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Mouse Models of Human Familial Paraganglioma

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1. Introduction

Tumor suppressor genes (TSGs) protect normal cells from tumorigenesis (Lasko et al., 1991; Sherr, 2004). Except in cases of haploinsufficiency, heterozygosity for a non-functional TSG allele protects a cell from tumor formation because the functional TSG allele produces a functional protein. Loss of heterozygosity (LOH) is a mechanism by which the remaining wild type tumor suppressor allele is lost, resulting in tumor formation (Lasko et al., 1991; Sherr, 2004). Loss of TSG expression may also occur by epigenetic silencing. The probability of a "second hit" follows a Poisson distribution with the number of tumors and time of incidence being variable in heterozygous carriers (Shao et al., 1999).

Many TSGs have been identified. Such genes play roles in many cellular functions including cell cycle checkpoint control, mitogenic signaling pathways, protein turnover, DNA damage, hypoxia and other stress responses (Sherr, 2004). Surprisingly, the *SdhB*, *SdhC*, and *SdhD* subunits of the metabolic enzyme succinate dehydrogenase (SDH), have also been identified as TSGs for neuroendocrine tumors such as paraganglioma (PGL) and pheocheomocytoma (PHEO).

PGLs are rare (1:300,000) tumors of neuroectodermal origin derived from paraganglia, a diffuse neuroendocrine system dispersed from the base of the skull to the pelvic floor (Baysal, 2002). PGLs are highly vascularized tumors that can originate in either the sympathetic or parasympathetic nervous systems (Baysal, 2002; Pacak et al., 2001).

Patients with PGL tumors that secrete catecholamines present symptoms of catecholamine excess including palpitations. The predominant clinical features of nonchromaffin PGLs are cranial nerve palsies and tinnitus; however, a small proportion of these nonchromaffin PGLs secrete catecholamines (Dluhy, 2002). A hereditary PGL predisposition is involved in at least 30% of cases (Maher & Eng, 2002; Bryant et al., 2003). Individuals with familial predisposition display at least 40% penetrance and a more severe presentation than those with the sporadic form of the disease. Extra-adrenal pheochromocytomas are estimated to be malignant in 40% of cases (Young et al., 2002). There is currently no effective cure for malignant PGL.

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Five genes encoding subunits of the succinate dehydrogenase (SDH) complex (*SdhA*, *SdhB*, *SdhC*, and *SdhD*) (Astuti et al., 2001; Baysal et al., 2000; Niemann & Muller, 2000; Burnichon et al.) or the enzyme responsible for *SdhA* flavination (Kaelin, 2009; Hao et al., 2009) have been identified as tumor suppressor genes in familial PGL. *Sdh* gene defects may also be the cause of sporadic head and neck PGLs where deletions at the same or closely related loci (11q13 and 11q22-23) are observed (Bikhazi et al., 2000). The remaining half of familial PGLs result from inherited mutations associated with von Hippel-Lindau (VHL) syndrome, multiple endocrine neoplasia type 2 (MEN 2), or neurofibromatosis genes (Inabnet et al., 2000; Bryant et al., 2003).

The SDH complex catalyzes the oxidation of succinate (Su) to fumarate (Fu) in the tricarboxylic acid (TCA) cycle and delivers the resulting electrons through various carriers to the ubiquinone pool of the electron transport chain. These electrons are ultimately donated to oxygen to generate water in the process that forms a proton gradient across the inner mitochondrial membrane for ATP production. The porcine SDH complex (Fig. 1) has been studied by X-ray crystallography (Sun et al., 2005). The largest subunit, SdhA, is a flavoprotein of 70 kDa that contains the SDH active site and FAD moiety. A smaller subunit, SdhB is an iron-sulfur protein of 30 kDa carrying three dissimilar iron clusters, $[2Fe\text{-}2S]^{2+,1+}$, $[4Fe\text{-}4S]^{2+,1+}$, and $[3Fe\text{-}4S]^{1+,0+}$. *SdhA/B* are anchored to the membrane by *SdhC* and *SdhD* (15 kDa and 12.5 kDa, respectively), which coordinate a heme group and possess a ubiquinone binding site essential for electron transport into the respiratory chain.

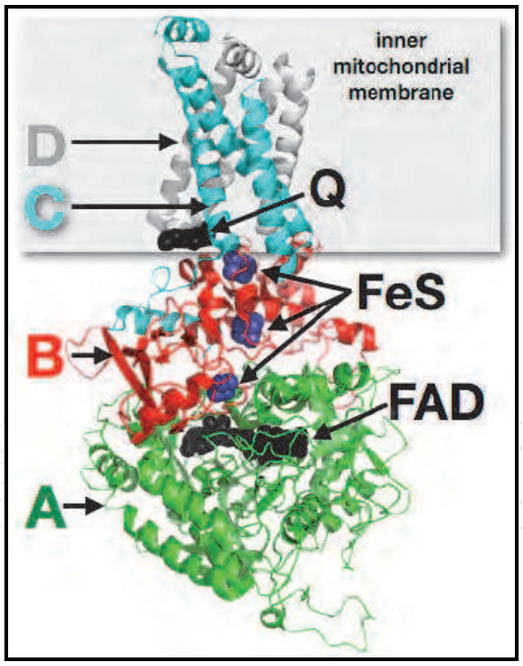

Fig. 1. X-ray crystal structure of SDH complex (Sun et al., 2005). Four subunits labeled and indicating the flavin of the catalytic A subunit (FAD), iron-sulfur clusters of the B subunit (FeS), and co-enzyme Q (Q) near the C and D subunits.

A broad spectrum of *Sdh* mutations has been reported in familial PGL. Mutations in *SdhB* and *SdhC* lead to non-imprinted autosomal dominant inheritance of familial PGL. Mutations in *SdhD* demonstrate imprinted paternal autosomal dominant inheritance (Baysal et al.,

2002). The range of mutations in SDH subunit genes identified in familial PGL suggests that loss of function of SDH subunits is the common cause of PGL.

Familial PGL is particularly fascinating because the causative genetic defects in SDH block the TCA cycle, enforcing upon the tumor an obligatory Warburg effect (Warburg, 1956). Thus, PGL tumor cells must apparently rely on glycolysis as an inefficient source of ATP. Familial PGL thus perfectly exemplifies the aerobic glycolysis commonly observed in cancer, and studies of PGL have the potential to reveal management strategies for all cancers that rely on glycolysis rather than the TCA cycle (Kaelin, 2009).

PGL causation may involve HIF1 activation and other epigenetic effects. Cells carefully regulate oxygen uptake, and respond to hypoxia by altering gene regulation. The master regulator of these responses is the heterodimeric basic helix-loop-helix transcription factor Hypoxia-Inducible Factor 1 (HIF1). HIF1 regulation involves oxygen-dependent prolylhydroxylation (PHD), ubiquitin ligation, and proteasomal degradation of the HIF1α subunit under normoxic conditions (Semenza, 2003). Prolylhydroxylation of HIF1α requires oxygen, iron, and 2-ketoglutarate (2KG), and the reaction produces succinate (Su) as a byproduct. If oxygen becomes limiting, prolylhydroxylation is inhibited, and HIF1α accumulates, translocates to the nucleus, and pairs with the constitutively expressed HIF1β subunit. Thus, HIF1 stability is directly regulated by oxygen. Hypoxic genes stimulated by HIF1 include transporters for increased glucose import (allowing anaerobic growth by glycolysis) and genes encoding angiogenesis factors. HIF1 activation is correlated with tumor aggressiveness and therapy resistance.

According to the succinate (Su) accumulation hypothesis (Lee et al., 2005; Maxwell, 2005; Selak et al., 2005; Smith et al., 2007; Favier & Gimenez-Roqueplo, 2010), the disruption of SdhB yields a catalytically inactive SdhA subunit and Su accumulates in the cell due to loss of SDH activity. Su diffuses to the cytoplasm where it acts as an inhibitor of the 2-ketoglutarate (2KG)-dependent prolyl hydroxylase (PHD) enzymes that use molecular oxygen as a substrate to hydroxylate HIF1α prolines for degradation when adequate oxygen is present. This class of enzyme reactions generates Su as a product, and is therefore susceptible to inhibition by elevated Su concentrations. Loss of SDH activity disables the TCA cycle and causes inappropriate HIF1 persistence due to Su inhibition of PHD enzymes. The resulting pseudohypoxic state is not tumorigenic in most cell types. However, it is hypothesized that chronic pseudohypoxic signaling is a mitogenic tumor initiator in neuroendocrine cells because these cells proliferate in a futile homeostatic attempt at a hormonal response to perceived hypoxia. Thus, inappropriate HIF1 persistence due to loss of SDH function in PGL drives tumorigenesis. HIF1 is therefore a novel target for therapy of PGL.

Our working hypotheses are shown in Fig. 2. We hypothesize that tumorigenic effects of succinate accumulation are not limited to inhibition of prolyl hydroxylation (McDonough et al., 2006), but also include inhibition of histone demethylation by Jumoni domain (JHDM) enzymes (Klose et al., 2006), and inhibition of 5-methylcytosine hydroxylation by TET1 (Tahiliani et al., 2009). Thus we are interested in model systems to probe how loss of SdhB acts as a tumorigenic trigger in neuroendocrine cells.

To date there have been limited opportunities to understand SDH dysfunction in such animal models. Although no human PGL cell lines exist, various studies have been undertaken using PGL tumor tissue samples to understand the underlying biochemistry and genetics (Benn et al., 2006). Unfortunately, such patient samples are not numerous and no systematic approach has been taken in understanding the pathological biochemistry of PGL.

Mutations in genes encoding *SdhB*, *SdhC*, or *SdhD* in *C. elegans*, *S. cerevisiae*, and mammalian cell lines have been utilized to examine the reactive oxygen species (ROS) hypothesis and the succinate accumulation hypothesis (Guo & Lemire, 2003; Ishii et al., 1998; Ishii et al., 2005; Lee et al., 2005; Oostveen et al., 1995; Selak et al., 2005). The only available mammalian PGL cell lines do not emulate the SDH familial form of PGL. For instance, rat PHEO cell line (PC12) (Tischler et al., 2004) and mouse PHEO cell lines (MPC) are available (Powers et al., 2000). However, PC12 cells are derived from a spontaneous PHEO tumor with functional Complex II and MPC cells are derived from neurofibromatosis [(*Nf1* +/- heterozygous] knockout mice (Tischler et al., 2004). During the course of this project a mouse model of *SdhD* deficiency was developed (Piruat et al., 2004). *SdhD* +/- mice were found to have decreased expression of *SdhD* and 50% SDH activity in various tissues relative to *SdhD* +/+ mouse tissues (Piruat et al., 2004). Although *SdhD* +/- mice were found to have carotid body glomus cell hyperplasia and organ hypertrophy, no PGL tumor formation was observed (Piruat et al., 2004; Bayley et al., 2009).

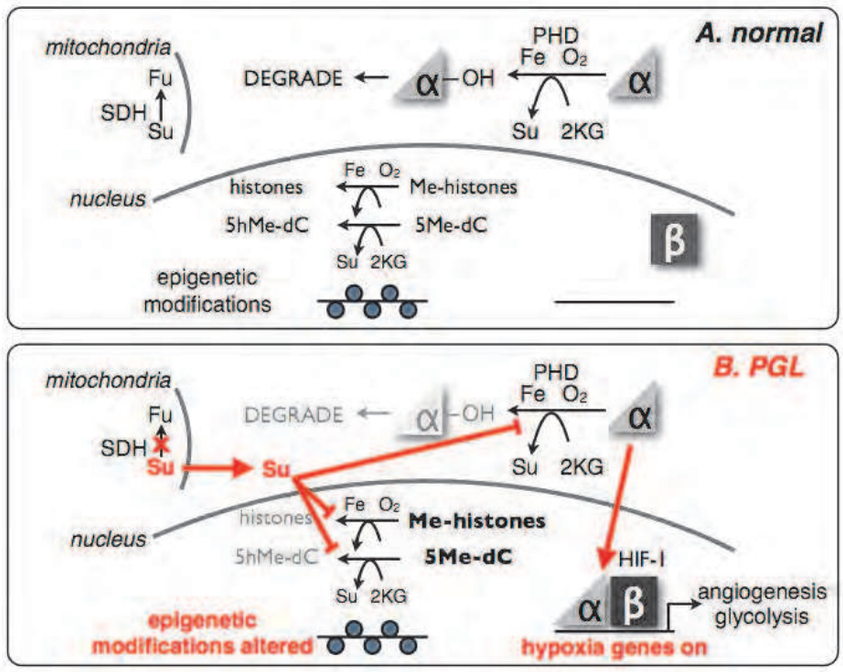

Fig. 2. A. Normal tumor suppressor functions of $Fe/O_2/2KG$-dependent dioxygenases in Hif-1α degradation and epigenetic regulation of histone methylation and 5-methylcytosine hydroxylation. B. Proposed effects of succinate inhibition in PGL. Simple genetic models of *Sdh* mutant PGL come in the form of model organisms that contain defects in SDH subunits.

We recently created and studied a yeast model lacking the *SdhB* subunit of Complex II (Smith et al., 2007). As expected for loss of a TCA enzyme, this yeast strain is dependent on glycolysis and is unable to survive on non-fermentable carbon sources. The yeast model has increased ROS and also shows accumulation of succinate. This succinate accumulation poisons at least two 2KG-dependent enzymes that produce succinate as a normal

byproduct. Succinate inhibition of such enzymes in mammalian systems (e.g. the 2KG-dependent prolyl hydroxylase that modifies HIF-1α and JHDMs) has been proposed as a completely novel metabolic mechanism of tumorigenesis. Further progress in understanding PGL and PHEO could be facilitated by development of animal models to allow testing of the ROS and succinate accumulation hypotheses and hypotheses related to environment, diet, and pharmaceutical interventions.

Human *SdhB* mutations are not associated with a parent-of-origin effect (Baysal, 2001). It has also been observed that both *SdhB*- and *SdhD*-linked PGL tumors tend to lose *SdhB* expression and have enhanced *SdhA* abundance (Douwes Dekker et al., 2003). Thus, *SdhB* disruption creates an obvious goal for genetic models. Analysis of causative *SdhB* mutations in human PGL suggests that total loss of *SdhB* function is the common feature (Baysal, 2001; Baysal, 2002; Eng et al., 2003).

Here we describe the generation of two heterozygous mouse lines carrying a disruption in one copy of *SdhB*. By analogy with human familial predisposition to PGL genetics (Baysal, 2001; Baysal, 2002), mouse strains heterozygous for functional *SdhB* are hypothesized to display no phenotype, but to be predisposed to PGL development due to random loss of the second *SdhB* allele during development. Based on human PGL genetics, it was hypothesized that loss of the second *SdhB* gene would be oncogenic only in neuroendocrine cells.

2. Materials and methods

2.1 Creation of an *SdhB* targeting vector

A recombinant targeting vector for mouse *SdhB* was designed and assembled according to standard procedures. *SdhB*-specific sequences were inserted into the commercial vector NTKV1901 (Stratagene) that carries *Neo* and *TK* genes for selection of targeted integrants. Briefly, two arms homologous to segments of the murine *SdhB* gene were amplified by PCR (Epicentre, Failsafe kit) from mouse genomic DNA with sets of primers containing two unique restriction sites. The left homologous arm (Scrambler A) was PCR-amplified with an upstream primer that contains a *Hin*dIII site, (LJM-2309: GCTAGCA$_2$GCT$_2$G$_2$AGATA-CAGCTCAGTCTGAGTG$_3$) and a downstream primer that contains a *Xho*I site, (LJM-2310: GCTAGC$_2$TCGAGCATC$_2$A$_2$CAC$_2$ATAG$_2$TC$_2$GCAC$_2$T). The Scrambler A PCR product was directly cloned into the targeting vector NTKV1901. The right homologous arm (Scrambler B) was PCR-amplified with an upstream primer containing a *Cla*I site (LJM-2311: GCTAGCATCGATG$_2$TG$_2$TGTC$_2$TGCTGTGCTGT$_3$GG) and a downstream primer containing a *Sac*II site (LJM-2312: GCTAGC$_3$GCG$_4$A$_3$G$_2$TG$_4$CAGACATAGTAC). The Scrambler B PCR product was first cloned into a pGEM-T Easy vector (Promega), then isolated with a *Sac*II digest and ligated into the targeting vector. Diagnostic *Not*I, *Hin*dIII/*Xho*I and *Sac*II restriction digests were performed.

2.2 Extension the *SdhB* targeting vector

A forward primer specific for *SdhB* intron seven that contains the *Sal*I restriction site (LJM-2599: ATATGTG$_2$TCAGTGCT$_4$C) and a reverse primer specific for a region downstream of *Sdh*B exon eight that contains a *Not*I restriction site (LJM-2595: GCTAGCGCG$_2$-C$_2$GC$_2$TA$_2$CTCACG$_2$A$_2$G$_3$CA$_2$G$_2$) were used to amplify a Scrambler B extension product by PCR (Epicentre, Failsafe kit). The product was cloned into the original targeting vector using standard procedures.

2.3 ES cell culture and transfection with targeting vectors

ES cells derived from C57BL/6 blastocysts (E3.5) were transfected with *Not*I linearized targeting vectors, and stable integrants were selected in Geneticin G418 medium as described (Hofker & van Deursen, 2002).

2.4 Southern blotting

PCR was used to generate a 200-bp probe with homology to intron two of the *SdhB* gene. The probe was labeled by random priming in the presence of [α−^{32}P]-dATP according to manufacturer's instructions (Roche). 10-20 µg of genomic DNA from ES cell clones was digested with *Sac*I (New England Biolabs) and the DNA was electrophoresed overnight at 40 V. Southern blotting of DNA was performed using standard procedures as described (Hofker & van Deursen, 2002).

2.5 Genetic analysis of 129SV/E *SdhB:β-Geo* disrupted ES cells

For expression analysis by RT-PCR RNA was harvested from *SdhB* +/- ES cells with Trizol reagent by standard procedures, and reverse transcribed with a pool of nonamers according to manufacturer's instructions (Epicentre). cDNA was amplified with a common forward primer specific to *SdhB* exon one (LJM-2684: $CGACG_2TCG_3TCTC_2T_2GA_2$) and either a *β-Geo*-specific reverse primer (LJM-2687: $AT_2CAG_2CTGCGCA_2CTGT_2G_3$) or an exon two-specific reverse primer (LJM-2685: GAGCTGCAGCAGCAGCTGTC) by PCR (Epicentre, Failsafe kit). For mapping of the gene integration point by PCR genomic DNA from *SdhB* +/- ES cells was precipitated with lysis/precipitation buffer [50 mM Tris-HCl (pH 8.0), 100 mM EDTA (pH 8.0), 100 mM NaCl, 1% SDS, 10 mg/ml proteinase K] and extracted with phenol:chloroform (1:1). A forward primer specific for *SdhB* exon one (LJM-2784: $AGCTGAC_2AGACA_2GAGTCACAG_2TGAT_2GACAGA$) and a reverse primer specific for the *β-Geo* marker (LJM-2787: $AGTATCG_2C_2TCAG_2A_2GATCGCACTC_2AGC_2AGC$) were used to amplify the region of the gene trap vector integration by PCR (Epicentre, Failsafe kit). The PCR product was purified and sequenced across the *β-Geo* marker to verify the exact *SdhB*:*β-Geo* junction.

2.6 Generation and husbandry of mice

Following genetic characterization, *SdhB* +/- ES cells were injected into C57/BL6 blastocysts and used to generate chimeric animals as described (Hofker & van Deursen, 2002). Animals were caged in groups of five, segregated by genotype and gender. Standard animal husbandry methods were used under IACUC protocol A29505 in the Mayo Clinic non-barrier mouse facility.

2.7 DNA extraction

DNA extraction from tail clippings was performed after overnight digestion in lysis/precipitation buffer [50 mM Tris-HCl (pH 8.0), 100 mM EDTA (pH 8.0), 100 mM NaCl, 1% SDS, 10 mg/ml proteinase K] at 55°C. DNA was precipitated with isopropanol, washed once in 80% ethanol and resuspended in sterile water.

2.8 Genotyping

To distinguish *SdhB* +/+ and *SdhB* +/- animals, genomic DNA was analyzed by PCR (Epicentre, Failsafe kit) with a common forward primer LJM-2826 (5'-

GTGTAGC$_3$TG$_2$CTGTC$_2$TG$_2$A$_2$CT$_2$GCTC) and differential reverse primers, LJM-2828 (5′-G$_2$CA$_3$C$_3$A$_4$G$_3$TCT$_3$GAGCAC$_2$AG) and LJM-2830 (5′-GTG$_3$AC$_2$TGCGTGACA$_3$GT GCATG$_2$AG), specific for *β-Geo* and intron one, respectively. *Bub1* Genotyping used standard PCR methods employing the Failsafe kit (Epicentre). The *Bub1* WT locus was amplified with a forward primer in exon eight (LJM-3169: CTG$_2$C$_2$TG$_2$A$_2$CT$_2$GCTATGTC) and a reverse primer in intron eight (LJM-3171: CG$_2$T$_3$CTCTGTATAGC$_3$TG$_2$C). The *Bub1* knockout allele was amplified with a forward primer in the neomycin cassette (LJM-3178: GCAGT$_2$CAT$_2$CAG$_3$CAC$_2$G$_2$AC) and the reverse primer LJM-3171. The *Bub1* hypomorphic allele was amplified with a primer set in the hygromycin cassette (forward primer, LJM-3172: CG$_2$A$_2$GTGCT$_2$GACAT$_2$G$_2$; reverse primer, LJM-3173: GTAT$_2$GAC$_2$GA-T$_2$C$_2$T$_2$GCG).

2.9 Histology

Tissue samples for histology were dissected and fixed in neutral-buffered 10% formalin (Sigma) for 24 h. Tissues were dehydrated, embedded in paraffin, and 10-µm slices were prepared with a microtome and mounted. Standard hematoxylin and eosin staining was used for initial histopathology. Bifurcations containing carotid bodies were dissected and fixed in formalin (Sigma) at 4°C for 16 h. Tissues were dehydrated and paraffin embedded, and 10-µm slices were obtained by using an RM2125 microtome (Leica Microsystems). Immunohistochemistry was performed according to standard procedures. For detection of glomus cells, tissues were immunostained with a rabbit polyclonal antityrosine hydroxylase (TH) antibody (Pel-Freez). After immunodetection with peroxidase-conjugated secondary antibody, tissue samples were counterstained with hematoxylin.

2.10 Quantitative RNA analysis by RT-PCR

RNA from liver or kidney tissue was harvested with the RNeasy Plus Mini kit (Qiagen), quantitated with a RiboGreen assay (Invitrogen) and reverse transcribed by random nonamers (Epicentre). cDNA was amplified by PCR (BioRad IQ SuperMix) with a forward primer (LJM-3115: ATGA$_2$CATCA$_2$CG$_2$AG$_2$CA$_2$TAC) and a reverse primer (LJM-3116: GAG$_3$TAGAT$_4$G$_2$AGACT$_3$GC) located downstream of the βGEO insertion site in exon four. Probe 5′-6-FAM/CACACGCAG$_2$ATCGACACG$_2$AC$_2$T/3BHQ-1 specific for exon four was used to monitor target amplification of either cDNA or an 82-bp synthetic DNA amplicon used to produce a standard curve in a BioRad iCycler.

2.11 Enzyme assays

SDH activity was assayed in kidney and liver extracts from juvenile mice of each genotype euthanized by cervical dislocation. SDH activity was measured as PMS-mediated reduction of the 2,6-dichloroindophenol dye in the presence of antimycin A, rotenone and cyanide, monitored at 600 nm as described (Kramer et al., 2005).

2.12 Metabolite analysis

Levels of TCA cycle metabolites were determined by acidification of urine and extraction of free acids into ethyl acetate. 2-keto acids (e.g. 2-KG) were first protected by oximation with hydroxylamine hydrate. After evaporation, the dry residue was silyated with N,O-bis-(trimethylysilyl)trifluoroacetamide and trimethylchlorosilane to produce volatile derivatives, and analyzed by capillary gas chromatography/mass spectrometry on an HP ChemStation instrument equipped with an HP-5 25 m column (ID 0.2 mm) using pentadecanoic acid as an internal standard. Metabolite levels were normalized to creatine.

3. Results

3.1 Generation of *SdhB* +/- mice

Two approaches were initially taken to generate an *SdhB* +/- mouse strain (Fig. 3). The first strategy involved the creation of a DNA targeting vector that would specifically replace part of exon three through part of intron four by homologous recombination with a neomycin resistance marker on a single *SdhB* allele (Fig. 3A). The targeting vector carried two arms of homology to *SdhB* (Scrambler A and Scrambler B) and two selectable markers – NEO (neomycin phosphotransferase) and TK (thymidine kinase) genes. These genes allow for selection of integrants and counterselection against non-homologous insertions, respectively. This construct was intended to exchange the selectable NEO marker for a segment of *SdhB* extending from part of exon 3 through part of intron 4. This strategy would create an *SdhB* mRNA with only a short reading frame, followed by nonsense codons. The resulting truncated polypeptide is analogous to loss-of-function products seen in human *SdhB* mutations causing PGL (Benn et al., 2006).

The targeting vector was constructed and verified with diagnostic restriction digests that confirmed the successful insertion and orientation of both *SdhB* homology regions into the targeting vector. Its size (13.6 kb) was confirmed by linearization with *Not*I, Scrambler A insertion was confirmed by a *Hin*dIII/*Xho*I digest, and Scrambler B insertion was confirmed by a *Sac*II digest. Molecular junctions were sequenced to confirm expected features of the targeting vector.

Mouse ES cells were electroporated with the linearized *SdhB* targeting vector, and NEO+ TK- colonies were selected and screened for targeted inactivation of one copy of *SdhB*. DNA was harvested from over 400 potential clones and analyzed by Southern blot. None of the clones showed evidence of homologous recombination at *SdhB*. Similar results were obtained even when the Scrambler B *SdhB* homology segment was increased in length.

We therefore adopted a second strategy (Fig. 3B). We obtained an *SdhB* +/- embryonic stem (ES) cell line that had been created by high throughput random gene trap integration by the Sanger Center gene trap consortium (Nord et al., 2006). Sequencing of an RT-PCR product derived from the integrated *β-Geo* marker gene revealed integration of the gene trap cassette in intron 1 of the mouse *SdhB* gene. The strong cleavage and poly(A) signals of the gene trap fragment should truncate *SdhB* mRNA after the short exon 1, resulting in a completely non-functional *SdhB* mRNA, analogous to that produced by disease-associated truncating alleles in humans. We confirmed the presence of the gene trap by PCR from random-primed cDNA prepared from the ES cells. PCR of genomic DNA from the ES cell line allowed us to map the precise location of the gene trap, and sequencing of PCR products showed the exact position of the trap reporter gene downstream of the exon 1/intron 1 junction (Fig. 4A).

The *SdhB* +/- ES cells were injected into blastocyts that were then implanted in pseudopregnant females. Multiple chimeric offspring were obtained, four of which transmitted the mutant *SdhB* allele in their germ lines. These founders were mated with C57BL/6 strain females. A PCR screening procedure was developed (Fig. 4B) to allow genotyping of (129SV/E x C57BL/6)F1 offspring generated from *SdhB* +/+ x chimeric *SdhB* +/- matings. This approach proved successful for generating the desired *SdhB* +/- mouse strain used to develop a colony of 55 *SdhB* +/+ and 55 *SdhB* +/- mice.

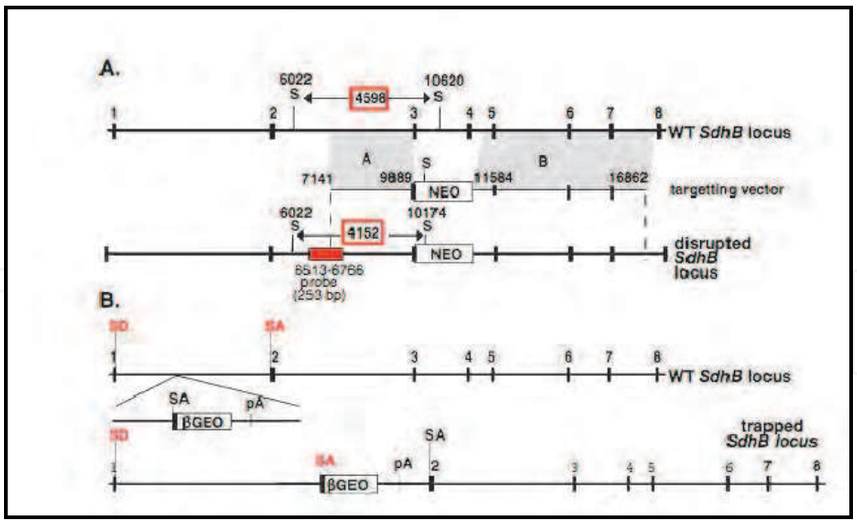

Fig. 3. *SdhB* knockout strategies. A. Targeted knockout approach. A targeting vector with homology regions encompassing intron two through part of exon three (shaded A) and encompassing part of intron four through intron seven (shaded B) flanking a neomycin cassette was used to target the WT *SdhB* locus and generate a disrupted *SdhB* locus. Numbers indicate position in *SdhB* locus from transcription start site. B. Gene trap vector approach. Gene trap vector contains a splice acceptor (SA) site upstream of the βGEO cassette (encodes β-galactosidase−neomycin phosphotransferase fusion protein) followed by a strong polyadenylation (pA) site inserted into intron one of *SdhB*. The SA site of the βGEO cassette will replace the SA site of exon two and will be spliced with the splice donor site of exon one.

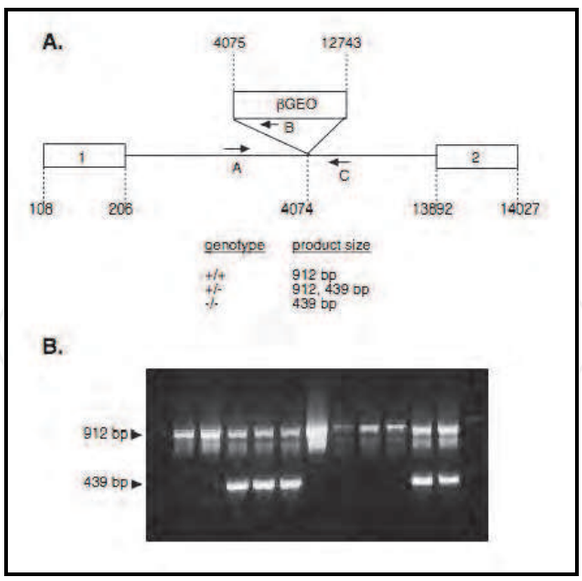

Fig. 4. *SdhB* genotyping PCR design. A. Schematic diagram showing the *SdhB* locus with or without a βGEO insertion in intron one. Primer set A/C amplifies the 912 bp WT locus and primer set A/B amplifies 439 bp from the disrupted locus. Numbers indicate position from the transcription start site in the wild type *SdhB* locus. B. PCR genotyping agarose gel showing *SdhB* +/+ vs. *SdhB* +/- amplification products from genomic DNA.

3.2 Characterization of *SdhB* +/- mice

We determined the viability of *SdhB* -/- mice. We hypothesized that the *SdhB* -/- condition would not support mammalian development due to lack of TCA cycle function. To test this hypothesis we crossed *SdhB* +/- heterozygotes and examined the genotypes of progeny. In the absence of *SdhB* -/- offspring, the prediction from Mendelian genetics is tbat 1/3 of progeny will be *SdhB* +/+ WT and 2/3 will be *SdhB* +/- heterozygotes. Breeding to test this hypothesis was performed until 107 offspring were genotyped. These offspring included 35 *SdhB* +/+ mice, 72 *SdhB* +/- mice with no *SdhB*(-/-) mice observed. This result implies that the *SdhB* -/- condition has an embryonic lethal phenotype. Indeed, it was previously reported that *SdhD* -/- mice die at 6.5 to 7.5 days post conception (Piruat et al., 2004). Evidence suggests that *SdhB* -/- mice also die around the time of organogenesis.
SdhB +/- mouse tissues were then characterized in terms of *SdhB* gene expression and SDH enzyme activity (Fig. 5).

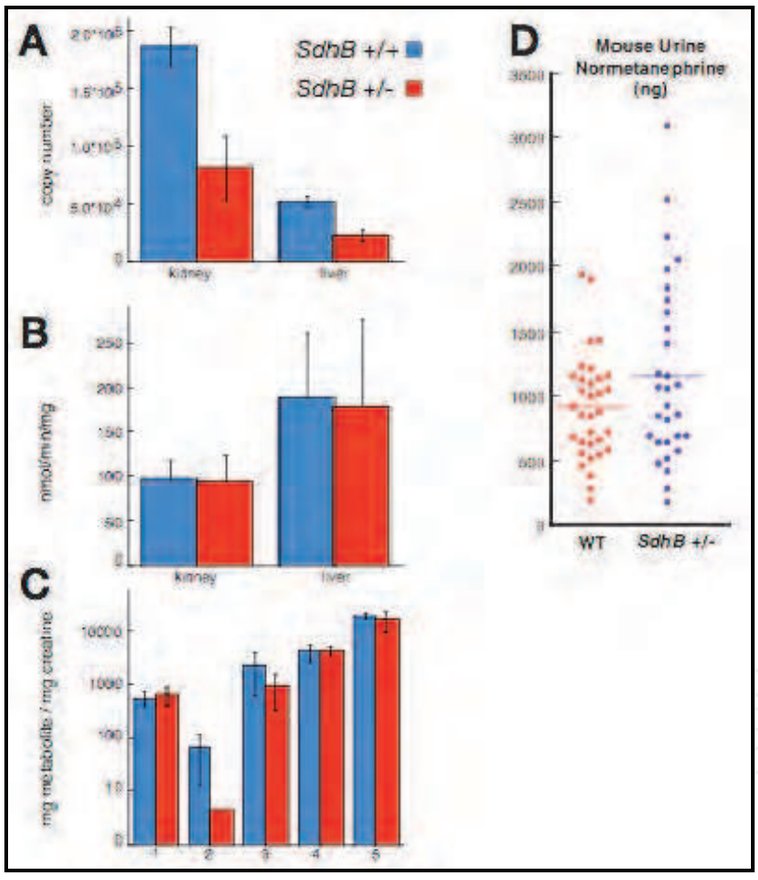

Fig. 5. Characterization of *SdhB* +/+ and *SdhB* +/- mice. A. *SdhB* expression levels (n=5). B. SDH activity (n=5). C. TCA metabolite levels in urine (n=3). 1, succinate; 2, fumarate; 3, malate; 4, citrate; 5, aconitate. Error basis indicates SEM. D. Results of 24-h urine normetanephrine screening in WT and *SdhB* +/- mice.

It was previously reported that *SdhD* +/- mice have a 50% decrease in *SdhD* expression and SDH activity (Piruat et al., 2004). We characterized *SdhB* expression and SDH activity in the liver and kidneys of *SdhB* +/- mice relative to *SdhB* +/+ mice. These organs have high

metabolic activity and are easily homogenized for assays. We found that *SdhB* gene expression was decreased by 50% (based on quantitative RT-PCR analysis; Fig. 5A). In contrast, no difference in SDH enzyme activity was detected in these tissues (Fig. 5B), suggesting translational compensation or other homeostatic mechanisms. Furthermore, TCA cycle metabolites (He et al., 2004) in tissue (Fig. 5C) and metanephrines in urine (Fig. 5D) were not different between mice with *SdhB*+/+ and *SdhB*+/- genotypes.

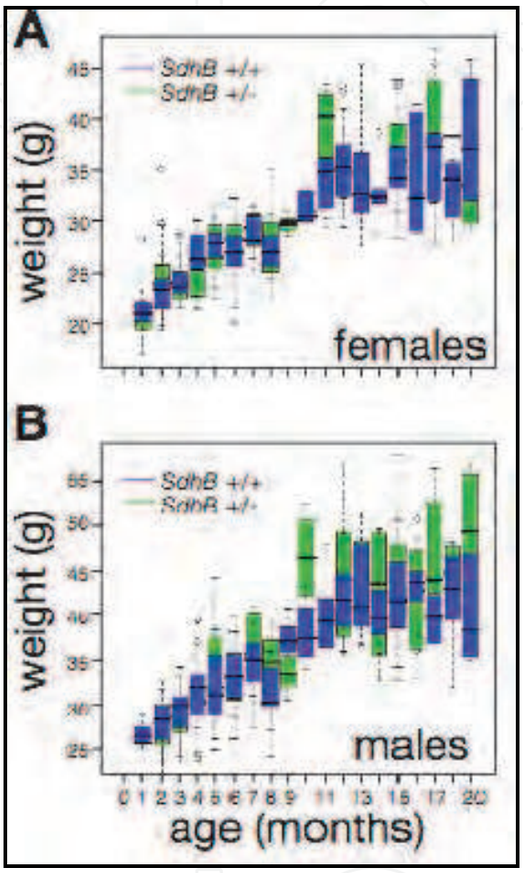

Fig. 6. Weight analysis for *SdhB* +/+ and *SdhB* +/- mice, by gender: A. Females. B. Males. The box represents the middle 50% of the weights (25-75%) and the horizontal bar represents the median weight for each group. The dashed lines encompass the remainder of the data, excluding outliers represented by circles.

Animals in the experimental colony were monitored by abdominal palpation and weight measurement. No abnormalities were detected in *SdhB* +/- animals. No significant differences were observed for weights of *SdhB* +/+ and *SdhB* +/- mice (Fig. 6). After one year, six *SdhB* +/+ and six *SdhB* +/- mice were euthanized and reviewed for gross and microscopic pathology of adrenal glands, heart, liver, lung, and kidneys. A detailed pathological analysis of carotid bodies investigated the possibility of neuroendocrine cell hypertrophy as has been suggested for *SdhD* +/- heterozygotes (Piruat et al., 2004). The results are shown in Fig. 7. Although there was a trend towards smaller carotid body size in *SdhB* +/- heterozygotes (Fig. 7A), the trend was not statistically significant (p values > 0.05 by t-test). This trend came mainly from male mice. Neuroendocrine cells of the carotid

bodies of males were detected by tyrosine hydroxylase (TH) staining. Again, a trend toward a reduced number of type I (TH+) cells was observed in the *SdhB* +/- carotid bodies (Fig. 7B). Again, this difference was not statistically significant. There was no carotid body hypertrophy or any sign of hyperproliferation (Fig. 7C). No PGL or PHEO tumors were observed in any mice, even in compound *SdhB* +/- *SdhD* +/- heterozygotes.

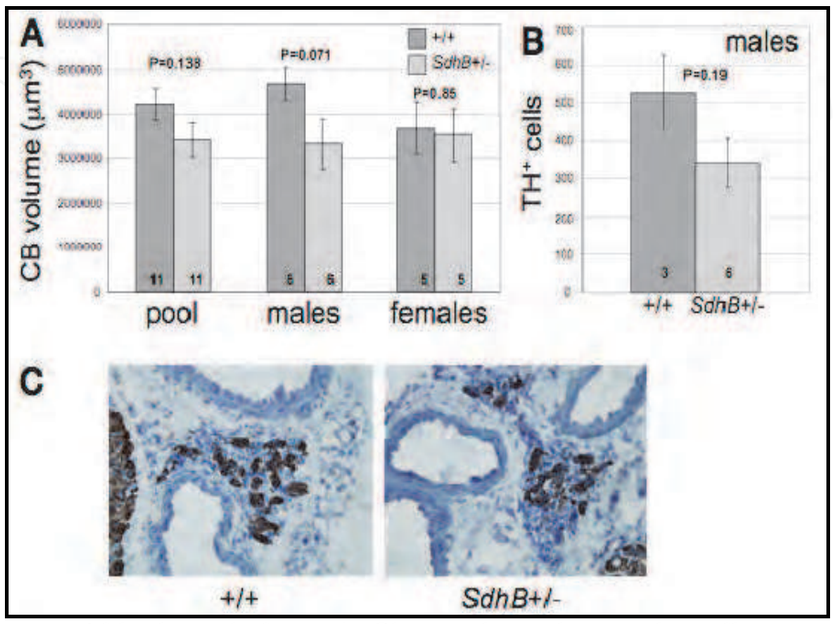

Fig. 7. Mouse carotid body pathology. A. Observed trend toward smaller carotid body size in *SdhB* +/- mice, not reaching statistic significance by t-test. The contribution to this trend comes mainly from males. B. Analysis of tyrosine hydroxylase positive (TH+) cells in carotid bodies from males. The trend toward a reduced number of TH+ cells in *SdhB* +/- male mice does not reach statistical significance. C. Examples of carotid body pathology. There is no evidence of carorid body hypertrophy or hyperproliferation in *SdhB* +/- mice.

3.3 Generation of *SdhB* +/- *Bub1* H/- mice

We hypothesized that the absence of PGL in *SdhB* +/- mice was due to the low rate of *SdhB* LOH. We therefore sought genetic methods to enhance this rate. Aneuploidy (abnormal chromosome content) is a hallmark of most solid tumors and cancer cell lines (Heim & Mitelman, 1995; Lengauer et al., 1997; Lengauer et al., 1997). Over 100 years ago Theodor Boveri postulated that chromosome instability (CIN) drives tumorigenesis. CIN is believed to be frequently responsible for TSG LOH (Bignold et al., 2006; Kops et al., 2005). More than 100 CIN genes have been identified in yeast (Kolodner et al., 2002; Nasmyth, 2002; Shonn et al., 2000). A novel mammalian gene of this type is *Bub1* (mouse chromosome 2), which plays a key role in the mitotic checkpoint.

Mitotic checkpoint proteins survey proper kinetichore attachment during mitosis. When the mitotic spindle is correctly attached to all kinetochores, Cdc20 activates the anaphase promoting complex (APC) to degrade securin, allowing release of separase to "unbind" sister chromatids and promote the transition from metaphase to anaphase (Kops et al., 2005). Mitotic checkpoint proteins produce a "stop anaphase" signal when kinetochores are not properly attached to the mitotic spindle during prometaphase. An APC inhibitory

complex is involved (Kops et al., 2005; Shah & Cleveland, 2000; Wang et al., 2001). The BUB1 protein has three putative molecular functions in this mitotic checkpoint (Acampora et al., 1999) as illustrated in Fig. 8.

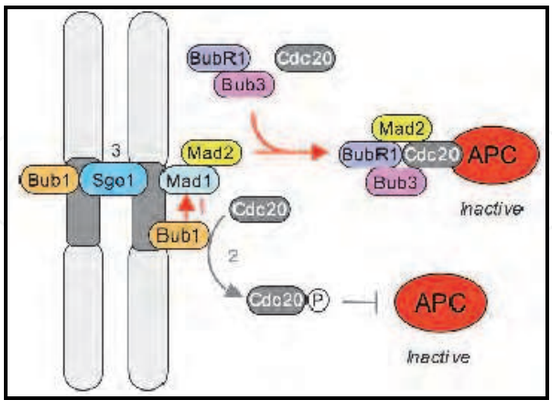

Fig. 8. Putative functions of *Bub1* in mitosis. 1. *Bub1* mediates Mad1/Mad2 binding to kinetochores, which allows for efficient formation of APC inhibitory complexes consisting of Mad2, BubR1, and Bub3. The APC inhibitory complex functions by sequestration of Cdc20. 2. *Bub1* phosphorylates Cdc20, thereby preventing APC activation. 3. *Bub1* is required for stability and centromeric localization of centromeric cohesin.

Loss of *Bub1* expression was predicted to result in aneuploidy and increased tumorigenesis. In support of this hypothesis, *Bub1* mutations were detected in 2/19 colorectal cancer cell lines with CIN (Cahill et al., 1998) and epigenetic silencing of *Bub1* is a frequent event in human colorectal carcinomas, with 30% exhibiting at least two-fold reduction in *Bub1* expression (Shichiri et al., 2002). In addition, *Bub1* mutations have been correlated with lung, pancreatic and rectal cancers (Gemma et al., 2000; Hempen et al., 2003; Imai et al., 1999).

The van Deursen laboratory has developed a series of mouse strains in which expression of *Bub1* protein is reduced in a graded fashion by different combinations of wild type, knockout, and hypomorphic alleles (Jeganathan et al., 2007). The *Bub1* hypomorphic allele [*Bub1*(H)] results from intron 9 insertion of the hygromycin B phosphotransferase gene (*Hyg*), resulting in a high level of premature transcription termination (van Deursen et al., 1994). Standard procedures and breeding yielded *Bub1* +/H, *Bub1* H/H, and *Bub1* H/- viable offspring. Western blotting showed graded reduction of *Bub1* levels across these strains in mouse embryonic fibroblasts.

Of the mice in the *Bub1* genotype series, the *Bub1* H/- strain shows the most dramatic phenotype. *Bub1* H/- mice display increased aneuploidy and tumorigenesis relative to the other *Bub1* genotypes. Analysis of 150 mitotic figures from *Bub1* H/- splenocytes showed 39% aneuploidy, compared to only 1% aneuploidy in *Bub1* +/+ animals (Jeganathan et al., 2007). *Bub1* H/- animals also suffer more tumors (52% tumor incidence in 530 days) than *Bub1* +/+ animals (33% tumor incidence in 772 days). Lymphoma and sarcoma incidence was higher and hepatocellular carcinoma incidence lower for *Bub1* H/- animals than for wild type animals.

Mutations in CIN genes can increase the rate of loss of entire chromosomes or chromosome segments during cell division, thereby accelerating LOH of tumor suppressor genes (Cahill et al., 1999; Cahill et al., 1998; Jallepalli & Lengauer, 2001). Indeed, it was shown in a cohort

of 30 *Bub1* H/- p53 +/- animals monitored for 100 days that 5 mice developed tumors. In contrast, 0/30 age-matched p53 +/- mice developed tumors during the same time period (Jeganathan et al., 2007). All tumors in *Bub1* H/- p53 +/- mice were thymic lymphomas, the most common tumor form in p53(-/-) mice (Donehower et al., 1992; Donehower et al., 1995; Jacks et al., 1994). Accelerated tumorigenesis resulted from p53 LOH as shown by PCR analysis of DNA from *Bub1* H/- p53 +/- lymphomas (Jeganathan et al., 2007; Jacks et al., 1994).

A two-part breeding program created a colony of 55 *SdhB* +/- *Bub1* H/- compound heterozygote mice (Fig. 9). In part 1, *SdhB* +/– females were crossed with *Bub1* H/- males to generate offspring of four genotypes (Fig. 9A). In part 2, offspring of these matings were crossed to generate the desired *SdhB* +/-*Bub1* H/- animals at the indicated expected frequencies (Fig. 9B). Multiplex PCR genotyping was developed to distinguish *Bub1* +, H and – alleles (Fig. 9C). The colony of *SdhB* +/- *Bub1* H/- compound heterozygote mice was monitored for PGL tumorigenesis. Age-dependent survival of this and related strains is shown in Fig. 10. As had been observed for *SdhB* +/- mice, no PGL tumors were observed in *SdhB* +/- *Bub1* H/- animals.

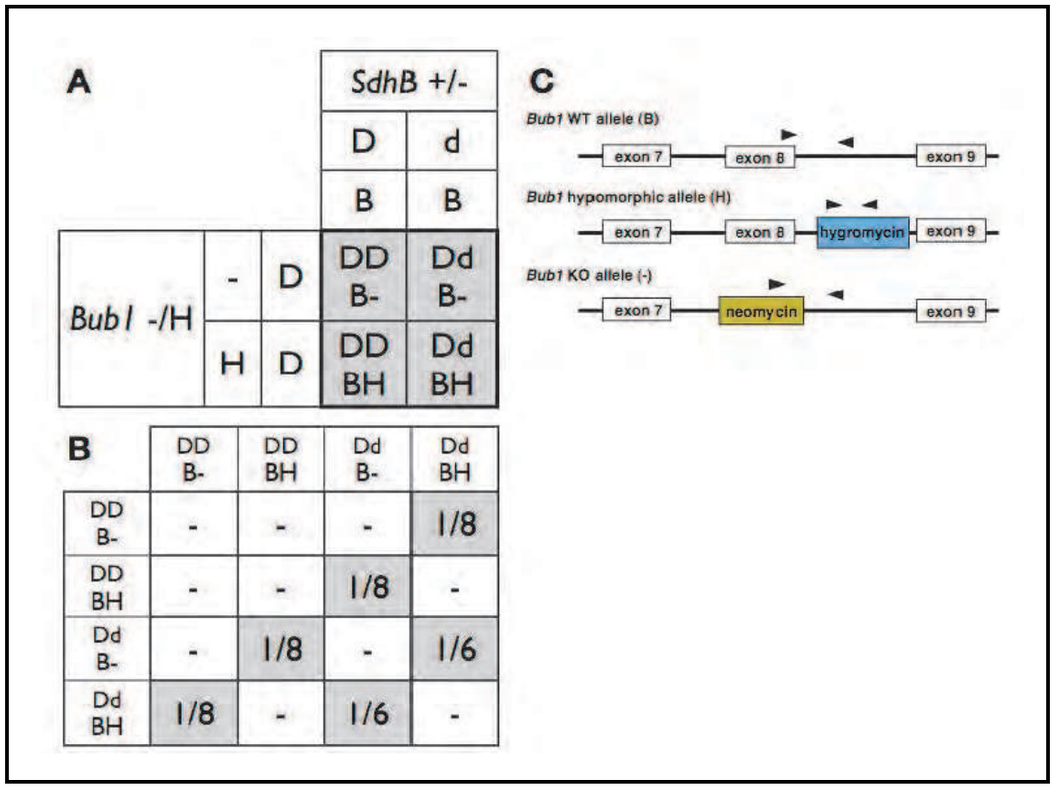

Fig. 9. *SdhB* +/- *Bub1* H/- breeding scheme. A. Initial cross providing offspring for brother-sister matings. B. Brother-sister mating scheme from (A), indicating expected frequencies of the desired *SdhB* +/- *Bub1* H/- genotype among offspring. Allele abbreviations: *SdhB* WT: D; *SdhB* gene trap disruption: d; *Bub1* WT: B; *Bub1* hypomorph, H; *Bub1* disruption: (-). C. *Bub1* Genotyping PCR design. Schematic diagram showing the *Bub1* locus with or without a hygromycin (hypomorphic allele) or neomycin (knockout allele) insertion. Primers used to amplify the alleles are designated by arrowheads.

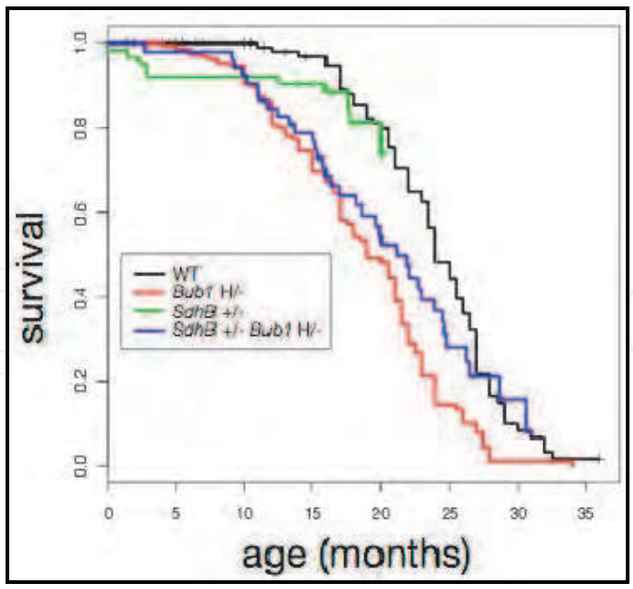

Fig. 10. Kaplan-Meier plot of mouse survival data.

4. Discussion

Spontaneous PGL is rare in mice (Jacks et al., 1994; Jacks et al., 1994). In a mouse model of human neurofibromatosis type 1, a dominant disease caused by inheritance of a mutant allele of NF1, a modest incidence of PHEO was observed (Jacks et al., 1994), but this genetic pathway to PGL appears unrelated to tumor suppression by *Sdh* genes.

We hypothesized that *SdhB* +/- mice would be predisposed to familial PGL. This hypothesis was based on the prevailing evidence that the *SdhB* +/- genotype predisposes humans to PGL, with high penetrance. We therefore developed a strain of *SdhB* +/- mice using mouse stem cells carrying a gene trap insertion in intron 1 of *SdhB*. Using these animals we showed that, as anticipated, the *SdhB* -/- genotype is not viable. Although we found that there is a significant reduction of SdhB expression in tissues of *SdhB* +/- mice relative to *SdhB* +/+ mice, there was no difference in SDH activity between the two groups in either liver or kidney. These results suggest that there is no phenotypic difference between *SdhB* +/- and *SdhB* +/+ mice, consistent with the absence of phenotype for humans heterozygous for *Sdh* germline mutations. However, unlike humans where there is high penetrance of PGL in *SdhB* +/- individuals, no PGL tumors were detected in *SdhB* +/- mice.

LOH of a TSG can be the rate-limiting step in tumor development (Lasko et al., 1991; Luo et al., 2000). The mechanism for LOH is not completely understood but involves chromosome loss, deletion and/or homologous interchromosomal mitotic recombination events (Henson et al., 1991; Shao et al., 2000; Stark & Jasin, 2003). Mitotic recombination refers to the reciprocal exchange of genetic material between nonsister chromatids in mitotic cells. Mitotic recombination occurs at a high frequency in humans and mice (Gupta et al., 1997; Holt et al., 1999; Shao et al., 1999). It has been suggested that mitotic recombination is the predominant pathway to LOH (Gupta et al., 1997; Luo et al., 2000; Shao et al., 1999; Shao et al., 2000).

Because mitotic recombination requires high nucleotide sequence homology, chromosomal divergence suppresses mitotic recombination and may modify cancer development by

lowering the rate of LOH (Shao et al., 2001). The use of inbred strains in this study (C57BL/6 x 129SVE)F1 should promote mitotic recombination. It has also been found that the spontaneous frequency of mitotic recombination increases with age (Grist et al., 1992). The penetrance of *SdhB* mutations in familial PGL is estimated to be 50% at age 30 and approaches 100% after 60 years of age (Neumann et al., 2004).

To address the possible limitation of LOH in converting somatic neuroendocrine cells from *SdhB* +/- to *SdhB* -/-, we developed a mouse colony of *SdhB* +/- mice bred onto the *Bub1* H/- genetic background previously shown to enhance aneuploidy. PGL tumorigenesis was not observed in this strain.

There are at least three possible explanations for these results. First, it is possible that the rate of somatic LOH at *SdhB* in neuroendocrine cells remains insufficient to drive PGL tumorigenesis within the lifespan of mice. Second, it is possible that PGL tumorigenesis takes place in *SdhB* +/- mice, but the subsequent rate of tumor growth is too slow to permit tumor detection within the mouse lifespan. Finally, it is possible that *SdhB* is not a tumor suppressor in murine PGL.

If either of the first two explanations are correct, it might be possible to enhance the rate of PGL tumorigenesis by developing a conditional knockout mouse strain where both copies of the *Sdh* gene for one SDH subunit are replaced by a knock-in construct that can be recombinationally inactived upon expression of a recombinase. Unlike standard conditional knock-out designs, it would be important to delete the *Sdh* subunit gene only at very low frequency, since most affected tissues would presumably be damaged by loss of the TCA cycle. Rather, an inefficient recombination that eliminated SDH in perhaps 1% of cells might be appropriate. This might be achieved either by low levels of neuroendocrine-specific expression of recombinase (e.g. from the tyrosine hydroxylase promoter), or by low levels of global recombinase expression. Unfortunately, mouse strains are not yet available for tissue-specific CRE expression in neuroendocrine cells.

Either low-efficiency conditional knockout strategy offers the prospect of dramatically increasing the early generation of neuroendocrine cells lacking SDH activity. Such a model would be hypothesized to increase the frequency of tumorigenesis, and allow a longer period for tumor growth.

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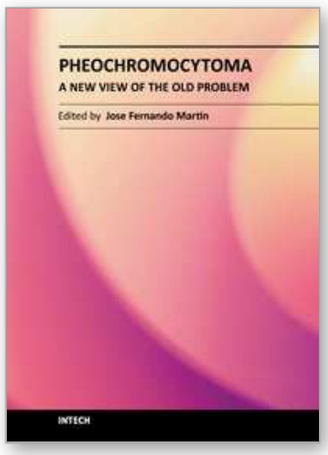

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The book is divided into six sections. The first three sections focus on the pathophysiology of the disease, showing anatomo- and histopathological aspects, experimental models and signaling pathways and programmed cell death related to pheochromocytoma. The fourth discusses some specific aspects of clinical presentation, with emphasis on clinical manifestations of headache and heart. The fifth section focuses on clinical diagnosis, laboratory and imaging, including differential diagnosis. Finally, the last section discusses the treatment of pheochromocytoma showing clinical cases, a case about undiagnosed pheochromocytoma complicated with multiple organ failure and other cases about catecholamine-secreting hereditary tumors. Thus, this book shows the disease "pheochromocytoma" in a different perspective from the traditional approach. Enjoy your reading.

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