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Mice Deficient in the *Fused* Homolog Do Not Exhibit Phenotypes Indicative of Perturbed Hedgehog Signaling during Embryonic Development

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Hedgehog (Hh) signaling plays a major role in multiple aspects of embryonic development. To understand how a single Hh signal is capable of generating distinct readouts in Hh-responsive cells requires elucidation of the signal transduction cascade at the molecular level. Key components that mediate Hh signal transduction downstream of the receptor include Fused (Fu), Suppressor of fused (Sufu), and Costal-2 (Cos2) or the vertebrate homologs Kif27/Kif7. Studies with both invertebrates and vertebrates have led to a model in which a protein complex composed of Fu, Sufu, and Cos2 controls the processing, activity, and subcellular distribution of the Ci/Gli transcription factors responsible for Hh target gene activation. These converging results obtained with different species reaffirm the prevailing view of pathway conservation during evolution. Genetic studies of Fu, Sufu, and Kif27/Kif7 in mice are required to provide further verification of Hh pathway conservation. To this end, we generated a gene-targeted allele of *Fu* in mice. Surprisingly, our analysis indicates that *Fu*-deficient mice do not exhibit any embryonic phenotypes indicative of perturbed Hh signaling. This could be due to either functional redundancy or Hh pathway divergence and clearly indicates greater complexity of Hh signaling in vertebrates.

Hedgehog (Hh) signaling plays a key role in inductive interactions in various tissues during animal development, and deregulation of the pathway in humans is associated with various congenital anomalies and tumors (33, 53, 87). The main players in the Hh signaling pathway appear to be conserved between invertebrates and vertebrates (29, 33, 48, 53). Moreover, many aspects of Hh signaling characterized so far are also conserved (29, 33, 48, 53). This leads to the view that little change in Hh pathway design has occurred over millions of years of evolution, and it is widely believed that principles derived from studies with simpler organisms will be applicable to more complex animal models.

In comparison to other major signaling pathways, the Hh pathway possesses several unique and unconventional features, specifically, lipid modification and transport of the ligand and signal transduction at the cell surface (33, 48, 50, 53). Hh signaling is initiated through Hh binding to Patched (Ptch) (51, 85), a 12-pass membrane protein of the sterol-sensing domain family (42). This interaction relieves Ptch repression of Smoothened (Smo), a seven-pass transmembrane protein (1, 85, 94), and allows Smo to activate the Hh signal transduction cascade. Despite intense studies, surprisingly little is known about the biochemical mechanism by which Smo activity is regulated by Ptch (14, 88).

Elucidating the signaling cascade downstream of Smo is crucial to our understanding of how distinct Hh readouts are generated in diverse developmental contexts, and a major player in this process is the putative serine/threonine kinase Fused (Fu) (73). Initially identified in genetic screens by its segment polarity phenotype (62), the essential role of *Fu* in invertebrate Hh signaling was firmly established through genetic studies with *Drosophila* and the nature of several classes of *fu* mutants was clarified through genetic interactions with *Suppressor of fused* (*Sufu*) (2, 25, 74, 91, 93). Interestingly, *Sufu*, which encodes a novel PEST domain protein (70), is dispensable for viability in flies and was identified as an extragenic suppressor of *fu* mutations rather than through mutant phenotypes (72, 74). Fu was shown to function in concert with the atypical kinesin gene *Costal-2* (*Cos2*) (75, 82), the transcription factor gene *Cubitus interruptus* (*Ci*) (65), and Sufu in transducing the Hh signal. The amino-terminal kinase domain of Fu (mutated in class I *fu* mutant flies) is proposed to primarily counteract Sufu (2, 25, 74, 91, 93), while the carboxyl-terminal domain (truncated in class II *fu* mutant flies) directly associates with Cos2 to oppose its activity (3, 4, 57). A cytoplasmic complex composed of Fu, Ci, Cos2, and a small amount of Sufu was also shown to be associated with Smo via Cos2 in an Hh-dependent manner through both biochemical and genetic studies (35, 49, 63, 76, 83, 84). In this model, Hh signal transduction leads to recruitment of the cytoplasmic protein complex to Smo and subsequent inhibition of Ci proteolysis, which would otherwise produce a transcriptional repressor of Hh target gene expression (48). Instead, Ci is converted into an activator by unknown mechanisms to activate Hh target genes (64). Fu and Sufu were proposed to exert opposite effects in controlling the processing, activity, and shuttling of Ci between the nucleus and the cytoplasm (43, 55, 97, 98). Sufu is believed to tether Ci in the cytoplasm and repress Hh signaling, which could be antagonized by Fu. While kinase activity of Fu has not been documented, Fu, as well as Sufu and Cos2, is phosphorylated in response to Hh signaling (49, 61, 92), raising the possibility that posttranslational modifications of Hh pathway components play a key role in regulating multiple steps of Hh signal trans-

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duction. Despite these insights, the central issues of how Ci proteolysis and activation are executed and controlled, the composition and transport of the cytoplasmic complex in response to Hh signaling, and the biochemical nature and consequences of protein-protein interaction in the protein complex remain largely unexplained.

In parallel with *Drosophila* studies, vertebrate homologs of Fu (58, 100), Sufu (19, 20, 41, 69, 86, 100), Cos2 (37, 38, 89), and Ci (32, 39, 40, 78, 95) were identified through either sequence analysis or functional studies. While mammals appear to contain a single *Fu* gene and a single *Sufu* gene, multiple vertebrate *Ci* and *Cos2* homologs have been reported, adding greater complexity to vertebrate Hh signaling. The activator and repressor activities of Ci are differentially distributed among three Gli proteins, the Ci homologs in mammals. Gli1 is a transcriptional activator, while Gli2 and Gli3 function as both activators and repressors of transcription (5, 17, 77, 80, 81). The relative contributions and combinatorial effects of Gli activator and repressor activities in distinct developmental contexts remain to be fully characterized (6–8, 13, 21, 31, 44, 52, 56, 68), but one may speculate that the complex genetic interactions and regulation of the three Gli proteins may be linked to modifications of Hh pathway design. Multiple members of the kinesin family (Kif), including *Kif27* and *Kif7*, are thought to be the vertebrate homologs of *Cos2* (37, 38; data not shown). This notion was further supported by biochemical and morpholino-mediated knockdown studies of *Kif7* in zebra fish, in which Kif7 was shown to function as an intracellular repressor of Hh signaling in conjunction with Sufu (89).

In vitro studies have independently revealed a role of vertebrate *Fu* in Hh signaling. Vertebrate Fu was shown to weakly synergize with Gli1/2 in activation of an Hh-responsive reporter (18, 58, 66), to affect Gli protein localization (58), and to antagonize the activity of Sufu (58). Moreover, a protein complex composed of Fu, Sufu, and Gli was identified, as well as the demonstration of opposite effects on the activity and subcellular distribution of Gli proteins exerted by Fu and Sufu (20, 22, 41, 54, 58, 69, 86). These independent studies with invertebrates and vertebrates strengthened the notion of conservation of the Hh pathway, and it is conceivable that genetic studies of *Fu*, *Sufu*, and *Kif27*/*Kif7* in mice will simply provide a final proof. It is expected that loss of mammalian *Fu* will recapitulate many aspects of reduced Hh signaling while mutations in *Sufu* will exhibit little or no effect, given the converging evidence from flies, zebra fish, and mice. To definitively test the conservation of the Hh signaling cascade downstream of Smo during evolution and to understand the role *Fu* plays in Hh signaling, we took a genetic approach in this study to inactivate vertebrate *Fu* in mice.

MATERIALS AND METHODS

Molecular biology. Standard molecular biology techniques, including molecular cloning, genomic DNA preparation, RNA isolation, reverse transcription (RT)-PCR, Southern analysis, and Northern analysis, were performed as previously described (59, 79). The sequences of the oligonucleotides (derived from mouse *Fu* genomic sequences) used in RT-PCRs are 5′ GTTTCTAGGCAAC TGAAGACTCTAGATCCTTT 3′ (*Fu* exon 1, sense strand), 5′ CTGGTCTTC AGGAAGTTTTCCATCATCTTCCAGAA 3′ (*Fu* exon 5, antisense strand), 5′ AGCTCTGTACTACCTGCATTCCCACCGCATCCTA 3′ (*Fu* exon 6, sense strand), 5′ CTTCTGCTTGGCTTCTTCAGCCATGAGTTTAC 3′ (*Fu* exon 9, antisense strand), 5′ CCACATCAACCTGGAGTGTGAACAAGGCTTCCC 3′ (*Fu* exon 11, sense strand), 5′ CTGCTCGCCAGTCCCGCGCAGCTGACTCT GGATG 3′ (*Fu* exon 12, antisense strand), 5′ CCGTCCACAGCTTCACACA AGACAGCAAAGCA 3′ (*Fu* exon 16, antisense strand), 5′ TGCTTTGCTGT CTTGTGTGAAGCTGTGGACGGA 3′ (*Fu* exon 16, sense strand), 5′ GTGA AAGTAGCAGATTGGGAAGAGTCCACTGA 3′ (*Fu* exon 20, sense strand), 5′ ACCAACCACATCCCTGATCAGGCCAGGCTTCCC 3′ (*Fu* exon 24, antisense strand), 5′ GGGATGTGGTTGGTTCAGAGGTGTGGACCATTCT 3′ (*Fu* exon 24, sense strand), and 5′ CTGATTCCCCAAAGAGAGCAAGGCTA ACTTCTCA 3′ (*Fu* exon 28, antisense strand).

Generation of targeted *Fu* mutant mice. To construct a positive-negative targeting vector for removing exons 1 to 5 of the *Fu* gene (the resulting allele is designated $Fu^{\Delta E1\text{-}5}$), a 4-kb fragment containing genomic sequences of the first 142 bp of intron 5 and upstream sequences was used as the 5′ region of homology (see Fig. 3). This region encompasses the first five exons, as well as sequences approximately 1.8 kb upstream of exon 1. Oligonucleotides containing one *loxP* site (5′ ATAACTTCGTATA GCATACAT TATACGAAGTTAT 3′) were inserted into the SalI site, located ~110 bp upstream of the 5′ untranslated region (exon 1) in the 5′ region of homology (see Fig. 3), resulting in destruction of the SalI site and creation of a BglII site to facilitate the identification of targeted embryonic stem (ES) cell clones. A 1.4-kb fragment containing genomic sequences (intron 5) immediately downstream of the 5′ region of homology was used as the 3′ region of homology and was inserted upstream of the MC1-*tk*-pA cassette (see Fig. 3). A cassette comprising FRT/PGK-*neo*-pA/FRT/loxP and β-galactosidase was inserted between the 5′ and 3′ homology regions (see Fig. 3). One *loxP* site was included at the 3′ end of FRT/PGK-*neo*-pA/FRT. E14Tg2A.4 (E14) feeder-independent ES cells (60) were electroporated with a SalI-linearized targeting vector and selected in G418 and fialuridine as previously described (36). Targeted ES clones were identified by Southern blotting using the 5′ and 3′ probes. The targeting frequency was approximately 2%. Heterozygous E14 ES cells were injected into blastocysts of C57BL/6 strain mice to generate germ line chimeras. Chimeric males were mated with β-*actin*::*Cre* mice (45) to remove sequences between the two *loxP* sites (including FRT/PGK-*neo*-pA/FRT) to generate $Fu^{\Delta E1\text{-}5}$ heterozygous animals. As a result, β-galactosidase was brought under the control of putative upstream *Fu* enhancers. Heterozygotes were identified by Southern blotting of tail tip DNA and were mated with C57BL/6, 129/Sv, 129/Ola, or Swiss-Webster females to maintain the *Fu* mutant allele in different genetic backgrounds. The majority of the analyses were performed in the Swiss-Webster mixed background. Homozygous mice were produced by crossing heterozygotes and identified by Southern blotting.

Histology and in situ hybridization. Embryo collection, histological analysis, whole-mount in situ hybridization using digoxigenin-labeled probes, and section in situ hybridization using ^{33}P-labeled riboprobes were performed as previously described (59, 99).

RESULTS

Mouse *Fu* is broadly expressed during embryogenesis. To better understand the potential role that *Fu* plays in vertebrate Hh signaling, we examined the temporal and spatial expression patterns of *Fu* in mouse embryos collected from 8.5 days postcoitus (dpc) to 18.5 dpc and compared the expression to that of other Hh pathway members such as *Shh* (23). By 8.5 dpc, when *Shh* is expressed at the axial midline including the notochord (Fig. 1A) (23), *Fu* is widely expressed at low levels (Fig. 1G and data not shown). At ~9.5 dpc, *Shh* is activated in the zone of polarizing activity of the forelimb (Fig. 1B) and its expression levels increase from 9.5 to 10.5 dpc, with expression extending to both limbs (Fig. 1C) (23). *Fu* continues to be broadly expressed (Fig. 1H), and at 10.5 dpc *Fu* expression can be observed in tissues including the limb (Fig. 1M), the neural tube (Fig. 1I and J), the somite (Fig. 1J), and the branchial arches (Fig. 1I). *Fu* expression persists in many tissues through later stages of embryogenesis (Fig. 1K and L and data not shown). Taken together, these findings suggest a potential role for *Fu* in Hh signaling since its expression domain overlaps those of Hh-responsive cells.

The *Fu* transcripts are alternatively spliced during mouse development. The mouse *Fu* genomic locus, located on chro-

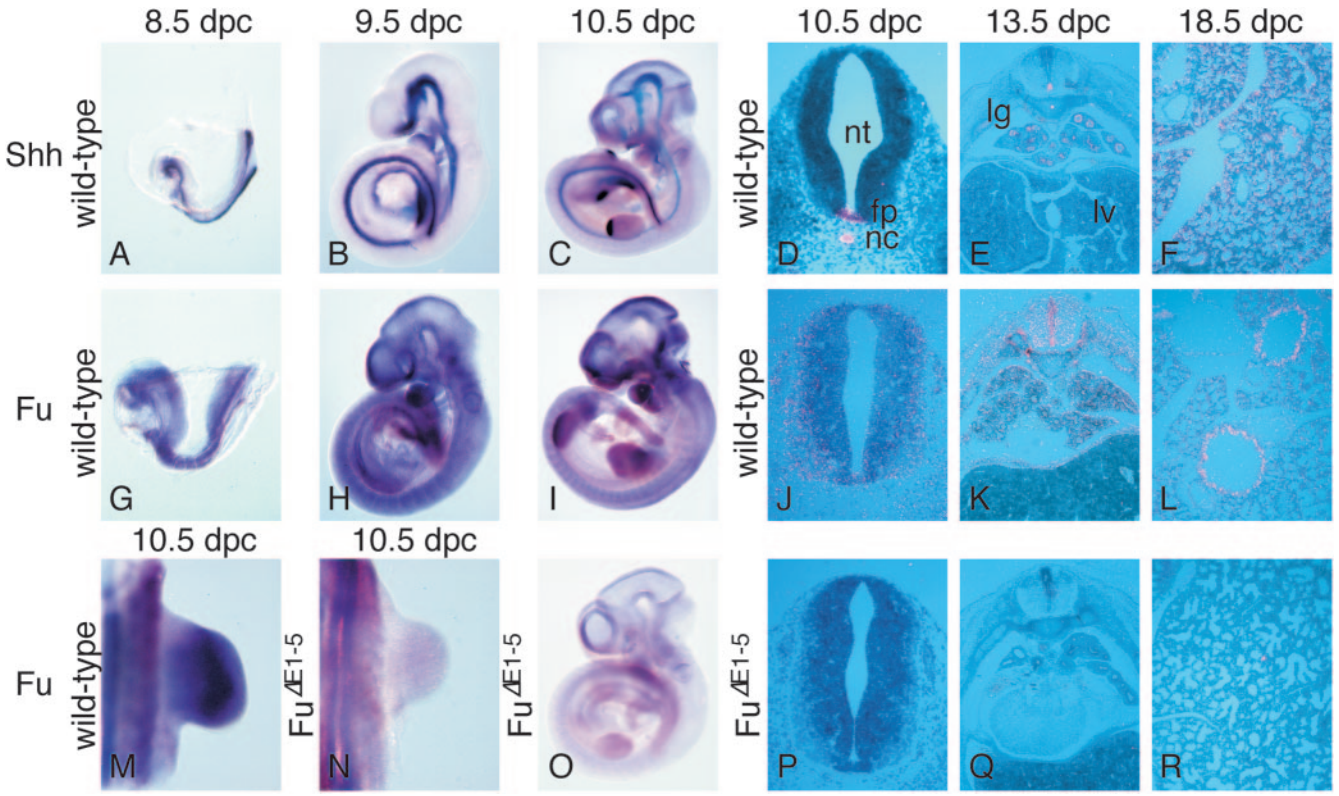

FIG. 1. Expression of *Fu* during mouse embryonic development. (A to C, G to I, and M to O) Whole-mount in situ hybridization using digoxigenin-labeled riboprobes on wild-type embryos at 8.5 (A and G), 9.5 (B and H), and 10.5 (C, I, and M) dpc and $Fu^{\Delta E1\text{-}5}$ embryos at 10.5 dpc (N and O). All views are lateral, with the exception of M and N, which are dorsal views of the forelimb. *Shh* is expressed in several signaling centers, while *Fu* is broadly expressed in wild-type embryos, including in domains of Hh-responsive cells. Only background *Fu* signal could be detected in $Fu^{\Delta E1\text{-}5}$ embryos. Embryos in panels A and G; B and H; C, I, and O; and M and N were photographed at the same magnification, respectively. (D to F, J to L, and P to R) Isotopic in situ hybridization using [^{33}P]UTP-labeled riboprobes (pink) on paraffin sections of 10.5-dpc wild-type (D and J) and $Fu^{\Delta E1\text{-}5}$ (P) embryos at the forelimb-heart level, 13.5-dpc wild-type (E and K) and $Fu^{\Delta E1\text{-}5}$ (Q) embryos, and 18.5-dpc wild-type (F and L) and $Fu^{\Delta E1\text{-}5}$ (R) lungs. Panels D, J, and P; E, K, and Q; and F and R were photographed at the same magnification, respectively. Panel L was photographed at a higher magnification than panels F and R. *Fu* signal is absent in $Fu^{\Delta E1\text{-}5}$ embryos. The *Fu* probe used for in situ hybridization is derived from *Fu* cDNA sequences that correspond approximately to exons 2 to 9. Probes derived from several other regions of *Fu* cDNA, including exons 2 to 5 and exons 27 and 28, gave identical expression patterns (data not shown). The *Fu* sense probes did not yield signals above the background (data not shown). nt, neural tube; fp, floor plate; nc, notochord; lg, lung; lv, liver.

mosome 1, consists of 28 predicted exons, and the translational start ATG codon of the *Fu* transcripts is predicted to reside in the third exon (Fig. 2C). The putative serine/threonine kinase domain located at the N terminus of Fu is encoded approximately by exons 3 to 9 (Fig. 2C). Analysis of mouse *Fu* cDNA sequences revealed multiple *Fu* splice variants. For instance, exons 13 to 15 are alternatively spliced and are not included in the shorter *Fu* transcripts (Fig. 2B and C). Interestingly, the first and second exons, which contain the 5′ untranslated region, are utilized alternatively in different *Fu* cDNA clones (Fig. 2B and C). We performed RT-PCR on RNA obtained from 10.5 dpc wild-type mouse embryos to verify the splice variants of the *Fu* transcripts and to determine their relative abundance during embryonic development. We found that longer *Fu* transcripts, containing exons 13 to 15, constitute the major species while transcripts without exons 13 to 15 (arrow in Fig. 2B, lane 4) are coexpressed at a much lower level. Alternative splicing of exon 8 or exon 23, previously reported for human *Fu* cDNA (66), was not detected using mouse 10.5 dpc RNA (Fig. 2B, lane 3 and 5). These results suggest that the *Fu* transcript, which includes all coding exons and encodes a protein of 1,262 amino acids, is the major product during mouse embryonic development.

Targeted disruption of mouse *Fu* results in postnatal lethality. To define the role *Fu* plays in vertebrate Hh signaling, we generated a gene-targeted allele of *Fu* in mice. We decided to delete the first five exons, since exons 3 to 5 are shared by different *Fu* splice variants and encode both the translational start and a portion of the highly conserved N-terminal kinase domain (Fig. 2C and 3) (58). In addition, ~110 bp upstream of exon 1 were also deleted in this targeting strategy, which could potentially contain the upstream promoter and regulatory elements (Fig. 3). We anticipated that no functional *Fu* transcripts or protein would be produced from the resulting targeted allele (designated $Fu^{\Delta E1\text{-}5}$).

To our surprise, animals homozygous for $Fu^{\Delta E1\text{-}5}$ were born alive and their appearance could not be distinguished from that of their wild-type littermates. The ratio of wild-type to $Fu^{\Delta E1\text{-}5/+}$ to $Fu^{\Delta E1\text{-}5}$ newborn pups approximates a 1:2:1 Mendelian distribution (data not shown). Homozygous $Fu^{\Delta E1\text{-}5}$ an-

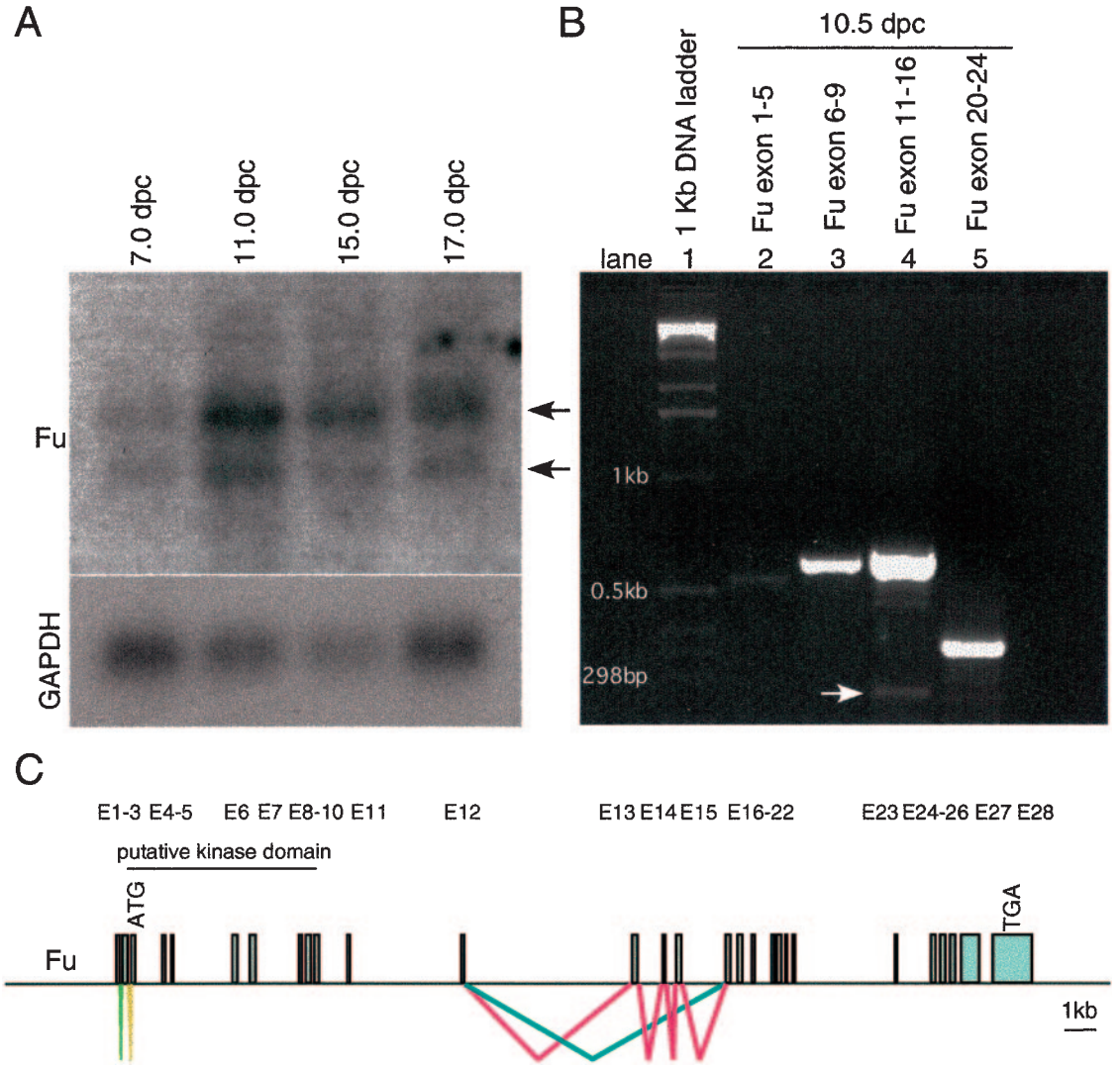

FIG. 2. Alternative splicing of *Fu* transcripts during mouse embryogenesis. (A) Northern blot analysis of poly(A)$^+$ RNA isolated from 7-, 11-, 15-, and 17-dpc wild-type mouse embryos (Clontech). Two major species of *Fu* transcripts, approximately 4.7 and 4.1 kb, respectively (arrows), were detected. In adult mice, *Fu* is mainly expressed in testis (data not shown). The *Fu* probe used for hybridization is derived from *Fu* cDNA sequences that correspond approximately to exons 2 to 9. The same membrane was rehybridized with a *GAPDH* probe, which serves as the loading control. (B) RT-PCR using RNA derived from wild-type 10.5-dpc mouse embryos in combination with primers spanning the indicated exons. Alternative splicing of the region containing exons 11 to 16 was detected by PCR. This was subsequently verified to be alternative splicing of exons 13 to 15 by sequencing. The white arrow points to the PCR product without exons 13 to 15, which is much less abundant than the upper band containing exons 13 to 15. No alternative splicing of exon 8 or exon 23, as reported for human *Fu* transcripts, was detected in this assay. A PCR product was amplified with primers derived from exons 1 and 5, respectively. Sequence analysis of the PCR product revealed that it contains exons 1 and 3 to 5, and whether it represents the dominant form during mouse embryogenesis remains to be further investigated. RT-PCR using primers derived from exons 2 and 5 failed to produce a product (data not shown). (C) Schematic diagram of the *Fu* genomic locus and possible alternative splicing. The *Fu* genomic locus consists of 28 exons (E1 to E28). The ATG translation start codon is predicted to reside in the third exon (E3), and the TGA stop codon is in the 28th exon (E28). Alternative splicing of exons 13 to 15 is labeled in red and blue, respectively, and potential alternative splicing of exons 1 and 2 is labeled with lime and olive, respectively. Splicing of common exons is not labeled. The longer *Fu* transcript, containing exons 13 to 15, appears to constitute the major species during mouse embryonic development based on the relative abundance of the transcripts.

imals continued to thrive after birth, but the majority of them became visibly smaller than their wild-type littermates around postnatal day 7 (data not shown and Fig. 4A). These runts failed to gain weight and appeared starved and emaciated, and the majority of them died before they reached 3 weeks of age. The lethality of $Fu^{\Delta E1\text{-}5}$ animals appears to be completely penetrant (with more than 50 animals examined so far). Examination of major organs did not reveal any obvious pathology in $Fu^{\Delta E1\text{-}5}$ mutants (Fig. 5, right panel, and data not shown). The findings of standard biochemical analyses of blood samples, including glucose levels, lipid profiles, electrolytes, and liver and renal function tests, are consistent with changes associated with starvation in $Fu^{\Delta E1\text{-}5}$ pups (data not shown). The cause of death of $Fu^{\Delta E1\text{-}5}$ animals remains to be further investigated.

***Fu* transcripts are barely detectable in gene-targeted $Fu^{\Delta 1\text{-}5}$ mice.** The unexpected phenotypes of *Fu*-deficient mice prompted us to investigate whether the targeted $Fu^{\Delta E1\text{-}5}$ allele

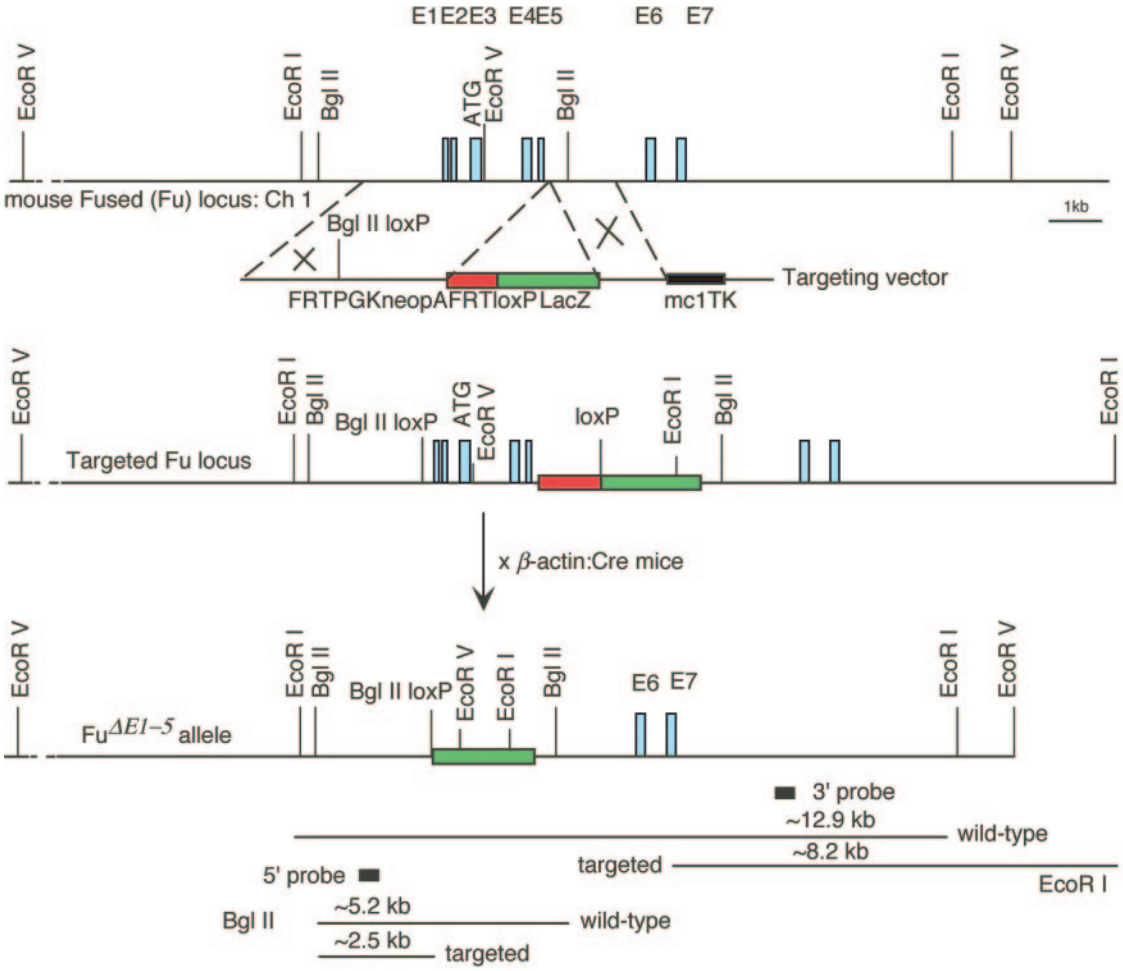

FIG. 3. Targeted disruption of the mouse *Fu* gene. The schematic diagram shows the *Fu* genomic locus, the targeting vector, and the mutant allele. The top line shows a partial restriction map of the mouse *Fu* genomic locus on chromosome 1. The *Fu* genomic locus consists of 28 exons (E1 to E28), and only the first 7 exons are shown for simplicity. The translation start ATG is predicted to reside in the third exon (E3). The regions between the dotted lines represent the 5′ and 3′ regions of homology used in gene targeting, respectively, and the symbol × indicates events of homologous recombination. Germ line-transmitting chimeric males carrying the targeted *Fu* locus were mated with β-*actin*::*Cre* mice to remove sequences between the two *loxP* sites (including the PGK-*neo*-pA selection cassette) and generate the $Fu^{\Delta E1\text{-}5}$ allele in which the first five exons of *Fu* are removed. As a result, β-galactosidase was brought under the control of putative upstream *Fu* regulatory elements. The locations of the fragments used as the 5′ or 3′ external probes in Southern blotting are shown, as well as the sizes of the restriction fragments detected for wild-type and targeted $Fu^{\Delta E1\text{-}5}$ alleles. The *Rnf25* (ring finger protein 25) gene is divergently transcribed immediately upstream of *Fu*, suggesting that targeted disruption of *Fu* could potentially remove regulatory elements that control *Rnf25* expression.

represents a null allele. Since the 5′ untranslated region of *Fu* and 110 bp of upstream sequence were deleted, we expected that *Fu* transcript levels would be greatly reduced or even completely absent in $Fu^{\Delta E1\text{-}5}$ embryos. Consistent with this, in situ hybridization of $Fu^{\Delta E1\text{-}5}$ embryos using a probe derived from genomic sequences deleted in $Fu^{\Delta E1\text{-}5}$ detected only background signals of *Fu* mRNA (Fig. 1N to R) compared to those of their wild-type littermates (Fig. 1M and I to L). Furthermore, Northern analysis of mRNA isolated from 10.5 dpc embryos revealed that *Fu* transcripts were not detectable in $Fu^{\Delta E1\text{-}5}$ embryos, contrasted with wild-type littermates (Fig. 4B). We also failed to detect any truncated *Fu* transcripts that could have resulted from aberrant splicing of the targeted $Fu^{\Delta E1\text{-}5}$ allele (Fig. 4B). To rule out the possibility that a trace amount of *Fu* transcript is produced but is beyond the detection limit of Northern analysis, we employed a sensitive PCR-based assay designed to detect any residual *Fu* transcripts. RT-PCR was performed using RNA isolated from 10.5 dpc embryos deficient in *Fu* or from wild-type controls, in combination with multiple primer pairs spanning different regions of the *Fu* transcripts (Fig. 4C). While PCR products of the predicted sizes were detected from RNA derived from wild-type embryos, no PCR products were generated using RNA obtained from $Fu^{\Delta E1\text{-}5}$ embryos (Fig. 4C). In contrast, PCR products corresponding to other genes such as *Shh* and *Fgf10* were detected at similar levels using RNA produced from either wild-type or $Fu^{\Delta E1\text{-}5}$ embryos (Fig. 4C). Nevertheless, it should be noted that a minute amount of PCR product, corresponding to a *Fu* transcript starting approximately at exon 19, could be detected when a higher number of PCR cycles was applied (Fig. 4C, lane 11, and data not shown). This could potentially represent a trace amount of truncated transcripts generated

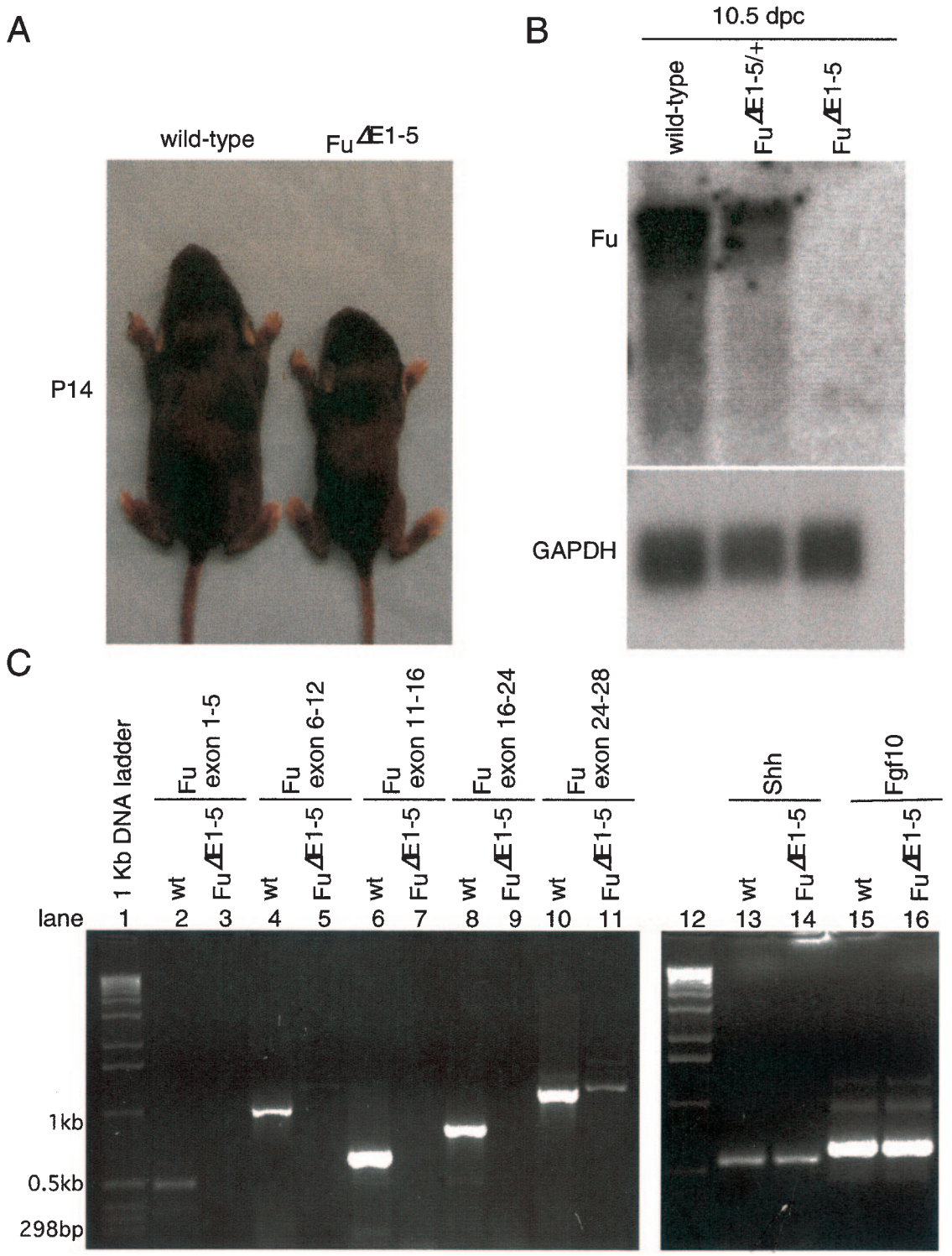

FIG. 4. $Fu^{\Delta E1\text{-}5}$ animals exhibit growth retardation and postnatal lethality, and *Fu* transcript levels are barely detectable in $Fu^{\Delta E1\text{-}5}$ embryos. (A) Wild-type and $Fu^{\Delta E1\text{-}5}$ animals photographed at postnatal day 14. The mutant is significantly smaller than its wild-type littermate. Some toes in both animals were clipped for numbering and genotyping. (B) Northern blot analysis of poly(A)$^+$ RNA isolated from 10.5-dpc wild-type, $Fu^{\Delta E1\text{-}5/+}$, and $Fu^{\Delta E1\text{-}5}$ embryos. The two *Fu* probes used for hybridization gave identical results, and they correspond approximately to the last 775 bp of *Fu* transcripts and the 624-bp genomic sequences (exons 2 to 5) deleted in the gene-targeted $Fu^{\Delta E1\text{-}5}$ allele (data not shown). A ~4.7-kb upper band with stronger intensity and a ~4.1-kb lower band were detected in both wild-type and $Fu^{\Delta E1\text{-}5/+}$ embryos but were completely absent in $Fu^{\Delta E1\text{-}5}$ embryos. The expression level of the housekeeping gene *GAPDH* serves as the loading control with the same membrane reprobed. (C) RT-PCR using RNA derived from 10.5-dpc wild-type (wt) and $Fu^{\Delta E1\text{-}5}$ mouse embryos in combination with primers spanning the exons indicated. While PCR products of the predicted sizes were detected from RNA derived from wild-type embryos, no PCR products were generated using RNA obtained from $Fu^{\Delta E1\text{-}5}$ mutants. The same number of PCR cycles (37 cycles) was employed for all the PCRs shown in this panel. The faint band, corresponding to exons 24 to 28, seen in $Fu^{\Delta E1\text{-}5}$ was not detected when a lower number of PCR cycles (less than 33) was applied (data not shown). PCR products corresponding to other genes such as *Shh*, *Fgf10*, and *GAPDH* (not shown) were detected at similar levels using RNA produced from wild-type or $Fu^{\Delta E1\text{-}5}$ mutants, suggesting that the absence of a *Fu* signal in $Fu^{\Delta E1\text{-}5}$ was not due to technical difficulties in RNA preparation or RT-PCR.

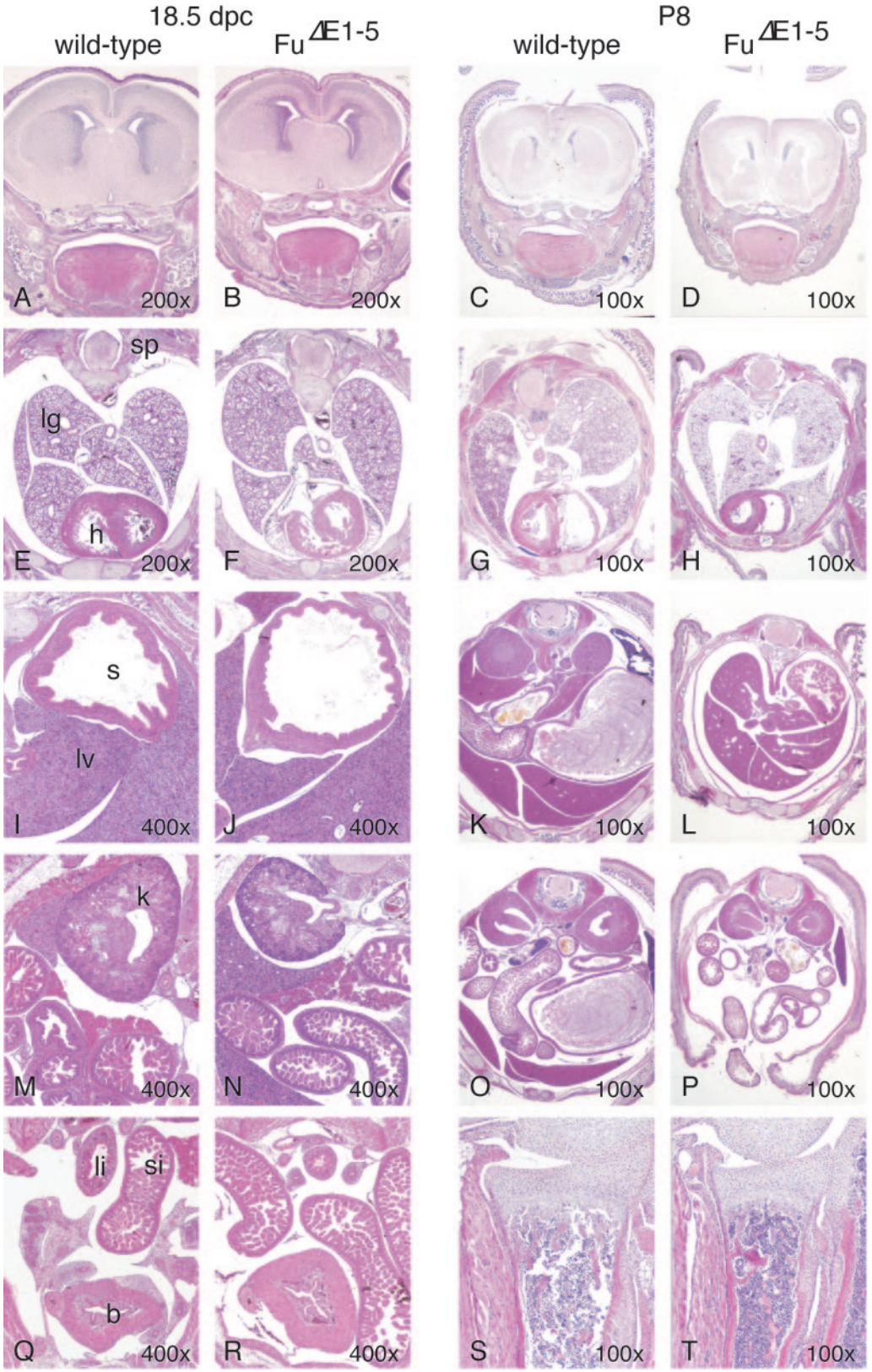

FIG. 5. Development of major tissues and organs does not appear to be affected in $Fu^{\Delta E1\text{-}5}$ mutants. (A to T) Hematoxylin-and-eosin-stained sections of major tissues and organs of wild-type and $Fu^{\Delta E1\text{-}5}$ animals at embryonic dpc 18.5 and postnatal day P8. A to D, coronal sections through the forebrain; E to H, cross sections through the thoracic cavity; I to R, cross sections through the abdominal cavity; S and T, longitudinal sections through the proximal ulna. Although $Fu^{\Delta E1\text{-}5}$ mutants were significantly smaller than their wild-type littermates at day P8, no obvious developmental defects or pathological changes in major tissues and organs were detected in $Fu^{\Delta E1\text{-}5}$ mutants. Multiple wild-type and $Fu^{\Delta E1\text{-}5}$ embryos were examined. In certain regions of the sections, the minor differences in morphology between wild-type and $Fu^{\Delta E1\text{-}5}$ animals were due to planes of sections. sp, spinal cord; lg, lung; h, heart; s, stomach; si, small intestine; li, large intestine; lv, liver; k, kidney; b, bladder. The magnification of each section is indicated.

from the $Fu^{\Delta E1\text{-}5}$ allele, but whether any residual truncated Fu protein was generated is not known. Taken together, these results indicate that *Fu* transcript levels are greatly reduced (or even absent) in $Fu^{\Delta E1\text{-}5}$ mutants, suggesting that little or no functional Fu protein was produced in $Fu^{\Delta E1\text{-}5}$ embryos.

Mice deficient in Fu do not exhibit phenotypes indicative of perturbed Hh signaling during embryogenesis. Mice homozygous for $Fu^{\Delta E1\text{-}5}$ appear to develop normally through embryogenesis, and their appearance cannot be distinguished from that of wild-type littermates. We performed a detailed histological analysis of mouse embryos collected between 9.5 and 18.5 dpc (Fig. 5, left panel, and data not shown). In contrast to *Shh* mutants, which exhibit defects in multiple tissues, including the developing neural tube, limb, hair follicle, gut, lung, pancreas, and kidney, due to defective Hh signaling (53), no significant morphological changes can be discerned in $Fu^{\Delta E1\text{-}5}$ animals compared to wild-type littermates (Fig. 5, left panel). There are also no skeletal defects due to defective *Ihh* signaling (53) in *Fu*-deficient embryos (Fig. 5, left panel, and data not shown). These results suggest that Hh signaling is not disrupted in $Fu^{\Delta E1\text{-}5}$ embryos.

It is possible that the Hh pathway is perturbed at molecular levels but the effects are subtle and cannot be discerned by our phenotypic analysis. To test this hypothesis, we performed in situ hybridization on wild-type and $Fu^{\Delta E1\text{-}5}$ mouse embryos collected between 9.5 and 18.5 dpc, focusing on Hh target genes including *Ptch1*, *Hip1*, and *Gli1* (16, 28, 32, 71). These genes are expressed in Hh-responsive tissues and are up-regulated in response to Hh signaling. For instance, while *Shh* is expressed in midline structures (23) (Fig. 6A), including the notochord and floor plate, *Ptch1*, *Hip1*, and *Gli1* are all expressed in the ventral neural tube (Fig. 6E, I, and M). Similarly, *Shh* is expressed in the epithelium of many developing organs, such as the gut (Fig. 6C) (9), while *Ptch1*, *Hip1*, and *Gli1* are expressed in the surrounding mesenchyme (Fig. 6G, K, and O). Expression patterns of Hh target genes in *Fu*-deficient embryos (Fig. 6F, J, N, H, L, and P) cannot be distinguished from those of their wild-type littermates, indicating that the Hh pathway is not perturbed due to loss of *Fu* during embryonic development.

DISCUSSION

The lack of embryonic phenotypes in $Fu^{\Delta E1\text{-}5}$-targeted mice came as a surprise, given the critical role that Hh signaling plays in embryonic development and the observation that a similar set of molecules appears to be employed for Hh signal transduction in both invertebrates and vertebrates (29, 33). It is clear that vertebrates contain more Hh pathway members due to gene duplication and in some cases even vertebrate-specific players that modulate Hh signaling (11, 16, 24, 30, 34). Nevertheless, many aspects of Hh signaling characterized so far are conserved (33). One can argue that the key issue of how lipid-modified Hh ligand is transported in the morphogenetic field requires further exploration to clarify the similarities and differences between vertebrates and invertebrates (12, 15, 27, 46, 67). It is, however, generally accepted that Hh signal transduction through Ptch and Smo on the cell surface is conserved as well as Hh target gene activation by the Ci/Gli family of transcriptional factors (33). The signal transduction cascade between Smo and Gli in vertebrates has been investigated to some extent in vitro (20, 22, 41, 58, 69, 86). An essential role of vertebrate *Fu* in Hh signaling was predicted from characterization of *Drosophila Fu* (2, 25, 26, 74, 75, 91–93) and further strengthened by studies of cultured cells in which vertebrate Fu was shown to weakly synergize with Gli1/2 in Hh activation (18, 58, 66), to affect Gli protein localization (58), and to antagonize the activity of Sufu (58). Moreover, morpholino-mediated knockdown of zebra fish Fu activities produces muscle phenotypes consistent with reduced Hh signaling (100). All of the available studies provided no evidence to suggest that vertebrates utilize a different strategy for Hh signal transduction downstream of Smo. In this regard, the lack of embryonic phenotypes in $Fu^{\Delta E1\text{-}5}$ mice is particularly puzzling.

It is possible that the lack of phenotypes in $Fu^{\Delta E1\text{-}5}$ embryos was simply due to the failure to generate a null allele of *Fu* since only the first 5 exons (out of 28) were deleted. However, several lines of evidence suggest that $Fu^{\Delta E1\text{-}5}$ is likely a null allele. No wild-type *Fu* transcripts were detected in $Fu^{\Delta E1\text{-}5}$ embryos by in situ hybridization, Northern analysis, or RT-PCR. Consistent with this observation, we failed to detect any β-galactosidase staining in $Fu^{\Delta E1\text{-}5}$ embryos (data not shown), suggesting that no functional *Fu* transcript was made. Nonetheless, it should be noted that a trace amount of truncated *Fu* transcript, possibly generated through alternative downstream transcriptional start signal or aberrant splicing, was detected from the $Fu^{\Delta E1\text{-}5}$ allele. It follows that a small amount of truncated Fu protein containing the distant region of Fu could potentially be produced, albeit in this case the Fu activity is unlikely to be preserved. The truncated Fu protein expressed in cultured cells displayed no effect in activating multimerized Gli reporters or opposing the activities of Sufu (N. Gao and P.-T. Chuang, unpublished data). The truncated Fu protein is also unlikely to exert dominant negative effects since overexpression of a truncated Fu protein lacking the N-terminal kinase domain failed to generate noticeable phenotypes in mice (R. Sutherland and P.-T. Chuang, unpublished data), which is different from the dominant negative effects exerted by a similar mutation in *Drosophila Fu* (3). Despite a lack of embryonic phenotypes in $Fu^{\Delta E1\text{-}5}$ mutants, these animals became emaciated after birth and eventually died of unknown causes. Whether the lethality reflects an essential requirement of Hh signaling during postnatal life or perturbation of other signaling pathways remains to be investigated.

The lack of embryonic phenotypes in $Fu^{\Delta E1\text{-}5}$ could also be attributed to functional redundancy between *Fu* and other potential *Fu* homologs. However, sequence analysis of vertebrate *Fu* has failed to identify additional *Fu* homologs. The similarity between fly and vertebrate *Fu* sequences predominantly resides in the putative serine/threonine kinase domain located at the N terminus (58). It is possible that the regions of *Fu* outside the kinase domain could have diverged extensively during evolution and sequence analysis alone may not be sufficient to identify additional *Fu* homologs in vertebrates. Instead, functional studies will be required to test the ability of any potential *Fu* homologs, which exhibit exceedingly limited sequence similarity to *Fu*, to compensate for the loss of *Fu* function in vitro and in vivo. Alternatively, loss of *Fu* could be compensated for by changes in expression levels or activities of other Hh pathway members, providing that the more complex

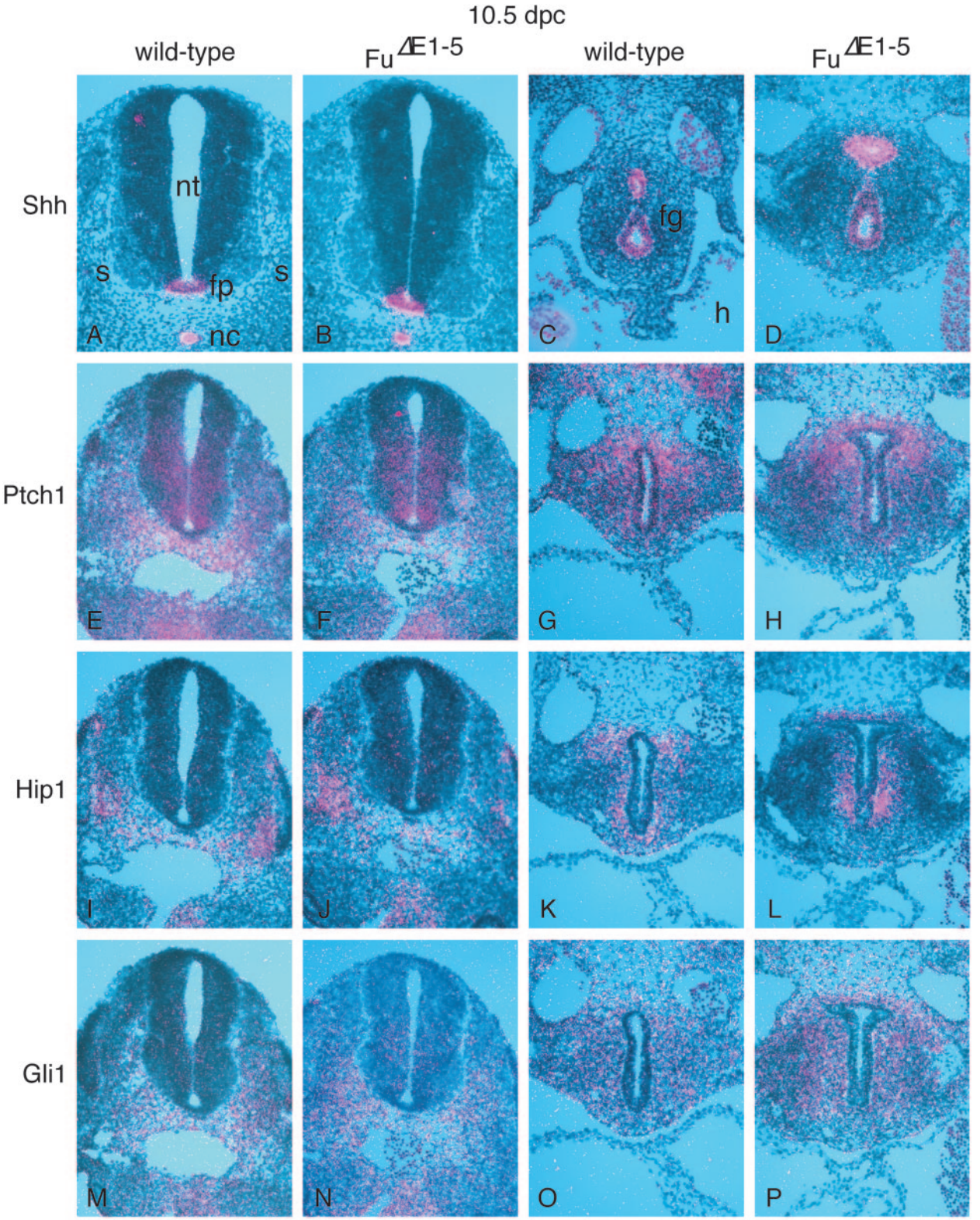

FIG. 6. Expression of Hh targets is not perturbed in $Fu^{\Delta E1\text{-}5}$ mutants. (A to P) Isotopic in situ hybridization using [^{33}P]UTP-labeled riboprobes (pink) on paraffin sections of wild-type (A, C, E, G, I, K, M, and O) and $Fu^{\Delta E1\text{-}5}$ (B, D, F, H, J, L, N, and P) 10.5-dpc embryos at the forelimb-heart level (A, B, C, D, G, H, K, L, O, and P) or the hind limb level (E, F, I, J, M, and N). The axis for orientation of the specimens is dorsal upward and ventral downward. *Shh* is expressed in several signaling centers such as the notochord and floor plate, while Hh targets, including *Ptch1*, *Hip1*, and *Gli1*, are expressed in the ventral neural tube and the somite in response to Hh signaling. *Shh* is also expressed in the epithelium of many developing organs, including the foregut endoderm, while *Ptch1*, *Hip1*, and *Gli1* are expressed in the surrounding mesenchyme. Multiple sections were examined in multiple rounds of in situ hybridization, and expression of Hh targets does not appear to be affected in $Fu^{\Delta E1\text{-}5}$ mutants. nt, neural tube; fp, floor plate; nc, notochord; s, somite; fg, foregut; h, heart.

vertebrate Hh pathway contains additional self-correcting mechanisms. For instance, compensatory down-regulation of *Sufu* or up-regulation of *Gli* in *Fu*-deficient mice may restore normal embryonic development. Further genetic and molecular analyses are required to test these hypotheses.

The design of the Hh pathway may have diverged during evolution. In the extreme case, *Fu* may have no role in vertebrate Hh signaling and *Sufu* has assumed a more prominent role in Hh signaling. Consistent with this notion, mice deficient in *Sufu* display dramatic embryonic phenotypes indicative of elevated Hh signaling (10; C. C. Hui, personal communication). Likewise, the function of vertebrate *Cos2* homologs may also have diverged during evolution, especially given the limited sequence similarity between Kif27/Kif7 proteins and *Drosophila* Cos2 (10, 37, 38, 89). In this scenario, the mechanism by which the Hh signal is transduced downstream of Smo would be significantly different between invertebrates and vertebrates (10). One can also surmise that the evolution of three Gli proteins with different activator and repressor functions and modified circuitry of Gli regulation has led to a corresponding change in the mechanics of Hh signaling downstream of Smo.

Another possibility of Hh pathway divergence would be that *Fu* is involved in Hh signaling but its relative contribution to Hh signaling differs between invertebrates and vertebrates, perhaps even between different vertebrate species. The ratio of combined Gli repressor and activator forms and their regulation may differ among species and one form may even predominate in certain tissues (47, 90, 96). This could contribute to an altered balance of *Fu* and *Sufu* function in Hh signaling in vertebrates to ensure that proper Hh responses are generated in diverse developmental contexts. Further experiments are required to distinguish between these models and to elucidate the mechanisms of Hh signal transduction in vertebrates.

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