NON-REDUNDANT FUNCTIONS OF THE MOUSE SSRP1 AND PFDN1

GENES REVEALED BY GENE TARGETING

AND GENE ENTRAPMENT

By

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To My Parents

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CHAPTER I

INTRODUCTION

Since the onset of modern genetics, mouse (*Mus musculus*) has provided a model for understanding mammalian gene functions and human diseases. The development, physiology and diseases of mice most resemble those of human beings and 99% of mouse genes have human orthologs (Bedell, Jenkins et al. 1997; Cox and Brown 2003). Classical genetic studies, so called "forward genetics", start with an inheritable trait and then characterize the mutation(s) responsible for the trait. Mouse embryonic stem cell technology enables "reverse genetics" in which gene functions are studied first by creating a mutation in a specific gene and then determining the phenotypes of mice bearing the mutation (Thompson, Clarke et al. 1989). Mutations may be induced in stem cells by homologous recombination that targets specific gene sequences (Doetschman, Gregg et al. 1987) or by gene entrapment that generates large collections of randomly mutagenized stem cell clones from which clones with mutations in specific genes of interest are selected for transmission into the mouse germline (von Melchner and Ruley 1989; Skarnes, Moss et al. 1995; Hicks, Shi et al. 1997). Mutant mouse models generated by gene targeting and gene entrapment have been used to delineate *in vivo* functions of large numbers of genes. The sequencing of the human and mouse genomes has identified most of the 25,000 or so genes required to specify mammalian organisms and has marked the beginning of the post-genome era of biomedical research (Lander, Linton et al. 2001;

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Venter, Adams et al. 2001; Waterston, Lindblad-Toh et al. 2002). Efficient mutagenesis strategies are required for functional studies of the many genes now characterized at the nucleotide level. My thesis will describe studies on deficient mouse models of the *Ssrp1* and *Pfdn1* genes, which were generated by conventional gene targeting and gene entrapment, respectively. This introduction will provide a brief review of major mutagenesis strategies and provide background and rationale for my thesis research.

Functional genomics in the post-genome era

A draft sequence of the human genome was completed in 2001 (Lander, Linton et al. 2001; Venter, Adams et al. 2001), while the mouse genome was sequenced in 2002 (Waterston, Lindblad-Toh et al. 2002). Now functional analysis of these 25,000 or so genes in the human and mouse genomes has become the major challenge. The Comprehensive Knockout Mouse Project Consortium was founded based on the premise that knockout mouse models should be made in a large scale, eventually covering all mouse genes. However, published knockouts correspond to only ~15% of the mouse genes (Austin, Battey et al. 2004). Therefore, generating knockouts for the remaining 85% of the genes necessitates efficient means for large-scale mutagenesis.

The following sections will briefly describe various mutagenesis strategies and their potential application in comprehensive analysis of mammalian gene functions.

Chemical mutagenesis

Chemical mutagens induce mutations at frequencies of $1.3 \times 10^{-5} \sim 1.5 \times 10^{-3}$ per locus and produce relatively specific spectrums of DNA sequence alterations. For example, ethyl-nitrosourea (ENU), an alkylating agent, a carcinogen and a super-mutagen (Russell and Montgomery 1982), induces single nucleotide transversions and transitions in the spermatogonial lineage of treated males. Through crosses and backcrosses of a treated male with wild type mice, dominant phenotypes and recessive phenotypes were examined among the immediate or later offspring, respectively (Bode 1988; Balling 2001). ENU mutagenesis screening of mutations on certain regions could take advantage of balancer chromosomes (Rinchik 1991). A balancer chromosome contains an inverted region that suppresses crossing over during mitotic recombination, a dominant genetic marker such as a coat color gene, and a recessive genetic marker that contributes to the death of mice homozygous for the balancer. Mice containing a balancer chromosome and an ENU-induced mutation on the corresponding region of a wild type chromosome could be selected by the coat color from offspring of the ENU-treated stud.

As a forward genetics approach, ENU mutagenesis allows large-scale screening for phenotypically important mutations. Moreover, different ENU-induced mutations in the same gene could lead to differing phenotypes, providing tools for detailed study of gene functions (Balling 2001). However, the mutation must first be mapped and then isolated by positional cloning (Beier and Herron 2004). Moreover, extensive back breeding required to analyze recessive mutations

is logistically demanding and limits the use of chemical mutagenesis for genome-wide analysis of gene function (Justice 2000; Balling 2001).

The impact of mouse embryonic stem cell technology

Mouse embryonic stem (ES) cell technology heralded a new era of mouse genetics. Derived from the inner cell mass (ICM) of pre-implantation mouse blastocysts (Evans and Kaufman 1981), ES cells can be cultured in vitro retaining pluripotency and can contribute to the germline of chimeras when injected into blastocysts (Bradley, Evans et al. 1984). Offspring of the chimeras can be intercrossed to analyze biological consequences of recessive mutations. The ability to transmit a mutation introduced into cultured ES cells into mice paved the way to analyze gene functions following gene targeting by homologous recombination, large-scale mutagenesis by gene entrapment, as well as chemical mutagenesis in ES cells (Chen, Yee et al. 2000; Vivian, Chen et al. 2002).

Homologous recombination and gene targeting

Genomic DNA fragments can be engineered and introduced into a mammalian cell to recombine with the endogenous homologous sequence. This process, termed homologous recombination, was first reported in mammalian cells in 1985 (Smithies, Gregg et al. 1985). The first successful targeting in mouse ES cells, which disrupted the hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene, was reported two years later (Doetschman, Gregg et al. 1987).

Germline transmission of a targeted HPRT allele in ES cells was first reported in 1989 (Thompson, Clarke et al. 1989).

A typical procedure of gene targeting starts with the construction of a targeting vector containing a selectable marker gene (and a negative selection marker in some designs), and flanking genomic sequences that are homologous to the desired chromosomal integration site (van der Weyden, Adams et al. 2002). The targeting vector is electroporated into ES cells, which is subsequently cultured in selective media (such as G418 for neomycin resistance). The resistant clones are screened for the presence of the desired recombinant allele by Southern blot or PCR analysis. Selected ES clones are injected into blastocysts (3.5 day embryos), which are then transferred to the uterus of a foster mother. Since targeted ES cells are male and are of 129 background, chimeric male pups with a high percentage of agouti coating have relative high possibility of germline transmission. These chimeras are crossed with wild type females to determine the efficiency of germline transmission. The resulting F1 heterozygotes can then be intercrossed to generate homozygous mutants (Fig. 1.1). As such, homologous recombination in ES cells, in concert with germline transmission, provides powerful means to generate gene deficient mice and therefore reveal gene functions in vivo.

Gene targeting studies can be enhanced by the use of recombinase systems such as Cre/loxP from the bacteriophage P1 (Lakso 1992; Orban 1992; Gu 1994),

A

Targeting vector

Wild type allele

Mutant allele

B

ES cells are transfected or electroporated with the targeting vector and cultured in selection media.

Correctly targeted ES cells identified by Southern blot or PCR analysis are injected into blastocysts.

Blastocysts implanted in a foster mother may give rise to chimeric pups.

Mice carrying the targeted allele can be bred from offspring of the chimera.
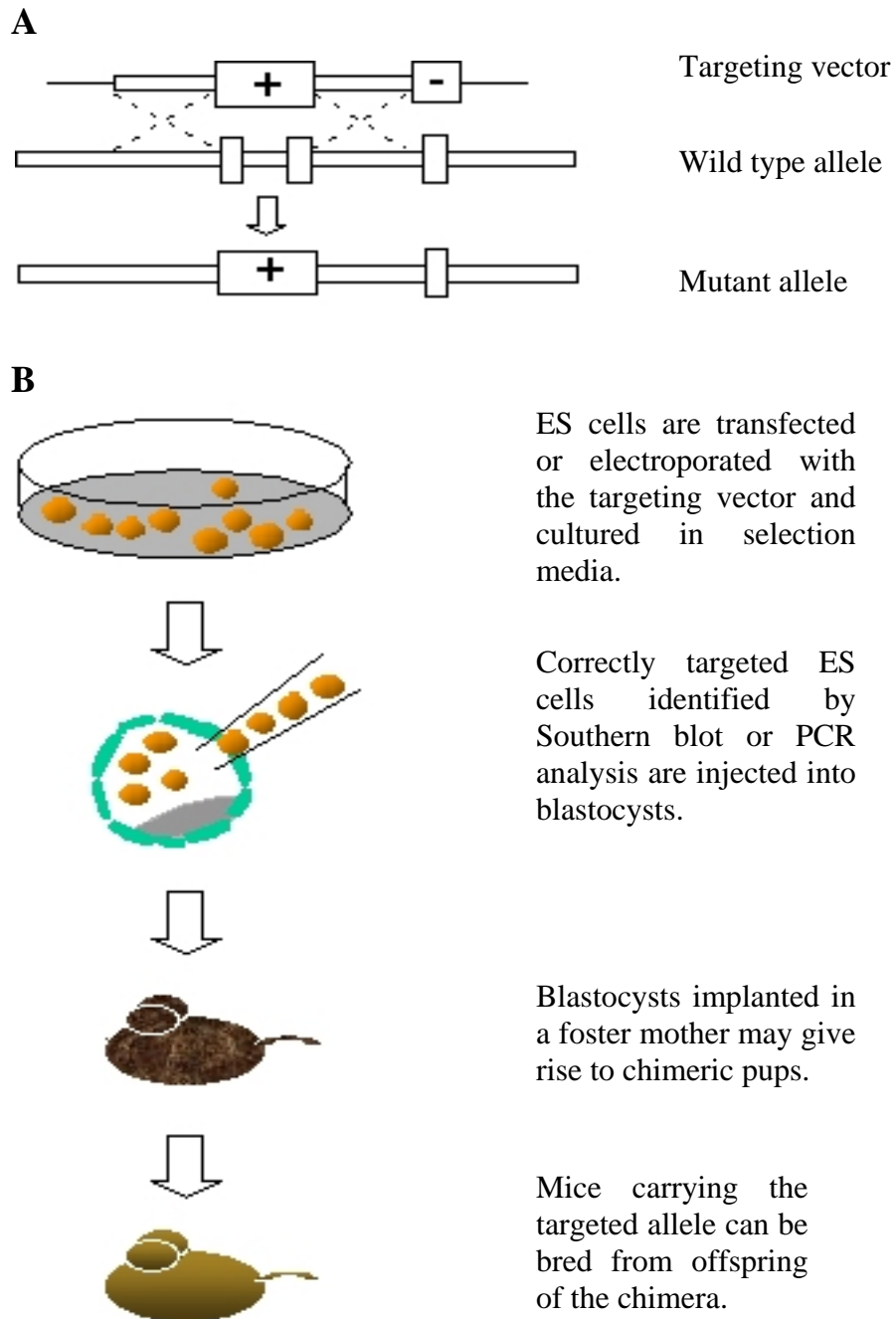
Figure 1.1. Schematic of gene targeting strategy.
A. Homologous recombination between the targeting vector and wild type gene generate the mutant allele.
+: Positive selection marker. -: Negative selection marker.
B. Targeted mouse ES cells and germline transmission.

and Flipase/FRT from the budding yeast *S. cerevisiae* (Farley, Soriano et al. 2000; Rodriguez, Buchholz et al. 2000). The resulting strategy, namely "conditional targeting", provides a means to study in vivo gene functions in a temporal and/or tissue-specific manner.

To date, gene targeting has contributed to unprecedented insights in gene functions and generated many disease models (Zambrowicz and Sands 2003). However, since the process of constructing a targeting vector and screening desired clones requires adequate knowledge of the gene structure and significant effort, some knowledge about the function of the target gene is required to provide a rationale for the knockout (Misra and Duncan 2002). Targeting attempts are often avoided for many genes that have been characterized only at the nucleotide level and for genes that are possibly required for the viability of mice or cells. Nevertheless, the Mouse Knockout Project validates the idea that all genes should be evaluated (Austin, Battey et al. 2004). Therefore, disrupting essentially all genes in the genome requires gene targeting and the complementation of strategies that are more efficient and economic.

Gene trapping

Taking a middle path between random and specific mutagenesis described above, gene entrapment randomly alters genes in ES cells. The gene trap is essentially a drug resistance gene incorporated into one or more exons that are allowed to integrate randomly throughout the genome. The appropriate selection generates cell clones in which the entrapment vector has inserted into endogenous

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genes. The mutagenized ES clones are used to generate mutant mice usually following selection or screening to select clones with mutations in specific genes based on sequence analysis or expression patterns (Evans 1998; Stanford, Cohn et al. 2001) (Figure 1.2).

Entrapment vectors include the enhancer trap, promoter trap, gene trap and polyA gene trap. Enhancer traps contain an autonomous minimal promoter and lacZ reporter gene, which is expressed when the vector inserts in regions influenced by cis-acting enhancer elements (Kothary, Clapoff et al. 1988). Since enhancers can act over large distances, a high proportion of inserted enhancer traps are expressed without disrupting cellular genes, thus undermining their use for insertional mutagenesis (Gossler, Joyner et al. 1989; Korn, Schoor et al. 1992).

Promoter trap vectors basically consist of a promoter-less reporter gene and/or selection marker. Insertion of the vector into an exon gives rise to a fusion transcript consisting of upstream exons of the occupied gene and the reporter/selection marker, thus selecting for highly mutagenic events (von Melchner and Ruley 1989; Reddy, DeGregori et al. 1991). However, by design, entrapment by promoter traps is limited to genes that are transcriptionally active in ES cells. In addition, exon definition frequently activates cryptic 3'splice sites which, when used in conjunction with the polyA site inserted by the vector, allow the selection/reporter gene to be expressed as a 3' terminal exon. Alternative splicing of the cryptic splice sites may result in hypomorphic mutations (Osipovich, White-Grindley et al. 2004; Osipovich, Singh et al. 2005).

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Figure 1.2. Gene entrapment vectors.
Representative enhancer trap (A), promoter trap (B), gene trap (C) and polyA gene trap (D) vectors with a lacZ reporter and neomycin resistance gene (neo) selecting for insertion events in (gene traps) or near (enhancer traps) cellular gene. Major functional elements are labeled; active promoters are indicated with arrows.

A. Enhancer trap

| | lac Z | pA | | neo | pA |

Endogenous regulatory element

| endogenous | | | | pA |

Transcripts: | endogenous | | lac Z | | neo |

B. Promoter trap

| lac Z | pA | | neo | pA |

| endogenous | | | | pA |

Transcripts: | endog | lac Z | | neo |

C. Gene trap

| SA | lac Z | pA | | neo | pA |

| endogenous | | | | pA |

Transcripts: | endogenous | lac Z | | neo |

D. polyA gene trap

| SA | lac Z | pA | | neo | SD |

| endogenous | | | | pA |

Transcripts: | endogenous | lac Z |

| neo | pA |
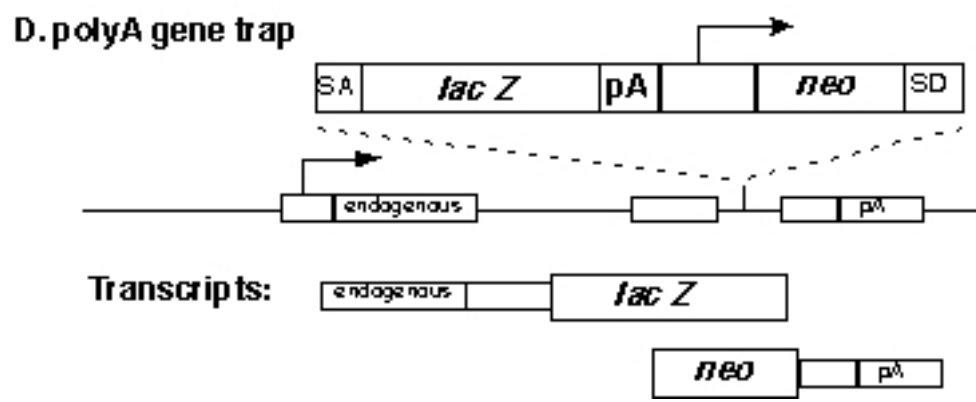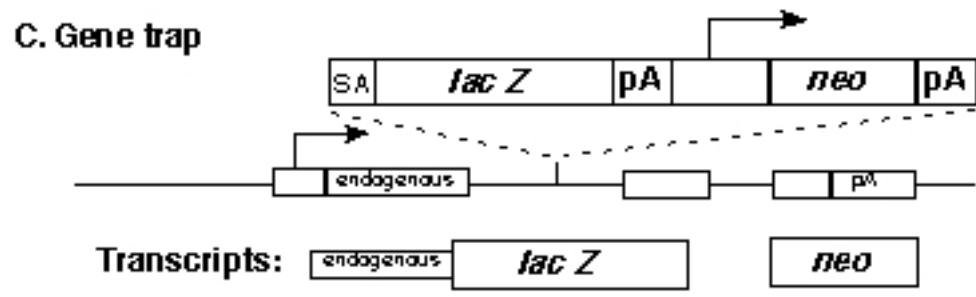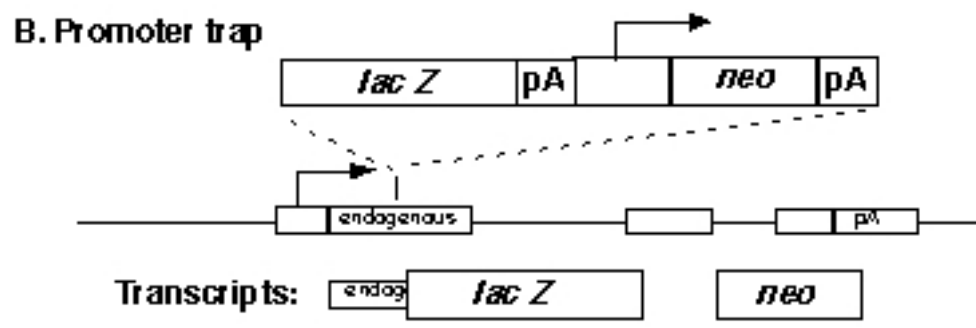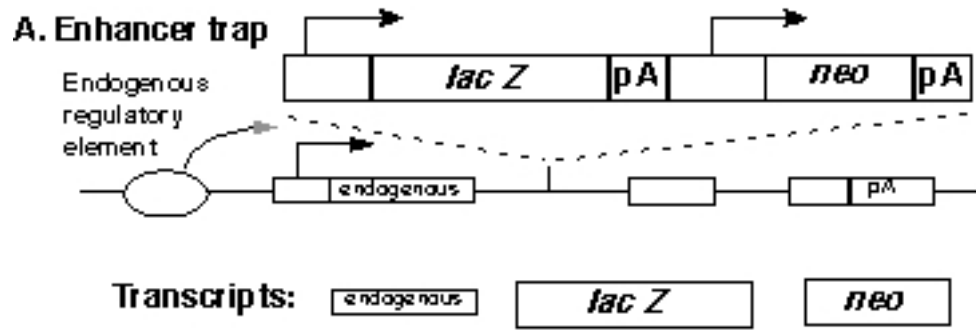
Gene trap vectors contain a splice acceptor and a promoter-less gene that is typically a reporter and selection marker (Gossler, Joyner et al. 1989). Designed to utilize endogenous promoter and enhancer elements by inserting into the intron of a trapped gene, gene trap vectors give rise to a fusion transcript of upstream exons and the reporter/selection marker, which can be used to identify trapped genes by rapid amplification of 5' cDNA ends (5'RACE) (Frohman 1988). Although the trapping efficiency is relatively high, the mutagenicity of gene traps is sometimes inhibited by alternative splicing (McClive, Pall et al. 1998).

PolyA gene traps consist of a mutator cassette consisting of a 3' terminal exon and a polyA trap cassette that contains an autonomous promoter and a selection marker flanked by a splice donor site. The insertion into an intron typically leads to two fusion transcripts. One corresponds to the upstream exons of the disrupted genes that splice to the mutator cassette; the other contains the selection marker spliced to the downstream exons (and polyA) of the occupied gene. Since the selection marker is expressed from an autonomous promoter, polyA gene traps can disrupt genes not expressed in ES cells. However, polyA gene traps preferentially insert in the last intron of the trapped gene, which allows fusion transcript to avoid nonsense-mediated decay. This can position the inserted gene trap downstream of the protein coding sequences without disrupting the expression of the occupied gene. (Baker and Parker 2004; Maquat 2004; Osipovich, Singh et al. 2005; Shigeoka, Kawaichi et al. 2005).

In general, gene trapping strategies provide gene-driven approaches that efficiently generate large-scale random mutations of genes. Moreover, gene entrapment can be coupled with DNA sequencing screens, as "tagged sequence mutagenesis". Gene entrapment is relatively economic in generating knockout models of large numbers of genes, including those that would be selected for mutagenesis by gene targeting. Therefore, gene trapping complements conventional gene targeting in the genome-wide knockout project.

Transposon-based mutagenesis

In addition to ENU mutagenesis, transposon-mediated mutagenesis shows promise for phenotype-driven mutagenesis screens (Miskey, Izsvak et al. 2005). A naturally occurring mobile genetic unit, the transposon, typically consists of flanking terminal inverted repeats (IRs), which contain binding sites for a sequence-specific transposase; and a transposable element (PE), which encodes the transposase, the only protein required for the transposon to move from one site in the genome to another (Izsvak, Ivics et al. 2000). Transposon-mediated mutagensis has been widely used in prokaryotes, *Drosophila* and Zebrafish. A synthetic transposon, *Sleeping Beauty* (SB), is active in mammalian cells and used for insertional mutagensis (Luo, Ivics et al. 1998; Carlson, Dupuy et al. 2003). Unlike chemical mutagenesis, transposon-induced mutations contain molecular tags that allow genes responsible for specific phenotypes to be easily identified. However, new transposon inserts occur mostly in close range of the original site, while transposons in general have limited packaging capacity (Dupuy, Fritz et al.

2001; Dupuy, Clark et al. 2002; Drabek, Zagoraiou et al. 2003). Practical use of transposon mutagenesis in mice will require new vectors that are not affected by these limitations. In this regard, the newly described piggyBac (PB) looks quite promising (Ding, Wu et al. 2005).

RNA interference

Recently, RNA interference techniques have greatly impacted genetics and cell biology in plants, *Drosophila*, *C. elegans*, and gradually also in mammalian cells and organisms (Geley and Muller 2004). In short, a 21nt RNA duplex called small interference RNA (siRNA), is recognized by RNA-induced silencing complex (RISC) when introduced into cells, and RISC activity degrades cellular RNA homologous to the siRNA sequence, thus repressing function of the corresponding gene (Agami 2002). To achieve long-term knockdown effects in mammalian cells, vector systems have been developed that use RNA polymerase I or III promoters to express siRNA, either from short hairpin RNAs (shRNA), or from two complementary short RNA strands (Brummelkamp, Bernards et al. 2002; Paddison, Caudy et al. 2002; Paddison, Caudy et al. 2002; Yu, DeRuiter et al. 2002). A transgenic knockdown mouse was first reported in 2002, with ubiquitous knockdown of EGFP expression (Hasuwa, Kaseda et al. 2002), and knockdown of endogenous targets was soon achieved using retroviral and lentiviral vectors (Hemann, Fridman et al. 2003; Rubinson, Dillon et al. 2003).

In principle, RNAi could provide a fast and economic alternative to existing mutagenesis strategies. However, a microarray gene profiling study

revealed considerable off-target gene regulation in cells transfected with a silencing siRNA (Jackson, Bartz et al. 2003). In addition, shRNA was recently reported in some mammalian cells to induce interferon responses, which was previously assumed to be induced only by longer dsRNA (Bridge, Pebernard et al. 2003). Therefore problems with knockdown specificity and interferon response limit the use of current RNA interference techniques for genetic studies in mice.

Historical context and rationale of the thesis project

Among the major mutagenesis strategies described above, gene targeting, in combination with gene trapping, appeared to be efficient strategies to generate knockout models for essentially all genes in the mouse genome. My dissertation describes the analysis of mice with mutations in *Ssrp1* and *Pfdn1*, generated by conventional gene targeting and gene trapping, respectively.

Chapter II describes the targeting of *Ssrp1*, which encodes the high-mobility-group (HMG) protein SSRP1/T160, a member of a conserved chromatin-remodeling complex (FACT/DUF/CP). SSRP1/T160 was first reported to bind cisplatin-DNA adduct (Bruhn, Pil et al. 1992), and a V(D)J recombination signal sequence (Shirakata, Huppi et al. 1991). Cisplatin, a chemotherapeutic agent, produces cisplatin-DNA adducts and kills rapidly growing cancer cells by triggering G2 cell-cycle arrest and apoptosis (Cohen and Lippard 2001). The role of the p53 tumor suppressor in this process is well established (Lowe, Ruley et al. 1993). By producing cells and mice deficient in SSRP1, I had hoped to analyze cellular responses to cisplatin-induced DNA damage, and to delineate the

underlying molecular mechanism for the cisplatin-induced apoptosis. In addition, *Ssrp1* deficient cells could be used to study the involvement of SSRP1 in the V(D)J recombination process, which was suggested by its binding ability to the V(D)J recombination recognition sequence. Moreover, the two processes might be fundamentally related, given the involvement of proteins involved in double strand break repair in V(D)J recombination, such as Ku70, Ku80, DNA ligase IV and XRCC-4 etc. (Ramsden and Gellert 1998; Nick McElhinny, Snowden et al. 2000).

My own work determined that *Ssrp*1 is required for cell viability (Cao, Bendall et al. 2003). During the course of these studies, *Ssrp*1 was found to be a highly conserved component of the chromatin remodeling complex FACT (facilitating chromatin transcription), involved primarily in transcription elongation (Orphanides, Wu et al. 1999). The binding of SSRP1 to cisplatin adducts and RSS may therefore reflect a conformation specific DNA binding activity that is not directly involved in cellular responses to cisplatin-induced DNA damage or V(D)J recombination. Furthermore, the requirement for *Ssrp1* in cell viability precluded the planned studies using *Ssrp1* deficient cells.

Chapter III describes the gene entrapment of *Pfdn*1 gene, which codes for the first subunit of molecular chaperone prefoldin. *In vitro* biochemical and structural studies established prefoldin as a co-chaperone of chaperonin TRiC (Tcp-1 ring complex) in the folding of cytoskeleton proteins such as actin and

Figure 1.3. A schematic of GTR1.3 polyA gene trap.

A. The insertion of GTR1.3 in *Pfdn*1.

The long terminal repeats (LTRs) direct integration of the retrovirus vector. Upstream exons (1-3) of the *Pfdn1* gene splice to the 3'Puro-LacZ mutator cassette. A neomycin resistance gene (Neo) expressed from the RNA polymerase II promoter (PolII) splices to exon 4 of *Pfdn1*. Fusion transcripts expressed by the occupied gene are indicated.

B. Selective cloning of Neo fusion transcripts. (From Lin, et al. manuscript submitted)

A NotI restriction site and intron were engineered in the neomycin resistance gene (Neo), enabling selective cloning of Neo fusion transcripts. Only 3'RACE products amplified from spliced fusion transcripts can generate a functional Neo resistance gene which confers Kanamycin resistance in *E. coli*. This design favors cloning of long and specific 3' RACE products and accelerates identification of trapped genes.

B
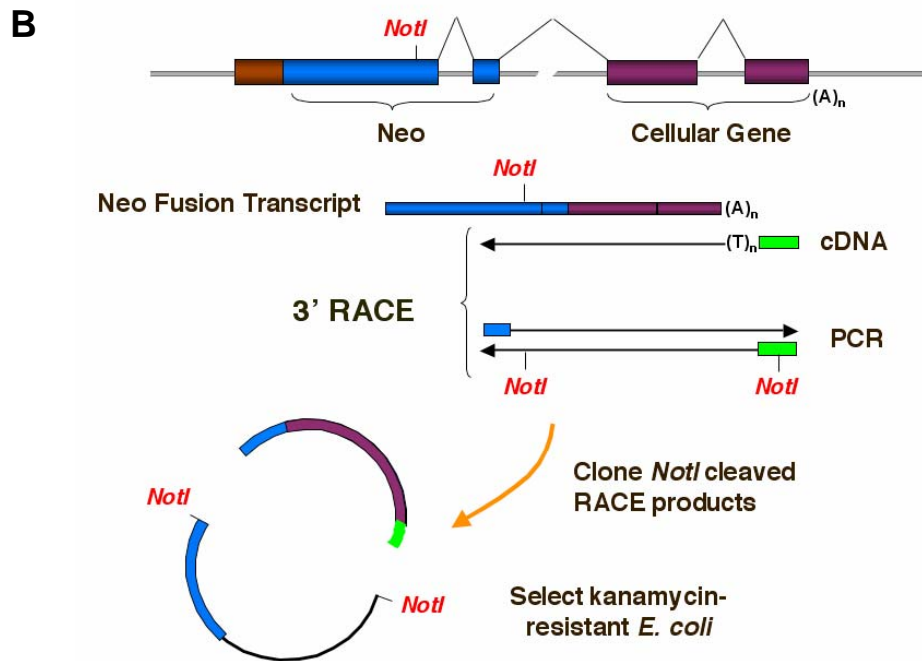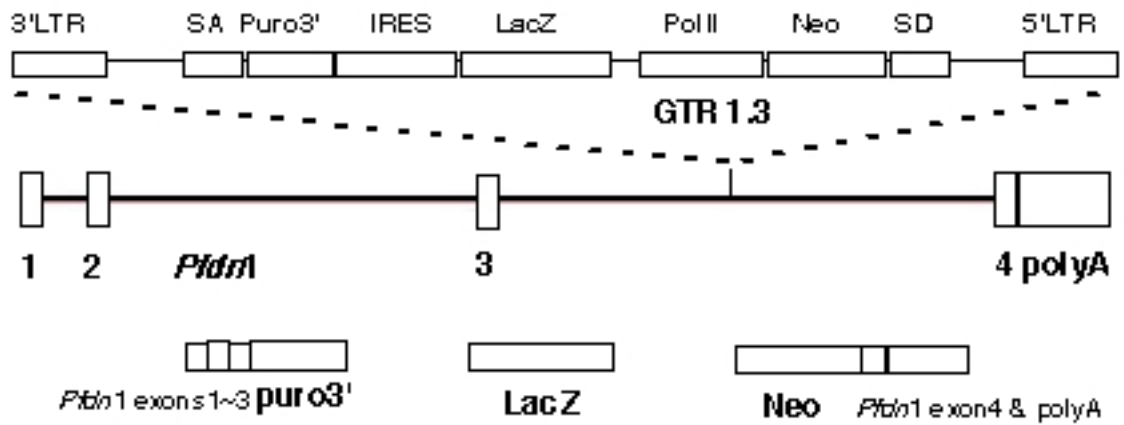
tubulin (Hansen, Cowan et al. 1999; Siegert, Leroux et al. 2000; Martin-Benito, Boskovic et al. 2002). Yeast lacking prefoldin function are viable, yet develop severe cytoskeletal defects, which are similar to the phenotype of temperature-sensitive TRiC mutants (Vainberg, Lewis et al. 1998).

An Embryonic Stem (ES) clone containing a mutation in *Pfdn*1 was identified from a gene entrapment library of 129sv ES cells (Lin et al. Manuscript submitted). GTR1.3 consists of a splicing acceptor (SA), 3' fragment of puromycin resistance gene (Puro3') followed by internal ribosome entry site (IRES) and *lacZ* reporter, PolII promoter, an intron-split Neomycin resistance gene (Neo) and a splicing donor (SD) (Fig. 1.3A). Trapped exons and polyAs were recovered in 3' RACE products, via an efficient selective cloning strategy on a split Neo gene (Fig. 1.3B). The location of retroviral insertion was identified by inverse polymerase chain reaction PCR. *Pfdn*1 mutant clones were injected into blastocysts and gave rise to germline chimeras.

GTR1.3 was inserted upstream of *Pfdn1* Exon4, which encodes a significant part of the coiled-coil domain. The function of this domain, which is essential to the interaction between prefoldin and its substrates, is very likely to be disrupted by the insertion of GTR1.3. In addition, the cytoskeleton defects in prefoldin-deficient yeast suggest the non-redundant function of *Pfdn1* in cytoskeleton maintenance. As such, I set my goal to study the *in vivo* functions of mammalian *Pfdn1* and the impact of loss of prefoldin function in *Pfdn1*-deficient cells.

My study revealed that the insertion of the polyA trap GTR1.3 ablated expression of Prefoldin 1. Loss of prefoldin function impaired cytoskeletal organization, as indicated in *Pfdn1*-deficient embryonic fibroblasts (MEFs) and B lymphocytes. Null mutant mice were marked by premature death, dwarfism, defective development in the central nervous systems (CNS), defective hematopoiesis and lymphopoiesis. Deficiencies of the *Pfdn*1 null mice indicate that *Pfdn*1 encodes non-redundant functions required for mouse development and survival, presumably by affecting cytoskeleton formation (Cao et al., 2005 manuscript in preparation). Gene entrapment of *Pfdn*1 and the deficiencies of *Pfdn*1 null mice will be described further in Chapter III.

CHAPTER II

SSRP1/T160: AN HMG-BOX PROTEIN REQUIRED FOR CELL VIABILITY

Introduction

Chromosomal DNA is packaged in a nucleic acid-protein structure known as chromatin. The highly ordered structure of chromatin protects genomic DNA from physical stress and provides a platform for a wide variety of transactions involving DNA. In addition to histones, the basic structural component of chromatin, additional chromatin-associated proteins such as the High Mobility Group (HMG) proteins manipulate DNA structure and facilitate the assembly of nucleoprotein complexes (Bustin and Reeves 1996). This chapter will describe my studies on one of the HMG-box proteins, Structural Specific Recognition Protein 1 (SSRP1). This introduction will provide the historical background on *Ssrp1* and provide the rationale for targeting *Ssrp* gene in mice.

V(D)J recombination and the discovery of mouse SSRP1/T160.

Mouse SSRP1/T160 was initially discovered as a potential regulator of lymphocyte-specific recombination. To fight against numerous pathogens, mammals develop a huge repertoire of lymphocytes that express antibodies with different antigen specificities. The diversity of the antibodies and receptors, made from a far smaller pool of genes, is provided by somatic gene rearrangements of V (variable), J (joining), and/or D (diversity) genes. This so called V(D)J recombination is mediated by recombinases encoded by recombination activating

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genes 1 and 2 (RAG-1, RAG-2). These lymphocyte-specific recombinases recognize DNA recognition signal sequences (RSS) located in the intervening DNA 3' of each V segment, 5' of each J segment and flanking both sides of each D segment. Additional accessory proteins that are widely expressed in many cell types are also involved in the breakage and re-ligation of immunoglobulin gene segments (Gellert 2002).

In the search for DNA binding components that might be involved in V(D)J recombination, Sakano's group screened a cDNA expression library from a mouse pre-B cell line with a probe containing a concatemer of the 12-bp RSS. Clone λT160 was isolated from this screen, and the corresponding gene encodes a protein of 80.6 kD. This protein bound to DNA probes containing RSS in southwestern assays, but not to a DNA probe containing a mutated RSS. (After the discovery of human SSRP1, T160 was identified as a mouse ortholog, hence mouse SSRP1/T160.) Homology searches revealed that SSRP1/T160 possesses a DNA-binding motif that has 56% sequence homology to the high mobility group domain (HMG box) of HMG1 (Shirakata, Huppi et al. 1991) (Figure 2.1.).

HMG box proteins play various roles from general chromatin maintenance (HMG-1, HMG-2) to transcription regulation (SRY, LEF-1) (Bianchi and Beltrame 2000). Despite reports indicating various new functions, members of this family share a common feature of manipulating DNA structure and facilitating the assembly of nucleoprotein complexes. Deletion of the HMG box
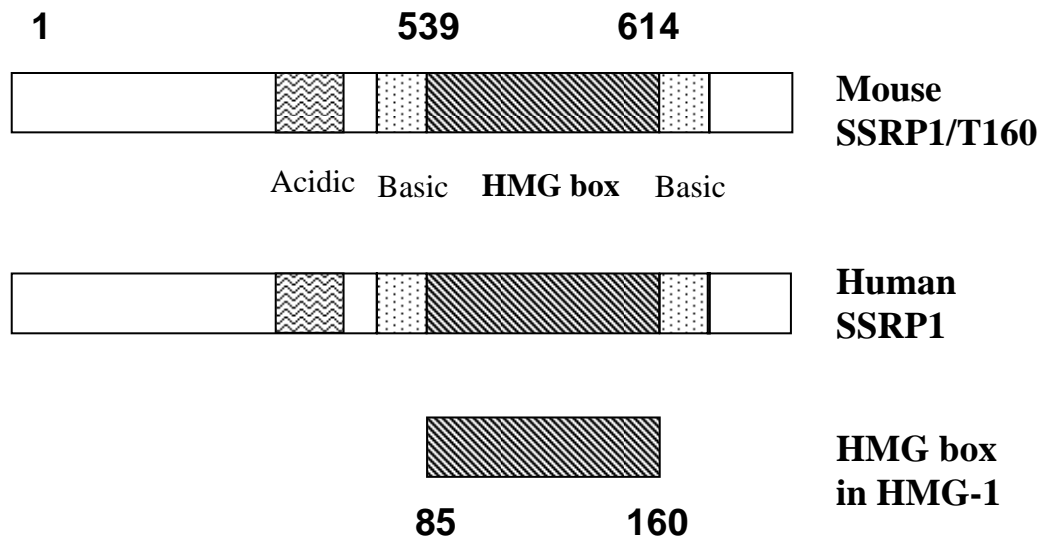
Figure 2.1 HMG box and major domains in Mouse and Human SSRP1.
Mouse SSRP1/T160 and human SSRP1 share 98% sequence identity,
highlighted by the acidic, basic and HMG box motifs that are all highly
homologous. HMG box (amino acid 539~614) of mouse SSRP1/T160 has 56%
sequence homology to HMG box (Amino acid 85~160) of HMG-1.

destroys the DNA binding activity of mouse SSRP1 (Shirakata, Huppi et al. 1991). The mouse *Ssrp1* gene is located near the *Rag-1* and *Rag-2* genes on mouse chromosome 2 (as their human homologs on Chromosome 11) (Huppi, Siwarski et al. 1993). This genetic evidence suggests a linkage between SSRP1 and RAG-1, RAG-2, and therefore its possible participation in V(D)J recombination.

The regulation of V(D)J recombination is interrelated to that of p53-mediated DNA damage response (Guidos, Williams et al. 1996). Accumulation of "Broken" V(D)J coding ends in DNA-dependent protein kinase (DNA-PK) deficient SCID lymphocyte precursors activates a p53-dependent DNA damage checkpoint. This suggests a DNA repair-related mechanism that distinguishes well-coordinated recombination from DNA damages in normal cells. Potential connections among V(D)J recombination, DNA damage response and SSRP1/T160, were especially interesting in light of the potential involvement of human SSRP1 in cellular response to DNA damage caused by anti-tumor agents described in the next section (Bruhn, Pil et al. 1992).

Human SSRP1 and cisplatin.

Human SSRP1 was discovered by screening a human B-cell cDNA expression library for proteins that bind DNA modified by cisplatin (cis-diamminedichloroplatinum (II)), a potent anti-tumor agent (Toney, Donahue et al. 1989; Bruhn, Pil et al. 1992). The protein expressed in one positive clone was tested for binding specificity in southwestern assays and bound strongly to DNA

probes modified by cisplatin, but did not bind to unmodified DNA or DNA modified with the clinically ineffective compound, trans-DDP (trans-diamminedichloroplatinum (II)). The full-length cDNA encoded an HMG box protein with 98% sequence identity to mouse T160. Since the protein bound cisplatin-modified DNA rather than a specific DNA sequence, it was named the Structure Specific Recognition Protein (SSRP1) (Bruhn, Pil et al. 1992). The specific recognition of cisplatin-modified DNA by SSRP1 suggests its involvement in cellular responses to cisplatin DNA damage.

Cisplatin has been a highly effective antitumor drug on a variety of cancers, including testicular, ovarian, head and neck cancer (Cohen and Lippard 2001). Although cisplatin reacts with various cellular components, the critical target for cisplatin is generally accepted to be DNA, as indicated by high sensitivity to cisplatin of cells with defects in DNA repair. The relatively low concentration of Cl ions in the cell facilitates hydrolysis of the chloride ligands of the drug. The hydrolyzed forms, once in the nucleus, generate mainly intrastrand d(GpG) and d(ApG) DNA crosslinks (Takahara, Rosenzweig et al. 1995). Such adducts bend the DNA duplex toward the major groove and unwind the DNA helix. This provides a distorted structure that probably mimics DNA structures to which HMG box proteins (SSRP1, HMG1, HMG2, Ixr1 etc.) normally bind (Brown, Kellett et al. 1993; Ohndorf, Whitehead et al. 1997).

The DNA damage caused by cisplatin, if not repaired, will cause defects in DNA replication and transcription, especially in rapidly growing tumor cells. Two models have been proposed to explain the role of HMG box proteins in the repair

of cisplatin induced DNA damage. In the "damage recognition" model, HMG box proteins may bind to cisplatin DNA adducts and act as a damage-recognition element, recruiting the repair complex to the site of damage. The "repair shielding" model proposes that the binding of HMG box proteins to the adduct blocks the repair complex, causing cell death in rapidly growing cells. Support for the "repair shielding model" is suggested by a report that a yeast strain containing an inactivated Ixr1 gene (a yeast homologue of mammalian *Ssrp1*) was half as sensitive to cisplatin as the parental strain (Brown, Kellett et al. 1993). *Ssrp1* deficient cell lines would facilitate studies to distinguish between these and other models in mammalian cells.

Cisplatin kills cancer cells, mainly, by triggering G2 cell-cycle arrest and apoptosis. The role of p53 tumor suppressor in this process is well established (Lowe, Ruley et al. 1993). p53 is a potent activator of apoptosis, (Stewart and Pietenpol 2001) and p53-deficient cancer cells are less sensitive to cisplatin (Gallagher, Cairney et al. 1997), while p53 - independent cell sensitivity to cisplatin is also documented (Zamble, Jacks et al. 1998). Another pathway depending on p73, the product of a p53-related gene, was also discovered in cisplatin-induced apoptosis (Gong, Costanzo et al. 1999). Unrepaired cisplatin DNA damage induces p73-mediated apoptosis, via activation of c-Abl tyrosine kinase, a mediator of cell-cycle arrest (Kharbanda, Ren et al. 1995; Gong, Costanzo et al. 1999). Put together, these findings indicated that cisplatin could induce two separate pathways dependent on p53 or p73.

As reported in 2001, the binding of HMG-1 to cisplatin DNA adducts is enhanced by interaction with p53 through its HMG box motif (Imamura, Izumi et al. 2001). SSRP1, in a complex with hSpt16, has also been reported to influence casein kinase 2-dependent phosphorlyation and the activity of p53 in UV irradiated cells (Keller, Zeng et al. 2001). This implies that HMG box proteins participate in cisplatin-induced apoptosis, possibly by recruiting specific protein to sites of adducted DNA.

Although cisplatin therapy has been relatively successful on cancers such as testicular and ovarian cancers, the drawbacks of this therapy, such as toxic side effects, drug resistance and limited efficacy in other cancers, have not yet been overcome. A better understanding of the underlying molecular mechanism may allow for the rational design of new anti-tumor therapies. *Ssrp1* deficient mice and/or cells will be useful reagents for studying the role of HMG box proteins in the mechanism of cellular response toward cisplatin damage.

SSRP1 is a component of chromatin remodeling complex FACT.

SSRP1 is also implicated in transcription elongation, as a component of FACT (facilitates chromatin transcription) complex (Orphanides, Wu et al. 1999). The mammalian FACT was initially characterized as an activity that prevented nucleosomes from inhibiting transcriptional elongation *in vitro*. Transcription of class II genes in vitro can be reconstituted on naked DNA with RNA Polymerase II and general transcription factors (GTFs) IIB, IID, IIE, IIF and IIH. However, chromatin templates, although competent for transcription activation, were

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defective for transcriptional elongation, due to the inhibitory effect of chromatin (for review, (Orphanides, Lagrange et al. 1996). Orphanides et al. screened Hela cell fractions for activities that would alleviate the inhibitory effect of chromatin on transcriptional elongation, using a reconstituted transcription system consisting of minimal transcription apparatus and a chromatin template assembled in *Drosophila* cell extracts (Orphanides, LeRoy et al. 1998). The transcription elongation factor was purified and named FACT (facilitates chromatin transcription). FACT was subsequently revealed to consist of two proteins of 140 and 80 kD. The smaller component is SSRP1/T160 and the larger component is an orthologue of the *S.cerevisiae* Spt16/Cdc68, an essential yeast protein implicated in transcription and cell-cycle regulation (Orphanides, LeRoy et al. 1998).

The role of FACT and SSRP1 in different organisms.

FACT complexes are abundant (~1,000,000 copies per nucleus) and highly conserved. Counterparts are designated CP in *S. cerevisiae*, DUF in *Xenopus* and FACT in *Drosophila*, mouse and human. Both components of the yeast CP complex, SPT16/CDC68 and POB3, are required for cell viability. Although similar to SSRP1, POB3 lacks an HMG box and relies on an HMG box protein, Nhp6a or Nhp6b, to recruit the yeast CP to chromatin (Brewster, Johnston et al. 2001; Formosa, Eriksson et al. 2001). Thus the functional orthologs of SSRP1 in yeast are encoded by two genes, POB3, which lacks HMG box, and the
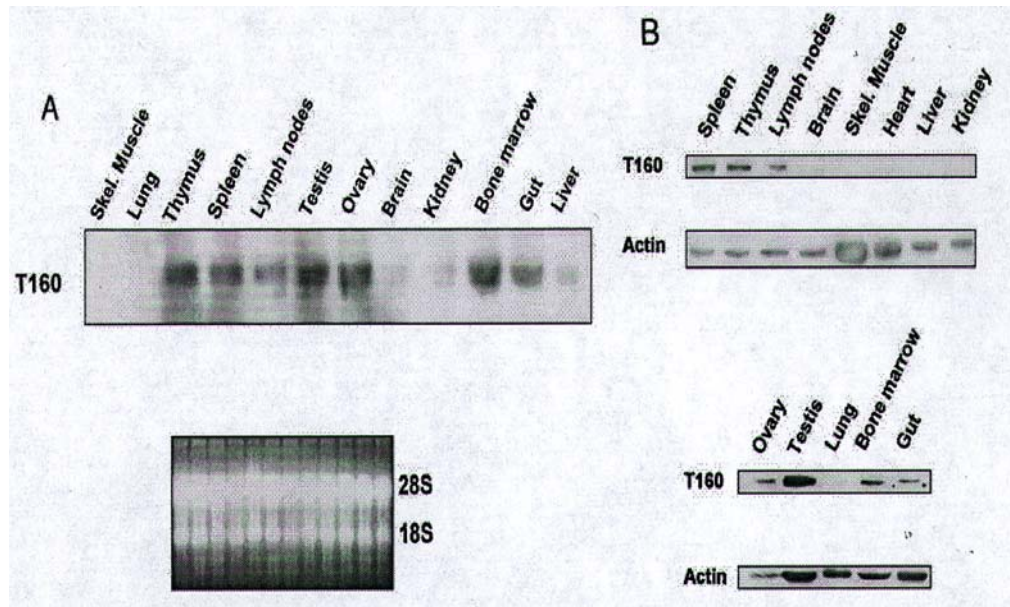
Figure 2.2. Mouse SSRP1/T160 is expressed ubiquitously. (From Hertel, De Andrea et al. 1999)

A. *Ssrp1* expression in various mouse organs and tissues assessed by Northern blot analysis.

B. SSRP1/T160 western blot analysis of samples from mouse organs and tissues.

genetically redundant Nhp6a/6b proteins that consist of little more than an HMG box. The *Xenopus* complex was purified as a duplex DNA-unwinding factor (DUF) that simulates DNA replication in ooctye extracts (Okuhara, Ohta et al. 1999). Drosophila FACT complexes are localized to transcriptionally active chromatin on polytene chromosomes (Kelley, Stokes et al. 1999).

Not necessarily independent of the FACT complex, SSRP1 is widely expressed in many tissues, such as thymus, spleen, lymph Nodes, bone marrow, testis and ovary and has been implicated in a variety of activities (Hertel, De Andrea et al. 1999) (Figure 2.2.). Confocal immuno-fluorescene analysis revealed co-alignment between SSRP1 speckles and DNA replication centers visualized by incorporated BrdU at mid or late S phase of the cell cycle, suggesting the involvement of SSRP1 in DNA replication (Hertel, De Andrea et al. 1999). SSRP1 has also been implicated in transcription control, for example, as a sequence-specific transcription factor of the embryonic ϵ globin gene (Dyer, Hayes et al. 1998). Other reports suggested SSRP1 as a coactivator working in concert with serum response factor (Spencer, Baron et al. 1999) and the p53-related p63 protein (Zeng, Dai et al. 2002).

In summary, SSRP1, most probably as a component of FACT, has been implicated in DNA recombination (Shirakata, Huppi et al. 1991), replication (Wittmeyer and Formosa 1997; Okuhara, Ohta et al. 1999; Wittmeyer, Joss et al. 1999), basal and regulated transcription (Brewster, Johnston et al. 1998; Evans, Brewster et al. 1998; Orphanides, Wu et al. 1999; John, Howe et al. 2000; Wada,

Orphanides et al. 2000), and DNA repair (Keller, Zeng et al. 2001; Yarnell, Oh et al. 2001; Keller and Lu 2002).

Embryonic lethality of *Ssrp1* deficient mice.

Shortly after the initial discovery of mouse SSRP1/T160, Dr. Eugene Oltz and colleagues targeted the gene via homologous recombination in mouse ES cells, in an attempt to obtain *Ssrp1* deficient mice for studies on the *in vivo* functions of *Ssrp1* gene, especially its role in V(D)J recombination and DNA damage response.

The targeting vector consisted of a Neomycin resistance gene (NEO) expressed from phosphoglycerol kinase (PGK) promoter flanked by sequences homologous to the *Ssrp1* gene. After homologous recombination, the vector replaced 3.9 kb of genomic sequences (located between *Kpn*I and *Xho*I sites) that contain exons 14-17 with the PGK-Neo cassette. The vector also contained a PGK-TK gene to select against non-homologous inserts (Fig. 2.3A). The mutant allele (designated *Ssrp1^t*) thus deletes the HMG box domain (Exon14, 15) along with 87 amino acids from the C-terminus. Targeted ES cells were identified by Southern blot hybridization to a flanking sequence probe (Fig. 2.3B).

Three *Ssrp1$^{+/t}$* ES clones independently injected into C57BL/6 blastocysts gave rise to germ line chimeras. *Agouti* offspring inheriting the targeted allele were identified by Southern blot, and the mutation was bred into a 129Xsv background for 3 generations and subsequently intercrossed to generate

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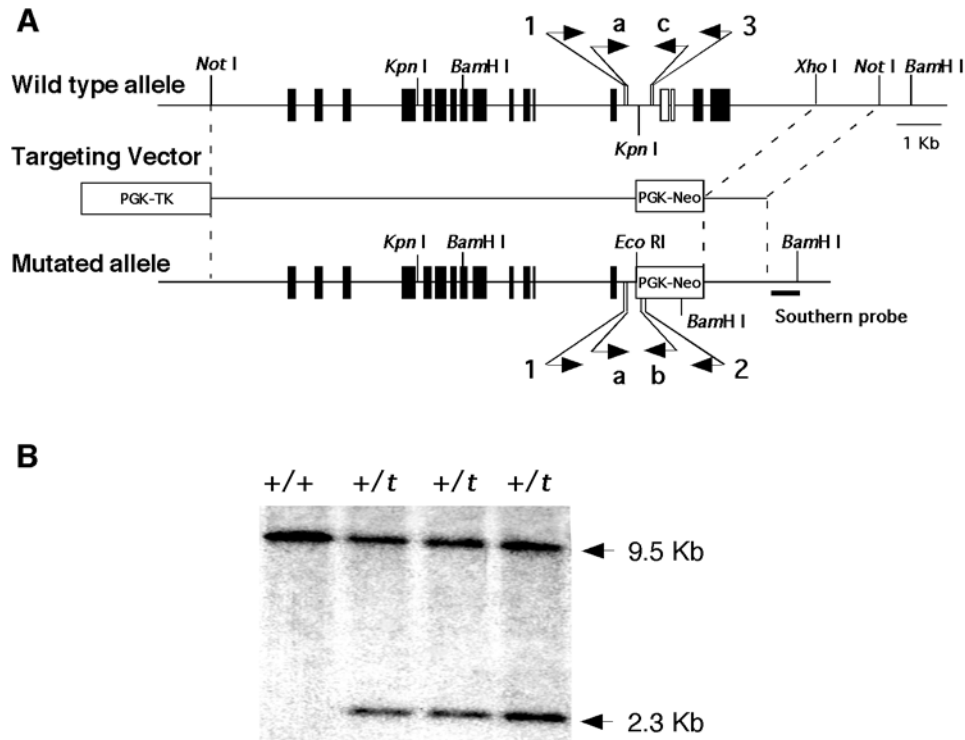
Figure 2.3. Targeted disruption of the *Ssrp1* gene.
A. A schematic diagram of the genomic organization of *Ssrp1* gene, the targeting vector and the mutated allele after the homologous recombination. The locations of sequences used as a probe for Southern blot hybridization and PCR primers (1, 2, 3, a, b, and c) are indicated. For positive selection, the targeting vector contained a neomycin resistance gene (Neo) expressed from the phosphoglycerol kinase (PGK) gene promoter. The Neo gene replaced sequences of the *Ssrp1* gene located between the *Kpn* I and *Xho* I sites. The targeting vector also contained a Herpes Simplex Virus thymydine kinase (TK) gene for negative selection.
B. Southern blot analysis showing correct targeting of the *Ssrp1* gene. Genomic DNA from ES cell clones isolated following positive/negative selection was digested with *Bam* HI and analyzed by Southern blot hybridization using the 3' flanking probe shown in Fig 2.3A. The 9.5 kb band represents the wild type (+) allele, and the 2.3 kb band corresponds to the correctly targeted allele (*t*).

homozygous mutant mice. However, no $Ssrp1^{t/t}$ mice were detected among offspring of heterozygote intercrosses.

Rationale for further study on *Ssrp1* mutation.

At the onset of my project, it was already clear that SSRP1/T160 is required for mouse viability although the timing of embryonic death was not known. Moreover, it remained unclear whether SSRP1/T160 would also be required for cell viability. If not, null cell lines could be isolated from homozygous mutant embryos for biochemical studies on gene functions. Even if SSRP1 mice died early in development, it might be possible to establish null ES cells from pre-implantation blastocysts. An abundant nuclear protein hnRNP C, for example, was required for late embryonic development, but not for cell viability (Williamson, Banik-Maiti et al. 2000). The isolation of hnRNP C null ES cells revealed the replacement of CARG in hnRNP complex and suggested the involvement of hnRNPc in the nuclear retention of unspliced RNA transcripts (Williamson, Banik-Maiti et al. 2000)and unpublished results).

If *Ssrp1* is not required for cell viability, *Ssrp1* deficient cells would be valuable reagents to study the cellular function of SSRP1/T160, especially in the context of V(D)J recombination and DNA damage response, as described earlier. The capacity to complete V(D)J recombination could be tested in *Ssrp1* deficient ES cells to determine whether SSRP1/T160 participated in this recombination. Sensitivity to various anti-tumor drugs could be tested in cells expressing different levels of SSRP1/T160. Furthermore, the role of SSRP1/T160 in

cisplatin-induced apoptosis pathways would be determined, in the context of p53 or p73 pathway. In addition, successful rescues from embryonic lethality were reported by crossing mutations in genes involved in cellular responses to DNA damage, including *BRCA1, Lig4*, and *XRCC4* into a p53-null background (Ludwig, Chapman et al. 1997; Frank, Sharpless et al. 2000; Gao, Ferguson et al. 2000). Similarly, crossing the *Ssrp1* mutation into the *p53*-null background may alleviate the lethal phenotype. If the lethality in *Ssrp1* deficient mice or cells was not relieved by the additional loss of p53, then *Ssrp1* is likely to function differently than other DNA repair proteins and independently of p53. Consequently, the effects of *Ssrp1* mutation in mice and cells were further studied as described in the rest of this chapter.

Results

Ssrp1 **expression is reduced in ES cells with the targeted mutation.**

ES cells with the targeted *Ssrp1* mutation were obtained from the laboratory of Dr. Eugene Oltz (Vanderbilt University). Four *Ssrp1*$^{+/t}$ ES clones independently injected into C57BL/6 blastocysts gave rise to germ line chimeras. *Agouti* offspring inheriting the targeted allele were identified by either Southern blot or PCR analysis, and the mutation was bred into a 129sv or C57BL/6 background for 3 generations before being intercrossed.

Since SSRP1 is expressed in many cell types, including ES cells, the effects of the targeted mutation on SSRP1 expression could be assessed by western blot analysis. As shown in Figure 2.4, clones containing the targeted allele expressed approximately half as much SSRP1 as wild-type cells. Moreover, truncated proteins potentially encoded by the targeted allele were not detected.

Ssrp1 **is required for early embryonic development.**

*Ssrp*1$^{+/t}$ mice were intercrossed in attempt to generate homozygous mutant mice. However, no *Ssrp*1$^{t/t}$ mice were detected among 220 offspring analyzed (Fig. 2.4 and Table 2.1). To determine the stage at which the homozygous mutant mice die, embryos from timed matings were genotyped after different times of gestation (Table 2.1). Again, none of the embryos that could be dissected from decidua at embryonic day (E) 6.5 to 8.5 was homozygous for the targeted allele.
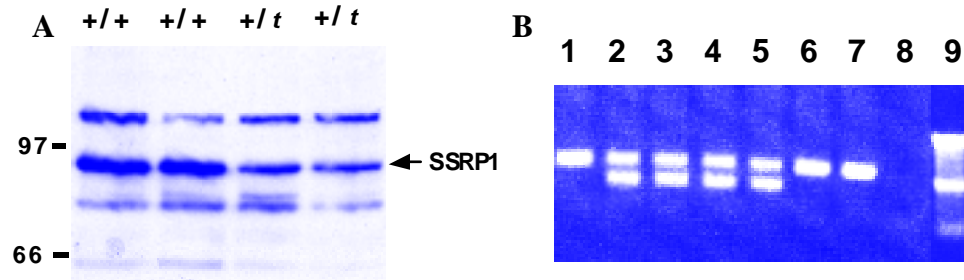
Figure 2.4. Targeting of *Ssrp1* assessed in ES cells and mice.
A. Western blot analysis of SSRP1 expression in wild type and heterozygous, targeted ES cells. Whole cell extracts from $Ssrp1^{+/+}$ and $Ssrp1^{+/t}$ ES cells were analyzed with antisera against the amino terminal region of SSRP1. The native, 86 KDa SSRP1 protein was detected (arrow) in both cell types, while no truncated proteins were detected in $Ssrp\ 1^{+/t}$ cells. The mobility of the 97 and 66 KDa molecular weight standards is shown.
B. PCR genotyping of wild-type and heterozygous mutant mice. Primers a, b and c (Fig 1A) were mixed and used to amplify sequences corresponding to the wild-type (374 nt) and targeted (250 nt) alleles. Tail DNA from 3 week-old offspring produced by crossing $Ssrp1^{+/t}$ heterozygotes (lanes 1-7) and a sample lacking DNA (lane 8) were amplified by PCR and fractionated on a 1.5% agarose gel, together with molecular weight markers (lane 19). 3 of the animals analyzed here were wild-type, 4 were heterozygous, while none was homozygous for the targeted allele.

However, embryos in 16 out of 60 decidua examined were nearly or completely resorbed and could not be genotyped. Moreover, their proportion (26.7%) was sufficient to account for the absence of homozygous mutants. Embryos were also examined at E5.5 in serial sections cut through intact decidua. Once again, the presumptive homozygous mutant embryos (4 out of 16) were almost completely resorbed (Fig. 2.5).

Table 2.1. Genotypes of offspring and embryos produced by *Ssrp1*$^{+/t}$ intercrosses. Mice heterozygous for the targeted mutation in the *Ssrp1* gene were mated and embryos and progeny of the indicated ages were genotyped.
*This total does not include 16 empty deciduas that could not be genotyped.

Age	+/+	+/t	t/t	*Total*
3 weeks	75	145	0	220
E7.5-8.5	13	31	0	44*
E3.5	32	54	29	115

The observed decidual reactions suggested that the mutant embryos die after implantation. To test this idea further, 105 pre-implantation, blastocyst-stage embryos (E3.5) were genotyped (Fig. 2.6) by using a nested PCR strategy (Fig. 2.3A). 29 homozygous mutant blastocysts were detected, together with 32 wild type and 54 heterozygous embryos—consistent with a Mendelian distribution (Table 2.1). The mutant blastocysts appeared morphologically

normal (Fig. 2.6). Taken together, these results suggest that $Ssrp1^{t/t}$ embryos die between implantation (E4.0) and E5.5.
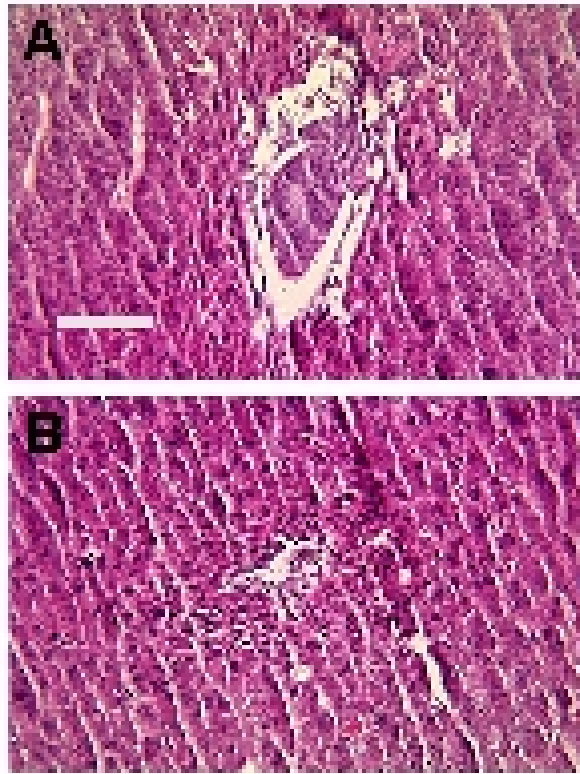
Figure 2.5. Histological sections of E5.5 embryos.
Decidua containing E5.5 embryos from $Ssrp1^{+/t}$ intercrosses were sectioned and stained with hematoxylin and eosin. Representative normal (A) and presumptive homozygous mutant (B) embryos are shown. The panels are reproduced at the same magnification (bar, 100 μm)
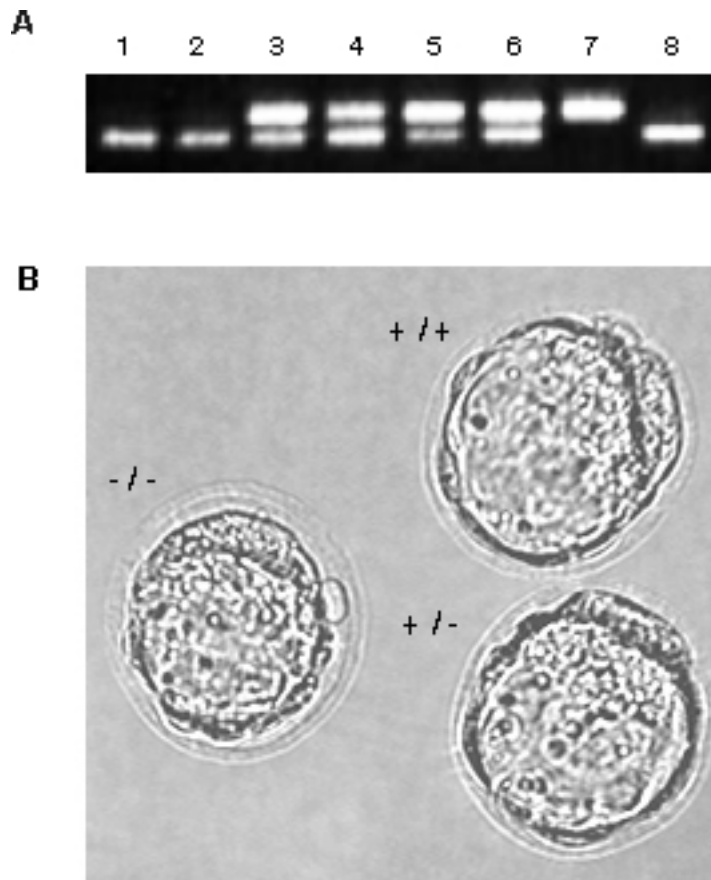
Figure 2.6. PCR genotyping of E3.5 blastocysts.
(A) Blastocysts from *Ssrp1* heterozygous intercrosses were analyzed by nested
PCR, using primers 1, 2, 3 and a, b, c (Fig 2.3.A) in the first and second rounds of
amplification, respectively. (B) Phase contrast photomicrographs of E3.5
blastocysts of the indicated genotypes are shown. *Ssrp1*$^{t/t}$ blastocysts appear
morphologically normal.

Defective outgrowth of ICM cells from *Ssrp1^{t/t}* blastocysts

Embryonic stem (ES) cell lines are readily derived from the inner cell mass (ICM) of E3.5 blastocysts, if the cells are maintained under conditions that suppress their differentiation. Cell lines homozygous for early embryonic lethal mutations can also be derived, when the affected genes are not required for the growth or viability of ICM cells. For example, ES cells deficient in hnRNP C and protein arginine methyltransferase 1 were successfully isolated in our laboratory, even though the mutant embryos died at or before E6.5 (Pawlak, Scherer et al. 2000; Williamson, Banik-Maiti et al. 2000). *Ssrp1*-deficient ES cells could provide a valuable system in which to study the biochemical functions of SSRP1. However, when blastocysts from *Ssrp1^{+/t}* intercrosses were cultured *in vitro*, none of the 12 resulting ES cell lines was homozygous for the targeted allele (data not shown). By contrast, eight lines were heterozygous for the mutant allele and four were wild-type. The numbers, while relatively small, are consistent with a Mendelian distribution, assuming homozygous mutant cells are selectively lost during cultivation. For reference, the probability (Chi square) of not recovering a homozygous mutant from among 12 progeny due to chance alone is 0.08.

The failure to recover homozygous mutant ES cell lines raised questions about whether SSRP1 is required for the growth or viability of ICM cells. To address this issue, 32 blastocysts from *Ssrp1^{+/t}* intercrosses were individually cultured on mouse embryonic fibroblast (MEF) feeder cells and examined daily. During the first 24 hours, all of the embryos attached and hatched from the zona
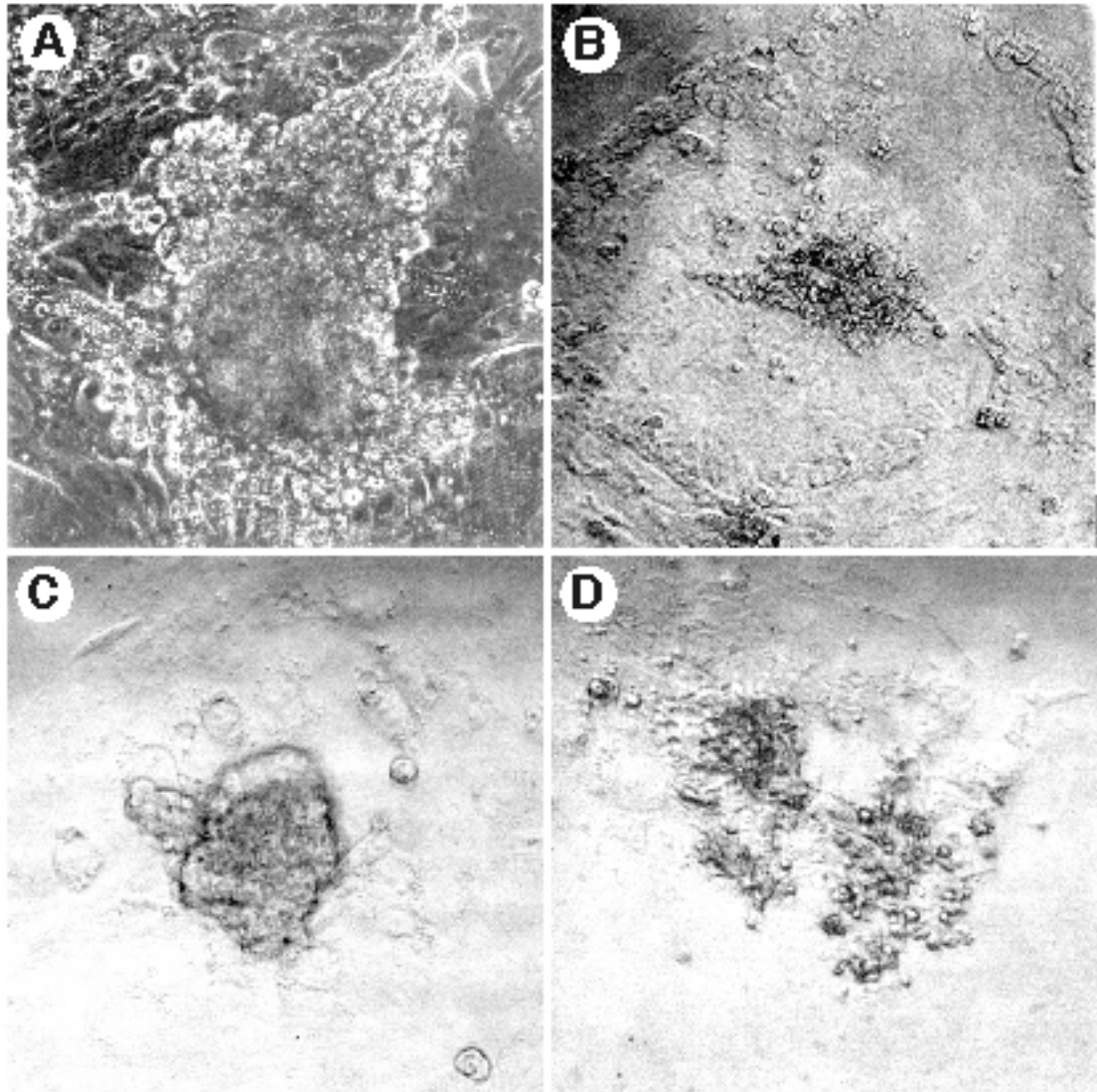
Figure 2.7. Defective outgrowth of *Ssrp1* homologous mutant blastocysts.
E3.5 blastocysts from *Ssrp1* heterozygous intercrosses were cultured *in vitro* for 4 days, both with (A, B) and without (C, D) mouse embryo fibroblast feeder layers. All embryos attached within 24 hours and hatched from their zona pellucidae. The inner cell mass (ICM) of $Ssrp1^{+/t}$ embryos produced adherent colonies of proliferating cells (A, C); whereas, ICM cells from $Ssrp1^{t/t}$ embryos died and/or detached from the culture vessel.

pellucidae. After 4 days of culture, the ICMs from 23 blastocysts formed well-delineated colonies on top of the more adherent trophoblast giant (TG) cells (Fig. 2.7A). ICM cells from the remaining 9 blastocysts, after limited proliferation, appeared to degenerate and/or detach from the dish (Fig. 2.7B). Cells from the 23 ICM-derived colonies were collected into drawn glass capillaries and were analyzed by PCR to determine their genotypes. As summarized in Table 2, eight of the colonies were wild type, 15 were heterozygous for the targeted allele, and none was homozygous for the mutation.

31 additional blastocysts were cultured without MEFs to eliminate the potential for contamination by wild-type feeder cells that might interfere with genotyping. After 3 to 4 days of culture, the ICM from 25 blastocysts formed colonies, although these were smaller than those produced on MEF feeder cells (Fig. 2.7C). ICM cells from the remaining 6 blastocysts degenerated and/or detached from culture vessel, instead of forming distinct colonies (Fig. 2.7D). Two of the defective outgrowths could not be genotyped, but the other four were $Ssrp1^{t/t}$. The genotypes of the 25 normal blastocyst-derived colonies included 7 wild-type, 18 heterozygotes, and no homozygous mutants (Table 2.2). Taken together, these results indicate that the murine $Ssrp1$ gene is necessary for the growth and/or survival of the ICM.

Table 2.2. Genotypes of blastocysts-derived colonies cultured with or without mouse embryo fibroblast feeder layers (MEFs). E3.5 day embryos produced by mating mice heterozygous for for the targeted mutation in the *Ssrp1* gene were cultured with and without MEF feeder layers and were genotyped by nested PCR after 4 days. *These colonies had degenerated and could not be genotyped.

Type	+/+	+/t	t/t	Unknown	Total
With MEFs					
Normal ICM colonies	8	15	0	0	23
Aberrant ICM colonies	0	0	0	9*	0
Without MEFs					
Normal ICM colonies	13	26	0	0	9
Aberrant ICM colonies	0	0	8	3	1

Increased numbers of apoptotic cells in Ssrp1-deficient blastocysts

SSRP1 protein has been reported to bind V(D)J recombination sequences and cisplatin adducts, suggesting a possible role in DNA repair and/or recombination (Malone, Clark et al. 1991; Shirakata, Huppi et al. 1991). DNA repair defects associated with the *Ssrp1* mutation could result in cell death by apoptosis and contribute to the rapidity with which *Ssrp1*-deficient cells lose viability both *in vivo* and *in vitro*. While *Ssrp1*-deficient blastocysts appear normal, freshly isolated E3.5 embryos were analyzed for the presence of apoptotic cells by terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL).

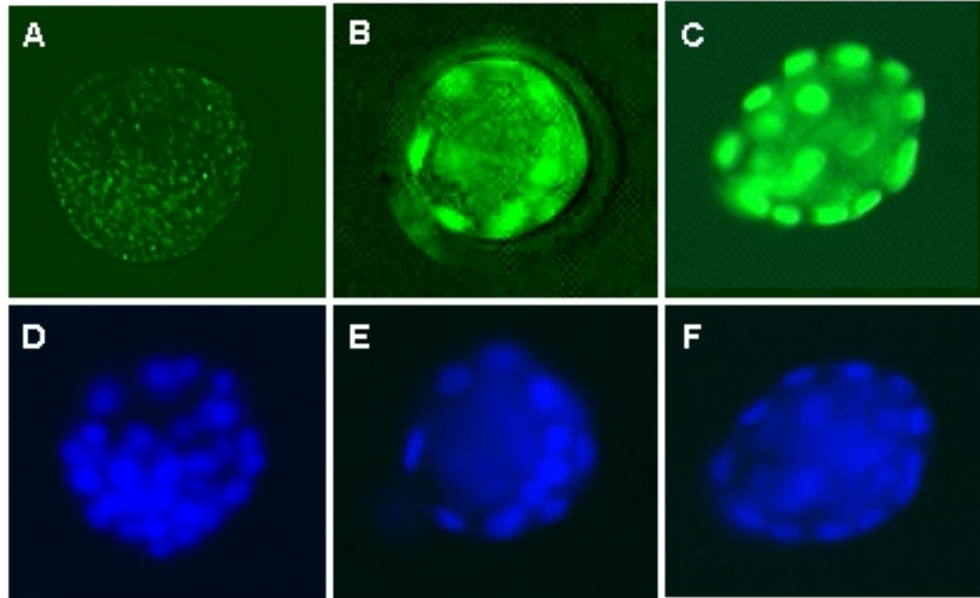**Figure 2.8. TUNEL staining of wild type and *Ssrp*1-deficient blastocysts.**
E3.5 blastocysts from *Ssrp1*$^{+/t}$ intercrosses were fixed, permeablized, and then stained with TUNEL mix (A to C) and with DAPI (D to F). The blastocysts were genotyped as *Ssrp1*$^{+/+}$ (A and D) and *Ssrp1*$^{t/t}$ (B and E). A DNase I treated *Ssrp1*$^{+/+}$ blastocysts (C and F) provided a positive control for DNA fragmentation.

73 blastocysts, of which 24 were wild-type and 20 were homozygous for the *Ssrp1* mutation, were stained, photographed, genotyped and the apoptotic cells were counted blind from coded photographs. Wild-type, heterozygous and mutant embryos averaged 1.2 ± 1.2, 1.7 ± 1.7, and 2.6 ± 3.4 TUNEL-positive cells per blastocyst, respectively, as illustrated in Figure 2.8. The difference between wild-type and mutant embryos, although relatively small, was statistically significant ($p < 0.04$), while the difference between heterozygous and wild-type embryos was not significant ($p < 0.09$). However, these differences do not appear to account for the growth and/or survival defects associated with the *Ssrp1* mutation.

p53-deficiency does not rescue embryonic lethality caused by the *Ssrp*1^t mutation.

As an important regulator of the cellular responses to DNA damage, the p53 tumor suppressor could interact with SSRP1 on several levels. First, a protein complex containing SSRP1 and SPT16 (FACT) has been reported to influence CK2-dependent phosphorylation and activity of p53 (Keller, Zeng et al. 2001). Since FACT enhances transcription elongation on chromatin templates *in vitro*, the complex has been postulated to activate p53 as part of a transcription-dependent mechanism for recognizing DNA damage. Second, several genes involved in the cellular response to DNA damage, including *BRCA*1, *Lig*4 and *XRCC*4, are required for embryonic development. Death of *BRCA*1, *Lig*4 and *XRCC*4 mutant embryos occurs at or before E7.5, E17.5, and E16.5, respectively, and requires active participation by the p53 tumor suppressor, which promotes

apoptosis in DNA repair-deficient cells (Ludwig, Chapman et al. 1997; Frank, Sharpless et al. 2000; Gao, Ferguson et al. 2000). As a consequence, inactivating mutations in p53 delay or prevent embryonic lethal phenotypes associated with these DNA repair deficiencies.

To assess the role of p53 in the death of *Ssrp*1-deficent embryos, mice with mutations in *Ssrp*1 and p53 were intercrossed. Since the *Ssrp*1 and p53 genes are unlinked, one in 16 offspring from mice doubly heterozygous for inactivating *Ssrp1* and p53 mutations is expected to be homozygous for both mutations if p53 deficiency rescues $Ssrp1^{t/t}$ embryos from embryonic death. However, none of the 106 pups analyzed, including 21 $p53^{-/-}$ offspring, was homozygous for the *Ssrp1* mutation. 65 E7.5 to E12.5 embryos were also genotyped, but none was homozygous for the targeted allele. Finally, p53 status had no discernable effect on the outgrowth of $Ssrp1^{t/t}$ blastocysts. Thus, of 25 blastocysts produced by intercrossing $Ssrp^{+/t}$ heterozygotes in a p53-null background, 8 $Ssrp^{+/+}$ and 12 $Ssrp^{+/t}$ gave rise to normal ICM-derived colonies. The remaining 5 blastocyts produced defective colonies, including 3 that were $Ssrp^{t/t}$ and 2 that could not be genotyped. These results indicate that p53 does not contribute to the growth and/or survival defects of *Ssrp1* mutant embryos, either *in vivo* or *ex vivo*.

Discussion

Mouse *Ssrp1* is an essential gene.

My results showed that murine *Ssrp1* is essential for the growth and survival of early embryonic cells both *in vivo* and *ex vivo*. Thus, while a number of high mobility group (HMG) chromatin-associated proteins are encoded by the mouse genome (Baxevanis, Bryant et al. 1995), none of these HMG-box family members appears able to compensate for the loss of *Ssrp1* function. *Ssrp*1 is required for the survival of post-implantation blastocysts and for the viability of cells of the ICM—phenotypes associated with genes essential for cell viability (Carlone and Skalnik 2001; Herceg, Hulla et al. 2001; Medghalchi, Frischmeyer et al. 2001). The results are consistent with studies linking *Ssrp1* expression with cell proliferation (Hertel, De Andrea et al. 1999) and with anti-sense ablation experiments implicating *Ssrp1* in the proliferation of murine fibroblasts (Hertel, Foresta et al. 1997). However, it is still possible that the gene is dispensable in some cell types, such as the case of *Brg1* gene (Bultman, Gebuhr et al. 2000).

The targeted mutation deleted 87 amino acids from the carboxyl-terminus of SSRP1, including the HMG box. Since the HMG box is required for DNA binding, the mutation is expected to inactivate SSRP1 function. The mutation was inherited as simple, recessive embryonic lethal trait; however, due to the severity of the growth and/or survival defects exhibited by *Ssrp1*-deficient embryos, I was unable to recover sufficient numbers of homozygous mutant cells to determine if the targeted mutation is a null allele. ES cells heterozygous for the

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mutation expressed approximately half as much SSRP1 protein as wild-type cells, and did not appear to express truncated forms of the protein. Moreover, heterozygous mice displayed no phenotypes that might result from the trans-dominant activity of a truncated protein.

SSRP1 and HMG protein family

While SSRP1 shares features in common with other HMG box proteins, the protein also has several unique features that distinguish it from other HMG family members. SSRP1 is most similar to HMG-1 within the HMG domain, but unlike HMG-1, SSRP1 is capable of binding DNA in a sequence specific manner (Dyer, Hayes et al. 1998). SSRP1 also contains only a single HMG domain, like the sequence-specific HMG box proteins, SRY and LEF-1/TCF-1a. Because of these and other features, SSRP1 was classified into a distinct subfamily of HMG domain proteins (Baxevanis, Bryant et al. 1995). Since targeted mutations in other HMG family members have thus far resulted in far more restricted phenotypes (Verbeek, Izon et al. 1995; Calogero, Grassi et al. 1999; Bianchi and Beltrame 2000; Staal and Clevers 2000; Britsch, Goerich et al. 2001; Kanai-Azuma, Kanai et al. 2002; Vasseur, Hoffmeister et al. 2002), the present study provides further evidence that *Ssrp*1 encodes unique, non-redundant functions.

Functions of SSRP1 implicated in this study

As a component of the phylogenetically conserved FACT/DUF/SPT16-POB3 complex, SSRP1 appears to assist in chromatin remodeling during

transcription initiation/elongation and DNA replication (Wittmeyer and Formosa 1997; Okuhara, Ohta et al. 1999; Orphanides, Wu et al. 1999; Wittmeyer, Joss et al. 1999; John, Howe et al. 2000; Wada, Orphanides et al. 2000). In *S. cerevisiae*, orthologous functions of SSRP1 appear to be supplied by POB3, which is similar to SSRP1 but lacks the HMG-box domain, and by Nhp6a/Nhp6b, genetically-redundant proteins consisting of little more than HMG boxes (Brewster, Johnston et al. 2001; Formosa, Eriksson et al. 2001). The phenotype of the *Ssrp1*[t] mutation in murine embyros provides additional evidence for phylogenetic conservation between SSRP1 and POB3, as POB3 is required for cell viability in *S. cerevisiae*.

Although initially discovered as a potential regulator of V(D)J recombination, the preferential binding of SSRP1 to the RSS probe but not the mutant probe with a single nucleotide mutation, is more likely the consequence of its secondary the structure rather than primary nucleotide sequence. Noteworthy, HMG-1 and HMG-2, which contain similar HMG box domains, are able to stimulate V(D)J cleavage at the site of RSS in *in vitro* assay (van Gent, Hiom et al. 1997). Thus the binding of SSRP1 on RSS appears to be related to the general ability of HMG proteins to bind distorted DNA conformations (Gariglio, Ying et al. 1997; Vasseur, Hoffmeister et al. 2002).

Several observations suggest SSRP1 may play a role in DNA repair. SSRP1 binds cisplatin DNA adducts (Malone, Clark et al. 1991), and SSRP1 has been reported to enhance the activities of the p53 tumor suppressor (Keller, Zeng et al. 2001; Keller and Lu 2002), and the p53-related protein, p63 (Zeng, Dai et al. 2002). As a component of the FACT complex, SSRP1 would be positioned to

activate p53-dependent responses to DNA damage at the sites of transcription or DNA replication. However, phenotypes caused by deficiencies in several DNA repair genes (e.g. *Brca*1, *Lig*4 and *XRCC*4) are less severe than those caused by loss of *Ssrp*1, and phenotypes resulting from these DNA repair defects, unlike *Ssrp1* deficiency, are reduced in severity in the absence of p53 (Ludwig, Chapman et al. 1997; Frank, Sharpless et al. 2000; Gao, Ferguson et al. 2000). Inactivation of Mdm2, a negative regulator of p53, also results in early embryonic lethality, but has no effect in p53 null animals (Montes de Oca Luna, Wagner et al. 1995). Thus, the phenotype of the $Ssrp1^t$ mutation and apparent lack of genetic interaction with p53, suggest that SSRP1 does not function primarily in DNA repair or as an upstream activator of p53.

In summary, while genetic inferences are necessarily indirect, *Ssrp1* appears to encode non-redundant functions that play essential roles in cellular metabolism.

Attempts at alternative genetic manipulations

While direct functional studies are hampered by the lack of *Ssrp1*-deficient cells, it should be possible to develop a conditional deficient cell line. A conditional complementation vector would be constructed that contains an *Ssrp1* expression cassette and a reporter gene (IRES followed by EGFP or mouse CD24) flanked by loxP sites, and a selectable marker (puromycin resistence gene). Ssrp1 heterozygous ES cells will be transfected with conditional vectors and selected in Puro selection media. Surviving cells, supposedly with stable incorporation of the

conditional vector, will be cultured in high concentration of G418 for loss-of-heterozygozity (LOH) (methods described in Donahue et al. manuscript submitted). The resulting clones would contain both mutated *Ssrp1* alleles, while cells live relying on SSRP1 expressed by the inserted conditional vector. This conditional null cell line, in the presence of Cre recombinase (cell-permeable Cre (Jo, Nashabi et al. 2001; Lin, Jo et al. 2004) directly delivered into the cell or Cre expressed by a transfected Cre expression plasmid (Li, Stark et al. 1996)), would delete the *Ssrp1* expression cassette in Cre/loxP mediated deletion and therefore lose *de novo* expression of SSRP1.

By design, the conditional *Ssrp1* deficient cell line would assist to delineate cellular functions of SSRP1 and cellular response to the sudden loss of the essential gene *Ssrp1*. However, whether conditional null cells, after Cre manipulation, would be viable long enough for the enrichment of null cells and subsequent functional studies, were potential caveats. Together with technical difficulties that hampered the construction of the conditional complementation vector, this approach was discontinued.

RNA interference strategy was also attempted to knockdown SSRP1 expression in various established human and mouse cell lines. An array of 21nt interfering sequences were designed and introduced into cells via plasmid or retroviral systems that express small hairpin RNA (shRNA) (Brummelkamp, Bernards et al. 2002; Yu, DeRuiter et al. 2002). Unfortunately, no cell line with ideal knockdown effect was obtained, partly due to a dilemma that renders the selection of efficient knockdown events intractable. Presumably, cells with

significantly reduced SSRP1 would have impaired proliferation and/or viability, therefore grow slower than the cells that are less affected by RNA interference and would be overwhelmed in culture.

Biochemical analysis of transcription elongation

I also conducted a number of experiments focused on the role of SSRP1 in transcription elongation. I hoped to employ systems in which gene expression is specifically activated at the level of transcription elongation. These involved activation of the HIV LTR promoter by TAT (Ping and Rana 2001; Fujinaga, Irwin et al. 2004) and UV activation of p21 transcription elongation (Espinosa and Emerson 2001; Espinosa, Verdun et al. 2003). Chromatin Immunoprecipitation (ChIP) assays (Kuo and Allis 1999) were used to determine if SSRP1 docks on the transcribed region prior to activating transcription elongation, or was recruited to the transcribed region as a cofactor of Polymerase II complexes engaged in elongation. This study was slowed by technical difficulties of ChIP assay. Moveover, during the course of my studies, several biochemical and genetic studies established the primarily function of FACT as a transcription elongation factor (Belotserkovskaya, Oh et al. 2003; Kaplan, Laprade et al. 2003; Saunders, Werner et al. 2003). *Drosphila* FACT was recruited to and tracked on polytene chromosomes with kinetics similar to RNA Polymerase II (Saunders, Werner et al. 2003). These advances undermined the potential significance of results that I might obtain. Therefore, these studies were discontinued.

Materials and Methods

Southern blot analysis

The *Ssrp1* genotype of ES cells and mice was assessed by Southern blot hybridization to a [^{32}P]dCTP-labeled, 0.7 kb *Bgl*II/*Bam*HI probe derived from genomic sequences located downstream of the 3' homolog region of the targeting vector, as described previously (Williamson, Banik-Maiti et al. 2000).

PCR genotyping

The *Ssrp1* genotype of E6.5 - E8.5 embryos was assessed by PCR, using a mixture of three primers:

a (5'CCGGCCCAGTAGGTATTTTC),

b (5'CAGACTGCCTTGGGAAAAGC)

and c (5'TCCCTCCAAGGAGCTATGTG).

Each 50 µl reaction mix contained 10 mM Tris-HCl (pH8.3), 5 mM KCl, 1.5 mM MgCl$_2$, 200 µM each deoxyribonucleoside triphosphate, each primer at 2 µM, and 2.5 U of Amplitaq (Roche). Reactions involved 30 cycles of denaturation (94°C, 1 min), primer annealing (59°C, 1min), and primer extension (72°C, 2 min).

The *Ssrp1* genotype of E3.5 blastocysts and blastocysts-derived colonies was assessed by nested-PCR. The first reaction used a mixture of 3 primers:

1 (5' AGGCTGGCTGTGACTTAGTG),

2 (5'ACTTGTGTAGCGCCAAGTG),

and 3 (5'CATCCGTGAGGGCTTACT),

as described above but for 20 cycles of denaturation (94°C, 1min), primer annealing (55°C, 1 min), and primer extension (72°C, 2 min). A second round of PCR used primers a, b, and c as described above in a 50 µl reaction.

Primers used for p53 PCR genotyping include:

X7 (5'TATACTCAGAGCCGGCCT)

X6.5 (5'ACAGCGTGGTGGTACCTTAT).

Neo19 (5'CTATCAGGACATAGCGTTGG)

The wild type allele was detected using X7 and X6.5 while the null allele (Lowe, Schmitt et al. 1993) detected with X7 and Neo19. Reactions involved 30 cycles of denaturation (94°C, 1 min), primer annealing (55°C, 1 min), and primer extension (72°C, 2 min) as described above.

Isolation and analysis of *Ssrp1* mutant cells

E3.5 blastocysts were isolated and used to derive ES cell lines as described earlier (Williamson, Banik-Maiti et al. 2000). For studies of blastocyst outgrowth, E3.5 embryos were cultured, either with or without irradiated mouse embryonic fibroblast feeder layers, in ES cell medium [Dulbeccco modified Eagle medium (Mediatech) supplemented with 15% Fetal Bovine Serum (heat-inactivated at 55°C for 30 min), 0.1 mM 2-mercaptoethanol, 100 mM nonessential amino acids, 100U/ml Penicillin-Streptomycin (Gibco BRL)].

The expression of SSRP1 protein in ES cell clones was assessed by Western blot analysis. Cell lysates were fraction by electrophoresis on an 8% SDS-polyacrylamide gel and transferred onto a PVDF membrane (NEN life

science), and SSRP1 proteins were detected by using rabbit polyclonal antibodies raised against the aminoterminus of the protein, as described elsewhere (Hertel, De Andrea et al. 1999).

Analysis of mutant embryos

The morphology of post-implantation embryos was assessed from serial sections (7 μm) of paraffin-inbedded deciduas stained with hematoxylin and eosin (Pawlak, Scherer et al. 2000). Apoptosis in pre-implantation embryos was detected with an *in situ* cell death detection kit (Roche). Blastocysts were fixed with 4% paraformaldehyde in PBS for 15 minutes and then permeablized for 30 minutes with 0.3% Triton X-100 and 1.5% bovine serum albumin in PBS. Blastocysts treated with 0.5 mg/ml DNase I for 10 minutes provided positive controls for DNA fragmentation. Embryos were incubated with the TUNEL reaction mix for 1 hour at 37°C, and cell nuclei were stained with DAPI (1 mg/ml in PBS) for 10 minutes. The embryos were viewed and photographed using a fluorescence microscope and then genotyped by nested PCR.

**FUNCTIONS OF MOLECULAR CHAPERONE PREFOLDIN REVEALED
IN PFDN1 NULL MICE GENERATED BY GENE ENTRAPMENT**

Introduction

An important cellular process is the conversion of nascent translation products into properly folded functional proteins. Although the three dimensional conformations of polypeptides are determined by their linear sequences, prompt and efficient protein folding in the crowded cellular environment often requires assistance of a group of proteins termed "molecular chaperones" (Hartl and Hayer-Hartl 2002). The maturation of cytoskeletal proteins such as actin and tubulin, involves a chaperone named prefoldin, a complex consisting of 6 subunits (Vainberg, Lewis et al. 1998). The mouse *Pfdn1* gene, which encodes the first subunit of prefoldin, was disrupted by a polyA gene trap vector in ES cells and the mutation was subsequently introduced into the mouse germline. This introduction will briefly describe background information about prefoldin and establish the rationale for studying *Pfdn1* function of *in vivo*.

Protein folding and chaperonin

To assist the proper folding of nascent peptides and to avoid protein misfolding and aggregation, molecular chaperones cooperate with each other in protein folding pathways. In eukaryotic cells, the majority of nascent peptides form a complex with Heat shock protein 70 (Hsp70s) (Eggers, Welch et al. 1997;

Pfund, Lopez-Hoyo et al. 1998), while others require the function of a chaperonin (Yaffe, Farr et al. 1992; Sternlicht, Farr et al. 1993).

Chaperonins are large, multi-subunit, cyclindrical protein complexes with a central cavity for the binding and folding of nascent chain substrates (Kim, Willison et al. 1994; Spiess, Meyer et al. 2004). There are two groups of chaperonins: Group I, includes the bacterial GroEL and mitochondrial Hsp60 proteins; and Group II, include the archaeal TF55, and eukaryotic TRiC/CCT/c-cpn [for T-complex polypeptide1 (Tcp1) ring complex or chaperonin-containing Tcp1 or cytosol chaperonin (Frydman, Nimmesgern et al. 1992; Gao, Thomas et al. 1992; Kubota, Hynes et al. 1994). In eukaryotic cells, TRiC processes the folding of approximately 10% of nascent proteins (Thulasiraman, Yang et al. 1999). Many TRic substrates are cytoskeletal components: α-and β-actin, α-,β- and γ- tubulin, actin-related protein V, cofilin and actin-depolymerizing factor 1 (Frydman, Nimmesgern et al. 1992; Gao, Thomas et al. 1992; Melki, Vainberg et al. 1993; Ursic, Sedbrook et al. 1994; Melki, Batelier et al. 1997). However non-cytoskeletal substrates have also been identified such as transducin, cyclin E, Von Hippel-Lindau (VHL) tumor tumor suppressor (Farr, Scharl et al. 1997; Won, Schumacher et al. 1998; Feldman, Thulasiraman et al. 1999), Cdc20 and several proteins containing tryptophanaspartic acid (WD) repeats (Camasses, Bogdanova et al. 2003; Siegers, Bolter et al. 2003).
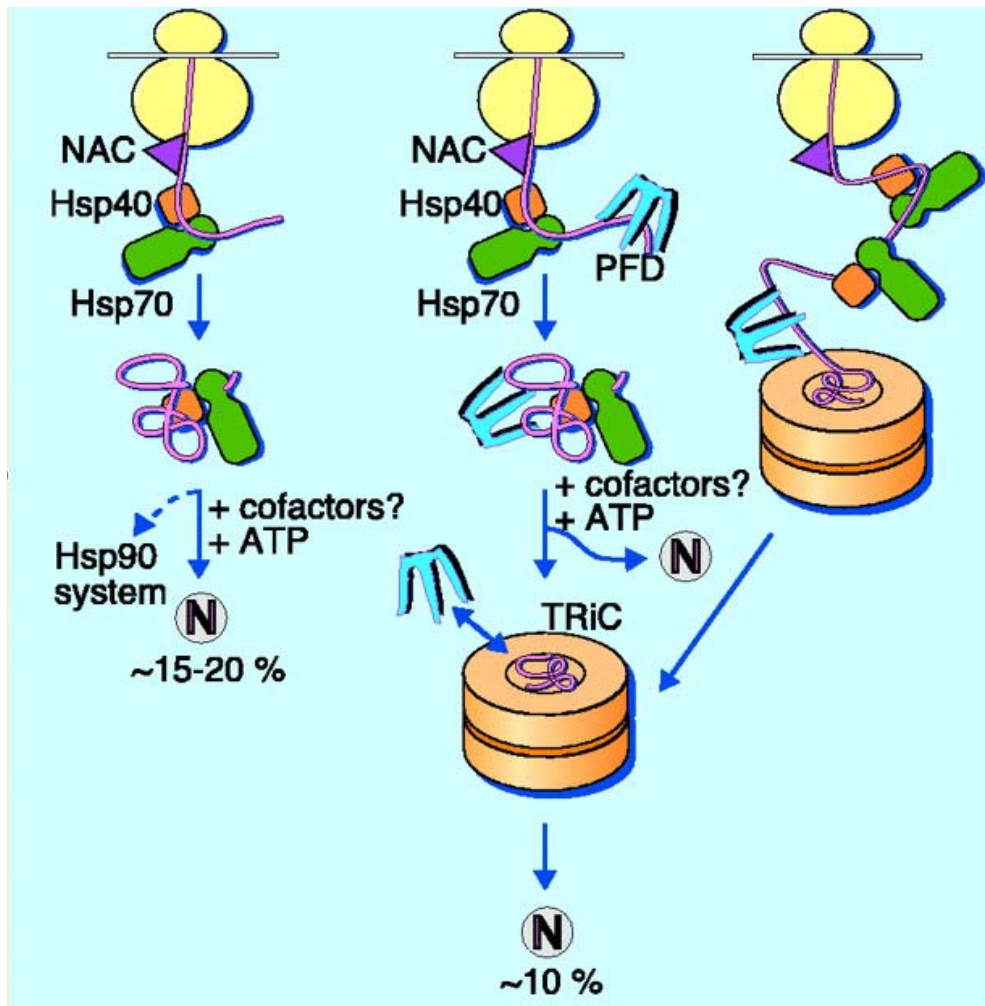
Figure 3.1 Protein folding pathways in eukaryotic cells.
(From Hartl, Hayer-Hartl et al. 2002)
The majority of newly synthesized polypeptides may fold
properly without further assistance. About 15 to 20% of
peptides reach their native states through a folding reaction
assisted by Hsp70 and Hsp40, and a fraction of these must be
transferred to Hsp90 for folding. About 10% of the chaperone-
assisted polypeptides are co- or posttranslationally passed on to
the chaperonin TRiC in a reaction mediated by PFD.

NAC: Nascent chain-associated Complex.
PDF: Prefoldin complex.
TRiC: Tcp-1 ring complex.
N: Native conformations of proteins.

A fraction of TRiC-mediated folding also requires one cytosolic chaperone prefoldin, a heterohexameric protein complex that is conserved in archaea, yeast (as GimC) but not bacteria (Geissler, Siegers et al. 1998; Vainberg, Lewis et al. 1998).

Prefoldin functions in the folding of cytoskeleton proteins

The prefoldin complex consists of 6 subunits, namely prefoldin 1-6, Prefoldin was initially purified for its ability to bind unfolded actin (Vainberg, Lewis et al. 1998). The yeast homologue of prefoldin the GimC complex, consists of Gim1 through Gim6 proteins. Deletion of these genes is not lethal, but leads to impaired function of the actin/tubulin cytoskeleton. The phenotype is similar to that of a temperature sensitive mutation of TRiC, which is required for cell viability (Vainberg, Lewis et al. 1998). In *in vitro* assays, prefoldin binds nascent chains of actin and tubulin, but not non-cytoskeletal substrates, and presents them to TRiC (Hansen, Cowan et al. 1999). (Figure 3.1 provides a schematic of the pathway). Yeast GimC delivers newly translated actin and tubulin to TRiC, as indicated by reduced rate of actin folding in cells lacking GimC function. (Siegers, Bolter et al. 2003). Structural studies revealed that prefoldin subunits form a pocket-like structure and interact with nascent peptides at the end of their coiled-coils domains (Siegert, Leroux et al. 2000; Martin-Benito, Boskovic et al. 2002). The interaction between prefoldin, unfolded actin

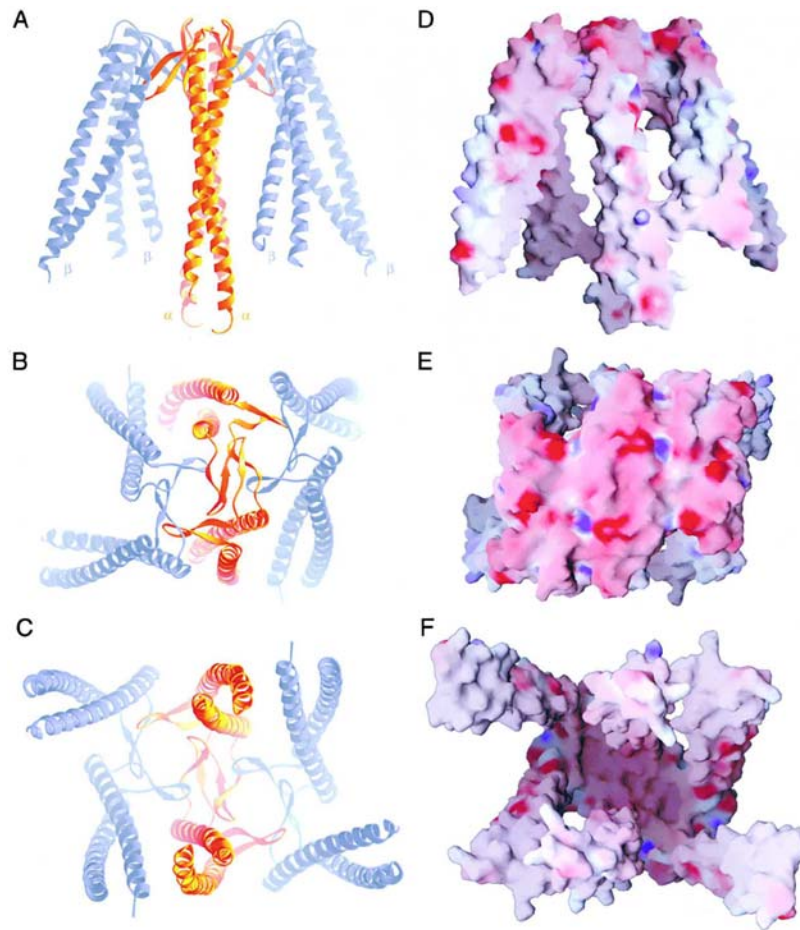**Fig. 3.2. Structure of the prefoldin heterohexamer complex.**
(From Siegert, Leroux et al. 2000)
(A-C) Ribbon representation of the prefoldin hexamer shown
from the side (A), top (B) and bottom (C). All ribbon diagrams
were generated using Ribbons. (D-F) The molecular surface of
the prefoldin hexamer. Red color indicates acidic and blue basic
surface regions. All surfaces were generated using GRASP.

and TRiC was also demonstrated by electron microscopy (Martin-Benito, Boskovic et al. 2002). (Figure 3.2) Collectively, these genetic, biochemical, kinetic and structural studies establish the cellular function of prefoldin as a co-chaperone that assists in TRiC-mediated folding of cytoskeleton proteins such as actin and tubulin (Figure 3.1).

Actin and tubulin, as basic components of the cytoskeleton, play fundamental roles in various cellular functions, such as, maintaining cell shape, providing a scaffold for docking and transport of cellular macromolecules, cell motility, cell adhesion and cell division. Actin is also involved in tissue-specific functions of the cytoskeleton, such as muscle contraction in smooth and cardiac muscle, outgrowth of axons and dendrites in neurons, and formation of neuronal and immunological synapses. However, deletion of the alpha-actins in mouse, does not lead to embryonic death. (Small, Rottner et al. 1999; Dustin and Cooper 2000; Luo 2002).

An Embryonic Stem (ES) clone containing a mutation in *Pfdn1* was identified from a gene entrapment library of 129sv ES cells generated using polyA entrapment vectors (Lin et al. Manuscript submitted). The sequence of the 3' RACE product from the p3b3A6 clone matched the fourth and last exon of *Pfdn1* (Figure 3.3 B), indicating that the retrovirus was inserted into the last intron. By design, *Pfdn1* -Vector fusion transcript expressed by the mutated allele should not contain Exon4, which encodes 27 amino acids of the 122 amino acids prefoldin, part of the coiled-coil domain that is required for interactions between

prefoldin and peptide substrates (Siegert, Leroux et al. 2000). Mice and/or cells containing this mutation would assist in delineating the *in vivo*i functions of mammalian *Pfdn1*. In addition, since the deletion of yeast prefoldin causes cytoskeletal defects, it would be interesting to know if *Pfdn1* mutants cause similar defects in mammalian cells and how these defects might be manifest in the whole animal.

In particular I wished to answer the following questions:

1) Does the insertion of GTR1.3 in the last exon of *Pfdn1* completely ablate *Pfdn1* expression or does the insert cause a hypomorphic mutation?

2) What is the effect of *Pfdn1* mutation on mouse development? The potential importance of a functional cytoskeleton suggests that loss of Prefoldin function could cause embryonic death, if not cell lethality.

3) What is the phenotype of mutant mice if they are viable? What organ systems are specifically affected?

4) If mutant cells are viable, do they have specific cellular defects? Is the cytoskeleton altered? Is actin maturation affected? Is the assembly or function of the cytoskeleton affected?

Disruption of the *Pfdn1* gene by gene entrapment

Pfdn1 was disrupted in embryonic stem (ES) cells by the insertion of the GTR1.3 gene trap retrovirus. Gene entrapment involved expression of a neomycin resistance gene from transcripts that splice from the GTR1.3 vector to the fourth and last exon of the *Pfdn1* gene (Figure 3.3 A). The sequence of the 3'RACE product derived from *Neo* fusion transcripts is shown in Figure 3.3 B. The mutation is expected to disrupt *Pfdn1* expression since the resulting *Pfdn1*-Vector fusion transcripts lack Exon 4, deleting 27 amino acids of the 122 amino acids prefoldin protein. The deleted sequences contain part of the coiled-coil domain that is thought to be required for interactions between prefoldin and peptide substrates (Siegert, Leroux et al. 2000). The location of the provirus in the third intron was determined by cloning genomic DNA sequences adjacent to the targeting vector by inverse PCR. The sequences of the inverse PCR product is shown in (Figure 3.3 C). PCR primers based on the flanking genomic DNA and 3'LTR sequences were used to genotype wild type and mutant alleles (Figure 3.3 A). ES cells containing the *Pfdn1* mutation were injected into C57BL/6 blastocysts and gave rise to germ line chimeras. *Agouti* offspring containing the mutated allele were identified by PCR analysis and intercrossed. The offspring of heterozygote intercrosses were PCR genotyped (Figure 3.3 D).
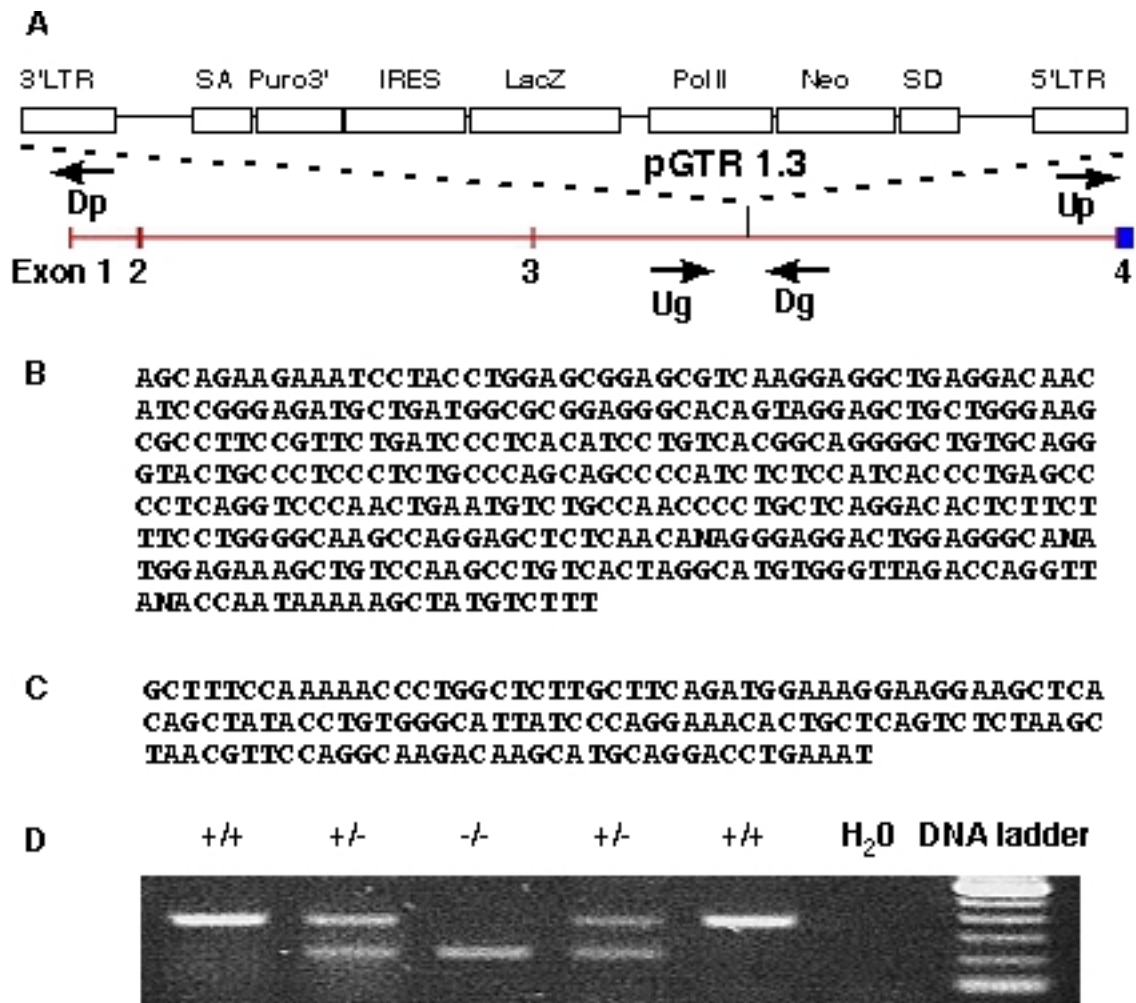
A

3'LTR   SA Puro3'   IRES   LacZ   Pol II   Neo   SD   5'LTR

pGTR 1.3

Dp   Up

Exon 1 2   3   4

Ug   Dg

B

AGC AGAA GAAA TCC TAC C TGGA GCGGA GCGTC AAGGA GGC TG AGGAC AAC
ATC CGGG AGA TG C TGA TGGCGC GGAGG GCACA GTAGG AGC TG C TGGG AAG
CGC C TTC CGTTC TGA TC CC TCA CATCC TGTCA CGGCA GGGGC TGTGC AGG
GTA C TGC CC TCC C TC TGCCCAG CAGCC CCATC TC TCC ATCAC CC TGA GCC
CC TCAGG TCCCA AC TGA A TG TC TGCCA ACCCC TGC TC AGGAC AC TC T TC T
T TC C TGGG GGCAA GCCAG GAGC TC TCAA CA NA GGGAGG AC TGG AGGGC A NA
TGG AGAA AGC TG TCCAA GCC TG TCAC TAGGCA TG TGGG T TAG AC CAG GTT
A NA CCAA TAAAA AGC TA TGTC T TT

C

GC T T TCCA AAAAC CC TGGC TC T TGC T TC AGA TGG AAAG GAAGG AAGC TCA
CAGC TA TA CC TGT GGGCA T TA TC CCAGG AAACA C TGC TCAGTC TC TAA GC
TAA CGTTC CAGGC AAGAC AAGCA TGCAG GACC TGAAAT

D

+/+   +/-   -/-   +/-   +/+   H₂0   DNA ladder

Figure 3.3. Disruption of the *Pdfn*1 gene by the insertion of GTR1.3.

A. A schematic diagram of the genomic organization of *Pdfn1* gene, and the insertion of the GTR1.3 gene trap retrovirus. The inverse PCR and genotyping primers corresponding to provirus (Up, Dp) and genomic (Ug, Dg) sequences are indicated.

B. Sequence of the 3' RACE product amplified from Neo-exon4 fusion transcripts.

C. Sequence of the inverse PCR product amplified with Up and Dp primers.

D. Three-primer PCR analysis of tail DNA samples from 3-week-old offspring of *Pfdn*1 heterozygous intercrosses. The 365bp band and 215bp band represent wild type (Ug and Dg) and mutant alleles (Ug and Dp), respectively.
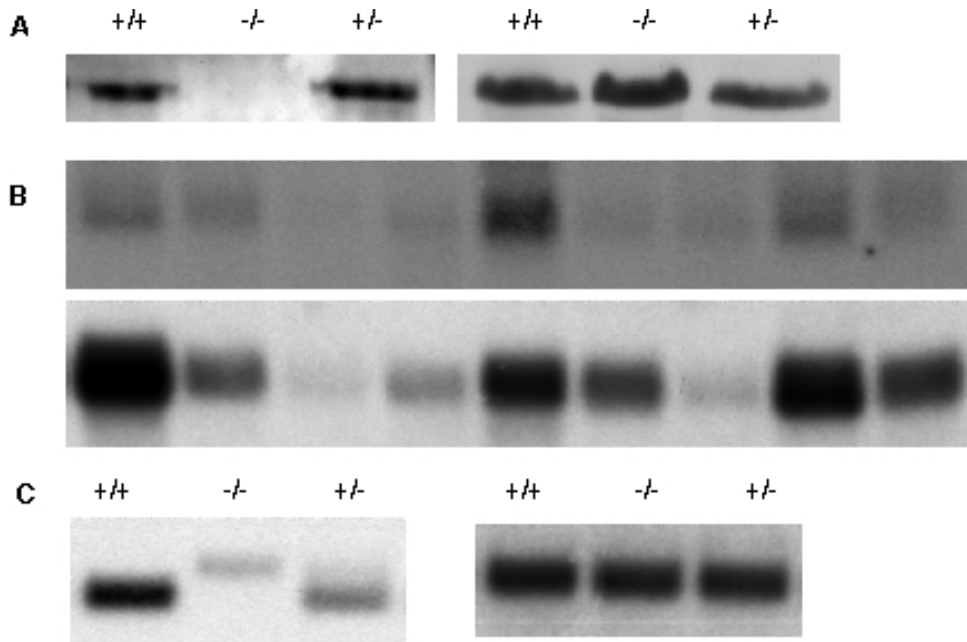
Figure 3.4. *Pfdn*1 is expressed in wild type, heterozygous but not homozygous mutant mice.

A. Western blot analysis of Prefoldin 1 expression in WT, heterozygous and homozygous mutant Mouse Embryo Fibroblasts (MEFs). 80μg of whole cell extracts from *Pfdn1* +/+, +/- and -/-cells were analyzed with antisera against the internal region of Prefoldin1 (Left panel). A 20 kDa protein was detected in both *Pfdn1* +/+ and +/- cells, but not *Pfdn1* -/- cells. The blot was stripped and reprobed with an anti-HnRNP C antibody to provide a loading control.

B. Northern blot analysis of *Pfdn1* transcripts in different tissues. From left to right: Muscle,Thymus, Spleen, Gut, Kidney, Liver, Lung, Heart, Brain. The blot was reprobed with a glyceroldehyde phosphate dehydrogenase (GAPDH) probe to provide a loading control (bottom)

C. Northern blot analysis of MEFs. 10μg of RNA from *Pfdn1*+/+, +/-, -/- MEFs were separated, transferred and detected with radio-labeled probe corresponding to 5' region of *Pfdn1* mRNA. A GAPDH re-blot is shown in the right panel.

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The complete disruption of *Pfdn1* expression was suggested by Western blot analysis of lysates from homozygous mutant MEFs. A 20 kDa band was detected in wild type and heterozygous MEF's but not in homozygous mutant MEFs (Figure 3.4 A left panel). Equal loading was confirmed by the levels of hRNP C detected in parallel Western Blots. (Figure 3.4A right panel).

Pfdn1 is ubiquitously expressed in mouse organs and tissues (Figure 3.4 B). Northern blot analysis of RNA from mutant MEFs, revealed the complete loss of the wild type *Pfdn1* transcript and the appearance of a larger transcript. (Figure 3.4 C). Expressed at lower levels, these transcripts presumably correspond to fusion transcripts generated by splicing among upstream *Pfdn1* exons and the 3' Puro-LacZ sequence carried by the provirus. However, Western blot analysis failed to detect altered proteins expected to be expressed from these transcripts.

Viability and growth of *Pfdn*1 mutant mice

36 of the 161 offspring produced by heterozygote intercrosses were homozygous for the *Pfdn1* mutation, close to a Mendelian ratio. *Pfdn1* -/- mice were smaller in size than wild type and heterozygous littermates (Figure. 3.5 A), grew more slowly (Figure 3.5 B), and most died before 5 weeks of age (Figure 3.5 C). Some homozygous mutants were distinguishably smaller at birth. Average weights of mutant mice at 2, 3 and 4 weeks were 3.9±0.6g (n=14), 5.3±1.0g (n=13) and 4.9±1.0g (n=10) respectively, while the wild type and heterozygous
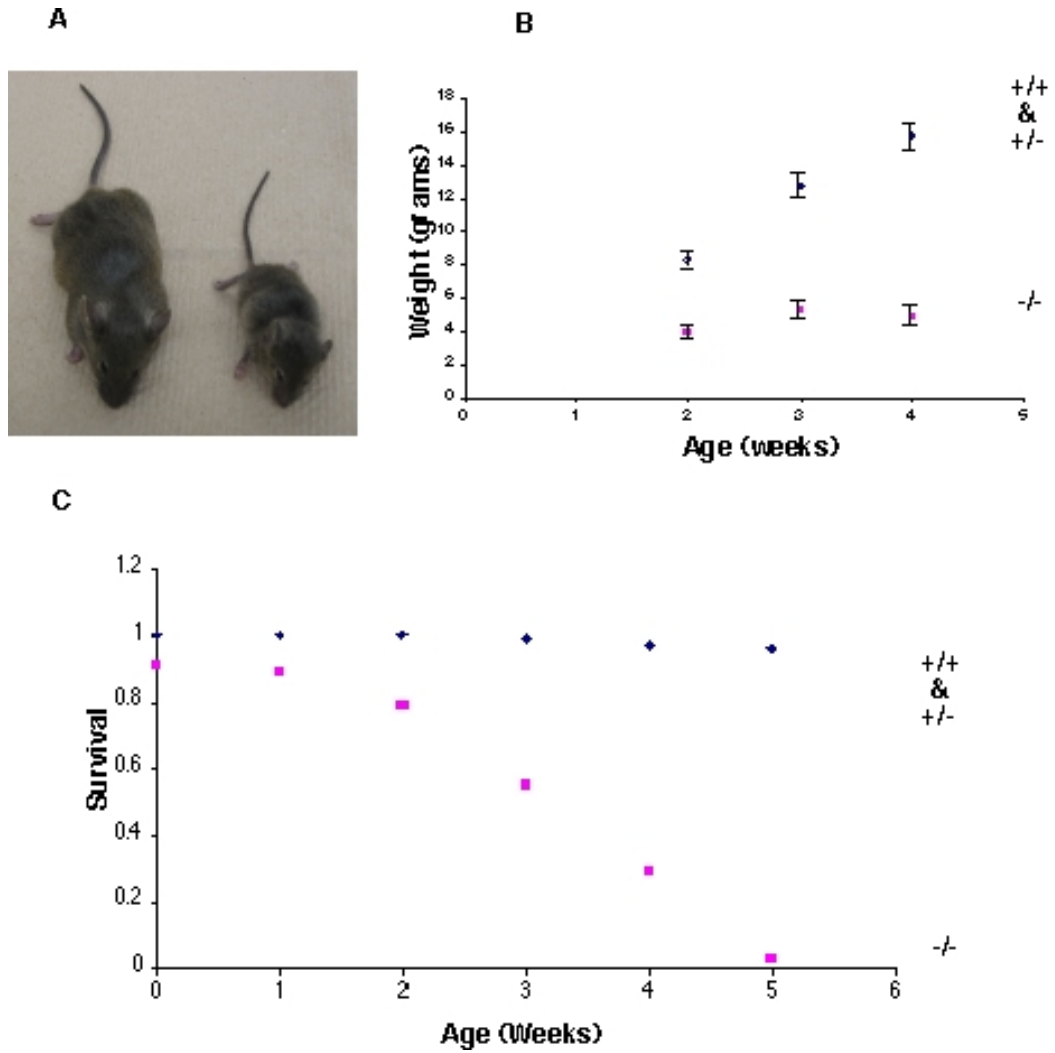
Figure 3.5. Growth and survival of *Pfdn1-/-* mice.
    A. *Pfdn1+/-* and -/- littermates at 4 weeks of age.
    B. Growth of *Pfdn1-/-* mice is severely retarded.
    C. Survival of offspring from *Pfdn1+/-* intercrosses.

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littermates weighed 8.3±0.8g (n=26), 12.7±1.8g (n=22) and 15.7±1.7g (n=17) respectively. The weight of *Pfdn1-/-* mice declined 0.3-0.7g (0.6±0.2g, n=5) during the last week before death, indicating physical wasting. In addition, ~40% homozygous mutant mice were observed with uncoordinated movement, indicating neuromuscular dysfunction.

Histological Analysis of *Pfdn*1 mutant mice

8 mutant mice and 8 control littermates 3 days to 5 weeks old were sacrificed and examined histologically. Sections from head, chest, hinder quarter and internal organs were stained with Hematoxylin and Eosin. All mutant mice had overall dwarfism and abnormalities in the brain and spleen. However, muscles, eyes, teeth, liver, kidney, gut, heart and lung of the mutants appeared normal. In addition, infected sinuses were seen in 3 mutant mice but not in control littermates.

Defective Commissures in Central Nervous System

Coronal sections through the brain of mice at 2 weeks and 4 weeks revealed disorganized and hypocellular Corpus Callosum (CC) in homozygous mutant mice (Figure 3.6 B). Dilated ventricles were also observed in mutant brains. However, this may be an effect of strain background and /or differences in tissue preparation. H&E stains of Sagittal sections, also revealed disrupted cerebellar commissures (cbc) in 4 week old mice (Figure 3.6 D).
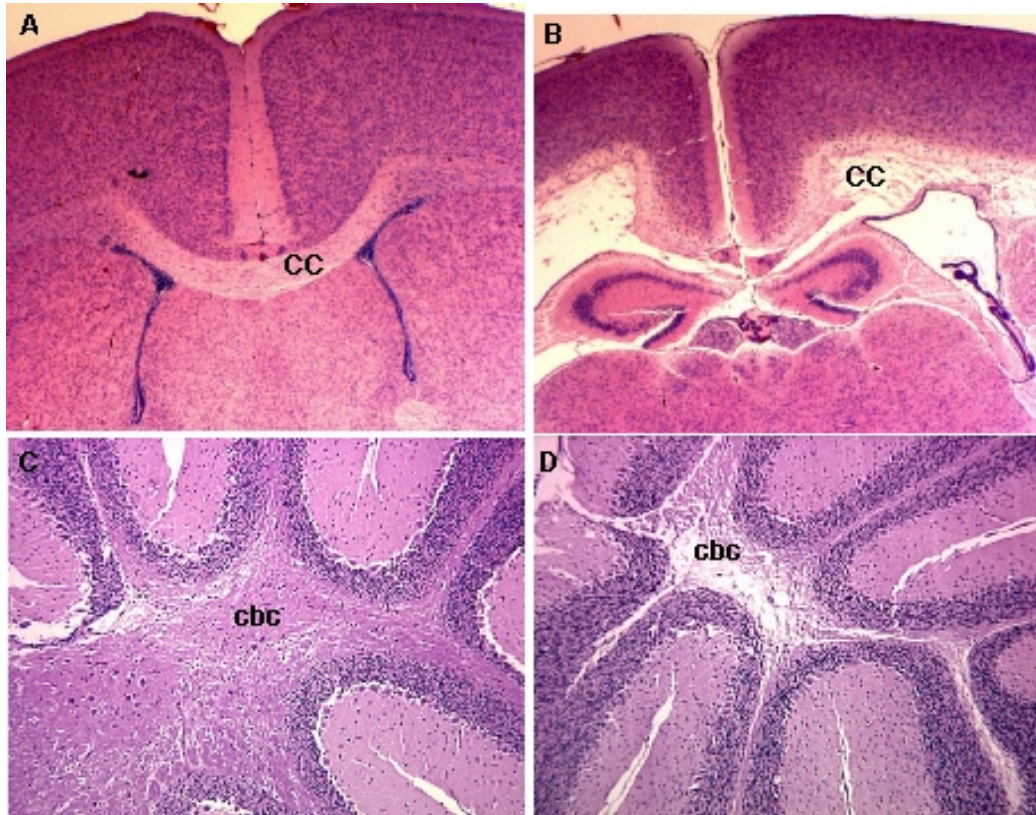
Figure 3.6. Defective neural commissures in *Pfdn1*-/- mice.
Coronal sections through the cerebrum (A.B.) or sagittal sectons through the cerebellum (C.D.) of 2 weeks old, *Pfdn1*+/- (A.C.) and *Pfdn1*-/- (B.D.) mice. Corpus callosum (CC) and cerebellar commissure (cbc) are indicated.

This cerebellum defect may be responsible for the uncoordinated movements of some mutant mice. While commissure defects are severe and consistent, general neuronal defects were not observed.

Defective hematopoiesis in *Pfdn*1-/- mice

There were fewer erythrocytes and white blood cells in the peripheral blood of Pfdn1-/- mice. Hemoglobin levels in mutant blood cells appeared to be normal. Although the reduction in erythrocyte counts was modest, micronuclei were prominent in ~2% of the mutant erythrocytes, as compared to 0.1% of wildtype and heterozygous mice (Figure 3.7 B). Micronucleated erythrocytes may result from defects in DNA synthesis annucleation and/or removal of micronucleated cells by spleen macrophages. Increased segmentation appeared in the nuclei of the homozygous mutant neutrophils (Figure 3.7 D).

Lymphopoiesis defects in *Pfdn1*-/- mice

The spleen and thymus were significantly smaller in *Pfdn*1-/- mice; 16.9 ± 4.4% (n=8) of the size of organs from littermate controls, or 36.7 ± 12.3% (n=8) of the size after normalizing against body weight. Total cell numbers retrieved from these organs were similarly reduced: 12.4 ± 7.1% (n=8) of control organs. In mutant spleens, the germinal centers were not clearly defined and the surrounding red pulp was disorganized (Figure 3.8 B). Thymuses from mutant mice were smaller but appeared to have normal histology.
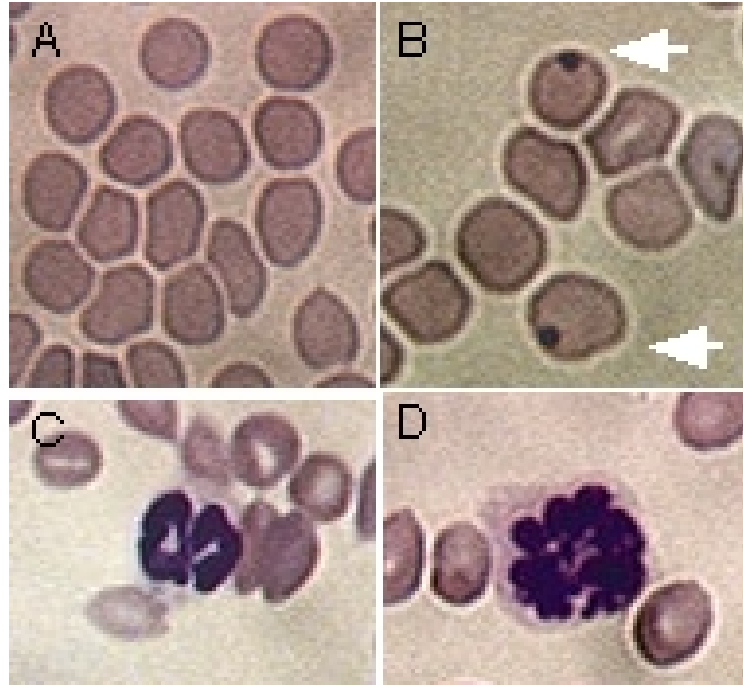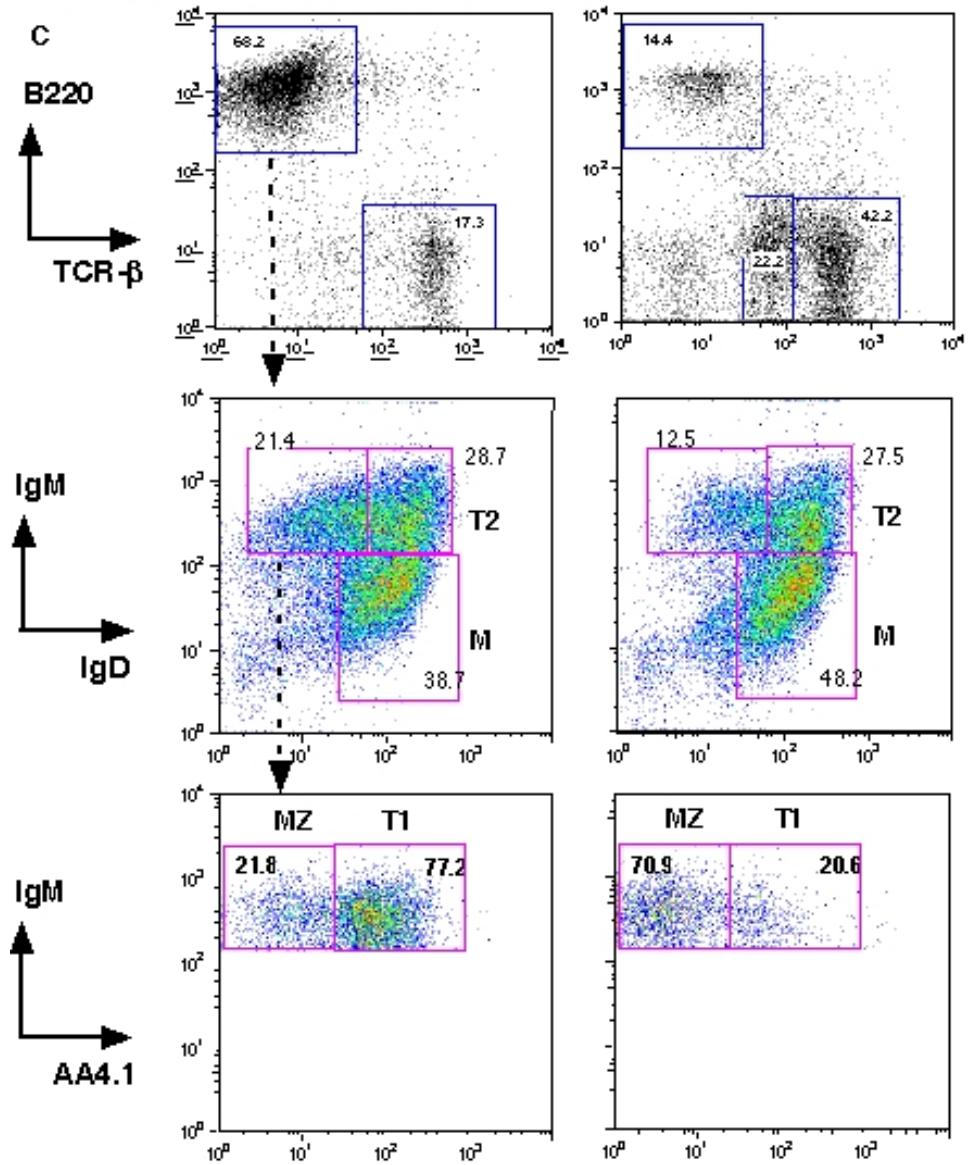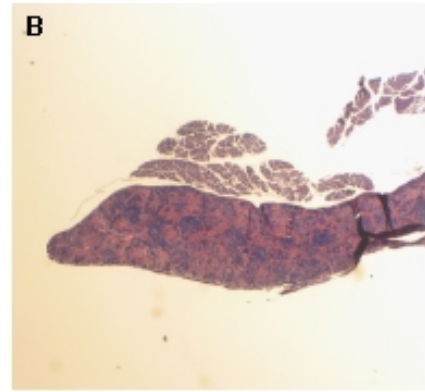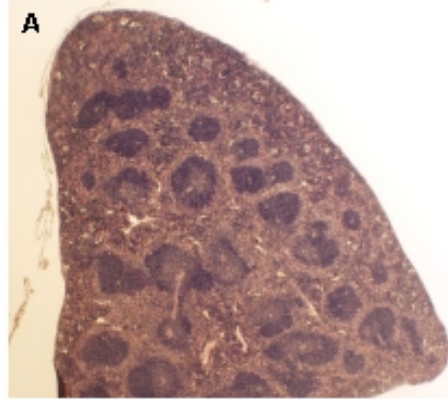
Figure 3.7. Hematopoietic defects in *Pfdn1*-/- mice.
Erythrocytes in wild type (A) appear normal whereas erythrocytes from
Pfdn1 deficient mice (B) frequently contain micronuclei (arrows).
Normal neutrophil (C) and abnormal nuclear segmentation in
neutrophil from *Pfdn1* deficient animal (D).

FACS analysis of spleen and bone marrow revealed significant decreases in the number and proportion of B cells in mutant mice; only 27.1 ± 7.6 % (n=5) spleen cells were B220 positive, as compared to 62.3 ± 7.8 % (n=5) for wild type or heterozygous littermates. Representative FACS profiles are shown in Figure 3.8 C. In concert with the dramatic change of architecture of the mutant spleen, the decreased percentage of B cells indicates defective B cell development in mutant mice. The number of T1 (IgM^{hi}, IgD^{lo}, $AA4.1^{hi}$) cells was reduced to a greater extent than mature B cells (IgM^{lo}, IgD^{lo}) (Figure 3.8 C lower panels), suggesting that the production or migration of B cell precursors from the bone marrow (BM) are reduced to a greater extent than B cell maturation..

B220$^+$ cells in the mutant BM were greatly reduced. In addition, among mutant B220$^+$IgM^{lo} cells, only 25.4 ± 4.1 % (n=4) were Pre-B (CD25^{hi}), as compared to 82.9 ± 1.3 % (n=4) for littermate controls. This indicates a severe reduction in B cell precursors in *Pfdn*1 mutant mice (Figure 3.8 D).

There appeared to be a higher percentage of TCR-beta positive T cells in mutant spleens, reflecting the relative lack of B cells. However, the percentages of CD4$^+$ cells were significantly higher. The ratio of CD4$^+$ to CD8$^+$ T cells in 5 mutant spleens was approximately 8 to 1, as compared to about 2 to 1 for WT and heterozygous littermates. Disproportionate numbers of CD4 T cells was also observed in the mutant thymus (Figure 3.8 E). Also strikingly, the percentage of CD4$^+$CD8$^+$ T cells was significantly lower in mutant thymus: 10.9 ± 3.1 % (n=5), than normal littermates: 77.7 ± 5.5 % (n=5).

A

B

C

B220

TCR-β

68.2   17.3

14.4

42.2

22.2

IgM

IgD

21.4   28.7   T2

38.7   M

12.5   27.5   T2

48.2   M

IgM

AA4.1

MZ   T1

21.8   77.2

MZ   T1

70.9   20.6
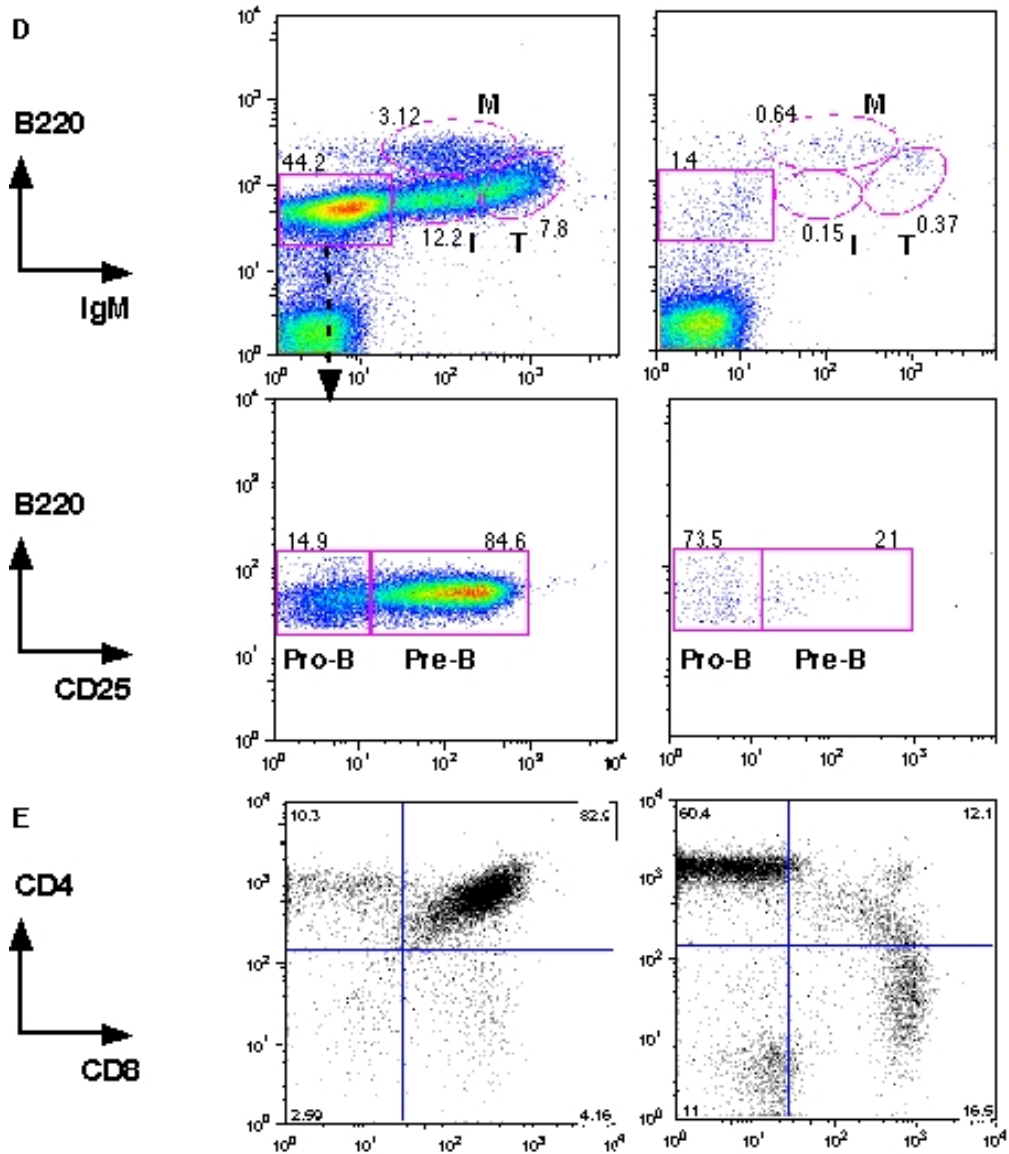
Figure 3.8. *Pfdn1*-/- lymphoid systems were defective.
A.B. Hematoxylin and Eosin staining of spleen sections from Pfdn1+/+(A.)
   and Pfdn1-/-(B.). GerminalCenter (GC).
C. Reduction of B cells, especially T1 B cells in mutant spleen. Splenocytes from control
   littermates (left panel) and mutants (right panel) were analyzed for B220 and TCR-β
   expression. IgM/IgD expression of B220+ cells were analyzed in the following panels.
   IgM^{hi}IgD^{lo} cells were further analyzed for AA4.1 expression. T1:Transitional 1 B cells;
   T2: Transitional 2 B cells; M: Mature B cells; MZ: Marginal Zone B cells.
D. Reduction of B220+ cells, especially Pre-B cells in mutant Bone Marrow. Bone
   Marrow cells from control littermates (left panel) and mutants (right panel) were
   analyzed for B220 and IgM expression. B220+IgM^{lo} cells were further analyzed for
   CD25 expression. M: Mature circulating B cells; T: Transitional B cells; I: Immature B
   cells.
E. Disproportion of T cell populations in mutant thymus. TCR positive cells were
   analyzed for CD4 and CD8 expression.

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In summary, loss of *Pfdn1* function impairs development of major lymphoid organs, and reduces the number of lymphocytes in early developmental stages, especially pre-B cells and CD4$^+$CD8$^+$ T cells.

Aberrant Actin cytoskeleton in *Pfdn1-/-* MEFs

It is well established in yeast that proper cytoskeleton formation requires prefoldin. Deletion of *pfd5 in* yeast, while not lethal, led to cytoskeletal alterations, as indicated by phalloidin staining morphological alterations and budding defects. (Vainberg, Lewis et al. 1998).

Mouse Embryo Fibroblast lines were isolated to study the impact of *Pfdn1* mutation on a mammalian cytoskeleton. Cells from E13.5 embryos produced from *Pfdn1*+/- intercrosses were cultured at near-confluent densities and passed 1:2 every two days. *Pfdn1-/-* MEF cells were viable, but appeared to grow more slowly than wild type cells. The average time for the doubling of *Pfdn1-/-* MEFs at an initial number of 6x10^5 exceeded 4 days, as compared to 2-3 days for wild type and heterozygous MEFs. Unlike conventional fibroblasts cultured. *Pfdn1-/-* MEFs appeared to be round and flat in shape (Figure 3.9).

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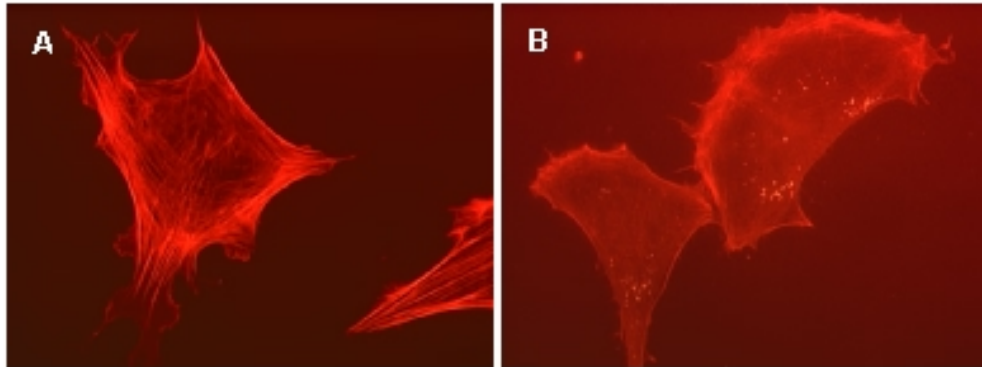
Figure 3.9 Actin filaments in *Pfdn1+/+* MEFs(A.) and *Pfdn1-/-*MEFs(B.).
Wild type and *Pfdn1* null MEFs were fixed, permeablized and stained with
Alexa 594- phalloidin. The stained actin filaments in null cells appeared to be
slimmer, shorter and less organized than those in wild type cells.

Phalloidin staining revealed abnormal actin filaments (F-actin) in *Pfdn1-/-* MEFs (Figure 3.9 B). F-actins in *Pfdn1* null cells were apparently shorter and less organized than in wild type cells. Moreover, instead of spreading throughout the cell actin filaments were mostly localized to the periphery of Pfdn1-deficient cells. Finally Pfdn1-/- cells contained cytoplasmic phalloidin-stained speckles. The abnormal localization and poor organization of F-actin is consistent with the phenotype of mutant yeast lacking prefoldin function (Vainberg, Lewis et al. 1998). Some defects in pfd5Δ yeast, such as impaired nuclear division in a small proportion of cells, were not observed in the established *Pfdn1* null MEFs, however, nuclear division defects were not examined during the process of cell establishment.

<center>**Discussion**</center>

Mutagenicity of GTR1.3 polyA trap

PolyA gene traps preferentially insert into the last intron of cellular genes, (REFs), which in some cases may fail to disrupt gene expression. However, the insertion of GTR1.3 in the last intron of *Pfdn1* completely ablated the *Pfdn1* expression as indicated by Western blot analysis. Although fusion transcripts containing *Pfdn1* upstream exons were present at low levels, fusion proteins expressed from these transcript were not detected.

Pfdn1 is not an essential gene

Previous studies have established the important role of prefoldin in the maturation of essential proteins such as actin and tubulin (Vainberg, Lewis et al. 1998; Hansen, Cowan et al. 1999; Siegert, Leroux et al. 2000; Martin-Benito, Boskovic et al. 2002; Siegers, Bolter et al. 2003). Considering the cytoskeletal defects caused by deletion of a yeast *pfd* gene (Vainberg, Lewis et al. 1998) mouse *Pfdn1* is surprisingly not an essential gene. The complete deletion of *Pfdn1* function did not result in embryonic death or obvious defects in organs such as heart, lung, liver, kidney, gut, eye, tooth, bone and muscle. Apparently, the basic cellular functions of the cytoskeleton such as cell division, cell shape maintenance, intracellular trafficking, etc. were not severely impaired.

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Neuronal defects in *Pfdn1* deficient mice

The pathogenesis of several neurodegenerative diseases, including Alzheimer's disease, Amyotrophic lateral sclerosis (ALS), giant axonal neuropathy, Corticobasal degeneration and parkinsonism linked to chromosome 17, have been linked to abnormal aggregation of cytoskeletal proteins. (REFs) Similarly the neuronal deficiencies in *Pfdn1* -/- mice could result from toxic effects of aggregated proteins that depend on *Pfdn1* for proper folding. However, cells affected by Pfdn1 deficiency, localized in the major Commissures were more limited than those affected by the common neurodegenative diseases. Similar commissure defects have been reported in deficient mouse models of genes regulating axon guidance, such as netrin and vax 1 (Serafini, Colamarino et al. 1996; Hallonet, Hollemann et al. 1998; Bertuzzi, Hindges et al. 1999; Deiner and Sretavan 1999; Kwon, Tsai et al. 1999). However, the defects were not identical and the underlying mechanism may differ. Instead of acting as a specific regulator of neuron specific activity such as axon guidance, Pfdn1 and/or its folding substrates are more likely to be involved through cytoskeleton reassembly. Efficient assembly and disassembly of actin cytoskeletons are required for the structure and motility of the growth cone involved in axon outgrowth and possibly for synapse formation (Allen, Shan et al. 2000; Jay 2000; Kuhn, Meberg et al. 2000). As in yeast, lack of prefoldin function may reduce the maturation rate of actin, although not the amount of native actin. In principles, the consequences of inefficient actin assembly would be magnified in rapidly

growing neuron filaments resulting in commissure defects (Dent and Gertler 2003); (Siegers, Waldmann et al. 1999).

Lymphocyte development and cytoskeleton

The deficient lymphopoeisis in *Pfdn1*-/- mice, in particular the reduction of pre-B cells in the bone marrow and of CD4+CD8+ T cells in the thymus appear to be caused by defects in the genesis and/or renewal of lymphocyte precursors, rather than blockages at later developmental stages. Increasing evidence indicate a role for the actin cytoskeleton in immunological synapse formation. For example, T cell activation is defective in mice lacking the Wiskott-Aldrich syndrome protein (WASP) (Snapper, Rosen et al. 1998). It is now well established that WASP, when recruited to activated T cell receptor (TCR), interacts with the Arp2/3 (Actin related protein 2 and 3) complex to regulate cytoskeletal reorganization of. Rapid actin polymerization provides motile force for clustering of T-cell receptors to form a synapse, while the network of actin filaments provides a scaffold for sustained cell signaling (Dustin and Cooper 2000)).

Cellular function of *Pfdn1* indicated by deficient MEFs

Pfdn1-/- MEFs were viable, but grew more slowly and were harder to establish as cell lines than wild type MEFs. Higher amounts of actin were detected in lysates of some early passage Pfdn1 -/- MEFs. However, these null cell strains failed to become immortalized cell lines and the actin over-expression

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was not observed in any established mutant cell lines. Thus while actin overexpression is not an intrinsic trait of mutant cells it is possible that the increased expression is a compensatory response to reduced levels of properly folded protein. This may result in even higher levels of proteins aggregation and toxicity that would select against cell expressing higher levels of the protein.

Materials and Methods

PolyA gene trapping

The construction of GTR1.3 retrovirus and polyA gene trap library, as well as the analysis of 3'RACE products, are described elsewhere (Lin et al. (Lin et al. submitted).

Sequences flanking one integrated provirus were isolated by inverse PCR. Briefly, 50-200 ng of genomic DNA from mouse tails was digested in 30 μl of digestion buffer with 5 units of *Ava*II (NEB). After overnight digestion at 37 °C, the reaction was terminated by incubation at 65°C for 10 min. Subsequently 15 μl of the digestion mix was ligated in a 20 μl reaction (T4 DNA ligase and buffer with 1mM ATP (NEB)) at room temperature for 10 min. 2μl of the ligation product was amplified by PCR in a 50 μl reaction (Roche) with primers:

Dp: CAGTCCTCCGATTGACTGAG and Up: GGGGTTGTGGGCTCTTTAT.

The amplification involved 35 cycles of incubation at 94°C for 1 min, 55°C for 1 min and 72 °C for 1 min.

PCR Genotyping

Mice and cells were genotyped by PCR as follows: 20-50 ng of genomic DNA was used as template in a 50μl reaction using the Roche PCR mix with primers:

Dg: TGGGATAATGCCCACAGGTA, Ug: AAGCACTCAGAGCAGCAAGTT, and Dp: CAGTCCTCCGATTGACTGAG.

The amplification involved 30 cycles of incubation at 94°C for 1 min, 55°C for 1 min and 72 °C for 1 min.

Histological analyses

Samples were fixed in 4 % paraformaldehyde embedded in paraffin and 10 μm serial sections were stained with hematoxylin and eosin.

Cell culture and staining

Embryos at E13.5 were isolated, minced and treated with 2.5% typsin-EDTA (Gibco BRL). Cell suspensions were washed and cultured in DMEM (Meditech), supplemented with 10% fetal calf serum (heat inactivated at $55^{o}C$ for 30 min) and 100 U/ml of penicillin-streptomycin (Gibco BRL). The primary MEFs were cultured at nearly confluent densities and passed at a 1:2 ratio until cell lines were obtained. MEFs were stained with alexa594-phalloidin as elsewhere described (Weaver lab staining paper)

Analysis of *Pfdn1* gene expression

The expression of Pfdn1 on mRNA transcripts in mice and cells was assessed by Northern blot hybridization to a [^{32}P]dCTP-labled probe derived from the full length *Pfdn1* cDNA, as described previously (Williamson, Banik-Maiti et al. 2000).

Prefoldin 1 protein expression in ES cell clones was assessed by Western blot analysis. Cell lysates were fractionated by electrophoresis on a 12% SDS-

polyacrylamide gel and transferred onto a PVDF membrane (NEN life science),
Prefoldin 1 proteins were detected by using donkey polyclonal antibodies raised
against an internal region of the protein (San Cruz Biotechnology), as described
previously (Williamson, Banik-Maiti et al. 2000).

Peripheral blood analyses and FACS

Peripheral blood samples were applied to glass slides and stained with
Wrights stain. Whole blood cell counts were obtained on a Coulter counter.
Single-cell suspensions were prepared from lymphoid organs and stained with
fluorescent antibodies (BD bioscience Pharmingen) following standard protocols.

CHAPTER IV

SUMMARY AND FUTURE PERSPECTIVES

Summary

My thesis work has been focused on characterizing the phenotypes of *Ssrp1* and *Pfdn1* deficient mice, generated by gene targeting and gene entrapment, respectively.

Prior to my study, SSRP1 has been implicated in V(D)J recombination (Shirakata, Huppi et al. 1991) and cell responses to DNA damage (Bruhn, Pil et al. 1992), while these two processes are not mutually exclusive, as illustrated by Ku70, Ku80, Lig4, and XRCC4, proteins involved in Non-homologous end joining (NHEJ) (Frank, Sharpless et al. 2000; Gao, Ferguson et al. 2000; Nick McElhinny, Snowden et al. 2000; Gellert 2002). In order to delineate the *in vivo* function of *Ssrp1*, especially its role in V(D)J recombination and DNA repair, *Ssrp1* was targeted via homologous recombination and the mutation was introduced into the germline. Targeting of *Ssrp1* disrupted the expression of the gene, lead to embryonic death shortly after implantation and the *ex vivo* death of Inner Cell Mass (ICM), indicating that *Ssrp1* is required for the growth and/or survival of pluripotent cells in early embryos. Apoptosis did not appear to be the major mechanism of early embryonic death. Moreover, loss of p53 did not rescue lethality of *Ssrp1* mutant embryos and cells, unlike genes involved in DNA repair or NHEJ. *Ssrp1* appears to encode non-redundant and p53-independent functions

that are necessary for cell viability, unlike a number of proteins involved in DNA repair. In subsequent studies, SSRP1 was identified as a component of a chromatin remodeling complex, FACT, that is involved in DNA replication, transcription elongation (Okuhara, Ohta et al. 1999; Belotserkovskaya, Oh et al. 2003; Kaplan, Laprade et al. 2003; Saunders, Werner et al. 2003). Moreover, Pob3, the yeast SSRP1 ortholog, is required for cell viability (Wittmeyer and Formosa 1997). Temperature sensitive (Ts) mutations in Pob3 produce phenotypes similar to histone mutations (Wittmeyer and Formosa 1997), implicating SSRP1 involved in the global maintenance of chromatin structure. In general, these observations were consistent with my results, indicating that *Ssrp1* encode non-redundant functions that play essential roles in cellular metabolism.

Another part of my thesis research started from the gene entrapment of *Pfdn1* gene by a *Pfdn1* encodes the first subunit of molecular chaperone prefoldin, which assists the folding of cytoskeletal proteins such as actin and tubulin (Vainberg, Lewis et al. 1998; Hartl and Hayer-Hartl 2002). Deletion of one or all of the six prefoldin genes in yeast, does not affect the cell viability, but reduces the rate of actin maturation, and leads to impaired cytoskeletal assembly and function (Vainberg, Lewis et al. 1998). To understand the function of *Pfdn1* in mammalian cells, and more importantly, the impact of potential cytoskeletal defects on various cell types in mice, an ES clone containing mutated *Pfdn1* was identified from the gene trap library and transmitted into the germline. Insertion of the entrapment vector in the last intron of *Pfdn1* ablated expression of the gene. *Pfdn1* -/- mice were born at nearly Mendelian ratios, but most died before 5

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weeks of age, after a period of stunted growth, physical wasting and neuromuscular dysfunction. Postnatal phenotypes of *Pfdn1* -/- mice include: (1) CNS defects involving the corpus callosum (CC) and cerebellar commissure (cbc); (2) hematopoietic defects consisting of micronucleated erythrocytes and neutrophils with abnormal nuclear segmentation; and (3) compromised immune systems with loss of B and T lymphocyte precursors. The organization and reassembly of actin cytoskeleton were impaired in *Pfdn1* -/- MEFs and B lymphocytes. These results suggest that *Pfdn1* encodes non-redundant, tissue-specific functions required for mouse development and survival, presumably by affecting cytoskeleton formation.

With the sequence of the human and mouse genomes the task of gene identification is largely complete. The focus now shifts to understanding gene function, both in the context of the whole organism and as a component of biochemical pathways. The problem has prompted efforts to disrupt all genes via the Knockout Mouse Project. The goal is to create null mutations in all genes either by gene targeting or by gene entrapment.

While my study included only two genes disrupted by gene targeting and gene entrapment, it provides certain perspectives for the methodology of functional genomics.

Gene targeting by gene targeting has the advantage of creating exactly the desired mutation. Thus the targeted mutation in *Ssrp1* deletes the 3' exams of one functionally essential HMG box. While entrapment mutations are less predictable, one insert in *Pfdn1* also appeared to cause a null mutation. However

gene targeting is fare more labor intensive and expensive thus we were able to target nearly 1000 genes in ES cells in less time and at a cost significantly lower than the cost of disrupting SSRPl.

Given the hours and expense gene targeting is best reserved for genes about which much is already known and where the mutation is likely to provide biological insights on gene function. However, predictions are frequently inaccurate. For example, cellular lethality of *Ssrp1* deficiency produced further studies on the *in vivo* functions of *Ssrp1* and biochemical analysis of SSRP1 deficient cells. Conversely given the known role of *Pfdn1* in actin folding in yeast made the gene a poor candidate for gene targeting. However the phenotype of *Pfdn1*-deficient mice predicts a novel role for the cytoskeleton in lymphocyte development.

Whether generated by gene entrapment or gene targeting the analysis of null mutations will identify genes that would benefit from conditional mutagenesis. Conditional gene targeting, via temporal or tissue-specific deletion of the target gene, is a powerful means to bypass and to dissect the role of the targeted gene in specific developmental periods or in specific cells or tissues.

Future Perspectives

My current studies on *Pfdn1* deficient mice suggested some of the essential functions of *Pfdn1* in tissues/systems that require *Pfdn1* the most. This preliminary characterization raises a number of questions to be addressed by future studies that take advantage of the knockout mouse model described in this dissertation.

The first category of future studies will be the biochemical analysis of protein folding in *Pfdn1* deficient cell lines. In yeast lacking one or more prefoldin/Gim genes, the maturation rate of actin is reduced, as assessed by the specific binding of DNaseI to native actin monomers (Siegers, Waldmann et al. 1999). It follows naturally to assess the folding rate of actin in *Pfdn1* deficient mammalian cells. Although very likely there will be a reduction, as suggested by the "downstream" cytoskeletal defects, how exactly the kinetics are affected will provide insight into how *Pfdn1* deficiency impacts the cytoskeleton. For example, if the reduction is less than that of mutant yeast, this may provide potential explanation to why not all cell types were affected and why the homozygous mutants were born alive. Additionally, since it is not yet certain whether disrupting *Pfdn1* also ablates the functions and/or structure of the prefoldin complex, comparing actin folding rates is one of the functional assays that will shed light on this question. As an experiment, RNA interference may be used to knockdown the expression of *Pfdn5*, which encode a subunit required for the prefoldin complex formation. Actin folding rates will be compared in a panel of cells, with *Pfdn1* deficiency and/or *Pfdn5* knockdown. The cell line(s) that

provides the lowest actin maturation rate potentially has the lowest or no function of prefoldin complex. If the *Pfdn1* deficient cell line has an intermediate rate, it is very likely that disruption of *Pfdn1* does not completely abolish the function of prefoldin complex.

Other than actin, folding of tubulin is known to require prefoldin. In addition, there are known substrates for TRiC complex, including proteins related to cytoskeleton, such as actin-related protein V, cofilin and actin-depolymerizing factor 1 (Frydman, Nimmesgern et al. 1992; Gao, Thomas et al. 1992; Melki, Vainberg et al. 1993; Ursic, Sedbrook et al. 1994; Melki, Batelier et al. 1997), all candidate substrates of prefoldin. The list of candidates may expand, even beyond the category of proteins related to cytoskeleton. For example, expression of *Pax5*, a regulator of B lymphocyte development, is correlated with *Pfdn1* in the brains of recombinant inbred mouse strains (refer to www.webqtl.org). Moreover, *Pax5* deficient mice have phenotypes similar to *Pfdn1* deficiency. Mice lacking of *Pax5* are also born at close to Mendalian ratio, with retarded growth in all and early death in some of them. Moreover, *Pax5* deficient mice defects are also largely restricted in the Central Nervous System and B lymphocyte development, although defect patterns are not the same as that of *Pfdn1* deficiency (Urbanek, Wang et al. 1994). *Pax5* is an example of immune/neuron specific regulators that might be substrates of prefoldin, and when unfolded, contribute to the defects in immune system and CNS in *Pfdn1* deficient mice.

As mentioned above the composition of prefoldin complexes has not be characterized in cells lacking *Pfdn1*, which is not required for formation of

prefoldin heterohexamer (Martin-Benito, Boskovic et al. 2002). For example the remaining 5 prefoldin subunits form complexes could with chaperonin activity. This possibility could be examined by Western blot analysis of prefoldin complexes fractionated on non-denaturing gels probed with antibodies against prefoldin 2 or 5, the two subunits required for complex formation. Multimeric prefoldins are expected to migrate with apparent molecular weights greater than the 30 kDa monomer. Further, actin folding activity can be measured using the DNaseI- actin binding assay, and the composition of the complexes can be assessed western blotting. It is intriguing whether multimeric prefoldin complexes can form in the absence of Pfdn1, or whether other subunits can substitute for Pfdn1 in the hexameric complex.

The lymphopoiesis defect in *Pfdn1* deficient mice was marked by the loss of B and T lymphocytes, especially the precursor cells. However, it remains unknown whether the lymphocyte defects are cell autonomous or environment-related. The proliferation and function of *Pfdn1* deficient lymphocytes in a mouse following transfer of mutant bone marrow into irradiated recipients. After 2~4 weeks of recovery, lymphocytes in the recipient mouse are derived from the transferred bone marrow cells. If lymphocytes defects in mice grafted with mutant lymphocytes would suggest that the lymphocyte defects are cell autonomous. If there is only mild or no difference, it is likely that the environment of Pfdn1-deficient mice contributes to the lymphocyte defects. Considering that loss of precursor cells is more severe than loss of mature lymphocytes in *Pfdn1* deficient cells, the lymphocyte defects are more likely to be cell autonomous.

The assembly and function of actin filaments were defective in *Pfdn1* deficient cells. Although the actin cytoskeleton defect might be the major cause of the immune comprised phenotype, it does not rule out possibilities of unfolded immune specific regulators that are substrates of prefoldin. One candidate substrate, Pax5 was discussed earlier in this chapter. Future studies on defective lymphocytes from *Pfdn1* deficient mice will address how actin cytoskeleton defects affect immune synapse formation and/or cell-cell interaction, and how these processes in turn affect the development and function of lymphocytes.

The commissure defects in *Pfdn1* deficient mice should lead to more detailed studies on the development of *Pfdn1* deficient neurons. Growth and orientation of an axon rely on rapid, organized assembly and disassembly of actin cytoskeleton in the growth cone (Jay 2000; Kuhn, Meberg et al. 2000). Axon outgrowth of a *Pfdn1* deficient neuron can be studied in *in vitro* cell cultures. The defective structure and/or function of actin cytoskeleton in the growth cone may contribute to the aberrant formation of the axon of *Pfdn1* deficient neurons. This may underlie why brain commissures, essentially bundles of neural fibers, were severely affected in *Pfdn1* deficient mice.

The commissure defect in *Pfdn1* deficient mice, was similar to netrin-1, DCC, vax1, p35 (Serafini, Colamarino et al. 1996; Hallonet, Hollemann et al. 1998; Bertuzzi, Hindges et al. 1999; Deiner and Sretavan 1999; Kwon, Tsai et al. 1999). However, the commissure in *Pfdn1* deficient mice was unique in that apparent cell death is involved. Future studies should probe the possible involvement of surrounding cells, such as glia cells. A defective cell-cell

interaction could trigger death of neurons. If the death is cell autonomous, there are three potential mechanisms:

1. Loss of *Pfdn1* leads to reduced levels of native actin monomers. The phenotype is reflected in cells that require rapid cytoskeleton assembly and reassembly.

2. *Pfdn1* deficiency may lead to a toxic accumulation of unfolded proteins, and some cells are more sensitive to this toxicity.

3. While prefoldin complexes are ubiquitous, some cells rely on the specific functions of prefoldin 1, and are more severely affected by the loss of *Pfdn1*.

While not mutually exclusive, these hypotheses could address the cell death of brain commissures, as well as defective hematopoiesis and lymphopoiesis. Future studies should test these three hypotheses.

In summary, *Pfdn1* deficient mouse provides a genetic model to study the *in vivo* functions of the prefoldin complex or subunit 1. The neuronal, lymphopoietic defects revealed in my studies would be a start point for future studies on the role of prefoldin in cytoskeleton formation, in the context of development and function of neuron and immune systems.

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