Cardiac and CNS defects in a mouse with targeted disruption of suppressor of fused

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Accepted 2 August 2005

Summary

The hedgehog (Hh) pathway is conserved from Drosophila to humans and plays a key role in embryonic development. In addition, activation of the pathway in somatic cells contributes to cancer development in several tissues. Suppressor of fused is a negative regulator of Hh signaling. Targeted disruption of the murine suppressor of fused gene (Sufu) led to a phenotype that included neural tube defects and lethality at mid-gestation (9.0-10.5 dpc). This phenotype resembled that caused by loss of patched (Ptch1), another negative regulator of the Hh pathway. Consistent with this finding, Ptch1 and Sufu mutants displayed excess Hh signaling and resultant altered dorsoventral patterning of the neural tube. Sufu mutants also had abnormal cardiac looping, indicating a defect in the determination of left-right asymmetry. Marked expansion of nodal expression in 7.5 dpc embryos and variable degrees of node dysmorphology in 7.75 dpc embryos suggested that the pathogenesis of the cardiac developmental abnormalities was related to node development. Other mutants of the Hh pathway, such as Shh, Smo and Shh/Ihh compound mutants, also have laterality defects. In contrast to Ptch1 heterozygous mice, Sufu heterozygotes had no developmental defects and no apparent tumor predisposition. The resemblance of Sufu homozygotes to Ptch1 homozygotes is consistent with mouse Sufu being a conserved negative modulator of Hh signaling.

Key words: Sufu, Suppressor of fused, Hedgehog, Mouse development, Left-right asymmetry

Introduction

Developmental pathways first elucidated by genetic studies in the fruit fly Drosophila melanogaster are conserved in vertebrates and often influence similar developmental processes. Many developmental genes continue to play an important role in the regulation of cell growth and differentiation after embryogenesis, and mutations in some of these genes can result in cancer in mammals.

Hedgehog (Hh) is a secreted molecule that influences the differentiation of a variety of tissues during development. Hh, its receptor ‘patched’ (Ptc) and many downstream members of the Hh signal transduction pathway were originally discovered by developmental biologists studying embryogenesis in Drosophila (Nusslein-Volhard and Wieschaus, 1980). This pathway lays down the basic framework of the embryo, determining anteroposterior relationships (‘segment polarity’) in developing structures. Secreted Hh diffuses freely to determine cell fates in a concentration-dependent manner. Ptc is a repressor of Hh signaling and constitutively inhibits smoothened (Alcedo et al., 1996; Stone et al., 1996; van den Heuvel and Ingham, 1996; Xie et al., 1998). Upon Hh binding, smoothened (Smo) is released from repression and signals downstream to a complex consisting of fused (Fu), suppressor of fused [Sufu], costal 2 (Cos2) and cubitus interruptus (Ci). Ci is the transcriptional effector of the pathway (Alexandre et al., 1996; Eaton and Kornberg, 1990; Forbes et al., 1993; Grau and Simpson, 1987; Preat, 1992; Preat et al., 1990; Robbins et al., 1997; Simpson and Grau, 1987; Sisson et al., 1997). When the pathway is switched on, Ci dissociates from the complex and translocates to the nucleus, resulting in the expression of target genes, including ptc. In the absence of signal, Ci is proteolyzed to a 75 kDa repressor form that inhibits Hh expression (Aza-Blanc et al., 1997; Methot and Basler, 1999). Elements of the Hh pathway are found in most multicellular organisms. In humans, mutations that lead to activation of the pathway are often associated with cancer. For example, PTCH, the hedgehog receptor, is mutated in Gorlin syndrome, an autosomal dominant disorder characterized by multiple basal cell carcinomas (BCC) and medulloblastoma (Hahn et al., 1996; Johnson et al., 1996). Gorlin syndrome patients exhibit generalized overgrowth, developmental defects of the central nervous system, polydactyly, and a variety of other birth defects. The phenotype in mice heterozygous for a null allele of patched (Ptc1) closely mimics the human disease. These mice are 10% larger than their wild-type littermates and have birth defects similar to those seen in humans (Aszterbaum et al., 1999; Goodrich et al., 1997; Hahn et al., 1998). They develop medulloblastoma, rhabdomyosarcoma, and BCC-like tumors. The Ptc1 homozygous mutant is embryonic lethal at day 9.0-10.5, and has an open neural tube and heart abnormalities.

Suppressor of fused, like patched, is a negative modulator of Hh signaling. It was first identified as a dominant second-site
suppressor of the fused phenotype in Drosophila (Preat, 1992). Drosophila Su(fu) reduces the nuclear accumulation of the activator form of full-length Ci, presumably through cytoplasmic retention of Ci (Methot and Basler, 2000; Wang et al., 2000). Similarly, human suppressor of fused (SUFU) sequesters the GLI proteins, homologs of Drosophila Ci, in the cytoplasm to inhibit Hh signaling (Kogerman et al., 1999; Stone et al., 1999). By analogy to Ptc1, Su(fu) may function as a tumor suppressor and play a role in the development of the central nervous system (CNS), limbs and heart. To study the role of Su(fu) in development and carcinogenesis, we constructed a mouse Su(fu) mutant by targeted disruption of the Su(fu) gene. We anticipated that Su(fu) heterozygotes, like Ptc1 heterozygotes, would be prone to BCC and medulloblastoma, exhibit increased size and a set of birth defects similar to those in Gorlin syndrome. Su(fu) homozygotes were predicted to be embryonic lethal, with neural tube defects and possible heart abnormalities.

**Materials and methods**

**Generation of Su(fu) mutant mice**

Su(fu) mutants were created by standard gene targeting techniques. A 3.6 kb fragment containing exons and introns 4, 5 and 6, and a 1.5 kb fragment containing the 3’ end of exon 7, intron 7 and most of exon 8, were generated by polymerase chain reaction (PCR) from 129/Sv mouse genomic DNA. The two arms were cloned into the targeting vector PGK-neo lox\textsubscript{P} on either side of the neomycin gene (neo) with neo oriented opposite to Su(fu). The linearized targeting vector was electroporated into 129/Sv embryonic stem (ES) cells, W9.5 (provided by Dr Colin Stewart), which were placed under neo selection to obtain 150 resistant clones. ES clones were screened by PCR with a reverse primer from the 5’ end of the neo cassette (TGCAATCCATCTGGTCAATTG) and a reverse primer from the extreme 3’ end of Su(fu) exon 8 (ACTTACGGAGCCCTGTCGTG) and a reverse primer from the 3’ end of Su(fu) exon 8 (ACTTACGGAGCCCTGTCGTG). Correct targeting was confirmed by Southern blot analysis with a [\textsuperscript{32}P]dCTP-labeled 300 bp PCR fragment containing the last 28 bp of exon 7 and the first 216 bp of intron 8. BamHI digests were predicted to yield a 9 kb fragment from wild-type DNA and an 11 kb fragment bearing the neo cassette from a correctly targeted gene. Successful targeting of the allele results in the deletion of an intron-exon junction and most of exon 7. One positive ES clone was identified from 150 G418-resistant colonies. C57BL/6 blastocysts were injected with the mutant 129/Sv ES cells and then implanted into CD1 pseudopregnant females to generate chimeras. Chimeras were bred with C57BL/6 females (Charles River Laboratories) to generate heterozygous offspring on a mixed genetic background, or to 129S1/SvImJ females (Jackson Laboratories) to establish heterozygotes on a pure genetic background.

**Mouse genotyping**

Genotyping was performed by: (1) PCR of a 700 bp fragment of the neo gene using a 5’ primer (ATGACTGGGCAACAGCACAG) and a 3’ primer (CGTACGGAGGCCGAGCGAG); (2) Southern blot analysis using a BamHI digest and the Su(fu) intron 8 probe described above to distinguish homozygotes from heterozygotes at 8.5 dpc or later; or (3) PCR of a 3.8 kb fragment from the 3’ end of intron 3 (ACTGCAATCTGGTCCGCTTC) to the 5’ end of exon 7 (ACTGACGGCTGCTAGTTG) to distinguish homozygotes from heterozygotes at stages earlier than 8.5 dpc, when the quantity of yolk sac DNA was insufficient for Southern analysis.

**Mouse embryo studies**

Embryos were obtained from timed intercrossing of heterozygous mice. Noon of the day of detection of the vaginal plug was considered as 0.5 dpc. Embryos were dissected in phosphate buffered saline (PBS).

**Northern blot hybridization**

Total RNA was isolated from whole 9.5 dpc wild-type and mutant embryos using RNaseasy Mini Kit (Qiagen). Approximately 15 \(\mu\)g of each RNA sample was electrophoresed through a 1.2% formaldehyde gel and transferred to a nylon membrane (Genescreen, Dupont). Hybridization in PerfectHyb Plus Hybridization Buffer (Sigma) was carried out at 65°C with either a 1.3 kb Su(fu) cDNA fragment with exons 1-12 up to the stop codon or an exon 12 fragment containing 924 bp that followed the stop codon as a probe. Equal loading of RNA in each lane was determined by the appearance of ribosomal bands on ethidium bromide-stained RNA gels, and by reprobing the same northern blots with a mouse Gapdh cDNA (bases 379 to 910) after stripping off the Su(fu) probes with 1% SDS.

**Western blot analysis**

Lysates from whole 9.5 dpc wild-type and mutant embryos were prepared in RIPA lysis buffer. 50 \(\mu\)g of protein was run on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Bio-Rad). Su(fu) protein expression was detected with a goat polyclonal Sufu antibody (Santa Cruz Biotechnology) that recognizes the carboxy terminus. Secondary antibody was horseradish-peroxidase-conjugated anti-goat (Santa Cruz Biotechnology). Equal protein loading was detected using a monoclonal β-actin antibody (Sigma).

**Histology**

Wild-type and mutant embryos were fixed overnight in 10% buffered formalin and then paraffin wax embedded. Transverse sections (4 \(\mu\)m) were stained with Hematoxylin and Eosin using standard histology techniques.

**RNA in situ hybridization**

Whole-mount RNA in situ hybridization was performed as described previously (Wilkinson and Nieto, 1993). A sonic hedgehog (Shh) digoxigenin-labeled riboprobe (Boehringer-Mannheim) was generated by in vitro transcription from a 700 bp PCR fragment containing a portion of exon 3, and a Pax7 probe was made similarly from an 800 bp PCR fragment containing a portion of exon 9 and the 3’ UTR. The Pitx2 probe was a 725 bp fragment from the 3’ end of the cDNA extending into the 3’ UTR; Dnalc1 (previously 1rd) was a 700 bp probe from the 3’ UTR; Ptc1 was a 540 bp fragment of exon 23; and Nodal was a 1.8 kb cDNA fragment encompassing most of the nodal gene (Zhou et al., 1993). Hybridized probes were detected with alkaline phosphatase (AP)-coupled anti-digoxigenin antibodies developed with BM Purple-AP substrate (Roche). For Shh and Pax7, 10 \(\mu\)m frozen transverse sections were prepared.

**Immunohistochemistry**

For whole-mount detection of acetylated tubulin, 7.75 dpc embryos were fixed overnight in 4% paraformaldehyde in PBS, then dehydrated on ice in a series of graded methanol and PBS solutions and stored in 100% methanol at -20°C. The embryos were rehydrated in a series of graded solutions of methanol and PBS with 0.2% Triton X100 (Kodak) (PBT), then treated for 1 hour with 3% bovine serum albumin (BSA) in PBT. They were incubated overnight at 4°C with a 1:200 dilution of mouse monoclonal anti-acetylated tubulin clone 6-IIB-1 (Sigma) in PBT-3% BSA, followed by three 45-minute PBT-3% BSA washes at room temperature, then incubated for 3 hours at room temperature with a 1:150 dilution of mouse monoclonal anti-actin antibody in PBT-3% BSA followed by three 45-minute washes in PBT at room temperature. They were mounted and cover-slipped on glass slides in ProLong Antifade medium (Molecular Probes) with the posterior of the embryo facing up, then viewed and photographed by confocal microscopy.

For sections through the neural tube, embryos were fixed overnight
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in 4% paraformaldehyde at 4°C, washed with PBS for one hour, and equilibrated in 30% sucrose overnight at 4°C. Transverse frozen sections (10 μm) were incubated in the indicated primary antibody for two hours and then incubated with corresponding secondary antibodies. Slides were mounted with ProLong Antifade mounting medium (Molecular Probes). Sections were examined with a Zeiss LSM 510 confocal microscope. Antibodies were mouse anti-Shh-N (Ericson et al., 1996), mouse anti-FoxA2 (Ericson et al., 1996), mouse anti-Nkx2.2 (Ericson et al., 1997a) and mouse anti-Pax6 (Ericson et al., 1997b). The anti-PTCH antibody was mouse anti-LSM 510 confocal microscope. Antibodies were mouse anti-Shh-N intermediate (Molecular Probes). Sections were examined with a Zeiss LSM 510 confocal microscope. Antibodies were mouse anti-Shh-N intermediate (Molecular Probes).

Exposure of mice to ionizing radiation

Isogenic Sufu heterozygotes and wild-type littermates at 2 months of age were exposed to one dose of 4 Gray (Gy) of whole-body ionizing radiation at 1.75 Gy/minute using a Mark I model 68A cesium-137 device. There were no deaths due to acute radiation exposure. Mice were monitored weekly for twelve months. After necropsy, tissue was fixed in formalin and embedded in paraffin. Sections were stained with Hematoxylin and Eosin. A veterinary pathologist examined the tissue sections.

Results

Germline transmission of the mutant allele and molecular characterization of the effects of gene targeting

Chimeras were bred to C57BL/6 animals and 129/Sv to create Sufu heterozygotes on mixed and pure 129/Sv backgrounds, and germline transmission was confirmed by PCR-based screening for the presence of the neo cassette and Southern blot analysis using a BamHI restriction digest of the Sufu gene (Fig. 1B). Another restriction enzyme, SphI, also verified correct targeting of the Sufu gene (data not shown). Heterozygous mice were viable and appeared grossly normal.

Intercrossing of heterozygous mice produced no live-born homozygous offspring. Analysis of timed stages revealed that Sufu homozygous mutants did not survive past 10.5 dpc. In order to determine the effects of gene targeting on mRNA and protein expression, we characterized the Sufu mutant transcript and protein in 9.5 dpc homozygous embryos.

Correct targeting of the Sufu gene would eliminate the intron 6/exon 7 junction and obliterate normal mRNA splicing in this region. A cDNA probe with the entire coding sequence of Sufu identified a 5.5 kb mRNA in wild-type mice, consistent with previous reports (Fig. 1C, upper panel). The Sufu homozygotes had aberrant 8.5 kb and 1.5 kb transcripts, and a transcript similar in size to the wild type. To help determine whether the 5.5 kb band in the mutant was truly identical to the wild-type band, the blot was reprobed with a fragment containing only the 3' end of the coding sequence. There was no band equal in size to wild type with this probe (Fig. 1C, middle panel). The Sufu homozygotes had aberrant 8.5 kb and 1.5 kb transcripts, and a transcript similar in size to the wild type. To help determine whether the 5.5 kb band in the mutant was truly identical to the wild-type band, the blot was reprobed with a fragment containing only the 3' end of the coding sequence. There was no band equal in size to wild type with this probe (Fig. 1C, middle panel). These results are consistent with the targeted gene producing three aberrant splice products. To confirm the loss of full-length Sufu protein, we performed western blot analysis with an antibody to the carboxy terminus of Sufu. The expected 54 kDa Sufu protein was observed in wild-type and heterozygous 9.5 dpc embryos (Fig. 1D). There was no detectable Sufu protein expression in

Fig. 1. Targeted disruption of the Sufu gene. (A) The Sufu gene consists of 12 exons and contains a putative PEST sequence. The carboxy terminus is required for Gli binding (Kogerman et al., 1999; Dunavea et al., 2003). In the targeting vector (middle), the intron 6-exon 7 junction and most of exon 7 was replaced with the neo gene. The fragment used as an external probe in Southern blotting for screening and genotyping is indicated. (B) Southern blot analysis of genomic DNA from 9.5 dpc wild-type, heterozygous and homozygous embryos. DNA was digested with BamHI and hybridized with an intron 8 probe that detects the 9 kb normal allele and the 11 kb targeted allele. (C) Northern blot analysis of 9.5 dpc whole embryo RNA using a full-length Sufu cDNA probe detected a 5.5 kb transcript in all genotypes, but aberrant 8.5 kb and 1.5 kb transcripts were detected in the homozygous mutant. A 3' probe did not detect any transcript in the homozygous mutant, but the expected 5.5 kb transcript was detected in wild-type and heterozygous embryos. Gapdh transcript is equal in all genotypes. (D) Western blot analysis of 9.5 dpc whole embryo lysates using an anti-Sufu antibody that detects the carboxy terminus. No Sufu protein expression was detected in the homozygous mutant, but the expected 54 kDa band was present in wild-type and heterozygous embryos. β-Actin expression was equal in all genotypes.
homozygous embryos. β-Actin expression was equivalent in all three genotypes.

**Detailed characterization of the heterozygous and homozygous mutant phenotype**

The Sufu mutant phenotype was virtually identical on a mixed 129/Sv;C57BL/6 background and on an inbred 129/Sv background. Unlike Ptch1 heterozygotes, which are consistently larger than their wild-type littermates and have a 5% rate of polydactyly, Sufu heterozygotes had no obvious abnormalities. Detailed necropsy showed no gross internal malformations in Sufu heterozygotes.

In addition to its role in the Hh pathway, Sufu has been shown to bind and inhibit the activity of β-catenin in tissue culture studies (Meng et al., 2001). This function is similar to that of adenomatous polyposis coli (APC), and APC heterozygous mutants in both mouse and human are prone to gastrointestinal polyps. Sufu mutants had no gastrointestinal polyps, indicating that this function is not significant in vivo or that loss of Sufu alone does not produce a strong enough effect to result in a visible phenotypic abnormality.

Homozygous Sufu mutants at 8.5 dpc grossly resembled their wild-type littermates. By 9.0-9.5 dpc, abnormalities first appeared with growth retardation, failure to undergo embryonic turning, abnormal somites and an open neural tube (Fig. 2B,C). Four out of 13 Sufu mutants at 9.5-10.5 dpc had L-looped (leftward) hearts (Fig. 2H), and one had indeterminate cardiac looping (Table 1). All wild-type littermates had normal D-looped hearts. The expression of Pitx2, normally found in the left lateral plate mesoderm at 8.5 dpc, paralleled the observed morphologic defects, with approximately one third of mutant embryos demonstrating absent or bilateral expression (Fig. 3C,D and Table 2). These data suggest an abnormal development of left-right asymmetry as the basis for the abnormal heart looping. Signals specifying left-right asymmetry originate at the node (Brennan et al., 2002; Levin et al., 1995), so the integrity of the node was examined. Nodal signaling at the node controls the laterality of left-right asymmetry (Brennan et al., 2002). In situ analysis for Nodal RNA at 7.5 dpc showed marked expansion of Nodal expression in six out of six Sufu–/– embryos analyzed (Fig. 3B). Cells in the node region of Sufu–/– embryos expressed left-right dynein (Dnahc11) RNA (data not shown). A more detailed examination of the node at 7.75 dpc with anti-acetylated tubulin antibody staining and confocal microscopy showed abnormally shaped nodes in five out of five embryos (Fig. 4B,C). Abnormalities varied from mild changes in shape, such as a diamond-shaped node instead of the normal more triangular node (Fig. 4B), to more extensive malformations such as multiple small nodes separated by intervening epithelial cells (Fig. 4C). At higher magnification, the node cells themselves appeared normal, and each node cell had a monocilium of the same length as that observed in wild-type nodes (Fig. 4D-F).

Additionally, 4 out of 14 mutants had hemorrhaging (aneurysms) in the diencephalic hemispheres (data not shown). This abnormality may be attributable to defects of the vasculature. Other evidence for such a defect was dilatation of the descending aorta (Fig. 2E,F; also visible in Fig. 6B). Hyperplasia of the myocardium was also observed (Fig. 2E,F).

Although some of the features of Sufu mutants resembled those seen in other mutants of the Hh pathway, none of the observed dysmorphology was a specific measure of Hh signaling. To further evaluate Hh pathway activity, we used

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<th>Direction of heart looping</th>
<th>Embryos examined n (%)</th>
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<tr>
<td>D-looped</td>
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</tr>
<tr>
<td>L-Looped</td>
<td>4 (31%)</td>
</tr>
<tr>
<td>Indeterminate</td>
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<th>Expression pattern</th>
<th>Embryos examined n (%)</th>
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<tr>
<td>Left</td>
<td>9 (69%)</td>
</tr>
<tr>
<td>Absent</td>
<td>3 (23%)</td>
</tr>
<tr>
<td>Bilateral</td>
<td>1 (8%)</td>
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</table>
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Fig. 3. Nodal and Pitx2 expression in wild-type and Sufu<sup>−/−</sup> mouse embryos. (A,B) Whole-mount RNA in situ using a digoxigenin-labeled Nodal riboprobe on 7.5 dpc embryos shows Nodal expression in the region of the node in a wild-type embryo (A) and marked expansion of the region of Nodal expression in a Sufu<sup>−/−</sup> embryo (B). (C,D) Whole-mount in situ hybridization using a Pitx2 digoxigenin-labeled riboprobe on 8.5 dpc embryos (posterior views) shows Pitx2 expression in the head folds and left lateral plate mesoderm in a wild-type embryo (C); Pitx2 expression is absent in the left lateral plate mesoderm of a Sufu<sup>−/−</sup> embryo (D).

In Drosophila, hh expression is repressed when the pathway is inactive. By analogy to Drosophila, loss of Sufu in the mouse should allow for de-repression of Shh expression. Whole-mount RNA in situ in 9.5 dpc embryos showed a dramatic upregulation of Shh expression throughout the brain and neural tube of Sufu homozygous mutants (Fig. 5B). Transverse sections showed that Shh expression expanded dorsally to encompass the ventral half of the neural tube (Fig. 5D). Likewise, the distribution of Shh protein, as determined by immunohistochemistry, was much broader than in wild-type embryos and encompassed most of the neural tube (data not shown). Although immaturity of Sufu mutants would be a possible explanation for persistent high levels of Shh in the brain, the increased distribution of expression in more caudal regions of the neural tube is not consistent with this explanation and indicates de-repression of the gene.

In addition to the effect on expression of Shh itself, loss of Sufu also removes a negative modulator of downstream Hh signaling. The combination of these two effects would be expected to strongly promote activation of the Hh pathway and upregulation of target genes. Ptch1 transcription is directly activated by Hh signaling and is normally restricted to ventral cells of the neural tube (Fig. 6C). In the Sufu mutant, Ptch1 protein was ectopically expressed throughout the ventricular zone (Fig. 6D).

Shh regulates the conversion of neural plate cells into ventralized neural progenitors. Markers of ventral cell types include Foxa2 and the Nkx2.2 homeodomain transcription factor (Hynes et al., 1995; Roelink et al., 1995). Normally, Foxa2 and Nkx2.2 are expressed in the floor plate, with Foxa2 expression being restricted to the ventral midline (Ang et al., 1993; Ruiz i Altaba et al., 1993; Sasaki and Hogan, 1993) (Fig. 6E,G). We observed a dorsal expansion of Foxa2- and Nkx2.2-expressing cells in the Sufu mutant (Fig. 6F,H). Pax6, a murine homeobox gene, is expressed in the lateral domain of the neural tube patterning as an assay for Hh effect in development. Shh determines cell fates in a concentration-dependent manner in the neural tube. Shh is initially produced in the notochord and then in the floor plate to modulate the differentiation of ventral cell types, such as motoneurons (Ericson et al., 1996).

Fig. 4. Abnormal node morphology in Sufu<sup>−/−</sup> mouse embryos. Immunofluorescent staining with anti-acetylated tubulin antibodies was performed on wild-type (A,D) and Sufu<sup>−/−</sup> (B,C,E,F) mouse embryos at 7.75 dpc. In wild-type embryos, the node is composed of tightly packed, monociliated cells and is roughly the shape of an isosceles triangle with the base oriented toward the posterior (yellow outlining in A). The cytoskeletons of cells surrounding the node are visible in A, which shows the normal size difference between node cells and their larger neighbors. Variable node dysmorphology (B,C) is seen in the Sufu<sup>−/−</sup> embryos. Five out of five 7.75 dpc Sufu<sup>−/−</sup> embryos examined displayed abnormal node architecture, ranging from mild changes in the shape of the node to irregular distribution of nodal cells. B shows an ovoid to diamond-shaped node in a Sufu<sup>−/−</sup> embryo. C shows three or more separate irregular islands of nodal tissue with flattened cells, atypical for a normal node, visible between the islands. (D-F) Higher magnification views show normal monocilia in a wild-type embryo (D), and in Sufu<sup>−/−</sup> embryos (E,F). Boxes in A-C outline the areas shown at higher magnification in D-F. All panels are oriented with anterior at the top and posterior at the bottom. Ant, anterior; Post, posterior. Scale bars: 50 μm in A-C; 10 μm in D-F.
ventricular zone (Fig. 6I). Low concentrations of Shh can activate Pax6 expression, but high concentrations repress expression (Ericson et al., 1997a). Consistent with the dorsal expansion of Shh expression, Pax6-expressing cells were detected in the extreme dorsal cells of the neural tube (Fig. 6J). A marker of dorsal cell types, was virtually absent from the extreme dorsal cells of the neural tube (data not shown). Pax7, whereas Pax6-positive cells were detected in the neural tube of Sufu mutants (Fig. 7B,D).

Consistent with morphological evidence for abnormal somite development, whole-mount analysis of Ptch1 expression provided evidence for excess Hh signaling in somites and the neural tube (Fig. 8B). These findings support an activation of the Hh pathway in non-neural tissues as well as in the neural tube.

Evaluation of Sufu heterozygotes for tumor predisposition

Ptch1 heterozygote knockout mice have a high incidence of tumors, including medulloblastoma, rhabdomyosarcoma and BCC (Aszterbaum et al., 1999; Goodrich et al., 1997; Hahn et al., 1998). To determine whether Sufu heterozygotes are susceptible to these tumors, we exposed these mice to a single dose of 4 Gy of ionizing radiation. Several studies have shown that ionizing radiation can increase the frequency of tumors and decrease tumor latency in animals carrying mutations in known tumor suppressor genes. In particular, this dose of radiation causes skin tumors in virtually 100% of Ptch1 mice (Aszterbaum et al., 1999). C57BL/6;129SV F1 progeny were generated by breeding chimeras to C57BL/6 females. Twenty Sufu heterozygotes and 21 isogenic wild-types were monitored weekly for tumors. One heterozygous mouse died of unknown causes. Otherwise, all animals survived and were necropsied twelve months after radiation exposure. None of the Sufu heterozygotes exhibited medulloblastoma, rhabdomyosarcoma or BCC (Table 3). Ovarian granulosa cell adenomas and hepatomas were observed in both wild-type and Sufu heterozygote mice. Bronchiolalveolar adenoma was observed in one Sufu heterozygote mouse; this tumor was not seen in wild-type littermates. However, the incidence of this tumor type is too low to draw any definitive conclusions. To determine whether genetic background could influence the tumor spectrum, we repeated this experiment with Sufu heterozygotes on a 129/Sv background. These mice have been monitored for eleven months and show no signs of tumor growth. Lack of medulloblastoma and BCC in Sufu heterozygotes suggests that loss of Sufu is not sufficient to induce these tumors on either of the two genetic backgrounds within the observed time frame.

Discussion

To study the role of Sufu in development and carcinogenesis, we constructed a mouse Sufu mutant by targeted disruption of the Sufu gene. We anticipated that Sufu heterozygotes, like Ptch1 heterozygotes, would develop BCC and medulloblastoma, and exhibit increased size and polydactyly, whereas Sufu homozygotes would exhibit embryonic lethality with neural tube and cardiac defects.

In general, our findings indicate that the phenotypic effect of Sufu disruption is milder than that of Ptch1 disruption. We found that Sufu heterozygosity confers no obvious developmental phenotype and that Sufu heterozygotes, unlike Ptch1 heterozygotes, have no increase in birth defects or tumor predisposition. Nevertheless, the homozygous mutants share many features. This study reveals several aspects of the role of Sufu in vertebrate development and the control of cell proliferation.

Conservation of function between Drosophila and mouse Sufu in Hh signaling

In Drosophila, the level of activity of the Hh pathway

Table 3. Tumor incidence following irradiation in 20 Sufu+/– and 21 wild-type littermates at 12 months

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Tumor type</th>
<th>Number of mice with tumor type</th>
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<tr>
<td>Wild type</td>
<td>Mammary gland adenoquamous carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>Wild type</td>
<td>Ovarian tubulostromal adenoma</td>
<td>1</td>
</tr>
<tr>
<td>Wild type</td>
<td>Ovarian granulosa cell adenoma</td>
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</tr>
<tr>
<td>Wild type</td>
<td>Hepatoma</td>
<td>3</td>
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<tr>
<td>Wild type</td>
<td>Small intestinal adenocarcinoma</td>
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<tr>
<td>Sufu+/-</td>
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</tr>
<tr>
<td>Sufu+/–</td>
<td>Bronchiolalveolar adenoma</td>
<td>1*</td>
</tr>
<tr>
<td>Sufu+/-</td>
<td>Hepatoma</td>
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*Same mouse.
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Mouse suppressor of fused mutant

Fig. 6. Excess Hh signaling and ventralization of the neural tube in Sufu–/– mutants. Cross-sections of the thoracic region stained with Hematoxylin and Eosin show the morphology of a normal neural tube of wild type (A) and the open neural tube of Sufu–/– mutants (B) at 9.5 dpc. Immunohistochemistry with markers of neural progenitor cells (anti-PTCH, anti-Foxa2, anti-Nkx2.2 and anti-Pax6) in wild-type (C,E,G,I) and Sufu–/– (D,F,H,J) 9.5 dpc embryos. Ptch1 (a Hh target gene product), Foxa2 and Nkx2.2 were expressed in the floor plate of wild-type embryos (C,E,G), with Foxa2 expression being restricted to the ventral midline. In Sufu–/– embryos (D,F,H), expression of ventral markers expanded to dorsal regions of the neural tube. Pax6 was expressed in lateral regions of the ventricular zone of the wild type (I), but its expression was repressed in Sufu–/– mutants (J). Cross-sections are of the thoracic region. Scale bars: 50 μm.

Fig. 7. Pax7 expression in wild type and Sufu–/– mutants. Whole-mount in situ hybridization using a Pax7 digoxigenin-labeled riboprobe on wild-type (A) and Sufu–/– (B) embryos at 9.5 dpc. The Sufu–/– embryo exhibited virtually no Pax7 expression throughout the brain and developing spinal cord compared with wild type. Cross-section of a wild-type embryo (C) demonstrates that Pax7 is normally expressed in the dorsal neural tube; a Sufu–/– embryo (D) displays virtually no expression throughout the neural tube. Cross-sections are of the thoracic region.

As might be expected, the upregulation of Shh expression in Sufu mutants could result solely from a lack of repression by Sufu, or from ligand-dependent Hh pathway activation due to dorsal diffusion of excess Shh protein. Future studies might include an analysis of Hh pathway target gene expression in Sufu–/–;wild-type chimeric embryos to determine how clones homozygous mutant for Sufu behave in a neural tube with a normal Shh gradient.

In vertebrates, Shh signals regulate embryogenesis by directing the transcription of target genes, including Ptch1. The negative-feedback loop is necessary to regulate the distribution of Shh protein and the level of Hh pathway activation. Ptch1 null embryos show upregulation of the Hh pathway and a relatively uncontrolled activity of Hh target gene transcription due to the lack of functional Ptch1 protein. We observed ectopic dorsal expansion of Ptch1 protein throughout the ventricular zone of Sufu mutants. In contrast to Ptch1 mutants, Sufu mutants have functional Ptch1 protein. The negative-feedback loop is intact, but loss of Sufu had the same effect as loss of Ptch1 in creating ventralization of cell types in the neural tube.

influences the maintenance of hh expression. When the pathway is inactive, the transcriptional repressor form of Ci represses hh. The presence of Hh ligand or the removal of an antagonist of Hh signaling allows for de-repression of hh expression. Theoretically, disrupting the Su(fu) gene would allow for ligand-independent Hh pathway activation in Su(fu) null cells. In agreement with this mechanism, Shh transcription is upregulated throughout the neural tube of Sufu mouse mutants. This expansion of the zone of Shh expression in Sufu mutants could result solely from a lack of repression by Sufu, or from ligand-dependent Hh pathway activation due to dorsal diffusion of excess Shh protein. Future studies might include an analysis of Hh pathway target gene expression in Sufu–/–;wild-type chimeric embryos to determine how clones homozygous mutant for Sufu behave in a neural tube with a normal Shh gradient.

As might be expected, the upregulation of Shh expression in Sufu mutants occurs in the regions where Sufu is expressed during mouse embryogenesis. Sufu expression is seen in the mouse embryo during mid-gestation in the forebrain, midbrain, hindbrain and developing spinal cord (Kogerman et al., 1999; Stone et al., 1999). Specifically, Sufu expression is observed within the neuroepithelium and dorsal ectoderm of the ventricular zone (Pearse et al., 1999; Simon-Chazottes et al., 2000).

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The Gli genes, vertebrate homologs of *ci*, are required for transduction of Hh signal. Among the three vertebrate Gli proteins, Gli3 appears to have largely a repressor function, and there is evidence that it undergoes proteolytic processing in vivo, analogous to that of *Drosophila* Ci (Ohlmeyer and Kalderon, 1998; Wang et al., 2000). Gli1 has an activator function, and Gli2 can exert both positive and negative functions in transcriptional regulation (Koebernick and Pieler, 2002), although a repressor role for Gli2 in vivo is not established (Bai and Joyner, 2001). Consistent with Gli3 being a negative regulator of Hh signaling, Gli3 mutant mice share some similarities with *Ptch1* mutant mice, having brain defects (Franz, 1994) and polydactyly (Hui and Joyner, 1993). Furthermore, *Ptch1* mutants, *Gli3* mutants (Goodrich et al., 1997; Ruiz i Altaba, 1998) and *Sufu* mutants all express ectopic Shh in the dorsal neural tube, suggesting that these loss-of-function mutants have an elevated Hh pathway activity.

Interestingly, in *Drosophila*, the loss-of-function mutant of *Su(fu)* is viable and displays an essentially normal phenotype (Prent, 1992), with very minor abnormalities in wing vein morphology (Ohlmeyer and Kalderon, 1998). *Su(fu)* overexpression mutants have no recognizable phenotype (Pham et al., 1995). These observations demonstrate that, under standard laboratory growth conditions, *Su(fu)* is not essential for normal development in *Drosophila*. There is no evidence that *Drosophila* *Su(fu)* alone has a powerful effect on cellular proliferation or differentiation. The requirement for Sufu in mouse, but not *Drosophila*, development suggests a divergence among organisms. The complexity of multicellular vertebrates and the need for a tighter control of signal transduction pathways may contribute to these disparities. Vertebrate morphological development may specifically require the use of Sufu for different functions that are essential to viability. In support of this model, there is growing evidence that genes conserved from *Drosophila* to vertebrates may acquire new interacting partners through evolution. Genetic screens in vertebrates have identified Hh pathway signaling components that do not seem to be necessary to Hh signaling in *Drosophila* (Bulgakov et al., 2004; Huangfu et al., 2003; Wolff et al., 2004), and, in addition, Sufu may function in other signaling pathways.

**Defects in left-right asymmetry and cardiac morphogenesis in mutants of the hedgehog pathway**

Hh signaling is involved in the developmental program that ensures that left-right asymmetry is nonrandom (Levin et al., 1995; Schneider and Brueckner, 2000). The embryonic node is a site of *Shh* expression and is essential in left-right determination (Pagan-Westphal and Tabin, 1998). In the chick, *Shh* is asymmetrically expressed, but *Shh* expression is not asymmetric in the mouse.

The phenotypes of mice with targeted disruption of Hh pathway genes indicate that Hh signaling may play a role at more than one stage of left-right determination. *Shh*+/− mice have a 10% incidence of reversed cardiac looping and an additional 30% incidence of abnormal positioning of the cardiac loop. *Pitx2* and other markers of left-sided signaling are expressed bilaterally in a portion of *Shh* mutants. There is evidence that hedgehog is required to prevent left determinants from being expressed on the right at a stage after initial asymmetric signaling is set up (Meyers and Martin, 1999).

Conversely, Hh pathway genes also act at an earlier stage at the level of the node, and left-right defects may be secondary to intrinsic abnormalities in this structure. *Smo*+/− embryos have much more profound cardiac abnormalities than *Shh*+/− embryos and closely resemble *Shh*+/−;*Ihh*−/− compound mutants (Zhang et al., 2001). These findings suggest that Shh and Ihh have redundant functions in left-right axis determination. Phenotypical features include a failure to undergo embryonic turning and a linear heart tube without right or left looping. *Pitx2* expression in the lateral plate mesoderm is absent in both *Smo*+/− embryos and *Ihh*−/−;*Shh*+/− compound mutants. Both *Smo*+/− and *Shh*+/−;*Ihh*−/− mutants have abnormal expression of genes normally expressed at the node and midline, including the Tgfβ growth factor *Gdf1* and *Nodal* (Zhang et al., 2001). Mutants deficient in dispatched 1, a molecule believed to mediate the release of soluble Hh protein necessary for communication with distant cells, have a phenotype similar to *Smo* mutants. There is nearly a complete absence of heart looping with an absence of *Nodal* expression in the left lateral plate mesoderm in *Disp1* mutants (Ma et al., 2002). It has been suggested that Hh lies upstream of the asymmetric calcium signals that are the earliest visible signs of left-right asymmetry in both mouse (Tanaka et al., 2005) and zebrafish (Sarmah et al., 2005).

Studies of chick development predict that loss-of-function mutations in both positive and negative members of the Hh signaling pathway might result in laterality defects (Levin et al., 1995), but a laterality defect in a mouse mutant with excess Hh signaling has not been reported previously. Although *Ptch1* mutants have cardiac anomalies (Goodrich et al., 1997), they reportedly establish a normal left-right axis (Zhang et al., 2001). *Sufu*+/− embryos presumably have excess, rather than a lack of, Hh signaling during the process of left-right determination. However, like *Smo* and *Disp1* mutants, they fail to undergo normal embryonic turning, and approximately one third have abnormal *Pitx2* expression and inverse cardiac looping. *Sufu* mutants also exhibit hyperplasia of the myocardium, an effect that might be predicted based on evidence from *Smo* mutants that Hh signaling controls early *Nkx2.5* expression in cardiac mesoderm (Zhang et al., 2001). *Nodal* is expressed dynamically in discrete regions of the developing mouse embryo. It is required for the induction of
mesendodermal cell types, and mouse Nodal mutants do not form a primitive streak (Conlon et al., 1994; Zhou et al., 1993). In addition, Nodal has been implicated in the formation and function of the mouse node. Asymmetric Nodal expression in the developing node and in the left lateral plate mesoderm determines normal left-right asymmetry. The defect that leads to left-right axis abnormalities in Sfu mutants appears to be marked expansion of Nodal expression with concomitant abnormal node morphology. Excess expression of Nodal in and around the developing node could plausibly lead to variable node dysmorphology, with more normal nodes leading to normal cardiac phenotypes and more disorganized nodes leading to randomized cardiac phenotypes via the disruption of normal left-right signaling. Milder defects might not affect the leftward nodal flow that initiates normal asymmetric left-right signaling, whereas more severe node malformations would lead to laterality defects. The variability of node dysmorphology could thus partially explain why fewer than half of the embryos examined exhibited abnormal cardiac looping. The node cilia appeared microscopically normal, although studies of ciliary motility in Sfu mutant embryos have not yet been done.

The molecular basis for the expansion of Nodal expression in 7.5 dpc embryos is not yet determined, and further studies will be necessary to clarify both the pathogenesis of the defect and its downstream effects. Nodal dysregulation at this embryonic age suggests an early function of Sufu, possibly through modulation of early Hh signaling. In support of this model, defects in the mouse homologs of the intraflagellar transport (IFT) components IFT172 and IFT88 result in altered Hh signaling, and abnormal nodes and left-right development (Huangfu et al., 2003). Another speculative explanation for the paradoxical lack of left-right abnormalities in Ptch1 mutants when compared with Sfu mutants is that Sfu might act through effectors other than the classical targets of the Hh pathway, i.e. through Notch or Wnt signaling, two pathways with known roles in Nodal expression. For example, there is evidence that Sfu is a negative regulator of β-catenin, a key member of the canonical Wnt signaling pathway (Meng et al., 2001). Mice mutant for Apc, another negative regulator of β-catenin, or Tcf3, a constitutive repressor antagonized by β-catenin, exhibit a variety of node abnormalities (Merrill et al., 2004) not dissimilar to those seen in the Sfu mutant. As in Apc and Tcf3 mice, the node defects in the Sfu+/- mouse could theoretically be due to upregulation of β-catenin signaling leading to an expansion of Nodal expression. Future studies will help to clarify the etiology of the expansion of Nodal expression, the node defect, and their downstream phenotypic sequelae.

Shh signaling and cancer predisposition

Ptch1 heterozygous mice phenotypically resemble humans with Gorlin syndrome, having developmental defects and cancer predisposition. Although loss of Sufu might be expected to generate a similar phenotype, adult Sfu heterozygous mice were grossly normal. They were equal in size to their wild-type littermates and had no developmental defects. Sfu heterozygotes did not develop tumors associated with Hh pathway activation.

In contrast to the mouse model, Sfu loss-of-function mutations in human heterozygotes can predispose to medulloblastomas (Taylor et al., 2002). Tumors in these individuals arise with loss of the normal allele. There are no prospective studies of human Sfu heterozygotes, and it is possible that these tumors are seen in only a minority of genetically affected individuals. A low-penetrance phenotype in humans would be consistent with the failure to identify medulloblastomas in the limited number of mice that have been studied thus far. Sfu mutation may not be a dominant mechanism in the development of these tumors, and other mutations might be required for tumorigenesis. Like mice heterozygous for Sfu mutations, there were no apparent developmental abnormalities in human Sfu heterozygotes. These data strongly support the notion that, in contrast to Ptch1, loss of one copy of Sfu is not sufficient to cause a phenotype related to Hh pathway activation. It would seem that Sfu functions as a modulator of Hh signaling but does not have the same impact as Ptch1 does, decreased activity of this gene product is likely to influence the cancer susceptibility in Ptch1 heterozygotes. Sfu+/-;Ptch1+/- mice might have a shorter tumor latency period, larger tumors or biologically more aggressive tumors, such as metastatic BCC. Predisposition to additional tumor types might be unmasked in this genetic background.

A.F.C. was the recipient of the Ford Foundation Fellowship and a UNCF-Merck Dissertation Fellowship. This work was supported by PHS grants (R01 CA57605 and R01 CA93908) and the Rachel Molly Markoff Research Award from the Brain Tumor Society to A.E.B. The monoclonal antibodies developed by T. Jessell, S. Brenner-Morton, and A. Kawakami were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA.

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