic studies of exposed women support a linear no-threshold dose response relationship down to doses as low as 100mSv.[88, 101, 103] Radiation-induced breast cancer risk is diminished by an early full-term pregnancy. Some authors speculate that because post-menopausal radiation exposure does not increase breast cancer risk, estrogen likely promotes tumorigenesis in breast tissue that has already been initiated by radiation.[104] Whether or not this is the case, it highlights the relationship between radiation exposure and estrogen exposure.[92]

Estrogen stimulation may be intimately linked with the development of radiation-induced breast cancer.[105] In addition to its stimulatory effects on proliferation, estrogen can cause a number of DNA lesions including DSB, and DNA repair variants such as ATM can act as modifiers in endometrial and breast cancers.[57, 106, 107] This suggests that

the female breast might experience a higher load of DNA double-strand breaks than other tissues, and that genetic susceptibility to breast cancer may occur as a result of the combined effects of radiation and estrogen.

Breast tissue is unique in that excess risk for cancer appears to be directly proportional to the cumulative radiation dose (sum of risks from individual small component doses).[108, 109] It follows that the breast will be more susceptible to the effects of lifelong exposure to very low or background levels of radiation. Radiation has many biological effects, including cell-cycle arrest or programmed cell death [110]; chromosomal breakage and instability, which can lead to gene amplification, deletion, and/or rearrangement;[111] alterations in gene expression;[112] and changes in microenvironment that may be conducive to initiation or promotion.[113]

1.6 Immediate and Delayed Genomic Instability in Breast Carcinogenesis

The DNA DSB is the most harmful lesion caused by radiation and possibly the most difficult to repair. It is becoming increasingly clear that an inability to respond properly to DSBs leads to genomic instability and promotes carcinogenesis. Misrepair of a DSB can result in a wide array of mutations, ranging from base substitutions or small deletions/insertions to amplifications, translocations, whole chromosome losses, and chromosomal aberrations.[114] These can result in proto-oncogene activation or tumor suppressor inactivation. DSBs occurring in highly repetitive regions of the genome have a tendency to trigger chromosomal fragility and subsequent aberrations.[115] DSBs can pair with inverted repeats to form large dicentric inverted dimers that cause gross chromosomal rearrangements and result in bursts of genomic instability.[116] Faulty repair

of DSBs involving the repetitive sequences of telomeres also results in chromosomal instability via break-fusion-bridge cycles and interstitial telomeric sequences.[117, 118] Furthermore, DNA DSBs can create selection pressure for the generation of mutations that abrogate the cell cycle checkpoint and apoptosis.[119] The direct role of DSBs in genomic instability and cancer can be inferred from the fact that cancer predisposition syndromes, which are characterized by genomic instability, are caused by mutations in DSB response pathways (ex. familial breast and ovarian cancer (BRCA1 & BRCA2), Cowden Syndrome (PTEN), Li Fraumeni syndrome (TP53), Ataxia-Telangectasia (ATM+/-), and Peutz-Jeghers syndrome (STK11)).[120, 121] These inherited cancer-prone conditions are characterized by an elevated sensitivity to the induction of chromosome damage in cells exposed to IR, indicating a defect in the ability to repair DNA damage.BRCA1, BRCA2, ATM, TP53 and STK11, as well as the newly identified breast cancer susceptibility genes CHK2, XPG and XRCC3, are all tumor suppressors that play a critical function in the signaling and/or repair of DNA DSBs.[122-127] Though these mutations are rare and highly penetrant, they support the hypothesis that less penetrant polymorphisms in DSB response genes are important modifiers of cancer risk in the general population. Consequently, the identification of DNA DSB repair polymorphisms linked with cancer risk represents an area of active research.

Chromosomal instability, common in familial and sporadic breast cancer patients, results from inefficient DNA repair.[128-130] Chromosomal radiosensitivity has been observed in significant proportions of patients with sporadic breast cancer (40% of patients with no family history versus 5%–10% of controls).[130-134] The level and pattern of chromosomal instability in sporadic breast cancer patients has recently been shown to

correlate with histologic tumor grade.[135] In addition, the more aggressive early neoplastic breast lesions (ductal carcinoma *in situ*) contain higher levels of genomic instability than lesions that are less likely to become malignant (atypical ductal hyperplasia).[136] These observations suggest that chromosomal instability is an early determinant for tumor progression that already exists in the early neoplastic *in situ* stage. Thus, genetic susceptibility to breast cancer appears to occur via a mechanism of radiation sensitivity and mechanisms responsible for maintaining chromosome stability and are likely to be important determinants of breast cancer development.[28] The relative risk for breast cancer development in Canadian fluoroscopy patients and Japanese atomic bomb survivors, beginning 5±10 yr after exposure, remained constant for at least several decades.[137] Radiation may thus continue to exert detrimental effects on breast tissues long after the initial exposure, suggestive of persistent genomic instability.

The role of radiation-induced chromosomal instability in carcinogenesis may be related to the high frequency of broad-based chromosomal instability observed in the progeny of irradiated breast stem cells. Delayed instability would put all genes at risk for mutation, leading to mutations in critical genes such as tumor suppressors or oncogenes. The mutator phenotype, as induced by radiation, may be responsible for the delayed carcinogenic effects observed in women with continued breast cancer risk decades after exposure to radiation. DNA DSB repair variants are compelling mutator gene candidates.

1.7 Breast Cancer and DNA Repair Polymorphisms

Many mutations involving HR DSB repair genes such as BRCA1/2 result in high-penetrance phenotypes that are associated with familial breast cancer.[138] In contrast, scant genetic evidence has been found linking severe defects in NHEJ genes with breast cancer. This is likely due to the requirement for NHEJ genes to maintain genomic stability and to avoid indefinite cell cycle arrest and eventual cell death, and/or premature non-breast cancer-related death (ie.lymphoma). Only subtle defects in low-penetrance alleles such as hypomorphic variants or mild polymorphisms would likely result in repair deficient phenotypes that are viable, thereby permitting sufficient lifespan for the development of breast cancer. Such hypomorphs have rarely been described in human populations. However, there is evidence that low-penetrance variants in NHEJ are responsible for the instability observed in breast cancer patients.[139] Dose-response relationships may be more apparent in subsets of women who harbor certain DNA repair variants. The radiation epidemiology data provide additional support for this hypothesis. Specifically, among A-bomb survivors exposed when <20 years of age, excess relative risk for early onset breast cancer (<35 years attained age) was 5.6x that for late onset breast cancer (13.5/Sv vs.2.41/Sv).[92] It has been speculated that a genetically susceptible subgroup with excess relative risk existed among these women.[140] Increased chromosomal radiosensitivity has been documented in early onset breast cancer patients without family history of breast cancer, and studies of women exposed for medical reasons have supported the hypothesis of increased carcinogenic radiosensitivity among certain subgroups.[141, 142]

1.8 Identifying Breast Cancer Susceptibility Genes – Current Approaches

The identification of DNA repair genes linked to breast cancer risk represents an area of active research. Current strategies for identifying DNA repair gene polymorphisms that enhance susceptibility to sporadic breast cancer are focusing on large–scale multigenic screens and pooled and meta-analyses to examine the combined effect of numerous low-penetrance polymorphisms on risk. This approach however is quite challenging because genetic mutations or polymorphisms are both rare and heterogeneous, and the human population is very genetically diverse. Because these studies require a large susceptible population, long-term and complete follow-up, carefully reconstructed estimates of lifetime dose and substantial appropriate control groups to ensure statistical integrity, they rarely provide sufficient statistical power or resolution to implicate the key interactions between environmental exposures and low-penetrance genes. Common polymorphisms in DNA repair genes that have been widely studied can only explain a small portion of risk and the genes truly responsible for radiation-induced breast cancer sensitivity remain elusive.[106, 143-145] Association studies may yield null results because they do not take into account relevant endogenous and exogenous exposures and interactions with other polymorphic genes.[66, 144, 146] However, a lack of association for particular genetic variants does not rule out their effects in modifying relationships between radiation exposures and breast cancer risk. Given the dynamic, multifactorial and multistep nature of the carcinogenic process, main effects from single variants would not be expected in the general population.

Nevertheless, genome-wide association studies and pooled and meta-analyses are beginning to yield reports of individual genotypes and genotype combinations in DNA

repair genes that modulate risk for sporadic breast cancer.[28,147-150] Risk for sporadic breast cancer has been associated with functional polymorphisms in XRCC1, XRCC2, XRCC3, and ERCC4/XPF.[63,69,150-155] A significant association has also been observed between increased breast cancer risk and a cooperative effect of single nucleotide polymorphisms in Ku70 and XRCC4, two NHEJ genes.[71]

It is likely that the incorporation of inter-individual genetic and environmental heterogeneity information into the multistage carcinogenesis model will enhance molecular epidemiologic approaches seeking to identify susceptible individuals. A recent study that benefited from detailed exposure data collected from a 2000-woman United States Radiologic Technologists (USRT) cohort identified significant modification of radiation-related breast cancer risk by five SNPs in variant DNA repair genes out of 55 SNPs evaluated.[65] *WRN* V114I significantly modified the association between cumulative breast dose and cancer risk and *BRCA1* D652N significantly altered the exposure-response relationship. Interestingly, the three other SNPs were in the same gene, *Prkdc*. *Prkdc* had rarely been evaluated in case-control studies because only a cDNA sequence of the gene was available, and no SNPs had been identified.[69] The few studies that did evaluate *Prkdc* yielded null results, likely due to inadequate dosimetry data.[65,71] However, the most recent findings implicating *Prkdc* are supported by evidence that DNA-PK (*Prkdc* protein product + Ku heterodimer) kinase activity is significantly lower in peripheral blood lymphocytes from breast cancer patients compared to controls.[156] Peripheral blood lymphocyte DNA-PK kinase activity also correlated inversely with tumor aggressiveness and metastasis.[157] An elegant hypothesis is that in this situation, the *Prkdc* variant would result in a hypomorphic protein product with diminished kinase

activity and, thus reduced DSB repair capacity. This would result in genome-wide chromosomal instability, particularly in circumstances of heightened background DSB induction as caused by radiation and estrogen exposure to the breast. The mutator phenotype would be more pronounced in the breast, causing accumulation of mutations, eventually permitting altered cells to escape control mechanisms.

The recent findings of a modifier effect on breast cancer risk by a variant *Prkdc* gene in the human population, along with diminished DNA-PK DSB repair capacity and kinase activity, is consistent with studies presented herein, showing that a spontaneously occurring *Prkdc* variant found in BALB/c mice is associated with susceptibility to radiation-induced mammary tumors.

1.9 Radiation Carcinogenesis in the BALB/c Mouse Model

Animal models provide alternative approaches to understanding radiation-genetic interactions. To date, most animal models for radiation-induced breast cancer have been developed using rodents. Mice can be bred to harbor several genotypic combinations of the genes of interest. As such, unique, renewable genetic constitutions can be created to incorporate both altered genes and background state. Early studies on radiation carcinogenesis in inbred mice showed a pattern of susceptibility that was strain-dependent.[158] Importantly, spontaneous and radiation-induced breast cancer risk in BALB/c mice was higher than in C57BL/6 and other common inbred strains examined. The Ullrich laboratory has used this inbred mouse model for a number of years to study radiation-induced mammary cancer. This mouse model capitalizes on naturally occurring differences in susceptibility between two inbred mouse strains, BALB/c (susceptible) and

C57BL/6 (resistant), to dissect the early cellular events driving the carcinogenic process. Several features of this model make it particularly well suited for human mammary carcinogenesis. First, unlike the majority of other rodent models that use viral or chemical carcinogens, this model employs radiation. Second, in preliminary studies comparing BALB/c, C57BL/6, and backcross progeny of their F1 hybrids, data suggested that BALB/c susceptibility is: a) caused by low-penetrance alleles or genetic polymorphisms, b) likely to be recessive, and c) a polygenic trait. These results mirror sporadic human breast cancer data that suggest a polygenic condition in which susceptibility to breast cancer is conferred by a large number of poorly penetrant and possibly recessive alleles.[159]

Interest in this model was initially based upon the observation that susceptibility to radiation-induced mammary carcinogenesis in BALB/c mice correlated with susceptibility to radiation-induced cytogenetic instability.[160-162] C57BL/6 mice, by contrast, were highly resistant to both radiation-induced instability and mammary carcinogenesis (Fig.1.2).[160,163] Radiation-induced chromosomal instability is often observed in DNA repair mutants such as SCID (severe combined immunodeficiency) mice, in which a truncating mutation in *Prkdc*^{SCID} results in deficient V(D)J recombination, as well as in poor post-irradiation DNA DSB repair. Evaluation of DSB repair in BALB/c mice revealed an intermediate phenotype between C57BL/6 and SCID, suggesting that a similar process may be involved.[164] Further studies identified a unique variant haplotype in *Prkdc*^{BALB}.[165] This haplotype consisted of two single nucleotide polymorphisms in the coding regions of the gene, which produced a full, variant protein product (DNA-PKcs). Backcross studies have demonstrated that *Prkdc*^{BALB} homozygous

progeny are sensitive to radiation-induced instability while heterozygotes are resistant. The backcross studies also indicated that although *Prkdc*^{BALB} homozygotes consistently develop radiation-induced preneoplastic mammary ductal dysplasias, lesion persistence and progression toward a neoplastic phenotype vary.

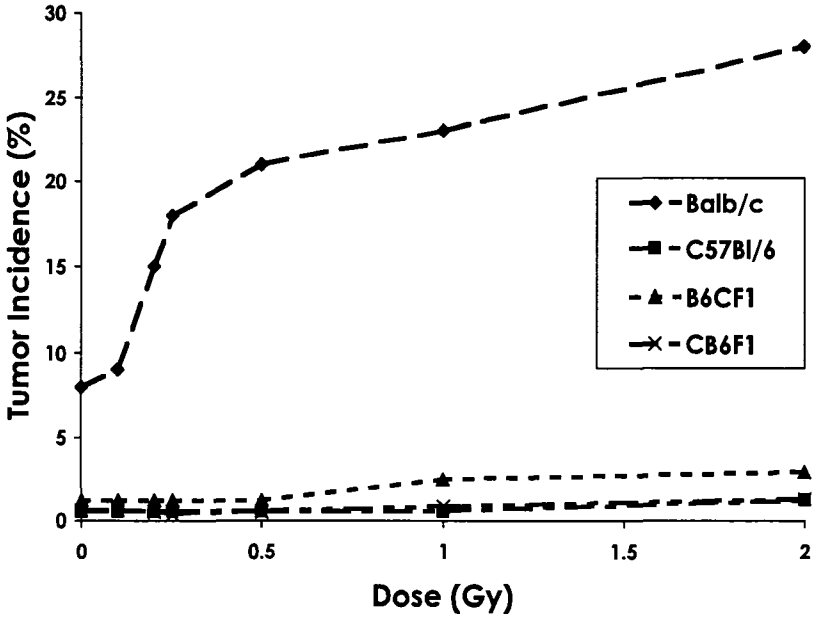

Figure 1.2: Frequency of Mammary Cancer Increases as a Function of Dose of γ-radiation in BALB/c but not C57BL/6 and BALB/c x C57BL/6 F1 Hybrids.[1]

Taken together, these results strongly suggested a role for *Prkdc* in radiation-induced cytogenetic instability and in preneoplastic lesions that subsequently regress. Modifier genes in the BALB/c background likely dictate whether or not preneoplastic lesions persist and progress to outright neoplasia. Additional studies were required to more directly examine the association between this locus and susceptibility to radiation-induced mammary tumorigenesis, as well as to elucidate potential molecular events linking cytogenetic instability with preneoplasias and neoplasias.

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2.0 BACKGROUND

The identification of specific genetic variants associated with radiation-related breast cancer risk represents an area of great interest to radioprotection policy-makers, health professionals and women in general. Radiosensitive subgroups of women would greatly benefit from modified diagnostic and therapeutic radiation protocols. With the advent of rapid genome sequencing techniques and chip microarray technology, large numbers of small changes in the genome can be rapidly detected, potentially correlated with risk, and become useful predictors of disease. The risk of developing breast cancer without a family history is closely linked with hormonal exposures and with a woman's ability to mitigate hormonal and radiation-induced DNA damage. Sporadic breast cancer could largely be explained by the synergistic interaction of clastogens such as radiation and estrogen, with low penetrance variants in DNA repair genes frequently found in the general population. *Prkdc* is one such candidate gene.

2.1 DNA-PKcs: Everybody's Protein

The protein product of *Prkdc*, DNA-PKcs (DNA-dependent Protein Kinase, catalytic subunit), is the catalytic subunit of the DNA-PK holoenzyme, which is composed of DNA-PKcs and a regulatory heterodimer (Ku70/Ku80)(reviewed in [2]). DNA-PKcs plays essential roles in V(D)J recombination, DSB repair, modulation of chromatin structure, telomere end-capping, transcriptional regulation, cell cycle control, apoptosis, innate immunity, and in cellular responses to genotoxic signals.[3-9] DNA-PKcs is a huge and dynamic macromolecule that interacts with DNA, RNA, and proteins, and assumes a wide variety of conformations. It is a member of the phosphatidyl-inositol 3-kinase-like

kinases (PIKK) family, a subcategory of PI-3 kinase (PI-3-K) superfamily that has protein kinase, rather than lipid kinase activity. Other members of the PIKK family include ataxia telangectasia mutated (ATM), ataxia telangectasia-related protein (ATR), mTOR, FKB12-rapamycin bindings proteins (FRAP), transformation/transcription domain associated protein (TRRAP) and hSMG-1 products, genes that are known to be involved in DNA repair and the control of genomic stability.[10] Of these, all but TRRAP have kinase activity. PIKK proteins all have a FAT (FRAP, ATM, and TRRAP) domain, followed by a kinase homology domain and a FATC domain in the C-terminal part of the sequence.[11]

2.1.1 Structure

DNA-PKcs is a serine/threonine protein kinase whose activity is most strongly activated upon association with DNA.[12] The gene for DNA-PKcs (*Prkdc*) is located on human chromosome 8 and mouse chromosome 16. The 13,506 base pair gene (87 exons) extends over about 200kb and contains nearly 100 introns.[13] It yields a 4,127 amino acid protein product, one of the largest known proteins made by human cells.[14] Similarly, in mice, the 12,674 bp transcript (86 exons) yields a 4,128-residue protein product. Due to the large size of DNA-PKcs (460 kDa), protein structure has been difficult to resolve. The amino acid sequence of DNA-PKcs is suggestive of two functional domains, one leucine zipper motif at amino acids 1500 to 1536 near the N-terminus of the protein, and the PI-3 kinase homology domain (PIKK domain) positioned at amino acids 3779 to 4098 near the C-terminus (figure 2.9).[15] Since not all members of the PI-3K superfamily have kinase activity, it is unclear whether the PI-3 kinase domain is responsible for DNA-

PKcs catalytic activity. However, the kinase domain is flanked on both sides by FAT and FATC domains occurring in combination, suggesting that they fold into a conformation that activates the catalytic site.[16]

Three-dimensional reconstructions using cryo-EM imaging, electron crystallography, and single particle-EM studies have been highly informative.[17-19] These have shown that DNA-PKcs is divided into three large regions: a head and a palm, connected by an arm. The structure contains an open channel large enough to accommodate double-stranded DNA. The head encloses a cavity that has two openings with a diameter suitable for internalization of single-stranded DNA. DNA induces conformational changes in the protein that can be observed as the arm acting as a hinge to enclose strands of DNA between palm and head.[20] Most significant among the conformational changes is the resultant interaction between the palm and head, which could activate kinase activity. [21] Recent sequence and structural analyses using repeat detection have revealed one HEAT (Huntingdon Elongation Factor 3, a subunit of protein phosphatase 2A, and TOR1) and one PFT (Protein Farnesyl Transferase) helical repeat motif in the N-terminus of DNA-PKcs.[11] Structural analyses correlating the degree of curvature in the repeat domains with the electron density map of DNA-PKcs indicate that the catalytic domain is located in the palm of the protein, that the HEAT repeats may correspond to the curved portion of the head, and that the PFT helical repeat region is located in the arm.[19] Cryo-EM reconstruction of DNA-PKcs at 15 A° resolution, sufficient to model several regions of this kinase, have implicated it in NHEJ.[19] DNA-dependent activation of the kinase at the site of the lesion is proposed to involve a structural rearrangement between the N-terminal HEAT-repeat-containing domain and the C-terminal kinase domain after DNA

binding. This reorganization of the molecule acts in combination with the repositioning of the FAT and FATC domains. They are directly linked to the catalytic domain and are proposed to regulate the conformation and activation of the kinase.[22] Domains for interaction with Ku, KIP and c-abl have been reported at the carboxyl terminus of DNA-PKcs, and domains for Lyn interaction were reported in the N-terminus region near the leucine zipper.[23, 24]

2.1.2 Regulation of DNA-PKcs Expression

The expression of DNA-PKcs is likely to be constitutive rather than transcriptionally regulated since the time required to transcribe the complete gene (~2 h) and the protein half-life (>24 h) are comparatively long.[25] *Prkdc* promoters (mouse and human) are found within a CpG island upstream and downstream of the transcriptional start site, 700bp away from the 5' end of the *MCM4* promoter (independent, divergently transcribed genes).[25, 26] MCM4 is a nuclear protein that regulates initiation of DNA synthesis during S phase to ensure that DNA is replicated only once per cell cycle. This head-to-head arrangement of *Prkdc/MCM4* is similar to that of *ATM/E14* and *BRCA1/NBR2*.Saito et al. suggest that this implies co-evolution of ATM family members and cell cycle regulating genes.

The GC content of the region (44-46%) places both DNA-PKcs and MCM4 in the moderately GC-rich H1 isochore (20% of human genes). Genes located in GC-rich isochores have shorter 5' UTRs and stronger avoidance of upstream start codons than GC-poor isochores, suggesting that both genes require high translation efficiency rather

than fine modulation of expression.[27] The promoter regions of *Prkdc* contain no TATA or CCAAT box sequences, also consistent with their role as housekeeping genes.

Although DNA-PKcs mRNA is constitutively expressed, significant regulation of protein abundance and activity can occur post-transcriptionally. Ninety-five percent of the genomic sequence (~16500 bp) of *Prkdc* is composed of small interspersed non-coding elements consisting primarily of *Alu* repeats. This low coding density (5.2%, 865 bp of cDNA) is observed in very few genes (BRCA1, APOC1, APOC4, BLYM).The high number of introns leaves room for production of several forms of polypeptide as a result of aberrant or alternative splicing of hnRNA. In fact, one alternative form of the protein that lacks a 93bp insert after nucleotide 11,445 in the region encoding the N-terminal portion of the PI kinase-homology domain, has been described in some T-lymphocyte and HeLa cell clones.[28, 29] This transcript accounts for a minor proportion of DNA-PKcs in most cell types. The radiosensitive cell line M059J, derived from a human malignant glioma, lacks DNA-PKcs due to reduced mRNA stability and lack of the alternatively spliced DNA-PKcs hnRNA transcript.[30] Regulation of mRNA stability and alternative splicing mechanisms could account for regulation of DNA-PKcs activity and for changes in DNA-PKcs mRNA levels as observed in response to NO.[31]

Regulation of DNA-PKcs levels also occurs via protein degradation. Proteasome-dependent downregulation of DNA-PKcs by degradation has been described in association with apoptosis. After cellular exposure to apoptosis causing agents, or after withdrawal of trophic factors, BCR-ABL triggers preferential degradation of DNA-PKcs (and not Ku) by an ICE-like protease (cysteine protease (CPP32)).[32-34]

2.1.3 Activation of the DNA-Dependent Protein Kinase

The leucine-rich region of DNA-PKcs imparts intrinsic affinity for nucleic acids that increases with decreasing ionic strength.[35,36] At physiologic salt concentrations, regulatory subunits (Ku70/80, Chk1) are required to recruit DNA-PKcs to DNA.[37] Proteins that may act as regulators of DNA-PKcs activity include Ku70/86, Chk1, c-Abl, EGFR and TRBP (thyroid hormone receptor-binding protein).[37-40] Intracellular localization can also regulate DNA-PKcs activity. DNA-PKcs kinase activity has been shown to be lower during M phase of the cell cycle due to predominant localization within the cytoplasm.[41] DNA-PKcs has an affinity for multiple configurations of DNA that interrupt its standard double helical conformation, including blunt ends, hairpin ends, structured single-stranded DNA, long single-stranded gaps, sequences for nuclear matrix attachment, DNA kinks, telomeres, and 4-way junctions.[42-47] DNA-PKcs is also activated by poly(rG) RNA in the presence of Ku86.[48] It can even be activated by regulatory proteins in the absence of DNA. Upon binding to nucleic acids and regulatory elements, DNA-PKcs undergoes substantial conformational changes that determine its function and activity.[2]

2.1.4 Phosphorylation Substrates and Other Interactions

DNA-PKcs phosphorylates a plethora of substrates *in vitro*, including Ku70/Ku80, Lig4, XRCC4, Artemis, H2AX, Histone H1, C1D, CHK2, Cds1, Sp1, p53, RNA Pol&II, mdm2, MHG, WRN, XRCC1, PARP-1, HSP90α (RNA Pol II), IGFBP3, vitamin D receptor, HSF1, c-Abl, c-fos, c-jun, c-myc, HDAC3, RPA2, Oct-1, Zic2, PDX-1, RNA helicase A, SV40 tag, AIRE and itself.[12, 42, 49-66] Although phosphorylation has not been

demonstrated, DNA-PKcs has been shown to coprecipitate with a variety of proteins such as E4-34K, r-hRPA, SEK1/MKK4, SAPK/JNK.[37, 67-69]

2.1.5 Functions

Accumulating evidence indicates that DNA-PKcs is a multi-functional protein that participates in and perhaps even orchestrates cellular responses to a variety of genotoxic stresses. Although it is best recognized for its intranuclear role in bringing together and protecting damaged dsDNA ends, DNA-PKcs also collaborates with a host of proteins to protect cells from both nuclear and cytoplasmic insults.

Evidence for the role of DNA-PKcs in transcriptional regulation is abundant. Signals inducing gene expression in response to genotoxic stresses such as IR originate from extracellular and cytoplasmic molecules and damaged DNA. The immediate-early response involves rapid activation of SAPK/JNK transcription factors via DNA-damage-independent mechanisms.[70] The delayed response is DNA-damage-dependent, and relies on DNA-PKcs to activate SAPK/JNK.[69] DNA-PKcs also modulates gene expression in other contexts. It phosphorylates the AIRE (AutoImmune Regulator, key mediator of the central tolerance for tissue-specific antigens) protein at Thr68 and Ser156 to influence its transactivation ability (transcriptional control of antigens in thymic medullary epithelial cells).[63] Phosphorylation of RNA helicase A at the MDR promoter activates transcription of multidrug resistance 1 (MDR1) and P-glycoprotein, a drug transporter that impedes efficacy of chemotherapy.[60] Phosphorylation of thyroid hormone receptor-associated HDAC3 enhances histone deacetylase activity, resulting in establishment of repressive chromatin at the target promoter.[59] Phosphorylation of Zic2 (zinc finger protein family,

essential for neurogenesis) permits complex formation with RNA helicase A and RNA polymerase II.[61] Phosphorylation of vitamin D receptor induces a conformational change that causes it to bind 1,25-dihydroxyvitamin D3 (1,25 vitaminD3) and inhibit expression of parathyroid hormone-related polypeptide (PTHrP).[64] Phosphorylation of PDX-1 by DNA-PKcs may drive PDX-1 degradation by the proteasome and subsequent reduction in transcriptional activation of insulin promoter and PDX-1 mediated gene expression.[66] Phosphorylation of Oct-1 by DNA-PKcs is required for transcriptional activity and its IR-induced survival signal.[71]

DNA-PKcs can influence cell cycle progression directly by phosphorylating Chk2 to activate it.[55, 65] Activated Chk2 mediates IR-induced inhibition of DNA synthesis and contributes to G2/M arrest.[72] By phosphorylating p53 and E2F-1, Chk2 also helps maintain sustained G1, G2/M arrest and apoptosis. It has long been know that DNA-PKcs regulates signals for apoptosis, and the mechanism most often invoked is phosphorylation of p53 (Ser 20 and Thr 18) to promote destabilization of its interaction with hdm2/mdm2 and accumulation of active p53.[42] Phosphorylation of p53 (Ser20) also stimulates transcriptional activation. Inactivation of DNA-PKcs by BCR-ABL results in marked DNA repair deficiency, increased sensitivity to IR, and resistance to apoptosis.[73] This mechanism could explain the accumulation of genetic mutations in cancers.[23]

Though DNA-PKcs is best recognized for its intranuclear response to DNA damage, it can also be found in the cytoplasm, where it mediates cell signaling. DNA-PKcs has been reported in association with Par3 and the tight junctions of epithelial cells.[74] DNA-PKcs can promote apoptosis by phosphorylating IGFBP-3 to facilitate its nuclear import (nucleo-mitochondrial shuttling of RXRα/NUr77).[53, 62] DNA-PKcs can also inhibit

receptor-initiated apoptosis, promoting cell survival. This contrasting effect may be tissue specific or depend on the nature of the stimulus.[75] Protein Kinase C epsilon (PKCε) is an antiapoptotic protein that is activated by extracellular TNFα, insulin and IR. Activation of DNA-PKcs by PKCε causes it to colocalize with Akt on the cell membrane and phosphorylate Akt at Ser473.[76, 77] The phosphorylation of Akt results in a ~10-fold increase in Akt activity. Akt (protein kinase B) is a cellular mediator that is constitutively activated in breast cancer, and is implicated in diabetes. Phosphorylation of Akt by DNA-PKcs is responsible for the survival signal induced by immunostimulatory DNA (CpG-DNA) to rescue B lymphocytes and dendritic cells from spontaneous apoptosis.[77] In response to bacterial DNA (unmethylated CpG-DNA) and damaging agents, DNA-PKcs activates IKKβ, leading to activation of NF-κB and induction of the innate immunity cytokines IL-6 and IL-12.[78] Thus, cytoplasmic DNA-PKcs mediates receptor-initiated apoptosis in response to immunostimulatory DNA, leading to stimulation of adaptive immunity. Furthermore, it recognizes bacterial DNA to stimulate the innate immune response. DNA-PKcs is also involved in mounting anti-viral responses.[79] Viruses such as HIV-1 and HSV-1 attempt to combat these immune responses by reducing DNA-PKcs expression levels.[79-81] In other cases, viruses appropriate DNA-PKcs to process their own DNA.[82-84]

Some suggest that DNA-PKcs evolved as part of an ancient innate immune system to detect foreign microbial DNA. Its evolutionary appearance coincides with the emergence of an adaptive immune system in vertebrates and with the requirement for the ability to resolve V(D)J hairpin intermediates. DNA-PKcs equivalents are rare in the genome of lower eukaryotes. Orthologs have been identified in *Anopheles gambiae* and *Apis*

mellifera, [85] and a homolog was also identified in the *Dictyostelium discoidum.*[86, 87] Thus the need for DNA-PKcs existed before the divergence of arthropods and vertebrates. However, DNA-PKcs evolved independently from Ku, suggesting that the two factors may have distinct functions apart from their common involvement in NHEJ and V(D)J recombination.

DNA-PKcs has recently been shown to mediate cellular responses to radiation initiated at the plasma membrane. In contrast to the nucleus, the plasma membrane is not traditionally considered to be a radiation target. However, the high efficiency of radiosensitization by inhibition of growth factor-dependent receptor tyrosine kinases (RTKs) suggests that these receptors are an important part of the radioprotective cellular defense system. Radiation activates multiple signaling pathways in cells. Which pathway is activated may depend on receptor expression and autocrine/paracrine factors (TGFα). Radiation can directly activate EGFR/ERBB, leading to activation of the Ras-MAPK/JNK signaling.[88] Radiation also indirectly activates EGFR via ROS and TGFα. Radiation causes cleavage of TGFα from the plasma membrane, and its release into the extracellular environment where it activates EGFR.[88] Reactive oxygen species generated by ionizing radiation inactivate redox-sensitive tyrosine phosphatases present at the plasma membrane, leading to increases in phosphorylated EGFR.[89,90] The phosphorylation status of EGFR is also modulated by PKCε/Akt. EGFR at the plasma membrane is located in lipid rafts and caveolae.[91] Lipid rafts, or detergent-insoluble glycolipid-enriched complexes (DIGs), are specialized cholesterol-rich membrane microdomains that incorporate proteins involved in endocytosis, cholestrol trafficking, and signal transduction.[92] DNA-PKcs is localized within the lipid rafts, where it interacts

with EGFR in response to ionizing radiation.[93-95] After nuclear translocation of the pEGFR/DNA-PKcs complex, EGFR phosphorylates DNA-PKcs at Thr2609. This leads to activation of DNA repair by DNA-PKcs and to expression of EGFR-responsive genes.[39, 95, 96] EGFR nuclear import and kinase activity are required for DNA-PKcs activity, DNA DSB repair and cell survival.[97-100] Clearly, the DNA-dependent protein kinase that initially made its mark as a DNA end-joining protein is now revealing a much more complex and diverse series of functions.

Because of the potential lethality of DNA DSBs, rejoining of damaged ends occurs in all organisms with DNA. In prokaryotes and lower eukaryotes, recombinational repair (HR) mechanisms predominate. Bacteria have very little non-coding DNA and thus require highly accurate repair mechanisms. However, when replication is inhibited, NHEJ may be required to repair DSBs. This is particularly true for bacteria that sporulate or spend a large portion of their life cycle in a stationary phase. In these organisms, a minimal NEHJ pathway evolved. For example, Ku and ligase homologs have been identified in *M.tuberculosis.*[101] Ku, Ligase 4, MRN, Pol μ, and FEN1 homologs exist in yeast as well. In bacteria and yeast, the key steps of NHEJ, bringing together and rejoining of broken ends can be accomplished using few reactions and molecules. The binding of Ku to broken ends limits nucleolytic degradation and juxtaposes ends via protein-protein interactions between end-binding factors. After minor or major processing, DNA ends can be ligated. The drawback is that NHEJ in prokaryotes and lower eukaryotes has very slow kinetics (hours).[102] The appearance of DNA-PKcs in lower eukaryotes may have served to accelerate the kinetics of NHEJ, allowing it to become the predominant repair pathway. DNA-PKcs accelerates NHEJ by acting as a

scaffold for and phosphorylating other proteins such as Ku70/80, Ligase 4, XRCC4, γ-H2AX, Artemis and C1D.[103] Importantly, the most relevant target for the enzymatic role of DNA-PKcs in NHEJ is DNA-PKcs itself. Binding of two Ku heterodimers to two opposite DNA ends recruits two DNA-PKcs molecules that form a synapse between opposite ends. The presence of DNA-PKcs at DNA ends interferes with efficient ligation.[104-107] Thus DNA-PKcs may protect DNA ends from premature processing until they are properly juxtaposed. DNA-PKcs then undergoes a series of autophosphorylation events and conformational changes that result in reversible kinase inactivation and dissociation from DNA/Ku, and ultimately determine successful completion of NHEJ.[108-110]

DNA-PKcs phosphorylates SQ/TQ motifs.[111] Mass spectrometry and solid phase protein sequencing studies have revealed many highly conserved autophosphorylation sites. There are two major clusters containing *in vitro* autophosphorylation sites. One, termed cluster ABCDE, contains sites S2612, S2624, T2609, T2620, T2638, and T2647.[112, 113] The other, the PQR cluster, contains sites S2023, S2029, S2041, S2053, and S2056.[114] Three of these sites are autophosphorylated *in vivo* and are conserved among human, horse, dog, mouse, chicken, and frog (T2609, T2638, and T2647).[115] Another conserved *in vivo* phosphorylation site is the recently identified T3950, located in a region similar to the activation loop in the PIK kinase domain.[116] Autophosphorylation within the kinase's activation loop (T3950) regulates kinase activity. Mutation at T2609 results in impaired DNA DSB end-joining and radiation sensitivity,[113] whereas mutations at T2638 and T2647 result in radiation sensitivity but do not affect end-joining,[117] suggesting that the two ends of the autophosphorylation domain

may regulate different interactions. Although not all DNA-PKcs autophosphorylation sites have been identified, a picture of autophosphorylation in NHEJ is emerging in which the sequential *trans* autophosphorylation of specific sites induces conformational changes, inactivates kinase activity, alters its affinity for DNA causing dissociation from the DNA/Ku complex, and makes DNA ends accessible to end-processing factors.[114] Perturbation of any of these steps leads to diminished end-joining efficiency and radiation sensitivity. After phosphorylated DNA-PKcs dissociates from DNA ends, it is dephosphorylated by protein phosphatase 5.[118] Phosphorylated DNA-PKcs does not have affinity for DNA and is inactive.[110] Protein phosphatase 5 inhibitors such as microcystin-LR require DNA-PKcs to inhibit DNA repair, suggesting that an excess of phosphorylated DNA-PKcs relative to the unphosphorylated form could cause radiation susceptibility.[119] One could hypothesize that excessive activation of DNA-PKcs by genotoxic and other stressors could potentially saturate dephosphorylation mechanisms, leading to depletion of unphosphorylated (active) DNA-PKcs and diminished overall repair and signaling capacity.[120]

The kinase activity of DNA-PKcs and autophosphorylation are required for DSB repair by NHEJ and radioresistance, but correlating *in vivo* function with specific phosphorylation substrates has been arduous.[121, 122] Early insights into the function of DNA-PKcs *in vivo* have principally come from analysis of the SCID mouse. The severe combined immunodeficiency (*scid*) mutation arose spontaneously in a C.BKa-*Igh*^{b}/IcrSmn (C.B-17) congenic mouse strain.[123] Mice homozygous for the *scid* mutation are severely deficient in functional B and T lymphocytes due to impaired recombination of antigen receptor genes and arrest in the early development of B and T lineage-

committed cells.[124] The mutation is a T to A transversion resulting in a nonsense mutation (Tyr4046) that truncates the protein at the last 83 amino acids of the carboxy terminus, while leaving a large portion of the kinase domain intact.[29, 125] Although the C-terminal region of the protein contains the PI-3 kinase motif, it is not clear that this is the catalytic center of the protein. It is suspected, though not proven, that the functional defect in *scid* mice is due to an unstable protein product that is rapidly degraded, rather than an inherent defect in kinase function.[126, 127]

Knockout mice have recently been generated for DNA-PKcs, Ku70, and Ku80. These mice are immunodeficient and sensitive to the DNA damaging effects of ionizing radiation.[127-129] The majority of DNA-PKcs and Ku-70 null mice also develop thymic lymphoma, as do irradiated *scid* mice, $p53^{-/-}$ *scid* mice, and a low percentage of unirradiated *scid* mice.[130-133] These mice also have impaired DNA DSB repair resulting in sensitivity to ionizing radiation.[134] Although DNA-PK-associated lymphomagenesis may result from aberrant lymphocyte maturation, an attractive model is that in the absence of Ku or DNA-PKcs, DSBs generated during V(D)J recombination are free to undergo inappropriate recombination events with other loci, some of which may have oncogenic capacity. This model is supported by the fact that the malignant lymphocytes of *scid* $p53^{-/-}$ mice harbor recurrent chromosomal translocations with breakpoints involving the IgH locus.[135] This is consistent with the hypothesis that the DNA-PK complex acts as a tumor suppressor that maintains genomic integrity by preventing inappropriate recombination events.

The study of *scid* mice has also revealed that DNA-PK plays an important role in telomere function and maintenance. Chromosomal fusions are often observed in the

context of shortened telomeres. However, *scid* mice, which have elongated telomeres compared to C57BL/6, C3H, and BALB, experience telomeric fusions (with retained telomeric sequence), suggesting that fusigenic potential is partly dependent on the structure of telomeric chromatin (telomeric "cap"), rather than telomere length.[136-138] Telomere caps consist of specialized nucleoprotein "loop" shaped structures at the physical ends of chromosomes that prevent joining between natural chromosome ends. DNA-PK holoenzyme can be found at the telomeres and is required for proper telomere capping.[138-141] DNA-PKcs kinase activity has recently been shown to be required to prevent telomere to telomere fusions as well as telomere to DSB fusions.[142, 143] Interstitial telomeric signals (ITS) are significantly increased in kinase-dead and autophosphorylation mutants compared to wild-type controls, in both unirradiated and irradiated cells.(Bailey & Williams, unpublished data) It is hypothesized that ITS induce genomic instability throughout the genome. Quantitative reductions of DNA-PKcs accomplished by RNA interference also result in telomere dysfunction and mutagenesis.[144] Importantly, even partial (50%-90% of normal) deficiencies in DNA-PKcs levels increase mutation frequency, indicating that heterozygosity or mild functional deficits may impart significant risk for acquiring additional mutations.[145]

Thus, the functions of DNA-PK in DSB repair versus telomere maintenance appear to be opposite. At chromosomal breaks, DNA-PK is required for rejoining, whereas at the natural chromosomal ends, it is required to prevent fusions. Although the precise regulatory pathways involved remain to be elucidated, this phenomenon is indeed intriguing because it represents an important mechanism for induction of chromosomal instability. While DNA-PKcs defects in DSB break repair leave broken chromosomes

open for mis-rejoining events, defects in telomeric end-capping leave chromosome ends open to be fused with other chromosome ends as well as with broken chromosomes. Such DSB repair defects and telomeric dysfunction results in multiple complex chromosome and chromatid aberrations including chromatid fusions, acentric chromosomes, dicentrics, translocations, and regional amplifications and deletions.[146] A defect in DNA-PKcs would induce chromosomal instability by virtue of improper telomere processing in addition to improper DSB repair, the effects of which could be synergistic, leading to an amplification of genomic instability, the driving force for cancer.

2.2 Characterization of susceptibility to radiation in the BALB/c Mouse

The BALB/c radiation carcinogenesis model has been used since 1977, when neutron carcinogenesis dose/dose rate experiments demonstrated that BALB/c mice are susceptible to the development of solid tumors, particularly lung, ovarian, and mammary carcinomas.[147] Early experiments demonstrated that 2 Gy of gamma irradiation nearly tripled mammary tumor incidence, increasing frequencies from 7% (spontaneous) to 20% (irradiated) (fig. 1.2).[148] BALB/c mice irradiated at 3-4 months of age develop mammary cancers relatively late in life (15-18 months of age; average lifespan ~24 months). The mammary glands of these mice appear normal until ~12-15 weeks of age, when discrete lesions classified as ductal dysplasias (abnormal terminal structures characterized by duct overcrowding, abnormal branching, and hyperplastic endbuds) appear. Histologically, lesions are epithelial hyperplasias consisting of papillary projections or infoldings of chords of epithelial cells into ductal lumina, they closely resemble ductal dysplasias

confirmed by serial transplantation experiments to have neoplastic potential, as well as "high-risk" ductal lesions associated with human breast cancer.[149] Ductal dysplasias develop many months after radiation exposure, and already contain multiple genetic changes that arose randomly and are not consistent between lesions. One explanation for delayed expression of dysplasias is that in a mature gland, normal epithelial cells inhibit the proliferation of individual abnormal cells.[150]

A cell transplantation system called the ductal dysplasia assay has been used to identify early radiation-induced cellular alterations in normal appearing mouse mammary glands. In the dysplasia assay, epithelial cells are isolated from the mammary gland of an irradiated donor mouse, dissociated into single-cell suspensions, diluted, and injected into the fat pad of young adolescent (3 week old) mice from which endogenous mammary epithelium has been removed (fig. 2.1). The cleared fat pad provides an ideal environment for proliferation of injected cells. Approximately 16 weeks after injection, the fat pad is completely filled by a normal mammary gland whose epithelial cell component arises from the donor mouse.[1] At ~16 weeks, when the mammary fat pad is full, growth regulatory signals are turned on, the endbuds regress, and active growth ceases.

Using this *in vivo* system, cells with altered growth potential can be detected in outgrowths as soon as 24 hours after irradiation of donor mice (fig. 2.2) [151] Cells with altered growth potential give rise to ductal dysplasias as they fill the cleared fat pad. Once the fat pad is filled and active growth stops, the dysplasias regress and are no longer detectable at 16 weeks. However, if cells are allowed to remain *in situ* in the irradiated animal for 1 to 16 weeks prior to removal and injection into recipients, ~12% of

dysplasias do not regress. These results suggest that cells giving rise to persistent dysplasias have undergone additional genetic alterations that promote their proliferation independent of growth inhibitory signals from surrounding cells.

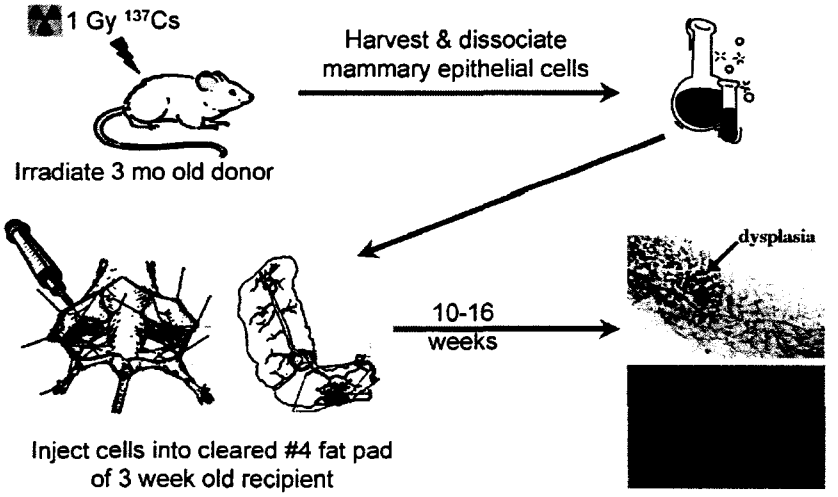

Figure 2.1: Cell dissociation and Ductal Dysplasia Assay

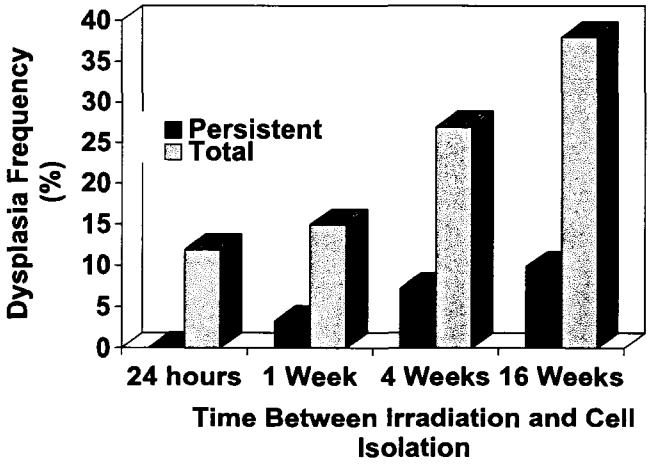

Figure 2.2: Frequency of Ductal Dysplasia in BALB/c mice as a Function of Time *in situ* after 1 Gray of γ-Irradiation, in 10 wk outgrowths (total) and 16 week outgrowths (persistent).[152, 153]

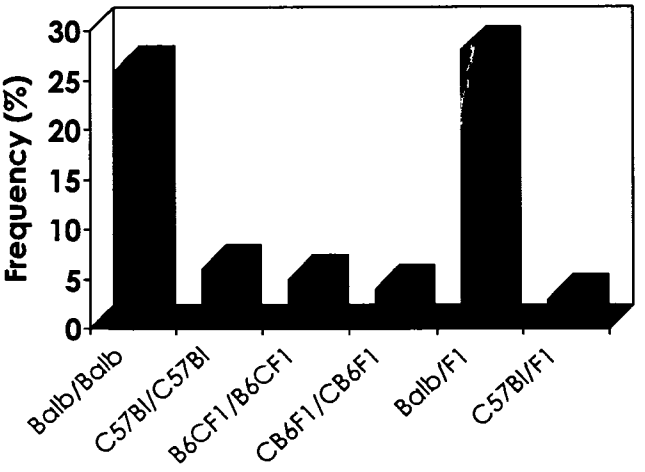

Figure 2.3: Ductal Dysplasia Frequency as a Function of Genetic Background of Donor and Recipient Mice. (Donor/Recipient; Donors irradiated with a total dose of 1G, Recipients nonirradiated) [1]

The dysplasia assay permits the direct quantification of the frequency of radiation-altered cells based on the number of cells injected because each outgrowth is derived from a single cell (clonogen) and a cell's inherent clonogenic potential is independent of genetic background and host environment.[1] Ullrich et ill-used this approach to demonstrate that variations in radiation susceptibility result from inherent differences in sensitivity of the mammary epithelial cells to radiation-induced transformation (fig. 2.3).[151] When cells from irradiated mice were transplanted into the cleared fat pads of multiple mice, 100% of BALB/c donors yielded ductal dysplasias after 10 weeks whereas none of the C57BL/6 donors did. These pioneering experiments comparing BALB/c and C57BL/6 mice demonstrated a clear, quantifiable difference in susceptibility to radiation-induced ductal dysplasia development between two inbred mouse strains, and led Ullrich to postulate that radiation-altered cells giving rise to persistent ductal dysplasias harbor molecular alterations which confer a high risk of developing mammary tumors.

It is well recognized that ionizing radiation damages DNA directly by causing frank breaks in the DNA backbone, or indirectly by reactive oxygen species. These breaks can

affect both strands, resulting in DSB and a fragmented karyotype, termed chromosomal instability. Chromosomal instability is a short-term effect in normal cells, owing to DNA repair processes that usually restore DNA integrity within 4-6 hours. Analyses comparing chromosomal instability as measured by chromatid-type aberrations in irradiated mammary epithelial cells isolated from BALB/c and C57BL/6 mice demonstrated a clear susceptibility in the BALB/c strain but not in C57BL/6. A correlation between latent expression of chromosomal damage *in vitro* and susceptibility to mammary tumors was also demonstrated (fig. 2.4).[154] BALB/c cells showed an increased frequency of total chromosomal aberrations immediately after irradiation. As expected, cells from both strains showed a rapid initial decline of aberrations, compatible with DNA damage repair. However, at 20 population doublings and thereafter, BALB/c cells exhibited a marked increase in chromosomal and specifically, chromatid aberrations. Delayed chromosomal aberrations were not observed in the C57BL/6 cells.

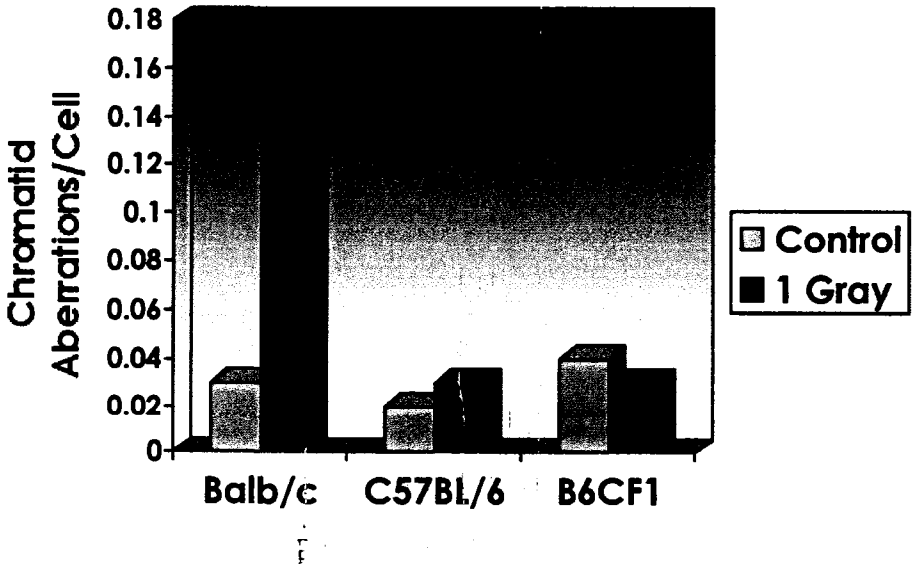

Figure 2.4: Cytogenetic Instability *In Vivo* as a Function of Genetic Background [154]

To determine whether this difference existed *in vivo*, where cells were not rapidly dividing, cells were allowed to remain *in situ* for 24 hours to 16 weeks after *in vivo* irradiation. Frequency of chromosomal aberrations increased with time *in situ*, demonstrating that radiation-induced instability can develop and persist within a mature fully differentiated tissue that is not undergoing rapid cell division (fig. 2.5).[154, 155]

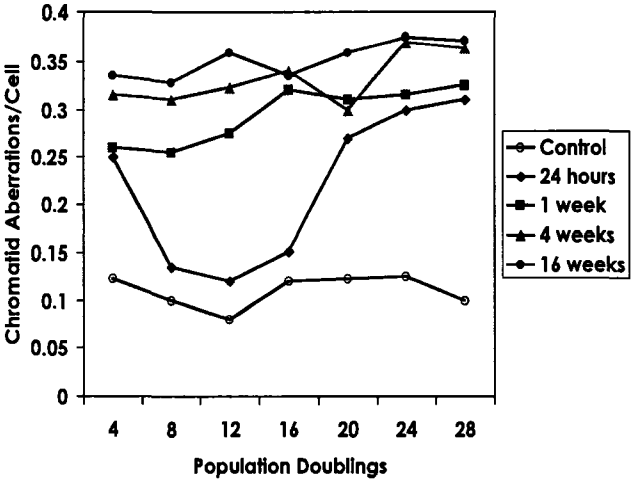

Figure 2.5: Cytogenetic Instability in BALB/c Mammary Epithelial Cells as a Function of Time *in vivo* Following γ-Irradiation. Mice were irradiated with a whole-body dose of 3 Gy. Mammary epithelial cells were removed at 24 hrs, 1 wk, 4 wks, or 16 wks after irradiation. Control: 0 Gy.[154]

Analyses of BALB/c x C57BL/6 F1 progeny and F1 x BALB/c backcross progeny demonstrated that susceptibility to radiation-induced ductal dysplasia and radiation-induced chromosomal instability is a heritable recessive trait. [156] Anticipating the need to identify potential candidate genes using genetic mapping, Ullrich analyzed cells from BALB/c and C57BL/6 mice for differences in their ability to repair DNA damage, cell cycle checkpoint control, and radiation-induced apoptosis. The only differences observed related to DNA repair efficiency. Cells from radiosensitive BALB/c mice exhibited

inefficient rapid phase end joining of gamma ray-induced DSBs whereas those from C57BL/6 mice exhibited repair kinetics similar to most other strains of mice. The defect in BALB/c was intermediate to that of SCID and C57BL/6 mice (fig. 2.6).

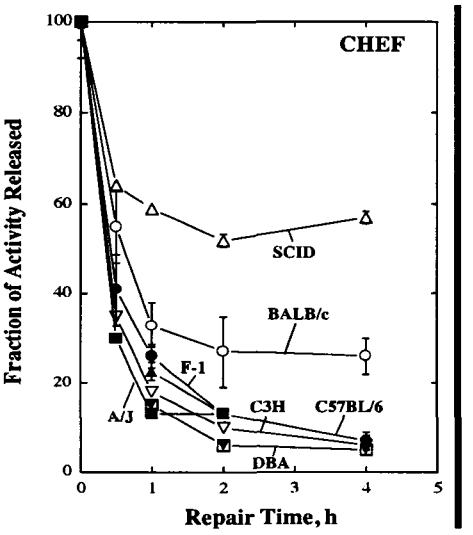

Figure 2.6: DNA DSB rejoining kinetics in γ-irradiated primary kidney cells from BALB/c, C57BL/6, F1, A/J, C3H, DBA, and SCID mice. Exponentially growing cells were irradiated with 50 Gy γ-rays and repaired at 37°C for various time periods. Data were obtained with clamped homogeneous electric field gel electrophoresis (CHEF). The means from 2-4 independent experiments are given; *bars*, SE (standard error).[157]

Since the *scid* mutation in *Prkdc* results in low tissue levels of DNA-PKcs and defective DSB repair, it is reasonable to postulate that low levels of DNA-PKcs in BALB/c cells might underlie the DSB repair defect in BALB/c mice. Examination of DNA-PKcs protein expression by SDS PAGE and western blot in various organs (mammary gland, heart, brain, kidney, liver, and spleen) revealed consistently reduced levels in BALB/c compared with other inbred mice except SCID (fig. 2.7).[157] Ku70 and Ku80 levels were normal. Intriguingly, DNA-PKcs tissue levels were lowest in the mammary gland.

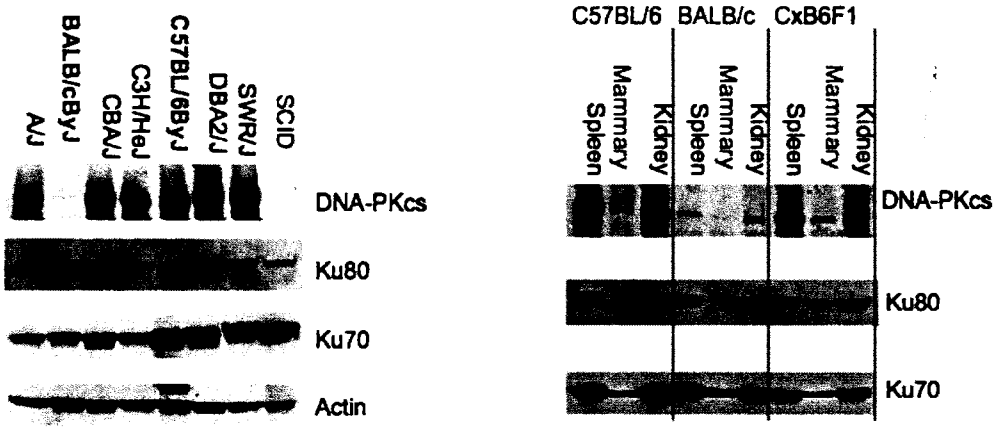

Figure 2.7: DNA-PKcs Expression and *Prkdc* Genotype. *Left*: expression of DNA-PKcs, Ku80, Ku70 in mouse kidney epithelium by western blot analysis according to strain; *Right*: expression in selected tissues.[157]

Reduced DNA-PKcs levels are likely due to diminished protein stability or increased protein degradation, since mRNA levels in BALB/c and C56BL/6 are similar.[7] DNA-PKcs kinase activity was also lower in cells from BALB/c mice than C57BL/6 and other strains, though this may reflect low overall tissue levels rather than inherent functional deficits in the variant BALB/c protein (fig. 2.8).

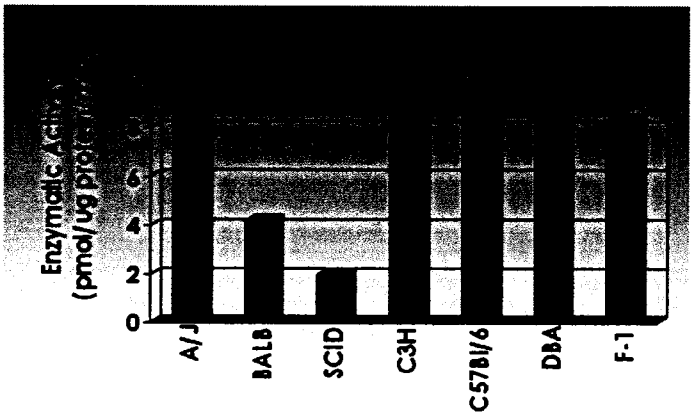

Figure 2.8: DNA-PKcs Kinase activity levels in whole-cell extracts derived from primary cells according to strain. The extracts were first pulled down with DNA-cellulose. The means from three experiments are given.(Ullrich et al., unpublished results)

Sequencing experiments comparing cDNA from BALB/c and C57BL/6 mice revealed two single nucleotide polymorphisms (SNPs) in the gene for DNA-PKcs (*Prkdc*) (fig. 2.9).[158] One SNP is an A to G transition at base 11530 resulting in a methionine to valine conversion at codon 3844 (M3844V), in the PI-3 kinase homology domain upstream of the *scid* mutation. The other SNP is a C to T transition at base 6418 resulting in an arginine to cysteine conversion at codon 2140 (R2140C) downstream of the putative leucine zipper domain and upstream of the autophosphorylation domain. Restriction endonucleases *Hph1* and *BsmB1* differentially cleave the BALB/c and C57BL/6 alleles for M3844V and R2140C, respectively. Using primers flanking each polymorphic site, Ullrich et al. developed a PCR/RFLP genotyping assay.

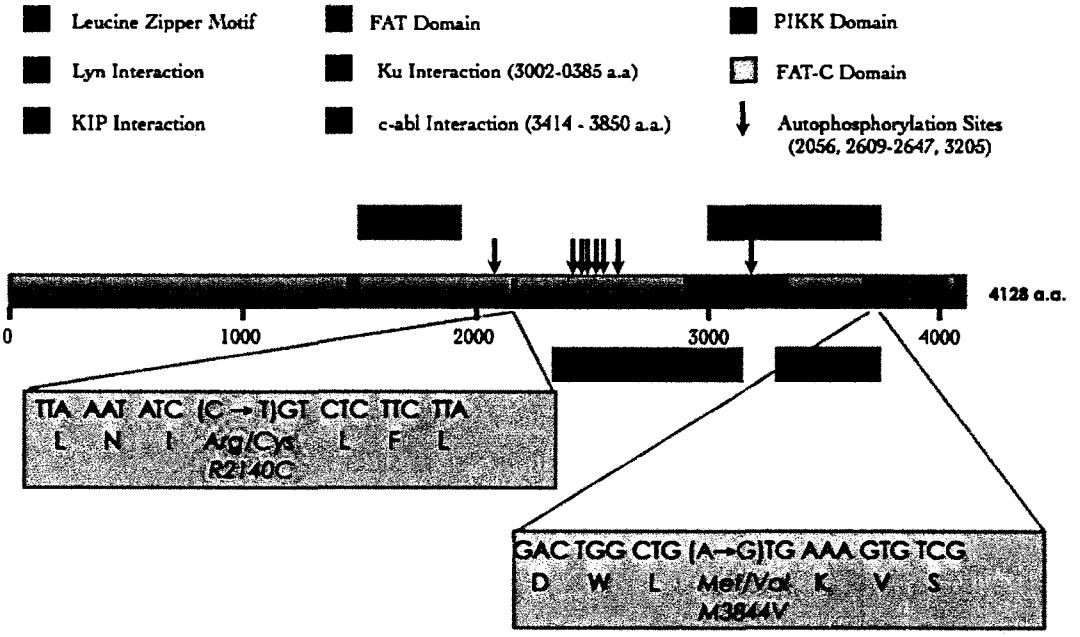

Figure 2.9: Two Single Nucleotide Polymorphisms in BALB/c *Prkdc*.[158] (Adapted from Dip, R. and H. Naegeli (2005).Faseb J 19(7):704-15.)

In a survey of over 70 inbred strains, M3944V and R2140C were found to be unique to BALB/c.(M. Weil, unpublished data) In F2 backcross progeny (BALB/c x C57BL/6 F1 progeny backcrossed to BALB/c), *Prkdc*$^{BALB/BALB}$ consistently associated with reduced DNA-PKcs tissue levels, reduced DNA-PKcs kinase activity, increased radiogenic chromosomal instability, and increased frequency of total ductal dysplasias, providing strong evidence that the variant *Prkdc* allele is responsible for those phenotypes.

Concordance of *Prkdc*$^{BALB/BALB}$ with persistent ductal dysplasias was more variable however (fig. 2.10). Neither *Prkdc*$^{BALB/C57BL/6}$ nor *Prkdc*$^{C57BL/6/C57BL/6}$ were associated with instability or ductal dysplasias, while *Prkdc*$^{BALB/BALB}$ was associated with persistent ductal dysplasias, but the association was variable between individual mice. These results indicate that although *Prkdc*$^{BALB/BALB}$ is required for radiation-induced chromosomal instability and total ductal dysplasias, modifying genes in the BALB/c background that do not consistently co-segregate with *Prkdc*^{BALB} allow the dysplasias to persist in the mature gland. If persistent ductal dysplasias are precursors of mammary neoplasms, BALB/c modifier genes are likely to be required to impart susceptibility to radiation-induced mammary tumors. One potential modifier gene is *Cdkn2a*, which encode the alternate splicing products p16^{INK4a} and p19^{ARF}. BALB/c mice harbor polymorphisms in *Cdkn2a* that are responsible for this strain's susceptibility to pristane-induced plasmacytomas.[159] However, testing of F2 backcross progeny for the allelic variants of *Cdkn2a* revealed no correlation between *Cdkn2a* status and susceptibility to radiation-induced instability and dysplasia (unpublished results).

Studies using FISH (fluorescence *in situ* hybridization) analysis with telomere-specific probes and a specialized cytogenetics technique called CO-FISH (chromosome orientation FISH) suggest that the role played by DNA-PKcs in the capping of telomeric ends is related to the expression of instability in BALB/c mice.[138] Metaphase spreads from F2 backcross mice from the linkage studies revealed greater frequencies of Robertsonian translocations in BALB/c cells than C57BL/6.

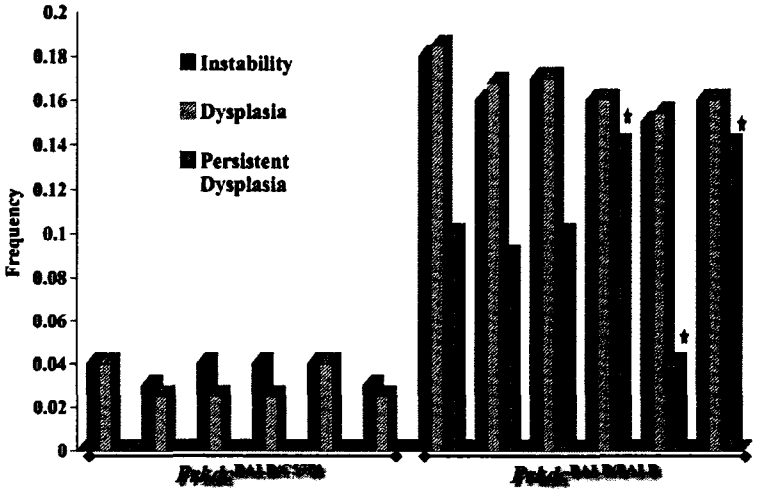

Figure 2.10: Radiation-induced genomic instability, total ductal dysplasia (10 wks), and persistent ductal dysplasia (16 wks) as a function of *Prkdc* Genotype. Mammary cells were isolated from individual CB6F1 x BALB/c backcross mice 4 weeks after irradiation. Each bar on the histogram represents an individual mouse.(Ullrich., unpublished results)

CO-FISH analyses of irradiated primary mammary epithelial cells from BALB/c and C57BL/6 demonstrated dose-dependent increases in telomere-DSB fusions in BALB/c but not in C57BL/6 mice (Bailey, unpublished results). These results suggest a potential mechanism of delayed instability whereby dysfunctional (uncapped) telomeres fuse with radiation-induced DSB resulting in chromosomes containing interstitial blocks of telomeric DNA, a form of chromosomal aberration that maybe transmissible to progeny cells.

Recent preliminary studies examining DNA-PKcs expression in CXB recombinant inbred (RI) strains and nearly congenic strains demonstrate linkage between *Prkdc*^{BALB} and decreased DNA-PKcs expression (Lila Ramaiah & Ullrich et al., unpublished results). CXB strains are RI mouse strains derived by 20 consecutive brother-sister matings of randomly selected CB6F2 progeny. The CB6F2 progeny are themselves derived from C57BL/6 and BALB/c parental strains such that the CXB RI strains are homozygous for either BALB/c or C57BL/6 at every locus. Thirteen of these strains exist, each with a unique mosaic of recombination points across its genome. The CXB RI strains have been genotyped for BALB polymorphisms in *Prkdc* (Ramaiah, unpublished), and DNA-PKcs levels have been measured. Strains that harbored the BALB/c allele of *Prkdc* (figs.2.11a & 2.11b) had low DNA-PKcs abundance (fig. 2.11c), whereas all those that harbored the C57BL/6 allele were normal. This is strong evidence that *Prkdc*^{BALB} is genetically linked with reduced DNA-PKcs levels.

In this model of radiation carcinogenesis, genomic instability is an early event that both precedes and causes mutation of critical genes. Cells with a "mutator phenotype" are more susceptible to the damaging effects of ionizing radiation because chromosomal aberrations continue to occur, even in the unirradiated progeny of irradiated cells (delayed instability). Although the exact mechanisms of genomic instability are unclear, defective DNA repair proteins appear to be involved. Genetic instability is normally low thanks to the protective effects of these "caretaker genes". The results described above support a model in which BALB/c cells possess a mutator phenotype due to expression of a defective caretaker gene (DNA-PKcs). When exposed to ionizing radiation, early and

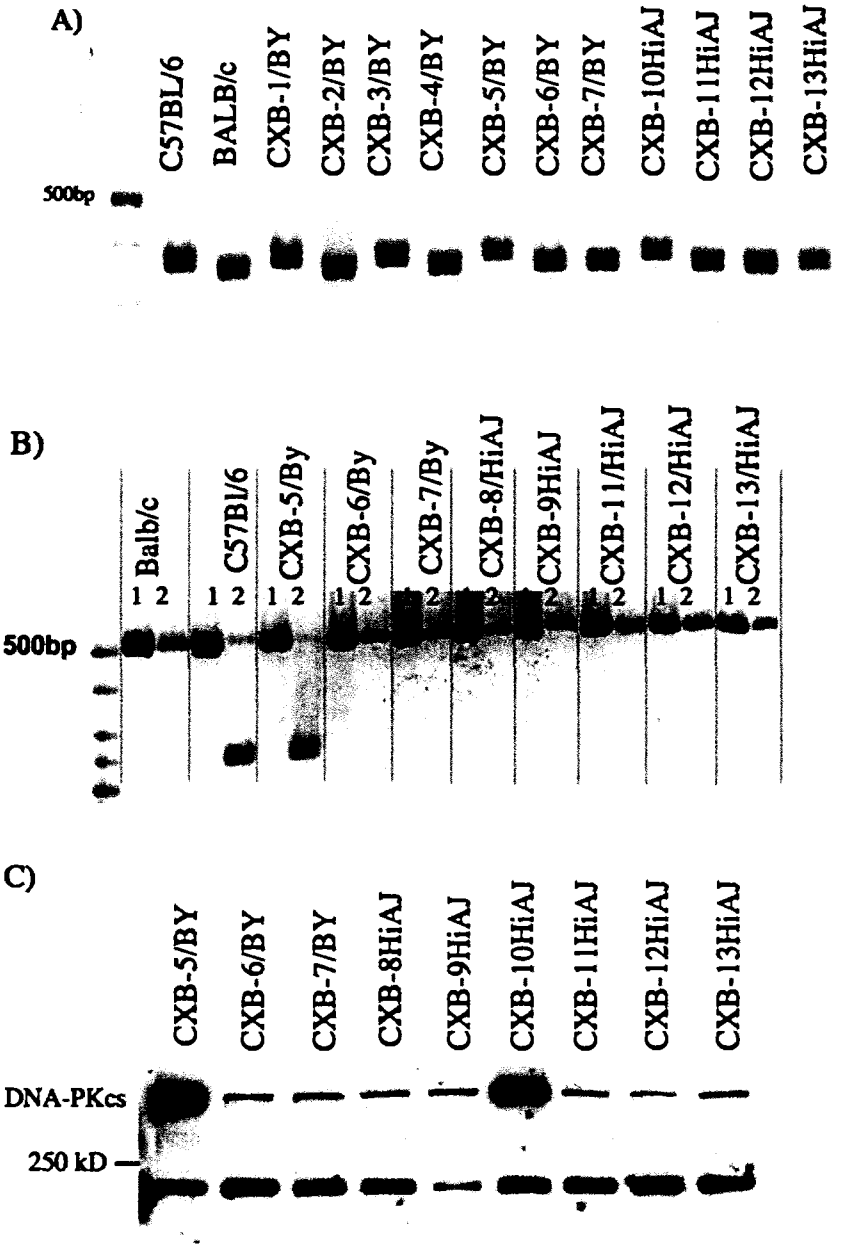

Figure 2.11: **A)** Genotyping RI Strains by PCR/RFLP at M3844V. Each lane contains Hph1-digested PCR product. RI strains harboring the $Prkdc^{BALB}$ allele at M3844V include CXB-2/By, CXB-4-By, CXB-6By, CXB-7By, CXB-11HiAJ, CXB-12HiAJ, and CXB-13HiAJ.**B)** Genotyping RI strains PCR/RFLP at C2140R.PCR products (lane 1) and products of PCR followed by BsmB1-digest (lane 2) run on a 3% agarose gel. PCR product. RI strains harboring the $Prkdc^{BALB}$ allele at C2140R include CXB-6By, CXB-7By, CXB-8By, CXB-9By, CXB-11HiAJ, CXB-12HiAJ, and CXB-13HiAJ.**C)** DNA-PKcs expression in RI strains. Diminished expression is observed in strains with $Prkdc^{BALB}$.(Ramaiah & Ullrich, unpublished results)

delayed instability ensues. Although all genes could potentially be affected, mutations in critical genes or "gatekeeper genes" such as p53 would give those cells a proliferative advantage compared to surrounding cells. This hypothesis is supported by mutational analyses of a preneoplastic cell line clonally derived from irradiated BALB/c mouse mammary tissue, in which mutations in p53 were acquired in the progeny of irradiated cells, several generations later.[160] P53 mutation frequency increased with additional passages, and mutations were acquired prior to developing a neoplastic phenotype.

Conventional theories of radiation carcinogenesis propose that radiation-induced cancers arise as a direct result of radiation-induced mutations. However, the data presented above supports a model that places genomic instability as the earliest event in the multi-step sequence of radiation carcinogenesis. According to our hypothesis, mutations in critical genes such as p53 arise due to genomic instability, as a secondary consequence of radiation-induced damage. This model is consistent with data from human breast cancer indicating that p53 mutation frequency increases as tumors progress, and that hypermutability, in the form of chromosomal instability, contributes to the pathogenesis of breast cancers.[161,162] The radiation-susceptible BALB/c mouse provides a unique model to study the genetic and molecular factors involved in radiation-induced cytogenetic instability and its role in mammary carcinogenesis. Ullrich et al. have demonstrated that a variant *Prkdc* gene exists in this strain and that the variant haplotype is associated with decreased DNA-PKcs kinase activity and heightened chromosomal instability after exposure to radiation. Further experiments were needed to dissect the functional consequences of the variant DNA-PKcs and to determine the extent of its role in radiation-induced mammary carcinogenesis.

2.3 Rationale of Dissertation

Sporadic breast cancer is a complex trait in which susceptibility is determined by the interplay between multiple genetic variants and environmental exposures to radiation and hormonal factors. The identification of genetic factors involved would greatly improve our ability to prevent and control this life-changing disease that affects one in eight women in the United States. Although association and genome-wide linkage studies can identify monogenic cancers caused by high penetrance genes such as BRCA1/2, genes causing sporadic breast cancer remain elusive. Novel animal models such as inbred and congenic mouse strains provide powerful alternatives as controllable, replicate systems that can be genetically and environmentally manipulated to reveal effects too subtle to be observed in the heterogeneous human population.

Previous investigations in the Ullrich laboratory described a heritable, naturally occurring susceptibility to radiogenic breast cancer in BALB/c mice. Preliminary data strongly implicated $Prkdc^{BALB}$ in susceptibility to radiation-induced chromosomal instability. Ullrich et al. initially demonstrated significant phenotypic differences between the susceptible BALB/c strain and a resistant (C57BL/6) strain, and provided evidence for a genetically determined link between susceptibility to radiation-induced mammary cancer, susceptibility to radiation-induced chromosomal instability, and the formation of specific preneoplastic lesions (ductal dysplasias). Two unique SNPs were identified in $Prkdc^{BALB}$. These correlated with decreased DNA-PKcs abundance and kinase activity, defective post-irradiation DNA DSB repair, and dysfunctional telomere capping. Preliminary backcross experiments demonstrated that $Prkdc^{BALB}$ homozygosity is significantly associated with susceptibility to radiation-induced chromosomal

instability and is required for the initiation of genomic instability in irradiated mammary epithelial cells. The characteristics of the variant form of the *Prkdc* gene in BALB/c mice (decreased DNA-PKcs protein expression and kinase activity, slow DNA DSB repair kinetics, and susceptibility to radiation-induced chromosomal instability and mammary cancer) suggest a direct mechanistic link between the *Prkdc* genotype and the susceptibility phenotype (fig. 2.12). Although there is strong genetic linkage between $Prkdc^{BALB}$ and radiation-induced chromosomal instability, the precise role of this allelic variant in radiation-induced mammary tumorigenesis is not as clear.

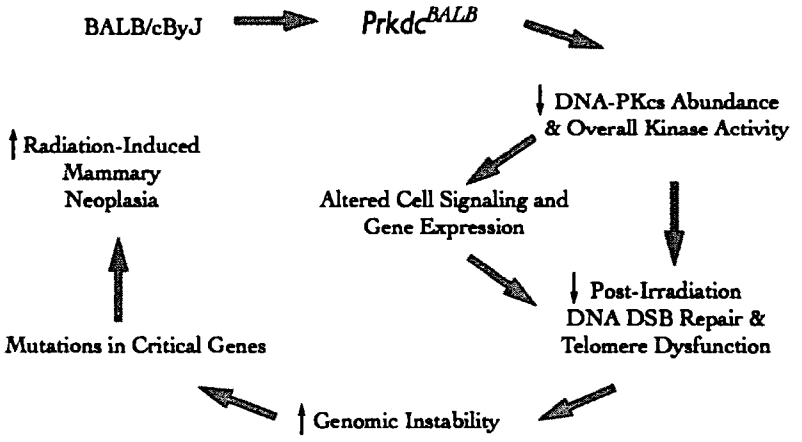

Figure 2.12: Proposed Model of Radiation-Induced BALB/c Mammary Tumorigenesis

Modifier genes in the BALB/c genome might contribute to tumor susceptibility in this strain. Assessing the contribution to susceptibility of $Prkdc^{BALB}$ from that of other BALB/c genes mandates the use of mice whose susceptibility allele ($Prkdc^{BALB}$) has been placed on a resistant strain background (ex.C57BL/6). Concurrently, determining the influence of BALB/c modifier genes on susceptibility requires the use of mice in which the resistant or wild type form of *Prkdc* ($Prkdc^{B6}$) has been placed on a BALB/c background. Here we describe the application of a direct genetic approach employing

two newly developed congenic mouse strains to examine linkage between the *Prkdc* locus and radiation susceptibility. Congenic strains are inbred strains in which a selected genomic region (*Prkdc*) has been replaced by the equivalent region from a donor strain through repeated backcrossing. Congenics are identical to their inbred partner at all loci except for the transferred locus and loci tightly linked to it. Congenic mice derived from the susceptible (BALB/c) and resistant (C57BL/6) strains would allow us to characterize the specific role of $Prkdc^{BALB}$ in radiation-induced mammary preneoplasia and neoplasia, and to estimate the extent of involvement of other BALB/c genes in producing the susceptibility phenotype. In contrast to "knock-in" technology, this approach has the advantage of preserving each parental strain's background genome without contamination from the genetically mixed and poorly defined 129 strains from which embryonic stem cells are derived. Using congenics, phenotypes produced by the parental strain's background would not be lost or diminished, unless they interacted with *Prkdc*. Identifying such interactions would validate the use of this model for sporadic breast cancer in humans. Furthermore, knock-in approaches are technically demanding and expensive. Analysis of congenic strains will allow us to confirm whether $Prkdc^{BALB}$ versus another BALB/c gene is responsible for each phenotype observed in BALB/c.

B6.C-*Prkdc*: BALB/c (C) *Prkdc* allele on the C57BL/6 (B6) background genome

C.B6-*Prkdc*: C57BL/6 (B6) *Prkdc* allele on the BALB/c (C) background genome

Marked strain differences in DNA-PKcs tissue expression and overall DNA-PKcs kinase activity exist in BALB/c mice compared with C57BL/6. BALB/c mice have 10-fold lower levels of DNA-PKcs in all tissues examined compared with other strains.

Overall kinase activity is also lower in cells isolated from BALB/c mice. These phenotypes correlate with two polymorphisms in *Prkdc*, the gene that codes for DNA-PKcs. Since both the polymorphisms and decreased kinase activity are unique to BALB/c mice (among strains examined), it is likely that the variant *Prkdc* is directly responsible for this phenotype. Analysis of congenic strains allowed us to confirm that $Prkdc^{BALB}$ and not another BALB/c gene is responsible for decreased DNA-PKcs levels in this strain.

Cells isolated from BALB/c mice have slow post-irradiation DNA double strand break repair kinetics due to a deficiency in the initial rapid phase of repair by non-homologous end-joining, in which DNA-PKcs participates. Since a functional DNA-PK is required for efficient DSB repair by NHEJ, polymorphisms resulting in a hypofunctional variant are likely to be involved. However, the precise contribution of the *Prkdc* allelic variant as opposed to other genes in the BALB/c genome is not well defined. Using congenic mice derived from the susceptible and resistant strains, we can characterize the specific role of $Prkdc^{BALB}$ in inefficient radiation-induced DNA damage repair. If $Prkdc^{BALB}$ is solely responsible for diminished DNA repair capabilities in BALB/c mice, C.B6-*Prkdc* mice should not demonstrate DNA repair dysfunction whereas B6.C-*Prkdc* mice should.

In this dissertation we describe the generation and genotypic/phenotypic characterization of two novel strains of mice carrying alternate genetic variants of the DNA repair gene *Prkdc* (DNA-PKcs). These strains serve as rodent models of sporadic and radiation-induced human breast cancer, providing proof of principle for the role of genetic polymorphisms in breast cancer susceptibility. Strains congenic for the common

(*Prkdc*^{B6}) and variant (*Prkdc*^{BALB}) alleles of *Prkdc* were developed and used to examine the functional consequences of *Prkdc*^{BALB}. We have characterized DNA-PKcs in these mice by quantifying DNA-PKcs protein expression, post-irradiation DSB repair, post-irradiation cell survival, breeding depression, and background and radiation-induced gene expression. Furthermore, we clearly demonstrate that *Prkdc*^{BALB} is sufficient and required for decreased DNA-PKcs protein expression and decreased cell survival after exposure to ionizing radiation. Using the congenic mice, we also present the first demonstration that *Prkdc*^{BALB} has significant effect on gene expression in unirradiated and irradiated mice. Future studies using the newly developed congenic strains will examine the extent of genetic linkage between the *Prkdc* locus and successive steps in the progression to mammary cancer, and will determine whether *Prkdc*^{BALB} alone is responsible for progression of radiation-induced cells to mammary cancer.

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3 GENERATION AND GENOTYPIC CHARACTERIZATION OF CONGENICS

Congenic strains are powerful tools in the investigation of complex traits. Congenic strains are inbred strains in which a selected genomic region has been replaced by the equivalent region from a donor strain through repeated backcrossing. They are essentially isogenic to their inbred partner providing background genome at all loci except for the transferred locus and loci tightly linked to it. This serves to eliminate much of the interference from genetic background that could confound the effect of the allele of interest on phenotype.[1] Thus, congenic strains permit characterization of a single locus without influence from genetic variation from the remainder of the genome.

Preliminary data strongly implicate *Prkdc*$^{\text{BALB}}$ in the phenotypic differences that exist between the radiation susceptible BALB/c strain and a resistant strain (C57BL/6). Strains congenic for the common and variant alleles of *Prkdc* facilitate the analysis of the genetic trait differences between BALB/c and C57BL/6. Congenic mice derived from the susceptible (BALB/c) and resistant (C57BL/6) strains allow us to characterize the specific role of *Prkdc*^{BALB} in radiation-sensitivity and to estimate the extent of involvement of other BALB/c genes in producing the susceptibility phenotype. Specifically, assessing the contribution to susceptibility of *Prkdc*^{BALB} from that of other BALB/c genes mandates the use of mice whose susceptibility allele (*Prkdc*^{BALB}) has been placed on a resistant strain background (ex.C57BL/6). Concurrently, determining the influence of BALB/c modifier genes on susceptibility requires the use of mice in which the resistant or wild type form of *Prkdc* (*Prkdc*^{B6}) has been placed on a BALB/c background.

In contrast to "knock-in" technology, the congenic approach has the advantage of preserving each parental strain's background genome without genetic contamination from embryonic stem cells. Strains from which embryonic stem cells are derived (129) are resistant to mammary carcinogenesis and therefore would not be useful in the study of events related to mammary cancer development. Furthermore, knock-in approaches are time-consuming, technically demanding and expensive. Congenics allow us to distinguish phenotypes caused by the parental strain's background from those caused by the *Prkdc* allele, and to identify phenotypes requiring interactions between the *Prkdc* and putative modifier genes in the parental background.

Here we describe the construction and initial characterization of two congenic strains derived from BALB/cByJ and C57BL/6J. Using high-density SNP genotyping, we precisely mapped the congenic interval and identified contaminating regions. The completed congenic strains were then used to directly link $Prkdc^{BALB}$ to phenotypic differences observed in BALB/c mice. The C.B6-*Prkdc* mice were used to answer the question: Is $Prkdc^{BALB}$ required for susceptibility to radiation-induced mammary cancer? The B6.C-*Prkdc* mice were used to answer the question: Is $Prkdc^{BALB}$ sufficient to confer susceptibility to radiation-induced mammary cancer? Both strains were also used to determine whether $Prkdc^{BALB}$ alone is responsible for the quantitative and functional DNA-PKcs deficiencies in BALB/c mice.

3.1 Inbred and Congenic Strains – Definitions and Nomenclature:[2]

Inbred strains are generated from ≥20 consecutive generations of brother-sister matings. They are presumed to be identical at all loci, having only 0.01 residual heterozygosity. They are maintained by continuous brother-sister mating. Inbred strains are designated by unique, brief, uppercase or roman type letters (ex.C3H). The number of inbreeding generations is indicated by the addition in parenthesis of F followed by the number of generations (ex.C3H(F150)). Abbreviations for the symbols of older or commonly used strains are useful for brevity (ex.C57BL/6=B6, BALB/c=C).

Substrains are inbred strains with established genetic divergence from one another due to early separation after completion of inbreeding or separation for more than 20 generations. Substrains are designated by the name of the parental inbred strain followed by a forward slash and a substrain designation (ex.C3H/He). One exception is BALB/c, which is a strain, not a substrain. The substrain designation or letters following the substrain refer to the person or institution originating the substrain (ex.C3H/HeH: derived at Harwell (H) from the Heston (He) substrain). Hybrids are the genetically identical progeny of two inbred strains, designated using the uppercase abbreviations of the two parents (maternal strain first), followed by F1 (B6CF1 or CB6F1).

Congenic strains are produced by repeated backcrosses to an inbred strain, with selection for a particular marker from the donor strain. They are regarded as congenic after a minimum of 10 backcross generations, counting the first hybrid as generation one. Congenic strains are designated with the background strain abbreviation separated by a period from the donor strain abbreviation (table 3.1). A hyphen separates the strain name from the italicized symbol of the differential allele introgressed from the donor strain.

Strain Name **Alt.Name** **(Abbrev.)**	**BALB/c** **(C)**	**C57BL/6J** **(B6)**	**B6.C-*Prkdc*^{C}** **B6.C-*Prkdc*^{BALB}** **(B6.C)**	**C.B6-*Prkdc*^{B6}** **C.B6-*Prkdc*** **(C.B6)**
Background	C	B6	B6	C
Prkdc Donor	C	B6	C	B6

Table 3.1: Nomenclature of Inbred and Congenic Strains

To avoid confusion concerning the donor *Prkdc* allele, we use the full designation BALB rather than its abbreviation (C), such that the variant BALB/c allele of *Prkdc* is designated *Prkdc*^{BALB}. In addition, because the C57BL/6 allele for *Prkdc* is the common form represented in most other inbred mouse strains, we do not designate the donor strain when referring to the common allele (C.B6-*Prkdc*). The number of backcross generations is indicated by the addition in parenthesis of N followed by the number of backcrosses (ex.C3H (N5)).

3.2 Materials and Methods

3.2.1 Congenic Breeding Protocol

The mating strategy used to generate congenic strains consisted of an initial cross between the BALB/c and C57BL/6, and backcrossing the progeny to the background parental strain to generate N2 progeny (fig. 3.1) These N2 progeny were selected for donor *Prkdc* sequence and backcrossed to mice of the background strain. Each generation was selected for those animals that inherited the donor allele of *Prkdc*. Strains were considered fully congenic after a minimum of ten backcross generations (N10).Congenic strains were then maintained by continued inbreeding of *Prkdc* homozygous progeny. Congenic strain development was carried out it three phases.

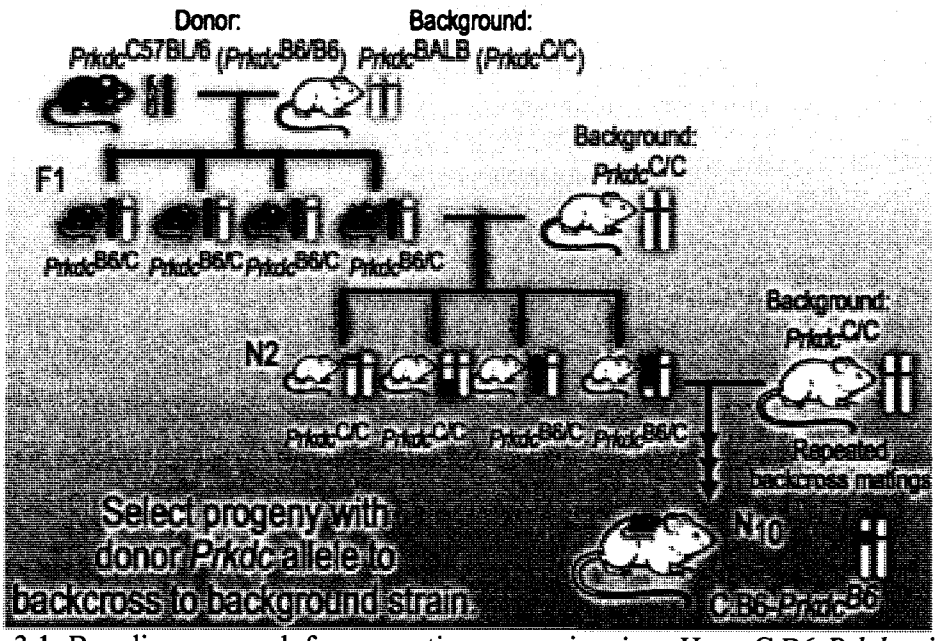

Figure 3.1: Breeding approach for generating congenic mice. Here, C.B6-*Prkdc* mice are being generated from BALB/c and C56BL/6 parents.

3.2.1.1 Phase I: From F1 to N7F3

The first phase of backcrossing was carried out by Dr. Christian Desaintes at the Radiobiology Unit in Boeretang, Belgium, using mice obtained from B&K Universal LTD (England). For both congenic strains, the breeding approach consisted of an initial outcross between a BALB/cByJ female and a C57BL/6J male, resulting in CB6F1 pups (fig. 3.2) For the C.B6 congenic strain, this was followed by a backcross of a female CB6F1 pup to a BALB/c male. After 4 additional consecutive backcrosses of female C.B6 pups carrying the donor *Prkdc* allele to BALB/c males, mice were inbred for three generations. For the B6.C congenic strain, female B6CF1 pups were backcrossed to a C57BL/6 male, followed by 3 consecutive backcrosses of female B6.C pups carrying the donor *Prkdc* allele to C57BL/6 males. Finally, male B6.C pups were backcrossed to a C57BL/6 female to ensure that the Y chromosome was derived from the background strain. Pups were selected for inbreeding if they were homozygous for the donor allele.

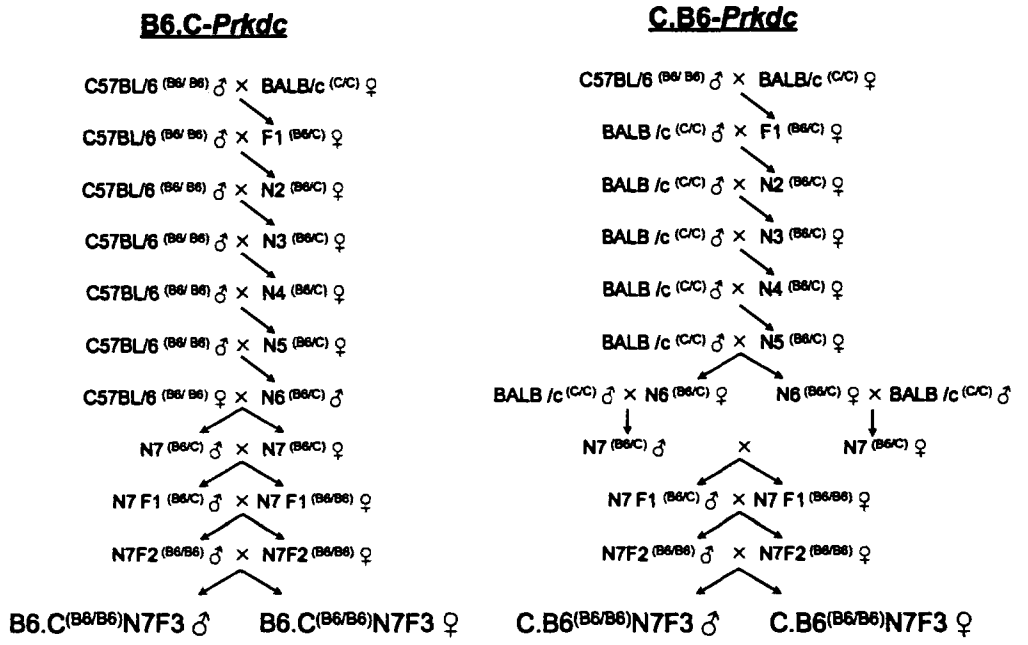

Figure 3.2: Backcrosses performed during Phase 1 of congenic strain generation.

3.2.1.2 Phase II: From N7F3 to N8

The second phase of backcrossing was carried out by Dr. Michael Weil, at the University of Texas M. D. Anderson Cancer Center in Houston, TX. This consisted of one additional backcross performed in a fashion similar to Phase I. Nearly congenic strains were maintained by repeated brother x sister matings.

3.2.1.3 Phase III: From N8 to completion of congenic strains

The third and final phase of backcrossing was carried out by Dr. Lila Ramaiah at Colorado State University, Fort Collins, CO. Congenic breeder pairs at N8F5 were received from Dr. M. Weil. Virgin female BALB/cByJ and C57BL/6ByJ mice used for backcrosses were obtained from The Jackson Laboratory, Bar Harbor, ME. All animals were maintained in the Colorado State University College of Veterinary Biomedical Sciences Laboratory Animal Resources (Painter Center) in accordance with federal regulations for animal care.

Backcrossing was commenced again as described in phase I, with the addition of marker-directed (speed congenics) breeder selection. Backcross progeny were positively selected for donor *Prkdc* and negatively selected for donor genome at loci outside *Prkdc*. Those with the least amount of donor genome contamination or with recombination breakpoints closest to *Prkdc* were selected for backcrossing and breeding. Once congenic strains had undergone a minimum of 10 backcross generations, they were inbred by repeated brother x sister matings. Breeding cages contained one male, and one or two females. Healthy pups were weaned and ear-tagged at 21-25 days of age. Poorly growing pups were weaned and ear-tagged at 26-28 days of age.

3.2.2 Allelic Discrimination

3.2.2.1 Tail DNA Isolation

High-molecular-weight mouse tail DNA was used as a template for PCRs. Tail biopsies were harvested from 10 to 21-day old mice according to approved CSU IACUC (Institutional Animal Care and Use Committee) protocols. DNA was extracted from tail biopsies using the DNeasy DNA Isolation Kit with modification (Quiagen, Cat.# 69506, Valencia, CA). DNA was eluted using PCR grade H_2O instead of Buffer AE. The concentration of DNA isolated was ~10μg/100μl as measured using a NanoDrop ND-100 Spectrophotometer (Wilmington, DE). The DNA samples were diluted to 25ng/μl by adding ~300μl PCR grade H_2O to each sample tube.

3.2.2.2 PCR/RFLP-based Genotyping for Prkdc and Cdkn2a SNPs

For genotyping at the *Prkdc* locus, a PCR/RFLP approach was used. DNA isolated from tail snips was subjected to PCR using two sets of primers, each flanking one polymorphic site. PCR amplifications were carried out in a MBS Satellite Thermal Cycler (Thermo Hybaid Waltham, MA). The PCRs were set up in a 25-µl reaction volume. Genomic DNA (1.5 µl, ~37.5 ng) was added to 23.5µl of master mix. Master mix contained 0.8 µmol each of forward and reverse primer (Macromolecular Resources, CSU), 2.5 units of Invitrogen DNA *Taq* polymerase (#18038, Carslbad, CA), 10x PCR Buffer (Invitrogen), 1.5 mM MgCl (Invitrogen), and 0.30 mM of each dNTP (Invitrogen). After an initial 5-min incubation at 95°C, the PCR thermocycling profile was as follows: 35 cycles of 95°C for 35 s, 49°C for 35 s, 72°C for 35 s, and a final extension for 10 min at 72°C. The PCR products were run on a 4% SeaKem LE (Lonza) or Metaphor agarose gel (1x TBE) and detected by SYBR Green staining. When differences in fragment length were too small to be resolvable by agarose gel electrophoresis, PCR products were resolved on 4-20% Novex precast polyacrylamide TBE gels (#EC62255, Invitrogen). All gels were visualized using an IS-2500 Digital imaging system with Kodak Image Analysis software, and were documented and saved in digital form. The primer sequences, annealing temperatures, restriction enzymes and cleavage product sizes were as follows:

***Prkdc* M3844V**

(F) 5' TGT CAC AAG AGG AGA AAG TGG C 3'
(R) 5' TGT ACA TTA GCA CAT AGG CTC C 3'

Annealing Temperature: 55°C
*Hph*I at 37°C for 3 h according to the manufacturer's instructions.
Cleavage of the PCR product with *HphI* gave rise to 37, 38, 515 and 442 bp fragments for $Prkdc^{BALB/BALB}$, 38, 479, and 515 bp fragments for $Prkdc^{B6/B6}$, and 37, 38, 442, 479, and 515 bp fragments for $Prkdc^{B6/BALB}$.

Alternate *Prkdc* M3844V Primers

(F) 5' ATG TTC TTT GCC ATG CAG T 3'
(R) 5' TTC TTC CCT CCC TTC TCA GTA 3'

Annealing Temperature: 55°C
*Hph*I at 37°C for 3 h according to the manufacturer's instructions.

***Prkdc* R2140C**

(F) 5' GCC ATG ATC CTT AGC AAG TG 3'
(R) 5' GCC TAA GGT AAG GTG CTG TA 3'

Annealing Temperature: 49°C
BsmBI at 55°C for 3 h according to the manufacturer's instructions
Cleavage of the PCR product with *BsmBI* gave rise to a 512 bp fragment for $Prkdc^{BALB/BALB}$, 260 and 252 bp fragments for $Prkdc^{B6/B6}$, and 512, 260, and 252 bp fragments for $Prkdc^{B6/BALB}$

***Cdkn2a* A134C (exon 1)**

(F) 5' ACT GAA TCT CCG CGA GGA AAG CGA ACT 3'
(R) 5' GAA TCG GGG TAC GAC CGA AAG AGT 3'

Annealing Temperature: 49°C
NlaIII according to the manufacturer's instructions
Cleavage of the PCR product with *NlaIII* gave rise to a 52 and 123 bp fragment for $Prkdc^{BALB/BALB}$, 52 and 70 bp fragments for $Prkdc^{B6/B6}$, and 52, 70, and 123 bp fragments for $Prkdc^{B6/BALB}$.[3]

***Cdkn2a* G232A (exon 2)**

(F) 5' GTG ATG ATG ATG GGC AAC GTT CA 3'
(R) 5' GGG CGT GCT TGA GCT GAA GCT A 3'

Annealing Temperature: 49°C
BsaAI according to the manufacturer's instructions
Cleavage of the PCR product with *BsaAI* gave rise to a 42 and 297 bp fragment for $Prkdc^{BALB/BALB}$, $Prkdc^{B6/B6}$ and $Prkdc^{B6/BALB}$.[3]

3.2.2.3 Resequencing-based Genotyping for Prkdc and Cdkn2a SNPs

When results from restriction digests were ambiguous, SNPs were sequenced. PCR products used for genotyping were directly run on a 1% agarose gel (1x TBE) and extracted using the Qiagen QIAquick Gel Extraction Kit (Cat# 28704) as per manufacturer's instructions. DNA was eluted from columns in 8 µl PCR grade H_2O. DNA was sequenced at Davis Sequencing (Davis, CA) using the same forward primers used to amplify the DNA.

3.2.2.4 PCR-based Genotyping using DMit Markers polymorphic between B6 and C

Microsatellites, or simple sequence repeats (SSR) are highly mutable polymorphic loci consisting of repeating units of 1 to 4 bp in length. Numerous microsatellite markers and single nucleotide polymorphisms exist for differentiating BALB/c and C57BL/6, and these, along with necessary protocols, are available on many mouse genome databases (Sloan-Kettering Mouse Project, MGI, Whitehead Institute, and Roche). Primer pairs for mapping (MapPairs/DMit markers), which are readily available commercially (Invitrogen), were used to amplify repetitive sequences of genome by PCR. Choice of markers was based on their ability to resolve BALB/c amplicon size from C57BL/6 amplicon size on high percentage agarose gels. Microsatellite polymorphisms were typed by sizing DNA amplification products on agarose gels. Amplicons were 50 to 400 nucleotides in length. Microsatellite analyses were performed using the PCR primers and conditions specified by Research Genetics (Hunstville, AL). Briefly, after an initial 5-min incubation at 95°C, the thermocycling profile was as follows: 35 cycles of 95°C for 35 s, 49°C for 35 s, 72°C for 35 s; and a final extension for 10 min at 72°C. The PCR

products were run on a 4% SeaKem LE or Metaphor agarose gel (1x TBE) and detected by SYBR Green staining. When differences in fragment length were too small to be resolvable by agarose gel electrophoresis, PCR products were resolved on 4-20% Novex precast polyacrylamide TBE gels (#EC62255, Invitrogen).

3.2.2.5 Resequencing-based Genotyping for SNPs polymorphic between B6 and C

PCR primers flanking SNPs were selected according to online mouse genome sequence databases (Ensembl, Blast, Primer3).(See DVD\genotpying\SNP resequencing\primers used) Primer sets were purchased from Macromolecular Resources (CSU). PCR cycling conditions were 94°C for 30 s, 49°C for 30 s, and 72°C for 30 s for 40 cycles, followed by a final extension at 70°C for 10 min. PCR products were directly run on a 1% agarose gel (1x TBE) and extracted using the Qiagen QIAquick Gel Extraction Kit (Cat# 28704) as per manufacturer's instructions. DNA was eluted from columns in 8 μl PCR grade H_2O. DNA was sequenced at Davis Sequencing (Davis, CA) using the same forward or reverse primers as those used to amplify the DNA. The following primers were generated for resequencing of microarray SNPs:

gnf13.035.637
(F) 5' CGC ACA CAC ACA CAC ACT TAC 3'
(R) 5' ATT CCT CCC TGA CAT CCT GAC 3'

rs13481764
(F) 5' TCT TTG GCA TTT TGG TTC TCT 3'
(R) 5' GAT TCT ACC CCC ATC AAG TCC 3'

rs13482398
(F) 5' CCG TTC CCT ACA CAT TGA CC 3'
(R) 5' TTT TAC TGT CTG TCG CCT TGA 3'

3.2.2.6 Microarray-based Genotyping for SNPs polymorphic between B6 and C

Undiluted tailsnip DNA was assayed by Dr. Michael Story (Dept. of Exper. Radiat., UT-MD Anderson Cancer Center, Houston, TX) for microarray SNP genotyping using the Mouse MD Linkage Panel (#GT-18-131, Illumina, San Diego, CA). The concentration of DNA was measured using a NanoDrop ND-100 Spectrophotometer (Wilmington, DE).

3.3 Results: Genotypic Characterization of Congenic Mice

3.3.1 Initial Haplotype Analysis in N8 Congenics: Traditional congenics failed to produce a recombination event on chromosome 16

Upon receipt at Colorado State University, nearly congenic strains at backcross eight were genotyped at the *Prkdc* locus to confirm the presence of the donor allele. They were also genotyped at two loci per chromosome using DMit markers to evaluate the extent of donor contamination and to guide future backcrosses. Homozygosity for donor allele at *Prkdc* was confirmed by PCR/RFLP SNP genotyping. BALB/c has two SNPs on chromosome 4 in *Cdk2na* that are responsible for altered cell cycle control and susceptibility to plasmacytomas in this strain.[3] Donor contamination by this allele needed to be excluded to ensure that it did not influence phenotype. The two *Cdkn2a* SNPs were genotyped by PCR/RFLP and/or SNP sequencing (fig. 3.3, Appendix Figure 1). The nearly congenic strains were confirmed to contain their appropriate background *Cdkn2a* allele. Initial genome-wide typing using DMit markers revealed donor contamination on chromosome nine. Higher density genotyping on chromosome 16 showed no recombination events around *Prkdc* (table 3.2).

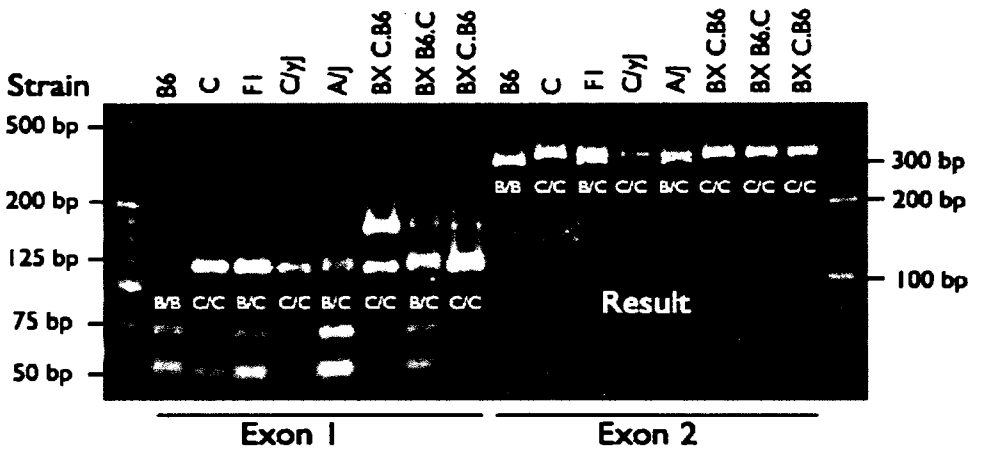

Figure 3.3: PCR/RFLP-based genotyping at two SNPs in *Cdkn2a* demonstrates presence of background allele in congenic strains.(C) BALB, (B6) C57BL/6.

3.3.2 Speed Congenics Dramatically Improved Strain Quality in only 2 Generations

In traditional congenic approaches, the percentage of background genome increases logarithmically with each successive backcross. Following ten generations of backcrossing, a congenic strain is statistically 99.9% identical to the background inbred strain at all loci except those linked to the transferred gene of interest. At eight backcross generations the congenic strain should be 99.61% identical to its background inbred strain. Our initial genotyping analyses did not show this to be the case. Specifically, the whole chromosome harboring our locus of interest had donor genome, i.e. recombination had failed to occur in all eight successive meiotic divisions.

NAME	CHROM.	POS (cM)	C.B6	B6.C
DXMit54	X	2.2		=
DXMit31	X	57.9		B6
D1Mit64	1	0		=
D1Mit294	1	3.3		
D1Mit464	1	113.7		B6
D2Mit277	2	56.8		
D2Mit265	2	91.8		B6
D3Mit130	3	4.4		=
D3Mit257	3	49.2		B6
D4Mit235	4	3.3		B6
D4Mit81	4	31.7		
D4Mit283	4	69.9		B6
D5Mit346	5	0		B6
D5Mit158	5	48.1		=
D5Mit99	5	73.2		=
D6Mit287	6	44.8		
D6Mit15	6	66.7		0
D7Mit246	7	12		B6
D7Mit362	7	67.8		B6
D8Mit224	8	16.4		
D8Mit280	8	74.3		B6
D9Mit67	9	13.1		
D9Mit75	9	39.3	B6	B6
D9Mit347	9	55.7		
D9Mit18	9	68.9		B6
D10Mit10	10	50.3		B6
D10Mit180	10	67.8		B6
D11Mit150	11	4.4		=
D11Mit339	11	31.7		B6
D11Mit338	11	78.7		B6
D12Mit37	12	1.1		B6
D12Mit101	12	44.8		B6
D13Mit218	13	2.2		B6
D13Mit262	13	47		B6
D14Mit11	14	3.3		B6
D14Mit186	14	16.4		0
D15Mit265	15	7.7		B6
D15Mit34	15	55.7		B6
D16Mit121	16	3.3	B6	
D16Mit182	16	3.3	B6	
D16Mit9	16	4.4	B6	
D16Mit131	16	6.6	B6	
D16Mit56	16	12	B6	
D16Mit34	16	12	B6	
D16Mit57	16	21.9	B6	
D16Mit211	16	24	B6	
D16Mit4	16	25.1	B6	
D16Mit59	16	25.1	B6	
D16Mit84	16	29.5	B6	
D16Mit222	16	33.9	B6	
D16Mit76	16	33.9	B6	B6
D16Mit189	16	40.4	B6	
D17Mit57	17	1.1		0
D17Mit88	17	29.5		B6
D18Mit66	18	0		B6
D19Mit59	19	0		B6
D19Mit109	19	1.1		B6
D19Mit6	19	57.9		B6

Table 3.2: Initial genome-wide genotyping using DMit markers revealed donor contamination on chromosome 9 and no recombinations on Chromosome 16..

To ensure the integrity of the congenic strains before generation N10, an aggressive marker-directed breeding program was initiated. The quality of the congenic strains was significantly improved with the use of marker-assisted (*i.e.,* speed congenic) protocols implemented in phase III of backcrossing. The number of progeny produced and genotyped at each generation was increased. Positive selection of breeders based on the presence of the donor *Prkdc* SNPs was continued as previously. Negative selection (speed congenics) involved genotyping positively selected pups for all genetic loci that had shown donor origin alleles outside of the *Prkdc* locus in progenitors. Backcross progeny were genotyped using all DMit markers that exhibited donor contamination in the previous generation, with particular focus on markers on chromosome 16 (carrying *Prkdc*) (fig. 3.4) (See DVD\genotyping\DMit marker assisted breeding)

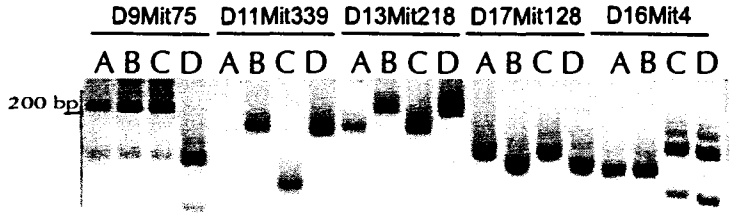

Figure 3.4: Genotyping using DMit Markers. Example of an Agarose Gel Electrophoretogram of PCR amplification products using DMit marker primers.
A) C57BL/6, B) C.B6-*Prkdc*, C) B6.C-*Prkdc*^{BALB}, and D) BALB/cByJ.

Breeders for each generation were selected based on microsatellite markers polymorphic between B6 and BALB. *Prkdc* is located at 15.5Mb (15,412,817 – 15,616,347 bp) on chromosome 16. The closest polymorphic DMit markers flanking *Prkdc* are located at 14.9 Mb upstream and 15.686 Mb downstream of *Prkdc*. Beyond these, a sufficient density of markers exists upstream, but not downstream; the next available downstream DMit marker is located at 23.36 Mb.

For fine genotypic mapping from 15.686 Mb to 23.36 Mb, we made use of known intronic SNPs located at 20.06 Mb and 22.6 Mb. Since no DMit markers existed for these loci, we genotyped SNPs by PCR/RFLP or by sequencing (ex. E in fig. 3.5). Sixty-four genome-wide DMit markers and 12 SNPs on chromosome 16 were assayed. The selection of animals for backcrossing at each generation was based on individual haplotype analysis of progeny and four criteria: 1) presence of donor *Prkdc* SNPs; 2) absence of donor genome outside *Prkdc*; 3) narrow recombination breakpoints; and 4) lower overall heterozygosity. For B6.C-*Prkdc*^{BALB}(N10), immediately prior to inbreeding, we performed one large (182 progeny) backcross focusing on chromosome 16, with the aim of generating congenic strains with donor sequences extending less than 1cM distal and proximal to *Prkdc*. The resulting strain pedigrees are shown in figures 3.6 and 3.7.

Mice at generation N10 or later were intercrossed. Progeny homozygous at *Prkdc* were selected for inbreeding. Marker-assisted breeder selection was also employed on inbreeding progeny until all loci were homozygous. Using this approach, we believe that very few donor genes other than *Prkdc* were acquired by the recipient strain. This was confirmed using a medium-density SNP array to precisely map the congenic boundaries and test for contaminating regions. The SNP microarray consists of 1,449 SNPs in 480 mouse strains. Among the 1,449 SNPs, 877 distinguish C57BL/6 and BALB/c alleles. They span the entire mouse genome with ~2 SNPs per 5 Mbs, equivalent to assessing genotypes at 3 cM intervals across the entire genome. We also performed a high-density screen on chromosome 16 immediately distal and proximal to *Prkdc* and located the recombination breakpoints in each congenic strain. A subset of congenic animals was evaluated in this manner. Minimal donor contamination was noted (figs. 3.8 & 3.9).

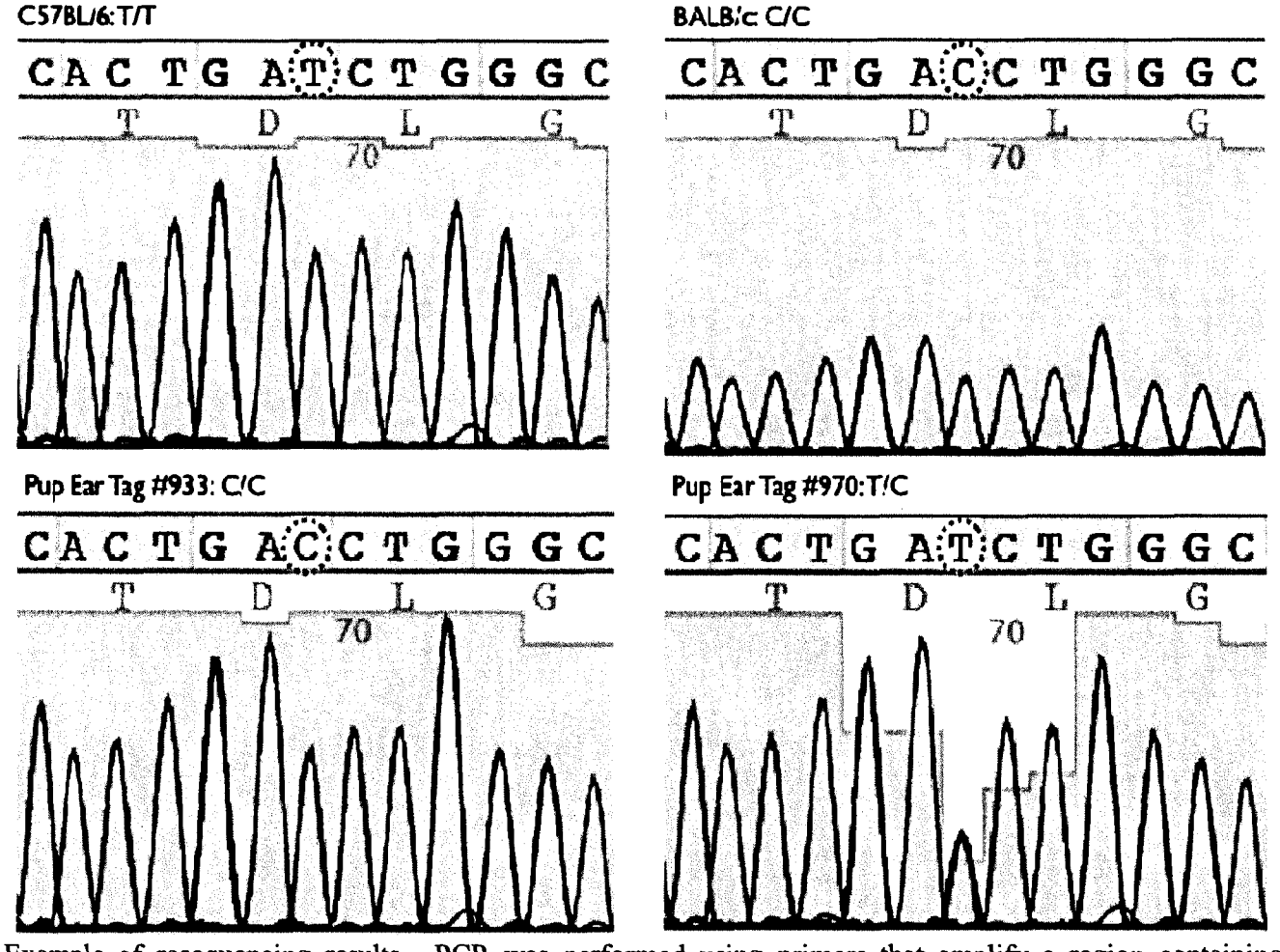

Figure 3.5: Example of resequencing results. PCR was performed using primers that amplify a region containing nucleotides polymorphic between BALB/c and C57BL/6. Pups (two C.B6 pups shown here) with minimal donor alleles were selected as breeders.

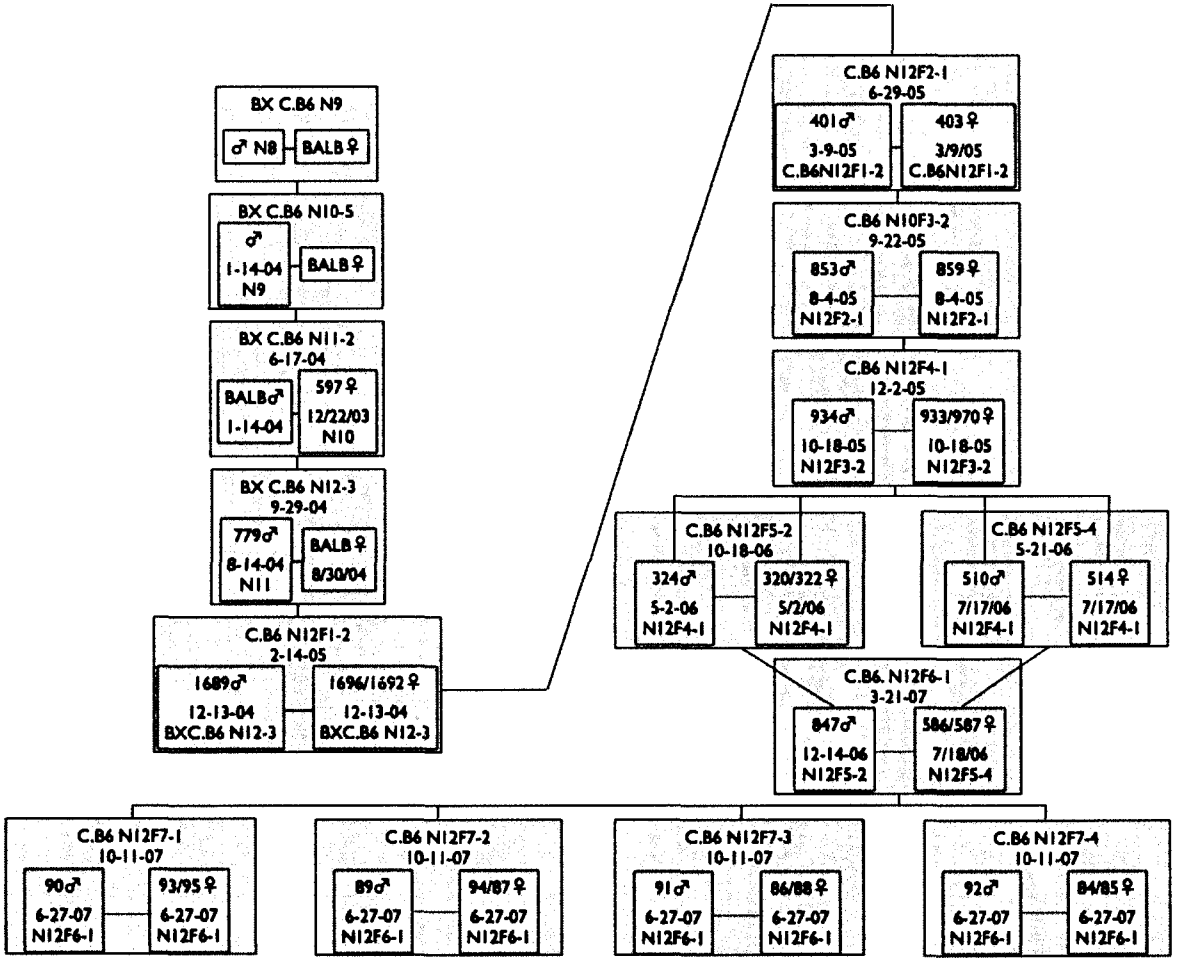

Figure 3.6: Schematic of individual animals mated to generate the C.B6-*Prkdc* congenic strain.

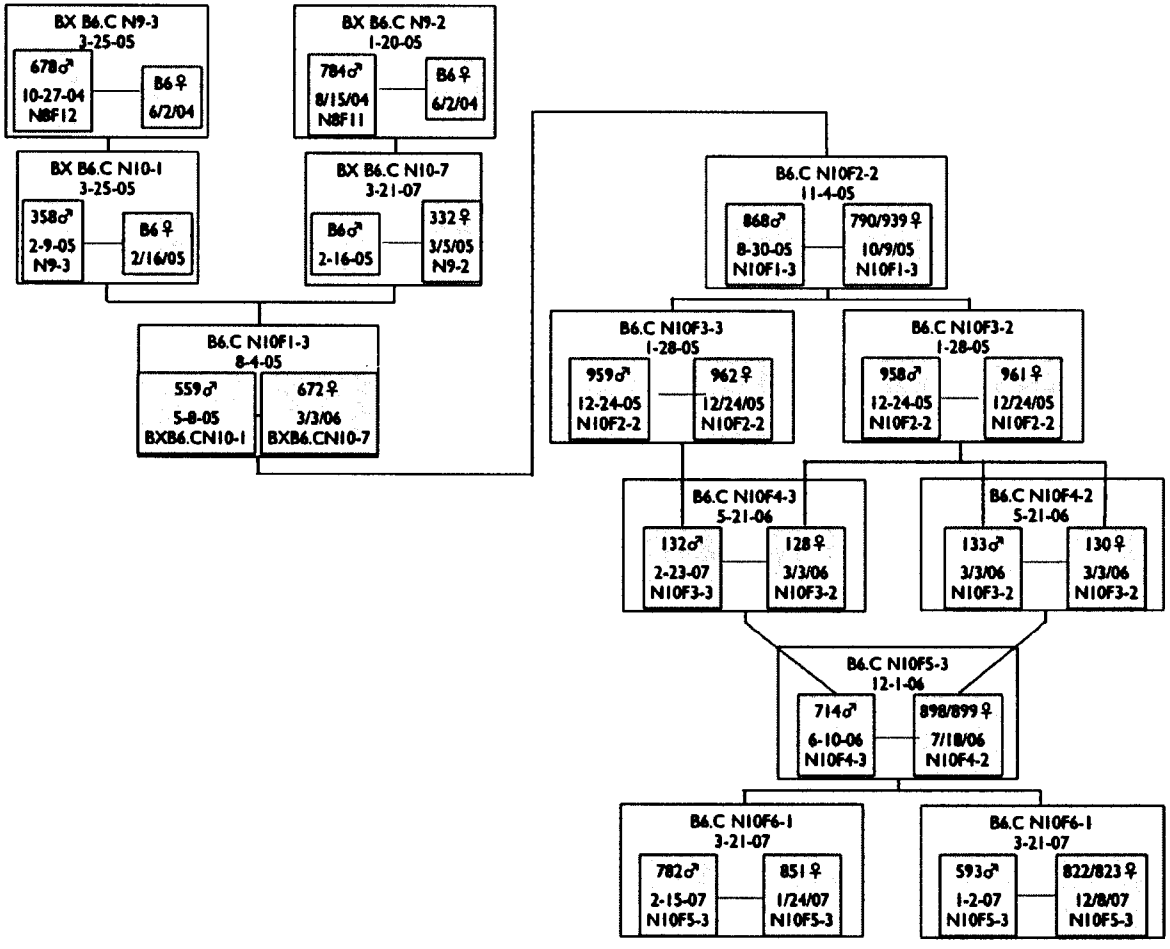

Figure 3.7: Schematic of individual animals mated to generate the B6.C-*Prkdc*^{BALB} congenic strain.

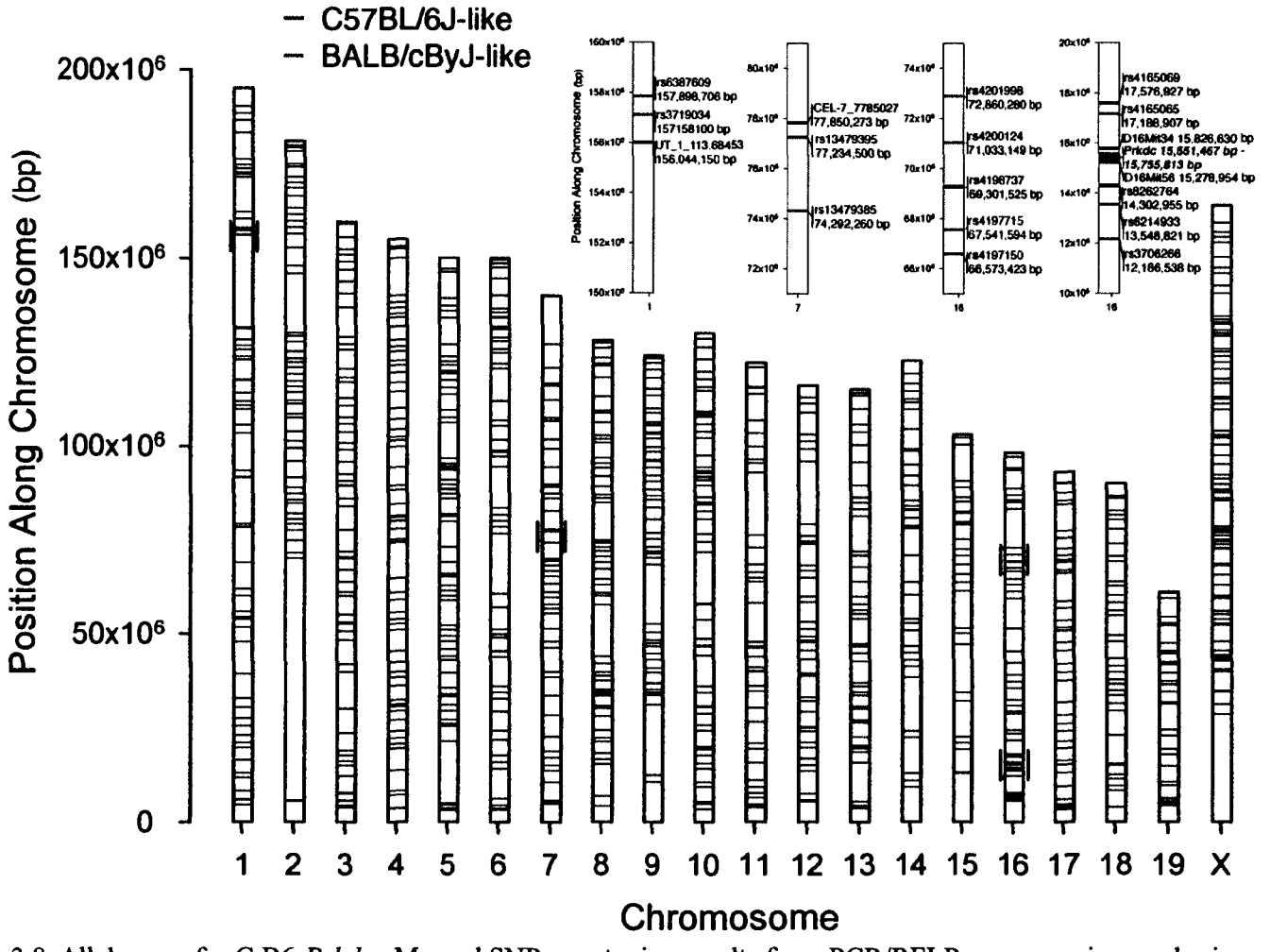

Figure 3.8: Allelogram for C.B6-*Prkdc*. Merged SNP genotyping results from PCR/RFLP, resequencing, and microarray.

Regions of Donor Genome Contamination: C.B6-Prkdc (N12F4)

Locus Name	Chr	Position (bp)	C	C	319	320	321	322	323	324	325	B6	B6	F1
UT_1_113.684537	1	156,044,150	B	B	B	B	B	B	B	H	H	A	A	H
CEL-7_77850273	7	77,850,273	H	B	B	B	B	H	H	B	H	A	A	H
rs4198737	16	69,301,525	H	B	B	H	H	B	H	H	H	A	A	A

Table 3.3: SNP Microarray detected three loci with contaminating SNPs in C.B6.

Two out of seven C.B6 mice examined were heterozygous on chromosome one at 156,044,150 bp (UT_1_113.684537).(Table 3.3) Flanking uncontaminated SNPs were at 131,376,437 upstream and 157,158,100 downstream. This region is large due to the low density of SNPs polymorphic between BALB/c and C57BL/6. Three out of seven C.B6 mice examined were heterozygous on chromosome 7 at 77,850,273 bp (CEL-7_77850273). Flanking uncontaminated SNPs were at 77,234,500 bp and 82,727,616 bp. False positives were seen in BALB/c and CB6F1, emphasizing the possibility of error in the results. This is plausible considering the inconsistency of findings among seven inbred brothers and sisters. These results should be confirmed by resequencing.

Five out of seven C.B6 mice examined were heterozygous on the distal end of chromosome 16 at 69,301,525 bp (rs4198737). Flanking uncontaminated SNPs were located at 67,541,594 bp and 71,033,149 bp. Given the high number of mice harboring this allele, it seems likely that the area of C57BL/6 contamination is real and may have persisted in later inbreeding generations.

Recombination breakpoints flanking the *Prkdc* donor region on chromosome 16 were determined by SNP resequencing. Flanking uncontaminated SNPs were at 13,548,821 bp (rs6214933) upstream of *Prkdc* and 21,592,383 bp (rs4165283) downstream of *Prkdc*. Thus, the recombination breakpoints around the *Prkdc* locus for C.B6-*Prkdc* lie between 13,548,821 bp and 21,592,383 bp, an 8 Mb region. This region contains approximately 164 known or potential genes.(See DVD\Genotyping\Genes in Congenic Region)

Results for rs4165069 (SNP resequencing) and rs4165065 (SNP microarray) are likely correct. Rather than representing a region of hyper-recombination, we believe that discrepancy is due to slight errors in SNP location mapping (which are often revised).

Regions of Donor Genome Contamination: B6.C-Prkdc^{BALB} (N12F4)

Locus Name	Chr	Position (bp)	C	C	776	777	891	892	B6	B6	F1
rs13481764	13	35994885	B	B	H	H	A	A	A	A	H
gnf13.035.637	13	36959616	A	A	H	H	B	B	B	B	H
rs4165065	16	17188907	B	B	B	B	B	B	A	A	H

Table 3.4: Three loci with contaminating SNPs in B6.C-*Prkdc*^{BALB}

By SNP microarray, two out of four B6.C mice examined were heterozygous on chromosome 13 at 35,994,885 bp (rs13481764) and 36959616 bp (gnf13.035.637).These results were confirmed by resequencing. Flanking uncontaminated SNPs were located at 34,496,620 bp upstream and 42,620,079 bp downstream. Two out of four B6.C mice examined were heterozygous on chromosome 14 at 112,310,948 bp (rs13482398) by SNP microarray. Resequencing at this locus indicated that B6.C congenics were homozygous for the C57BL/6 allele, contradicting SNP microarray data.(See Appendix Figure 1 for resequencing results.) Therefore, outside of chromosome 16, donor contamination existed only on chromosome 13.

All four B6.C mice examined were homozygous for the donor allele on chr. 16 at 17,188,907 bp (rs4165065). This locus is within the *Prkdc* donor region for this strain. Recombination breakpoints flanking the *Prkdc* donor region on chromosome 16 were determined by SNP resequencing. Flanking uncontaminated SNPs were at 13,548,821 bp (rs6214933) upstream of *Prkdc* and 23,382,939 bp (rs4165334) downstream of *Prkdc*. Thus the recombination breakpoints around the *Prkdc* locus for C.B6-*Prkdc* lie between 13,548,821 bp and 23,382,939 bp, an 9.8 Mb region, a region containing ~207 known and potential genes. (See CD\Genotyping\Genes in Congenic Region)

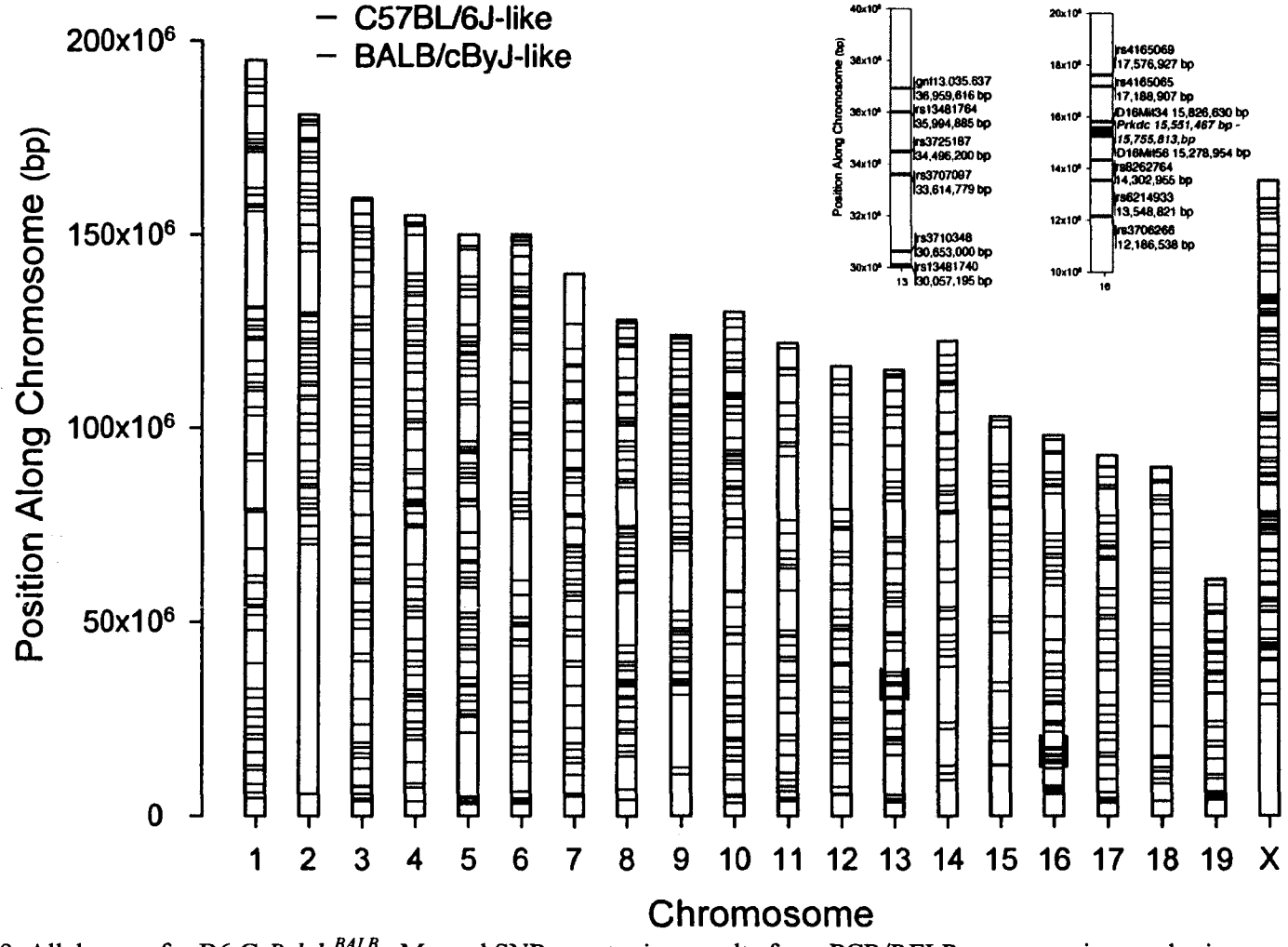

Figure 3.9: Allelogram for B6.C-*Prkdc*^{BALB}. Merged SNP genotyping results from PCR/RFLP, resequencing, and microarray.

3.4 Discussion & Future Directions

The marker-directed breeding approach used in phase III succeeded in generating two highly congenic mouse strains: C.B6-*Prkdc* and B6.C-*Prkdc*^{BALB}. Ten and twelve backcross generations, for B6.C-*Prkdc*^{BALB} and C.B6-*Prkdc*^{B6} respectively, were completed in the construction of the congenic strains. Although 10 backcross generations are required for a strain to be considered fully congenic, the speed congenics method used in phase III imposed higher stringency in the selection of progeny, resulting in rapid removal of excess donor genome.

Genome-wide SNP haplotype analysis revealed potential areas of donor genome contamination. Resequencing at the loci will confirm the results. The current data suggests that donor contamination may exist on the distal end of chromosome 16 in the C.B6-*Prkdc* congenic strain, between 67 Mb and 71 Mb. We have also determined that the congenic region extends from 13.5 Mb to 21.6 Mb for C.B6, and from 13.5 Mbp to 23.4 Mb for B6.C. The recombination breakpoints are concordant with high resolution maps of mouse chromosome 16 which indicate that a large region of low crossover frequency may exist from 13 Mb to 16 Mb.[4] (Figure 3.10) It can reasonably be inferred that these regions contain donor genetic sequences that could potentially impact phenotype.

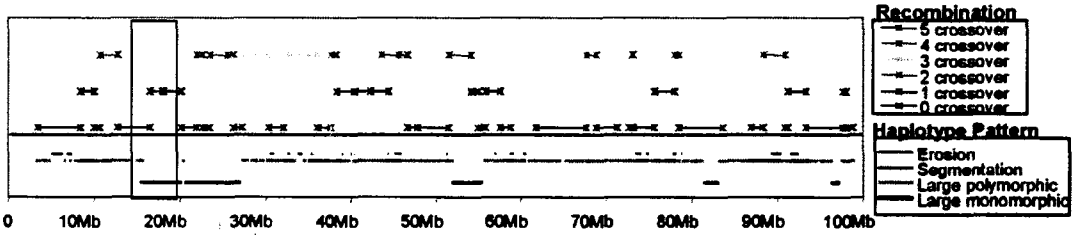

Figure 3.10: Recombination crossovers (top panel) and haplotype pattern (bottom panel) on mouse chromosome 16.[4]

The genetic material contained within the congenic regions in both strains contains approximately ~207 known and putative genes. Among these, the following had functions relating to phenotypes we were interested in examining: radiation response (Snai2), DNA metabolism (Cdc35l, Mcm4, Rfc4, and Top3b), cell cycle control (Cdc45l, Crkl, Mapk1, Ndel, Ranbp1, Sept5, and Thpo), apoptosis (Airm3, Bfar, Ppm1f, Rrn3, Snai3), and chromatin remodeling (Cebpd, Etv5, Gsc2, Hic2, Hira, Map6d1, Mcm4, Ndel, Rfc4, Snai2, Tbx1, Thap7, Top3b). Contamination by these genes would only influence our results if they were polymorphic between BALB/c and C57BL/6. Whether or not that is the case remains to be determined.

Results from the genome-wide SNP analysis suggest that outside of chromosome 16, genetic sequence is primarily of the recipient haplotype. The few contaminant alleles that surfaced might represent analytical error or might be real donor sequence that may or may not have been propagated in subsequent generations. Should they represent real donor sequence, inference of haplotype at these loci may not be appropriate. Haplotype inference assumes that the genome is composed of discrete blocks (chromosomal segments with limited diversity) separated by hotspots of recombination and describes the entire segment according to the allelic combinations of few SNPs within it. Because large-scale linkage disequilibrium patterns are usually similar between low-density SNP sets, haplotypes of SNP markers have demonstrated efficacy in QTL and single gene trait mapping. However, the concept of the haplotype block is not useful or reliable, and inference about variation between SNPs is not possible. Large-scale, high resolution mapping studies demonstrate an emerging complexity in haplotype structure.[5-7] Haplotype blocks often disappear, continually fragmenting into sub-blocks with

increasing SNP density. Local recombination rates may be stochastic or may be influenced by the complex interplay of many factors, most of which are unknown. Haplotype structure is fragile and soft rather that stable and solid. Even for high-density (2 kb) SNP marker sets, different blocks might arise depending on the marker set used.[4,7] Therefore, one cannot generalize local SNP findings to predict the haplotype of individual SNPs within a "block".

Our genome-wide scan revealing minimal donor haplotype contamination provides sufficient evidence that our strains, developed by marker-directed backcrossing, are truly congenic for the common and BALB/c variant alleles of *Prkdc*. Phenotypic differences that persist between the congenics and their recipient inbred partners can be interpreted to be a consequence of the introgressed allele. The BALB/c inbred mouse is highly studied for its susceptibility in pathologic processes involving DNA repair and acquired & innate immunity. Because DNA-PKcs is a caretaker in these processes, mice congenic for $Prkdc^{BALB}$ could be useful to many investigators. To facilitate their widespread use, the congenic strains will be submitted to The Jackson Laboratory (TJL) for cryopreservation. It is our hope that TJL will make these strains available to interested investigators.

ENDNOTES

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4 PHENOTYPIC CHARACTERIZATION OF CONGENIC MICE

Genetic tumor predisposition in BALB/c mice is suspected to arise in part from two SNPs in the *Prkdc* gene. However, subtle effects due to $Prkdc^{BALB}$ must be distinguished from effects due to other genes in the BALB/c background. This can be accomplished by comparing animals in which differences in the genetic background have been eliminated as experimental variables. By placing the variant allele into the genome of the resistant strain, and the common (wild-type) allele into the genome of the susceptible strain, we could perform a direct comparison between the variant and wild type strains that differ only at the *Prkdc* locus. Phenotypic differences could then be linked with the genotype of background genome or the allele at the *Prkdc* locus. Congenic strains are inbred strains in which a selected genomic region (*Prkdc*) has been replaced by the equivalent region from a donor strain through repeated backcrossing. The congenics are identical to their inbred partner at all loci except for the transferred locus and loci tightly linked to it. Congenic mice derived from the susceptible (BALB/c) and resistant (C57BL/6) strains would allow us to characterize the specific role of $Prkdc^{BALB}$ in radiation-induced mammary preneoplasia and neoplasia, and estimate the extent of involvement of other BALB/c genes in producing the susceptibility phenotype. The congenic strains were used to directly link $Prkdc^{BALB}$ to functional defects in DNA-PKcs. Mice whose susceptibility allele ($Prkdc^{BALB}$) was placed on a resistant strain background (ex. C57BL/6) allowed us to assess the contribution to susceptibility of $Prkdc^{BALB}$ compared to that of other BALB/c genes. The B6.C-$Prkdc^{BALB}$ strain allowed us to assess whether $Prkdc^{BALB}$ is **sufficient** to confer susceptibility to radiation. Concurrently, mice in which the resistant or wild type form of *Prkdc* ($Prkdc^{B6}$) had been placed on a BALB/c

background allowed us to determine the influence of BALB/c modifier genes on susceptibility. The C.B6-*Prkdc* strain allowed us to assess whether $Prkdc^{BALB}$ is **required** to confer susceptibility. Furthermore, phenotypic differences between congenic and recipient inbred partners must be a consequence of the donor genomic region.

In the previous chapter we described the construction of two strains congenic for the common and for the BALB/c variant forms of *Prkdc*. Here we present the evaluation of these congenic strains in comparison to the parental strains for various endpoints involving or requiring DNA-PKcs expression and function. By western immunoblot we demonstrate reduced DNA-PKcs protein expression levels in congenic mice carrying $Prkdc^{BALB}$. Using a complete coverage GeneChip mouse microarray (Affymetrix) we identify genes that are differentially expressed *in vivo* (constitutive and radiation-induced) between the parental and congenic strains. *Prkdc*-associated differential cell survival after exposure to IR is demonstrated using a clonogenic survival assay. *In vitro* DSB repair is evaluated using three different methods: disappearance of γ-H2AX foci, comet assay, and a modified FAR assay (PFGE followed by Southern blot). Finally, parental and congenic strains are evaluated for differences in reproductive physiology. Parental BALB/c, C57BL/6, human skin fibroblasts, and SCID cells are used as controls.

4.1 Materials & Methods

4.1.1 Mice

Five-week-old virgin female BALB/cByJ, C57BL/6J, CB6F1 and SCID mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Congenic mice were generated by repeated backcross matings as described in the previous chapter. All animals were maintained at the CSU Laboratory Animal Resources Painter Center. Mice were housed according to the standards described in the Animal Welfare Act regulations, the Guide for the Care and Use of Laboratory Animals (Guide) and the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching and covered daily husbandry, space requirements, environmental control (i.e. light, temperature, humidity, noise, ventilation), social environment, sanitation, waste management, security, veterinary medical care, water quality, pest control and animal identification and records. No more than 5 same-sex mice were housed per filter-top cage. Purina pellet mouse feed and water were offered *ad libitum.* Bedding was changed weekly.

4.1.2 Cell Culture

Immortalized neonatal human skin fibroblasts (HSF), were purchased from Cascade Biologics (HDF C-0004-5C; Portland, OR). Immortalized mouse DNA-PKcs knockout cells (PK-/-) were kindly provided by Dr.H.Nagasawa.[1] Primary mouse kidney fibroblasts (KFB) were isolated from age-matched mice as previously described with modifications.[2, 3] Both kidneys were mechanically minced (in 10 μl sterile M199) to 1-2 mm fragments on a glass plate using two sterile scalpels. Minced tissue was transferred to a sterile Erlenmeyer flask containing 20-30 ml of 199 medium containing collagenase

(200 units/ml, Type III, Worthington). Tissue was enzymatically digested for 3-5 hours in a 37°C shaking water bath. Digested tissue was washed 6 times by centrifugation at 4°C (1500rpm) using sterile PBS containing 0.5% FBS. The cells were plated in α-MEM containing 15% fetal bovine serum (FBS), penicillin/streptomycin (P/S), and Fungizone (≈one kidney/100mm dish with 15ml medium) at 37°C, in humidified air containing 5% CO_2. After three days, the plates were washed with sterile PBS, and media was changed. Cells were subcultured (1:3) on the following day. All cell cultures were maintained exponentially growing in α-MEM containing 15% FBS/P/S, and incubated at 37°C in an atmosphere of 5% CO_2 in air. Primary cells at passage 1-10 were used for experiments.

4.1.3 Protein Isolation

Total cell lysates were extracted as previously described, with modifications.[2] Approximately $1x10^7$ 75% confluent cells were harvested by trypsinization, followed by immediate enzymatic block using cold serum-containing media, and by two washes in cold PBS. Cells were resuspended in 100 µl of lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2mM EDTA, 2 mM EGTA, 25 mM NaF, 25 mM β-glycerophosphate, 0.2% Triton X-100, 0.3% NP-40, 0.1 mM Na-ortho-vanadate; freshly added 0.1 mM PMSF, 5 µg/ml leupeptin/aprotinin) and transferred to a 1.5 ml microcentrifuge tube. After a 10-minute incubation on ice with intermittent flicking of the tube, lysates were spun down at top speed for 10 minutes at 4°C. Supernatants were transferred to a new tube for storage at -80°C. Protein concentrations were determined by spectrophotometry using the Lowry-based BioRad DC Protein Assay (SmartSpec 3000 Spectrophotometer, #170-2501, BioRad).

4.1.4 SDS-Page/Western Blotting

Total DNA-PKcs protein levels were quantified as previously described with modifications.[3] Protein electrophoresis and transfer were performed in the X-cell SureLock Mini-Vertical Electrophoresis system per manufacturer's instructions (Invitrogen). After a 5-minute denaturation at 95°C in NuPage loading buffer containing 0.5 M DTT, 66 μg of total protein were electrophoretically separated on SDS/polyacrylamide gels (precast 4-12%, NuPAGE Novex Tris-Glycine, Invitrogen) at 110 V for 2 hours. Separated proteins were then transferred (43-hours, 10 volts, 4°C) to a nitrocellulose membrane in 20% methanol/TBST. After a 5-hour block in 7% non-fat dry milk/TBST at RT, membranes were probed with antibodies (mouse monoclonal anti-DNA-PKcs Ab-4 NeoMarkers and anti-actin C2, Santa Cruz-8432). Primary antibody (40μl of 200μg/ml in 8 ml TBST with 3% milk) was incubated on the membrane at RT for 2 hours. After 4 – 6 washes in TBST lasting a total of 30 minutes, the membrane was incubated with HRP-conjugated secondary antibody (goat antimouse F(ab') Amersham, 1:1000 in TBST/3% milk) at RT for 2 hours. ECL Plus™ western blotting detection reagent (ECL Plus, #RPN2106 Amersham Biosciences) was used to detect labeled protein by chemiluminescence using a Storm scanner (Storm 840 Phosphorimager, Amersham Pharmacia Biotech Ltd., Buckinghamshire, England). ImageQuant Software (Molecular Dynamics Version 5.1) was used for quantification of band fluorescence intensity.

4.1.5 Irradiation and Repair

Irradiations were carried out using a Shepherd 6000 Ci ^{137}Cs self-shielded cabinet irradiator (2.5 Gy/min). For studies of DNA DSB rejoining kinetics less than two hours, cells were irradiated on ice. For repair times longer than 2 hours, cells were irradiated at room temperature (RT). Repair was allowed to proceed at 37°C in air: CO_2 (95:5).

4.1.6 Immunofluorescence for γ-H2AX

Primary kidney fibroblasts obtained from 7-month-old virgin female mice at passage three were plated at high density in four or 8-well slide chambers (Falcon Culture Slides, #BD354108, BD Biosciences). Cultures grew to confluency within one day, and were irradiated (1 Gy, 2.5 Gy/min.) and incubated for repair. At each time point, slides were fixed with fresh 4% formaldehyde for 6 minutes at RT. After three 10 minute washes in PBS, cells were permeabilized in 0.2% Triton for 6 minutes and blocked in 5% milk for 30 minutes. Incubation in primary antibody (anti-γ-H2AX, 1:500 in 5% milk, #05-636, Upstate, Lake Placid, NY) was for one hour, at RT, with shaking, followed by 2 washes in PBS. Incubation in secondary antibody (goat anti-mouse, FITC-conjugated) was in 5% milk for 1.25 hours at RT, with shaking, in the dark. After four washes in PBS, chambers were removed; slides were mounted in anti-fade/DAPI and coverslipped. Signal was detected by epifluorescence imaging (Zeiss AxioScop, MetaSystems Isis FISH Imaging, Germany). Digital images were stored as ".tiff" files.(See DVD\DSB repair by gamma H2AX) Foci were enumerated in at least 100 randomly selected cells per group. Foci were all included in the enumeration since background was low and there was low variability in focus size.

4.1.7 Neutral Single Cell Gel Electrophoresis (Comet Assay)

Cells were irradiated (50 Gy, 2.5 Gy/min) in T25 flasks containing cell culture media. At each repair point, cells were evaluated in the Comet Assay using the Trevigen Comet Assay reagent kit as per manufacturer's instructions (Trevigen, #4250-050, Gaithersburg, MD). Briefly, cells were suspended in Trevigen LM Agarose and layered onto Trevigen glass slides. After a 30-minute incubation in cell lysis buffer, slides were subjected to a short electrophoretic run in neutral buffer (1x TBE). Electrophoresis causes the migration of unwound or fragmented DNA out of the nucleus of the cells, resulting in a characteristic comet-like appearance. Single strand breaks were excluded from the analysis by using neutral rather than alkaline buffer, so as to not denature dsDNA molecules. Slides were incubated in 70% ethanol for 5 minutes and air-dried. Slides were fluorescently stained with 25 µl 1000x SYBR Green immediately prior to image analysis. Measurement and quantitative analysis of an extended dynamic range of fluorescence intensities yielded measurements of cellular (tail and head) DNA content, distribution, and damage (Loats Associates, Inc. Comet Assay Analysis System, Westminster, MD). At least 100 randomly selected cells were analyzed per sample.

4.1.8 Modified FAR (Fraction of Activity Released) Assay: PFGE/Southern Blot

A flow diagram of the modified FAR assay procedure is presented in Figure 4.1. Cells were irradiated and incubated for repair in vented flasks or in agarose plugs suspended in media. At each repair time point, cells were harvested by trypsinization, blocked with cold 5ml 10% FBS α-MEM, and washed once with 10 ml serum-free α-MEM. The resultant pellet was resuspended in 0.6% Type IX-A agarose/serum-free

media for a final cell concentration of 1-5 x 10^5 cells/100μl plug, then embedded into a 100μl BioRad plug mold, and immediately cooled to solidify at 4°C (Agarose Type IX-A, Ultra-low Gelling Temperature, Sigma). Agarose plugs were then immersed in ice-cold lysis solution (0.01M Tris, 0.5M EDTA, 2% Sarcosyl, and 0.2mg/ml proteinase K) or in culture media for repair (2 plugs/ml lysis solution) for 1 hour on ice, followed by an overnight incubation at 37°C. For the initial repair point (0 hr), cells were embedded in agarose plugs, immersed in ice-cold lysis buffer, and irradiated while in lysis buffer on ice. After overnight lysis, plugs were washed three times in 1x TE buffer. To avoid streaking, plugs were incubated in electrophoresis buffer prior to PFGE (pulsed field gel electrophoresis).

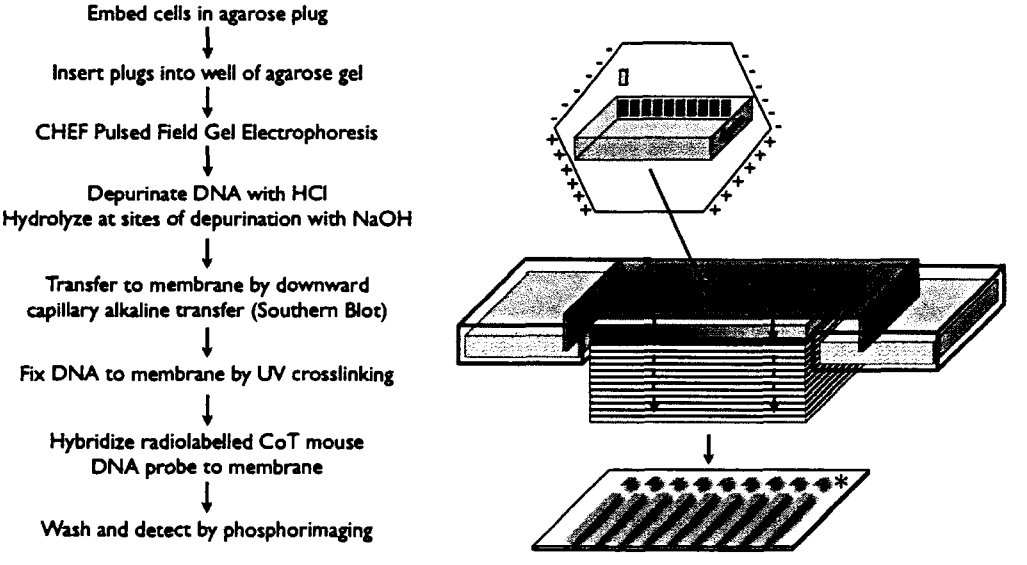


Figure 4.1: Diagrammatic representation of modified FAR assay (PFGE/Southern blot).

Plugs were inserted into the wells of a 0.7% high strength ultra-pure agarose gel. Empty well space was filled with the same agarose, and the entire gel was covered with a layer of agarose. PFGE was run on a contour-clamped homogeneous electric field (BioRad, CHEF II System, #170-3619) in 0.5x TBE buffer at 25°C using 40V, pulse

times of 75 minutes, and a total run time of 24 hours. This procedure separated DNA fragments by size, leaving intact or repaired DNA in the plugs (wells) while broken or unrepaired DNA migrated through the lanes.

The gel was then incubated in 0.25 M HCl for 15 minutes to partially depurinate the DNA. After several washes in H_2O, DNA in the gel was hydrolyzed at sites of depurination using 0.4 N NaOH for a 30-minute incubation, to yield 1.1 kb long DNA fragments that could be transferred to a GeneScreen membrane by downward capillary alkaline transfer (GeneScreen NEN Life Science Products). The blot was performed for 3 to 5 days using 0.4 M NaOH as a buffer. After transfer, the membrane was washed in 2x SSC for 1-2 minutes to remove residual agarose. DNA was fixed to the membrane by UV crosslinking (Stratalinker, exposure at 254 nm, 1200 $\mu W/cm^2$). After a 30 minute prehybridization at 42°C in fresh BLOTTO (50% formamide, 0.25% nonfat dried milk, 6x SSC), the membrane was probed overnight at 42°C with BLOTTO containing denatured [α-^{32}P]dCTP-CoT_1 mouse DNA (Gibco Standard labeling protocol 18187-013). Radiolabelled probe was generated by random priming of CoT_1 mouse DNA and purified with Qiagen Nucleotide Removal Kit. The membrane was washed twice in 2x SSC and once in 2xSSC/1% SDS for 30 minutes at 42°C. A phosphoimaging screen was exposed to the radiolabelled membrane and analyzed on a Storm phosphorimager. Analysis was volume per area, with background correction. Using the generated volume reports, fraction of activity released was calculated as [lane]/[lane + well].

4.1.9 Clonogenic Survival

Cells were plated at $5x10^5$ cells/T75. When cells reached 90% confluency (exponentially growing), media was changed to IL-MEM (Isoleucine-deficient media; Media: Gibco #04-5033EL, Additives: 10% triple dialyzed FCS, P/S). IL-MEM was changed after one population doubling times. After 2 population doubling times, synchronized cells were harvested by trypsinization, washed in 20% FBS alpha-MEM, and resuspended in 20% FBS alpha-MEM for a final concentration of $1x10^4$ cells/ml. Cells were aliquoted for irradiations according to the table below (table 4.1). Irradiations occurred in a sequential/additive manner at RT and cells were immediately plated. Plating densities were determined based on results from preliminary experiments such that for each dose, there would be 50-100 colonies/plate. All points were done in triplicate. Media was changed after 10 days. After 21 days, plates were stained with 1% crystal violet in 70% EtOH. Colonies were included in enumerations if they contained more that 50 non-pyknotic cells.

DOSE (Gy)	Cells to plate	**Vol. to add /plate**	Aliquot vol. (ml)	media/plate
0	2000	**200 µl**	0.6 or 0.9	19.8 ml
0.5	4000	**400 µl**	1.2 or 1.8	19.6 ml
1	6000	**600 µl**	1.8 or 2.7	19.4 ml
2	10,000	**1 ml**	3 or 4.5	19 ml
4	14,000	**1.4 ml**	4.2 or 6.5	18.6 ml
6	20,000	**2 ml**	6 or 9	18 ml
Total/strain	56,000*3=168,000	5.6 ml * 3 = 16.8 ml	16.8 or 25.4	54.4*3 = 170ml

DOSE (Gy)	Cells to plate	**Vol. to add /plate**	Media/plate
0	1000	**100 µl**	19.9 ml
0.5	2000	**200 µl**	19.8 ml
1	3000	**300 µl**	19.7 ml
2	5,000	**500 µl**	19.5 ml
4	7,000	**700 µl**	19.3 ml
6	10,000	**1 ml**	19 ml
Total/strain	28,000 * 3=84,000	2.8 ml * 3 = 8.4 ml	57.2 ml * 3 = 171.6ml

Table 4.1: Clonogenic survival assay set-up.

4.1.10 Gene Expression Microarray

Tissue from virgin female mice aged 2 to 3.5 months was harvested 4 hrs after irradiation (2 Gy, 2.5 Gy/min). Three mice per strain were irradiated simultaneously in 50 ml polyethylene tubes. Livers and spleens were incubated overnight in 2 ml RNA Later (rotating shaker, 4°C). Tissues were submitted to Dr. M. Story at the Univ. of Texas Southwestern Medical Center. Samples were hybridized to Illumina whole genome expression BeadChip Mouse-6 V1.1 arrays. Pair-wise comparisons for statistical significance were done using Significance Analysis of Microarrays, Stanford, CA.

4.1.11 Reproductive Physiology

The congenic and parental strains were assessed for breeding characteristics, including number of litters and litter size. Breeding rounds per breeder pair numbered from 0 to 10 and lasted an average of 4.6 months (1–14 mo). Animal age and breeding time were calculated on the basis of documented breeder set-up dates, birth dates, and breeder pair termination dates. Because deaths prior to weaning were not documented, the number of litters per breeder pair and litter size were calculated based on progeny known to have survived after weaning. Matings were included in an analysis only if all data was available and if breeders homozygous at *Prkdc*. Consequently, the results presented do not constitute a comprehensive analysis of all matings performed during the course of this study. Also, results from inbreedings and backcrosses were pooled and classified according to the strain of either the male or of the female partner. The number of litters was corrected for breeder time spent together (months) and for the number of females per cage (1 or 2) by dividing the total number of litters by the number of months spent together and by the number of females per breeder cage.

4.2 Results

4.2.1 ***Prkdc^{BALB} is required and sufficient to decrease DNA-PKcs protein expression***

Cells isolated from BALB/c mice have low DNA-PKcs protein levels and low DNA-PKcs kinase activity, which correlate with polymorphisms in *Prkdc*. Since the polymorphisms and decreased kinase activity are both unique to BALB/c mice, it is reasonable to predict that *Prkdc^{BALB}* may be directly responsible for the altered phenotype. Analysis of congenic strains allowed us to confirm that *Prkdc^{BALB}* alone, without requirement of or contribution from modifier BALB/c genes, is mot likely to be responsible for decreased DNA-PKcs levels. DNA-PKcs expression was quantified by SDS-PAGE/western blot using kidney fibroblasts isolated from the congenic strains B6.C-*Prkdc^{BALB}* and C.B6-*Prkdc*. Low DNA-PKcs protein expression in B6.C-*Prkdc^{BALB}* and BALB/c indicates that *Prkdc^{BALB}* is sufficient to decrease DNA-PKcs protein expression (figure 4.2 and table 4.2).

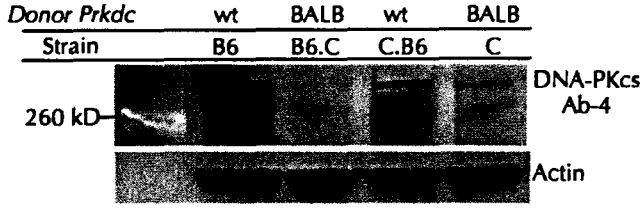


Figure 4.2: Total DNA-PKcs expression in congenics as determined by western blot.

Strain	Volume	Background	%	Bckgrnd Type	Vol. (Bckgrnd-Corr'd)
B6	428849.81	8018.17	25.2	Local Average	420831.64
B6.C	18317.97	3986.307	1.08	Local Average	14331.66
C.B6	1134711.76	6875.8	66.67	Local Average	1127835.96
C	120221.1	4406.771	7.06	Local Average	115814.33

Table 4.2: Chemiluminescence Band Intensity of Western Blot for DNA-PKcs in Congenic Strains– ImageQuant-generated volume report. (Intensity units are relative pixel intensity (unitless) generated by ImageQuant software)

The present result demonstrating that *Prkdc*^{BALB} is responsible for diminished DNA-PKcs protein expression in BALB/c mice confirms previous observations in CXB strains (figure 2.11). Given that no differences in *Prkdc* mRNA expression have been observed between BALB/c and C57BL/6,[4] diminished transcription is not involved. Wild-type DNA-PKcs is constitutively expressed, activated by damaged DNA, and inactivated by autophosphorylation. The two BALB/c amino acid substitutions could reduce DNA-PKcs expression and kinase activity via numerous mechanisms. The amino acid conversions could have a direct effect on function or could alter the tertiary structure of the protein. Similarly, the truncating mutation in *Prkdc*^{scid} is known to result in decreased overall kinase activity, however the precise mechanism has not been demonstrated.[5] Potential mechanisms include decreased protein stability or increased protein degradation, decreased DNA binding affinity, decreased specific kinase activity, altered autophosphorylation, or inhibition of dephosphorylation. These mechanisms are not necessarily mutually exclusive as one may affect the other. For example, excessive autophosphorylation could induce protein degradation as well as transcription levels. Future studies should aim to quantify DNA-PKcs specific kinase activity and protein metabolism. Challenges that must be overcome will include the large size and very low levels in mouse tissue of DNA-PKcs. These issues will best be addressed using the congenic strains developed here.

4.2.2 Prkdc^{BALB} is required for deficient radiation-induced DSB rejoining in BALB/c

Cells isolated from BALB/c mice have slow post-irradiation DNA DSB repair kinetics due to a deficiency in the initial rapid phase of repair by NHEJ, in which DNA-PKcs participates. Since functional DNA-PK is required for efficient DSB repair by

NHEJ, polymorphisms resulting in a hypomorphic variant are likely to be involved. However, the precise contribution of the *Prkdc* allelic variant, in contrast to other genes in the BALB/c genome, was not clear. Using congenic mice derived from the susceptible and resistant strains, we proceeded to examine whether $Prkdc^{BALB}$ is responsible for diminished radiation-induced DNA damage repair. If $Prkdc^{BALB}$ is solely responsible for diminished DNA repair capabilities in BALB/c mice, C.B6-*Prkdc* mice should not demonstrate DNA repair dysfunction whereas B6.C-$Prkdc^{BALB}$ mice should. To directly determine the role of $Prkdc^{BALB}$ in altered repair of radiation-induced DNA damage we examined DNA DSB rejoining in the congenic mice using three assays: disappearance of γ-H2AX foci, neutral comet assay, and a modified FAR assay.

4.2.2.1 $Prkdc^{BALB}$ and Post-Irradiation Disappearance of γ-H2AX Foci

Upon exposure of cells to IR, DNA damage signals are activated that recruit repair elements to the site of damage.[6, 7] One of the earliest events is the phosphorylation of H2AX. H2AX is an isoform of the canonical core histone H2A.[8] Its levels vary from 2 to 25% of the histone H2A pool. Like other H2A histones, it can be phosphorylated on Ser1, acetylated on Lys5, and ubiquitinated on Lys188.[9] The X isoform of H2A has a short COOH terminal tail with a highly conserved serine residue (Ser139) that is rapidly phosphorylated upon exposure to IR. After a wave of phosphorylation that spreads to each side of the break, phosphorylated H2AX (γ-H2AX) can be observed as nuclear foci in the ~2Mb region flanking DSB.[10] Phosphorylation of H2AX causes restructuring of chromatin to concentrate and retain proteins and DNA ends in the vicinity.[11] Gamma-H2AX foci colocalize with and precede the formation of IR-induced foci (IRIF, micro-

domains containing 100's to 1000's of molecules such as ATM, BRCA1, 53PB1, MDC1, RAD51, and MRN, that accumulate around DSBs).[10, 12] In yeast, elimination of the H2AX tail impairs NHEJ. In p53 knockout mice, H2AX deficiency causes radiosensitivity, chromosomal instability, and tumor susceptibility, emphasizing the importance of this signal in DNA homeostasis.[13-16]

Some studies have shown a linear, one-to-one relationship between the number of γ-H2AX foci and the number of DSBs from 1mGy to 100Gy (35 DSB/Gy).[17, 18] Foci are also induced by replication-associated breaks, apoptosis, dysfunctional telomeres, meiotic recombination, and CSR (class switch recombination).[19] As DSBs are repaired, γ-H2AX foci disappear, presumably via dephosphorylation. The persistence of γ-H2AX foci at unrepaired breaks provides a useful indicator of repair.

To evaluate DNA DSB rejoining kinetics in our congenic mice, we quantified γ-H2AX foci in cells incubated for repair after exposure to IR. BALB/c and B6.C-*Prkdc*^{BALB} tended to have more residual foci than C57BL/6J and C.B6-*Prkdc* after a 4-hour incubation for repair (figures 4.3 and 4.4). The distribution of remaining foci was also skewed, as cells from BALB/c and B6.C-*Prkdc*^{BALB} often contained very high numbers of residual foci and larger foci (not quantified, pictured in Appendix Figure 2). The kinetics of focus disappearance appear similar between the 1 hour and 4 hour time points, suggesting that induction of foci is higher in BALB/c and B6.C. Alternatively, rapid DSB repair and disappearance of foci in C57BL/6 and C.B6 prior to the 1-hour time-point may account for the differences in focus induction. This would be consistent with the slow kinetics of repair observed in DNA-PKcs-deficient cell lines.[20] Experiments enumerating γ-H2AX foci at 15, 30, and 45 minutes post-IR would help

confirm whether or not DSB repair with fast kinetics occurs within 1 hour of irradiation in C57BL/6 and C.B6, but not in BALB/c and B6.C.

Since induction of foci at the one-hour time point is adequate in BALB/c and B6.C, altered DNA repair in these strains is not likely to be a consequence of diminished H2AX phosphorylation. H2AX is phosphorylated at an SQE motif, which is the consensus *in vitro* PIKK phosphorylation sequence.[15] ATR-Chk1 phosphorylate H2AX at stalled replication forks and in response to UV light.[21, 22] ATM phosphorylates H2AX in response to uncapped telomeres[19] and meiotic recombination.[23] Although DNA-PK knockouts have normal H2AX phosphorylation in response to DSB, phosphorylation in response to IR occurs by either ATM or DNA-PKcs.[23] Inhibition of both kinases by wortmannin inhibits H2AX phosphorylation.[23, 24] Many genotoxins have been reported to induce H2AX Ser139 phosphorylation.[25] Its use as a specific equivalence marker of IR-induced DSBs is often questioned since the DSBs induced by IR do not always correlate with foci in terms of complexity, location, and timing.[26-28] Furthermore, dephosphorylation by HTP-C does not occur immediately after DSB repair.[29, 30] All the same, DNA-PKcs does seem to play a role in H2AX phosphorylation in response to IR, and our results support this hypothesis. Furthermore, the functional variant $Prkdc^{BALB}$ is associated with diminished resolution of γ-H2AX foci. This may represent failure to rejoin broken ends by NHEJ, or inhibition of γ-H2AX dephosphorylation.

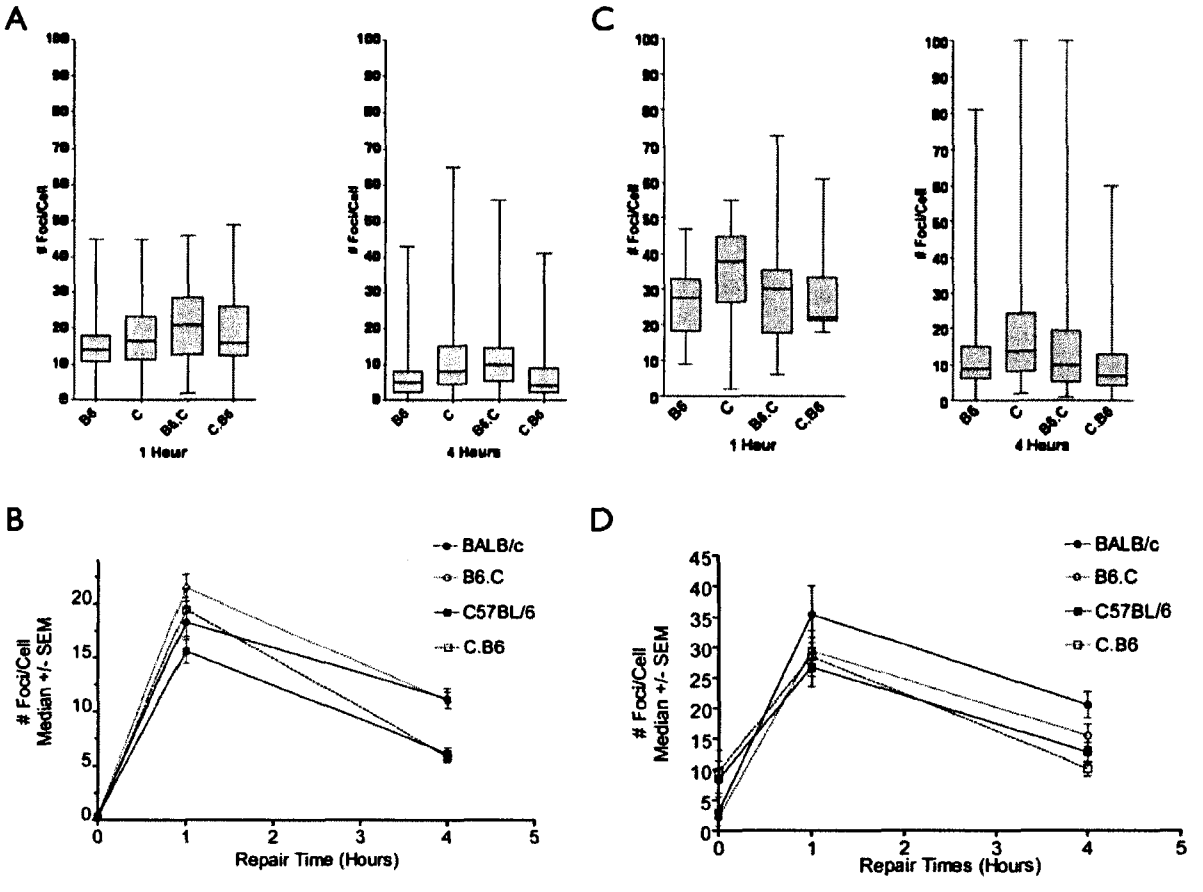


Figure 4.3: Disappearance of γ-H2AX foci over time (4 hours). Results from two separate experiments: Exp.1 (left, A and B) and Exp.2 (right, C and D). Box-Whisker Plots (A & C) indicate distribution of number of foci/cell (maximum, 3rd quartile, median, 1st quartile, and minimum).

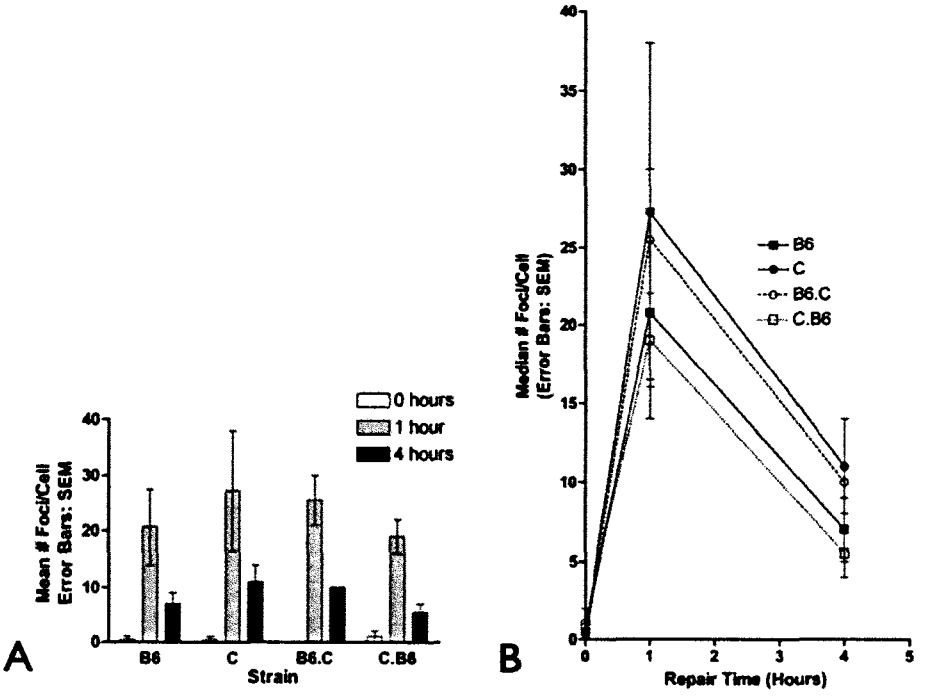


Figure 4.4: Disappearance of γ-H2AX foci after exposure to 1 Gy (2.5 Gy/min). Mean of experiment 1 and experiment 2 from figure 4.3. Primary kidney fibroblasts were irradiated in flasks and allowed to repair at 37°C. γ-H2AX foci detected by immunofluorescence were enumerated in at least 100 cells. (A) according to strain. (B) over time.

4.2.2.2 Neutral Comet Assay

We examined DSB repair using neutral single cell gel electrophoresis (comet assay). In this assay, single cells are evaluated for DSBs according to the propensity of DNA to be eluted from the nucleus by an electric field. This occurs primarily due to break-induced relaxation of the DNA super-helix. A neutral electrophoresis buffer prevents DNA denaturation to distinguish DSBs from S-phase breaks and single stranded breaks. In a preliminary study comparing %Tail DNA and Tail Moment we found less heterogeneity (smaller CV) among triplicates for %Tail DNA. Tail moment is an

analytical strategy that takes into account the distribution of DNA in the tail (tail length). It is calculated as the percent DNA in the tail multiplied by the distance between the means of the head and tail distributions.[31] In this author's opinion, tail moment has less specificity for double strand breaks because it is highly dependent on random DNA supercoil relaxation as opposed to numbers of breaks. Percent tail DNA is less influenced by supercoiling. This is supported by our observation that between sample variation is greater for tail moment than for %Tail DNA. Tail moment is measured in arbitrary units that differ between image analysis systems, whereas % Tail DNA is more meaningful, easier to conceptualize, and comparable between studies performed on different systems. In view of this, %Tail DNA was the quantitative method selected for our analysis. Percent DNA in comet tails increased exponentially with dose, with significant overlap between dose groups, particularly at low doses. This degree of overlap brought into question the utility of this assay in detecting subtle differences in repair. In our hands, the best approach for detecting differences in repair by comet assay was with an exposure of 50 Gy, and comparison of medians between strains (fig. 4.5).

Using the neutral comet assay we were able do detect slight differences in DSB levels after 4 hours of repair in BALB, indicating deficient repair of DSB in BALB/c relative to C57BL/6. The magnitude of differences between BALB/c and C57BL/6 were small and difficult to resolve, emphasizing the low inherent sensitivity of this assay.

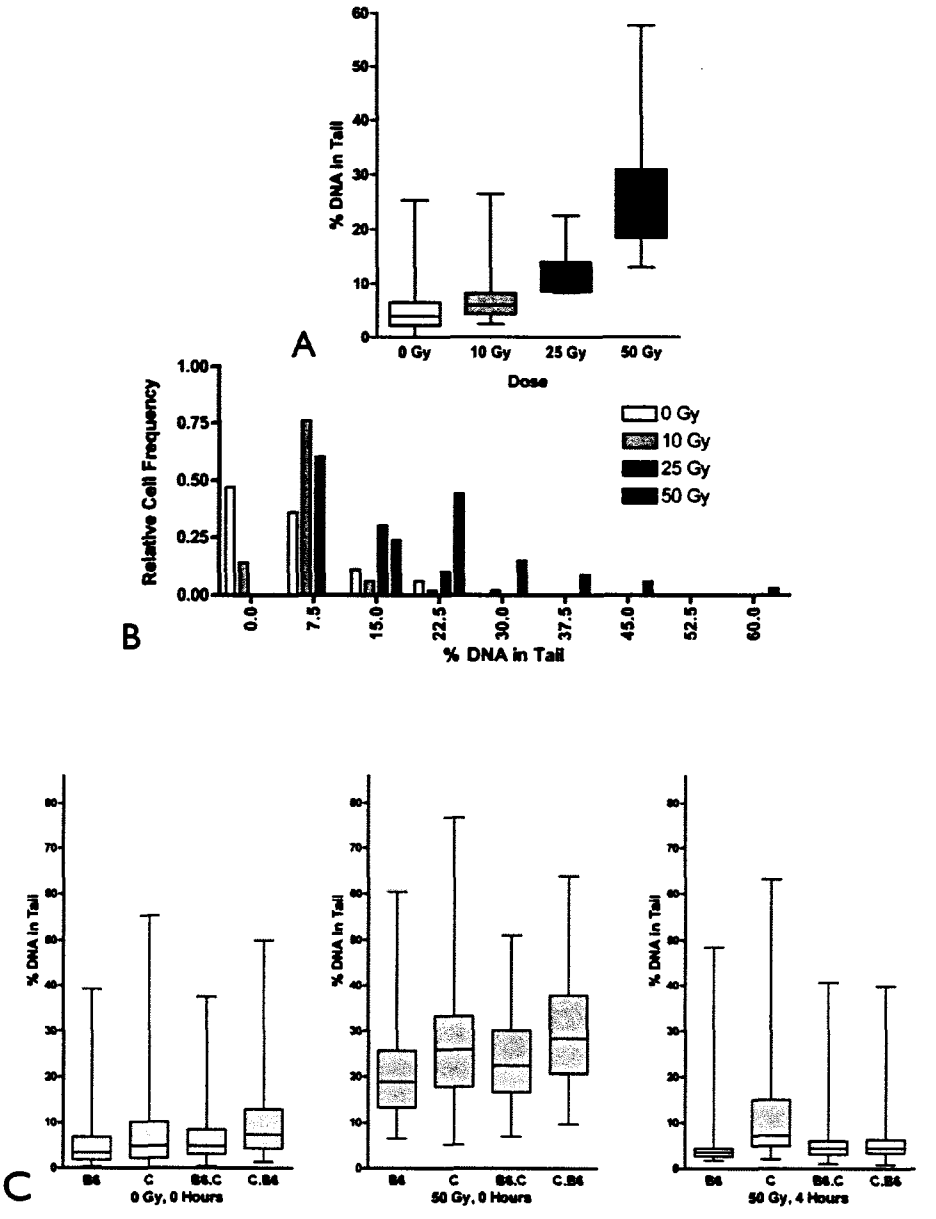


Figure 4.5: Neutral Comet Assay. (A & B) Dose response curve using the neutral comet assay. (A) Box-Whisker Plot indicating distribution of number of %DNA in Tail (maximum, 3rd quartile, median, 1st quartile, and minimum). (B) Frequency distribution for dose response. (C) Box-Whisker Plots of %Tail DNA according to strain. Left: background %DNA in Tail. Middle: Induction by 50Gy. Right: % DNA in Tail after 4-hour repair.

No differences were observed in the congenic strains using this assay, which conflicts with results obtained using the γ-H2AX focus disappearance assay. Many possibilities could account for this discrepancy. One of them is that modifier BALB background genes in addition to *Prkdc* are required for impaired DSB rejoining but not for disappearance of foci. Another possibility is that real differences in end joining could not be discerned due the technical limitations of this assay. The ability to analyze single cells has the advantage of revealing subpopulations of susceptible cells. However, the drawback is that relatively few cells can be evaluated, not providing sufficient statistical power for detection of differences in heterogeneous samples with overlap between groups. In fact, random heterogeneity between experimental units is a common feature of this assay.[31] Another limitation of the comet assay is the requirement for viable single cells (non-apoptotic or necrotic) that have not been heavily damaged during cell preparation. Though primary kidney fibroblasts do not have a tendency to undergo apoptosis after irradiation, their harvesting requires aggressive trypsinization that may damage a proportion of cells. The comet assay is particularly sensitive to cellular damage incurred during tissue disaggregation.[32] The γ-H2AX focus disappearance assay may be more reliable because it does not require that cells be harvested or manipulated. The disadvantage of the focus disappearance assay is that it is an indirect method for quantifying DSBs. Finally, the comet assay provides no information on fragment size. Elution of DNA out of the nucleus is a function of supercoil relaxation, not fragment size. Factors affecting DNA supercoiling as opposed to actual breaks could further obscure results.

4.2.2.3 DSB Repair by FAR Assay

The method of choice for quantification of double strand breaks in mammalian cells is pulsed-field gel electrophoresis (PFGE). This assay quantifies the amount of DNA DSB damage in cells embedded in agarose and assayed for migrating DNA using gel electrophoresis. It can be considered a bulk method for performing the comet assay on millions of cells in a single true measurement of DSBs. PFGE can resolve molecules 5 kbp up to 5.7-10 Mbp, much larger than is possible under a unidirectional field system. PFGE can be used to separate the large DNA fragments induced by radiation from intact or repaired DNA. Post-irradiation DSB repair kinetics can be determined by measuring the time-dependent decrease in the fraction of fragmented DNA that is eluted into an electrophoresis gel.[33, 34]

The contribution of *Prkdc*^{BALB} to slow repair was assessed by modified FAR assay in both congenic strains and in the parent inbred strains. Specific protocols differ in their DNA detection methods, cell lysis protocols, electrophoresis, and in their DSB frequency quantification methods. DNA can be radiolabelled with ^{14}C prior to irradiation and repair, or it can be detected after the PFGE by fluorometry (SYBR Green/EtBr) or by Southern blot. Cell lysis can be performed cold instead of hot to avoid break formation at heat-labile sites and to better document fast repair kinetics. Many calculations also exist for quantifying DSBs. The protocols used depend on the model system and experimental requirements. We employed the standard quantification approach, which is the FAR (fraction of activity released) method. This method measures the amount of DNA migrating into the lane relative to the total amount of DNA (DNA_{lane} + DNA_{well}). The total amount of DNA can also be determined by running a paired plug irradiated at 600

Gy with each test sample. To avoid direct damage to DNA caused by radioisotopes, we chose to label the DNA after electrophoresis. We quantified DNA by fluorometry of EtBr-labelled gels or by phospholuminometry of Southern blots.

Quantification of total DNA in plugs by fluorescence imaging of a plug irradiated with 600 Gy was not useful in our hands due to variability in area to be quantified from one gel to the next, and to the relatively high volume (intensity) reported using this method. We found that the results obtained using fluorescence intensity on a Storm imager were highly dependent on surface area analyzed; as surface area increased, intensity increased dramatically, even with background correction. Values obtained differed dramatically (orders of magnitude) between test lanes and 600Gy plugs samples. Analysis of two PFGE/fluorometry gels by measuring amounts of DNA in lanes only revealed variable induction of breaks and differences at the 4 hours repair time point. Differences in repair between our control samples, BALB/c and C57BL/6, were observed. Without normalization for maximum break induction before repair, C.B6 repaired DSB as effectively as C57BL/6 (fig. 4.6). B6.C had an intermediate repair phenotype between BALB/c and C7BL/6. With normalization for maximum break induction before repair, differences between BALB/c and C57BL/6 were still observed, but both congenic strains seemed to have deficient repair compared to C57BL/6 (fig. 4.7). This observation is the result of wide differences in measurement of induced breaks. Using this technique, it was not clear how results should be interpreted. We decided not to pursue this approach, feeling that fluorometry for EtBr-stained DNA was not the best DNA quantification technique due to saturation effects seen in wells, and the inability to measure actual total amount of DNA with the test sample plug.

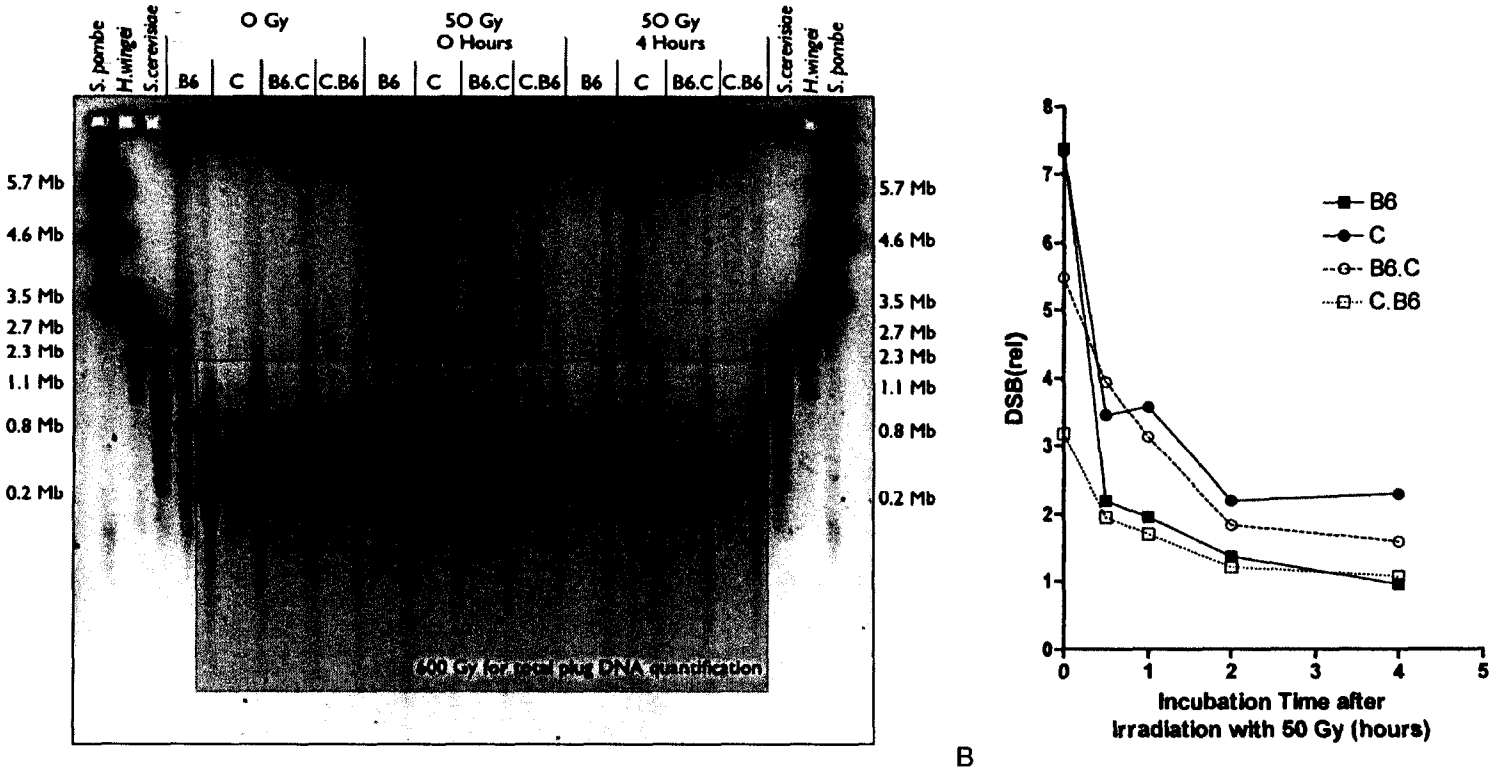


Figure 4.6: Modified FAR Assay (Exp.1). Total DNA in plugs quantified by fluorescence imaging of a paired plug irradiated with 600 Gy. (A) Electrophoretogram showing paired plugs. (B) Double strand breaks (after background correction) decrease with repair time. Repair in BALB (C) is deficient when compared to C57BL/6 (B6) and C.B6. B6.C has intermediate repair.

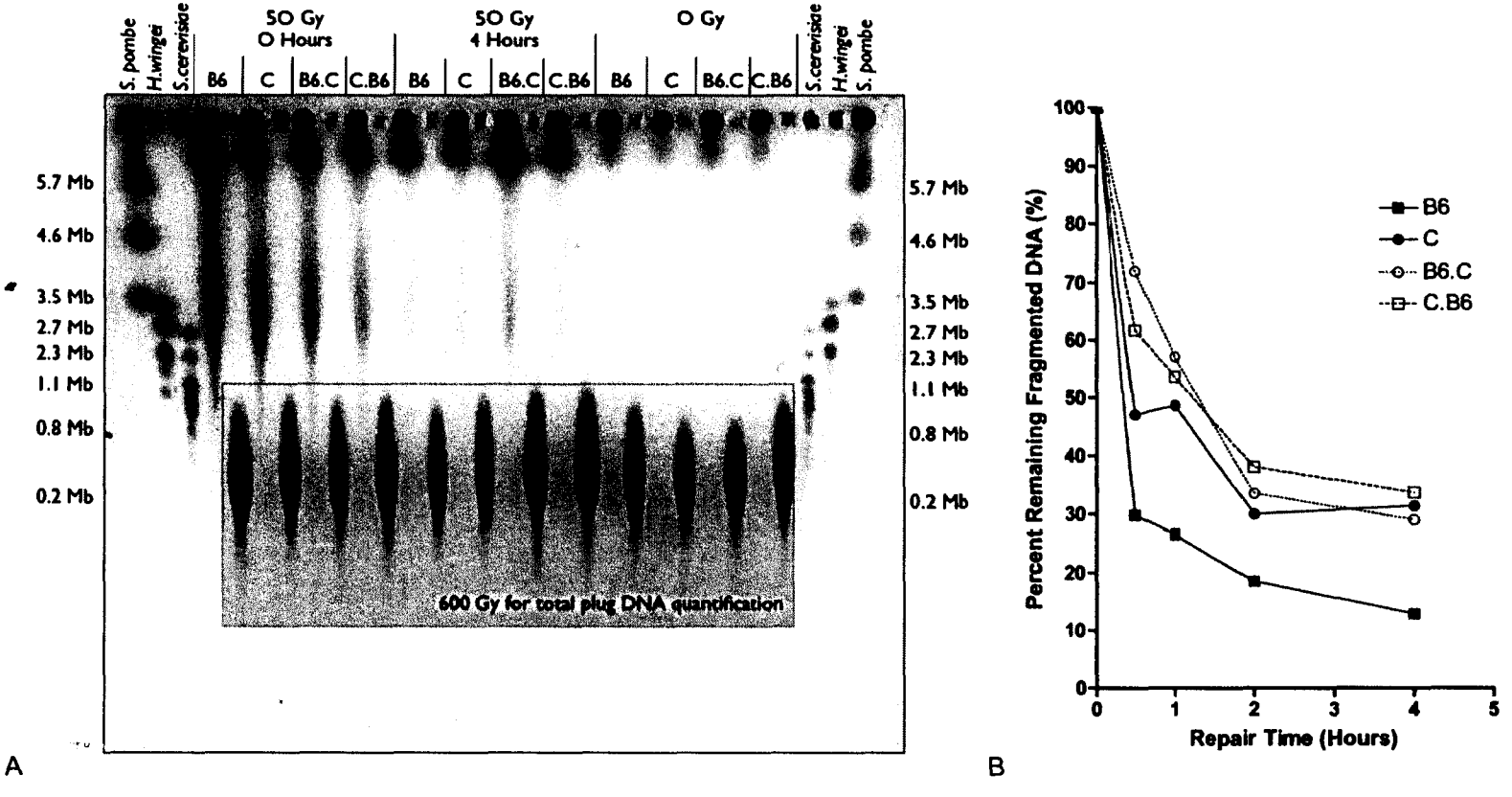
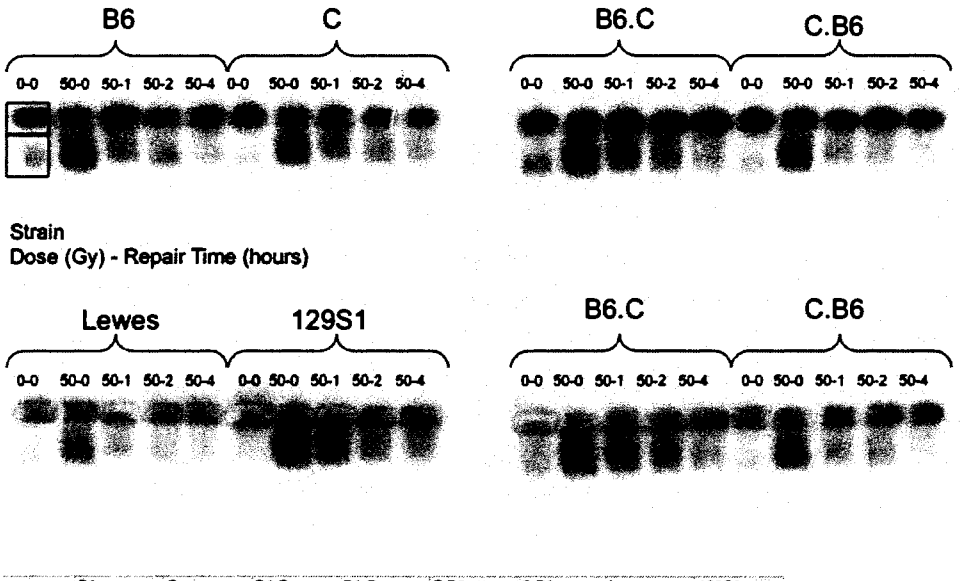


Figure 4.7: Modified FAR Assay (Exp 2). Total DNA in plugs quantified by fluorescence imaging of a paired plug irradiated with 600 Gy. (A) Electrophoretogram showing paired plugs. (B) Percent DNA remaining in lane normalized to maximal break induction prior to repair. Repair in BALB (C), B6.C and C.B6 is deficient when compared to C57BL/6 (B6)

Probing for DNA after Southern blot of PFGE gels is a rapid technique that minimizes use of radioisotope, is highly sensitive and accurate, and does not expose cultured and repairing cells to IR. Use of a short electrophoretic protocol also diminishes quantification artifacts from surface area differences (figure 4.8A).

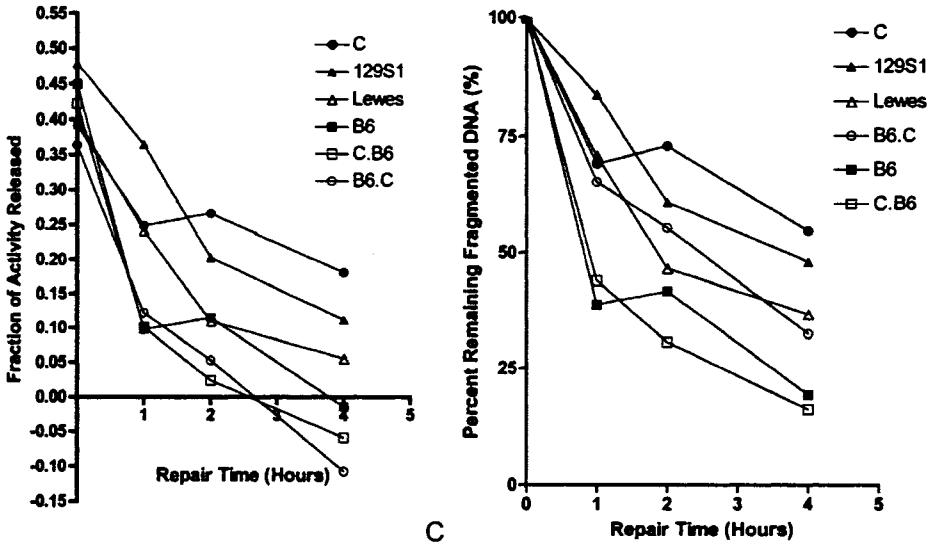
Interpretation of results was highly dependent on the quantitative method used. Background correction consisted of an initial subtraction of intensity in a lane and well of equal volume as those for test samples using a blank plug, followed by subtraction of intensity in the 0 Gy lane for each sample. This yielded fraction of activity released, as seen in figure 4.8B. Kinetics of repair could be compared between strains by performing a transformation of data, normalizing for maximal breakage induced with 50 Gy. After normalization, C.B6 followed the same repair kinetics as C56BL/6 and B6.C followed intermediate repair kinetics (figure 4.8C). This result could not be repeated in subsequent gels due to technical difficulties (uneven probe binding onto membrane).

Taken together, the DSB repair findings from γ-H2AX focus disappearance, neutral comet assay, and FAR assay seem to suggest that repair in C.B6-*Prkdc* is not diminished. Thus, $Prkdc^{BALB}$ appears to be responsible for diminished repair in BALB/c. Results for B6.C were rather equivocal, ranging from normal to intermediate or deficient, depending on the assay used. This could be due to technical difficulties encountered in the development of each assay, to the different endpoint actually measured, or to the multiple functions of DNA-PKcs and its BALB/c variant. It is likely that some of the functions of DNA-PKcs rely on interactions with modifier genes in the BALB/c genome, which were not present in the B6.C-$Prkdc^{BALB}$ mice. This might be reflected in the variability of our observed results.



	B6	C	B6.C	B6.C	C.B6	C.B6	Lewes	129S1
-1	21.87625	15.89788	32.37781	63.22202	17.01071	35.79827	26.44355	32.24894
0	100	100	96.87544	103.1245	87.53593	112.4641	100	100
1	38.73497	69.14825	49.7205	80.85137	39.38681	48.53236	70.85326	83.87553
2	41.64489	72.94572	39.65887	71.10506	23.48228	37.69706	46.63479	60.89109
4	19.28907	54.70929	19.99427	44.83302	14.0624	18.18657	36.63111	48.06223

A



B C

Figure 4.8: Modified FAR assay: PFGE followed by Southern blot. (A) Membrane probed with radiolabelled mouse CoT DNA shows decreased DNA in lane with increasing DNA repair time in each strain. A shorter run time minimizes error due to different surface area between lane and well. Quantification of DNA was by phosphorimaging (table) to yield relative luminescence. (B) Absolute fraction of activity released after background correction. (C) Normalized FAR after background correction.

Among over 100 inbred strains evaluated, the M3844V SNP in *Prkdc* has only been detected in the recently derived LEWES/Ei and DDO strains and in the old laboratory strain DW/J. (Unpublished data, M. Weil) We evaluated DSB repair using the FAR assay (Southern blot technique) in Lewes and 129SS1/SvImJ strains. Lewes had an intermediate phenotype while 129S had BALB/c-like. This is consistent with intermediate and diminished DNA-PKcs expression levels detected by western blot in Lewes and 129S1/SvImJ, respectively (Kristin Askin, unpublished data).

4.2.3 $Prkdc^{BALB}$ is required and sufficient to decrease clonogenic survival in BALB/c

To further characterize radiosensitivity in the congenic strains, we evaluated clonogenic survival in primary fibroblasts after exposure to increasing doses of IR. Significant differences in survival between BALB/c and C57BL/6 were observed at all doses (figure 4.9). After 1 Gy, the difference between the two phenotypes only marginally increased with increasing dose. Survival in the congenic strains was a direct function of the donor *Prkdc* allele. Radiosensitivity in B6.C-$Prkdc^{BALB}$, like BALB/c, was significantly greater than C57BL/6, indicating that $Prkdc^{BALB}$ alone can reduce cell survival. In contrast, C.B6-*Prkdc* was less radiosensitive than BALB/c, with sensitivity following C57BL/6, indicating that $Prkdc^{BALB}$ is required for the clonogenic radiosensitivity phenotype in BALB/c mice.

Clonogenic survival is an endpoint that implicates many cellular functions. Cells may undergo reproductive death due to permanent cell cycle arrest because of mis/unrepaired DNA, they may have entered apoptosis in response to IR, or improper rejoining of broken ends may have resulted in lethal genetic mutations. DNA-PKcs is

involved in all of these cellular processes. However, it has previously been shown that cell killing is directly linked to the induction of DSB and dicentrics.[35] Therefore, replicative cell death due to un/misrepaired DNA is the most likely cause of clonogenic radiosensitivity in *Prkdc*$^{BALB/BALB}$ cells.

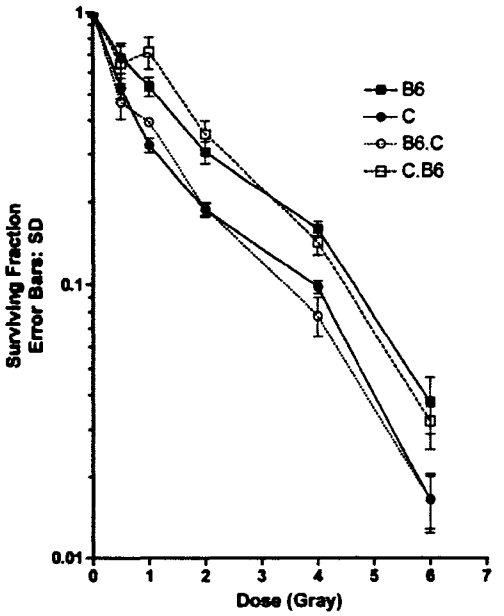


Figure 4.9: Clonogenic survival of primary kidney fibroblasts is linked to *Prkdc* allele.

4.2.4 Differential Gene Expression

Multiple signaling pathways are activated in the complex molecular responses to genotoxic stresses such as DNA damage. Radiation induces changes in gene expression at very low doses that produce little toxicity, indicating that surviving cells contribute to the radiation response. This is particularly relevant for human populations exposed to background radiation. Deficits in the ability to induce the proper transcriptional responses could render some individuals more susceptible to the damaging effects of radiation.

Transcriptional responses occur in genes involved in a generalized response to cellular injury, with altered expression of p53-responsive proteins (CDKN1A/p21,

GADD45), growth factors (MAP kinase pathway, EGFR), cytoskeletal elements, and cytokines (TNFα, IL6, TGFβ), as well as in DNA repair genes. Radiation causes p53-mediated upregulation of *CDKN1A, GADD45A,* and *MDM2* to induce the requisite cell cycle delays, apoptosis, and p53-regulatory feedback. [36] The expression profile is dependent on dose, dose-rate, and time, with immediate as well as delayed gene expression changes. Dose rate-dependent changes tend to induce apoptosis whereas dose-rate independent changes involve cell cycle regulation.[37]

Given the complexity of DNA-PKcs and its potential role in regulating gene expression, it became relevant that we examine effects on as many target and downstream genes as possible using cDNA microarray technology. Mice were irradiated with 2 Gy of γ-rays (2.5Gy/min). Spleens and livers harvested 4 hours after irradiation were probed by microarray for differences in gene expression. Hierarchical clustering analysis confirmed our suspicions that tissue and genetic background would be the strongest determinants of global gene expression (figure 4.10).

Interestingly, donor *Prkdc* allele was a stronger determinant of global gene expression than was exposure to radiation, indicating that $Prkdc^{BALB}$ has a greater impact on overall gene expression than does exposure to 2 Gy of IR. Thus the consequences of $Prkdc^{BALB}$ appear to extend beyond the ability to properly signal radiation responses. Contaminating donor genes within the congenic region would also be responsible for this effect if they were polymorphic between BALB/c and C57BL/6. This remains to be determined.

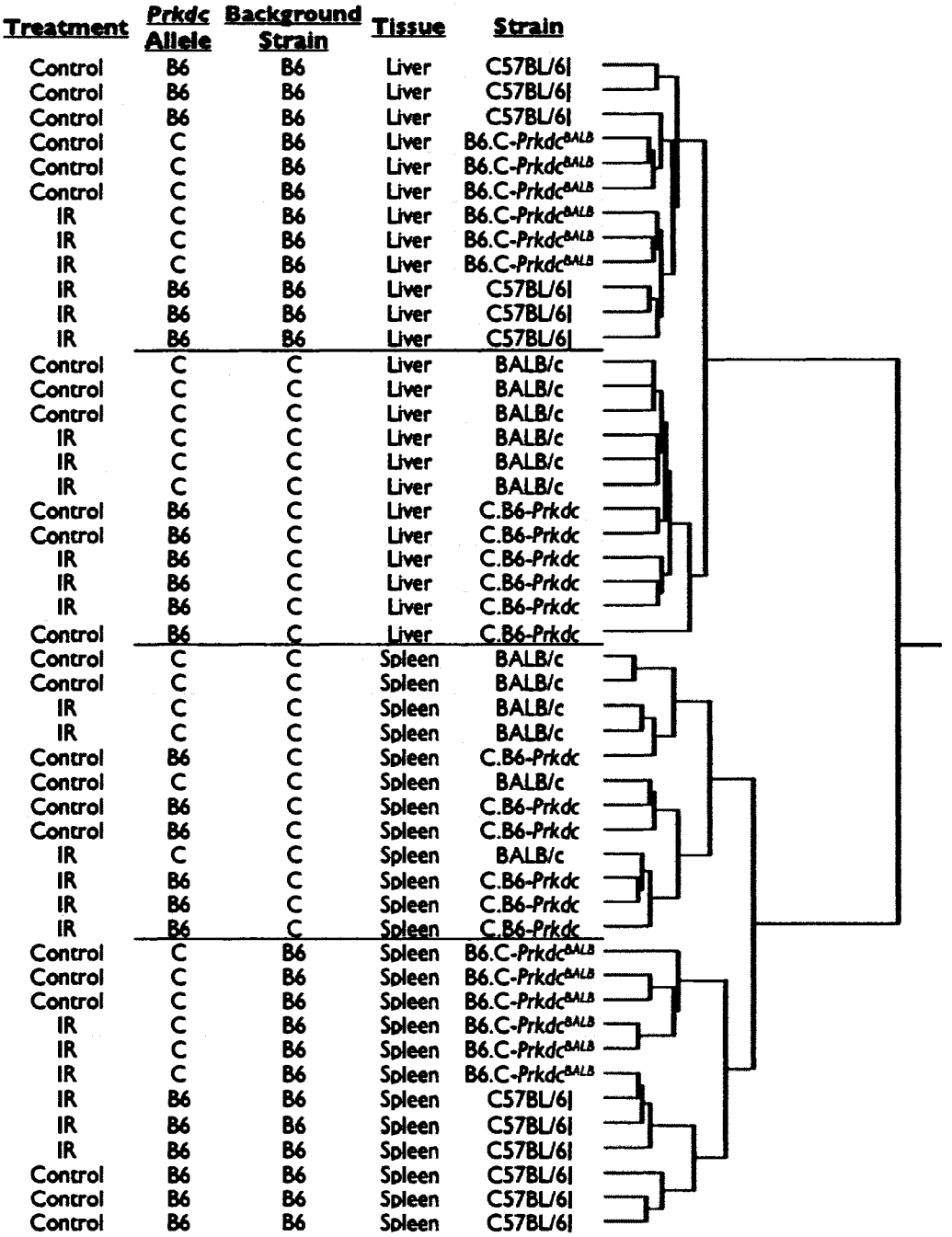


Figure 4.10: Hierarchical clustering of microarray gene expression results. Tissue and genetic background are the strongest determinants of global gene expression, followed by donor *Prkdc* allele and radiation exposure.

We probed unirradiated mice for genes that are differentially transcribed between each congenic and its inbred partner. Differences were observed in constitutively expressed genes for each congenic and inbred recipient pair (figure 4.11). These differences reflect the radiation damage-independent functions of DNA-PKcs, and further demonstrate that the BALB/c variant alters cellular functions in the absence of radiation.

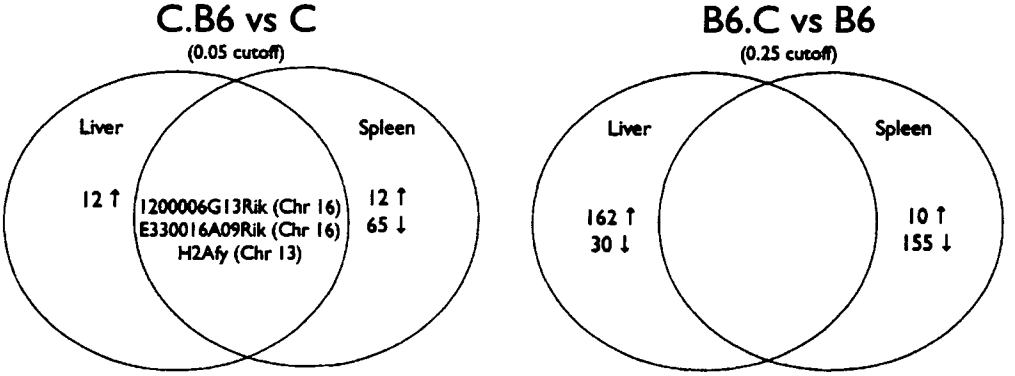


Figure 4.11: Venn diagram showing the number of differentially transcribed constitutively expressed genes between each congenic and its inbred partner. Samples were hybridized to microarrays of murine cDNAs.

In both liver and spleen, the common allele but not the BALB/c variant of *Prkdc* was associated with induction of expression of histone H2AY (murine core histone macro H2A.1). This variant histone replaces conventional H2A to locally repress transcription. Two other overexpressed genes were located on chromosome 16 at ~13.8Mb, within the recombination breakpoints of the C.B6 strains. These genes, involved in amino acid metabolism, likely represent genetic contaminants within the congenic region from the C57BL/6 donor genome.

In the spleen, *Abcc1* was overexpressed in B6.C vs. B6, and underexpressed in C.B6 vs. C. Thus, this gene is likely to be differentially expressed between BALB/c and C57BL/6, with upregulation associated with a BALB/c donor allele and downregulation with a C57BL/6 donor allele. The *Abcc1* gene is located just downstream of *Prkdc* on chromosome 16, within the congenic region for both congenic strains. Therefore, this result is a contaminating effect not likely to be a consequence of the *Prkdc* allele.

The DSBs generated by radiation appear to further activate the transcriptional regulatory activities DNA-PKcs. *Prkdc*-dependent gene expression changes specific to radiation were observed in both the liver and spleen. Radiation induced upregulation of 5 genes in all strains and all tissues (figure 4.12 and table 4.3). Down-regulation was not consistently observed in all strains and tissues. (See DVD\Gene Expression\Final Results)

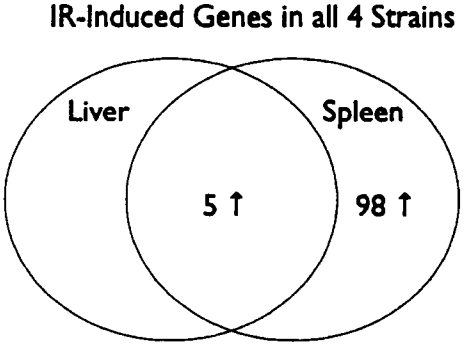


Figure 4.12: Venn diagram showing the number of genes up or down regulated by two Gy (2.5 Gy/min) γ-radiation in liver and/or spleen in all four strains.

Tissue		LIVER				SPLEEN			
Gene	**Description**	**B6**	**B6.C**	**C**	**C.B6**	**B6**	**B6.C**	**C**	**C.B6**
Ccngl	cyclin G1	2.60	2.22	2.30	3.31	1.72	8.30	6.32	7.63
Cdknla	cyclin-dependent kinase inhibitor 1A (P21)	27.11	25.28	11.78	17.29	18.18	31.76	17.96	15.38
Eda2r	ectodysplasin A2 isoform receptor	7.13	6.91	8.29	6.91	4.24	5.01	4.94	5.33
Phlda3	pleckstrin homology-like domain, family A, member 3	5.77	6.79	6.15	5.25	12.15	14.31	11.47	11.05
Polk	polymerase (DNA directed), kappa	2.18	2.42	3.24	3.17	4.28	4.54	6.45	4.33

Table 4.3: Genes upregulated by 2 Gy (2.5 Gy/min) γ-radiation in both liver and spleen in all 4 strains (Fold increase).

Few genes responded in all tissues examined and gene expression patterns were specific for each tissue. A similar pattern of downregulation versus upregulation according to tissue was observed in all strains. Many genes were upregulated in all strains in the spleen but not the liver in response to radiation. These comprised genes from a wide variety of ontologies. Of interest were upregulation in genes involved in DNA repair (Polk, Ercc5), regulation of cell cycle progression (Rbl2, Ccngl, Cdknla), p53 regulation (mdm2), induction of apoptosis (Phlda3, Trp53inpl, Tnfrsf6, Acvrlb, Bax), proteolysis (Ephxl, Mmp2, Gne, Plau, Encl), amino acid phosphorylation (Plk2, Ikbke, Mapkapk3), cell differentiation (Serpine2), regulation of transcription (Sox21), and NfκB activation (Plk2, Eda2r). These results demonstrate that radiation induced a stimulus for cell cycle arrest, DNA repair, and p53-dependent apoptosis independent of the *Prkdc* allele. Trp53inpl, Bax, and Ccngl were also elevated in the liver in three out of 4 strains.

Differences in induction of gene expression were observed between BALB/c and C57BL/6 (figure 4.13). In the liver, 27 genes were upregulated in C57BL/6 but not BALB/c, while 3 were upregulated and one was down-regulated in BALB/c but not

C57BL/6.In the spleen, 495 and 288 genes were up-regulated and down-regulated, respectively, in C57BL/6 but not BALB, whereas 83 and 115 were up- and down-regulated in BALB but not C57. In the liver, two of these differences could be attributed to background genome. In the liver, radiation-induced expression of Mmrn2 and Icam1 was observed in C57BL/6 and B6.C, but not in C.B6 or BALB, indicating that background genome dictates upregulation of these genes in response to radiation.

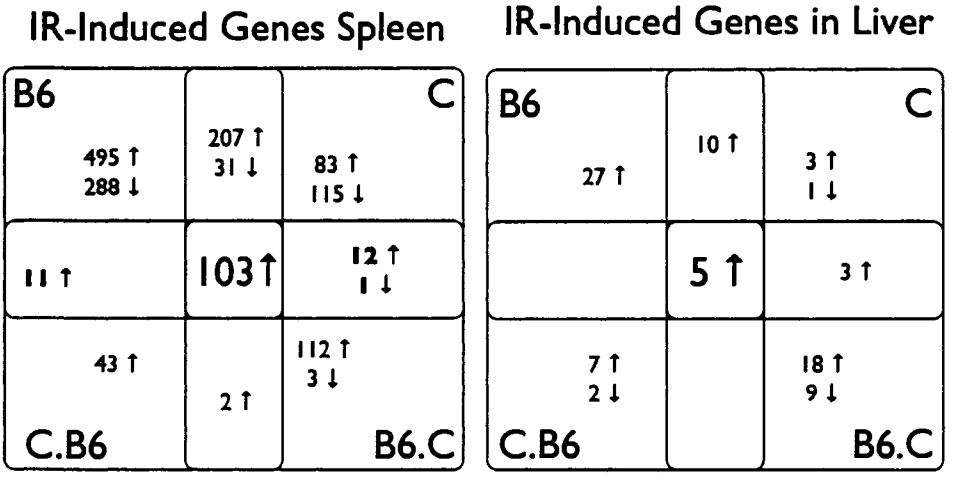


Figure 4.13: Venn Diagrams showing the number of genes up- or down-regulated in spleen (left) and liver (right).

Correlations with donor *Prkdc* allele were identified for genes that were differentially expressed between BALB/c and C57BL/6 (tables 4.4, 4.5, and 4.6) In the liver, radiation induced Mdm2 expression in BALB/c and B6.C but not in C57BL/6 or C.B6. However, mdm2 expression was induced in all strains in the spleen. In the spleen, 13 genes were upregulated and one was downregulated in BALB/c and B6.C but not C57 or C.B6. Eleven genes were upregulated in C57BL/6 and C.B6 but not in BALB or B6.C.

Gene	Description	GO Biological Process	GO Molecular Function	B6.C	BALB
0610010E21Rik				2.25	2.15
2310005E10Rik	RIKEN cDNA.		oxidoreductase activity	1.70	1.87
4632419K20Rik	RIKEN cDNA			1.79	1.88
9530055J05Rik	hypothetical protein	carbohydrate metabolic process	alpha-L-fucosidase activity catalytic activity cation binding hydrolase activity, acting on glycosyl bonds	2.50	3.23
Adn	Mus musculus adipsin (Adn), mRNA.	complement activation, alternative pathway immune response innate immune response proteolysis	complement factor D activity hydrolase activity peptidase activity serine-type endopeptidase activity	51.77	21.44
D130078K04Rik	GRP1 binding protein GRSP1 (FRAGMENT) homolog		binding cytoskeletal protein binding	2.58	2.45
Glipr2	GLI pathogenesis-rel 2			2.03	1.95
LOC231081	similar to spermidine/ spermine N1-acetyltransferase			1.83	2.48
Nr1h3	nuclear receptor subfamily 1, group H, member 3	cellular lipid metabolic process electron transport negative regulation of transcription, DNA-dependent	copper and zinc ion binding ecdysteroid hormone receptor activity electron carrier activity ligand-dependent nuclear receptor activity protein and DNA binding receptor activity sequence-specific DNA binding transcription factor activity transcription regulator activity	2.02	2.15
Rab40c	Rab40c, member RAS oncogene family	intracellular signaling cascade protein transport small GTPase mediated signal transduction	GTP binding nucleotide binding	2.00	1.83
Snai3	snail homolog 3 (Drosophila)	electron transport regulation of transcription, DNA-dependent	copper & zinc ion binding electron carrier activity heme binding nucleic acid binding transcription factor activity	2.38	1.97
Trrp7	transient receptor protein 7	biological_process calcium ion transport ion transport sodium ion transport transport	ADP-ribose diphosphatase activity calcium channel activity calcium ion binding hydrolase activity receptor activity sodium channel activity sodium ion binding	1.97	2.18
Gdf3	growth differentiation factor 3 (Gdf3)	growth	cytokine activity growth factor activity	0.29	0.40

Table 4.4: IR-induced genes differentially expressed between BALB/c and C57BL/6 that correlate with *Prkdc*^{BALB}. (Spleen)

Gene	Description	GO Biological Process	GO Molecular Function	B6.C	C
Mdm2	transformed mouse 3T3 cell double minute 2	protein catabolic process, ubiquitination traversing start control point of mitotic cell cycle	ligase activity metal ion binding protein binding ubiquitin-protein ligase activity zinc ion binding	2.06	2.44
Ddit4l	DNA-damage-inducible transcript 4-like	biological_process	molecular_function	2.51	2.25
Ddit4	DNA-damage-inducible transcript 4	biological_process	molecular_function	3.18	2.85

Table 4.5: IR-Induced genes differentially expressed between BALB/c and C57BL/6 that correlate with *Prkdc*^{BALB}.(Liver)

Gene	Description	GO Biological Process	GO Molecular Function	B6	C.B6
LOC385062	Sim. to hypothetical protein			4.23	3.05
Fv1	Friend virus susc. 1	response to virus		1.92	2.49
C030014C12Rik				3.04	1.79
9630028G16Rik	16 days neonate cerebellum			4.37	2.08
C230078J11Rik	weakly similar to prostatic steroid-binding protein chain C1 precursor [Rattus norvegicus]			3.12	2.21
4933435F18Rik				2.02	1.86
Cldn10	claudin 10, transcript variant 2		structural molecule activity	1.83	4.70
BC024537	cDNA sequence			1.91	1.81
Klk24	kallikrein 24	proteolysis	Hydrolase & peptidase serine-type endopeptidase tissue kallikrein activity	0.48	3.13
A330023F24Rik	RIKEN cDNA gene			2.00	1.88
1700067I02Rik	RIKEN cDNA gene			1.92	1.76

Table 4.6: IR-induced genes differentially expressed between BALB/c and C57BL/6 that correlate with *Prkdc*$^{C57BL/6}$. (Spleen)

For many genes, expression was induced in three out of four strains. This was interpreted to indicate a false negative result in the strain without induction. Such genes were not included in the tables presented above. For other genes, induction specific to the *Prkdc* allele occurred in one tissue whereas induction occurred in all 4 strains in the other tissue. Although this may also represent erroneous results, the genes were included here for the sake of completeness. The chromosomal location of all genes was identified to exclude genes contained within the congenic region. Confirmation of differential gene expression among the congenic and parental strains must be conducted by RT-PCR analysis.

4.2.5 Reproductive physiology

Differences in reproductive performance have been documented between BALB/c and C57BL/6. One striking difference present in the historical data is a higher frequency of non-productive matings in BALB/c mice relative to C57BL/6 (table 4.7).

	BALB/cByJ	**BALB/cJ**	**C57BL/6J**
Mice weaned/dam	24.3	16.6	21.8
Litters/dam	4.10	3.60	3.8
Mice/litter	6.8	4.9	6.6
% males @ weaning	44.5	51.4	51.2
% matings non-productive	19.9	44.4	12.6

Table 4.7: Reproductive Performance in BALB/c and C57BL/6J: Historical Data [38]

The congenics exhibited breeding depression when compared to parental strains. Differences between BALB/c and C57BL/6 were not identified, likely resulting from the low sample size for BALB/c. A statistically significant decrease in litter size was observed in both congenic strains relative to the C57BL/6 parental strain. There was a non-statistically significant decrease in the number of litters produced in B6.C relative to C.B6.

Dam Strain	C57BL/6			BALB/c			B6.C-*Prkdc*^{BALB}			C.B6-*Prkdc*		
Parameter	**Mean**	**Range**	**N**	**Mean**	**Range**	**N**	**Mean**	**Range**	**N**	**Mean**	**Range**	**N**
Time to 1st litter (mo)	1.5	1-3	14	**1.1	1-1.5	5	**1.2	1-2	15	2.5	1-12	17
# Litters/month	0.68	0.22-1.3	10	1.1	0.67-1.3	5	0.66	0-1.3	10	0.84	0-2.0	11
# Litters/month/dam	0.49	0-0.8	11	1.1	0.67-1.3	5	0.57	0-1.3	10	0.84	0-2	11
# mice weaned/dam	15.53	2-31	15	14.89	0-29	9	13.76	0-46	17	14.65	0-49	20
# Pups/litter	5.4	1-9	13	5.1	0-9.5	9	*4.1	0-7	17	*3.7	0-7	21
% males/litter	73	0-93	15	57	0-93	9	69	0-93	17	61	0-92	20
Female age @ mating (mo)	3.8	1-7	13	2.0	1-3	5	2.4	1-6	16	3.6	1-13	21
% non-productive matings	0		15	11		9	12		17	15		20

* One-sample T-test: significant difference from C57BL/6, CI = 99%. B6.C: p= 0.017; C.B6:p=0.0009
** One-sample T-test: significant difference from C57BL/6, CI = 99%. C: p= 0.0161; B6.C:p=0.0019

Table 4.8: Reproductive Performance in BALB/c, C57BL/6J and congenics according to dam strain.

Sire Strain	C57BL/6			BALB/c			B6.C-*Prkdc*^{BALB}			C.B6-*Prkdc*		
Parameter	**Mean**	**Range**	**N**	**Mean**	**Range**	**N**	**Mean**	**Range**	**N**	**Mean**	**Range**	**N**
Time to 1st litter (mo)	1.5	1-2.5	12	1.8	1-3	4	1.4	1-3.0	22	2.5	1-12	17
# Litters/month	0.6	0.2-1	12	0.33	0.33-0.33	4	0.68	0-1.3	15	0.87	0-2.0	13
# Litters/month/dam	0.60	0.2-1	12	0.33	0.33-0.33	4	0.53	0-1.3	15	0.87	0-2	13
# mice weaned/dam	23	8-37	12	***5.3	2-10	4	***13	0-46	25	***15	0-49	23
# Mice/litter	6.3	0.20-8.3	12	5.3	2.0-10	4	*4.4	0-9.0	25	*4.0	0-9.5	27
% males @ weaning	92	0-95	12	67	0-89	4	**80	0-93	25	**83	0-93	23
Male Age @ mating (mo)	2.2	1.0-5.0	12	2.8	2.0-5.0	4	2.7	1.0-7.0	24	3.2	1.0-11	23
% Non-productive matings	0		12	0		4	8		25	13		23

* One-sample T-test: difference from C57BL/6, CI = 99%.B6.C: p=0.0002; C.B6: p<0.0001.
** Wilcoxon Signed Rank Test: difference from C57BL/6, non-parametric, CI = 99%.B6.C: p>0.0001; C.B6: p<0.0002
*** One-sample T-test: difference from C57BL/6, CI = 99%.BALB: p = 0.0029; B6.C: p=0.0001; C.B6: p<0.0084.

Table 4.9: Reproductive Performance in BALB/c, C57BL/6J and congenics according to sire strain.

Embryonic lethality is associated with double-deficiency for Artemis and DNA-PKcs but not in ATM/Artemis double-deficient mice suggesting that DNA-PKcs carries out functions in normal development.[39] We analyzed reproductive performance in the congenic strains to determine whether the $Prkdc^{BALB}$ was associated with altered fertility and embryogenesis (tables 4.8 and 4.9). Time to first litter was slightly greater in mice carrying the common *Prkdc* allele when compared to mice homozygous for the $Prkdc^{BALB}$ allele (statistically significant). Frequency of productive litters was diminished in B6.C compared to C.B6. However, this finding was not statistically significant and did not appear to be linked to the *Prkdc* allele since it was not replicated in BALB/c mice. The low sample size for BALB/c breeders precludes a conclusive interpretation from being made on this matter. What can be concluded is that successive generations of marker-based selective breeding may result in diminished reproductive performance because breeders are selected for genotype rather than overall parental fecundity.

4.3 Discussion and Future Directions

In our phenotypic characterization of newly developed mouse strains congenic for common and variant alleles of *Prkdc*, we have demonstrated that $Prkdc^{BALB}$ is required and sufficient to decrease DNA-PKcs protein expression, confirming previous observations in CXB strains. We have also shown, using three different DSB repair quantification methods, that the repair of radiation-induced DSB is not diminished in C.B6-*Prkdc*, indicating that $Prkdc^{BALB}$ is required for reduced radiation-induced DSB rejoining in BALB/c. Deficient DSB repair was most likely to be responsible for decreases in clonogenic survival, which was a direct function of the donor *Prkdc* allele. We clearly demonstrated by using the newly developed congenic strains that $Prkdc^{BALB}$ is

both sufficient and required for the clonogenic radiosensitivity phenotype of BALB/c mice.

GeneChip microarray analysis of differential constitutive and radiation-induced gene expression revealed that tissue and genetic background were the strongest determinants of global gene expression and that $Prkdc^{BALB}$ has a greater impact on overall gene expression than did radiation. Differences observed in constitutively expressed genes for each congenic and inbred recipient pair reflect the radiation damage-independent functions of DNA-PKcs, and further demonstrate that the BALB/c variant alters cellular functions in the absence of radiation. Though contaminating donor genes within the congenic region could be responsible for the effect, our results suggest that the consequences of $Prkdc^{BALB}$ extend beyond the ability to properly signal radiation responses. For example, association of the common *Prkdc* allele but not $Prkdc^{BALB}$ with induction of histone H2AY expression suggests that *Prkdc* regulates this variant histone to locally repress transcription in the absence of radiation.

Radiation induced a stimulus for cell cycle arrest, DNA repair, and p53-dependent apoptosis independent of the *Prkdc* allele. Upregulated genes included: DNA repair (Polk, Ercc5), regulation of cell cycle progression (Rbl2, Ccng1, Cdkn1a), p53 regulation (mdm2), induction of apoptosis (Phlda3, Trp53inp1, Tnfrsf6, Acvr1b, Bax), proteolysis (Ephx1, Mmp2, Gne, Plau, Enc1), amino acid phosphorylation (Plk2, Ikbke, Mapkapk3), cell differentiation (Serpine2), regulation of transcription (Sox21), and NfκB activation (Plk2, Eda2r).

Prkdc-dependent differences in gene expression were also demonstrated to occur in response to radiation. $Prkdc^{BALB}$ was associated with Mdm2 induction in the liver. In the spleen, 13 genes were upregulated and one was downregulated in association with $Prkdc^{BALB}$ and eleven genes were upregulated in association with the common *Prkdc* allele. Though RT-PCR confirmation of differential gene expression among the congenic and parental strains is necessary, our results suggest that *Prkdc* may play a role in constitutive and DNA damage-induced apoptotic and transcriptional responses.

Finally, a brief retrospective analysis of reproductive physiology revealed that successive generations of marker-based selective breeding resulted in diminished reproductive performance. No reproductive endpoints examined were linked with the *Prkdc* allele in a manner that was statistically significant.

Future studies should aim to quantify DNA-PKcs specific kinase activity and protein metabolism. Challenges that must be met will include the large size and very low levels in mouse tissue of DNA-PKcs. Cytogenetic instability should also be evaluated, with particular emphasis on telomeres. We expect that $Prkdc^{BALB}$ will be strongly linked with cytogenetic instability because rejoining of incorrect DNA ends originates exclusively from slowly rejoining double strand breaks.[40] We believe that the main role of $Prkdc^{BALB}$ is in radiation-induced initiation of mammary epithelial cells, an early event in the multi-step process of mammary tumorigenesis. The congenic strains developed and characterized in this study will be a useful tool for clarification of the role of *Prkdc* and genomic instability in the initiation and progression of radiation-induced mammary carcinogenesis in the BALB/c mouse.

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5 CONCLUSIONS AND DISCUSSION

The wide spectrum of molecular pathologies observed in human cancers emphasizes both their etiologic diversity and their inherent genomic instability. Early systems categorizing cancer-related genes as oncogenes or tumor suppressors only explained a fraction of carcinogenic events or were only valid for specific types of cancers. These systems also could not account for variability in expressivity between individuals. It has become clear in recent decades that most cancers arise as a consequence of the synergism of multiple genetic and extragenetic (environmental) factors. For most individuals, cancer risk is determined by the interaction between an individual's genome-wide set of allelic variants and low doses of carcinogen exposures. An improved understanding of these interactions will eventually lead to reduced cancer mortality and morbidity by enabling the identification of preventative and therapeutic approaches that are tailored to each individual or patient.

Because of the broad allelic and lifestyle diversity among human populations, epidemiologic studies infrequently yield clear and conclusive answers concerning the roles of specific allelic and environmental interactions in carcinogenesis. Inbred mice provide a means for examining physiologic, genetic, and environmental interactions that would be extremely difficult to analyze directly in humans. They constitute well-characterized, genetically identical experimental units that have extensive synteny with human genomes and many physiologic similarities to humans.[1, 2]

Genetically engineered mice (GEM) such as knockouts and knock-ins are most frequently used to study the role of specific genes in carcinogenesis. However, these models do not realistically reproduce the type or spectrum of genetic variability in human

populations. Tissue- and developmental stage-specific requirements and expression levels of knocked-in/out genes often dramatically modify phenotypes in ways that do not correlate with naturally-occurring genetic deficiencies responsible for complex traits. GEM provide valuable *in vivo* molecular function and pathway information, but they do not always represent good models for studying cancer susceptibility because they usually result in high penetrance phenotypes. A more suitable approach to study the influence of allelic variants is to capitalize on the rich naturally occurring variation in complex traits that exist among the many available inbred mouse strains. QTL mapping is one approach that uses genome scans of large outcrosses to attempt to statistically link inheritance of disease-related phenotypes with the parental origins of DNA in segregating crosses. However, this approach suffers from one major drawback: although it can readily identify broad genetic loci, finely localizing underlying genes is an arduous process. Congenic mice provide a powerful adjunct or alternative to QTL mapping for fine mapping of loci that contribute to complex traits. In congenic mice, a single allele contributes to a phenotypic difference between the background strain and the congenic strain, in effect "Mendelizing" the complex trait. As a result, specific genes that confer risk can be identified and the magnitude of their effect can be ascertained.

Naturally occurring differences in susceptibility to radiation-induced mammary cancer have been documented between BALB/c (susceptible) and C57BL/6 (resistant), and are concomitant with susceptibility to radiation-induced ductal dysplasia and genomic instability, defective telomere capping, slow DNA double strand break repair, reduced DNA-PKcs kinase activity, and reduced DNA-PKcs protein expression.[3-13] We hypothesized that an allelic variant of the gene that encodes DNA-PKcs (*Prkdc*)

occurring in BALB/c mice might produce one or more of these phenotypes, with or without interaction with allelic variants in other BALB/c genes. We constructed two mouse strains congenic for the variant ($Prkdc^{BALB}$) and common ($Prkdc^{B6}$) alleles of *Prkdc* and probed these for phenotypic differences that existed between the parental strains (BALB/c and C57BL/6). Because the validity of our results was greatly dependent upon the quality of the congenic strains, we used marker-assisted breeder selection and assessed strain purity by probing for 877 polymorphic markers at every 3cM across the genome. Both newly developed congenic strains were highly uncontaminated. Rare instances of donor genome outside the *Prkdc* locus were inconsistent between different individual inbred mice and likely represented false positive results, though residual heterozygosity is also possible. This will be confirmed in future studies by resequencing SNPs at all loci that had possible contamination.

The congenic region around *Prkdc* extends from 13.5 Mb to 21.6 Mb for C.B6-*Prkdc*, and from 13.5 Mbp to 23.4 Mb for B6.C-$Prkdc^{BALB}$. The recombination breakpoints are concordant with a large region of low crossover frequency from 13 Mb to 16 Mb.[14] The congenic region contained ~207 known or putative genes. The presence of these genes may be responsible for the gene expression clustering results, indicating that donor allele was a stronger determinant of differential gene expression than radiation exposure. This may be a direct consequence of differences in expression of the genes themselves, or may reflect their effect on the expression of other genes (in trans).

Contaminating donor genetic sequence resulted in differences in the constitutive expression of three genes located within this region. One of these, *Abcc1* (ATP binding cassette, subfamily C, MRP1, multi-drug resistance-associated protein), is a plasma

membrane transporter that is overexpressed in the multidrug resistant small cell lung cancer cell line NCI-H69. This transporter contributes to defense against xenobiotics and endogenous toxic metabolites, leukotriene-mediated inflammatory responses and dendritic cell function, and protection from the toxic effect of oxidative stress.[15] We are the first to demonstrate differential expression of *Abcc1* in BALB/c versus C57BL/6. We have shown that BALB/c genomic sequence at the A*bcc1* locus results in upregulation whereas C57BL/6 genomic sequence results in lower *Abcc1* expression. This finding must be confirmed by RT-PCR. MRP1, the protein product of *Abcc1*, is involved in abolishing the toxic effects of peroxides by enabling the efflux of glutathione disulfide (GSSG) from the cell when GSSG reductase activity becomes rate limiting. MRP-1 also removes glutathione conjugates formed in cells at high oxidative state. BALB/c-associated *Abcc1* overexpression may be responsible for resistance to bystander effects observed in this strain and in B6.C congenics (K. Askin, A. Williams, and T. Hagelstrom, unpublished data). The impact this genetic contamination has on our results is questionable. It is unlikely to affect DNA-PKcs protein expression and DNA repair examined using the FAR assay. For DSB repair examined by comet assay and γ-H2AX, and for the clonogenic survival assay, it would cause diminished susceptibility via its protective effects, rather than heightened susceptibility as we observed.

To provide direct evidence that $Prkdc^{BALB}$, as opposed to other BALB/c genes, caused decreased DNA-PKcs abundance, congenic mice were tested for DNA-PKcs levels in kidney tissue by SDS-PAGE/Western blot. Inbred C.B6-*Prkdc* mice had C57BL/6-like levels of DNA-PKcs expression, whereas B6.C-$Prkdc^{BALB/BALB}$ had levels equivalent to that of BALB/c mice. This corroborates data obtained using CXB RI

strains, and is strong evidence that *Prkdc*^{BALB} is genetically linked to reduced DNA-PKcs levels and, by consequence, with decreased kinase activity. Diminished kinase activity associated with *Prkdc*^{BALB} must be confirmed using the congenic strains.

DSB rejoining was examined in the congenic and parental strains using three methods: γ-H2AX focus disappearance, single cell gel electrophoresis (comet assay), and modified FAR assay (PFGE/southern blot).Our preliminary results examining disappearance of γ-H2AX foci suggest that *Prkdc*^{BALB} may be required for efficient DSB rejoining as detected by phosphorylated H2AX. Diminished disappearance of γ-H2AX foci was linked with *Prkdc*^{BALB}, representing either a failure to rejoin broken ends by NHEJ or inhibition of γ-H2AX dephosphorylation associated with this allele. In the comet assay, both congenic strains repaired DNA as efficiently as C57BL/6. In the FAR assay, repair was efficient for the common *Prkdc* allele (C57BL/6 and C.B6), and intermediate in B6.C. Thus, repair was consistently normal for C.B6, indicating that slow repair in BALB/c requires *Prkdc*^{BALB}.

Results in the B6.C-*Prkdc*^{BALB} congenics were not as straightforward, ranging from deficient (BALB/c-like), to intermediate, to normal (C57BL/6-like), suggesting that *Prkdc*^{BALB} may not be sufficient for diminished repair in BALB/c, and that a requirement for modifier genes depends on the endpoint examined. Alternatively, technical factors and assay limitations could be responsible for the discordant results. Differences in cell cycle stage could also be responsible. Quiescent cells (G0) defective in NHEJ are exquisitely sensitive to radiation.[16] Cells defective in NHEJ are also more sensitive to radiation during G1, compared to S and G2/M phases.[17] Exponentially growing cells were utilized for the comet and FAR assays, because well-dispersed cells must be

enumerated for each of these assays, whereas highly confluent cultures could be used for the γ-H2AX assay. It is likely that the exponentially growing cell cultures contained a higher proportion of cells in S and G2/M phases when they were less reliant on NHEJ to repair their DNA. Therefore, the impact of *Prkdc*^{BALB} on radiation sensitivity would be less apparent using the electrophoretic DNA separation assays in comparison to the γ-H2AX assay. The influence of cell cycle on the ability to repair DNA DSB in response to radiation could be better examined in the congenics using an assay that quantifies repair of potentially lethal damage (PLDR), such as cytogenetic analysis for premature chromosome condensation over time. Premature chromosome condensation reflects the level of chromosome breaks in interphase nuclei, a method that would be amenable to studying subtle NHEJ defects in quiescent cells. This approach is likely to yield more consistent results when evaluating polymorphic NHEJ genes.

Although repair defects were difficult to quantify in B6.C-*Prkdc*^{BALB}, most likely due to heterogeneity of cell cycle stage, the consequences of deficient repair were readily observed in the clonogenic survival assay. Survival in the congenic strains was a direct function of the donor *Prkdc* allele, demonstrating that *Prkdc*^{BALB} is both required and sufficient to reduce clonogenic survival in irradiated BALB/c cells. Though differences in apoptosis could theoretically be responsible for this effect, our use of fibroblasts, which are quite resistant to radiation-induced apoptosis, makes an apoptotic mechanism unlikely. Increased replicative death due to deficient repair is consistent with the DNA DSB repair differences observed among the congenic and parental strains.

Gene expression profiles suggested that radiation-induced gene expression could also be responsible for differences in cell survival observed among the four strains. Many genes were upregulated in all strains in response to radiation. Of interest were upregulation in genes involved in DNA repair (Polk, Ercc5), regulation of cell cycle progression (Rbl2, Ccng1, Cdkn1a), p53 regulation (mdm2), induction of apoptosis (Phlda3, Trp53inp1, Tnfrsf6, Acvr1b, Bax), proteolysis (Ephx1, Mmp2, Gne, Klk24, Plau, Enc1), amino acid phosphorylation (Plk2, Ikbke, Mapkapk3), cell differentiation (Serpine2), regulation of transcription (Sox21), and NfκB activation (Plk2, Eda2r). Radiation upregulated Cdkn1a (p21) and cyclin G1 in all four strains, suggesting that p53-mediated gene induction involved in cell cycle arrest was normal and independent of the *Prkdc* allele. Our findings parallel those in the published literature. Radiation has been shown to induce expression of cell cycle genes (Cdkn1a, Ccng1, BTG2, ELK4 and mdm2), apoptosis genes (Fas, TNFSF6/9, Trailr2, TRID and bbc3), and DNA repair genes (p48, XPC, gadd45, PCNA).[18, 19] Specific profiles are highly dependent on dose, dose-rate [19], time, cell cycle stage, and tissue.[20] At high doses (~4Gy), apoptosis and cell proliferation genes tend to be differentially regulated, whereas at low doses (~0.2 Gy), changes in genes involved in cell-cell signaling, signal transduction, and DNA damage response tend to predominate.[21] Our results reflected both categories. Maximal induction of radiation-responsive genes occurs four hours after radiation exposure at high doses (20Gy), and 2-3 hours after treatment with low doses (50 cGy).[20] Early response genes return to background levels after 24 hours, while different gene changes persist longer. Our examination of gene induction after 4 hours was most likely to reveal potential transcriptional consequences of $Prkdc^{BALB}$.

Intriguingly, more genes were responsive to radiation in the spleen than in the liver, consistent with published data.[20] This may reflect the spleen's greater sensitivity to radiation-induced apoptosis.[22] Though p53-responsive elements are induced in all tissues, the outcome is highly tissue-dependent. Epithelial tissues are more likely to respond in conjunction with growth factor, intercellular, and extracellular signals than are lymphoid tissues.[23-25] Therefore, the results we obtained from liver may more closely mimic those of the mammary gland.

Subtle differences in levels of upregulation of p53 response elements were noteworthy. For example, although in the spleen, mdm2 was upregulated in all four strains, in the liver it was only upregulated in BALB/c and B6.C. Mdm2 is an E3 ubiquitin ligase that negatively regulates p53 transcriptional activity and targets p53 for proteasome degradation. It is a p53 responsive gene responsible for preventing uncontrolled cell death in response to radiation.[26, 27] Therefore, in the liver, $Prkdc^{BALB}$ may confer enhanced survival and diminished cell cycle arrest in response to radiation. The lack of a cell cycle arrest could reduce the time needed to repair breaks and result in chromosomal instability. *In vitro* differential expression of cell cycle and apoptotic genes has previously been associated with reduced DNA-PK activity in peripheral blood lymphocytes and the SX9 cell line.[28, 29] Our examination of DNA-PKcs-associated differential gene expression did not identify the same genes, probably because our analysis was *in vivo* and used a different cell type.

Our investigation of differential radiation-induced gene expression according to *Prkdc* allele suggests that $Prkdc^{BALB}$ may play an important role in immunity. Some genes that were differentially expressed after irradiation between $Prkdc^{BALB}$ and $Prkdc^{B6}$

are involved in the innate immune response, including functions in complement activation (*Adn*) and response to virus (*Fv1* and *Mdm2*). *Adn* (adipsin, Complement factor D) was strongly upregulated in BALB/c and B6.C-$Prkdc^{BALB}$ mice, but was not in C67BL/6 or C.B6-*Prkdc*. Complement factor D is an extracellular endopeptidase that is required for the alternative pathway of complement activation. Although it is not clear how *Adn* and *Prkdc* are related, the strong overexpression of *Adn* in association with $Prkdc^{BALB}$ emphasizes the importance of this allelic variant in innate immunity.

Mdm2, which was upregulated in $Prkdc^{BALB}$ mice, is activated by Akt1 to suppress p53-dependent apoptosis in response to immunostimulatory DNA.[30] This process relies on DNA-PKcs-dependent activation of Akt, [31, 32] indicating that $Prkdc^{BALB}$ may collaborate in the suppression of virally induced apoptosis. In contrast, *Fv1* was induced in congenic mice carrying $Prkdc^{B6}$ but not $Prkdc^{BALB}$ in response to radiation. *Fv1* is a cell surface antigen that determines the susceptibility of cells to retroviral infection (including Friend leukemia virus) by restricting interaction with viral capsid sequences.[33] Thus, radiation may generate DSB products that resemble viral DNA and induce an immuno-stimulatory response, upregulating expression of *Fv1*. Interestingly, gp70 (*Fv1* product) co-precipitates with DNA-PKcs and enhances γ-radiation-induced apoptosis in a DNA-PK-dependent manner.[34] A positive feedback mechanism could exist between DNA-PKcs and *Fv1*, such that gp70 activates DNA-PKcs, and DNA-PKcs upregulates *Fv1* expression in response to radiation and viral infection. Our results suggest that the latter effect may be diminished in BALB/c and B6.C-$Prkdc^{BALB}$. Taken together, these results suggest that $Prkdc^{BALB}$ may diminish apoptotic and transcriptional responses to immuno-stimulatory or radiation-induced DSB DNA compared to $Prkdc^{C57BL/6}$.

C57BL/6 and BALB/c have long served as important animal models for immunologists because of innate differences in their immune responses.C57BL/6 is the prototypical Th1-responding strain, whereas BALB/c is the prototypical Th2 responding strain.[35] In addition, differences exist in their regulation MHC I expression and NK cell activation.[36] This results in vast differences in their ability to respond to pathogens, with BALB/c mice being susceptible to a number of bacterial agents such as *Leishmania.* Our gene expression results suggest that *Prkdc* may constitute a locus that modifies innate immunity in BALB/c mice. Such strain differences in innate immunity could alter immunosurveillance mechanisms and partly explain BALB/c susceptibility to cancer. Strain differences in DNA repair could further diminish immunosurveillance capabilities in cancer cells. Genomic instability would generate unrecognizable epitopes that evade recognition by the immune system (enhanced immunoediting).

Genomic instability, the hallmark of cancer cells, is hypothesized to be central to carcinogenesis. It allows cells to acquire the multiple mutations that result in cancer initiation, promotion, and progression.[37] Individuals with genomic instability are said to have a mutator phenotype, which may be acquired or inherited.[38] The causes of genomic instability are those which increase DNA damage or decrease a cell's ability to repair it. Decreased DNA-PKcs kinase activity is associated with increased genomic instability in human and mouse (BALB/c) cells as well as cancer patients.[8, 39, 40] We have demonstrated that differences in DNA-PKcs protein expression, DNA-repair, and clonogenic cell survival correlate with the BALB/c variant of *Prkdc*. The identification of a heightened genomic instability in the congenic strain B6.C-*Prkdc*^{BALB} would confirm the role of this variant in susceptibility to radiation-induced mammary carcinogenesis in

BALB/c, and would support its continued use as a model of sporadic breast cancer in humans. The human *Prkdc* gene has recently been probed for functional variants or polymorphisms and these have been associated with heightened risk for radiation-induced breast cancer. [41] It is not clear why functional polymorphisms of DNA repair genes would persist in the population. The $Prkdc^{BALB}$ allele has been detected in some wild-derived mouse inbred strains, suggesting that this allele could occur and persist in natural populations. Its persistence could be related to a protective role in immunity or other functions. Alternatively, the allele could be linked to a protective gene by chromosomal proximity or by functional interaction. A hypomorphic *Prkdc* variant could also occur and persist in the human population, and predispose to radiation-induced breast cancer. Further study of the BALB/c model is likely to provide more insight into sporadic breast cancer in humans. It is likely that modifier genes in the BALB/c genome modify susceptibility to radiation-induced mammary cancer.

Additional phenotypic studies using the congenic mice developed here will further delineate the role of $Prkdc^{BALB}$ versus modifier genes in radiation-induced breast cancer, and provide insight into the mechanisms of diminished DNA-PKcs kinase activity in BALB/c mice. The two single nucleotide polymorphisms in $Prkdc^{BALB}$ result in amino acid conversions that could have a direct effect on function or alter the tertiary structure of the protein. This could diminish kinase activity by decreasing protein stability or increasing protein degradation, DNA binding affinity, specific kinase activity, or autophosphorylation. These mechanisms are not necessarily mutually exclusive as one may affect the other. Further investigations using the congenic mice are required to answer these questions.

A thorough understanding of the mechanisms of action of DNA-PKcs and its functional polymorphisms will be useful to determine radiation-susceptibility as well as to induce it. DNA-PKcs is being actively investigated in the development of noncytotoxic radiation-sensitizing drugs. Selective DNA-PKcs inhibitors have the potential to enhance tumor control by radiotherapy and chemotherapy without the inadvertent systemic toxicities that arise with drugs such as cisplatin, Taxol, and mytomycin C.[42-45] Current approaches for inhibiting DNA-PK include decreasing protein expression, inhibiting repair complex recruitment, and inhibiting kinase activity.[45] Decreased expression has been achieved using antisense oligonucleotides. Aptamers and peptide antagonists have also been designed to block binding of regulatory units to DNA-PKcs. Most successful have been modifications of small molecule PI3-K inhibitors such as wortmannin and Ly294002, that are commonly used in the laboratory to inhibit a variety of PIKKs. However, their therapeutic range and cytotoxicity are not amenable to *in vivo* use in patients. Newer molecules such as quercetin and vanillin derivatives, NU7026, NU7441 and OK-1035 are attractive alternatives because they have very high specificity for DNA-PK but still lack the *in vivo* efficacy that would be required for tumor radiosensitization in cancer patients, primarily due to pharmacokinetic issues. Nevertheless, transient inhibition of DNA-PK is a very attractive option for radio- and chemo-sensitization of tumors with few cytotoxic sequelae to normal tissues, and represents a growing area of R&D investment in the pharmaceutical industry.

In conclusion, we have described the application of a direct genetic approach employing two newly developed congenic mouse strains to examine linkage between the *Prkdc* locus and radiation susceptibility. We have generated two novel strains of mice carrying common and variant alleles of the DNA repair gene *Prkdc*. Using these strains we have determined that there exist functional consequences of the naturally occurring BALB/c allelic variant of the DNA repair gene *Prkdc* which modulate and even diminish the ability of cells to maintain genomic homeostasis. Furthermore, we have demonstrated that $Prkdc^{BALB}$ has unique functional consequences that are radiation-independent. These newly-developed congenic strains serve as valid rodent models of sporadic and radiation-induced human breast cancer, and provide proof of principle for the role of genetic polymorphisms in breast cancer susceptibility.

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6 APPENDIX

Appendix Figure 1a: Resequencing at *Cdkn2a* Exon 1: Congenic Strains

Appendix Figure 1b: Resequencing at *Cdkn2a* Exon 1: RI Strains

Appendix Figure 2a: Resequencing at rs13481764, contaminant SNP on chr. 13, confirms microarray result.

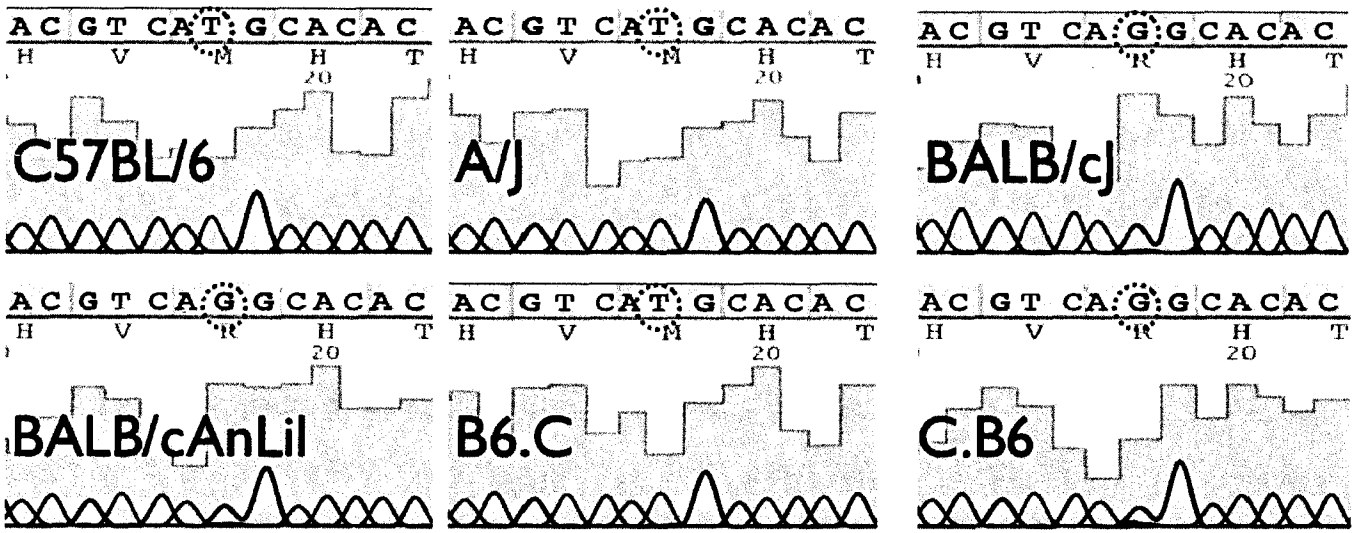
Appendix Figure 2b: Resequencing at gnf13.035.637, contaminant SNP on chr. 13, confirms microarray result.

Appendix Figure 2c: Resequencing at rs13482398, contaminant SNP on chr. 14, contradicts microarray result (T/C).

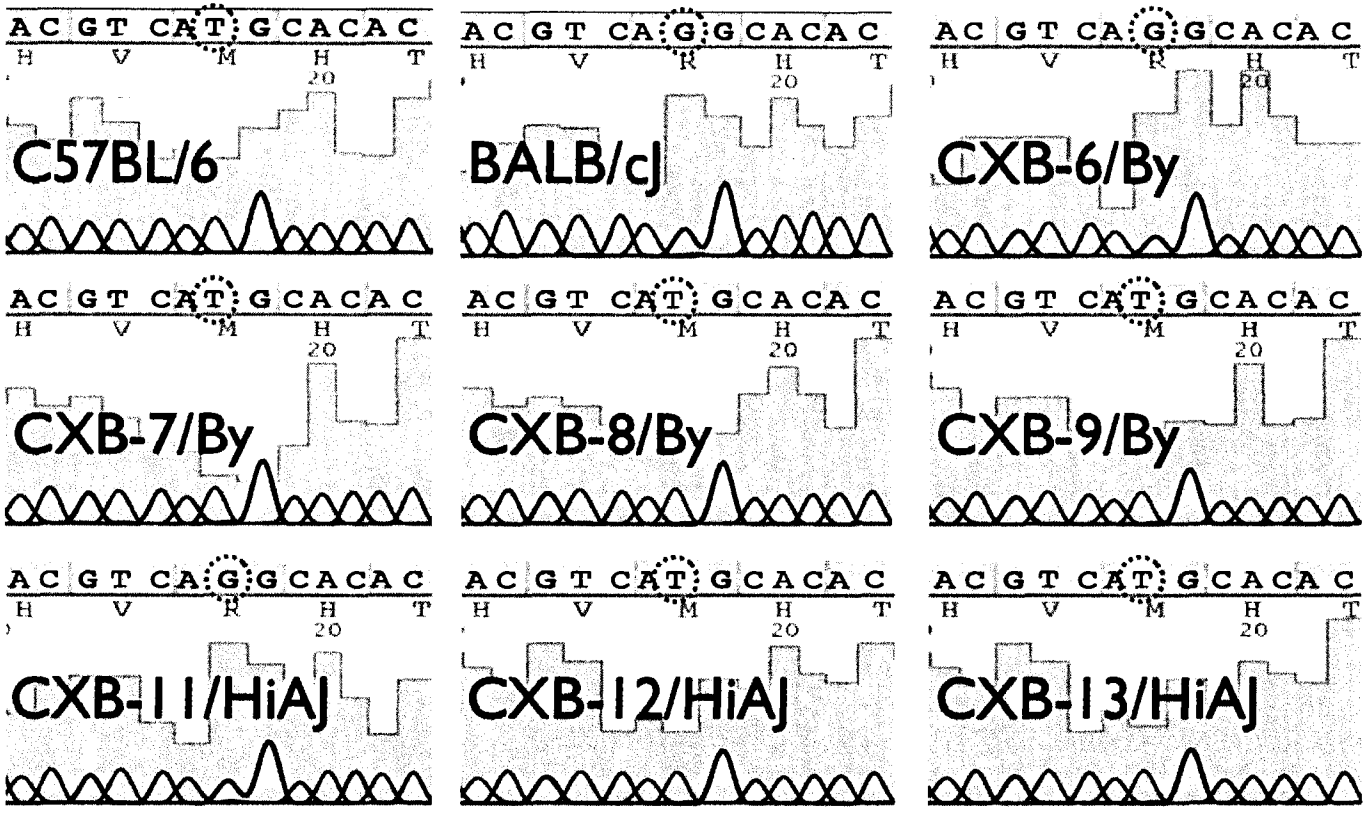
Appendix Figure 3a: γ-H2AX foci in individual cells. Background (0Gy) and maximally induced (1 Gy, 1 hour).

Appendix Figure 3b: Disappearance of γ-H2AX foci in individual cells after 1 Gy γ-radiation and 4 hours of repair. Though foci are difficult to quantify individually, residual foci are more evident in BALB/c (C) and B6.C-*Prkdc*^{BALB} than C57BL/6 (B6) and C.B6.

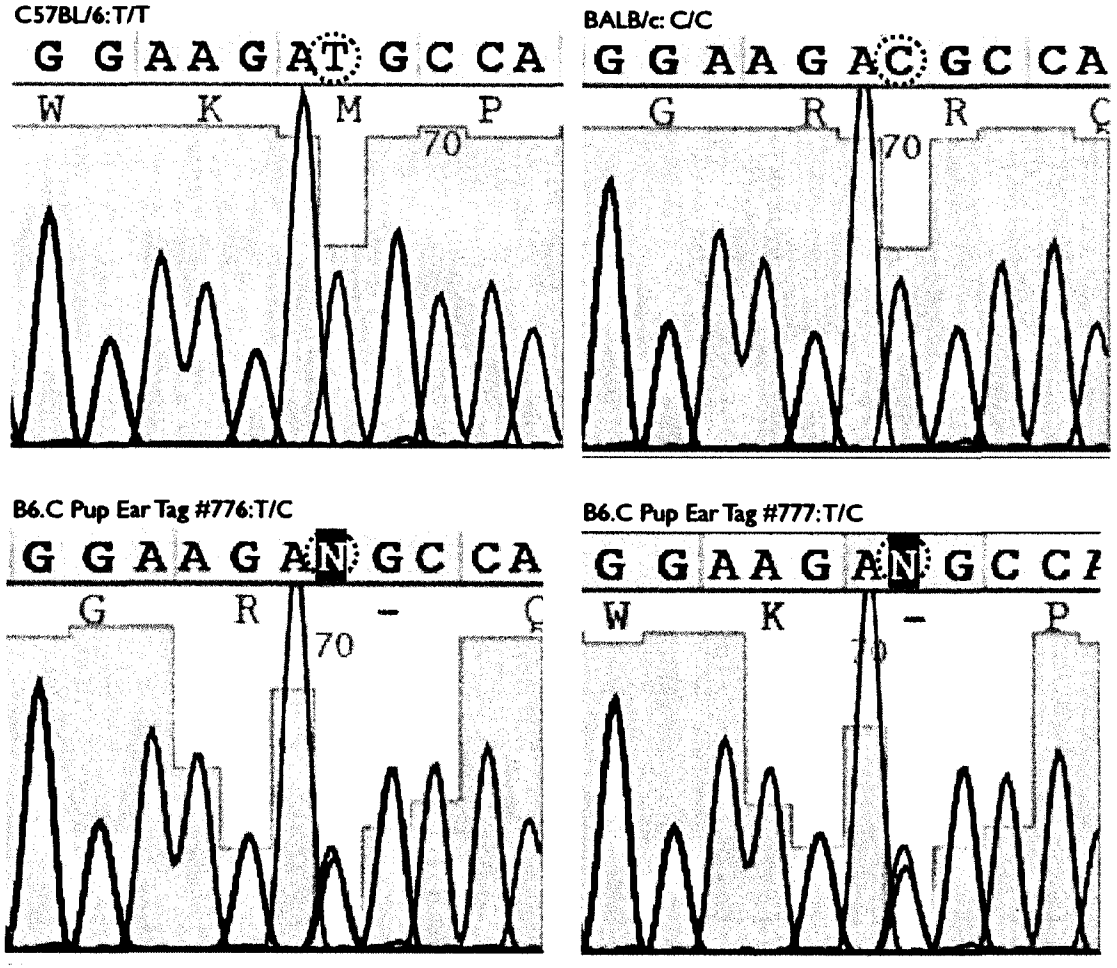
Appendix 4: List of Abbreviations



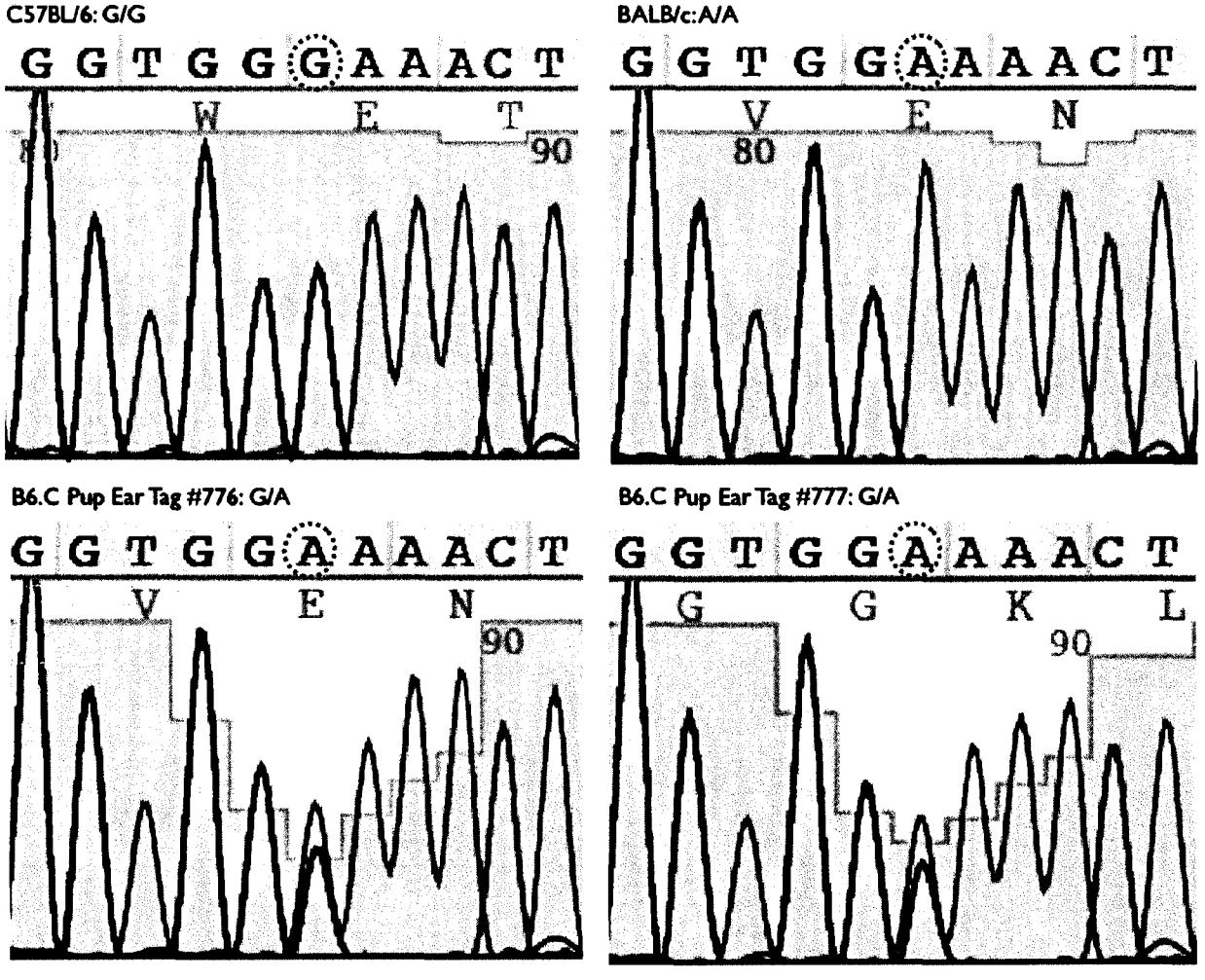
Appendix Figure 1a: Resequencing at *Cdkn2a* Exon 1: Congenic Strains



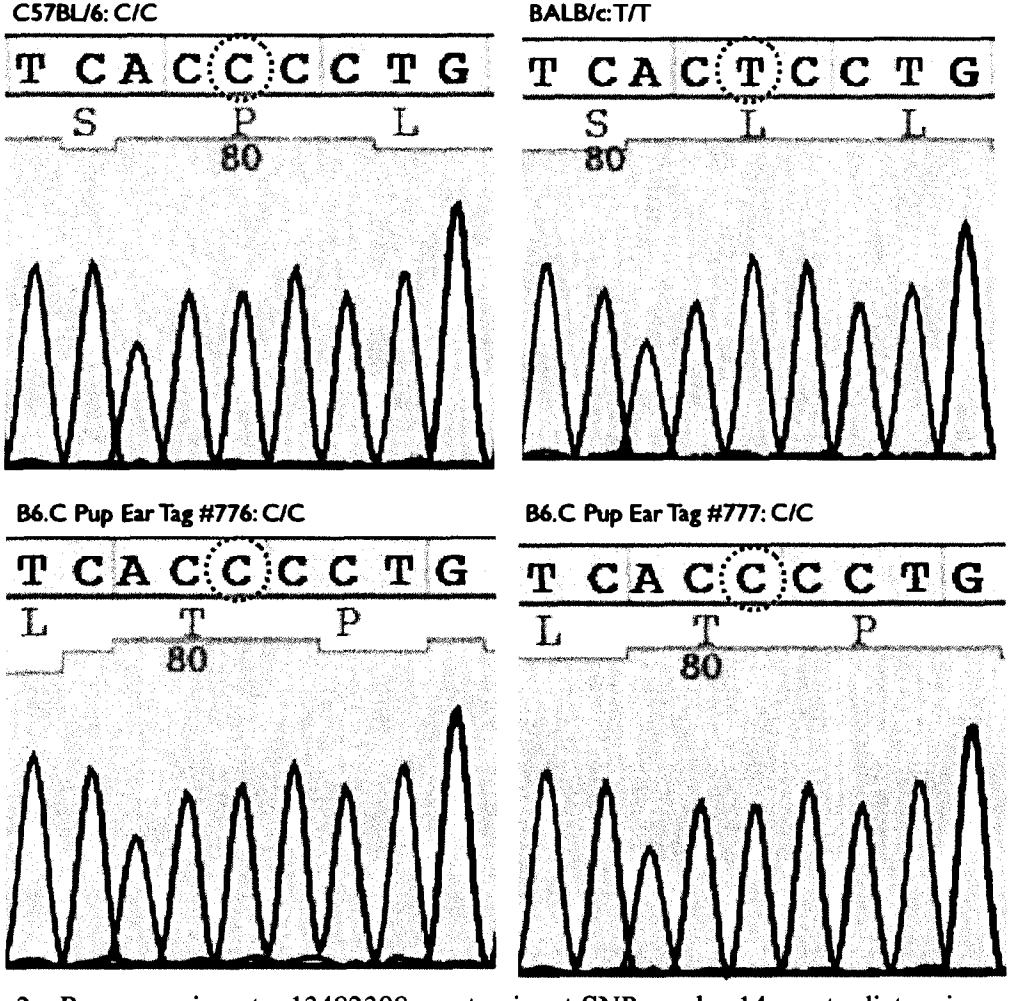
Appendix Figure 1b: Resequencing at *Cdkn2a* Exon 1: RI Strains.



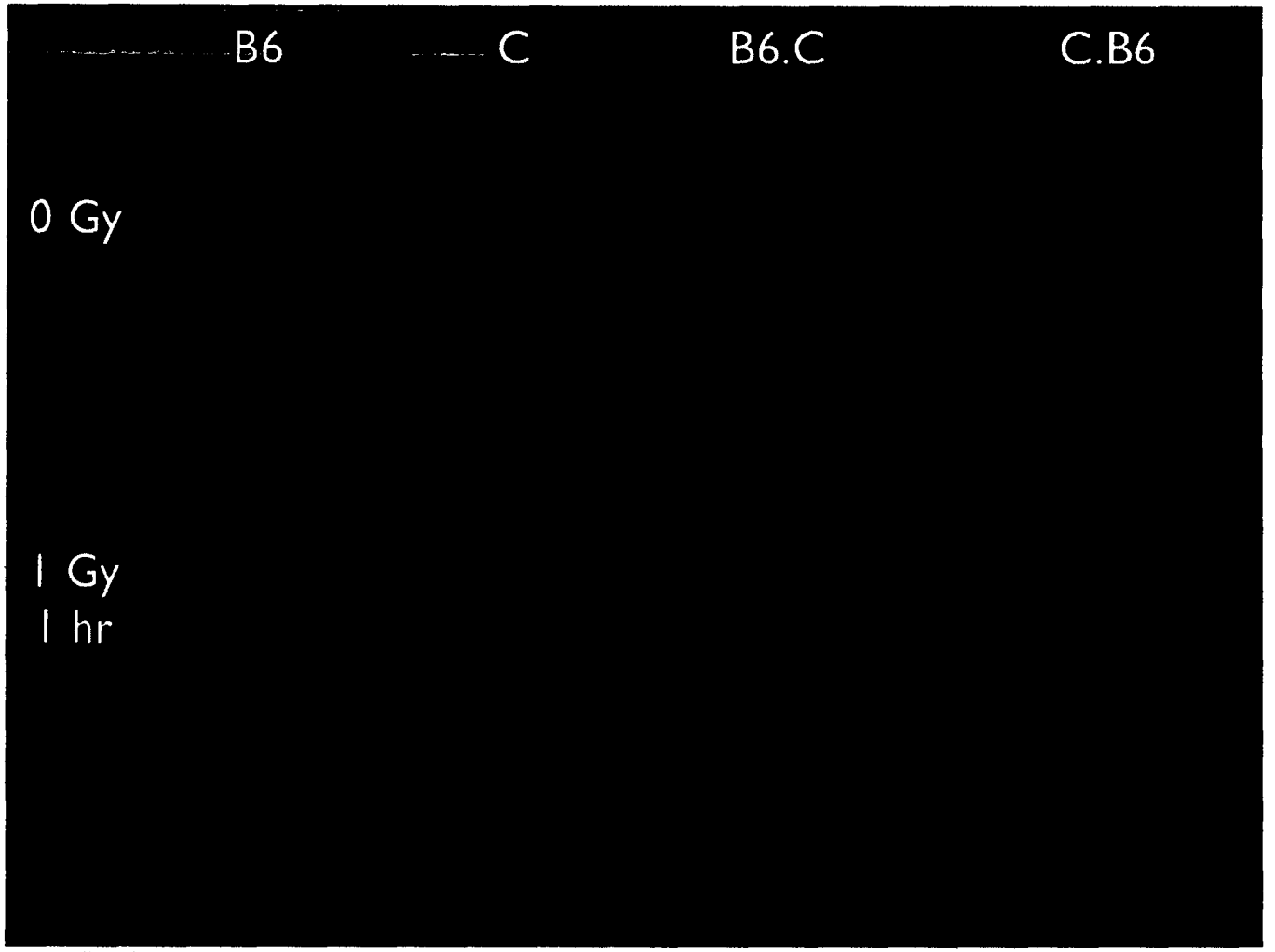
Appendix Figure 2a: Resequencing at rs13481764, contaminant SNP on chr. 13, confirms microarray result.



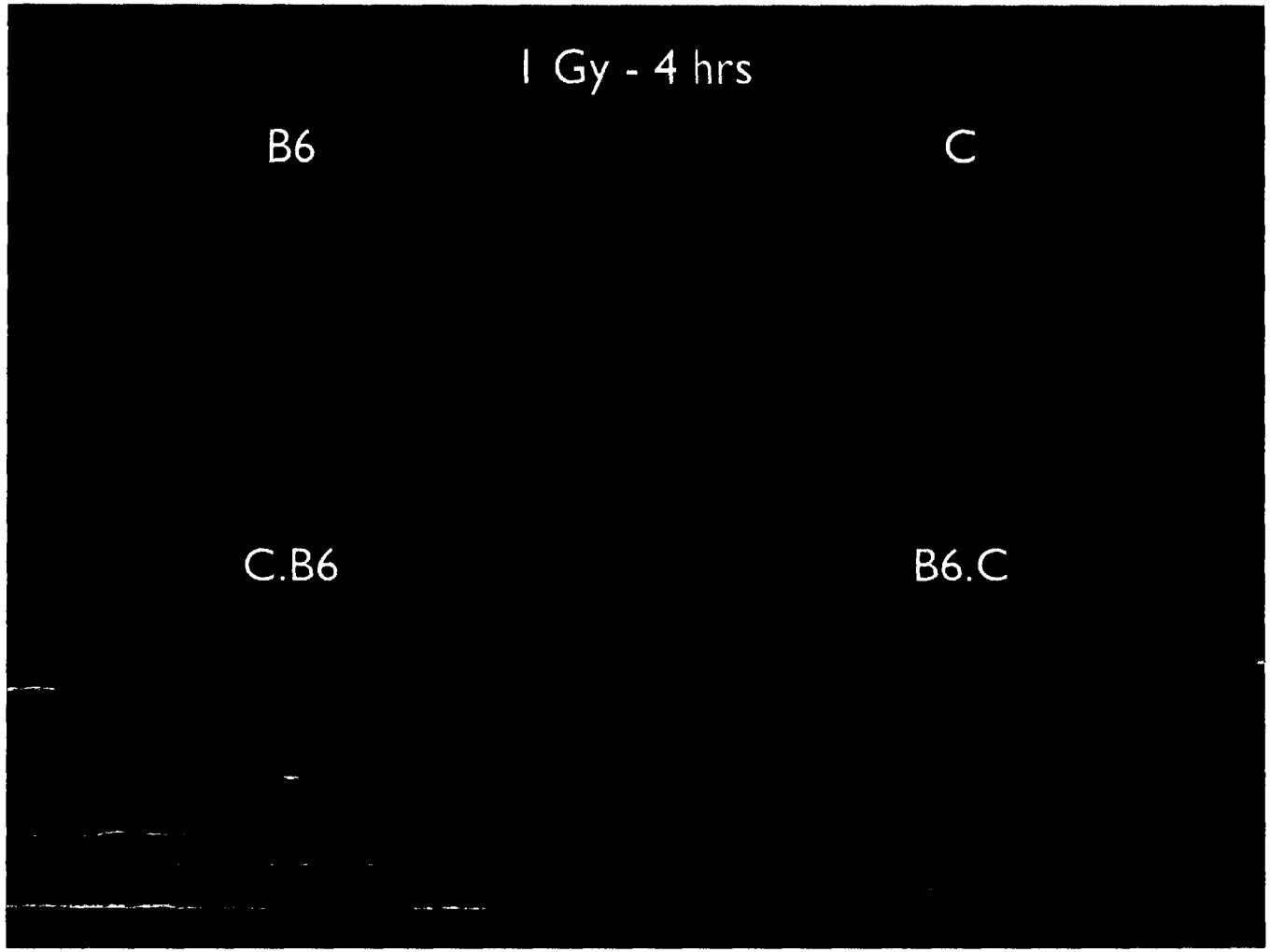
Appendix Figure 2b: Resequencing at gnf13.035.637, contaminant SNP on chr. 13, confirms microarray result.



Appendix Figure 2c: Resequencing at rs13482398, contaminant SNP on chr. 14, contradicts microarray result (T/C).



Appendix Figure 3a: γ-H2AX foci in individual cells. Background (0Gy) and maximally induced (1 Gy, 1 hour).



Appendix Figure 3b: Disappearance of γ-H2AX foci in individual cells after 1 Gy γ-radiation and 4 hours of repair. Though foci are difficult to quantify individually, residual foci are more evident in BALB/c (C) and B6.C-*Prkdc*^{BALB} than C57BL/6 (B6) and C.B6.

Appendix 4
LIST OF ABBVIATIONS

ACS	American Cancer Society
ADP	adenosine diphosphate
ARF	alternate reading frame
AT	ataxia telangiectasia
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	ataxia telangectasia-related protein
B6	short for C57BL/6 inbred mouse strain
BCR	breakpoint cluster region
BER	base excision repair
BLM	Bloom syndrome homolog (human) mouse gene
BLOTTO	bovine lacto transfer technique optimizer
bp	base pair
BPDE	benzo a-pyrene-diol-epoxide
C	short for BALB/c inbred mouse strain
CCC	Comprehensive Cancer Control
CD	cluster of differenciation
CDC	Centers for Disease Control
cDNA	complementary deoxyribonucleic acid
CHEF	clamped homogeneous electric field
cM	centimorgan
CO_2	carbon dioxide
CO-FISH	chromozome orientation - fluorescence *in situ* hybridization
COMT	catechol-*O*-methyltransferase
CpG	cytosine and guanine separated by a phosphate
CSR	class switch recombination
CSU	Colorado State University
CV	coefficient of variation
DAPI	4',6-diamidino-2-phenylindole
DCPC	Division of Cancer Prevention and Control
DNA	deoxyribonucleic acid
DNA-PKcs	DNA-Dependent Protein Kinase, catalytic subunit
DSB	double strand break
EDTA	ethylenediamine tetraacetic acid
EGFR	epidermal growth factor receptor
EM	electron microscopy
ER	estrogen receptor
EtBr	ethidium bromide

FAR	fraction of activity released
FAT	focal adhesion targeting
FATC	the FRAP, ATM, TRRAP proteins found at the C-terminal end of the PIK-related kinases
FBS	fetal bovine serum
FCS	fetal calf serum
FISH	fluorescent *in situ* hybridization
FITC	fluorescein isothiocyanate
FRAP	FKB12-rapamycin bindings protein
GC	guanine-cytosine content
GEM	genetically engineered mouse
GSSG	glutathione disulfide
GSSG	glutathione disulfide
GST	glutathione S-transferase
Gy	gray
HCC	hepatocellular carcinoma
HDAC	histone deacetylase
HEAT	Huntingdon Elongation Factor 3, a subunit of protein phosphatase 2A and TOR1
HPV	human papillomavirus
HR	homologous recombination
HRP	horseradish peroxidase
HSF	human foreskin fibroblasts
HSF1	Heat shock factor protein 1
HSP90α	inducible heat shock protein 90
HSV-1	herpes simplex virus 1
IACUC	Institutional Animal Care and Use Committee
IGFBP-3	insulin-like growth factor binding protein 3
IL	interleukin
IR	ionizing radiation
IRIF	ionizing radiation induced foci
ITS	interstitial telomeric signals
kb	kilo base (1,000 b)
kbp	kilo base pair (1,000 bp)
kDa	kilodalton
LET	linear energy transfer
Lig	ligase
LMP	low molecular weight polypeptide
MDR	multidrug resistance
MEM	minimal essential media

MGI	Mouse Genome Informatics website run by The Jackson Laboratory
MRN	MRE11–RAD50–NBS1 protein complex
MRP	multi-drug resistance-associated protein
MTHFR	methyltetrahydrofolate reducatse
mTOR	mammalian target of rapamycin
NCCCP	National Comprehensive Cancer Control Program
NER	nucleotide excision repair
NHEJ	non-homologous end joining
NK	natural killer cell
NO	nitric oxide
P/S	penicillin/streptomycin
PAGE	polyacrylamide gel electrophoresis
PAH	polycyclic aromatic hydrocarbon
PARP	Poly ADP-ribose polymerase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFT	protein farnesyl transferase
PFGE	pulsed field gel electrophoresis
PI-3	phosphoinositide 3-kinase (PI-3-K)
PIK	phosphoinositide kinase
PIKK	phosphoinositide 3-kinase–related kinase
PK	protein kinase
PLDR	potentially lethal damage repair
PR	progesterone receptor
Prkdc	gene encoding DNA-PKcs
$Prkdc^{B6}$	common *Prkdc* allele (C57BL/6)
$Prkdc^{BALB}$	variant *Prkdc* allele (BALB/c)
QTL	quantitative trait locus
RFLP	restriction fragment length polymorphism
ROS	reactive oxygen species
RR	relative risk
SAM	Significance Analysis of Microarrays
SCID	severe combined immunodeficiency
SDS/PAGE	sodium dodecyl sulfatee /polyacrylamide gel electrophoresis
SNP	single nucleotide polymorphism
SOD	superoxide dismutate
SSB	single strand break
SSR	simple sequence repeats
Sv	sievert

TJL	The Jackson Laboratory
TRRAP	transformation/transcription domain associated protein
USRT	United States Radiologic Technologists
UTR	untranslated region
UV	ultraviolet
WRN	Werner syndrome
XP	xeroderma pigmentosum
XRCC	X-ray repair complementing