Fancd2 IN EPITHELIAL CANCER AND DNA REPAIR

by

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A DISSERTATION

Presented to the Department of Molecular and Medical Genetics

and the Oregon Health & Science University

School of Medicine

in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

July, 2005

School of Medicine
Oregon Health & Science University

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ACKNOWLEDGEMENTS

The work presented in this doctoral dissertation represents the efforts of many individuals at Oregon Health & Science University (OHSU) as well as collaborators at other institutions. Alan D'Andrea, Steven Meyn, and Milton Finegold have been long time collaborators of Dr. Markus Grompe and contributed greatly to the scientific ideas explored in this body of work. Of course, none of these experiments would have been possible without members of the FA scientific community within OHSU including Cynthia Timmers, Jim Hejna, Robb Moses, Grover Bagby, Susan Olson, Amy Newell, Yasmine Akkari, Andreas Rothfuss, Henri van de Vrugt, Meenakshi Noll, Barbara Cox, Sean Baker, and Kevin McCabe. Cynthia Timmers deserves special recognition for her efforts in cloning *FANCD2* and providing expert assistance, training and friendship to a young and somewhat naive first year graduate student. This project could not have happened without her efforts and encouragement. Finally, I'd like to give a special thanks to Mushen Al'Dhalimy who brings humor, great work ethic and a calming presence to what sometimes feels like a hectic place to work.

I spent a great deal of time in the lab over the past 6 years. However, I wouldn't have survived without a great group of friends on the outside. I've been blessed with amazing housemates including Paul Meyer, David Jacobson, Jeff Koch, and Ryan Stout. David introduced me to the Northwest Visitors (and Storm Shirley) who have provided wonderful companionship on some exciting musical adventures. Ryan introduced me to the members of JIM which led to psYchic and finally to whor$hack. My many teammates have been a source of incredible joy. I feel very lucky to have found such a wonderful group of friends within the Portland ultimate community.

My family has also provided wonderful support, love, and fun before and during my time at OHSU. It's been a special treat to be very close to Heather, Jay, Duncan and Tessa in Seattle. Thank you for your generosity, understanding, inspiration, curiosity, love and willingness to nod your head and smile when I've attempted to explain the scientific basis for the experiments I've done.

It's been an honor and a privilege to train under the auspices of Markus Grompe. He has been a patient and understanding leader while maintaining high expectations. Markus has allowed me to present the work included this dissertation at national meetings and encouraged me to pursue my training with the best and brightest in the scientific world. Markus has also been a fine example by balancing the challenges of working in the lab with trips to the Hillsdale Pub, bike rides to work, and an interesting sense of humor. He has also maintained an understanding that vibrancy and fruitfulness within the lab can be strengthened by happiness provided by pursuits outside of the lab.

Finally, I'd like to thank the faculty members at OHSU who served on my thesis committee: Mitchell Turker, Stephan Lanker, Mike Liskay, Hua Lu, Maureen Hoatlin, and Gary Thomas.

LIST OF ABBREVIATIONS

AML- acute myelogenous leukemia

AT- ataxia telangiectasia

ATM- ataxia telangiectasia mutated

ATR- ataxia telangiectasia and rad3 related

BASC- BRCA1-associated genome surveillance complex

BLM- Bloom helicase

BMT- bone marrow transplantation

BRCA2- breast and ovarian cancer susceptibility gene 2

BRDU- bromodeoxyuridine

CPM- counts per minute

DAPI- 4',6-diamidino-2-phenylindole

DMEM- Dulbecco's modified eagle medium

DNA-PK- DNA dependant protein kinase catalytic subunit

DSB- double strand break

DTT- dithiothreitol

ERCC1- excision repair cross complementing rodent repair group 1

FA- Fanconi anemia

FAAP- Fanconi anemia associated protein

FANCD2- Fanconi anemia complementation group D2

FBS- fetal bovine serum

GCR- gross chromosomal rearrangement

GCSF- granulocyte colony stimulating factor

GFP- green fluorescent protein

GMCSF- granulocyte-macrophage colony stimulating factor

GVHD- graft versus host disease

H+E- hematoxylin and eosin

HDR- homology directed repair

HMT- 4'-hydroxymethyl-4, 5', 8-trimethylpsoralen

HPV- human papilloma virus

HSC- hematopoietic stem cell

HSP70- heat shock protein 70

ICL- DNA interstrand crosslink

IFAR- international Fanconi anemia registry

IFN- interferon

IL- interleukin

IR- ionizing radiation

LOH- loss of heterozygosity

MDS- myelodysplastic syndrome

MEF- mouse embryonic fibroblast

MEN1- multiple endocrine neoplasia type 1

MMC- mitomycin c

MMCT- micro-cell mediated chromosome transfer

NADPH- nicotinamide adenine dinucleotide phosphate

NHEJ- non-homologous end joining

NLS- nuclear localizaton signal

PBS- phosphate buffered saline

PCR- polymerase chain reaction

PKR- double-stranded RNA dependent protein kinase

PRKDC- protein kinase, DNA activated, catalytic subunit

RACE- rapid amplification of cDNA ends

RB- retinoblastoma

RDS- radioresistant DNA synthesis

ROS- reactive oxygen species

SCID- severe combined immunodeficient

SCC- squamous cell carcinoma

SCP3- synaptonemal complex protein 3

SDS-PAGE- sodium dodecyl sulfate poly acrilamide gel electrophoresis

SSA- single strand annealing

STGC- short tract gene conversion

STAT1- signal transducer and activator of transcription 1

SV40- simian virus 40

TERC- telomerase RNA component

TNF alpha- tumor necrosis factor alpha

TRP53- transformation related protein 53

TUNEL- terminal nick end labeling

USP1- ubiquitin specific protease 1

XPF- xeroderma pigmentosum group F

XRCC9- x ray cross-complementing rodent repair group 9

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ABSTRACT

Fanconi anemia (FA) is a genetic disorder characterized by hypersensitivity to DNA damage, bone marrow failure, congenital defects, and cancer. To further investigate the *in vivo* function of the FA pathway, mice with a targeted deletion in the distally acting FA gene *Fancd2* were created. Similar to human FA patients and other FA mouse models, *Fancd2* mutant mice exhibited cellular sensitivity to DNA interstrand crosslinks (ICLs) and germ cell loss. In addition, chromosome mispairing was seen in male meiosis. However, *Fancd2* mutant mice also displayed phenotypes not observed in other mice with disruptions of proximal FA genes. These include microphthalmia, perinatal lethality and epithelial cancers, similar to mice with *Brca2/Fancd1* hypomorphic mutations. These additional phenotypes were not caused by defects in the Atm (ataxia telangiectasia mutated) mediated S phase checkpoint which was intact in primary *Fancd2* mutant fibroblasts.

To further investigate the role of the FA pathway in tumor prevention, *Fancd2* mutant mice were crossed to mice with a null mutation in the tumor suppressor gene, *Trp53*. The tumor spectrum in *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice included sarcomas expected in *Trp53* heterozygotes, as well as mammary and lung adenocarcinomas that occur rarely in *Trp53* heterozygotes. These tumors occurred earlier than in *Fancd2*$^{-/-}$ control mice. Therefore, the *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice represent an improved model for the study of adenocarcinoma in FA. In addition it was found that *Fancd2*$^{-/-}$ mouse embryonic fibroblasts (MEFs) but not *Fancd2*$^{-/-}$/*Trp53*$^{-/-}$ MEFs arrest following DNA damage. Therefore, *Trp53* is required for an S phase checkpoint activation observed in *Fancd2*

mutant cells. *Fancd2*$^{-/-}$/*Trp53*$^{-/-}$ cells also showed an increase in aneuploidy and had multiple gross chromosomal rearrangements (GCRs).

In addition to a hallmark sensitivity to drugs that induce ICLs, cells from FA patients and mouse models are modestly sensitive to ionizing radiation (IR). IR induces a variety of DNA lesions including double strand breaks (DSBs). To investigate the role of the FA pathway in repair of IR induced DNA damage, we generated *Fancd2*$^{-/-}$/*Prkdc*$^{sc/sc}$ double mutant mice. *Prkdc*$^{sc/sc}$ mutant mice have a defect in non-homologous end joining (NHEJ) and are sensitive to IR induced DNA damage. Double mutant animals and cells were more sensitive to IR than *Prkdc*$^{sc/sc}$ mutants. In addition, double mutant cells had reduced colony forming ability following electroporation of the restriction enzyme, *PvuII*. This indicates that DSBs are the specific type of IR induced DNA damage to which the cells are sensitive. We conclude that Fancd2 operates in a DSB repair pathway that is distinct from NHEJ.

In many respects, *Fancd2* mutant mice represent an improved mouse model of the human disease, FA. Mice lacking functional Fancd2 share many phenotypes described in previously generated FA mice but have an increased incidence of epithelial tumors that is accelerated by heterozygosity at *Trp53*. The phenotype of *Fancd2* knockout mice supports a role for the FA pathway in preventing genomic instability by functioning in a DNA repair pathway in response to ICLs and DSBs.

Chapter One: Introduction

1.1. **Clinical description of Fanconi anemia**

The first description of FA was published in 1927 by the Swiss pediatrician, Guido Fanconi (Fanconi 1967). The disease is rare and has an estimated incidence of 3 in 1,000,000, members of the general population (Tischkowitz and Dokal 2004). It has been diagnosed in many ethnic groups. However, founder mutations in Ashkenazi Jews, Afrikaans of South Africa, black populations of southern Africa and Spanish gypsy families have contributed to a higher incidence in these populations (Rosendorff et al. 1987; Verlander et al. 1995; Callen et al. 2005; Morgan et al. 2005). The inheritance pattern is recessive, both autosomal and x-linked (Meetei et al. 2004a; Tischkowitz and Dokal 2004). FA is characterized by congenital defects, progressive pancytopenia and a predisposition to a variety of malignancies. Multiple complementation groups exist (FA-A, B, C, D1, D2, E, F, G, I, J, and L) and all share a similar yet often variable phenotype (Alter 1993; Levitus et al. 2004).

Most FA patients are born with at least one congenital abnormality. These may include short stature, skeletal defects, microphthalmia, microcephaly, skin pigmentation abnormalities, male hypogonadism, cardiac defects, renal defects, hearing deficits and mental retardation (see table 1) (Tischkowitz and Hodgson 2003). The phenotype, even among patients with identical pathogenic mutations, can be quite variable as illustrated by a consanguineous family with four patients harboring the same *FANCA* mutation (Koc et al. 1999).

Table 1. Physical abnormalities of Fanconi anemia patients

Abnormality	Frequency
Skeletal (radial ray, hip, vertebral scoliosis, rib)	71%
Skin pigmentation (cafe au lait, hyper- and hypopigmentation	64%
Short stature	63%
Eyes (microphthalmia)	38%
Renal and urinary tract	34%
Male genital	20%
Mental retardation	16%
Gastrointestinal	14%
Cardiac abnormalities	13%
Hearing	11%
Central nervous system (hydrocephalus, septum pellucidium)	8%
No abnormalities	30%

Taken from (Tischkowitz and Hodgson 2003)

FA is the most common type of inherited bone marrow failure syndrome (Tischkowitz and Dokal 2004). The hematological complications of FA have a variable age of onset but the median is 7 years (Auerbach and Allen 1991; Butturini et al. 1994). Macrocytosis and pancytopenia are often evident in the first decade of life. The hematological disease is likely due to a defect in hematopoietic stem cells or early progenitors, as all blood lineages are affected. In one study the median age at death was 13 years (Butturini et al. 1994). A recent study found that the presence of certain congenital abnormalities were prognostic for the development of bone marrow failure (Rosenberg et al. 2004). Patients with radial defects had a 5.5 fold increased risk of bone marrow failure compared to cases with normal radii.

Some patients may appear normal and diagnosis for FA only occurs at the time of onset of bone marrow failure. The diagnostic test for FA takes advantage of the sensitivity

of patients' cells to drugs that induce ICLs (Auerbach and Wolman 1976). Exposure to these drugs, such as DEB, cause radial formation and chromosome breaks. FA and normal cells have distinct ranges of sensitivity thus making diagnostic testing feasible (Auerbach 1993). A new diagnostic test for FA is based on immunoblotting for the downstream FA protein, FANCD2, and can be accompanied by retroviral complementation to classify patients into complementation groups (Shimamura et al. 2002; Shimamura and D'Andrea 2003). This method for complementation group identification may be preferred to the laborious cell fusion studies of the past.

Myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML) are common in FA patients. Three recent studies used different data sets and statistical methods to analyze cancer risk in FA patients but came to similar conclusions regarding patients' risk of developing AML or MDS. The first, a literature review from 1927-2001, found 1300 documented FA cases of which 9% had primary AML and 7% had MDS (Alter 2003). The second used a retrospective cohort of North American FA patients and identified 9 cases of leukemia in 145 patients (Rosenberg et al. 2003). The cumulative risk for leukemia was 10% at age 48. The third study analyzed patients in the International Fanconi Anemia Registry (IFAR) and identified 120 hematological neoplasms in 754 subjects with a risk of hematological neoplasm of 33% by age 40 (Kutler et al. 2003a). A summary of the three studies noted the crude risk of leukemia (exclusive of MDS) to be between 5% and 10%, while crude risk of MDS is about 5% (Alter et al. 2003).

AML is the most common malignancy in FA but patients, particularly those that do not succumb to bone marrow failure, have a 50 fold increased risk of developing solid

tumors (Rosenberg et al. 2003). Hepatic tumors and squamous cell carcinoma (SCC) of the aerodigestive tract and vulva are common (Alter 2003; Kutler et al. 2003a; Rosenberg et al. 2003). Patients have shown toxicity associated with traditional bone marrow transplantation (BMT) conditioning regiments and although these have been modified, FA patients who receive BMT still have a heightened risk of solid tumors. The age-specific hazard for developing squamous cell carcinoma (SCC) of the head and neck is 4.4 fold higher for patients who receive BMT than for those who did not (Rosenberg et al. 2005). Although patients with mutations in *BRCA2* (complementation group D1) have an elevated incidence of brain tumors and may have an increased predisposition to solid tumors early in childhood, patients from all other groups have an equal risk for developing malignancies (Offit et al. 2003; Hirsch et al. 2004).

Inactivation of FA genes has also been implicated in a variety of cancers among the general population. Taniguchi and coworkers showed that 18% of the primary ovarian cancers they screened had defects in the FA pathway due to silencing of the *FANCF* gene (Taniguchi et al. 2003). Inherited mutations in *FANCC* and loss of heterozygosity (LOH) for the wild-type allele have been identified in early onset pancreatic cancer (van der Heijden et al. 2003; Couch et al. 2005). The most striking connection between FA and cancer is the fact that inheritance of germ line mutations in *BRCA2/FANCD1*, predispose individuals to a number of malignancies including breast and ovarian cancer (Tavtigian et al. 1996). It also bears noting that some cases of familial ovarian cancer have been linked precisely to the region where *FANCD2* resides in the human genome (Sekine et al. 2001; Simsir et al. 2001; Zhang and Xu 2002). Thus, inactivation of FA genes in otherwise healthy individuals, may contribute to malignancy in a variety of tissues.

Treatment for FA patients includes supportive care for bone marrow failure but the only curative therapy currently available is BMT. Patients displaying anemia are often treated with the oral androgen, oxymetholone, with the effect of raising red cell and platelet counts (Shimamura 2003). Androgen therapy involves specific side effects including increased risk of hepatic tumors. Patients may also respond to treatment with growth factors such as, G-CSF and GM-CSF but the risk of hematological malignancy may increase (Guinan et al. 1994; Rackoff et al. 1996). Most patients will eventually require further treatment involving BMT.

The cell source for transplantation may be from umbilical cord blood or bone marrow. Patients who receive BMT from HLA-compatible sibling donors, using modified conditioning regiments of low dose IR and a reduced dose of cyclophosphamide, have a 2 year survival rate of 75% (Guardiola et al. 1998; Socie et al. 1998). This modified regiment has improved post-BMT survival but may contribute to increased risk of graft versus host disease (GVHD). FA patients conditioned with the modified regiment had double the risk of developing acute GVHD compared to a cohort of non-FA patients receiving a matched BMT using the standard conditioning regiment (Guardiola et al. 2004).

For those patients without a suitable matched sibling, unrelated matched BMT has been attempted. Results have been predictably poor with one study reporting a low 33% survival rate at 3 years (Guardiola et al. 2000). Recent reports have demonstrated that a fludarabine based conditioning regiment, that excludes IR, may improve the outcome of both related and unrelated matched donor BMT (de la Fuente et al. 2003). A number of

studies have reported positive results including decreased GVHD and increased survival when using fludarabine based conditioning regiments (Tischkowitz and Dokal 2004).

The selective growth advantage of genetically corrected FA cells makes the disease an ideal candidate for gene therapy. *Fancc* knockout mice showed phenotypic correction of MMC sensitivity when treated with *Fancc* retrovirus (Gush et al. 2000; Noll et al. 2001). However, retroviral based therapy in human patients has shown limited success (Liu et al. 1999). Promising reports have come from experiments using lentiviral gene delivery methods in mice (Galimi et al. 2002). Recent unpublished results using non-viral naked DNA gene delivery techniques have been reported. The *Sleeping Beauty* transposon is effective in the treatment of murine models of hemophilia B and hereditary tyrosinemia type 1 and may have utility in the treatment of FA (Montini et al. 2002; Ohlfest et al. 2004). Non-viral mediated gene delivery methods may be ideal for the treatment of FA due to low cost of plasmid preparation, lack of viral induced immune response, lack of insertional mutagenesis and lack of *ex vivo* manipulation of HSCs (Markus Grompe, unpublished results).

1.2. Molecular genetics and biochemistry of Fanconi anemia

FA is genetically heterogeneous consisting of at least 11 complementation groups, FA-A, B, C, D1, D2, E, F, G, I, J, and L that have been identified by cell fusion studies (Levitus et al. 2004). A number of gene identification methods have been used to identify FA genes including expression cloning (*FANCC*), micro-cell mediated chromosome transfer (MMCT) (*FANCD2*) and mutation screening in candidate genes (*BRCA2/FANCD1*) (Strathdee et al. 1992; Timmers et al. 2001; Howlett et al. 2002). To date, nine causative genes have been identified including *FANCA*, *B*, *C*, *D1/BRCA2*, *D2*, *E*, *F*, *G/XRCC9*, and *L/PHF9* (Strathdee et al. 1992; Lo Ten Foe et al. 1996; de Winter et al. 1998; de Winter et al. 2000a; de Winter et al. 2000b; Timmers et al. 2001; Howlett et al. 2002; Meetei et al. 2003a; Meetei et al. 2004a). The majority of patients fall into complementation groups FA-A (65%), FA-C (15%), and FA-G (10%) with the other groups being relatively rare (D'Andrea and Grompe 2003) (see table 2).

Table 2. Genetic subtypes of Fanconi anemia

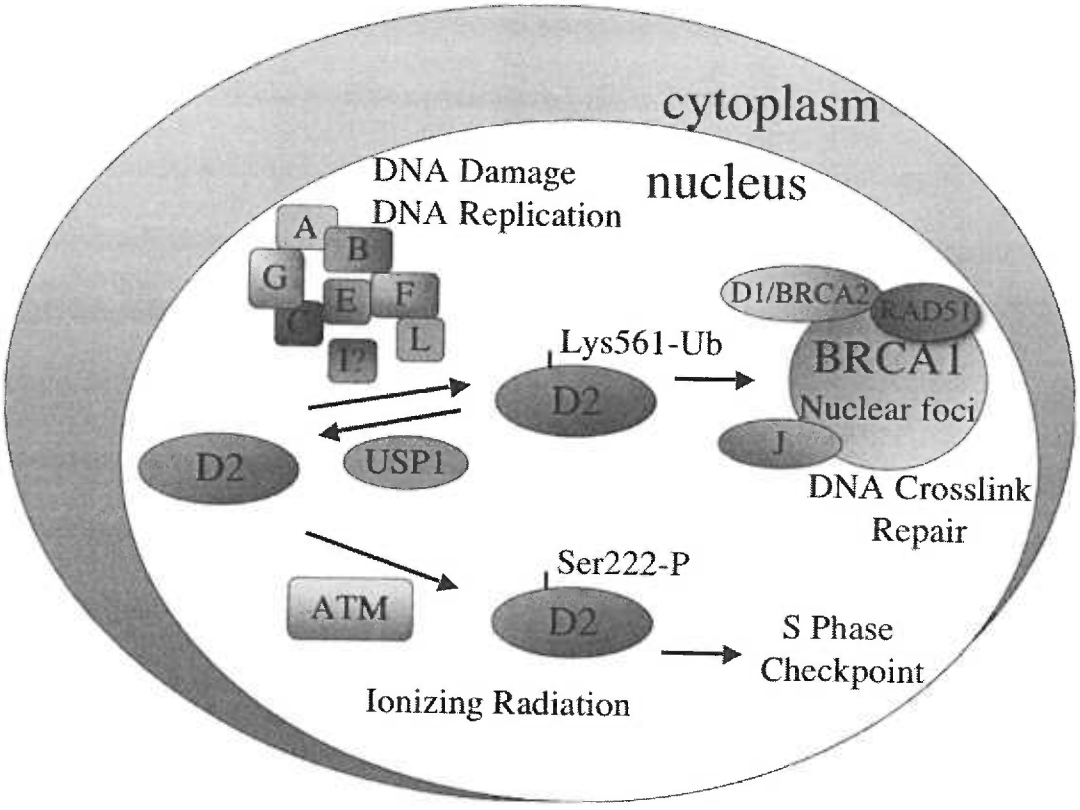
Complementation Group	Reference Cell Line	Defective Gene	Relation to FANCD2	% of Patients in Group
FA-A	HSC72	*FANCA*	Upstream	~65%
FA-B	HSC230	*FANCB*	Upstream	rare
FA-C	HSC536	*FANCC*	Upstream	~10%
FA-D1	HSC62	*BRCA2/FANCD1*	Downstream*	<5%
FA-D2	PD20	*FANCD2*	----------	<5%
FA-E	EUFA130	*FANCE*	Upstream	<2.5%
FA-F	EUFA121	*FANCF*	Upstream	<2.5%
FA-G	EUFA143	*FANCG/XRCC9*	Upstream	~10%
FA-I	EUFA592	*FANCI*	Upstream	rare
FA-J	EUFA543	*FANCJ*	Downstream*	rare
FA-L	EUFA868	*FANCL/*	Upstream	rare

(adapted from (Levitus et al. 2004))

*BRCA2 and FANCJ may also function independent of the FA pathway

The precise function of the FA proteins remains uncertain, however many clues point to a role for the proteins in a common pathway that responds to DNA damage and is required for genomic stability (D'Andrea and Grompe 2003). The cloning of *FANCC* in 1992 opened the door for a molecular understanding of the pathway, however the lack of homology to other proteins offered no clues to a potential function (Strathdee et al. 1992). The identification of *FANCA* led to the demonstration that FANCA and FANCC bind one another and form a complex in the nucleus (Kupfer et al. 1997). Subsequently, FANCB, FANCE, FANCF, FANCG, and FANCL have been shown to exist as part of the FA core complex (Figure 1) (Garcia-Higuera et al. 1999; de Winter et al. 2000c; Medhurst et al. 2001; Pace et al. 2002; Taniguchi and D'Andrea 2002; Meetei et al. 2004a).

Figure 1. Model of FA Pathway



A functional FA core complex is required for the monoubiquitination of FANCD2 that occurs following DNA damage and during S phase of the cell cycle (Figure 1) (Garcia-Higuera et al. 2001; Taniguchi et al. 2002a). FANCL, a member of the FA complex, is an E3 ubiquitin ligase and is likely responsible for this modification of FANCD2 (Meetei et al. 2003a; Meetei et al. 2004b). The protein responsible for deubiquitination of FANCD2, USP1, was recently identified (Nijman et al. 2005). Interestingly, knockdown of the *USP1* mRNA by siRNA results in a hyper-accumulation of monoubiquitinated FANCD2 and a decreased sensitivity to MMC, thus further implicating monoubiquitinated FANCD2 in the response to ICLs. Monoubiquitinated FANCD2 forms nuclear foci that co-localize with the DNA repair proteins, BRCA1 and RAD51 (Garcia-Higuera et al. 2001; Taniguchi et al. 2002a).

While the precise nature of the interaction between FANCD2 and BRCA1 in nuclear foci is unclear, it is known that BRCA1 is required for the formation of monoubiquitinated FANCD2 and IR induced FANCD2 nuclear foci (Garcia-Higuera et al. 2001). BRCA1 is a tumor suppressor gene that is mutated in familial breast and ovarian cancer and is believed to function as a "caretaker" of the genome (for review see (Deng and Brodie 2000; Deng and Scott 2000)). BRCA1 may function as a coordinator of multiple repair processes as it forms a large complex with many proteins termed BRCA1-associated genome surveillance complex (BASC) (Wang et al. 2000). It is involved in many cellular processes including DNA damage repair, cell-cycle regulation, transcription, and chromatin remodeling via its interaction with multiple proteins. The exon 11 encoded portion of BRCA1 is the largest, encompassing 60% of protein, and contains two nuclear localization signals (NLS). Proteins that interact directly and/or

indirectly with the exon 11 encoded portion of BRCA1 include RAD50, RAD51, RB, and c-MYC (Scully et al. 1997; Wang et al. 1998; Aprelikova et al. 1999; Zhong et al. 1999). The C terminus contains two BRCT (BRCA1 C terminal) domains that interact directly or indirectly with p53, BRCA2, RB, and a histone deacetylase complex (Chen et al. 1998; Ouchi et al. 1998; Zhang et al. 1998; Chai et al. 1999; Yarden and Brody 1999).

Cells lacking BRCA1 share certain phenotypes with cells that lack RAD51 or BRCA2, including hypersensitivity to IR, chromosomal aberrations, and early embryonic lethality (Lim and Hasty 1996; Sharan et al. 1997; Shen et al. 1998). These defects suggest that BRCA1 may function directly in DNA repair. This idea is supported by the work of Moynahan *et al* who observed gene targeting defects at the *Rb* locus in *BRCA1* deficient ES cells (Moynahan et al. 2001a). The mutant cells also showed impaired homology directed repair (HDR) of DSBs that may account for the genetic instability observed in *BRCA1* mutant cells.

FANCD2 has also been shown to interact with BRCA2/FANCD1 both in yeast two hybrid experiments and *in vivo* in chromatin (Hussain et al. 2004; Wang et al. 2004). BRCA2/FANCD1 binds single stranded DNA, interacts directly with RAD51, and modulates its activity (Marmorstein et al. 1998; Davies et al. 2001; Yang et al. 2002). Cells lacking functional BRCA2/FANCD1 are deficient in HDR of DSBs, adding further evidence that the FA pathway may function to repair DNA by homologous recombination (Moynahan et al. 2001b; Tutt et al. 2001). BRCA2/FANCD1 and the yet unidentified FANCJ act downstream of other FA proteins, as FANCD2 is monoubiquitinated in cell lines from FA-D1 and FA-J patients whereas FANCI has been placed upstream of

FANCD2 based on the lack of monoubiquitinated FANCD2 in FA-I cell lines (Figure 1) (Howlett et al. 2002; Levitus et al. 2004).

In addition to its interaction with the upstream nuclear complex, FANCD2 has been shown to interact with proteins that are important for the cell's response to IR. Cells from ataxia-telengiectasia (AT) and Nijmegan breakage syndrome (NBS) have mutations in *ATM* and *NBS1* respectively, and are sensitive to IR (Chun and Gatti 2004; Matsuura et al. 2004). In response to IR, FANCD2 is phosphorylated at Serine 222 by ATM (Figure 1) (Taniguchi et al. 2002b). This phosphorylation is required for the establishment of an intra-S-phase checkpoint but is not required for FANCD2 monoubiquitination or FANCD2 recruitment to nuclear foci. FANCD2 phosphorylation by ATM in wild-type cells, following treatment with MMC, is dependent on NBS1 (Nakanishi et al. 2002). While *ATM* and *NBS1* mutant cells are both hypersensitive to IR, FA cells are only modestly sensitive. Thus, the relevance of FANCD2's interaction with these proteins remains uncertain.

FANCD2 has also been shown to be the target of a second DNA damage signaling kinase, AT-related rad 3 (ATR). *ATR* is mutated in the cells of Seckel syndrome patients that show a similar chromosome instability resulting in radial formation following treatment with MMC (O'Driscoll et al. 2003). ATR functions upstream of a signaling pathway that is necessary for an ICL induced S phase arrest and is required for FANCD2 phosphorylation that occurs following ICL induction (Pichierri and Rosselli 2004). Furthermore, ICLs trigger ATR and FANCD2 to co-localize in nuclear foci. ATR is also required for efficient monoubiquitination of FANCD2 (Andreassen et al. 2004). Altogether, the downstream FA protein, FANCD2, receives multiple inputs from at least

three distinct DNA damage response pathways and may function as a central node in the response to various types of DNA damage.

While a definitive role for FANCD2 in DNA repair has not been demonstrated, activated FANCD2 associates with chromatin, thus placing it in the correct cellular location for this putative function. The first indication that FANCD2 may be functioning in chromatin came indirectly via its interaction with MENIN (Jin et al. 2003), a tumor suppressor that directly binds DNA (La et al. 2004). Inactivation of MENIN causes the human tumor syndrome, multiple endocrine neoplasia type I (MEN1), associated with tumors of the parathyroid, pancreatic islets, and the pituitary (Agarwal et al. 2004). Menin is localized to chromatin and menin's association with FANCD2 is enhanced by IR.

FANCD2 also interacts with other chromatin associated proteins. Activated FANCD2 is targeted to chromatin where it interacts directly with BRCA2/FANCD1 (Wang et al. 2004). FANCD2 mutant cells are deficient in the assembly of damage inducible BRCA2/FANCD1 foci and also deficient in the loading of BRCA2/FANCD1 into chromatin.

While identification of FA genes and biochemical investigations of their encoded protein's function has offered clues to the function of the pathway, there are still at least three unidentified genes corresponding to complementation groups FA-I, FA-J, and FA-M (Levitus et al. 2004). A recent biochemical approach led to the identification of *FANCL* and *FANCB*. These proteins were isolated as part of a multi-protein nuclear complex termed BRAFT, that contains BLM helicase, topoisomerase III alpha, replication protein A, and other unidentified FA-associated proteins (FAAP) (Meetei et al. 2003b). Further

biochemical investigations may lead to the identification of genes mutated in other FA complementation groups. Based on the helicase activity of BLM, Meetei *et al* have suggested that this complex may facilitate unwinding of DNA. Further investigations into the precise biochemical function of these newly identified FA proteins may yield insights into the function of the FA pathway.

1.3. Potential functions of the Fanconi anemia proteins

FA cells have been shown to have a heightened sensitivity to agents that induce ICLs. This well accepted phenotype forms the basis for a diagnostic test and likely contributes to the pathology associated with the syndrome. However, other phenotypes described for FA cells support roles in cellular processes that regulate oxygen free radicals, telomere length and hematopoiesis (Stark et al. 1993b; Leteurtre et al. 1999; Pagano and Youssoufian 2003).

FA cells have increased sensitivity to oxygen and chromosomal breaks decrease in a low oxygen environment (Joenje et al. 1981). Some ICL agents produce reactive oxygen species (ROS) and FA cells may be sensitive due to an impaired ability to process ROS (Pagano and Youssoufian 2003). Other studies have also demonstrated links between FA proteins and proteins involved in cellular oxygen metabolism. Yeast two hybrid experiments have identified a potential association between FANCC and NADPH cytochrome p450 reductase as well as FANCG and cytochrome p450 2E1 (CYP2E1) (Kruyt et al. 1998; Futaki et al. 2002). These enzymes are involved in the generation of ROS and their interactions with FA proteins may explain the increased oxygen sensitivity in FA cells.

The anemia observed in FA patients has led many investigators to propose a primary role for FA proteins in hematopoiesis. Hematopoiesis was found to be defective in bone marrow from FA patients by using an *in vitro* long term culture assay (Stark et al. 1993b). FA cells also show changes in the levels of certain growth factors which may be required for proper functioning of bone marrow including reduced levels of interleukin 6 (IL-6), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin 1 beta

(IL1-β) and increased tumor necrosis factor alpha (TNF-α)(Rosselli et al. 1992; Stark et al. 1993a; Rosselli et al. 1994).

In biochemical assays FA proteins interact with signaling molecules found in the cytoplasm. This association may explain the bone marrow failure in FA patients. FANCC interacts with heat shock protein 70 (HSP70), an association shown to be required for suppression of the pro-apoptotic interferon-inducible double-stranded RNA-dependent protein kinase (PKR) (Pang et al. 2002). FANCC also binds STAT1, an interaction required for growth signaling following stimulation with interferon gamma (IFN-γ) (Pang et al. 2000). These interactions with molecules involved in preventing pro-apoptotic signaling and enhancing pro-survival/growth signaling may account for the decreased hematopoiesis observed in FA patients.

Lensch *et al* have suggested that these initial signaling abnormalities may lead to an increase in apoptosis of progenitor cells (Lensch et al. 1999). In the context of heightened genomic instability due to inactive FA genes, mutations in tumor suppressor genes may occur, allowing some cells to escape apoptosis. These clones may have a selective advantage and progress to MDS or AML. It is interesting to note that the chromosomal deletions observed in AML of non-FA patients are similar to those seen in AML of FA patients. Thus the same anti-apoptotic selective pressure may be functioning in both settings.

Human FA patients display significantly shortened telomeres in peripheral blood cells, supporting a role for the FA proteins in telomere maintenance (Leteurtre et al. 1999; Adelfalk et al. 2001; Hanson et al. 2001). It is unclear whether the shortening is due to increased cycling of HSCs or due to a direct role in telomere maintenance. A relative

increase in breaks at telomeres in FA cells versus controls, suggests that the FA proteins may function directly at telomeres in either the prevention or repair of telomeric chromosome breaks (Callen et al. 2002). In contrast, investigations of telomere dynamics in *Fancg* KO mice have revealed no defects in telomere end capping, telomerase function, or telomere length even in the presence of DNA damage (Franco et al. 2004). One explanation for the lack of observed defects in *Fancg* mutant mice may be differences in telomere length between mice and humans. To address this difference Franco *et al* crossed *Fancg* knockout mice to *Terc*$^{-/-}$ mice for five generations to create *Fancg* mutant mice with short telomeres. No detectable telomere defects were observed in this cohort of mice either. Therefore, rather than a primary defect in telomere maintenance, the observed telomere shortening in FA patients may be due to a secondary effect caused by increased cycling of the hematopoietic progenitor cells.

Although the FA proteins may function in oxygen metabolism, hematopoiesis, and telomere maintenance, the most agreed upon function for the FA proteins is in a DNA damage response pathway (D'Andrea and Grompe 2003). The FA pathway could function in repair of a variety of DNA lesions but the hallmark sensitivity to cross-linking agents suggests an important role in repair of ICLs. ICLs represent a particularly toxic genetic lesion because both strands of the DNA helix are involved. Repair of ICLs requires components of multiple DNA repair pathways: nucleotide excision repair proteins, XPF and ERCC1, are needed for an unhooking step that is followed by a homologous recombination step to provide the required genetic material for repair (McHugh et al. 2001).

Recent experiments investigating ICL repair in mammalian cells show that they are incised throughout the cell cycle and processed to DSBs during S phase (Rothfuss and Grompe 2004). The initial incision step during ICL repair creates single strand breaks, both 5' and 3' of the lesion, and allows for further processing. The FA pathway is likely not required for the incision step as monoubiquitinated Fancd2 is not detected at this time. However, the presence of monoubiquitinated FANCD2 correlates with the appearance of DSBs and suggests that activated FANCD2 may play a role in the processing of these DNA lesions. Interestingly, monoubiquitinated FANCD2 is detectable very quickly following IR, leading to the hypothesis that the FA pathway may respond specifically to DSBs. The idea that FA proteins function in the repair of DSBs is further supported by reports that FA knockout mice have a modestly increased sensitivity to IR (Noll et al. 2001; Yang et al. 2001; Houghtaling et al. 2003) and plasmid based studies which demonstrate an impaired processing of DNA double stranded ends in FA cells (Escarceller et al. 1997; Donahue and Campbell 2002).

DSBs are particularly toxic DNA lesions for which multiple repair proteins have evolved. Two major pathways exist in eukaryotic cells, homology directed repair (HDR) and non-homologous end joining (NHEJ) (Valerie and Povirk 2003; Pfeiffer et al. 2004). HDR is the major pathway for repair of DSBs in budding yeast while NHEJ is the preferred pathway in mammalian cells (Liang et al. 1998; Haber 2000). HDR subclasses in mammalian cells include single-strand annealing (SSA) and short-tract gene conversion (STGC), both of which have specific protein requirements (Ivanov et al. 1996; Karran 2000; Thompson and Schild 2001; West 2003).

NHEJ requires a distinct set of proteins including the main components, XRCC4/DNA ligase IV and DNA-PK, comprised of the DNA-PK catalytic subunit (DNA-PK) and the Ku70/80 heterodimer (Valerie and Povirk 2003). The requirement for DNA-PK in NHEJ is supported by the identification of severe combined immune deficient (scid) mice, which have reduced DNA-PK activity due to a nonsense mutation in *Prkdc* (Blunt et al. 1996). The NHEJ defect in these mice contributes to impaired V-D-J immunoglobulin recombination and increased sensitivity to IR induced DSBs (Blunt et al. 1995).

The FA field has slowly uncovered clues that suggest a specific role for FANCD2 in DNA repair by a homology dependent mechanism. FANCD2 is directed to nuclear foci containing the central HDR protein RAD51 and may modulate its function (Taniguchi et al. 2002a). The functional significance of this colocalization remains unclear as loss of FANCD2 does not perturb RAD51 foci formation (Godthelp et al. 2002; Houghtaling et al. 2003; Ohashi et al. 2005; Yamamoto et al. 2005). However, FANCD2 was shown to interact directly with BRCA2/FANCD1, a protein that binds to and modulates the activity of RAD51 (Davies et al. 2001; Yang et al. 2002; Hussain et al. 2004). Recent experiments using chromosomally integrated reporter constructs have demonstrated a role for both human and chicken FANCD2 in HDR of DSBs (Nakanishi et al. 2005; Ohashi et al. 2005; Yamamoto et al. 2005). Finally, FANCD2 has been shown to bind Holliday junctions and double strand DNA substrates *in vitro* (Park et al. 2005). While the precise role of the FA pathway in DNA repair is unclear, evidence is mounting to support its function in DNA repair by a homology dependant mechanism.

1.4. **Previous mouse models of Fanconi anemia**

One approach to investigate the function of a human gene product is to genetically engineer a mutation in the mouse and examine the phenotypic consequences. Mouse models of human disease can also provide access to mutant tissues that may not be retrievable from patients. Furthermore, by generating mice with deletions in multiple genes, researchers can investigate the functional relationship of these genes. Mouse models are also useful for preclinical trials of potential therapies, long-term cancer incidence studies, chemoprevention experiments, and gene therapy studies. Homologs for many of the FA genes have been identified in mice and a number of mouse models of the disease have been generated.

FANCC was the first human FA gene identified as well as the first mouse homolog identified (Wevrick et al. 1993). Mouse *Fancc* shares 79% amino acid identity with its human homolog and is capable of complementing the MMC sensitivity of human *FANCC* mutant patient cell lines. Analysis of *Fancc* mRNA levels revealed expression in all mouse tissues examined. *Fancc* expression levels have also been examined in the developing embryo (Krasnoshtein and Buchwald 1996). Expression was observed in the mesenchyme at 8-10 days post coitus and cells from both osteogenic and hematopoietic lineages at 13-19.5 days post coitus. Interestingly, high expression was observed in rapidly dividing progenitors, such as chondro- and osteo-progenitor cells, but was down regulated in differentiated cells, such as chondrocytes and osteocytes.

Two groups have reported similar findings for *Fancc* knockouts (table 3) (Chen et al. 1996; Whitney et al. 1996). *Fancc*$^{-/-}$ mice were born in the expected Mendelian ratios with normal gross morphology. Male and female *Fancc* mutants have a reduced number

of germ cells and impaired fertility. The germ cell number in *Fancc* mutant mice was reduced as early as embryonic day 12.5, suggesting that Fancc functions prior to meiosis and is required for mitotic proliferation of primordial germ cells (Nadler and Braun 2000). As expected, cells from the knockout mice have an increase in chromosomal breaks and aberrations following treatment with DNA cross-linkers. In addition, Whitney *et al* observed a heightened sensitivity to IFN-γ in progenitor cells from *Fancc* mutants. Unlike human FA patients, *Fancc* mutant mice do not develop anemia.

Fanca shares 65% amino acid identity with its human homolog, has a ubiquitous expression pattern in the embryonic and adult mouse (with highest levels in lymphoid tissue, testis and ovary) and is capable of complementing the MMC sensitivity of human FA-A cells (van de Vrugt et al. 2000; Wong et al. 2000). Morphologically normal knockouts of *Fanca* are born in the expected Mendelian ratios, do not develop anemia and have a phenotype similar to *Fancc* mutant mice, including hypogonadism and sensitivity to cross-linkers (table 3) (Cheng et al. 2000; Noll et al. 2002). More recently, Wong, *et al.* generated a *Fanca* knockout that was similar to previously described *Fanca* mutant mice but had strain specific phenotypes including growth retardation, microphthalmia, and craniofacial malformations (Wong et al. 2003).

Fanca/Fancc double mutant mice show no evidence for additional phenotypes beyond those observed in single mutants (Noll et al. 2002). The similarity in a number of phenotypes supports a model in which Fanca and Fancc are part of a multi-subunit nuclear complex. *Fancg* knockout mice have also been generated (table 3). Consistent with a protein that functions as a member of a multi-subunit complex with Fanca and

Fancc, *Fancg* mutants share a nearly identical phenotype with *Fanca* and *Fancc* knockouts (Yang et al. 2001; Koomen et al. 2002).

Although mice lacking *Fancl* were not generated deliberately, they were identified in an insertional mutagenesis screen based upon their lack of germ cells and termed germ cell deficient (gcd) (table 3) (Pellas et al. 1991). Subsequently a novel gene, *Pog*, was shown to be identical to that disrupted in gcd mice (Agoulnik et al. 2002). *Fancl/Pog* knockouts have reduced numbers of germ cells, reduced body weight and a strain specific embryonic lethality. Cells from *Fancl/Pog* knockouts also show sensitivity to DNA cross-linkers (Meetei et al. 2003a).

Table 3. Previous knockout mice for Fanconi anemia

Gene	Complementation Group	Similarities to Human Patients	Differences from Human Patients	Reference
Fanca	FA-A	Reduced germ cells MMC sensitivity	No malformations No anemia No tumors	1, 2, 3
Fancc	FA-C	Reduced germ cells MMC sensitivity Slight IR sensitivity	No malformations No anemia No tumors	4, 5, 6
Fancg	FA-G	Reduced germ cells MMC sensitivity Slight IR sensitivity	No malformations No anemia No tumors	7, 8
Fancl/Pog	FA-L	Reduced germ cells MMC sensitivity	No malformations No anemia No tumors	9, 10

1 (Cheng et al. 2000), 2 (Noll et al. 2002), 3 (Wong et al. 2003), 4 (Whitney et al. 1996), 5 (Chen et al. 1996), 6 (Noll et al. 2001), 7 (Yang et al. 2001), 8 (Koomen et al. 2002), 9 (Agoulnik et al. 2002), 10 (Meetei et al. 2003a)

While no mouse models of FA display spontaneous hematological complications, anemia has been induced in *Fancc* knockouts by delivering non-lethal doses of MMC (Carreau et al. 1998). Acute exposure to MMC resulted in marked bone marrow

hypoplasia and degeneration of proliferative tissues, but sub-lethal doses caused a progressive decrease in all peripheral blood cell types. Anemia was also induced in FA mice by crossing *Fancc* mutant mice to *Sod1* mice that lack Cu/Zn superoxide dismutase (Hadjur et al. 2001). Hepatocytes of *Fancc/Sod1* double mutant mice displayed microvesicular steatosis and bone marrow hypocellularity that was accompanied by significant decreases in peripheral blood erythrocyte and leukocyte numbers. Finally, 3 week old *Ercc1* knockout mice display multilineage cytopenia and fatty replacement of the bone marrow similar to very old wild-type mice (Prasher et al. 2005). Interestingly, although *Ercc1* has not been identified as an FA gene, its encoded protein product appears to function in early steps of ICL repair (McHugh et al. 2001).

Mutations in any of the mouse FA genes that function upstream of *Fancd2*, result in a similar phenotype that is generally mild. The knockout mice are born in expected Mendelian ratios, are morphologically similar and have no anemia or cancer predisposition. In contrast, mice lacking functional Brca2/Fancd1, which operates downstream of Fancd2, and Brca1, which is known to co-localize in DNA damage inducible nuclear foci with Fancd2, have more severe phenotypes. Mutations in either *Brca1* or *Brca2/Fancd1* that disrupt the majority of the C terminus of the protein cause an early embryonic lethal phenotype (table 4) (Ludwig et al. 1997; Sharan et al. 1997).

Partial viability is observed in *Brca2/Fancd1* mutant mice when the truncated protein retains the Rad51 interacting BRC motifs. 10% of $Brca2^{Tm1Cam}$ mice (Friedman et al. 1998) survive to birth whereas an average of 20% (depending upon the genetic background) of $Brca2^{Tr2014}$ mice (Connor et al. 1997) survive to birth. Both of these

Brca2/Fancd1 mutants are infertile, have a markedly reduced lifespan and succumb to thymic lymphomas.

The phenotype of other *Brca2* knockout mice is less severe. $Brca2^{\Delta 27/\Delta 27}$ mutants that retain all of the BRC motifs but lack exon 27 show perinatal lethality. Only 67% of the expected mutants are present at genotyping (McAllister et al. 2002). These mice are fertile, show decreased ductal side branching in the mammary gland and have a reduced lifespan due to an increase in a variety of tumors. Lymphoma, sarcoma, adenoma as well as carcinoma of mammary, gastric, endometrial, and lung epithelial cells form with a long latency of between 12 and 17 months. While approximately 25% of homozygous mutants develop carcinomas none were observed in $Brca2^{\Delta 27/+}$ heterozygotes. The elevated incidence of carcinoma in $Brca2^{\Delta 27/\Delta 27}$ mutant mice, suggests that the C terminus of Brca2, particularly exon 27, functions to suppress tumor formation.

Similar to *Brca2/Fancd1* mutations that lack the majority of the protein, *Brca1* mutations that delete the C terminal half of the protein result in early embryonic lethality (Hakem et al. 1996; Liu et al. 1996; Ludwig et al. 1997). However, mutations that retain the majority of the N terminal and C terminal domains but lack the portion encoded by exon 11, result in mid-embryonic lethality (Gowen et al. 1996; Shen et al. 1998; Xu et al. 2001). In addition, a $Brca1^{1700T}$ mutant that retains the first but deletes the second BRCT motif in the C terminus of the protein results in a mid-embryonic lethal phenotype (Hohenstein et al. 2001). Therefore, expression of mutant protein that retains the C terminal domain of Brca1 can extend the mutants' lifespan and suggest a functional importance for the BRCT domains of Brca1.

Combining the *Brca1*$^{\Delta 223\text{-}763}$ mice generated by Gowen *et al* with *Trp53*$^{-/-}$ deficiency rescued the embryonic lethal phenotype and resulted in 3 animals that survived for between 10 and 12 weeks but succumbed to thymic lymphomas (Cressman et al. 1999a). These mice had severe growth retardation, males lacked spermatogenesis, and females had defects in mammary development. Similarly, combining the *Brca1*$^{\Delta 11/\Delta 11}$ mice with heterozygosity (*Trp53*$^{+/-}$) or homozygosity (*Trp53*$^{-/-}$) of deficiency for Trp53 resulted in a near complete rescue of the embryonic lethal phenotype. However, the majority of these animals succumbed to mammary carcinoma (Xu et al. 2001). These findings indicate that Trp53 may function to eliminate cells that have undergone damage due to loss of functional Brca1. Loss of p53 can extend the lifespane of mice with otherwise lethal mutations but may also contribute to an increased cancer risk.

Brca1$^{Tr/Tr}$ mice, which have a truncating mutation at amino acid 924, express no truncated or wild-type transcript but do express an endogenous *Brca1* splicing variant that lacks exon 11 (Ludwig et al. 2001a). Unlike other *Brca1* exon 11 mutations that result in low expression of a *Brca1* splicing variant lacking exon 11, this allele is expressed at near wild-type levels. The mice are viable but display developmental abnormalities including a mild growth defect, kinky tails, and skin pigmentation defects. 85% of the mutant *Brca*$^{Tr/Tr}$ animals developed tumors including lymphoma, sarcomas, and carcinoma of mammary, lung, liver, and endometrial tissues. Thus, protein interactions mediated by the exon 11encoded portion of Brca1 seem to be particularly important for the prevention of a variety of tumors in the mouse.

Table 4. Knockouts of *Brca1* and *Brca2/Fancd1*

Mutation	Mutation position	Viability	Developmental Defects	Tumorigenesis	Reference
Brca1^{ex2}	exon 2 insertion	EL 7.5 d	n/a	n/a	1
Brca1$^{5-6}$	exon 5 truncation	EL 7.5 d	n/a	n/a	2
Brca1^{ko}	exon 11 insertion	EL 7.5-8.5 d	n/a	n/a	3
Brca1$^{\Delta 223-763}$	exon 11 spliced product	EL 10-13.5 d	n/a	n/a	4
Brca1$^{\Delta 223-763}$, *Trp53*$^{-/-}$	exon 11 spliced product	3 viable mice	severe growth retardation, mammary gland defects, infertile	thymic lymphomas	8
Brca1$^{11-/-}$	exon 11 Insertion	EL 8.5-9.5 d	n/a	n/a	5
Brca1$^{\Delta 11/\Delta 11}$	exon 11 spliced product	EL 12.5 d 1% viable	severe growth retardation	n/a	6
Brca1$^{\Delta 11/\Delta 11}$, *Trp53*$^{+/-}$ or *Trp53*$^{-/-}$	exon 11 spliced product	65-80%	None	mammary AC; few lymphoma, few ovarian AC	6
Brca1^{1700T}	truncation after BRCT1	EL 10.5 d	n/a	n/a	7
Brca1$^{Tr/Tr}$	exon 11 insertion	viable on 129 strain	mild growth defects, kinky tail, skin pigmentation defects	mammary AC, lung AC, HCC, endometrial, colon, lymphoma, sarcoma,	9
Brca2^{Brdm1}	exon 10	EL 7.5 d	n/a	n/a	10
Brca2^{ex11}	5' exon 11	EL 8.5 d	n/a	n/a	11
Brca2^{Tr2014}	truncation after BRC7	~20%	small, kinky tail, no male germ cells	thymic lymphoma	12
Brca2^{Tm1Cam}	truncation after BRC3	~10%	not assessed	thymic lymphoma	13
Brca2$^{\Delta 27/\Delta 27}$	exon 27 deletion	67%	mammary gland defects	mammary AC, lung AC, gastric, endometrial, lymphoma, sarcoma,	14

(Adapted from (Moynahan 2002))

1 (Ludwig et al. 1997), 2 (Hakem et al. 1996), 3 (Liu et al. 1996), 4 (Gowen et al. 1996), 5 (Shen et al. 1998), 6 (Xu et al. 2001), 7 (Hohenstein et al. 2001), 8 (Cressman et al. 1999a), 9 (Ludwig et al. 2001a), 10 (Sharan et al. 1997), 11 (Ludwig et al. 1997), 12 (Connor et al. 1997), 13 (Friedman et al. 1998), 14 (McAllister et al. 2002)

To prevent the embryonic lethal phenotype observed in the majority of *Brca1* and *Brca2/Fancd1* germ line mutants, tissue specific deletion of the genes can be engineered. Mouse models in which deletion of a gene is restricted to mammary tissues, have been created by using the promoters, WAP (whey acidic protein) or MMTV-LTR (mouse mammary tumor virus-long terminal repeat). These mammary specific promoters drive expression of CRE recombinase, which deletes genetic material flanked by loxp sites. A variety of engineering strategies have been employed to inactivate either *Brca1* or *Brca2/Fancd1* in mammary tissue (table 5). All of these mice succumb to tumors, highlighting the important role of Brca1 and Brca2 in the prevention of cancer (Xu et al. 1999; Brodie et al. 2001; Ludwig et al. 2001b).

Brca1$^{Ko/Co}$ (*Brca1*$^{\Delta 11}$) mice, in which deletion of *Brca1* in mammary epithelium is accomplished by MMTV driving Cre, had a 25% incidence of mammary AC in 150 mice (Brodie et al. 2001). The average tumor latency was 11 months. Interestingly, crossing these mice to p53 heterozygotes increased the overall tumor incidence from 25% to 100% and decreased the tumor latency by approximately 3 months. Thus, deficiency for Trp53 can accelerate the mammary tumor phenotype of Brca1 conditional knockout mice.

The conditional KO of *Brca2/Fancd1* generated by Ludwig *et al* resulted in 20 out of 26 *Brca2*$^{flox/-}$/Wap^{cre} mice developing mammary tumors (Ludwig et al. 2001b). Tumors began to form at approximately 13 months. Nearly half of the mice analyzed in this study had more than one mammary tumor with 10 of 20 having 2-4 tumors. Interestingly, a conditional knockout of *Brca2/Fancd1* (K14cre/*Brca2*$^{F11/F11}$) that deletes exon 11 but retains N terminal and C terminal function does not result in an increased incidence of mammary tumors (Jonkers et al. 2001). However, 72% of

K14cre/*Brca2*$^{F11/F11}$ mice crossed to *Trp53*$^{F2\text{-}10/F2\text{-}10}$ developed mammary tumors. 55% of K14cre/*Brca2*$^{F11/+}$; *Trp53*$^{F2\text{-}10/F2\text{-}10}$ mice that retain one wild-type allele of *Brca2/Fancd1* in mammary tissue developed mammary tumors. 7 of 16 tumors in this group retained the wild-type allele of *Brca2/Fancd1,* suggesting that haploinsufficiency for Brca2/Fancd1 in the context of loss of Trp53 function, can lead to mammary tumors in mice. Finally, 31% of K14cre/*Brca2*$^{F11/F11}$; *Trp53*$^{F2\text{-}10/+}$ mice develop mammary tumors. All tumors examined by Southern blot had undergone LOH for the wild-type allele of *Trp53*. Again, deficiency of Trp53 seems to cooperate with loss of tumor suppressor genes to accelerate tumor formation.

Table 5. Tumorigenesis in conditional knockout of *Brca1* and *Brca2/Fancd1*

Conditional Mutation	Cre promoter	Tumorigenesis	Tumor Latency	Reference
Brca1$^{Ko/Co}$ (*Brca1*$^{\Delta 11}$)	MMTV^{cre}	mammary (25%)	~11 mo	1
Brca1$^{Ko/Co}$; *Trp53*$^{+/-}$	MMTV^{cre}	mammary (100%)	6-8 mo	1
Brca2$^{flox/-}$	Wap^{cre}	mammary (77%)	~13 mo	2
Brca2$^{F11/F11}$	K14^{cre}	None	n/a	3
Brca2$^{F11/F11}$, *Trp53*$^{F2\text{-}F10/F2\text{-}F10}$	K14^{cre}	mammary (72%)	~6 mo	3
Brca2$^{+/F11}$, *Trp53*$^{F2\text{-}F10/F2\text{-}F10}$	K14^{cre}	mammary (55%)	~10 mo	3
Brca2$^{F11/F11}$, *Trp53*$^{+/F2\text{-}F10}$	K14^{cre}	mammary (31%)	~12 mo	3

1 (Brodie et al. 2001), 2 (Ludwig et al. 2001b), 3 (Jonkers et al. 2001)

As both Brca1 and Brca2/Fancd1 are known to function in maintaining genomic stability, the tumors that formed in conditional knockouts have been analyzed for their relative degree of aneuploidy and chromosome aberrations. Mammary tumors from *Brca2*$^{flox/-}$/Wap^{cre} mice demonstrate various degrees of genetic abnormalities as analyzed

by metaphase karyotype analysis (Ludwig et al. 2001b). *Brca1*$^{Ko/Co}$/*Trp53*$^{+/-}$ mammary tumors were aneuploid and contained structural abnormalities (Brodie et al. 2001). Some of these tumors were also examined by spectral karyotyping (SKY) and found to contain chromosome losses and gains, chromatid breaks, insertions, deletions, and multiple rearrangements. Therefore, the increased incidence of tumors in *Brca1/2* conditionally mutant mice may be caused by loss of "genome caretaker" function of these proteins.

Overall, the phenotype of *Brca1* and *Brca2/Fancd1* mutants is much more severe then either *Fanca*, *c*, *g*, or *l* knockout mice. While loss of the majority of *Brca1* or *Brca2/Fancd1* gene results in an embryonic lethal phenotype, a variety of mutations have been engineered that result in improved viability. Loss of *Trp53* can extend the life span of *Brca1* and *Brca2/Fancd1* mutants but may also increase the tumor incidence. Finally, although no tumors are observed in any of the knockout mice mutant in upstream FA genes, many of the *Brca1* and *Brca2/Fancd1* mutants and conditional knockouts are tumor prone.

The experiments described in this thesis were undertaken to further investigate the *in vivo* function of mouse *Fancd2*. In chapter two, the phenotypic consequences of a null mutation in mouse *Fancd2* are described. We investigate whether complete loss of Fancd2 results in a phenotype identical to other FA mice or if loss of Fancd2 leads to additional phenotypes not observed in previous mouse models of FA. We show that loss of Fancd2 results in a qualitatively similar phenotype to other FA knockout mice. However, unlike FA mouse models lacking the upstream nuclear core complex, *Fancd2* knockouts develop a variety of epithelial tumors. In chapter three, we describe an improved model for studying cancer in FA by combining loss of *Fancd2* with

heterozygosity for a deficiency of *Trp53* and begin to explore the role of Fancd2 and Trp53 in maintaining genomic integrity. In chapter four, we investigate whether Fancd2 acts in a DNA repair pathway that is distinct from NHEJ. We show that Fancd2 functions in DSB repair and the maintenance of genomic stability which may explain the increased incidence of epithelial tumors observed in *Fancd2* knockout mice. Chapter 5 provides a summary of the findings and suggests experiments to further explore the biology of the FA pathway.

Chapter Two: Epithelial Cancer in **Fancd2** *Knockout Mice*

2.1 Abstract

FA is a genetic disorder characterized by hypersensitivity to DNA damage, bone marrow failure, congenital defects, and cancer. To further investigate the *in vivo* function of the FA pathway, mice with a targeted deletion in the distally acting FA gene *Fancd2* were created. Similar to human FA patients and other FA mouse models, *Fancd2* mutant mice exhibited cellular sensitivity to ICLs and germ cell loss. In addition, chromosome mispairing was seen in male meiosis. However, *Fancd2* mutant mice also displayed phenotypes not observed in other mice with disruptions of proximal FA genes. These include microphthalmia, perinatal lethality and epithelial cancers, similar to mice with *Brca2/Fancd1* hypomorphic mutations. These additional phenotypes were not caused by defects in the Atm mediated S phase checkpoint which was intact in primary *Fancd2* mutant fibroblasts. The phenotypic overlap between *Fancd2* null and *Brca2/Fancd1* hypomorphic mice is consistent with a common function for both proteins in the same pathway, regulating genomic stability.

2.2. Introduction

FA is an autosomal recessive disorder characterized by cellular sensitivity to DNA damage, bone marrow failure and predisposition to cancer (Fanconi 1967; D'Andrea and Grompe 2003). Patients may also have congenital malformations including short stature, small gonads, microphthalmia, and skeletal defects (Alter 1993). At least 11 complementation groups exist (FA-A, B, C, D1, D2, E, F, G, I, J, and L) and all share a similar yet often variable phenotype (Alter 1993; Levitus et al. 2004). The genes for 9 of these have been identified (*FANCA*, *B*, *C*, *D1/BRCA2*, *D2*, *E*, *F*, *G/XRCC9*, and *L/PHF9*) (Strathdee et al. 1992; Lo Ten Foe et al. 1996; de Winter et al. 1998; de Winter et al. 2000a; de Winter et al. 2000b; Timmers et al. 2001; Howlett et al. 2002; Meetei et al. 2003a; Meetei et al. 2004a).

The precise functions of the FA, BRCA1 and BRCA2 proteins remain unclear but there is evidence for overlapping roles. FANCA, FANCC, FANCE, FANCF, and FANCG interact in a multisubunit nuclear complex (Kupfer et al. 1997; Garcia-Higuera et al. 1999; de Winter et al. 2000c; Medhurst et al. 2001). Genes constituting this complex act epistatically as shown by the redundant phenotypes of *Fanca, Fancc, Fancg,* and *Fanca/Fancc* double knockout mice (Noll et al. 2002). FANCD2 acts downstream of the nuclear complex. After DNA damage (Garcia-Higuera et al. 2001) and during the S phase of the cell cycle (Taniguchi et al. 2002a) FANCD2 is mono-ubiquitinated at Lys_{561} but not in cells lacking any member of the upstream FA complex (Garcia-Higuera et al. 2001). Mono-ubiquitination of FANCD2 is required for its co-localization with RAD51 and BRCA1 in nuclear foci. FANCD2 can also be phosphorylated at Ser_{222} by ATM. This modification requires wild-type nibrin (Nakanishi et al. 2002) and has been reported to

mediate the damage induced arrest of DNA synthesis in SV40 transformed fibroblasts (Taniguchi et al. 2002b).

The identification of BRCA2 as an FA protein has suggested a potential function for the pathway. BRCA2 binds single stranded DNA (Yang et al. 2002), interacts directly with RAD51 (Marmorstein et al. 1998), and modulates its activity (Davies et al. 2001). Cells lacking functional BRCA2 are deficient in homology directed repair of DNA double strand breaks (Moynahan et al. 2001b; Tutt et al. 2001) and therefore the FA pathway may function to modulate DNA repair by homologous recombination.

Mouse genetics has been useful for investigating the function of the FA pathway. Knockouts of *Fancc* (Chen et al. 1996; Whitney et al. 1996), *Fanca* (Cheng et al. 2000; Noll et al. 2002), and *Fancg* (Yang et al. 2001; Koomen et al. 2002) all share a defect in germ cell development and demonstrate cellular sensitivity to agents that produce ICLs. In contrast to human FA patients, however, FA mice do not develop anemia, do not have skeletal defects, nor are they at an increased risk for developing cancer. Targeted deletions have also been generated for the murine homolog of *BRCA2*. While homozygous null mutants lacking Brca2 (Sharan et al. 1997) die at mid-gestation, mice homozygous for a truncating mutation at the C-terminus of *Brca2* are viable and show some phenotypic overlap with *Fanca, Fancc,* and *Fancg* knockout mice (Connor et al. 1997; McAllister et al. 2002). Here, we report the phenotype of mice homozygous for a null allele of the *Fancd2* locus.

2.3. Materials and Methods

Targeting vector design and generation of Fancd2 deficient mice

A Lamda phage library of mouse cDNA (Lambda FIX II, Stratagene, La Jolla, CA) was screened with a 3' 3.0 kbp R.A.C.E. fragment of mouse *Fancd2* cDNA. DNA was purified from positive phage clones and a 7.9 kbp *BamH*I fragment of genomic DNA was sub-cloned into the *S. cerevisiae/E. coli* shuttle vector, YCplac22-TK (Gietz and Sugino 1988), to generate YCplac22-TK+*BamH*I fragment. This 7.9 kbp fragment was further subcloned, sequenced, and found to contain exons 21-28 of the mouse *Fancd2* gene. The following primer pairs were used to amplify sequence flanking exons 26 and 27 and the PCR products were cloned into the plasmid pRAY-1 (Storck et al. 1996) to generate pRAY-1+flanking arms: 5'-TCAGCCTCACATGGAGTTTAACG-3'/5'-GTGTGGACACTAACCTCACTCGC-3' and 5'-TTTCCTGTCCTCATCTGCG-3'/5'-TGCAAAGAGAGAAATGACTCAAG-3'. pRAY-1+flanking arms and YCplac22-TK+*BamH*I fragment were co-transfected into yeast strain MW3317-21A (-trp, -ura) and selected in -ura/-trp dropout media. Clones were screened for a recombination event between the two plasmids that replaced mouse exon 26 and 27 with the neo-ura cassette from pRAY-1. YCplac22-TK+*BamH*I (del exon26/27) was linearized and injected into 129S4 ES cells. gDNA was isolated from clones that were doubly resistant to G418 and gancyclovir, digested with *Xba*I and Southern blotted using a 333 bp probe which binds 266-599 bps 3' of the *BamH*I site marking the 3' most end of genomic mouse sequence in the targeting vector (see figure 2). The probe was amplified from total mouse genomic DNA using the following PCR primers pairs: 5'-GAGTTGTTCTTTTGTATGCCCGTC-3'/5'-GTCTTGTATCTGCTTTCCTCTGCC-3' and cloned into pCR-Blunt II-TOPO

(Invitrogen, Carlsbad, CA). The probes were digested out with *EcoRI*. Four highly chimeric males were produced from blastocyst injections of correctly targeted ES cells.

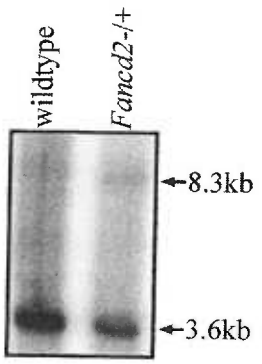


Figure 2. Southern blot analysis using genomic DNA from targeted ES cell clones digested with XbaI. Homologous integration of the targeting plasmid eliminates an XbaI site in intron 26 resulting in a non-targeted band at 3.6 kb and a targeted band at 8.3 kb.

Animal Husbandry

Chimeric animals were crossed to C57BL/6J mice and homozygous *Fancd2* knockout mice on a mixed 129S4, C57BL/6J background were generated. Founder animals were also backcrossed for 6 generations to pure C57BL/6J mice to generate a population of homozygous *Fancd2* knockout mice on the C57BL/6J background. A strain of congenic 129S4 *Fancd2* mutants was generated by breeding a germ line chimera with 129S4 females. To generate *Fancd2* mutants which lacked the neo-ura knockout cassette, a *Fancd2* heterozygous male was crossed to a female mouse in a mixed C57BL/6J, DBA background containing the transgene encoding Cre recombinase driven by a testes specific promoter [Jackson Labs strain # 3466, B6;D2-TgN(Sycp1-Cre)4Min].

PCR genotyping

50 ng of gDNA was prepared from toes as previously described (Miller et al. 1988) and used as a template in PCR (Mullis and Faloona 1987) to genotype mice- a forward

primer, MG968-5'-TCAGCCTCACATGGAGTTTAACG-3', and two reverse primers, MG1007-5'-AATTCGCCAATGACAAGACGC-3' (amplifies a mutant band of 556 bp) and MG1008-5'-CAGGGATGAAAGGGTCTTACGC-3' (amplifies a wild-type band of 303 bp). The reaction conditions were 94° for 3 minutes; 37 cycles of 94°C for 25 seconds, 48°Cfor 25 seconds, and 72°C for 35 seconds; and a final extension at 72° C for 2 minutes. For detection of the *Fancd2* mutant allele lacking the neo-ura cassette, the same reaction was used but primer MG1007 was replaced with primer MG1280-5'-GCTACACAGCATTGCCCATAAAG-3' (amplifies a mutant band of 459 bp).

Histology

Tissues fixed in 10% phosphate-buffered formalin for 24 hours, pH 7.4, were dehydrated in 100% EtOH and embedded in paraffin wax at 58 ° C. Four micron sections were rehydrated and stained with Hematoxylin-Eosin.

Meiotic Chromosome Staining

Surface spreads of pachytene and diplotene spermatocytes from male mice between the ages of 16 and 35 days old were prepared as described by Peters, *et al.* (Peters et al. 1997). A polyclonal goat antibody to the mouse Scp3 protein was used to visualize axial elements and synaptonemal complexes in the meiotic preparations (Plug et al. 1997). The anti-Rad51 rabbit polyclonal antibody (Ab-1) was purchased from Oncogene Research Products. Antibody incubation and detection procedures were a modification of the protocol of Moens *et al.* (Moens et al. 1987) as described by Keegan *et al.* (Keegan et al. 1996). Combinations of Donkey-anti rabbit and Donkey-anti goat IgG secondary

antibodies conjugated to either FITC or TRITC were used for detection (Jackson ImmunoResearch Laboratories, West Grove, PA). All preparations were counterstained with 4', 6' diamino-2-phenylindole (DAPI, Sigma, St. Louis, MO) and mounted in a DABCO (Sigma, St. Louis, MO) antifade solution. The preparations were examined on a Zeiss Axioplan-2 microscope (63X Plan Apochromat and 100X Plan Apochromat oil-immersion objectives). Each fluorochrome (FITC, TRITC and DAPI) image was captured separately as an 1344x1024 pixel 12-bit grey scale source image via OpenLab software (Improvision, Lexington, MA) controlling a cooled-CCD camera (Hamamatsu Orca ER) and the separate grey scale images were cropped, 24-bit pseudocolored and merged using Adobe Photoshop.

Cell Growth Assays

Primary (passage 2) mouse embryonic fibroblasts (MEFs) were prepared from pregnant mice at between 12-15 days post-coitus and seeded in quadruplicate at a density of 500 cells/well in 96 well plates. Cells were grown in Dulbecco's modified eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 15% fetal bovine serum (FBS) (HyClone, Logan, UT) and 1x penicillin/streptomycin (Invitrogen, Carlsbad, CA). Cells were treated with increasing doses of MMC (Sigma, St. Louis, MO) (0-640ng/ml) or 4'-hydroxymethyl-4, 5', 8-trimethylpsoralen (HMT) (Sigma, St. Louis, MO) plus UVA (0-0.5ng/ml), as previously described (Akkari et al. 2000). IR was administered at a dose of 145.9 rads/minute from a ^{137}Cs source. Following treatment, cells were allowed to grow for 5 days at 37° C in 5% CO_2. Plates were frozen at –80° C. Total DNA was quantified using CyQuant (Molecular Probes, Eugene, OR) and compared to untreated controls as

an indication of cell growth as previously described (Cheng et al. 2000). Quantification of DNA using CyQuant was performed using a Fluorstar plate reader (BMG Lab Technologies, Durham, NC) according to manufacturer's instructions.

Radioresistant DNA Synthesis (RDS) Assay

The RDS assay was performed as previously described with modifications (Painter and Young 1980; Taniguchi et al. 2002b). Primary MEFs (wild-type and *Fancd2*, p2) or ear (*Atm*, p3) fibroblasts were seeded at a cell density of 75,000 per 60 mm dish. Cells were pre-incubated in media containing 10nCi/ml thymidine [methyl-^{14}C] (catalog# NEC568) (NEN Life Sciences, Boston, MA) for 24 hours to control for total DNA content and then incubated for 24 hours in non-radioactive media. Following DNA damage cells were incubated for 30 minutes at 37° C with 5% CO_2 to allow arrest of DNA synthesis and then incubated in media containing 2.5 μCi/ml thymidine [methyl-^{3}H] (catalog #NET027) (NEN Life Sciences, Boston, MA) for 3 hours. Cells were post-incubated for 30 minutes at 37° C with 5% CO_2 in non-radioactive media to remove unincorporated thymidine pools, washed three times in ice cold phosphate buffered saline (PBS) and fixed at room temperature in 90% methanol. Cells were scraped onto Whatman QF/C filters and air dried for 10 minutes. Radioactivity was measured using a Beckman LS6500 scintillation counter. DNA synthesis is represented as the ratio of ^{3}H:^{14}C cpm corrected for channel crossover and is represented as a percentage of untreated controls. Experiments were performed in quintuplet. Data shown is mean +/- standard error of the mean (SEM).

Immunoblotting of Brca2, Rad51 and Fancd2

Preparation of whole cell lysates: Testes from 4-6 week old mice belonging to complementation groups A, C, D2 and control mice or MEFs were homogenized with an eppendorf homogenizer containing 500µl of lysis buffer (1x PBS, 1% Nonidet P-450, 0.5% sodium deoxycholate and 0.2% SDS) containing 20 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, and 1µg aprotinin. The suspension was passed through a 27 gauge needle a few times to shear the DNA and centrifuged for 20 minutes at 15,000 x g and 4° C to remove the debris. The supernatant was stored at –80° C. Total protein from each of the samples was analyzed in triplicates using the Lowry assay. For immunoprecipitation and Western blotting of Brca2, mouse monoclonal Brca2 (Ab-1) antibody (Oncogene Research Products) was used for the human cells and a rabbit polyclonal anti-Brca2 (H-300) antibody was used for the mouse samples. Anti-Rad51 antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) was used on the mouse testicular samples for both immunoprecipitation (IP) and western blotting.

Immunoblot Analysis of Rad51: Testicular lysates were resolved on pre-cast Nupage 10-12% Bis-Tris gels (Invitrogen, Carlsbad, CA). 100 µg of lysate was denatured by heating at 70° C for 10 minutes after the addition of appropriate amount of 4x Nupage sample buffer and 10x reducing agent. The proteins were transferred to Immobilon-P Transfer membranes (Millipore Corporation, Billerica, MA) and Rad51 was detected using a 1:500 dilution of anti-Rad51 antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), a 1:2,000 dilution of anti-rabbit secondary antibody (Amersham Life Science, Piscataway, NJ) and the ECL detection system (Amersham Life Science, Piscataway, NJ).

Immunoblot analysis of Fancd2: Testicular lysates were resolved on pre-cast Nupage 3-8% gradient gels (Invitrogen, Carlsbad, CA). MEF lysates were resolved on a 5% Tris-glycine gel. 100 μg of the lysates was denatured by heating at 70° C for 10 minutes after the addition of appropriate amount of 4x Nupage sample buffer and 10x reducing agent. The proteins were transferred to Immobilon-P Transfer membranes (Millipore Corporation, Billerica, MA) and Fancd2 was detected using a 1:5000 (testes) or 1:1000 (MEFs) dilution of anti-FANCD2 polyclonal antibody and 1:10,000 dilution of anti-rabbit secondary antibody (Amersham Life Science, Piscataway, CA) and the ECL system (Amersham Life Science, Piscataway, CA).

Immunoprecipitation of Rad51 and Brca2 from testicular lysates:

Preparation of Protein A Sepharose: The media (Amersham Life Science, Piscataway, CA) was washed three times with the lysis buffer described above and centrifuged at 12,000 x g for 20 seconds. The supernatant was discarded between washes. A 50% slurry was prepared by mixing equal volumes of media and lysis buffer, and was stored at 4° C.

Cell lysis: For Brca2, VU423 (Howlett et al. 2002) and Hela cells were used as controls. The culture medium was removed and the cells were washed twice with ice cold PBS. The dishes were placed on ice and the cells were collected with a cell scraper. Tissues were lysed in 100 μl of lysis buffer and incubated on ice for 10-15 minutes with occasional shaking. The cells were further disrupted by passage through a 21 gauge needle. The lysates were centrifuged at 12,000 x g for 10 minutes at 4° C to remove particulate matter and the supernatant was transferred to a fresh tube.

Immunoprecipitation: 1 mg (1 ml) of whole cell or tissue extract was transferred to a 2 ml centrifuge tube. The lysate was precleared by adding 1 μg of the appropriate normal mouse, rat, rabbit or goat IgG together with 20 μl of Protein A sepharose conjugate. Incubation was done at 4° C for 1 hour and pelleted by centrifugation at 2,500 rpm at 4° C for 5 minutes. The precleared supernatant was transferred to a fresh eppendorf tube.

Coupling of antigen to the antibody: The above supernatant was aliquoted to 500 μl and the respective primary antibodies (anti-Rad 51, H-300 for mouse Brca2 and Ab-1 for human BRCA2) were added to a concentration of 5 μg and mixed on a rotary shaker for 1 hour at 4° C.

Precipitation of the immune complexes: 50 μl or Protein A Sepharose 4 Fast Flow suspension (50% slurry, described above) was added to the antigen-antibody complex and mixed on a rotary shaker for 1 hour at 4° C. The complex was centrifuged at 12,000 x g for 20 seconds and both the pellet and the supernatant were saved to compare bound versus unbound protein. The pellet was washed three times with 1 ml lysis buffer and once with wash buffer (50 mM Tris, pH 8.0).

Dissociation and analysis. Final pellets were suspended in 30 μl of 4x Nupage sample buffer (Invitrogen, Carlsbad, CA) containing 1x DTT. The samples were heated to 95° C for 3 minutes and centrifuged at 12,000 x g for 20 seconds to remove the beads. The supernatant was removed and analyzed by immunoblot as described above.

Rad51 foci formation immunocytochemistry:

Primary wild-type and *Fancd2*$^{-/-}$ MEFs were seeded on 18mm cover slips and grown in DMEM + 15% FBS. Cells were (untreated or treated with 12 Gy IR) were fixed 6 hours

after treatment with 4% paraformaldehyde for 10 minutes at room temperature and permeabilized with PBS + 0.5% Triton X-100 for 5 minutes. Cells were blocked overnight in PBS-Tween 20 (0.25%) + 2% BSA at 4°C. Anti-human RAD51 antibody (Oncogene Research Products) was used at a dilution of 1:1,000 in PBS-Tween 20 (0.25%) + 2% BSA for one hour at room temperature. Cells were washed 4 x 5 minutes in PBS-Tween 20 (0.25%). Anti-rabbit-Cy3 conjugated secondary antibody (Upstate Biotechnology, Waltham, MA) was used at a dilution of 1:2,000 in PBS-Tween 20 (0.25%) for one hour at room temperature. Cells were washed 4 x 5 minutes in PBS-Tween 20 (0.25%). Coverslips were mounted in a DAPI containing antifade solution (Molecular Probes, Eugene, OR) and analyzed by fluorescence microscopy. Images were captured using Openlab software. Results are the mean of two independent experiments in which 200 cells were counted per experiment. Cells were scored positive if they contained greater than 5 foci per nucleus.

2.4. **Results**

Generation of Fancd2 knockout mice

Murine Fancd2 is highly homologous to human FANCD2 and consists of 44 exons (Genbank accession # BC042619 or XM132796). The targeting construct (figure 3) was designed to delete exons 26 and 27 (220 bp) of the 44 exon gene and is predicted to disrupt the reading frame of the mRNA resulting in a premature nonsense codon at 54 basepairs downstream of the exon 25 and exon 28 junction. Chimeric males were bred to generate Fancd2 heterozygotes and crossed to produce Fancd2 mutants and controls in the syngeneic 129S4, the congenic C57BL/6J, and a mixed 129S4, C57BL/6J strain background (figure 4).

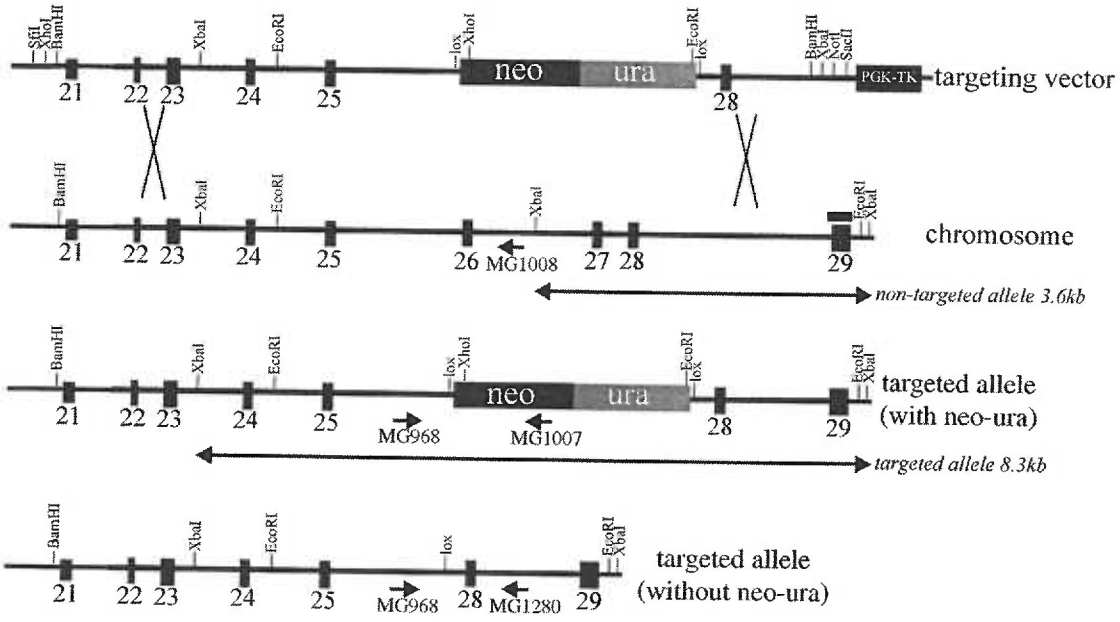


Figure 3. Schematic of targeting vector. Wild-type *Fancd2* locus, targeted allele (with neo-ura cassette), targeted allele (without neo-ura cassette) are shown. Homologous integration of the targeting plasmid replaces *Fancd2* exons 26 and 27 with a neo-ura cassette flanked by lox sites. The binding site for the probe is indicated by a black bar.

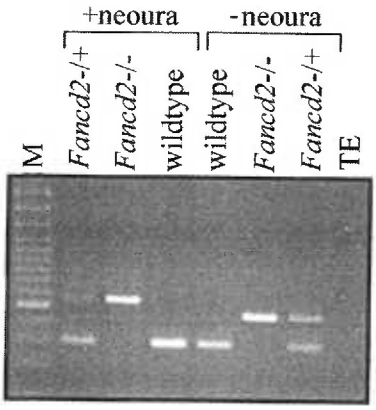


Figure 4. *Fancd2* knockout PCR genotyping using genomic DNA from wild-type, heterozygous, and mutant tail as template. Mutant *Fancd2* alleles with and without the neo-ura cassette are shown. The mutant band is 556 bp (with neo-ura) or 459 bp (without neo-ura). The wild-type band is 303 bp in length

To confirm that the targeted allele of *Fancd2* was a null mutation, RT-PCR was performed using mRNA generated from *Fancd2* mutant and wild-type testes. A product of the predicted size was amplified from wild-type testes and a product lacking exons 26 and 27 of Fancd2 was amplified from *Fancd2* mutant testes, indicating that some mRNA is transcribed from the mutant allele (data not shown). We next performed Western blots on protein extract prepared from mouse bone marrow, testes and mouse embryonic fibroblasts (MEFs) using an N-terminus specific antibody (kindly provided by Alan D'Andrea) (data not shown, figure 5 and figure 6). Both isoforms of Fancd2 (Fancd2-S, 156 KD and Fancd2-L, 162 KD) were detectable in wild-type testes extract. Only Fancd2-S was observed in extract from *Fanca* mutant and *Fanca/c* double mutant testes. Full length Fancd2 was also observed in Western blots using protein extract from wild-type MEFs and wild-type bone marrow (figure 6 and data not shown). Importantly, no full length Fancd2 or any bands of the predicted truncated protein's molecular weight (~88kD) were observed in protein extract from testes, bone marrow, or MEFs. Thus we conclude that the targeted allele of *Fancd2* is a null mutation at the protein level.

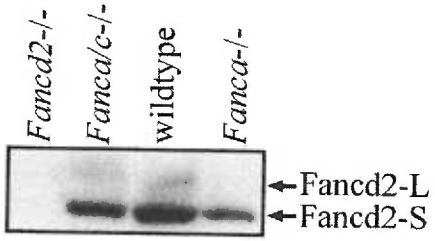


Figure 5. Western blot with anti-Fancd2 antibody on protein lysate from *Fancd2* mutant, *Fanca/c* double mutant, wild-type, and *Fanca* mutant testes. Fancd2-S (155 kd) and Fancd2-L (162 kd) are visible in wild-type extracts. Only Fancd2-S is detectable in *Fanca/c* mutant and *Fanca* mutant extracts. No signal is detectable in *Fand2* mutant extract.

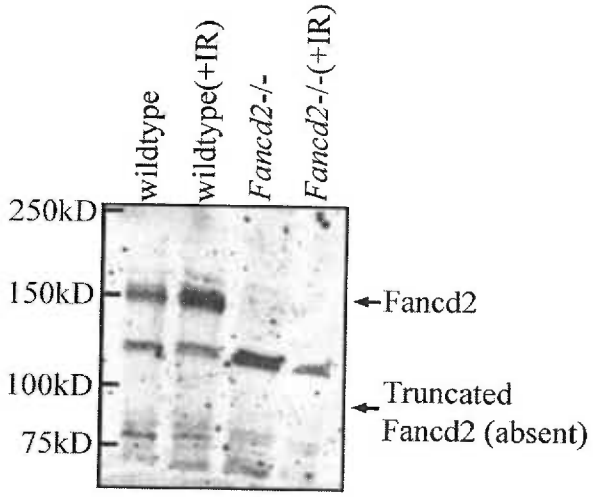


Figure 6. Western blot with anti-Fancd2 antibody on protein lysate from wild-type and *Fancd2* mutant MEFs either untreated or irradiated with 10 Gy. Fancd2 (155/162kD) is detected in wild-type extracts but absent from *Fancd2* mutant extracts. The absence of a band at 88kD, corresponding to the predicted size of truncated Fancd2, is indicated.

Fancd2 *knockouts display phenotypes similar to* Fanca, Fancc, *and* Fancg *knockouts*

Upon gross examination, *Fancd2* mutants on the 129S4 background were indistinguishable from their littermates. However, *Fancd2*$^{-/-}$ mice on the C57BL/6J background were smaller than controls, similar to *Fancc* mice (Manuel Buchwald, personal communication). Mutants were consistently smaller than controls at each data point analyzed. At day 5, mutant males weighed 2.5 +/- 0.7 g (n=9) and non-mutant control males weighed 3.1 +/- 0.4 g (n=23) ($p< 0.05$, t-test) indicating delayed development in utero. At day 40 (adulthood) mutant males weighed 18.3 +/-1.2 g (n=3)

and non-mutant control males weighed 21.2 +/-1.0 g (n=19) ($p<.01$, t-test). A similar result was found for female mutants and controls. We conclude that *Fancd2* mutant mice display a developmental delay in utero as well as post-natally.

Individual testes from adult (age 2-6 months) *Fancd2* mutant and control animals on the 129S4 background were also examined. The average weight per testis from *Fancd2* mutants (23+/-4 mg, n=20) was smaller than that of controls (90+/-19 mg, n=13) ($p< 0.001$, one-way ANOVA). Interestingly, the testicular weight of *Fancd2* mutants was also significantly reduced compared to *Fanca* (78+/-13 mg, n=14) ($p< 0.001$, one-way ANOVA) and *Fancc* mutants (56+/-15 mg, n=14) ($p< 0.001$, one-way ANOVA) on the same strain background (Noll et al. 2002). Despite the weight difference, histological examination of adult *Fancd2* mutant testes showed a similar mosaic pattern of normal and abnormal tubules. This characteristic pattern consists of apparently normal tubules, abnormal tubules that lack all germ cells and abnormal tubules that contain germ cells but have increased numbers of apoptotic pachytene spermatocytes along with a relative paucity of diplotene spermatocytes and spermatids (figure 7A-D).

Ovaries from adult females were also abnormal and contained a markedly reduced number of developing follicles (data not shown.) Importantly, there was a lack of germ cells in testes of newborn *Fancd2* mutants (data not shown), as previously also reported for *Fancc* mice (Whitney et al. 1996). This finding, together with the observed deficiency of spermatogonia in adult males (figure 7B), provides evidence that the FA pathway may be important for the survival of early germ cells. Alternatively, the phenotype might also be caused indirectly as a result of an inability to repair DNA damage that occurs constitutively.

The excess of apoptotic spermatocytes seen in some *Fancd2* tubules suggested that loss of Fancd2 function may lead to aberrant germ cell development during meiosis I. To follow meiotic events, we examined surface spreads of mouse spermatocytes using polyclonal antibodies against Rad51 and against Scp3, a component of the axial elements of unsynapsed chromosomes and of synaptonemal complexes that form between homologous chromosomes (Dobson et al. 1994). *Fancd2*$^{-/-}$ mice display the same meiotic pairing abnormalities as those seen in other FA mice (S. Meyn, unpublished data). The earliest substages of meiotic prophase (leptonema and early zygonema) appear normal in *Fancd2*$^{-/-}$ spermatocytes, as monitored by Scp3 and Rad51 staining. However, there was an increased frequency of synaptic abnormalities beginning in mid-zygonema. Synapsis between homologues is normally initiated while axial elements are still forming. As a result, unpaired axial elements in wild-type zyogtene spermatocytes are typically short, with nearly full length but unpaired axial elements uncommon (figure 7E). Unpaired axial elements in most zygotene *Fancd2*$^{-/-}$ nuclei tended to be longer than normal (figure 7F) and some zygotene *Fancd2*$^{-/-}$ nuclei contained multiple full length axial elements that had aligned with their homologues but failed to properly synapse (figure 7G). The majority of *Fancd2*$^{-/-}$ pachytene nuclei appeared to be normal. However there was an excess of *Fancd2*$^{-/-}$ pachytene nuclei with autosomal axial elements that had failed to synapse and/or with non-homologues that had attempted to pair (figure 7H). These observations suggest that, while the FA pathway is not absolutely required for meiosis I, it acts to promote efficient and accurate pairing of homologues.

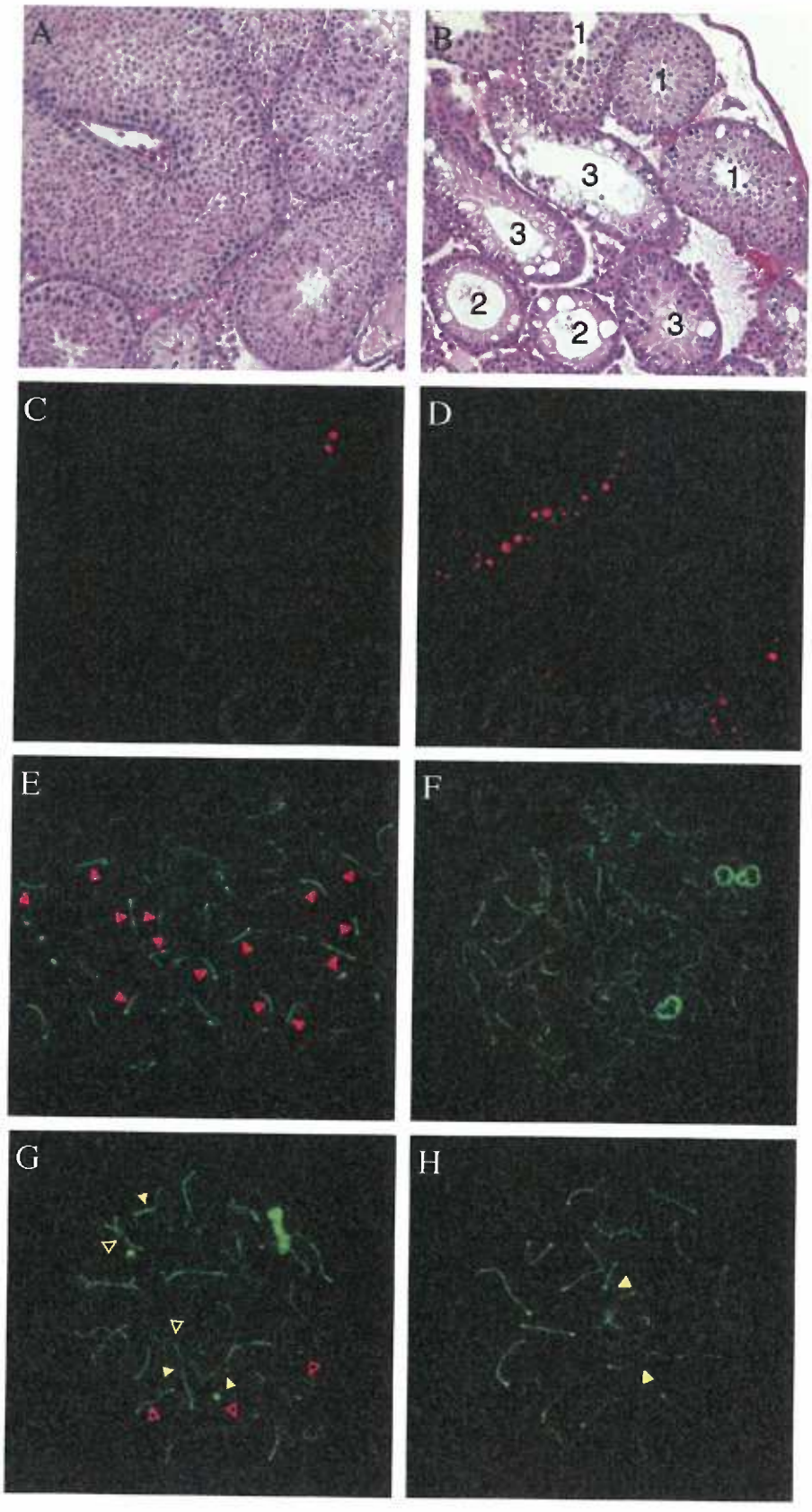

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Figure 7. Germ cell and meiotic pairing abnormalities in *Fancd2*$^{-/-}$ mice.
(A and B) H&E staining of testis sections from 7 week old wild-type (A) or *Fancd2*$^{-/-}$ (B) littermates. All stages of spermatogenesis are seen in the wild-type adult testes. *Fancd2*$^{-/-}$ testes show a mosaic pattern of normal appearing tubules (tubule 1) tubules with vacuolated Sertoli cell cytoplasm and no germ cells (tubule 2) and tubules with vacuolated Sertoli cell cytoplasm and decreased numbers of spermatocytes and spermatids (tubule 3) (20x magnification).
(C and D) TUNEL staining of testes sections from 7 week old wild-type (C) and *Fancd2*$^{-/-}$ (D) littermates. Occasional cells were labeled in wild-type tubules near the basement membrane while *Fancd2*$^{-/-}$ testes containing tubules with multiple numbers of apoptotic spermatocytes (arrow). The nuclei of healthy cells fluoresce blue due to DAPI staining, while apoptotic cells are TUNEL labeled with Cy3-dCTP and fluoresce red (20x magnification).
(E, F, G and H) meiotic pairing abnormalities in *Fancd2*$^{-/-}$ spermatocytes detected by indirect immunofluorescence staining for Scp3 (green) and Rad51 (red). (E) zygotene wild-type nucleus; (F) Zygotene *Fancd2*-/- nucleus with unusually long unpaired axial elements; (G) late zygotene *Fancd2*$^{-/-}$ nucleus with multiple synaptic abnormalities; and (H) pachytene *Fancd2*$^{-/-}$ nucleus with unpaired (open arrowheads) and mispaired homologues (closed arrowheads) (100x magnification).

Fancd2 *knockouts display phenotypes not observed in previous mouse models of FA*

Fancd2 mutants also had phenotypes not previously observed in *Fanca*, *Fancc*, or *Fancg* knockout mice. Pups were born in near expected Mendelian ratios from crosses of heterozygous 129S4 breeders, however a significant deviation from the expected ratios was observed on the C57BL/6J background strain (data not shown). Only 16.5% (50/303), versus the expected 25%, of the pups present at the time of genotyping (postnatal day 5) were mutant ($p< 0.005$, χ^2 test). Although perinatal lethality has not been previously reported for FA knockout mice, it has been described in mice homozygous for a C-terminus truncating mutation in *Brca2* in which a ~33% reduction of mutant animals was noted (McAllister et al. 2002). The lack of this phenotype in the 129S4 strain suggests the presence of a modifier locus in either the 129S4 or C57BL/6J strain, which affects the penetrance of the perinatal lethality phenotype.

An increased incidence of eye abnormalities was observed for *Fancd2* mutants in the C57BL/6J background (figure 8). 32 of 41 (78%) *Fancd2* mutants examined had microphthalmia. Consistent with previous reports, non-mutants in the C57BL/6J population also showed a low incidence (3.2%) of microphthalmia (Kalter 1968). To determine if the phenotype was due to altered expression of nearby genes, also referred to as position effects (Olson et al. 1996), and not the *Fancd2* mutation itself, the neo-ura cassette was removed by breeding to transgenic mice expressing Cre recombinase (see experimental procedures). Consistent with our previous finding, nine of 19 (47%) $Fancd2^{-/-}$ pups with the allele lacking the neo-ura cassette but none of the controls had microphthalmia. This confirmed that Fancd2 is capable of modifying the microphthalmia predisposition of C57BL/6J mice.

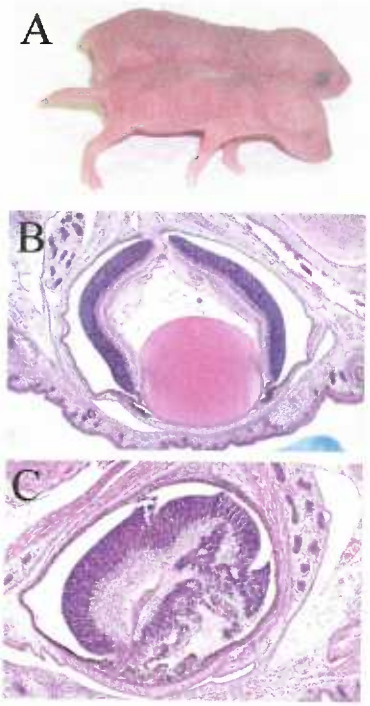

Figure 8. *Fancd2* mutants are predisposed to microphthalmia. (A) *Fancd2* mutants are smaller than wild-type controls at post-natal day 1. Microphthalmia is visible in *Fancd2* mutants. (B) Histology of eye from a wild-type control pup (post-natal day 1) (50x magnification). (C) Representative histology of eye from a $Fancd2^{-/-}$ pup (post-natal day 1) shows a disorganized retina with lack of a distinct chamber or lens development (100x magnification).

To determine whether *Fancd2* mutants are tumor prone, a cohort of animals in the mixed and 129S4 background were monitored long-term. Animals were sacrificed if

abnormal growths were obvious or if they appeared otherwise unhealthy. A final sacrifice of all animals occurred at 18-24 months for those in the mixed background and 7-17 months for those in the 129S4 background. To date, 10 of 54 *Fancd2* mutants, 3 of 28 *Fancd2* heterozygote controls (p=ns), and 1 of 14 wild-type controls (p=ns) have developed adenomas (p= .0385 for *Fancd2* mutant versus controls) or carcinomas (p= .0328 for *Fancd2* mutant versus controls) at between 3 and 23 months of age (figure 9A+B). Tumors occurred in multiple different epithelial cell types (summarized in table 6). Paraffin embedded sections of these tumors were stained with H+E (figure 9C-F) and classified by histological examination. Abnormal cell proliferation was confirmed by immunohistochemistry with an antibody to phosphorylated histone H3, a marker of mitosis (data not shown). The increased incidence of tumors in *Fancd2* mutants but not other FA mice, demonstrates that complete loss of Fancd2 function is necessary for the presentation of a cancer phenotype. The occurrence of hepatic adenomas in 3 of the heterozygous controls suggests that *Fancd2* heterozygotes may also be at an increased risk for carcinogenesis. However, this data is not statistically significant and a larger study will be necessary to demonstrate this definitively. Importantly, although some controls developed hepatic adenomas, no carcinomas were observed in these mice.

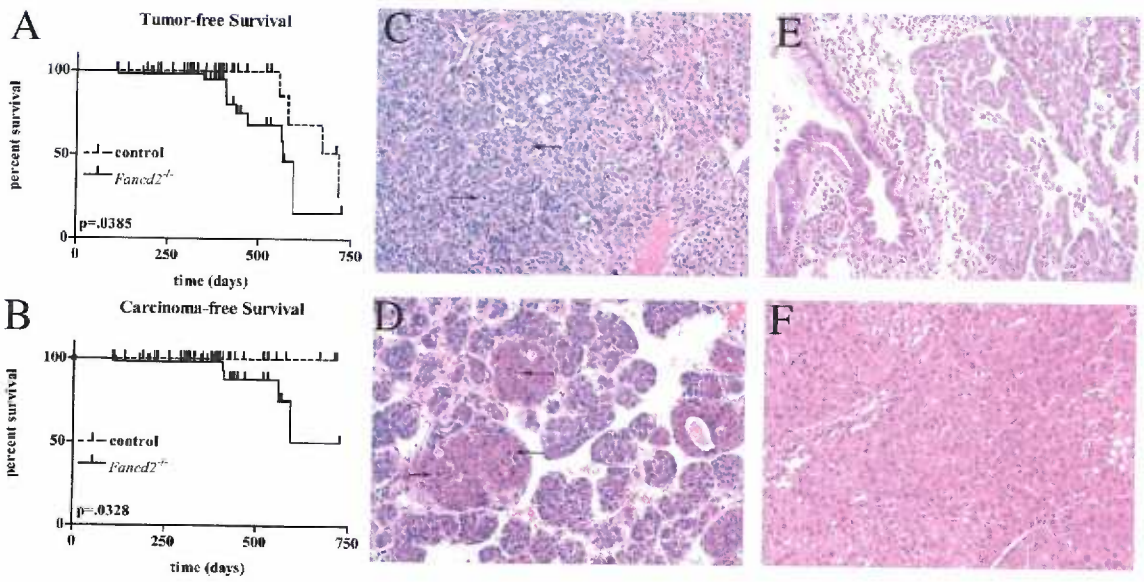

Figure 9. Histology of carcinomas and survival curves for *Fancd2* mutants and controls. Tumor-free (A) (both adenoma and carcinoma) and carcinoma-free (B) (only carcinoma) survival curves are shown for the combination of mixed and 129S4 populations. P values were determined by Logrank test. (C-F) H+E stained paraffin embedded sections of tumors from *Fancd2*$^{-/-}$ animals (200x magnification). Arrows indicate mitotic cells with condensed chromatin. (C) Ovarian adenocarcinoma with large acini lined by tall columnar cells having abundant eosinophilic cytoplasm and highly pleomorphic hyperchromatic nuclei (129S4, 3 months). (D) Mammary adenocarcinoma with large clusters of mitotically active and pleomorphic cells were found, ranging from columnar cells to nests of small, undifferentiated cells in clusters (mixed, 13 months). (E) Lung adenocarcinoma of broncho-alveolar origin, with rows of columnar epithelial cells having a high nucleus to cytoplasm ratio lining septa, sometimes forming papillae or filling air spaces (129S4, 13 months). (F) Hepatocellular carcinoma was multinodular and well differentiated. The neoplastic cells varied from being smaller than normal hepatocytes to 2 to 3 times larger (mixed background, 18 months).

Table 6. Increased incidence of tumors of epithelial cell origin in *Fancd2* mutant mice

Genotype	Background	Sex	Age (months)	Tumor Type
Fancd2$^{-/-}$	129S4	female	3 months	ovarian adenocarcinoma
Fancd2$^{-/-}$	129S4	male	13 months	lung adenocarcinoma
Fancd2$^{-/-}$	mixed	female	14 months	ovarian adenoma
Fancd2$^{-/-}$			13 months	mammary adenocarcinoma
Fancd2$^{-/-}$		male	15 months	hepatic adenoma
Fancd2$^{-/-}$			19 months	hepatic adenoma*
Fancd2$^{-/-}$			19 months	adenoma of fundic mucosa*
Fancd2$^{-/-}$			19 months	hepatocellular carcinoma**
Fancd2$^{-/-}$			19 months	bronchoalveolar carinoma**
Fancd2$^{-/-}$			19 months	bronchoalveolar carinoma**
Fancd2$^{-/-}$			18 months	hepatocellular carcinoma
Fancd2$^{-/-}$			18 months	gastric adenoma
Fancd2$^{-/-}$			13 months	facial seborrheic keratosis
Fancd2$^{-/-}$			19 months	B cell lymphoma**
Fancd2$^{+/-}$	mixed	female	22 months	ovarian cystadenoma
Fancd2$^{+/-}$			18 months	hepatic adenoma
Fancd2$^{+/-}$		male	23 months	hepatic adenoma
wild-type	mixed	male	19 months	hepatic adenoma

*hepatic adenoma and adenoma of fundic mucosa occurred in the same *Fancd2* mutant
**B cell lymphoma and 3 primary tumors occurred in the same *Fancd2* mutant

Fancd2 *and* Fancc *knockouts share a similar DNA damage response profile*

FANCD2 acts downstream of the FA nuclear complex and thus it may act as a convergence point between multiple upstream DNA damage response pathways (Garcia-Higuera et al. 2001). Furthermore, FANCD2 is phosphorylated by ATM following IR (Taniguchi et al. 2002b). These observations suggested that cells lacking FANCD2 might be more sensitive to DNA damage, particularly IR, than cells lacking either FANCA, FANCC, or FANCG. To test this hypothesis, primary MEFs were prepared and their sensitivity to various DNA damaging agents was measured in cell growth assays. Cells were treated with increasing doses of MMC, IR, HMT+UVA. As expected, *Fancd2* mutant cells displayed a heightened sensitivity to MMC induced DNA damage as compared to wild-type controls (figure 10A). *Fancd2* and *Fancc* mutant cells were

equally sensitive to HMT+UVA induced ICLs (figure 10B). However, neither *Fancc* nor *Fancd2* mutant fibroblasts showed a differential sensitivity to IR when compared to wild-type controls (figure 10C).

To further investigate the role of FANCD2 in the response to IR, we also exposed *Fancd2* mutants and controls to whole body irradiation at doses of 9, 10 and 11 Gy (figure 10D and data not shown). We hypothesized that while neither Fancd2 nor Fancc seems to function in response to IR in fibroblasts, a role for Fancd2 may be revealed in whole animals. At a dose of 10 Gy, 11 of 14 controls survived whereas only 3 of 14 *Fancd2* mutants survived ($p< 0.005$, Logrank test). No *Fancd2* mutants survived following a dose of 11 Gy while 4 of 6 control animals survived. Thus, the LD_{50} for *Fancd2* mutants was estimated to be 9.5 Gy compared to 11 Gy for non-mutant littermate controls (proportional difference ~1.15) that is identical to that previously reported for *Fancc* mutants (proportional difference of ~1.2) (Noll et al. 2001). While the *Fancd2* mutants had a moderate *in vivo* IR sensitivity similar to *Fancc* mutants, the lack of a differential sensitivity of *Fancd2* mutant MEFs or a major *in vivo* sensitivity of whole animals to irradiation argues against a significant role for Fancd2 in cellular responses to IR.

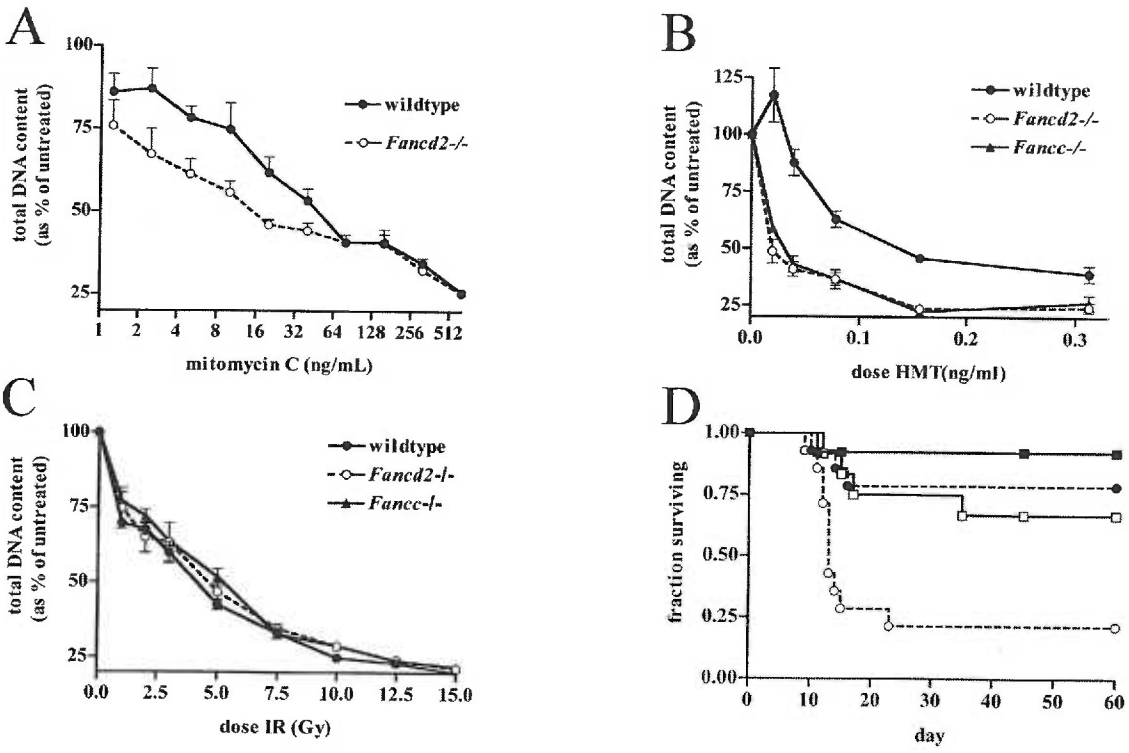

Figure 10. DNA damage sensitivity in *Fancd2*$^{-/-}$ MEFs and mice. Primary MEFs were challenged with increasing doses of MMC (A), HMT+UVA (B), or IR (C). Closed circles indicate wild-type control MEFs, open circles indicate *Fancd2* mutant MEFs, and triangles indicate *Fancc* mutant MEFs. Experiments were performed in quadruplicate. Each data point represents the mean +/- standard error of the mean (SEM). (D) Survival curve of control (closed circles) and *Fancd2* mutant (open circles) mice following a dose of 9 (solid line) and 10 (dashed line) Gy of whole body irradiation.

Primary Fancd2 *mutant fibroblasts have an intact ATM dependent S phase checkpoint*

Recent experiments have demonstrated that FANCD2 is phosphorylated at Ser_{222} by the DNA damage signaling kinase, ATM. Using mutant cDNA constructs of human FANCD2, it was shown that this modification was necessary for the arrest of DNA synthesis after DNA damage, an ATM dependent function (Taniguchi et al. 2002b). In addition, others have reported a defect in S phase arrest in response to HMT+UVA in multiple FA cell lines (Centurion et al. 2000; Sala-Trepat et al. 2000). These findings

prompted the investigation of the status of the checkpoint in primary *Fancd2* mutant MEFs.

Primary (passage 2) *Fancd2* mutant and wild-type MEFs, and primary (passage 3) Atm mutant ear fibroblasts were treated with 10 Gy of IR and their ability to inhibit DNA synthesis was measured. While Fancd2 mutant and wild-type cells inhibited DNA synthesis equally, Atm mutant cells did not (figure 11A). As a control, both Fancd2 mutant and wild-type cells were then pre-incubated in 10mM caffeine, an inhibitor of ATM (Sarkaria et al. 1999). Inhibition of Atm in these cells was capable of inducing a radio-resistant DNA synthesis phenotype similar to Atm mutant cells (figure 11B).

Furthermore, both *Fancd2* mutant and wild-type cells were equally capable of inhibiting DNA synthesis following treatment with 0.5 ng/ml of HMT+UVA, whereas *Atm* mutant cells were not and continued to incorporate DNA following damage (figure 11B). Together, these experiments demonstrate that primary *Fancd2* mutant MEFs have an intact Atm dependant S phase checkpoint.

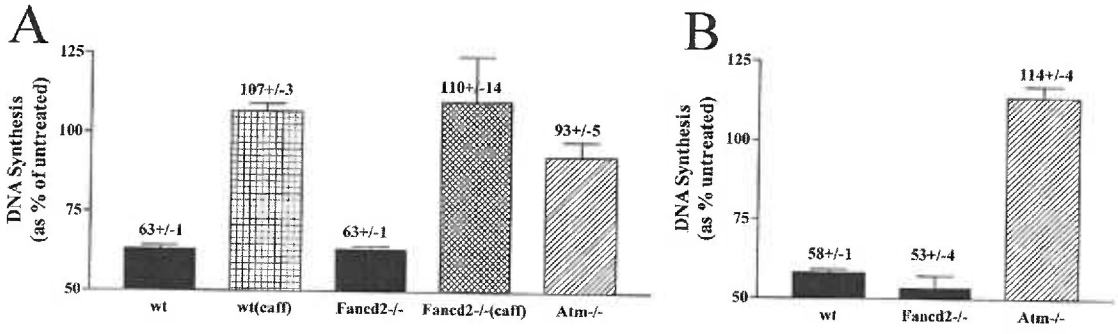

Figure 11. Primary *Fancd2*$^{-/-}$ MEFs have an intact S phase checkpoint. DNA synthesis was measured 30 minutes after treatment with 10 Gy of IR (A) or 0.5 ng/ml HMT+UVA (B) and is represented as the percentage of untreated controls. As a control, cells were also pretreated with 10 mM caffeine (caff). Experiments were performed in quintuplet. Error bars indicate SEM.

Expression and interaction of RAD51 and BRCA2 is normal in Fancd2 *mutants*

Cells harboring mutations in genes implicated in control of homologous recombination repair of DNA damage, including BRCA1, BRCA2, and RAD51 are sensitive to DNA cross-linkers (Yu et al. 2000; Moynahan et al. 2001a). After treatment with ICLs these cells show gross rearrangements involving non-homologous chromosomes, a phenotype identical to cells from all FA complementation groups. RAD51 functions in the process of homologous recombination (Baumann et al. 1996). BRCA2 interacts directly with RAD51 (Marmorstein et al. 1998) and cells lacking wild-type BRCA2 have increased levels error-prone HDR of DSBs, suggesting BRCA2 may function in error-free repair (Tutt et al. 2001). As FANCD2 acts downstream of the FA complex, it was hypothesized that it may function to modulate the stability of RAD51 and BRCA2 or their interaction.

To investigate this hypothesis, immunoblots with testicular lysates from *Fancd2* mutant, *Fancc* mutant, and control mice were prepared. Immunoblotting revealed no significant differences in the steady state level of Rad51 (Figure 12A). Immunoprecipitation of Brca2 from these lysates probed with the same Brca2 antibody also showed no significant differences in expression of Brca2 (data not shown). We next asked whether Fancd2 plays a role in modulating the interaction between Rad51 and Brca2. Immunoprecipitation of Brca2 was performed on whole cell lysates from *Fancd2* mutant, *Fancc* mutant, and wild-type mouse testes. An anti-Brca2 antibody but not control IgG was capable of immunoprecipitating Rad51 from whole cell lysates of testis from *Fancd2* mutant, *Fancc* mutant, and wild-type animals (figure 12B). We conclude

that the protein levels and interaction of Rad51 and Brca2 are normal in *Fancd2* mutant and *Fancc* mutant mice.

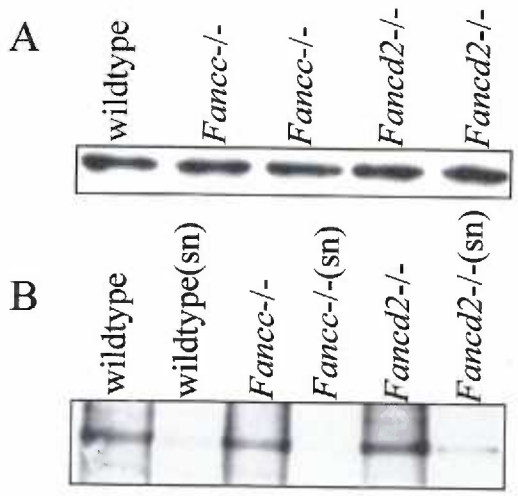

Figure 12. Interaction of Rad51 and Brca2 is normal in *Fancd2*$^{-/-}$ and *Fancc*$^{-/-}$ cells. Whole cell lysates from mouse testis of the indicated genotypes were separated by SDS-PAGE and probed with an antibody against Rad51. (B) Anti-Brca2 antibodies were used to immunoprecipitate protein complexes. Complexes were separated by SDS-PAGE and probed with an antibody against Rad51. SN indicates supernatant.

Finally, we performed immunocytochemistry experiments designed to examine whether Rad51 foci form normally in the nuclei of Fancd2 null fibroblasts following DNA damage (figure 13). Primary wild-type and *Fancd2*$^{-/-}$ MEFs were scored for the presence of Rad51 foci. An equal number of untreated wild-type (36%) and *Fancd2*$^{-/-}$ (32%) MEFs contained greater than 5 foci per nucleus. Similarly, no difference in the level of Rad51 foci was observed between wild-type (51%) and *Fancd2*$^{-/-}$ (58%) MEFs 6 hours after treatment with IR. We find that Rad51 foci formation is unaltered in Fancd2 null fibroblasts when compared to wild-type controls.

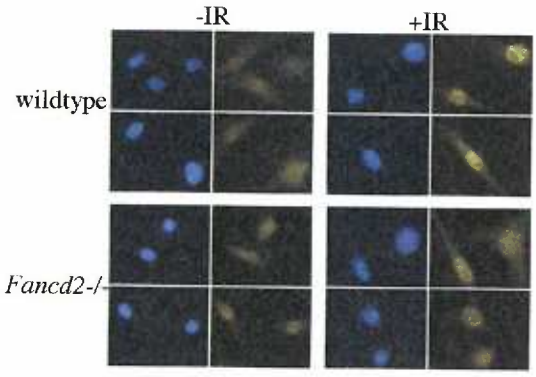

Figure 13. Rad51 foci formation is normal in *Fancd2*$^{-/-}$ cells. Rad51 foci formation in wild-type and *Fancd2*$^{-/-}$ MEFs either untreated (-IR) or following 12 Gy ionizing radiation (+IR). Cell nuclei were counter-stained with DAPI (blue).

2.5. **Discussion**

The phenotype of human FA patients indicates that the FA pathway is important for normal embryonic development, the maintenance of genomic stability, and the preservation of several types of stem cells (D'Andrea and Grompe 2003). The recent discovery that the breast cancer gene, *BRCA2*, is an FA gene (Howlett et al. 2002) has connected this rare disorder to a common form of cancer. Nonetheless, the precise function(s) of the FA pathway remains unknown (D'Andrea and Grompe 2003). We and others have previously created strains of mice with targeted deletions of the murine *Fanca*, *Fancc* and *Fancg* genes, all components of the FA nuclear complex. These mutants have very similar phenotypes (Chen et al. 1996; Whitney et al. 1996; Cheng et al. 2000; Yang et al. 2001; Koomen et al. 2002; Noll et al. 2002), supporting a model where the components of the complex participate in a common function. To determine whether Fancd2 participates only in this function *in vivo* or has additional roles, we generated a strain of mice with a null allele in this gene.

Earlier studies have suggested that Fancd2 may have unique roles that are distinct from the other FA proteins. First, it acts downstream of the FA nuclear complex and is the target of the mono-ubiquitination mediated by the complex (Garcia-Higuera et al. 2001; Timmers et al. 2001). Hence, the integrity of the FA nuclear complex is not perturbed in FA-D2 cells unlike in other complementation groups (Garcia-Higuera et al. 1999; de Winter et al. 2000c). Second, FANCD2 is the only FA protein known to form nuclear foci after DNA damage and to co-localize with the repair proteins BRCA1 and RAD51 (Garcia-Higuera et al. 2001; Taniguchi et al. 2002a). Finally, FANCD2 is directly phosphorylated by ATM in response to IR but not in response to ICLs (Nakanishi et al.

2002; Taniguchi et al. 2002b). Other FA proteins are not known to be targets of the ATM kinase.

Despite these biochemical differences, human FA-D2 patients do not differ significantly clinically from FA patients belonging to other complementation groups (Timmers et al. 2001). However, FA-D2 is a rare complementation group and all human patients reported to date have at least one mutant allele that could have some residual function and therefore, the phenotype of a true *FANCD2* null mutation remained uncertain. The *Fancd2* mutant mice reported here display all the features of previously reported strains of FA knockout mice, but also have important differences.

Fancd2, ionizing radiation and the Atm signaling pathway

Ser_{222} of human FANCD2 is phosphorylated by ATM in response to IR and this post-translational modification has been correlated to increased IR sensitivity in immortalized fibroblasts (Taniguchi et al. 2002b). In addition, phosphorylation of Ser_{222} was associated with radiation induced arrest of DNA synthesis. Therefore, in order to test whether Fancd2 played an important role in the response to IR, the survival of isogenic, primary cells from *Fancc* mutant and *Fancd2* mutant cells was compared. Interestingly, Fancd2 null cells were not differentially sensitive to IR or ICLs compared to Fancc null cells. Furthermore, while mild hypersensitivity to IR was observed *in vivo* (LD_{50}=~9.5Gy), this sensitivity was also not greater than that observed for *Fancc* mutant mice (Noll et al. 2001).

In addition, and in contrast to *Atm* mutants, Fancd2 deficient cells did not display radiation resistant DNA synthesis. Therefore, in murine primary cells, Fancd2 is not

necessary for the Atm mediated S phase arrest following IR. Overall, the phenotype of *Fancd2* mutant mice is significantly different from Atm null mice, which display marked radiation sensitivity, immune deficiency and hematologic tumors (Xu et al. 1996). Together, our observations suggest that Fancd2 does not play a significant role in Atm mediated physiologic responses to IR. Perhaps, human FANCD2 and murine Fancd2 differ is this aspect of their function, although the proteins are highly homologous and $Serine_{222}$ is conserved between the species. Alternatively, FANCD2 may play a role as a mediator of ATM function in only some specific circumstances, for example T-antigen immortalized fibroblasts.

Novel phenotypes in Fancd2 *knockout mice*

While *Fancd2* mutant mice do not differ measurably from *Fancc* mutants in terms of their response to DNA damage, they have some features not seen in *Fanca*, *Fancc* or *Fancg* mutant animals. These phenotypes include microphthalmia, perinatal lethality, more severe hypogonadism and tumor development. Normal function of the FA nuclear complex is necessary for the mono-ubiquitination of FANCD2 in response to DNA damage or DNA replication (Garcia-Higuera et al. 2001; Taniguchi et al. 2002a). Therefore, two basic models exist to explain the divergence of phenotypes between mutants in *Fancd2* and nuclear complex genes. First, non-ubiquitinated Fancd2 protein, as present in *Fanca*, *Fancc* and *Fancg* knockout mice, may have some residual activity in the function common to the FA pathway. In this model, knockouts of the nuclear complex genes would be similar to hypomorphic mutations of the *Fancd2* gene. The phenotypes would be the result of deficiency in the same biochemical function, but they would be

more severe in *Fancd2* mutant mice. In the second model, Fancd2 is a multifunctional protein with a domain that functions in the FA pathway and other domains that mediate unrelated functions. The additional phenotypes of *Fancd2* mutants would then be due to deficiency of these additional functions.

Most of the features observed in Fancd2 null mice can be interpreted as more severe manifestations of the qualitatively similar defects seen in other FA knockouts. This is most obvious with the germ cell defects. Although testicular weight is clearly more affected in *Fancd2* mutant mice, the histology is similar to other FA models. Similarly, we have observed microphthalmia in the mutant offspring of some *Fancc* breeding pairs (M. Grompe, unpublished data), indicating that this is not a truly novel phenotype but a more complete manifestation of a defect common to all FA mice. The increased susceptibility of FA cells to apoptotic cell death after DNA damage could provide a common explanation for the observed germ cell deficiency, microphthalmia, small size and perinatal lethality. In the case of these developmental defects, the cells would be responding to spontaneous DNA damage. Overall, the phenotype of *Fancd2* mutant mice does not convincingly establish the existence of additional functional domains in the Fancd2 protein.

Fancd2, Brca2 and carcinomas

Although human patients with FA develop a variety of cancers, tumors have never been reported in the other FA knockout mice even when followed to very late ages. In contrast, it is well established that mice with a truncating mutation in *Brca2* are at an increased risk of a variety of neoplasms (Connor et al. 1997; McAllister et al. 2002).

Interestingly, the tumor spectrum of the *Brca2* hypomorphic mice was comparable to that seen in *Fancd2* mutants (McAllister et al. 2002). In both cases, a predisposition towards epithelial cancers was seen and the age of onset was similar. *Brca2* hypomorphic mice also have other similarities such as an FA-like germ cell defect, small size and perinatal lethality. Taken together, this extensive phenotypic overlap is consistent with the hypothesis that the carboxy-terminal domain of Brca2 functions in the same pathway as Fancd2.

Human BRCA2 is known to interact with and modulate the activity of RAD51, a central player in homologous recombination and *Brca2* mutant cells are known to have defects in error-free recombination (Moynahan et al. 2001b; Tutt et al. 2001). Therefore, the identification of *Brca2* as an FA gene supports a role for the FA pathway in recombination DNA repair. This hypothesis is strengthened by the observation that chromosomes in the pachytene stage of meiosis mispair, as described here. One potential function for the FA pathway could be to control the interaction between RAD51 and BRCA2 and thereby modulate homologous recombination events at sites of DNA damage. However, Western blot analysis of *Fancd2* mutant tissues showed normal Rad51 levels as well as normal interaction of the Brca2 and Rad51 proteins as determined by co-immunoprecipitation. Furthermore, Rad51 foci formation following DNA damage was normal in *Fancd2*$^{-/-}$ MEFs. Thus, our data do not support a simple model in which the FA pathway controls the stability/rate of Brca2/Rad51 interaction.

In humans, heterozygosity for inactivating *BRCA2* germ line mutations is associated with breast, ovarian and pancreatic cancer, all tumors of epithelial origin. Currently, it is unclear why these tissues are particularly affected, but it is interesting to

note that epithelial tumors in rodents have been associated with telomere shortening (Artandi et al. 2000; Chang et al. 2001). Human FA patients are known to display significantly shortened telomeres in hematopoietic cells (Adelfalk et al. 2001; Brummendorf et al. 2001; Hanson et al. 2001) and it is therefore interesting to speculate that the FA pathway could be involved in telomere maintenance in some tissues.

The high incidence of epithelial tumors observed in *Fancd2* mutants here also raises the issue whether FANCD2 could be an important gene in some human epithelial cancers, especially of the breast and ovary. Importantly, some cases of familial ovarian cancer have been linked precisely to the region where *FANCD2* resides in the human genome (Sekine et al. 2001; Simsir et al. 2001; Zhang and Xu 2002). Future studies of human patients with these disorders will be needed to determine whether FA genes play a role in sporadic or inherited human cancers.

2.6. Acknowledgements

Cynthia Timmers, Ph.D. (Department of Molecular and Medical Genetics, OHSU) cloned the *Fancd2* cDNA, designed the targeting construct and was critical in guiding the creation of the targeting plasmid for the *Fancd2* knockout mouse. Dr. Timmers also performed the Southern blot to confirm homologous integration of the targeting construct and designed the PCR genotyping reaction. The engineering of the *Fancd2* knockout mouse would not have been possible without her expert guidance. **Steve Jones, Ph.D.** (Department of Cell Biology, University of Massachusetts Medical School, North Worcester, MA) performed the ES cell electroporation. **Meenakshi Noll, Ph.D.** (Department of Molecular and Medical Genetics, OHSU) performed the Fancd2 western blot on mouse testis protein extract (figure 5). She also performed the Rad51 western blot and the Brca2 immunoprecipitation/Rad51 western blot (figure 12). She also provided *Fancc* knockout mice used to generate *Fancc* mutant MEFs. **M. Stephen Meyn, Ph.D.** (Department of Molecular and Medical Genetics, University of Toronto, Toronto, Canada) performed the TUNEL assay on *Fancd2* and wild-type testes sections (figure 7), the immunochemistry to detect pairing abnormalities in *Fancd2* mutant testes (figure 7) and generated figure 7. **Milton J. Finegold, M.D.** (Department of Pathology, Texas Children's Hospital, Baylor College of Medicine, Houston, Texas) performed histological examination of testes, ovaries and tumors samples (figure 9) from *Fancd2* knockout mice. **Alan D'Andrea, M.D.** (Dana Farber Cancer Institute, Boston, MA) kindly provided Fancd2 antibodies used for western blots. This work was supported by NHLBI Program Project Grant 1PO1HL48546 awarded to Markus Grompe, M.D.

Chapter Three:

Heterozygosity for Trp53 *accelerates tumor phenotype of* Fancd2 *knockout mice*

3.1. **Abstract**

FA is an autosomal recessive disease characterized by progressive bone marrow failure and an increased susceptibility to cancer. FA is genetically heterogeneous, consisting of at least 11 complementation groups, FA-A through L, including FA-D1 (*BRCA2*) and D2. We have previously reported an increased incidence of epithelial tumors in *Fancd2* knockout mice. To further investigate the role of the FA pathway in tumor prevention, *Fancd2* mutant mice were crossed to mice with a null mutation in the tumor suppressor gene, *Trp53*. The tumor spectrum in *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice included sarcomas expected in *Trp53* heterozygotes, as well as mammary and lung adenocarcinomas that occur rarely in *Trp53* heterozygotes. These tumors occurred earlier than in *Fancd2*$^{-/-}$ control mice. Therefore, the *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice represent an improved model for the study of adenocarcinoma in FA. In addition it was found that *Fancd2*$^{-/-}$ MEFs but not *Fancd2*$^{-/-}$/*Trp53*$^{-/-}$ MEFs arrest following DNA damage. Therefore, *Trp53* is required for the S phase checkpoint activation observed in *Fancd2* mutant cells. *Fancd2*$^{-/-}$/*Trp53*$^{-/-}$ cells showed an increase in aneuploidy and had multiple gross chromosomal rearrangements.

3.2. **Introduction**

FA is an inherited bone marrow failure syndrome associated with an increased incidence of cancer (Tischkowitz and Hodgson 2003). Patients often present with multiple abnormalities including short stature, microphthalmia, radial ray defects, infertility, and pigmentation defects. The majority of patients succumb to bone marrow failure. AML is the most common malignancy but patients, particularly those surviving following treatment by bone marrow transplantation, also have an increased risk of solid tumors including aerodigestive and gynecological carcinomas (Alter 2003; Alter et al. 2003). FA is genetically heterogeneous consisting of at least 11 complementation groups, FA-A, B, C, D1, D2, E, F, G, I, J, and L (Levitus et al. 2004). The genes for 9 of these have been identified (*FANCA*, *B*, *C*, *D1/BRCA2*, *D2*, *E*, *F*, *G/XRCC9*, and *L/PHF9*) (Strathdee et al. 1992; Lo Ten Foe et al. 1996; de Winter et al. 1998; de Winter et al. 2000a; de Winter et al. 2000b; Timmers et al. 2001; Howlett et al. 2002; Meetei et al. 2003a; Meetei et al. 2004a). Although patients with mutations in *BRCA2* (complementation group D1) have an elevated incidence of brain tumors and may have an increased predisposition to solid tumors early in childhood, patients from all other groups have an equal risk for developing malignancies (Offit et al. 2003; Hirsch et al. 2004).

The precise function of the FA pathway remains uncertain. However much evidence points to a role for the proteins in response to DNA damage and in maintenance of genomic stability (D'Andrea and Grompe 2003). Following DNA damage and during S phase of the cell cycle FANCD2 is monoubiquitinated and forms nuclear foci that colocalize with known DNA repair proteins, BRCA1 and RAD51 (Taniguchi et al.

2002a). This posttranslational modification is dependent upon a nuclear complex of FA proteins and is required for the targeting of FANCD2 to chromatin where it facilitates the loading of BRCA2 into a chromatin complex (Garcia-Higuera et al. 2001; Wang et al. 2004). FANCL has been implicated as the ubiquitin ligase responsible for the modification of FANCD2 (Meetei et al. 2003a; Meetei et al. 2004b). BRCA2 and the yet unidentified FANCJ act downstream of other FA proteins, as FANCD2 is monoubiquitinated in cell lines from FA-D1 and FA-J patients whereas FANCI has been placed upstream of FANCD2 based on the lack of monoubiquitinated FANCD2 in FA-I cell lines (Howlett et al. 2002; Levitus et al. 2004). Recent studies have suggested that the FA pathway functions in the repair of DNA damage, particularly ICLs, which are processed to DSBs during S phase of the cell cycle (Rothfuss and Grompe 2004).

Mouse models of FA faithfully represent some aspects of the human condition. *Fanca* (Cheng et al. 2000; Noll et al. 2002; Wong et al. 2003), *Fancc* (Chen et al. 1996; Whitney et al. 1996), *Fancg* (Yang et al. 2001; Koomen et al. 2002), and *Fancd2* (Houghtaling et al. 2003) mice share many phenotypes including reduced fertility, small size and a cellular sensitivity to interstrand cross-linkers. The most striking phenotype of the *Fancd2* mice is an elevated incidence of epithelial cancer including hepatocellular, mammary, lung, and ovarian adenocarcinoma that typically develop with a late onset (Houghtaling et al. 2003).

Trp53 is a tumor suppressor gene coding for a transcription factor that has been implicated in regulating cell cycle control, apoptosis and repair of damaged DNA. Mice harboring homozygous mutations in *Trp53* are viable but develop malignant lymphomas by 6 months of age (Donehower et al. 1992; Jacks et al. 1994). *Trp53* heterozygous mice

are also susceptible to tumors, predominantly sarcomas and lymphomas, which develop by 18 months of age. However, mammary adenocarcinoma or other tumors of epithelial origin are rare in *Trp53* heterozygous mice of any strain, accounting for less than 10% of their tumors (Harvey et al. 1993).

There is evidence to suggest that loss of *p53* function may facilitate tumor development in FA patients and mouse models. Recent studies by two groups have identified a higher proportion of HPV positive SCC among FA patients than among controls indicating that loss of functional p53 facilitates the development FA tumors (Kutler et al. 2003b; Lowy and Gillison 2003). Mice harboring mutations in *Brca1* or *Brca2* have been intercrossed to *Trp53* mutant mice with the general trend of increasing tumor incidence and decreasing tumor latency (Cressman et al. 1999b; Xu et al. 1999; Brodie et al. 2001; Jonkers et al. 2001; Xu et al. 2001). Finally, *Fancc* mutant mice heterozygous at *Trp53* developed blood and solid malignancies that appear earlier than mice with mutations in *Trp53* alone (Freie et al. 2003). Taken together these reports indicate that the FA proteins and p53 cooperate in the prevention of various types of tumors. In this study we demonstrate that *Fancd2* and *Trp53* cooperate in the prevention of tumors, specifically mammary adenocarcinoma.

3.3. **Materials and Methods**

Animal Husbandry

Fancd2 mutant mice, previously described by our laboratory, were maintained on a 129S4 background (Houghtaling et al. 2003). *Trp53* mutant mice, obtained from the laboratory of Dr. Allan Bradley, were maintained on 129S4 background (Donehower et al. 1992). *Fancd2*$^{+/-}$/*Trp53*$^{+/-}$ breeding pairs were crossed to generate *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ animals and littermate controls. Animals were examined three times per week for tumor development and sacrificed if tumors were present or if animal was otherwise unhealthy. Animals were housed at the Oregon Health & Science University Department of Animal Care according to an approved IACUC protocol.

Survival Curves

Tumor free and epithelial cancer free survival curves were generated using Prism software (Graphpad Software, Inc., San Diego, CA www.graphpad.com). Statistical significance between genotypes was determined using built-in analysis for survival curves consisting of a Logrank test yielding a p value.

Tumor Histology

Tumors fixed in 10% phosphate-buffered formalin for 24 h (pH 7.4) were dehydrated in 80% ethanol and embedded in paraffin wax at 58°C. Then 4-μm sections were rehydrated and stained with hematoxylin and eosin (H&E).

Southern blot analysis

Genomic DNA (gDNA) was extracted from tumors or tail as previously described (Miller et al. 1988). Southern blot analysis was performed as previously described (Donehower et al. 1992). Briefly, gDNA was digested with *BamHI* and separated on 0.8% agarose gel and transferred to Hybond N+. The radiolabeled probe consisted of exons 2-6 of *Trp53*. *Trp53* mutant and wild-type bands were normalized to the pseudogene and the relative intensity compared to determine if LOH had occurred.

MEF growth experiments

Primary MEFs were generated from pregnant *Fancd2*$^{+/-}$/*Trp53*$^{+/-}$ or *Fancd2*$^{+/-}$/*Trp53*$^{-/-}$ crossed to *Fancd2*$^{+/-}$/*Trp53*$^{+/-}$ males on the 129S4 background at between 12.5-14.5 days gestation. Passage 1 MEFs were seeded in triplicate at a density of 4200 cells/cm^2 on 12 well plates. Cells were grown in DMEM (Hyclone, Logan, UT) supplemented with 15% FBS (HyClone, Logan, UT), 1x penicillin/streptomycin (Mediatech, Inc, Herndon, VA), and 1x L-glutamine (Mediatech, Inc, Herndon, VA). Cells were treated as previously described with slight modification (Akkari et al. 2000). To induce DNA cross-linking, cells were incubated in the dark for ten minutes in Hank's balanced salt solution (HBSS) + 2% FBS containing 0.0 or 0.2 ng/ml HMT (Sigma, St. Loius, MO). Cells were irradiated with UVA for 30 minutes and HBSS + HMT was replaced with complete media. At 3, 5, and 7 days post treatment, cells were washed in PBS, trypsinized, and resuspended in 2.0 ml complete media. Cells were counted using a Coulter Multisizer II according to manufacturer's instructions. Cell number is represented as percentage of cells present on day of treatment (day 0). Error bars indicate mean +/- SEM.

BrdU Detection

Passage 1 MEFs were seeded at a density of 4200 cells/cm^2 on glass coverslips in a 6 well plate and treated as described above to induce ICLs. BrdU (Sigma, St. Louis, MO) was added to media at a final concentration of 50uM for 2 hours. Cells were washed in PBS and fixed for 2 minutes in 60% ethanol/2.5% paraformaldehyde/4% glacial acetic acid. Cells were washed 3 x 2 minutes in PBS and denatured in 0.07 N NaOH for 3 minutes. Cells were washed 3 x 2 minutes in PBS (pH 8.5) and blocked in PBS + 0.5% Tween-20 for 10 minutes. Anti-BrdU antibody (Becton-Dickinson, San Jose, CA) was diluted 1:10 in blocking solution and added to cells for 30 minutes. Cells were washed in PBS 3 x 2 minutes and FITC conjugated donkey anti-mouse secondary antibody (Jackson Immunoresearch, West Grove, PA) diluted 1:100 in PBS + 0.5% Tween-20 was added for 30 minutes. Cells were washed 3 x 2 minutes in PBS. Cover slips were mounted in SlowFade Light Antifade solution with DAPI (Molecular Probes, Eugene, OR) and analyzed by fluorescent microscopy. Images were captured using OpenLab software at 20x magnification.

Phosphorylated Histone H3 detection

Passage 1 MEFs were seeded at a density of 4200 cells/cm^2 on glass coverslips in a 6 well plate and treated as described above to induce ICLs. Cells were fixed in 4% paraformaldehyde, washed three times in PBS for 2 minutes and permeabalized in PBS+0.1% Triton x-100 for 3 minutes. Cells were blocked in PBS+1% bovine serum albumin. Cells were washed in PBS for 15 minutes. Cells were incubated for 1.5 hours at

room temperature in anti-phosphorylated histone H3 antibody (Upstate biotechnology, Lake Placid, NY) diluted 1:100 in blocking solution. Cells were washed 3x5 minutes in PBS. Cells were incubated for 30 minutes in Cy3 conjugated goat-anti-rabbit antibody (Jackson Immunoresearch, West Grove, PA) diluted 1:100 in blocking solution for 30 minutes. Cells were washed 3x5 minutes in PBS. Cover slips were mounted in SlowFade Light Antifade solution with DAPI (Molecular Probes, Eugene, OR) and analyzed by fluorescent microscopy. Images were captured using OpenLab software at 20x magnification.

Cytogenetics

MEFs were plated on 100-mm dishes in DMEM+10%FBS and treated as described above to induce ICLs or mock treated. After 5 days, colcemid (Sigma, St. Louis, MO) was added at a final concentration of 150 ng/ml. After 4–6 hours, cells were digested with trypsin, placed in hypotonic medium consisting of 5% FCS and 75 mM KCl, and fixed to slides. Slides were G-banded by treatment with 10% trypsin for 45–55 s followed by Wright's stain (Fisher Scientific, Pittsburgh, PA) for 2 min 30 s. Cells were observed using a Nikon E800 fluorescence microscope, and captured using CytoVision software from Applied Imaging.

3.4. **Results**

Heterozygosity for Trp53 *accelerates tumor phenotype of* Fancd2 *knockout mice*

To test the hypothesis that Fancd2 and Trp53 cooperate in tumor prevention we intercrossed *Fancd2*$^{+/-}$ and *Trp53*$^{+/-}$ mice on the 129S4 strain background. A cohort of 20 *Trp53*$^{+/-}$ and 22 *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice were monitored for 20 months and compared to *Fancd2*$^{-/-}$ or control (*Fancd2*$^{-/+}$ or wild-type) animals on the 129S4 strain background. Animals were sacrificed if tumors were palpable or if they were otherwise unhealthy.

A total of 15 tumors were identified in 13 of 20 (65%) Trp53$^{+/-}$ control mice (figure 14 and table 7). The median age of tumor free survival for these mice was 468 days and the majority of tumors were either lymphoma or sarcoma as previously reported (Harvey et al. 1993; Jacks et al. 1994). One *Fancd2*$^{+/-}$/*Trp53*$^{+/-}$ mouse developed two independent carcinomas and an adenoma of a pancreatic islet (table 7).

Heterozygosity for *Trp53* had a significant effect on the incidence and origin of tumors in the *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice compared to the *Fancd2*$^{-/-}$ mice. 15 of 22 (68.2%) animals developed a total of 23 tumors (table 7). The mice had a median age of tumor free survival of 404 days (figure 14). 15 of 23 (65.2%) tumors involved non-epithelial tissue whereas the remaining 8 of 23 (34.8%) involved epithelial components of lung, ovary, or mammary glands (table 7 and figure 15). 6 of the 22 *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice had multiple independent tumors compared to only 1 of 20 controls.

The incidence of epithelial cancer was particularly striking when only female *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice were compared to controls (figure 14C). 10 of the 22 *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice in the experimental group were female. 6 of these 10 (60%) developed at least one adenocarcinoma. Of the14 tumors that formed in female *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$

mice, 8 (57.1%) involved epithelial tissue. The female *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice begin to develop adenocarcinoma at a time when *Fancd2*$^{-/-}$ females have not yet developed epithelial cancer. This difference was statistically significant and demonstrates that heterozygosity at *Trp53* can accelerate the tumor phenotype of *Fancd2* mutant mice (figure 14C).

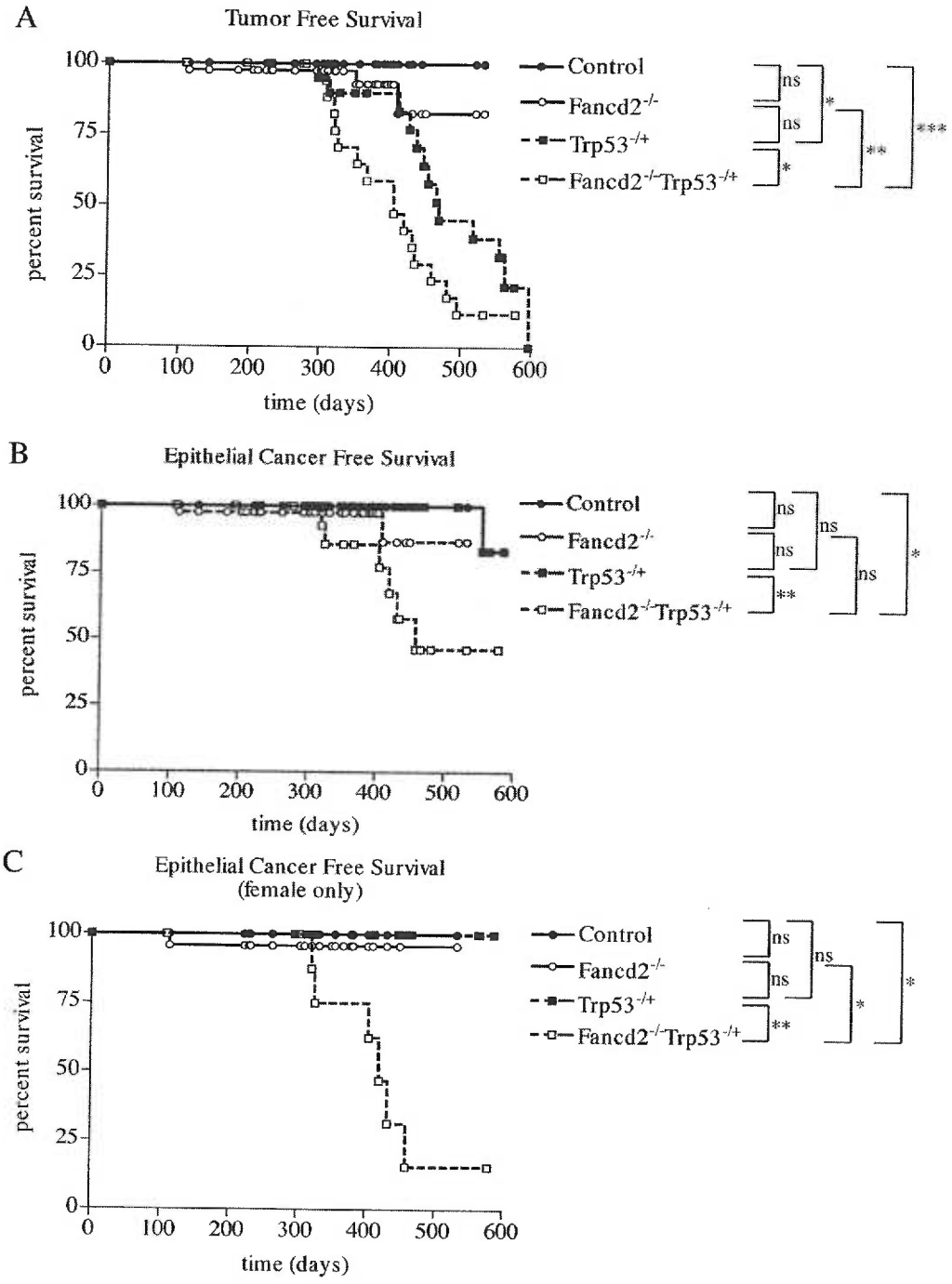

Figure 14. Heterozygosity for *Trp53* accelerates formation of tumors in *Fancd2*$^{-/-}$ mice (A) Tumor free survival curves include all tumor types observed. (B) Epithelial cancer free survival curves show an increased incidence of epithelial tumors in *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice compared to *Trp53*$^{+/-}$ or control mice. (C) Epithelial cancer free survival curves for female mice show a significant increase in tumors in *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice compared to *Fancd2*$^{-/-}$, *Trp53*$^{+/-}$ or control mice. *Fancd2*$^{-/-}$ and control genotype curves were adapted from (Houghtaling et al. 2003). Statistical significance between curves was determined using Logrank test. * = ($p<0.05$), ** = ($p<.01$), *** = ($p<.001$), ns = ($p>.05$).

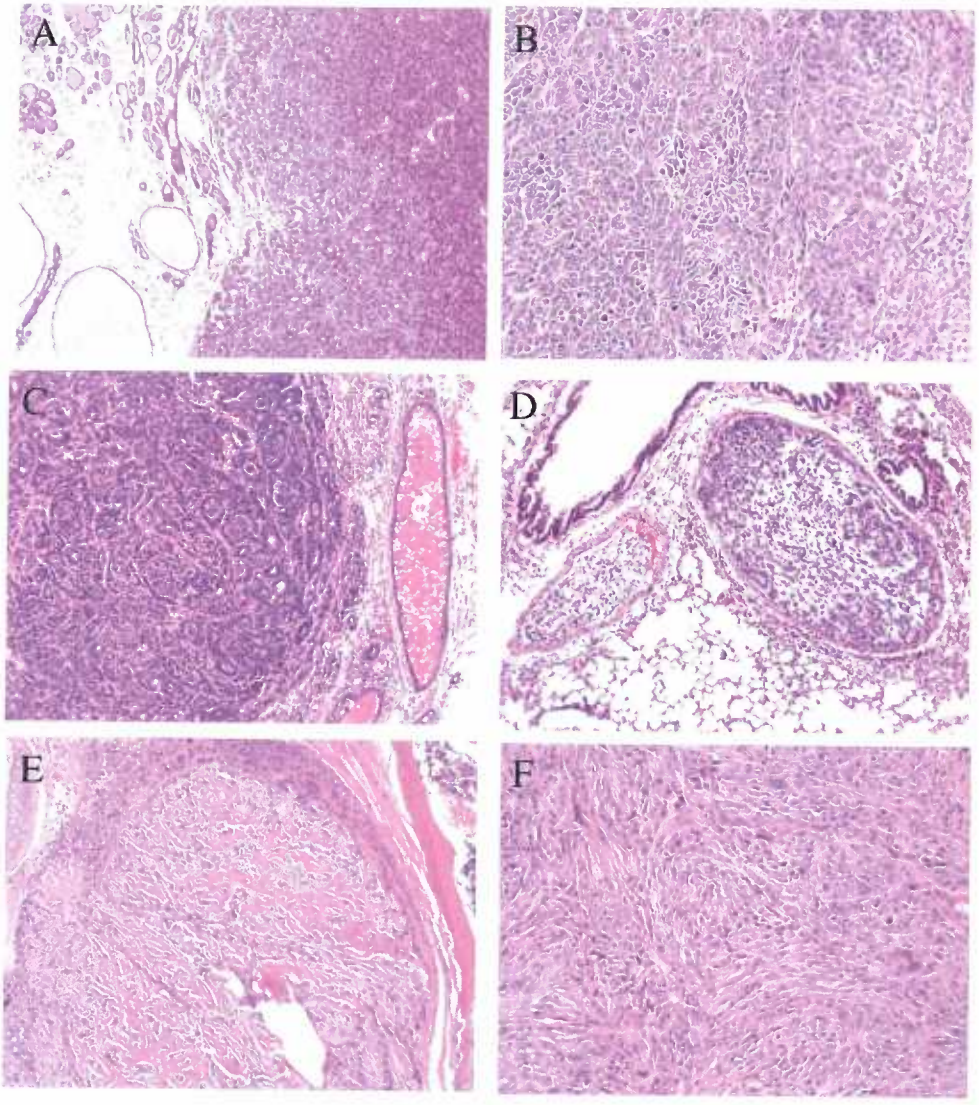

Figure 15. Examples of tumor histology from *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice.
(A) Mammary adenocarcinoma occurred in 10 month old female mouse. The carcinoma, seen on the right side, had a sheet-like growth pattern with some gland formation and compressed the residual normal parenchyma, seen on the left side (x100). (B) Diffusely effacing the ovary, a primary ovarian adenocarcinoma occurred in a 13 month old mouse. Numerous mitoses were present (x200). (C) Mammary adenocarcinoma, which had obvious gland formation with pushing borders and a lymphocytic response, occurred in a 15 month old female mouse (x100). (D) Lung tumor embolic to pulmonary arteries occurred in 13 month old mouse (x100). (E) Osteosarcoma occurred in 10 month old *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mouse. Malignant osteoid was rimmed by atypical cells with nuclear pleomorphism and hyperchomasia (x100) (F) Malignant fibrous histiocytoma with highly pleomorphic spindled to epithelioid cells had scattered atypical mitoses. This tumor contained no evidence of differentiation and occurred in a 10 month old mouse (x200).

Table 7. Tumor spectrum from *Fancd2*$^{-/-}$, *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ and control mice

Genotype	Sex	Age (months)	Tumor Type
Fancd2$^{-/-}$	male	13	lung adenocarcinoma
	female	3	ovarian adenocarcinoma
Fancd2$^{+/-}$*Trp53*$^{+/-}$	male	14	MFH (abdomen)
		15	tumor (source unknown)
	female	10	B cell Lymphoma
		14	osteosarcoma (scapula)
		15	lymphoma
		15	*lymphoma*
		15.5	osteosarcoma (pelvis)
		18	bronchoalveolar carcinoma@ hepatocellular carcinoma@ adenoma (pancreas)@
		20	lymphoma
Trp53$^{+/-}$	male	14.5	lymphoma
		17	MFH (abdomen)
	female	10	MFH (uterus)
		18.5	lymphoma
Fancd2$^{-/-}$ *Trp53*$^{+/-}$	male	10	MFH (perirectal)
		10	sarcoma
		11	MFH
		12	rhabdosarcoma
		12	MFH (head)# osteosarcoma (spine)#
		14.5	sarcoma (dermis)
		16	osteosarcoma (source unknown)
		16.5	lymphoma
	female	10	mammary adenocarcinoma[3]** osteosarcoma (rib cage)**
		11	mammaryadenocarcinoma[4]
		13	myxoma-myxosarcoma (shoulder)### ovarian adenocarcinoma### lung adenocarcinoma###
		13	MFH (back)
		14	mammary adenocarcinoma[1]## ovarian stromal tumor##
		14	mammary adenocarcinoma*** osteosarcoma (source unknown)***
		15	mammary adenocarcinoma[2]* mammary carcinosarcoma* ovarian stromal cell tumor*

@Bronchoalveolar carcinoma, hepatocellular carcinoma, adenoma occurred in same mouse; # Malignant Fibrous Histiocytoma and Osteosarcoma occurred in same mouse; ## Mammary Adenocarcinoma and Ovarian Stromal Tumor occurred in same mouse; ### Myxoma-myxosarcoma, Ovarian Adenocarcinoma, and Lung Adenocarcinoma occurred in same mouse; * Mammary Adenocarcinoma, Mammary Carcinosarcoma, and Ovarian Stromal Cell Tumor occurred in same mouse; ** Mammary Adenocarcinoma and Osteosarcoma occurred in same mouse; *** Mammary Adenocarcinoma and Osteosarcoma occurred in same mouse, 1, 2, 3, 4 are tumors represented in Figure 16

Data for *Fancd2*$^{-/-}$ mice was adapted from previous publication (Houghtaling et al. 2003)

Loss of Heterozygosity (LOH) at Trp53 *contributes to formation of solid tumors in* Fancd2$^{-/-}$/Trp53$^{+/-}$ *mice*

In cells heterozygous for a mutant allele of a tumor suppressor gene, LOH can result in loss of the wild-type allele and contribute to tumor formation. LOH for the wild-type allele of *Trp53* was observed in 55% of tumors from *Trp53*$^{+/-}$ mice in one study (Harvey et al. 1993). In mouse models with conditional loss of *Brca1* or *Brca2* and heterozygosity at *Trp53* most of the mammary tumors that formed had lost the wild-type allele of Trp53 (Brodie et al. 2001; Jonkers et al. 2001).

We analyzed the status of the wild-type copy of *Trp53* in tumors from *Fancd2*$^{-/-}$*/Trp53*$^{+/-}$ and *Trp53*$^{+/-}$ mice by Southern blot analysis (Figure 16). Comparison of intensity between wild-type and mutant *Trp53* bands shows that 4 of 4 mammary adenocarcinomas from *Fancd2*$^{-/-}$*/Trp53*$^{+/-}$ mice had undergone LOH at *Trp53*. The absence of complete loss of the wild-type band in genomic DNA from the mammary AC may be due to contaminating normal tissue or to a mix of tumor material in which not all cells have undergone LOH. In addition to the 4 mammary adenocarcinomas, 8 of 9 osteosarcomas or soft tissue sarcomas from *Fancd2*$^{-/-}$*/Trp53*$^{+/-}$ mice exhibited loss of the wild-type copy of *Trp53*. 5 of 7 osterosarcomas or soft tissue sarcomas examined from control *Trp53*$^{+/-}$ mice had also undergone LOH at *Trp53*. Thus, consistent with previous reports (Harvey et al. 1993), LOH may not be necessary for tumor formation in cells heterozygous for *Trp53*, but may be a necessary step for the development of certain tumor types, such as mammary adenocarcinoma in *Fancd2*$^{-/-}$*/Trp53*$^{+/-}$ animals.

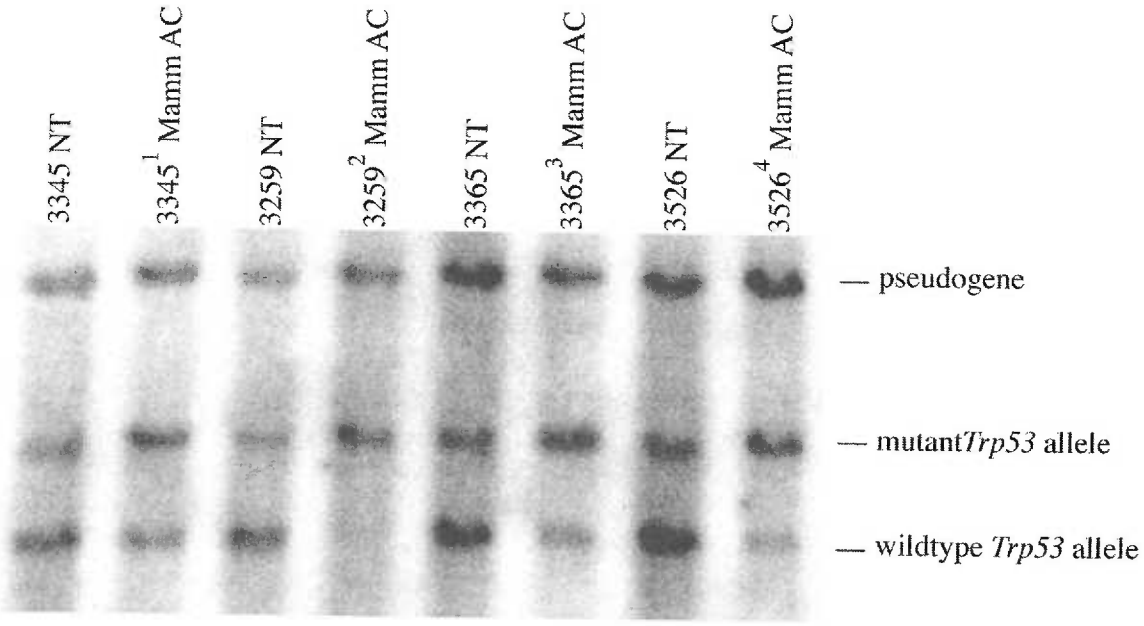

Figure 16. Mammary adenocarcinomas from *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice undergo loss of heterozygosity for the wild-type allele of *Trp53*. This representative Southern blot shows near equal hybridization at wild-type and *Trp53* mutant bands in non-tumor (NT) genomic DNA (tail) but reduced hybridization for the wild-type band in genomic DNA from mammary adenocarcinomas (Mamm AC). Numbers 1 through 4 refers to mice in table 7.

Primary Fancd2$^{-/-}$/Trp53$^{-/-}$ *MEFs continue to proliferate following DNA damage*

Both wild-type and FA cells are capable of repairing ICLs and arrest with near 4N DNA content in late S phase (Akkari et al. 2000; Akkari et al. 2001). To examine the role of Trp53 in this late S phase arrest, primary mouse embryonic fibroblasts (MEFs) deficient in *Fancd2*, *Trp53*, or both genes and *Fancd2*$^{+/-}$/*Trp53*$^{+/-}$ control MEFs were treated to induce ICLs. Following a dose of 0.2 ng/ml HMT+UVA, *Fancd2*$^{+/-}$/*Trp53*$^{+/-}$, *Fancd2*$^{-/-}$, and *Fancd2*$^{-/-}$/*Trp53*$^{-/-}$, but not *Trp53*$^{-/-}$ MEFs stop accumulating in cell number (figure 17B) relative to mock treated controls (figure 17A).

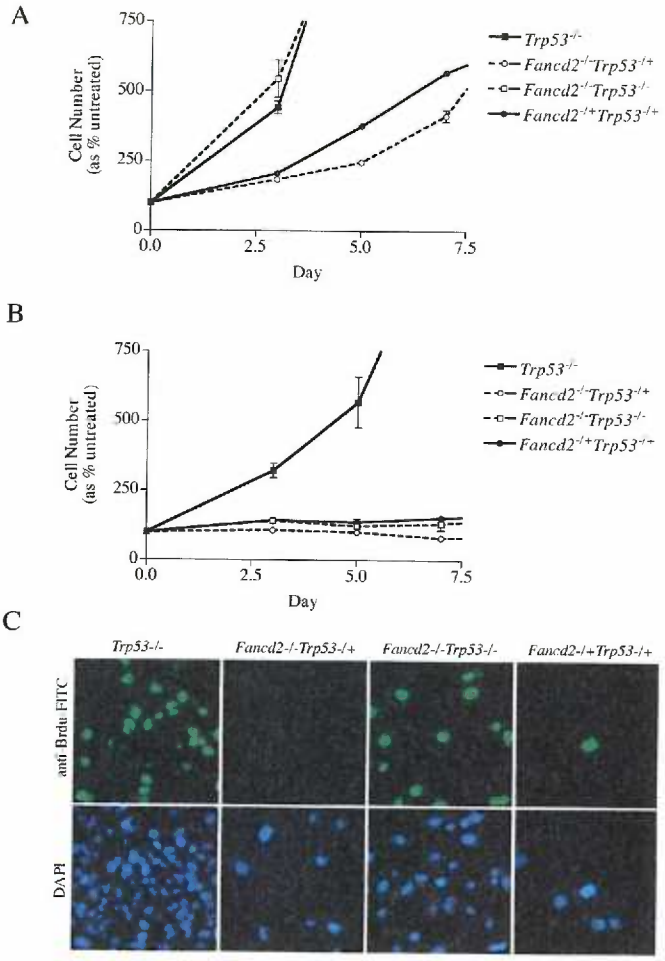

Figure 17. Cell growth kinetics of primary *Fancd2/Trp53* MEFs. Cell number is represented as a percentage of total cells at time of treatment. Cells were mock treated with 0 ng/ml (A) or treated with 0.2 ng/ml (B) HMT+UVA. Following treatment with HMT+UVA *Trp53*$^{-/-}$ cells continue to increase cell number whereas *Fancd2*$^{-/-}$*/Trp53*$^{+/-}$, *Fancd2*$^{-/-}$*/Trp53*$^{-/-}$, *Fancd2*$^{+/-}$*/Trp53*$^{+/-}$ and MEFs stop increasing cell number. Data points represent mean +/- SEM. At 5 days after induction of DNA cross-links, cells were allowed to incorporate BrdU to detect cycling cells. (C) BrdU positive cells were scored using an antibody against BrdU. Nuclei were counterstained with DAPI. *Fancd2*$^{-/-}$*/Trp53*$^{-/-}$ and Trp53$^{-/-}$ MEFs continue to incorporate BrdU following treatment whereas *Fancd2*$^{-/-}$*/Trp53*$^{+/-}$ and *Fancd2*$^{+/-}$*/Trp53*$^{+/-}$ MEFs do not.

To investigate whether *Fancd2*$^{-/-}$*/Trp53*$^{-/-}$ MEFs had undergone a cell cycle arrest, cells were allowed to incorporate BrdU 5 days after treatment and immunocytochemistry was performed to detect cells in S phase. While mock treated *Fancd2*$^{+/-}$*/Trp53*$^{+/-}$ and *Fancd2*$^{-/-}$ MEFs incorporated BrdU, their treated counterparts failed to incorporate BrdU (table 8), consistent with previous results showing that wild-type and FA cells undergo a cell cycle arrest following induction of ICLs (Akkari et al. 2000; Akkari et al. 2001). *Trp53*$^{-/-}$ MEFs continued to incorporate BrdU following treatment, demonstrating that they have actively replicating DNA, consistent with their increase in cell number. The

percentage of treated *Fancd2*$^{-/-}$/*Trp53*$^{-/-}$ MEFs that were positive for BrdU was similar to *Trp53*$^{-/-}$ MEFs in multiple independent experiments, indicating that these cells are also replicating DNA. We also performed immunocytochemistry to identify cells in mitosis. Consistent with the BrdU data, both *Trp53* mutant and double mutant cells failed to stop progression through the cell cycle (table 8). The seemingly paradoxical result of double mutant cells continuing to progress through the cell cycle but failing to increase in cell number, can be explained if these cells die following attempts to divide after induction of DNA damage that cannot be repaired. Thus the cell cycle arrest observed in both wild-type and Fanconi cells is dependent upon Trp53.

Table 8. Measure of primary MEFs in S phase and M phase at day 5 after ICL induction

	0.0 ng/ml HMT+UVA	*0.2 ng/ml HMT+ UVA*
Fancd2$^{-/-}$*Trp53*$^{+/-}$		
S phase (Brdu+)	26/131 (19.1%)	7/109 (6.4%)
M phase (pH3+)	6/246 (2.4%)	0/219 (0.0%)
Fancd2$^{+/-}$*Trp53*$^{+/-}$		
S phase (Brdu+)	20/114 (17.5%)	5/95 (5.3%)
M phase (pH3+)	39/528 (7.4%)	12/504 (3.0%)
Fancd2$^{-/-}$*Trp53*$^{-/-}$		
S phase (Brdu+)	90/376 (23.9%)	81/254 (32.0%)
M phase (pH3+)	91/547 (16.6%)	43/245 (17.6%)
Trp53$^{-/-}$		
S phase (Brdu+)	91/280 (32.5%)	43/121 (35.5%)
M phase (pH3+)	83/593 (14.0%)	57/278 (20.5%)

To further investigate the effects of continued cycling with unrepaired DNA damage, we performed detailed cytogenetic analysis on *Trp53* mutant and *Fancd2/Trp53* double mutant MEFs 5 days after mock treatment or treatment with 0.2 ng/ml HMT+UVA. In two independent, blinded experiments the treated double mutant MEFs were identified as the most genetically abnormal sample. Both the untreated and treated

double mutant samples contained numerous GCRs (figure 18A). These included deletions, translocations, and chromosomes rearranged beyond recognition (markers). An average of 0.4 GCRs were identified per cell in untreated *Trp53* mutant MEFs and 0.3 GCRs were identified per cell in treated *Trp53* mutant MEFs. Loss of *Fancd2* contributed greatly to the genetic instability observed in double mutant cells as shown by an average of 1.3 GCRs identified per metaphase in untreated double mutant MEFs and a striking 2.9 GCRs identified per metaphase from treated double mutant MEFs. Metaphase spreads were also analyzed for total chromosome number (figure 18B). All cells examined showed a moderate increase in aneuploidy however most of the untreated or treated *Trp53* mutant and untreated double mutant cells contained near diploid (2N) or tetraploid (4N) genomic content. In contrast, nearly all treated double mutant MEFs showed marked aneuploidy. Thus, both Fancd2 and Trp53 cooperate in maintaining genomic integrity.

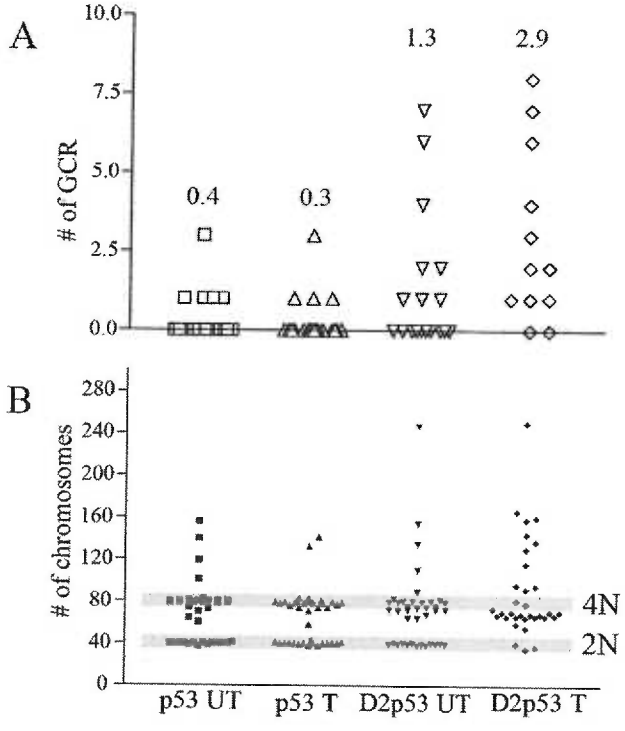

Figure 18. *Fancd2*$^{-/-}$/*Trp53*$^{-/-}$ MEFs show an increase in GCRs and aneuploidy.
(A) *Fancd2*$^{-/-}$/*Trp53*$^{-/-}$ MEFs show an increase in the total number of GCRs per cell. The GCRs included deletions, translocations, and rearranged marker chromosomes. The mean number of GCRs per cell is shown above the individual scatterplot ($p<0.001$ for p53T vs D2p53T).
(B) The majority of cells from both untreated (UT) and treated (T) Trp53$^{-/-}$ (p53) and untreated *Fancd2*$^{-/-}$/*Trp53*$^{-/-}$ (D2p53) contain a near diploid (2N) or tetraploid (4N) number of chromosomes. However, the majority of cells from treated (T) *Fancd2*$^{-/-}$/*Trp53*$^{-/-}$ cells have a total number of chromosomes that reflects marked aneuploidy.

3.5. **Discussion**

Useful model of spontaneous adenocarcinoma

Here we describe an improved model of epithelial cancer in FA mice. *Fancd2*$^{-/-}$*/Trp53*$^{+/-}$ mice provide a useful model of solid tumor formation in FA patients. All FA patients are at an increased lifetime risk of developing a variety of solid tumors (Alter 2003; Alter et al. 2003). Understanding this risk will become increasingly important as treatments for the hematological complications of FA improve and more patients survive bone marrow failure. While the tumor spectrum in *Fancd2*$^{-/-}$*/Trp53*$^{+/-}$ mice is not identical to that observed in human patients, this is the first animal model of FA in which a significant fraction of the tumors observed originate in epithelial tissues. Thus, *Fancd2*$^{-/-}$*/Trp53*$^{-/+}$ mice are a useful model for testing the efficacy of potential chemoprevention regimens.

The difference in incidence of adenocarcinoma, particularly mammary adenocarcinoma, between female and male *Fancd2*$^{-/-}$*/Trp53*$^{+/-}$ mice is particularly striking and suggests testable hypotheses for future experiments. Although the most obvious hypothesis involves the larger population of target cells in female versus male mice, other potential explanations exist. Perhaps androgens are protective and prevent the development of adenocarcinoma. If this is correct, castrated male *Fancd2*$^{-/-}$*/Trp53*$^{+/-}$ mice may develop mammary adenocarcinoma at an increased rate. Alternatively, estrogen may promote the development of tumors in the female *Fancd2*$^{-/-}$*/Trp53*$^{+/-}$ mice. If female hormones are tumor promoting factors, male *Fancd2*$^{-/-}$*/Trp53*$^{+/-}$ mice given estrogen should develop tumors at a similar rate as female *Fancd2*$^{-/-}$*/Trp53*$^{+/-}$ mice. Investigating the cause of the different incidence between male and female *Fancd2*$^{-/-}$*/Trp53*$^{+/-}$ mice will

be particularly important given that androgen therapy is often used in the treatment of anemia associated with FA (Frohnmayer 2000)

Many of the pleiotropic phenotypes we have previously described in *Fancd2* mutant mice, including small size, microphthalmia, and reduced germ cell number, may be attributed to reduced function of stem cells that give rise to the affected tissues (Houghtaling et al. 2003). It has been proposed that increased turnover of HSCs in FA patients results in the shortened telomeres observed in peripheral blood mononuclear cells and correlates with the onset of aplastic anemia (Ball et al. 1998; Leteurtre et al. 1999; Hanson et al. 2001). Other investigators have demonstrated increased breakage at telomeric repeats and an increased frequency of chromosome end fusions in FA lymphocytes (Callen et al. 2002). Thus, it remains possible that increased telomere shortening and/or breakage predispose FA patients to myeloid malignancies. The existence of mammary stem cells has been demonstrated by serial transplantation studies in mice (Deome et al. 1959; Daniel et al. 1968) and deregulation of normal self renewal in mammary stem cells has been proposed as a mechanism by which mammary adenocarcinoma forms (Dontu et al. 2003). Thus, it is reasonable to consider that loss of the FA pathway may increase genomic instability in mammary stem cells and predispose to mammary adenocarcinoma. This possibility could be further investigated in the future by serially passaging mammary stem cells from *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ females and controls. Mammary progenitor cells from *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ females may form mammary tumors at an increased frequency compared to controls when transferred to recipient animals. Alternatively, mammary tumors from *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice could be analyzed for the presence of particular cell surface markers such as Sca-1, associated with mammary stem

cells to further substantiate this theory (Welm et al. 2003).

Finally, the tumor spectrum of *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice is markedly different from that of *Fancc*$^{-/-}$/*Trp53*$^{+/-}$ mice (Freie et al. 2003). While approximately a third of *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice developed adenocarcinoma, *Fancc*$^{-/-}$/*Trp53*$^{+/-}$ mice do not develop adenocarcinoma and only 1 ovarian tumor was observed in a population of 22 animals (the representation of males and females was not indicated). This difference in phenotype is important for at least two reasons. First, it demonstrates that a null mutation in *Fancd2* causes a more severe phenotype in mice than a null mutation in *Fancc*. This observation supports a model in which both isoforms of Fancd2 (Fancd2-S and Fancd2-L) perform qualitatively similar functions but that complete loss of both isoforms, as observed in *Fancd2* null mice, is required to reach a critical threshold at which certain phenotypes, such as mammary adenocarcinoma, are observed. Second, it suggests that FA patients, with mutations in genes proposed to function downstream in the FA pathway may be at an increased risk of developing solid tumors. Increased surveillance for solid tumors may be warranted for patients from complementation group D2 as has been proposed for FA patients from group D1 (Hirsch et al. 2004).

Trp53 is required for S phase arrest following DNA damage

We have generated primary MEFs deficient in either *Fancd2*, *Trp53*, or both genes, and controls and shown that *Trp53* is required for the cell cycle arrest following induction of ICLs. *Trp53*$^{-/-}$ and double mutant cells continued replicating DNA following induction of ICLs whereas *Fancd2* and control cells were capable of arresting their cell cycle following a dose of 0.2 ng/ml HMT+UVA. It is reasonable to assume that cells

lacking the appropriate checkpoints to halt cycling may compound the unrepaired DNA damage by dividing before repair has occurred. Cytogenetic analysis of treated double mutant MEFs, revealed many gross chromosomal rearrangements, including deletions and translocations as well as marked aneuploidy. It is likely that loss of the FA pathway leads to unrepaired lesions and an inability to arrest and successfully repair contributes to the genomic instability leading to adenocarcinoma observed in *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice.

3.6. **Acknowledgements**

Laura Granville, M.D. and **Milton Finegold, M.D.** (Department of Pathology, Texas Children's Hospital, Baylor College of Medicine, Houston, Texas) performed the histology on tumor sections shown in figure 15. **Yumi Torimaru** and **Yassmine Akkari, Ph.D.** performed the cytogenetics studies (figure 18) in the cytogenetics laboratory directed by **Susan Olson, Ph.D.** (Department of Molecular and Medical Genetics, OHSU).

Chapter Four: Fancd2 in DNA Double Strand Break Repair

4.1 **Abstract**

FA is a multigenic recessive disease resulting in bone marrow failure and increased cancer susceptibility. Cells from FA patients and mouse models are sensitive to ICLs and FA mice are moderately sensitive to IR. Both kinds of damage induce DNA DSBs. To date 9 genes in 11 complementation groups have been identified, however, the precise function of the FA pathway remains unclear. Many of the proteins form a nuclear complex necessary for the mono-ubiquitination of the downstream protein, Fancd2. To further investigate the role of the FA pathway in repair of DSBs, we generated *Fancd2*$^{-/-}$/*Prkdc*$^{sc/sc}$ double mutant mice. *Prkdc*$^{sc/sc}$ mutant mice have a defect in NHEJ and are sensitive to IR induced DNA damage. Double mutant animals and primary cells were more sensitive to IR than either single mutant suggesting that Fancd2 operates in DSB repair pathway distinct from NHEJ. *Fancd2*$^{-/-}$/*Prkdc*$^{sc/sc}$ double mutant cells were also more sensitive to DSBs generated by a restriction endonuclease. The role of Fancd2 in DSB repair may account for the moderate sensitivity of FA cells to irradiation as well as FA cells sensitivity to ICLs that are repaired via a DSB intermediate.

4.2 **Introduction**

FA is a rare autosomal and X-linked recessive disease resulting in birth defects, bone marrow failure and cancer susceptibility (D'Andrea and Grompe 2003). Cells from patients and FA mouse models share a common phenotype of chromosomal instability, particularly following treatment with agents that induce ICLs (Sasaki and Tonomura 1973). At least 11 complementation groups exist, FA-A, B, C, D1, D2, E, F, G, I, J, and L (Levitus et al. 2004) and 9 causative genes have been identified including *FANCA, FANCB, FANCC, FANCD1/BRCA2, D2, E, F, G/XRCC9,* and *L/PHF9* (Strathdee et al. 1992; Lo Ten Foe et al. 1996; de Winter et al. 1998; de Winter et al. 2000a; de Winter et al. 2000b; Timmers et al. 2001; Howlett et al. 2002; Meetei et al. 2003a; Meetei et al. 2004a). Many of the FANC proteins interact to form a multisubunit nuclear complex required for the monoubiquitination of FANCD2 at lysine 561 that occurs following DNA damage and during the S phase of the cell cycle (Garcia-Higuera et al. 2001; Taniguchi et al. 2002a).

FANC proteins have been suggested to have a role in the repair of DSBs based on reports that FA mice have a modestly increased sensitivity to IR (Haneline et al. 1999; Noll et al. 2001; Yang et al. 2001; Houghtaling et al. 2003) and plasmid based studies which demonstrate an impaired processing of broken DNA ends in FA cells (Escarceller et al. 1997; Lundberg et al. 2001; Donahue and Campbell 2002). DSBs are particularly toxic DNA lesions for which multiple repair systems have evolved. Two major pathways exist in eukaryotic cells, homology directed repair (HDR) and non-homologous end joining (NHEJ) (Valerie and Povirk 2003; Pfeiffer et al. 2004). HDR is the major pathway for repair of DSBs in yeast while NHEJ is the preferred pathway in mammalian

cells (Liang et al. 1998; Haber 2000). HDR subclasses include single-strand annealing (SSA) and short-tract gene conversion (STGC), both of which have specific protein requirements (Ivanov et al. 1996; Karran 2000; Thompson and Schild 2001; West 2003). NHEJ also requires a distinct set of proteins including the main components, XRCC4/DNA ligase IV and DNA-PK, comprised of the DNA-PK catalytic subunit (DNA-PK) and the Ku70/80 heterodimer (Valerie and Povirk 2003). The requirement for DNA-PK in NHEJ is supported by the phenotype of severe combined immune deficient (scid) mice, which have reduced DNA-PK activity due to a nonsense mutation in *Prkdc* (Blunt et al. 1996). The NHEJ defect in these mice contributes to impaired V-D-J immunoglobulin recombination and increased sensitivity to IR induced DSBs (Blunt et al. 1995).

The FA field has slowly uncovered clues that suggest a specific role for FANCD2 in repair of DSBs by a homology dependent mechanism that is distinct from NHEJ. First, the end joining activity deficient in FA extracts is not blocked by wortmannin in normal extracts indicating that it is biochemically distinct from NHEJ (Lundberg et al. 2001). Second, FANCD2 is directed to nuclear foci containing the central recombination protein, RAD51 (Taniguchi et al. 2002a). The functional significance of this finding remains unclear, as vertebrate cells lacking FANCD2 have normal levels of RAD51 foci formation (Godthelp et al. 2002; Houghtaling et al. 2003; Ohashi et al. 2005; Yamamoto et al. 2005). Third, FANCD2 was shown to interact directly with FANCD1/BRCA2, which binds to and modulates the activity of RAD51 (Hussain et al. 2004). Fourth, recent experiments using chromosomally integrated reporter constructs have demonstrated a role for both human and chicken FANCD2 in HDR of DSBs (Nakanishi et al. 2005;

Ohashi et al. 2005; Yamamoto et al. 2005). Finally, FANCD2 has been shown to bind Holliday junctions and double strand DNA substrates *in vitro* (Park et al. 2005).

In this study we undertook a genetic approach to examine the epistatic relationship between *Fancd2* and *Prkdc*. We show that Fancd2 operates in a DSB repair pathway that is distinct from *Prkdc*, thus *Fancd2* and *Prkdc* are non-epistatic for DSB repair. We also show that NHEJ plays little to no role in the repair of ICLs. Finally, we show that $Fancd2^{-/-}/Prkdc^{sc/sc}$ double mutant cells are more sensitive to DSBs generated by a restriction endonuclease than *Fancd2* complemented control cells demonstrating that Fancd2 functions specifically in the repair DSBs rather than other types of DNA damage generated by irradiation.

4.3 **Materials and Methods**

Animal husbandry

Fancd2$^{+/-}$ on a C57/Bl6J background (Houghtaling et al. 2003) were crossed to *Prkdc*$^{sc/sc}$ (B6.CB17-*Prkdc*^{sc}/SzJ) purchased from The Jackson Laboratory (Bar Harbor, Maine) to generate *Fancd2*$^{+/-}$/*Prkdc*$^{+/sc}$ breeders. Genotyping was performed as previously described for *Fancd2* mice (Houghtaling et al. 2003) and *Prkdc*^{sc} mice (Blunt et al. 1996; Araki et al. 1997). *Fancd2*$^{+/-}$/*Prkdc*$^{+/sc}$ breeders were intercrossed to generate *Fancd2*$^{+/-}$/*Prkdc*$^{sc/sc}$ breeders that were crossed to generate *Fancd2*$^{-/-}$/*Prkdc*$^{sc/sc}$ and *Prkdc*$^{sc/sc}$ animals. Animals were maintained according to an approved IACUC protocol in the Department of Animal Care at Oregon Health & Science University.

Cell line generation

Primary mouse ear fibroblasts (MEFs) were prepared from 4 week old mice by digestion of tissue in collagenase/dispase (Sigma, St. Louis, MO) for one hour and collagenase (Sigma, St. Louis, MO) for one hour at 37 C. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT) supplemented with 15% fetal bovine serum (FBS) (HyClone, Logan, UT) and 1x penicillin/streptomycin (P/S) (Invitrogen, Carlsbad, CA). Cells were immortalized by transfection with a plasmid expressing SV40 T antigen and passaged (Wood et al. 1987). Immortalized cell lines were grown in DMEM supplemented with 10% bovine calf serum (BCS) (HyClone, Logan, UT) and 1x P/S. Immortalized cells were complemented by infecting with pMMP-puro-*mFancd2* retrovirus or pMMP-puro-empty retrovirus and selecting in 3.0 ug/mL puromycin as previously described (Ory et al. 1996; Naf et al. 1998; Garcia-Higuera et al. 1999).

DNA damage assays

Primary (passage 2) and immortalized cells were seeded in quadruplicate at a density of 500 cells/well in 96-well plates. Cells were treated with increasing doses of MMC (Sigma, St. Louis, MO) or HMT+UVA (Sigma, St. Louis, MO), as previously described (Akkari et al. 2000). IR was administered at a dose of 142.5 rad/min from a ^{137}Cs source. Following treatment, cells were allowed to grow at 37°C/5% CO2 for 5 days. Plates were frozen at –80°C. CyQuant (Molecular Probes, Eugene, OR) was used to measure total DNA content as an indication of cell number as previously described (Houghtaling et al. 2003).

Whole body irradiation

Fancd2$^{-/-}$*/Prkdc*$^{sc/sc}$ and *Prkdc*$^{sc/sc}$ littermate controls were irradiated with 420 cGy at a dose of 142.5 cGy/min from a ^{137}Cs source at between 6 and 8 weeks of age. Following irradiation animals were monitored daily, weighed and sacrificed if they appeared moribund. Survival curves were generated using Prism software (GraphPad Software, Inc., www.graphpad.com). Statistical significance between genotypes was determined using built-in analysis for survival curves consisting of a log rank test yielding a p value.

Restriction endonuclease electroporation

10^4 MEFs of the indicated genotypes were resuspended in 200 uL serum free DMEM and combined with 5, 10 or 20 units (50uL) of *PvuII* (Roche, Indianapolis, IN) diluted in the appropriate 1x buffer/PBS. *PvuII* was heat inactivated after dilution in appropriate 1x

buffer/PBS for 18 hours at 80 C. The mixture was electroporated in a 0.4 cm cuvette using a BioRad Gene Pulser (BioRad, Hercules, CA) at a field strength of 0.75 kV per cm and 960 microfarads. Following electroporation, 1000 cells were plated on 100mm plate in duplicate in DMEM + 10% FBS. Cells were grown for 10 days and colonies were stained with a 1% methylene blue solution (6.25% EtOH). The number of colonies from electroporation with 5, 10, or 20 units of enzyme was compared to the number of colonies from electroporation with 0 units of enzyme.

Green fluorescent protein electroporation/FACS

20 ug of recombinant GFP (BD Biosciences Clontech, Mountain View, CA) was electroporated into 2x10e6 MEFs using the conditions described above. Cells were washed in DMEM and resuspended in PBS containing 10 ug/mL propidium iodide. Analysis of mock electroporated and rGFP electroporated MEFs was performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

4.4 **Results**

Fancd2 operates in a DSB repair pathway that is distinct from Prkdc-dependent NHEJ

We have previously described a role for Fancd2 in repair of ICLs however fibroblasts from Fancd2 knockout mice were not differentially sensitive to IR (Houghtaling et al. 2003). In addition, *Fancd2*$^{-/-}$mice were only moderately more sensitive than littermate controls to whole body irradiation (Houghtaling et al. 2003). To investigate the relationship between the FA pathway and NHEJ we crossed *Fancd2* mutant mice to *Prkdc*$^{sc/sc}$ (scid) mice. *Prkdc*$^{sc/sc}$ mice are radiosensitive due to reduced activity of the catalytic subunit of Dna-pk, a central component of the NHEJ repair pathway (Blunt et al. 1995; Pastwa and Blasiak 2003). We predicted that if Fancd2 functions in a repair pathway that is separate from NHEJ, double mutant animals may be more sensitive to IR than *Prkdc*$^{sc/sc}$ mutants. To investigate this possibility double mutant *Fancd2*$^{-/-}$/*Prkdc*$^{sc/sc}$ mice and controls on a C57/Bl6J background were generated.

Primary ear fibroblasts prepared from *Fancd2*$^{-/-}$/*Prkdc*$^{sc/sc}$ mice and controls, were subjected to increasing doses of the HMT+UVA in a cell growth assay (figure 19A). As expected for cells lacking an intact FA pathway, both *Fancd2*$^{-/-}$ and *Fancd2*$^{-/-}$/*Prkdc*$^{sc/sc}$ MEFs were highly sensitive to HMT+UVA induced ICLs. *Prkdc*$^{sc/sc}$ cells were no more sensitive than wild-type controls and double mutant cells were no more sensitive than *Fancd2*$^{-/-}$ cells. Taken together, these results indicate that NHEJ plays little or no role in the repair of HMT+UVA induced ICLs in primary mouse fibroblasts.

The same cells were also exposed to increasing doses of IR in a similar cell growth assay. In agreement with previous results comparing the sensitivity of *Fancd2* mutant MEFs and wild-type controls on a 129S4 background, *Fancd2* mutant MEFs on

the C57/Bl6J background were no more sensitive to IR than wild-type controls (figure 19B) (Houghtaling et al. 2003). As expected for cells with impaired NHEJ, *Prkdc*$^{sc/sc}$ cells were more sensitive to IR than *Fancd2*$^{-/-}$ or wild-type controls. Interestingly, *Fancd2*$^{-/-}$ */Prkdc*$^{sc/sc}$ fibroblasts were clearly more sensitive to DNA damage from IR than *Prkdc*$^{sc/sc}$ cells.

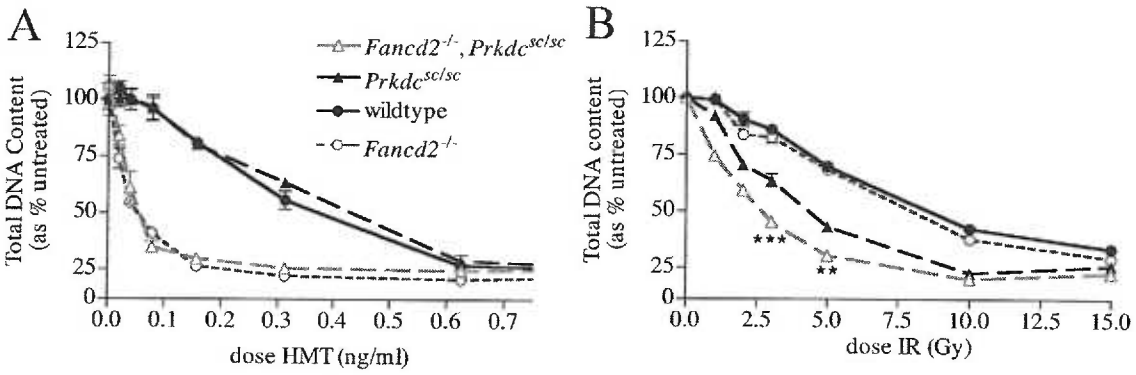

Figure 19. DNA damage sensitivity of *Fancd2*$^{-/-}$*/Prkdc*$^{sc/sc}$ cells and controls. Primary MEFs of the indicated genotypes were plated in quadruplicated and treated with increasing doses of HMT+UVA (A) or IR (B). Total DNA content was measured as an indication of cell growth. Double mutant cells were more sensitive to IR than *Prkdc*$^{sc/sc}$ MEFs. Both double mutant and *Fancd2*$^{-/-}$ MEFs were equally sensitive to HMT+UVA. These experiment were repeated three times with identical results. At 3.0 Gy dose, p=.001 (***) for *Fancd2*$^{-/-}$*/Prkdc*$^{sc/sc}$ vs *Prkdc*$^{sc/sc}$. At 5.0 Gy dose, p<.005 (**) for *Fancd2*$^{-/-}$*/Prkdc*$^{sc/sc}$ vs *Prkdc*$^{sc/sc}$.

To further investigate the role of Fancd2 in the response to IR we next exposed 6-8 week old *Fancd2*$^{-/-}$*/Prkdc*$^{sc/sc}$ mice and sex matched *Prkdc*$^{sc/sc}$ littermate controls to 4.2 Gy whole body irradiation (figure 20). We have previously described a modest *in vivo* radiosensitivity phenotype in *Fancd2* mutant mice in the mixed 129S4;C57/Bl6J background in which the LD_{50} for *Fancd2* mutant mice (LD_{50}= 9.5 Gy) was reduced compared to controls (LD_{50}=11Gy) (Houghtaling et al. 2003). *Fancd2*$^{-/-}$*/Prkdc*$^{sc/sc}$ mice on a C57/Bl6J background had a markedly reduced survival following irradiation compared to *Prkdc*$^{sc/sc}$ controls. Whereas only 4 of 17 *Prkdc*$^{sc/sc}$ controls died following 4.2 Gy of

whole body irradiation, 3 of 3 *Fancd2*$^{-/-}$*Prkdc*$^{sc/sc}$ mice died within 10 days after receiving the same dose. This finding, together with the *in vitro* cell growth assays, establishes the important role for Fancd2 in the response to IR induced DNA damage and demonstrates that Fancd2 functions in a repair pathway that is distinct from NHEJ.

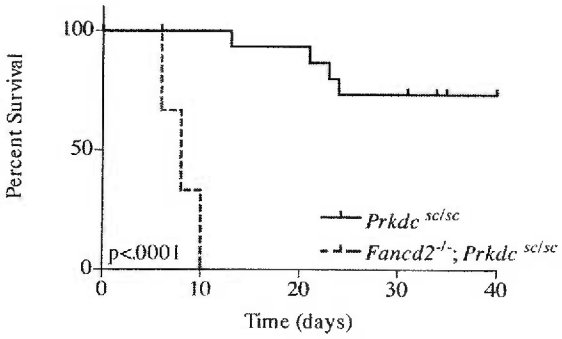

Figure 20. *In vivo* IR sensitivity of *Fancd2*$^{-/-}$*/Prkdc*$^{sc/sc}$ mice. *Fancd2*$^{-/-}$*/Prkdc*$^{sc/sc}$ and littermate control *Prkdc*$^{sc/sc}$ mice were irradiated with 4.2 Gy of whole body irradiation. Double mutant mice were significantly more sensitive than *Prkdc*$^{sc/sc}$ controls.

Fancd2 functions in repair of restriction endonuclease induced DSBs

The hypersensitivity of double mutant MEFs to IR suggests that Fancd2 functions in repair of IR induced DNA damage. Because IR induces various types of DNA damage we sought to test whether Fancd2 functions specifically in repair of IR induced chromosomal DSBs. We measured the colony forming ability of SV40 immortalized *Fancd2*$^{-/-}$*/Prkdc*$^{sc/sc}$ MEFs and *Fancd2*$^{-/-}$*/Prkdc*$^{sc/sc}$ MEFs retrovirally corrected with *Fancd2* cDNA following electroporation of the blunt cutting restriction endonuclease, *PvuII*. Complemented cells were no longer sensitive to MMC induced ICLs as measured by a cell growth assay (data not shown).

Restriction endonucleases have previously been shown to induce chromosomal DSBs resulting in reduced cell viability (Winegar et al. 1989; Giaccia et al. 1990; Costa et al. 1993; Kinashi et al. 1995; Harvey et al. 1997). Double mutant cells had a reduced colony forming ability relative to *Fancd2* corrected controls following electroporation of

5, 10, or 20 units of *PvuII* (figure 21). Heat inactivated *PvuII* resulted in a colony forming ability comparable to mock electroporated cells (data not shown). Our results are in agreement with previous reports demonstrating a role for the FA pathway in response to restriction endonuclease induced DSBs (Donahue and Campbell 2002).

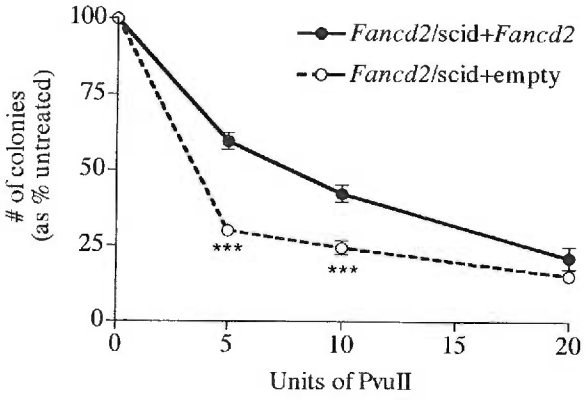

Figure 21. Fancd2 functions in repair of *PvuII* induced DSBs. Immortalized *Fancd2*$^{-/-}$/*Prkdc*$^{sc/sc}$ (open circles) and *Fancd2* corrected *Fancd2*$^{-/-}$/*Prkdc*$^{sc/sc}$ cells (closed circles) were electroporated with 0, 5, 10, or 20 units of *PvuII* and plated. Each data point represents the combined mean +/- SEM for three independent experiments performed in duplicate (*** = $p < .001$).

To control for potential variability in protein uptake between cell lines, both genotypes were electroporated with recombinant GFP and analyzed by FACS. The level of GFP positive cells relative to mock electroporated cells was approximately equal in both uncorrected double mutant cells (42.4%) and *Fancd2* corrected double mutant cells (47.8%) (figure 22). Thus, the reduced colony forming ability of double mutant cells was not due to increased uptake of restriction endonuclease but rather reduced repair of chromosomal DSBs induced by the restriction endonuclease.

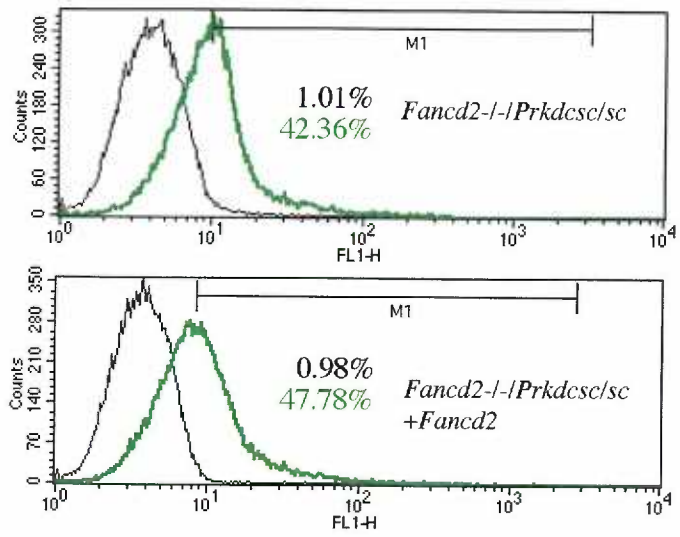

Figure 22. Electroporation of rGFP and FACS analysis. *Fancd2*$^{-/-}$*/Prkdc*$^{sc/sc}$ and *Fancd2* corrected *Fancd2*$^{-/-}$*/Prkdc*$^{sc/sc}$ take up near equal amounts of rGFP. Region M1 was normalized for each genotype such that ~1% of mock electroporated cells were scored as GFP postitve.

4.5 **Discussion**

We have demonstrated that Fancd2 functions in a pathway for the repair of IR induced DNA damage both *in vitro* and *in vivo*. Furthermore, the reduced colony forming ability of *Fancd2*$^{-/-}$/*Prkdc*$^{sc/sc}$ cells relative to *Fancd2* corrected controls following electroporation of a blunt-cutting restriction endonuclease, demonstrates that Fancd2 functions specifically in the repair of chromosomal DSBs as opposed to other types of damage induced by IR. In previous reports we were not able to demonstrate a differential sensitivity to IR between fibroblast derived from FA mutant mice and controls (Noll et al. 2001; Houghtaling et al. 2003). In addition, the *in vivo* sensitivity of *Fancd2* and *Fancc* mutant mice was only moderately increased relative to mutants, such as scid mice, that have a pronounced sensitivity to IR. The lower radiosensitivity of Fancd2 mutants compared to scid mice suggests that the FA pathway is less important in the repair of IR damage than NHEJ. This finding is consistent with previous observations that multiple DSB repair pathways exist in mammalian cells and that different species have varying dependencies on each pathway (Jeggo 1998).

Because NHEJ is the major DSB repair pathway in mammalian cells, we hypothesized that a role for the FA pathway might only be appreciable *in vivo* in the absence of a functional NHEJ repair pathway. Indeed, we observed an increased sensitivity to IR induced damage for *Fancd2*$^{-/-}$/*Prkdc*$^{sc/sc}$ mice and cells compared to *Prkdc*$^{sc/sc}$ mice and cells. A number of recent experiments using reporter based repair substrates have suggested that Fancd2 functions in homology directed repair of DSBs (Nakanishi et al. 2005; Ohashi et al. 2005; Yamamoto et al. 2005). In addition, previous reports using plasmid based studies have suggested that an end joining defect in FA

nuclear extracts is distinct from NHEJ (Lundberg et al. 2001). The experiments described in this paper are to our knowledge the first that have taken a genetic approach to demonstrate that *Fancd2* is non-epistatic with *Prkdc in vivo* for repair of DSBs in mammalian cells.

In addition to demonstrating a role for the FA pathway in repair of IR induced DSBs we have shown that NHEJ plays little to no role in the repair of HMT+UVA induced ICLs. This finding is consistent with models of ICL repair that invoke multiple repair pathways, but not NHEJ, in the processing of ICLs (Dronkert and Kanaar 2001; McHugh et al. 2001). Although the precise steps of ICL repair in mammalian cells have yet to be fully understood, genetic and molecular biological studies have implicated XPF/ERCC1, a component of the nucleotide excision repair pathway, in the incision and unhooking of ICLs. A recombination step or translesion synthesis (TLS) is then predicted to complete the repair of ICLs (McHugh et al. 2001; Niedzwiedz et al. 2004). Recent experiments from our lab have investigated the details of ICL repair and show that ICLs are incised throughout the cell cycle but processed to a DSB intermediate only during S phase (Rothfuss and Grompe 2004). Incision at ICLs is independent of FA pathway activation. However, the presence of active FANCD2 correlates with the appearance of DSBs, suggesting that FANCD2 may function in this later step of ICL repair.

The upstream FA protein, FANCC, has been suggested to act in both HDR and TLS for the repair or ICLs in chicken DT40 cells (Niedzwiedz et al. 2004). While our data cannot rule out a role for the FA pathway in TLS, the reduced colony forming efficiency in cells lacking Fancd2 following electroporation of *PvuII*, supports a role for the protein in HDR of DSBs because blunt-ended DSBs are not expected to be repaired

by TLS. Based on our demonstration that Fancd2 functions in repair of a subset of DSBs, it is intriguing to speculate that the specific defect in ICL repair observed in FA cells involves an inability of the FANCD2 to process the DSB intermediates that are generated during repair of ICLs.

In addition to variations among species in their relative use of HR versus NHEJ in the repair of DSBs, variations in the activity of each throughout the cell cycle have been documented. In chicken DT40 cells NHEJ plays a major role in G1 and early S while recombinational repair is preferentially used in late S/G2 phase (Takata et al. 1998). Activation of Fancd2 and its colocalizaton with HR proteins in nuclear foci occurs specifically in S phase of the cell cycle in wild-type cells (Taniguchi et al. 2002a). It will be interesting to compare the sensitivity of *Fancd2*$^{-/-}$*/Prkdc*$^{sc/sc}$ cells and *Fancd2* complemented double mutant controls to IR at various times during the cell cycle. One prediction is that double mutant cells irradiated during G1 phase of the cell cycle will not be more sensitive than complemented control cells. In contrast, double mutant cells may be more sensitive than complemented control cells when irradiated during S phase of the cell cycle when the FA pathway is most active.

In these studies we have used primary fibroblasts and whole animals to compare the relative radiosensitivity of *Fancd2*$^{-/-}$ and wild-type cells. While we did not observe a difference in primary cells, we did observe a difference in whole animals, suggesting that specific cell types, such as hematopoietic stem cells, may have a greater dependence on the FA pathway for repair of DSBs. It is attractive to speculate that the FA pathway may function specifically in long-lived progenitors or stem cells and these cells, when deficient in the FA pathway, may acquire the genetic mutations required to progress

towards cancer. Regardless of the precise nature of the defect in FA cells, Fancd2's role in repair of spontaneously generated DSBs may explain the genomic instability and cancer predisposition observed in human FA patients and *Fancd2* knockout mice.

4.6. **Acknowledgements**

The primary cells used in these experiments were tested by **Yassmine Akkari, Ph.D.** in the cytogenetics lab directed by **Susan Olson, Ph.D.** at Oregon Health & Science University. The *Fancd2* retrovirus used to complement *Fancd2*$^{-/-}$/*Prkdc*$^{scid/sc}$ cells was provided by **Toshi Taniguchi, M.D.** of the Dana Farber Cancer Institute. FACS analysis was performed by the expert ebay purchaser, **Craig Dorrell, Ph.D.**

Chapter 5: Summary and Conclusions

The human disease, FA, is very rare affecting approximately 3 in 1,000,000 members of the general population worldwide. The study of rare genetic disorders may lead to improved therapies for patients, or perhaps a cure. Like many investigations of rare disorders, the study of the proteins disrupted in Fanconi anemia, has led to a better understanding of their normal biology in unaffected members of the population. In the studies described in this body of work we have generated a mouse model of the disease to further investigate the function of the FA pathway.

We have created a null mutation in the downstream FA gene, *Fancd2*, and show that mice lacking a functional protein share many of the phenotypes described in human patients and previously generated FA knockout mice. These include developmental defects of the germ cells, a modest *in vivo* sensitivity to IR, and a cellular sensitivity to interstrand cross-linkers. *Fancd2* knockouts also show additional phenotypes not previously described in other FA knockouts including small body size, microphthalmia, perinatal lethality, and an increased incidence of epithelial tumors. We have improved on the model of cancer in *Fancd2* mice by crossing them to mice heterozygous for *Trp53* deficiency and show that heterozygosity for *Trp53* accelerates the epithelial tumor phenotype of female *Fancd2* mutant mice. Finally, we have investigated the epistatic relationship of *Fancd2* and *Prkdc* by generating $Fancd2^{-/-}/Prkdc^{sc/sc}$ double mutant mice. We show that while NHEJ does not participate in repair of ICLs, Fancd2 is required for a portion of DSB repair and functions in a repair pathway, both *in vitro* and *in vivo*, which is distinct from NHEJ.

Phenotype of Fancd2 *knockout mice*

Multiple knockouts of genes functioning upstream in the FA pathway have been generated including, *Fanca*, *Fancc*, *Fancg* and *Fancl* genes (Chen et al. 1996; Whitney et al. 1996; Cheng et al. 2000; Yang et al. 2001; Agoulnik et al. 2002; Koomen et al. 2002; Noll et al. 2002). These mutants have very similar phenotypes, in agreement with their interaction in a multi-protein complex necessary for the post-translational modification of Fancd2. To determine whether Fancd2 participates only in this function *in vivo* or has additional roles, we generated a strain of mice with a null allele in this gene.

Earlier studies have suggested that Fancd2 may have unique roles that are distinct from the other FA proteins. First, it acts downstream of the FA nuclear complex and is the target of the mono-ubiquitination mediated by the complex (Garcia-Higuera et al. 2001; Timmers et al. 2001). Hence, the integrity of the FA nuclear complex is not perturbed in FA-D2 cells unlike in other complementation groups (Garcia-Higuera et al. 1999; de Winter et al. 2000c). Second, FANCD2 is the only FA protein known to form nuclear foci after DNA damage and to co-localize with the repair proteins BRCA1 and RAD51 (Garcia-Higuera et al. 2001; Taniguchi et al. 2002a). As knockouts of *Brca2* and *Rad51* result in an embryonic lethal phenotype it was possible that a null mutation in *Fancd2* might also result in embryonic lethality. Finally, FANCD2 is directly phosphorylated by ATM in response to IR but not in response to ICLs (Nakanishi et al. 2002; Taniguchi et al. 2002b) and disruption of *Fancd2* might lead to phenotypes similar to *Atm* knockouts. The *Fancd2* mutant mice reported here display all the features of previously reported strains of FA knockout mice, but also have important differences.

These differences include microphthalmia, perinatal lethality, more severe hypogonadism and tumor development. Two basic models exist to explain the divergence of phenotypes between murine mutants in *Fancd2* and nuclear complex genes. First, non-ubiquitinated Fancd2 protein, as present in *Fanca*, *Fancc*, *Fancg*, and *Fancl* knockout mice, may have some residual activity in the function common to the FA pathway. In this model, knockouts of the nuclear complex genes would be similar to hypomorphic mutations of the *Fancd2* gene. The phenotypes would be the result of deficiency in the same biochemical function, but they would be more severe in *Fancd2* mutant mice. In the second model, Fancd2 is a multifunctional protein with a domain that functions in the FA pathway and other domains that mediate unrelated functions. The additional phenotypes of *Fancd2* mutants would then be due to deficiency of these additional functions.

Most of the features observed in Fancd2 null mice can be interpreted as more severe manifestations of the qualitatively similar defects seen in other FA knockouts. This is most obvious with the germ cell defects. Although testicular weight is clearly more affected in *Fancd2* mutant mice on the 129S4 background, the histology is similar to other FA models on the same background. Similarly, we have observed microphthalmia in the mutant offspring of some *Fancc* breeding pairs (M. Grompe, unpublished data). Furthermore, a *Fanca* mutant (*Fanca*^{tm1Hsc}) reported at the same time as our *Fancd2* knockout mice, had qualitatively similar developmental defects including small size, microphthalmia and craniofacial malformations (Wong et al. 2003). Similar to *Fancd2* knockouts these phenotypes were only manifest on the C57/BL6 strain but not the 129S6 strain (*Fancd2* knockouts were generated on the 129S4 background). Although our *Fancd2* knockouts had a higher incidence of microphthalmia compared to *Fanca*^{tm1Hsc}

mutants (78% vs 30.3%), it is not a truly novel phenotype but a more complete manifestation of a defect common to all FA mice (Houghtaling et al. 2003; Wong et al. 2003).

The increased susceptibility of FA cells to apoptotic cell death after DNA damage could provide a common explanation for the observed germ cell deficiency, microphthalmia, small size and perinatal lethality. In the case of these developmental defects, the cells would be responding to spontaneous DNA damage. Overall, the phenotype of *Fancd2* mutant mice does not convincingly establish the existence of additional functional domains in the Fancd2 protein.

Epithelial cancer in Brca2 *and* Fancd2 *knockout mice*

Although human patients with FA develop a variety of cancers, tumors have not been reported in the other FA knockout mice even when followed to very late ages. In contrast, it is well established that mice with a truncating mutation in *Brca2* are at an increased risk of a variety of neoplasms (Connor et al. 1997; McAllister et al. 2002). Interestingly, the tumor spectrum of the *Brca2* hypomorphic mice was comparable to that seen in *Fancd2* mutants (McAllister et al. 2002). In both cases, a predisposition towards epithelial cancers was seen and the age of onset was similar. *Brca2* hypomorphic mice also have other similarities such as an FA like germ cell defect, small size and perinatal lethality. Taken together, this extensive phenotypic overlap is consistent with the hypothesis that the carboxy-terminal domain of Brca2 functions in the same pathway as Fancd2.

Human BRCA2 is known to interact with and modulate the activity of RAD51, a central player in homologous recombination and *Brca2* mutant cells are known to have defects in error-free recombination (Moynahan et al. 2001b; Tutt et al. 2001). Therefore, the identification of *Brca2* as an FA gene supports a role for the FA pathway in HDR. This hypothesis is strengthened by the observation that chromosomes in the pachytene stage of meiosis from *Fancd2* knockout mice mispair, as described here. One potential function for the FA pathway could be to control the interaction between RAD51 and BRCA2 and thereby modulate homologous recombination events at sites of DNA damage. However, Western blot analysis of *Fancd2* mutant tissues showed normal Rad51 levels as well as normal interaction of the Brca2 and Rad51 proteins as determined by co-immunoprecipitation. Furthermore, Rad51 foci formation following DNA damage was normal in *Fancd2*$^{-/-}$ MEFs. Thus, our data do not support a simple model in which the FA pathway controls the stability/rate of Brca2/Rad51 interaction.

In humans, heterozygosity for inactivating *BRCA2* germ line mutations is associated with breast, ovarian and pancreatic cancer, all tumors of epithelial origin. Currently, it is unclear why these tissues are particularly affected, but it is interesting to note that epithelial tumors in rodents have been associated with telomere shortening (Artandi et al. 2000; Chang et al. 2001). Human FA patients are known to display significantly shortened telomeres in hematopoietic cells (Adelfalk et al. 2001; Brummendorf et al. 2001; Hanson et al. 2001) and it is therefore interesting to speculate that the FA pathway could be involved in telomere maintenance in some tissues. The high incidence of epithelial tumors observed in *Fancd2* mutants raises the issue whether FANCD2 could be an important gene in some human epithelial cancers, especially of the

breast and ovary. Importantly, some cases of familial ovarian cancer have been linked precisely to the region where *FANCD2* resides in the human genome (Sekine et al. 2001; Simsir et al. 2001; Zhang and Xu 2002).

To improve upon the model we crossed *Fancd2* mutant mice to mice heterozygous for *Trp53*. Similar to other mouse models of spontaneous cancer, heterozygosity for *Trp53* accelerated the tumor phenotype of *Fancd2* knockouts. While the tumor spectrum in *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice was not identical to that observed in human patients, it is the first animal model of FA in which a significant fraction of the tumors observed originate in epithelial tissues.

The exact reason why heterozygosity for *Trp53* accelerates the tumor phenotype of *Fancd2* knockout mice is unclear. To further investigate the relationship of Fancd2 and Trp53 we generated double mutant cells and show the Trp53 is required for an S phase checkpoint in both wild-type and *Fancd2* mutant cells. This defect could contribute to cancer development *in vivo*. Alternatively, the loss of Trp53's apoptotic response function could contribute to the growth of tumors in the mice. Cytogenetic analysis of double mutant MEFs treated to induce ICLs, revealed many GCRs, including deletions and translocations as well as marked aneuploidy. As the wild-type allele of *Trp53* is lost in all of the adenocarcinoma analyzed from *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice, it is possible that loss of the FA pathway leads to unrepaired lesions and an inability to arrest and repair these lesions. Continued cycling of cells with unrepaired DNA damage may contribute to the genomic instability leading to adenocarcinoma observed in *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice.

The tumor spectrum of *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice is markedly different from that of *Fancc*$^{-/-}$/*Trp53*$^{+/-}$ mice (Freie et al. 2003). While approximately a third of *Fancd2*$^{-/-}$

/Trp53$^{+/-}$ mice developed adenocarcinoma, *Fancc*$^{-/-}$*/Trp53*$^{+/-}$ mice do not develop adenocarcinoma and only 1 ovarian tumor was observed in a population of 22 animals (the representation of males and females was not indicated). This difference in phenotype is important for at least two reasons. First, it demonstrates that a null mutation in *Fancd2* causes a more severe phenotype in mice than a null mutation in *Fancc*. This observation is consistent with the model described above in which both isoforms of Fancd2 (Fancd2-S and Fancd2-L) perform qualitatively similar functions but that complete loss of both isoforms, as observed in *Fancd2* null mice, is required to reach a critical threshold at which certain phenotypes, such as mammary adenocarcinoma, are observed. Again, this model is distinct from a second, in which the increased severity of the phenotypes is attributed to multiple domains of Fancd2 operating in distinct pathways. Second, the presence of tumors in *Fancd2* mutant mice but not *Fancc* mice, suggests that FA patients with mutations in genes proposed to function downstream in the FA pathway may be at an increased risk of developing solid tumors. Increased surveillance for solid tumors may be warranted for patients from complementation group D2 and J as has been proposed for FA patients from group D1 (Hirsch et al. 2004).

Fancd2 in DNA repair

While many functions have been hypothesized for the FA pathway, the most agreed upon is a role in DNA repair (D'Andrea and Grompe 2003). Cells from FA patients and mouse models share a common sensitivity to DNA cross-linkers and a less pronounced sensitivity to IR induced DSBs. We have taken a genetic approach to investigate the relationship between Fancd2 and Dna-pk, a central component of NHEJ.

We have shown that Fancd2 functions in the repair of IR induced DNA damage both *in vitro* and *in vivo*.

In previous reports we were not able to demonstrate a differential sensitivity to IR between fibroblast derived from FA mutant mice and controls (Noll et al. 2001; Houghtaling et al. 2003). In addition, the *in vivo* sensitivity of *Fancd2* and *Fancc* mutant mice was only moderately increased relative to mutants, such as scid mice, that have a pronounced sensitivity to IR. The difference in radiosensitivity between *Fancd2* mutant and scid mice suggests that the FA pathway does not play a major role in the repair of IR damage. This finding is consistent with observations that multiple DSB repair pathways exist in mammalian cells and that different species have varying dependencies on each pathway (Jeggo 1998).

A number of recent experiments using reporter based repair substrates have suggested that Fancd2 functions in HDR of DSBs (Nakanishi et al. 2005; Ohashi et al. 2005; Yamamoto et al. 2005). In addition, previous reports using plasmid based studies have suggested that an end joining defect in FA nuclear extracts is distinct from NHEJ (Lundberg et al. 2001). The experiments described herein are to our knowledge the first that have taken a genetic approach in mammals to demonstrate that *Fancd2* is non-epistatic with *Prkdc in vivo* for repair of DSBs.

In addition to demonstrating a role for the FA pathway in the cellular response to IR induced DSBs, we have shown that NHEJ plays little to no role in the repair of HMT+UVA induced ICLs. This finding is consistent with models of ICL repair that invoke multiple repair pathways, but not NHEJ, in the processing of ICLs (Dronkert and Kanaar 2001; McHugh et al. 2001). Although the precise steps of ICL repair in

mammalian cells have yet to be fully understood, biochemical and molecular studies have implicated XPF/ERCC1, a component of the nucleotide excision repair pathway, in the incision and unhooking of ICLs. Recombination steps or translesion synthesis are then predicted to complete the repair of ICLs (McHugh et al. 2001; Niedzwiedz et al. 2004).

Recent experiments from our lab have investigated the details of ICL repair and show that ICLs are incised throughout the cell cycle but processed to a DSB intermediate only during S phase (Figure 23) (Rothfuss and Grompe 2004). Incision at ICLs is independent of FA pathway activation, however the presence of active FANCD2 correlates with the appearance of DSBs, suggesting that FANCD2 may function in this later step of ICL repair. Based on our demonstration that Fancd2 functions in repair of DSBs, it is intriguing to speculate that the specific defect in ICL repair may involve an inability of the FA pathway to process the DSB intermediates that are generated during repair of ICLs.

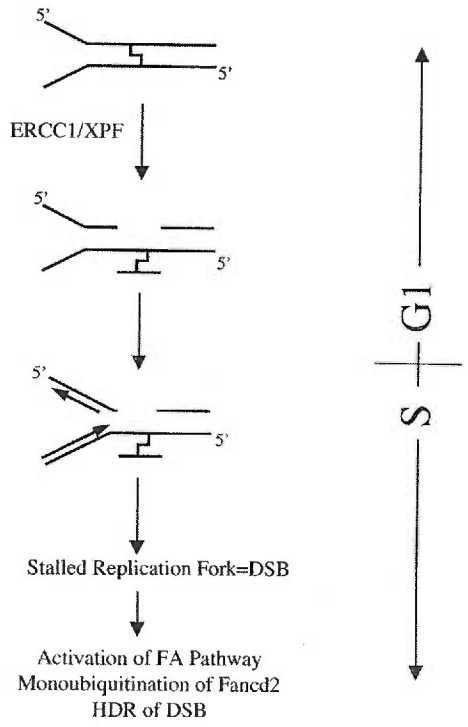

Figure 23. Model for repair of ICLs. ERCC1/XPF unhooks ICLs throughout the cell cycle. During S phase, a replication fork encounters the gap and generates a DSB. The presence of DSBs correlates with the monoubiquitination of Fancd2 and activation of the FA pathway. Fancd2 may function with other proteins to repair the DSB intermediated by HDR (modified from (Rothfuss and Grompe 2004).

The phenotype of human FA patients and mouse models of the disease indicates that the FA pathway is important for normal embryonic development, the maintenance of genomic stability, and the preservation of several types of stem cells (D'Andrea and Grompe 2003). The FA pathway may function specifically in long-lived progenitors or stem cells. These cells, when deficient in the FA pathway, may acquire the genetic mutations required to progress towards cancer. Regardless of the precise nature of the defect in FA cells, Fancd2's role in repair of spontaneously generated DSBs may explain the genomic instability and cancer predisposition observed in human FA patients and *Fancd2* knockout mice.

Future Directions

The generation and phenotypic characterization of *Fancd2* knockout mice has led to multiple insights into the role Fancd2 in developmental processes and tumor prevention. Double mutant $Fancd2^{-/-}/Prkdc^{sc/sc}$ mice have facilitated investigations into the specific role of the FA pathway in DNA repair. Our findings, particularly those demonstrating that Fancd2 functions in prevention of a variety of epithelial tumors in the mouse, have led us to imagine many future experiments.

Fancd2 mutants, like $Fanca^{tm1Hsc}$ mutant mice, display microphthalmia and small size on the C57/BL6 background but not the 129S4 or 129S6 background. Furthermore, the germ cell defect is more pronounced on the C57/BL6 background for both *Fancd2* and *Fancc* mutant mice (Markus Grompe, personal communication). In contrast, while a variety of tumors were observed in *Fancd2* mutants on the mixed or 129S4 background, none were seen on the C57/BL6 background. The presence of some phenotypes on the

C57/BL6 background that are absent on the 129S4 background suggests that a locus in either strain is capable of modifying the penetrance of these phenotypes. Mapping this modifer gene may provide insight into the phenotypes observed in FA knockout mice.

The difference in incidence of adenocarcinoma, particularly mammary adenocarcinoma, between female and male *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice is particularly striking and suggests testable hypotheses for future experiments. Although the most obvious hypothesis involves the larger population of target cells in female versus male mice, other potential explanations exist. Perhaps androgens are protective and prevent the development of adenocarcinoma. If this is correct, castrated male *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice may develop mammary adenocarcinoma at an increased rate. Alternatively, estrogen may promote the development of tumors in the female *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice. If female hormones are tumor promoting factors, male *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice given estrogen should develop tumors at a similar rate as female *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice. Investigating the cause of the different incidence between male and female *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$mice will be particularly important given that androgen therapy is often used in the treatment of anemia associated with FA (Frohnmayer 2000)

The high incidence of epithelial tumors in *Fancd2*$^{-/-}$/*Trp53*$^{-/+}$ female mice will also allow for testing the efficacy of potential chemoprevention regimens. We have begun investigations examining whether the nitroxide antioxidant and superoxide dismutase (SOD) mimetic, tempol, prevents or delays adenocarcinoma in a cohort of *Fancd2*$^{-/-}$ /*Trp53*$^{+/-}$ female mice. Tempol has previously been shown to increase the tumor-free surivival of both *Atm* mutant mice and C3H mice (Mitchell et al. 2003; Schubert et al. 2004).

Many of the pleiotropic phenotypes we have previously described in *Fancd2* mutant mice, including small size, microphthalmia, and reduced germ cell number, may be attributed to reduced function of stem cells that give rise to the affected tissues (Houghtaling et al. 2003). The existence of mammary stem cells has been demonstrated by serial transplantation studies in mice (Deome et al. 1959; Daniel et al. 1968) and deregulation of normal self renewal in mammary stem cells has been proposed as a mechanism by which mammary adenocarcinoma forms (Dontu et al. 2003). Thus, loss of the FA pathway may increase genomic instability in mammary stem cells and predispose to mammary adenocarcinoma. This possibility could be further investigated by serially passaging mammary stem cells from *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ females and controls. Mammary progenitor cells from *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ females may form mammary tumors at an increased frequency compared to controls when transferred to recipient animals. Alternatively, mammary tumors from *Fancd2*$^{-/-}$/*Trp53*$^{+/-}$ mice could be analyzed for the presence of particular cell surface markers such as Sca-1, associated with mammary stem cells (Welm et al. 2003).

My thesis work has demonstrated a role for Fancd2 in DSB repair and shown that Fancd2 participates in a DSB response pathway that is distinct from NHEJ, one major pathway for repair of DSBs. There is evidence for varying dependence on HDR and NHEJ for DSB repair between species (Jeggo 1998). In addition to variations among species in their relative use of HR versus NHEJ, variations in the activity of each throughout the cell cycle have been documented. In chicken DT40 cells NHEJ plays a major role in G1 and early S while recombinational repair is preferentially used in late S/G2 phase (Takata et al. 1998). Activation of Fancd2 and its colocalizaton with HR

proteins in nuclear foci occurs specifically in S phase in wild-type cells suggesting that the FA pathway may function during this portion of the cell cycle (Taniguchi et al. 2002a). It will be interesting to compare the sensitivity of *Fancd2*$^{-/-}$/*Prkdc*$^{sc/sc}$ cells and *Fancd2* complemented double mutant controls to IR at various times during the cell cycle. One prediction is that double mutant cells irradiated during G1 phase will not be more sensitive than complemented control cells. In contrast, double mutant cells may be more sensitive than complemented control cells when irradiated during S phase of the cell cycle when the FA pathway is most active. The use of mouse models to investigate FA has yielded many insights to the function of FA pathway. Continued efforts aimed at gene identification, biochemical investigations of FA protein function and preclinal therapy studies using mouse models will shed more light on the function of these proteins and may lead to improved therapy for FA patients.

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