Characterization of a Dchs1 mutant mouse reveals requirements for Dchs1-Fat4 signaling during mammalian development

Yaopan Mao¹, Joanna Mulvaney², Sana Zakaria², Tian Yu²,³, Katherine Malanga Morgan¹, Steve Allen⁴, M. Albert Basson²,³, Philippa Francis-West²,*† and Kenneth D. Irvine¹,*†

SUMMARY

The Drosophila Dachsous and Fat proteins function as ligand and receptor, respectively, for an intercellular signaling pathway that regulates Hippo signaling and planar cell polarity. Although gene-targeted mutations in two mammalian Fat genes have been described, whether mammals have a Fat signaling pathway equivalent to that in Drosophila, and what its biological functions might be, have remained unclear. Here, we describe a gene-targeted mutation in a murine Dachsous homolog, Dchs1. Analysis of the phenotypes of Dchs1 mutant mice and comparisons with Fat4 mutant mice identify requirements for these genes in multiple organs, including the ear, kidney, skeleton, intestine, heart and lung. Dchs1 and Fat4 single mutants and Dchs1 Fat4 double mutants have similar phenotypes throughout the body. In some cases, these phenotypes suggest that Dchs1-Fat4 signaling influences planar cell polarity. In addition to the appearance of cysts in newborn kidneys, we also identify and characterize a requirement for Dchs1 and Fat4 in growth, branching and cell survival during early kidney development. Dchs1 and Fat4 are predominantly expressed in mesenchymal cells in multiple organs, and mutation of either gene increases protein staining for the other. Our analysis implies that Dchs1 and Fat4 function as a ligand-receptor pair during murine development, and identifies novel requirements for Dchs1-Fat4 signaling in multiple organs.

KEY WORDS: Fat, PCP, Kidney, Mouse, Dchs1, Lung, Sternum, Vertebræ, Ear, Heart

INTRODUCTION

Studies in Drosophila have identified a Fat signaling pathway, which includes the transmembrane receptor Fat and its transmembrane ligand, Dachsous (Ds) (for reviews, see Reddy and Irvine, 2008; Sopko and McNeill, 2009). Fat and Ds are both large (5147 and 3503 amino acids, respectively) proteins with multiple cadherin domains in their extracellular regions. Fat signaling is also regulated by the kinase Four-jointed (Fj), which modulates binding between Fat and Ds (Brittle et al., 2010; Ishikawa et al., 2008; Simon et al., 2010). There are two distinct downstream branches of Fat signaling, one regulating planar cell polarity (PCP), and the other regulating transcription. The transcriptional pathway (Fat-Hippo signaling) interconnects with the Hippo pathway, which regulates transcription through the co-activator protein Yorkie (Yki) (for a review, see Oh and Irvine, 2010). The basis for Fat-PCP signaling is not well understood, but both the myosin Dachs and the transcriptional co-repressor atrophin, have been implicated in this branch (Fanto et al., 2003; Mao et al., 2006; Matakatsu and Blair, 2008).

Homologs of most of the key players in Drosophila Hippo signaling are conserved in mammals, together with their regulatory interactions (for reviews, see Reddy and Irvine, 2008; Zhao et al., 2010). Moreover, as in Drosophila, the mammalian Hippo pathway plays a crucial role in growth control and oncogenesis. By contrast, the extent to which Fat signaling is conserved in mammals is unclear. The relationship between Drosophila and mammalian Fat signaling is also complicated by the presence of multiple orthologs. Mammals encode four Fat-related proteins (Fat1, Fat2, Fat3 and Fat4), two Ds-related proteins (Dchs1 and Dchs2), a single Fj-related protein (Fjx1) (Rock et al., 2005) and two homologs of the Fat modulator Lowfat (Lix1 and Lix1-L) (Mao et al., 2009). However, mammals do not have a clear Dachs homolog. Drosophila have single ds and fj genes, but two Fat genes (fat and kugelei, which is also known as fat2 or fat-like). The two Drosophila Fat genes have similar extracellular domains, but their cytoplasmic domains are unrelated, and they influence distinct processes (Castillejo-Lopez et al., 2004; Viktorinova et al., 2009). Among mammalian Fat proteins, only Fat4 shares similarity to Drosophila Fat in its cytoplasmic domain (Rock et al., 2005; Tanoue and Takeichi, 2005). Gene-targeted mutations in two murine Fat genes, Fat1 and Fat4, have been reported. Defects in renal glomeruli have been described for Fat1 mutants (Ciani et al., 2003), whereas defects in the development of the inner ear, kidney and neural tube were described for Fat4 mutants (Saburi et al., 2008). None of these phenotypes linked Fat4 to Hippo signaling, but they do appear to link Fat4 to the regulation of PCP (Saburi et al., 2008).

PCP encompasses a range of processes that involve the polarization of cellular structures or cellular behaviors within the plane of a tissue (for reviews, see Simons and Mlodzik, 2008;
Strutt, 2008). Genes that influence PCP were first identified in *Drosophila*, and most *Drosophila* PCP genes can be placed into one of two pathways: a Frizzled (Fz)-dependent pathway or a Fat-dependent pathway. Crosstalk between these pathways has been identified in some contexts, and some forms of PCP can be influenced by both pathways, but there are also cases where each pathway acts independently (Lawrence et al., 2007; Simons and Mlodzik, 2008; Strutt, 2008). Studies of Fz-PCP homologs in mammals have uncovered several requirements for this pathway. These include oriented cell movements that drive convergent extension during gastrulation, orientation of stereocilia in the inner ear, hair patterning, neuronal morphology and cochlear elongation (for reviews, see Simons and Mlodzik, 2008; Wang and Nathans, 2007). The reported effects of a Fat4 mutation on the inner ear, neural tube and kidney are consistent with influences on PCP in mammals (Saburi et al., 2008). Fat4 mutants exhibit smaller kidneys with renal cysts, which were suggested to result from defects in the elongation of kidney tubules due to effects on oriented cell divisions (Saburi et al., 2008).

We report here a characterization of requirements for the murine Dchs1 gene. Dchs1 mutant mice exhibit phenotypes previously described in Fat4 mutants, including kidney cysts and reduced cochlear elongation. We also identify additional requirements for Dchs1 and Fat4 in diverse organs, including the skeleton, kidney, lung, heart and intestine. These observations expand the range of processes known to require Fat signaling during mammalian development. Moreover, the similarity between Dchs1 and Fat4 phenotypes across a wide range of organs, together with reciprocal influences of Fat4 and Dchs1 on protein staining, identifies Dchs1 and Fat4 as a ligand-receptor pair for mammalian Fat signaling. Characterization of abnormalities in the kidneys of Dchs1 and Fat4 mutants further reveals a requirement for Dchs1-Fat4 signaling in growth and branching during early kidney development, and a novel influence on the viability of epithelial cells in the developing kidney.

**MATERIALS AND METHODS**

**Targeting vector**

*Dchs1* DNA was amplified from 129/SvImJ genomic DNA using Takara PrimeSTAR-HS polymerase in three parts: *Dchs1* exon2 plus partial introns (2936 bp), left arm (3997 bp) and right arm (3991 bp). These were cloned into pNZTK2 [a gift from R. Palmer (University of Washington, Seattle)], where the *lacZ* gene was replaced by a PGK-Neo marker and adjacent loxP and FRT sites from p-loxP-2FRT-PGKneo (Transgenic Core, University of Michigan, MI, USA). Another loxP site was introduced by homologous recombination into the targeting vector BAC clone (Fjx1, University of Michigan, Ann Arbor), where the *Fjx1* probe corresponding to exon 2 was cloned by PCR. The *loxP* sites were 527 bp 5′ to exon 2, and 516 bp 3′ to exon 2.

**Mutant mice**

Electroporation of ES cells (AB2.2, 129SV/EV) and production of chimeras was performed by the Mouse ES Core at Baylor College of Medicine, Houston. Positive clones were screened by Southern blotting (Fig. 1B) and PCR (Fig. 1C). Exon 2 was deleted in vivo by crossing *Sox2-Cre* mice, and a *Dachs1* probe corresponding to exon 2 was cloned by PCR. Quantitative PCR was performed on 10 µm sections of *paraformaldehyde-fixed tissue processed into wax or 40 µm tissue slices embedded in low-melting point agarose according to standard protocols, using DIG-labeled probes: *Spry1* (Minowada et al., 1999), *Gad6* (Heilmich et al., 1996), *Wnt11* (Majumdar et al., 2003), *Foxd1* (a gift from D. Wellik (University of Michigan, Ann Arbor)), *Fjx1* and *Dachs2* (Rock et al., 2005). A *Dachs1* probe corresponding to exon 2 was cloned by PCR.

**RESULTS**

**A gene targeted mutation in Dachs1**

To investigate requirements for Fat signaling in mammals, we sought to create and characterize mice with mutations in genes that encode murine homologs of *Drosophila* Fat and Ds. Among the two Ds-related proteins in mice, we focused on Dchs1 over Dchs2 both because it is more widely expressed, and because it exhibits greater sequence similarity to *Drosophila* Ds (see Fig. S1 in the supplementary material) (Rock et al., 2005). As a gene targeting strategy, we opted for an approach based on excision of the second exon, which encodes the first 593 amino acids of Ds (Fig. 1A). Its deletion removes the first six cadherin domains, and the initiator methionine and signal peptide, and thus prevents expression of Dchs1 protein. Targeting of a conditional deletion construct was confirmed in ES cells, and later in transgenic mice, by Southern blotting (Fig. 1B) and PCR (Fig. 1C). Exon 2 was deleted in vivo by crossing to *Sox2-Cre*-expressing mice, and a *Dachs1* mutant mouse line was established. Excision of exon 2 was confirmed by PCR (Fig. 1C).
**Fig. 1. A gene-targeted mutation in Dchs1.** (A) Map of the Dchs1 locus and targeting strategy. Line below number coordinates represents Dchs1 transcription unit; thin lines are introns; thick lines are exons; gray represents untranslated regions; black represents the open reading frame. Thick line above indicates region of homology in the targeting construct. Lines below show, at higher resolution, a region of the targeting construct and correctly targeted allele (T), with Neo cassette flanked by FRT sites (orange triangles) and exon 2 flanked by loxP sites (purple triangles). Expression of recombinases deletes sequences between these sites, to generate the neo-deleted allele (D) and the correct allele (T), with Neo cassette flanked by FRT sites (orange triangles) and exon 2 flanked by loxP sites (purple triangles). Expression of recombinase alone generates linearizates (C) and mutants. (B) Southern blot genotyping of XbaI-digested ES cell DNA with a probe 3’ to the targeted region, +, wild-type allele; T, targeted allele. (C) PCR genotyping of mice, alleles are as described in A,B. Colored bars indicate which PCR primers were used for genotyping, as in A. (D) P0 wild-type (top, wt) and Dchs1 mutant (bottom) mice. (E) F7 mice wild-type (right) and Dchs1 mutant (left) mice.

*Dchs1* mutant mice are born at the expected Mendelian ratio; e.g. from multiple litters, 42 of 170 pups (24.7%) were homozygous mutant for *Dchs1*. Mutant pups are similar in size to their wild-type siblings, and their external morphology is largely normal, except that they often have a bent spine and curly tails (Fig. 1D). Both of these phenotypes are also observed in *Fat4* mutants (Saburi et al., 2008). However, in contrast to what was reported for *Fat4*, we found that if not euthanized, and if given a chance to nurse by removal of wild-type siblings, some *Dchs1* mutant mice could survive for a couple weeks. These *Dchs1* mutant pups exhibited some signs of postnatal development, such as hair formation, but failed to grow, remaining similar in size to newborn pups (Fig. 1E).

**Influence of *Dchs1* mutants on known PCP phenotypes**

To investigate the possibility that *Dchs1* influences PCP, and to determine whether it might do so in conjunction with *Fat4*, we examined phenotypes previously associated with *Fat4* mutants. One of the best characterized examples of PCP in vertebrates is in the cochlea, where PCP genes are required both for the polarized orientation of the stereocilia, and for cochlear elongation (for a review, see Chacon-Heszele and Chen, 2009). The cochleae of *Dchs1* mutant mice were ~20% shorter than wild-type cochlea (Fig. 2B,J), and a similar reduction was observed in *Fat4* (Fig. 2C,J) (Saburi et al., 2008). Examination of stereocilia throughout the length of the cochlea did not reveal obvious differences in stereocilia orientation between *Dchs1* mutant mice and their wild-type siblings (Fig. 2E,G and data not shown). Quantification of stereocilia orientation within each of the four rows of hair cells at 50% cochlea length revealed subtle (less than two degrees) differences in the average orientation of stereocilia (Fig. 2G,J; see Tables S1 and S2 in the supplementary material). We performed similar analyses on *Fat4* mutants, and on *Dchs1* *Fat4* double mutants, and in most cases the difference in average stereocilia orientation was less than two degrees. However, for the outermost row of hair cells (OHC3), the average difference in orientation compared with wild-type was four degrees for *Fat4* and *Dchs1* *Fat4* double mutants (Fig. 2F,H,I; see Tables S1 and S2 in the supplementary material).

Another prominent PCP defect in vertebrates is a wider and open neural tube, which has been attributed to convergent-extension defects and misoriented cell divisions. *Fat4* mutants do not have open neural tubes, but have an increased width-to-height ratio; we observed a modest, but statistically significant, increase in the width-to-height ratio of *Dchs1* neural tubes at P0 (Fig. 3N).

One of the most striking phenotypes observed in *Fat4* mutants was the appearance of small cystic kidneys (Saburi et al., 2008). *Dchs1* mutants also have small cystic kidneys (Fig. 3B,E,J), and the overall size and morphology of the kidney appears similar to that in *Fat4* mutants (Fig. 3A,F), although the number and size of cysts in *Dchs1* mutants appears to be less than in *Fat4* mutants (Fig. 3B,E, compare with 3C,F). By immunostaining of P0 kidneys with markers of tubules and ducts, including the ascending loop of Henle, distal convoluted tubules, connecting tubules and collecting ducts, we observed that these were present, but appeared to be slightly more dilated and less straight in *Dchs1* mutants when compared with wild type (Fig. 3G,H), consistent with studies of *Fat4* mutant kidneys (Saburi et al., 2008). To examine whether an increase in tubule width could be detected at earlier stages of kidney development, we examined distal collecting ducts (E-cadherin-positive tubules) in E15.5 kidneys. A modest, but
statistically significant, increase in the number of cells around the circumference of tubules was detected in Dchs1 mutants when compared with wild type (Fig. 3K-M). Thus, although in some instances the effects are subtle, we observed phenotypes in the ear, neural tube and kidney of Dchs1 mutants that are similar to those in Fat4 mutants and that might reflect influences on PCP.

**Dchs1 and Fat4 mutants exhibit defects in multiple organs**

The results described above identify several shared phenotypes for Dchs1 and Fat4, consistent with the possibility that they are functionally linked. To extend this analysis, and to identify additional requirements for Dchs1-Fat4 signaling in mammals, we examined multiple internal organs in P0 mice mutant for Dchs1 or Fat4.

Examination of the skeleton revealed an obvious defect in the sternum, which is both wider and shorter in Dchs1 or Fat4 mutants, and also exhibits an abnormal pattern of ossification (Fig. 4A-C). In addition to the substantial increase in the width of the sternum, a more modest increase in the width of the vertebral column is evident in Dchs1 or Fat4 mutants, but only in the lumbar and posterior thoracic region (Fig. 4F-H). These vertebrae were also narrower along the anterior-posterior axis. In addition, there are dual ossification centers and the centrum at the midline is malformed.

Analysis of P0 Dchs1 and Fat4 mutant lungs revealed a decrease in size (Fig. 4J-O-Q and data not shown). The intestines of Dchs1 and Fat4 mutants were also shorter than their wild-type siblings. Measurements of the intestinal length, from the stomach to the rectum, revealed that on average the intestine in Dchs1 mutants was 72% of its length in wild-type siblings; a similar reduction was observed in Fat4 (Fig. 4E). These reductions in the size of certain internal organs (intestine, lung and kidney), are not simply reflections of decreased body size, because newborn pups did not differ significantly in overall size and other organs (e.g. skeleton, liver and heart) were not significantly smaller in Dchs1 or Fat4 mutants. Although the hearts were not smaller, heart morphogenesis was affected, as Dchs1 and Fat4 mutants exhibit defects in atrial septation (Fig. 4K-M). In both Dchs1 and Fat4 mutants, the septum primum was often thinner and the ostium secundum was much larger than in wild-type littermates (Fig. 4K-M). Together, these observations establish that Dchs1 and Fat4 are broadly required for the morphogenesis of multiple organs.

**Dchs1 and Fat4 double mutants resemble single mutants**

The similar phenotypes of Fat4 and Dchs1 mutants across multiple organs imply that they function together within the same pathway, consistent with the possibility that they function as a ligand-receptor pair. However, as there are multiple Fat- and Ds-related genes in mammals, we also considered the possibility that other Dchs or Fat genes might participate in these processes. As a genetic test of this, we examined double mutant animals. If Dchs1 and Fat4 function in the same pathway as a dedicated ligand-receptor pair, then we would expect that Dchs1 Fat4 double mutants should appear identical to single mutants. By contrast, if they acted in parallel pathways, or in concert with paralogues, then double mutants would be expected to have more severe phenotypes. As noted above, both cochlea elongation and stereocilia orientation in Dchs1 Fat4 double mutants was similar...
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To that in single mutant animals (Fig. 2). We also examined the skeletal, heart and lung phenotypes of Dchs1 Fat4 double mutants, and in all cases they appeared similar to those in single mutants (Fig. 4 and data not shown). Thus, we infer that Dchs1 and Fat4 influence these processes by acting within the same pathway, and that paralogous Dchs and Fat genes do not make significant contributions.

Influence of Dchs1 and Fat4 mutants on kidney growth and branching

One of the most obvious phenotypes in Dchs1 or Fat4 mutants is the reduction in kidney size (Fig. 3). Kidney development initiates around E10.5, when the ureteric epithelium buds off from the Wolffian duct and begins to invade the surrounding metanephric mesenchyme (for a review, see Costantini and

Fig. 3. Kidney and neural tube phenotypes in Dchs1 mutants. Paired mutant and wild-type images are always at the same magnification. (A-F) Hematoxylin and Eosin stains on P0 kidneys of wild-type (A,D), Dchs1 (B,E) and Fat4 (C,F) mutants at lower (A-C) and higher (D-F) magnification. Arrows indicate examples of cysts. (G,H) Sections of P0 kidneys stained for Tamm-Horsfall protein (THP, blue), which marks ascending loop of Henle and distal convoluted tubules, Aquaporin 2 (AQP2, green), which marks connecting tubule and collecting duct, and E-cadherin (E-cad, red), which marks the collecting duct. (I,J) Whole kidneys from P0 mice to indicate the difference in size and shape. (K,L) Sections through representative tubules in E15.5 embryos, stained for E-cadherin (red), DNA (Hoechst, blue) and aPKC (green). (M) Histogram showing mean number of cells around the circumference of E15.5 tubules; error bars indicate s.e.m. The difference in the means is significant (t-test, P = 0.014). (N) Histogram of mean width to height ratio in P0 neural tubes sectioned at the position of the heart and lungs; error bars indicate s.e.m. The difference is significant (t-test, P < 0.0001).

Fig. 4. Requirement for Dchs1-Fat4 signaling in internal organs. Paired mutant and wild-type images are always at the same magnification. (A-D) Sternums from P0 skeletal preparations of the indicated genotypes, stained with Alcian Blue (for cartilage) and Alizarin Red (for bone). (E) Histogram of average length of intestines in Dchs1 and Fat4 mutants compared with wild type; error bars indicate s.d. The differences between mutants and wild type are significant (t-test, P < 0.01). (F-I) Lumbar vertebrae from P0 skeletal preparations of the indicated genotypes. (J) Histogram indicating the relative size of selected lung lobes (superior, middle and left) in Dchs1 mutants compared with wild-type siblings. (K-N) Hearts from P0 mice of the indicated genotypes, stained with Hematoxylin and Eosin. Arrows indicate enlarged ostium secundum in the septum primum (SP). The right atrium (RA), left atrium (LA), septum secundum (SS), aorta (AO) and pulmonary artery (PA) are marked. (O-R) Example of superior lung lobes from P0 mice of the indicated genotypes.
Kopan, 2010; Dressler, 2009). During normal kidney development, kidney growth is coupled to branching of the ureteric epithelium. We examined early kidney growth and branching from E10.5-E13.5 by confocal microscopy of kidneys stained with E-cadherin (E-cad), which marks the ureteric epithelium, and Pax2, which marks both the ureteric epithelium and surrounding metanephric mesenchyme. The initiation of kidney development at E10.5 appeared normal in Dchs1 and Fat4 mutant kidneys (Fig. 5A-D). However, by E11.5, the branching of the ureteric epithelium in mutants lags behind that of wild-type siblings (Fig. 5E-H). By E12.5, the reduced kidney growth and ureteric branching is substantial, and in some regions the ureteric epithelium also appears wider and more irregular than in wild type (Fig. 5J-L). When quantified, mutants had approximately half as many ureteric bud tips as wild-type siblings (Fig. 5I). Similar phenotypes were observed in Dchs1 and Fat4, although the Fat4 was slightly more severe. The difference in growth continues until E13.5, by which time mutant kidneys are dramatically reduced in size compared with wild type (Fig. 5M-P). However, as the fold difference in size between wild-type and mutants does not appear to increase further from E13.5 to E15.5 (Fig. 5Q-T), nor from E15.5 to P0 (Fig. 3), it appears that later stages of kidney growth are less affected by Dchs1 or Fat4 mutation.

**Analysis of gene expression in Dchs1 and Fat4 mutant kidneys**

The reduction in size and branching during early kidney development observed in Fat4 or Dchs1 mutants is reminiscent of that reported in Wnt11 mutants (Majumdar et al., 2003). Wnt11 is expressed in the epithelial bud tips, and is involved in a reciprocal signaling loop with Gdnf, which is expressed in the surrounding mesenchyme, such that Wnt11 and Gdnf expression are mutually dependent during early kidney development (Majumdar et al., 2003). To examine whether the influence of Dchs1 or Fat4 mutants on early kidney development might involve effects on Wnt11 or Gdnf, we examined their expression in E12.5 kidneys, both by in situ hybridization, and by quantitative RT-PCR, but no significant differences were observed (Fig. 6A-F and data not shown). The Gdnf receptor Ret was also expressed at normal levels by quantitative RT-PCR (Fig. 6A,B). To explore potential effects on other genes involved in early kidney development, we also examined the expression of the receptor tyrosine kinase inhibitor Sprouty1 (Basson et al., 2006), which inhibits branching, and the transcription factor Foxd1, which is expressed in kidney stromal cells that are required for growth and branching (Hatini et al., 1996), but neither was visibly affected (Fig. 6G-J). We also examined several other proteins linked to pathways implicated in kidney development, Hippo signaling or PCP, but none was significantly affected (see Fig. S2 in the supplementary material).
**fj** is a downstream target of Fat signaling in *Drosophila*, and it has been reported that expression of its mammalian homologue, *Fjx1*, is elevated in the kidneys of *Fat4* mutants at P0 and E16.5 (Saburi et al., 2008). To examine whether *Fjx1* expression is regulated by *Fat4* or *Dchs1* during stages when they influence kidney growth, we assayed *Fjx1* expression by in situ hybridization and RT-PCR in E12.5 kidneys.

**Influence of Dchs1 and Fat4 mutations on kidney cell proliferation and apoptosis**

To investigate the cellular basis for the reduction in kidney size, we analyzed cell proliferation and cell death during early stages of kidney development. We focused this analysis on *Fat4* mutants, because the early reduction in growth was more evident than for *Dchs1*. A significant reduction in cell proliferation, revealed by staining mitotic cells with phospho-histone H3 (PH3) was identified in both mesenchymal and epithelial cells of E11.75 *Fat4* kidneys (Fig. 6M-Q). Cell death was analyzed by staining for cleaved caspase 3 (Cas3), which is a marker of apoptosis. Occasional spots of Cas3 staining were detected within both the epithelium and the mesenchyme of wild-type kidneys. By contrast, *Fat4* mutant kidneys, and to a lesser extent *Dchs1* mutant kidneys, exhibited striking clusters of Cas3 staining within the ureteric epithelium (Fig. 6R-AA). Cas3 staining within the mesenchyme was not visibly affected. The clusters of increased Cas3 staining were detected as early as E11.5 (Fig. 6X,Y), and were always found in the center of the epithelium. Thus, the reduction in kidney size in *Fat4* and *Dchs1* mutants is associated with both increased cell death and decreased proliferation. To investigate whether this unusual pattern of apoptosis occurred in other organs in *Fat4* mutants, we examined E11.5 lungs, but no significant difference was detected between wild type and mutants (see Fig. S3 in the supplementary material).

**Dchs1 and Fat4 are expressed in mesenchymal cells**

Some analysis of *Dchs1* and *Fat4* expression has been reported (Rock et al., 2005), but their expression during early stages of kidney development has not been characterized. In situ hybridization to...
expressed most prominently in a medial domain adjacent to Dchs1 (Fig. 7K,L; see Fig. S4 in the supplementary material). The specificity of the Dchs1 expression was confirmed by using a probe corresponding to the region deleted in mutant animals (Fig. 7C,D; data not shown). We also extended analysis of Dchs2 expression by characterizing its expression in E9.5, E10.5 and E12.5 embryos. Dchs2 was readily detected in the nervous system, but not in the cochlea, kidney, lung, somite or intestine (see Fig. S4 in the supplementary material; data not shown).

To explore the localization of Fat4 and Dchs1 protein, we performed antibody staining, using previously described antisera (Ishiuchi et al., 2009). We focused on the kidney and lungs, as these have significant defects in Dchs1 and Fat4 mutants. Consistent with the in situ hybridization analysis, both Dchs1 and Fat4 were detected in mesenchymal cells of E12.5 kidneys (Fig. 8) and lungs (see Fig. S3 in the supplementary material). Immunostaining of mutant tissue confirmed the specificity of the immunostaining for both proteins, although some faint background staining was detected in epithelial cells (Fig. 8C,D; see Fig. S3 in the supplementary material). Double staining kidneys with anti-Pax2 revealed that Fat4 and Dchs1 are expressed at highest levels in the Pax2-negative mesenchymal cells, although low levels were also detected in Pax2-positive mesenchymal cells (Fig. 8). Using a different anti-Fat4 sera, Saburi et al. (Saburi et al., 2008) reported that Fat4 localizes to cilia in cultured MDCK cells. However, we found that Fat4 and Dchs1 are distributed around the periphery of E12.5 kidney and lung mesenchymal cells (Fig. 8E,F; see Fig. S3 in the supplementary material), and saw no evidence for preferential localization to cilia (see Fig. S5 in the supplementary material).

**Reciprocal influence of Dchs1 and Fat4 on protein staining**

The similar phenotypes of Dchs1 and Fat4 mutants, together with the ability of Dchs1 and Fat4 to mediate aggregation of L cells (Ishiuchi et al., 2009), and studies of their *Drosophila* homologues Ds and Fat, suggest that Dchs1 and Fat4 function as a ligand-receptor pair. In *Drosophila*, mutation of *ds* can influence the levels or localization of Fat, and mutation of *fat* can influence the levels or localization of Ds, although the effect varies in different locations (Ma et al., 2003; Mao et al., 2009; Strutt and Strutt, 2002). If Dchs1 and Fat4 directly interact with each other in vivo, as suggested by their shared phenotypes, then mutation of one gene might influence the levels or localization of the other. Indeed, RNAi-mediated knockdown of Fat4 in the cerebral cortex was reported to decrease staining for Dchs1, whereas RNAi-mediated knockdown of Dchs1 increased staining for Fat4 (Ishiuchi et al., 2009). In the developing kidney and lung, we found both that mice mutant for Dchs1 exhibited stronger staining for Fat4, and that mice mutant for Fat4 exhibited stronger staining for Dchs1 (Fig. 8G-J; see Fig. S3 in the supplementary material). As these reciprocal influences on expression levels were not detected when Dchs1 and Fat4 transcripts were quantified by RT-PCR (Fig. 6A,B), we infer that this effect is post-transcriptional. We hypothesize that interaction between Dchs1 and Fat4 might promote their endocytosis and subsequent degradation, as is the case for other ligand-receptor pairs.

**DISCUSSION**

**In vivo evidence that Dchs1 functions as a ligand for Fat4**

One striking result from our creation and analysis of a mutation in the murine Dchs1 gene is the realization that Dchs1 and Fat4 have similar mutant phenotypes in diverse organs. This observation
provides an in vivo argument that Dchs1 functions as a ligand for Fat4. This conclusion is further supported by the reciprocal influences that mutations in these genes have on each other’s immunostaining. Moreover, we infer from the similar phenotypes of these mutations, as well as that of Dchs1 Fat4 double mutants, that they function as a dedicated ligand receptor pair – that is, at least for the phenotypes we identified, they do not have significant interactions with Dchs2 or Fat1-3. The absence of detectable Dchs2 expression in many organs where Fat4 plays important roles further supports the conclusion that its contribution to Fat4 regulation is limited. Fat4 phenotypes do appear to be slightly stronger than Dchs1 phenotypes in some instances. However, fat mutant phenotypes are generally stronger than ds phenotypes in Drosophila, even though there is only a single ds gene, which suggests that Fat proteins might have some ligand-independent activity. The tail and skeletal phenotypes of Dchs1 and Fat4 mutants appear similar to the classical mouse mutant screw tail (MacDowell et al., 1942), but as this mutant is lost, it was not possible to test for allelism.

Although genetic characterization of Dchs1 and Fat4 implies that they function within the same signaling pathway, like their Drosophila homologs, there are intriguing differences. In Drosophila, Ds and Fat are expressed and function within epithelial cells. In mice, by contrast, Dchs1 and Fat4 are predominantly, if not exclusively, expressed in mesenchymal cells. In Drosophila, Fat is expressed relatively broadly, whereas Ds is expressed in a more restricted pattern, overlapping Fat, but in gradients that are important for its effects on Fat signaling (Casal et al., 2002; Rogulja et al., 2008; Simon, 2004; Strutt and Strutt, 2002; Willecke et al., 2008; Yang et al., 2002). Conversely, in mammals, Dchs1 expression is not obviously graded. Finally, in Drosophila, Ds-Fat signaling regulates two distinct downstream branches, one that

Fig. 8. Dchs1 and Fat4 protein expression in kidneys. All panels show E12.5 kidneys, stained for E-cad (green), Pax2/5/8 (Pax2, blue) and either Dchs1 or Fat4 (red), as indicated. Panels marked by prime symbols show the Dchs1 or Fat4 channel only of the stain on the left. Scale bars are in the top right-hand corner; asterisk (B,H) indicates mesenchymal cells surrounding the ureter with strong Fat4 expression. (A,A’) Wild-type littermates from Dchs1 mutant stock, stained for Dchs1. (B,B’) Wild-type littermates from Fat4 mutant stock, stained for Fat4. (C,C’) Dchs1 mutant, stained for Dchs1. Some staining is detected in the epithelia, which we infer represents non-specific background from the antisera. (D,D’) Fat4 mutant, stained for Fat4. Some staining is detected in the epithelia, which we infer represents non-specific background from the antisera. A,C and B,D are matched pairs, dissected from the same litters, and fixed, stained and imaged under identical conditions. (E,E’) Higher magnification of Dchs1 staining. (F,F’) Higher magnification of Fat4 staining. (G,G’) Wild-type littermates from Fat4 mutant stock, stained for Dchs1. (H,H’) Wild-type littermates from Dchs1 mutant stock, stained for Fat4. (I,I’) Fat4 mutant, stained for Dchs1 (note increased expression relative to G). (J,J’) Dchs1 mutant, stained for Fat4 (note increased expression relative to H). G,J and H,J are matched pairs, dissected from the same litters, and fixed, stained and imaged under identical conditions.
influences Hippo signaling and one that influences PCP (for a review, see Reddy and Irvine, 2008). Although it remains possible that Dchs1-Fat4 signaling affects Hippo signaling in limited contexts, or acts redundantly with other inputs, phenotypes associated with tumor suppressors in the Hippo pathway, such as enlarged livers and epidermal hyperplasia (Lee et al., 2008; Song et al., 2010; Zhou et al., 2009), were not observed in Dchs1 or Fat4 mutants. Moreover, we did not detect effects on the Hippo pathway components Yap or Lats1.

Requirements for Dchs1-Fat4 signaling during mammalian development

Initial studies of Fat4 mutant mice identified requirements in the cochlea, tail, neural tube and kidney (Saburi et al., 2008). In addition to establishing that Dchs1 and Fat4 have similar biological functions, we have now significantly expanded the range of organs known to require the normal function of these genes, including the skeleton, heart, lung and gastrointestinal tract, and we also characterized a requirement during the early growth and branching of the kidney. Thus, we conclude that Dchs1-Fat4 signaling is broadly required across multiple organ systems during mouse development.

In Drosophila, the Fz-PCP and Fat-PCP pathways can function independently, but also interact in some contexts (for reviews, see Lawrence et al., 2007; Simons and Mlodzik, 2008; Strutt, 2008). Our results, together with those of Saburi et al. (Saburi et al., 2008), are consistent with the possibility that at least some Dchs1-Fat4 phenotypes reflect modulation of PCP, and further imply that mammals have a Fat-PCP pathway that can act independently from Fz-PCP signaling, yet has overlapping roles. Both Dchs1-Fat4 and Fz-PCP mutants can affect neural tube width, cochlear elongation, stereocilia orientation, development of kidney tubules, intestinal elongation, lung growth and tail morphology (Cervantes et al., 2009; Chacon-Heszele and Chen, 2009; Karner et al., 2009; Saburi et al., 2008; Simons and Mlodzik, 2008; Wang and Nathans, 2007; Yates et al., 2010). However, for some of these, such as the influence on stereocilia orientation or neural tube width, the influence of Dchs1-Fat4 signaling is very minor compared with what has been observed in Fz-PCP pathway mutants. Fz-PCP signaling also has other phenotypes that we could not detect in Dchs1 mutants (not shown), such as effects on hair polarity (Wang and Nathans, 2007). Conversely, Dchs1 and Fat4 exhibit novel phenotypes. The simultaneous shortening and widening of the sternum and vertebrae is suggestive of an influence on a convergent extension-like process, which might thus involve PCP. Consistent with this possibility, a similar sternum phenotype occurs in mice mutant for the PCP effector Fus (Gray et al., 2009). The abnormal ossification of the sternum is probably a secondary consequence of the increased width, as the normal pattern, with alternating bands of cartilage and bone, is thought to be directed by signaling from the ribs, which locally inhibit ossification (Chen, 1953). Whether the atrial septation defect reflects a PCP process is unknown, but this phenotype is of potential clinical significance, as these defects are a common form of congenital heart disease in humans (Warner et al., 2001), and thus far only a few gene mutations have been implicated in atrial septation defects. In sum, our observations identify important novel functions for Dchs1-Fat4 signaling in mammals.

Dchs1-Fat4 signaling during early kidney development

We identified an important early role for Dchs1-Fat4 signaling in the survival of kidney epithelial cells, and in the proliferation of epithelial and mesenchymal cells. These effects could account for both the reduced growth and the reduced branching of the kidney, because kidney growth and branching are interlinked. Apoptosis was detected within the epithelium, in striking clusters concentrated in the center, a phenotype that to our knowledge has never been described or observed previously in any other mouse mutants. As Dchs1 and Fat4 expression is only detected at background levels in epithelial cells, we infer that the influence of these genes on epithelial morphogenesis is indirect. For example, they might influence the production or transmission of a signal from mesenchymal or stromal cells. The early kidney phenotype of Dchs1 or Fat4 mutants has similarities with Wnt11 mutants, but Wnt11 is required for normal levels of Gdnf expression in mesenchymal cells (Majumdar et al., 2003), whereas we did not observe significant effects on the expression of Gdnf or Wnt11, which suggests that Dchs1-Fat4 signaling and Wnt11 signaling act independently.

Intriguingly, two other branching organs, the lungs and the salivary glands (S.Z., Y.M., P.F.-W. and K.D.I., unpublished), are also decreased in size in Dchs1 and Fat4 mutants, which suggests that this pathway might have a general role in the development of branching organs. The coordinated growth and branching of both kidneys and lungs is dependent upon reciprocal signaling between mesenchymal and epithelial cells (Cardoso and Lü, 2006; Dressler, 2009), and based on their expression we infer that Dchs1 and Fat4 function within mesenchymal cells. Fz-PCP signaling has also been implicated in lung branching and growth, as Vangl2 and Celsr1 mutants have smaller lungs with reduced branching (Yates et al., 2010). But these genes are expressed preferentially in lung epithelial cells, which suggests that they influence lung branching through a distinct mechanism.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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References


Characterization of a Dchs1 mutant


