Atmin mediates kidney morphogenesis by modulating Wnt signaling

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The DNA damage protein and transcription factor Atmin (Asciz) is required for both lung tubulogenesis and cilio genesis. Like the lungs, kidneys contain a tubular network that is critical for their function and in addition, renal ciliary dysfunction has been implicated in the pathogenesis of cystic kidney disease. Using the Atmin mouse mutant Gasping6 (Gpg6), we investigated kidney development and found it severely disrupted with reduced branching morphogenesis, resulting in fewer epithelial structures being formed. Unexpectedly, transcriptional levels of key cilia associated genes were not altered in AtminGpg6Gpg6 kidneys. Instead, Gpg6 homozygous kidneys exhibited altered cytoskeletal organization and modulation of Wnt signaling pathway molecules, including β-catenin and non-canonical Wnt/planar cell polarity (PCP) pathway factors, such as Daam2 and Vangl2. Wnt signaling is important for kidney development and perturbation of Wnt signaling pathways can result in cystic, and other, renal abnormalities. In common with other PCP pathway mutants, AtminGpg6Gpg6 mice displayed a shortened rostral-caudal axis and mis-oriented cell division. Moreover, intercrosses between AtminGpg6+/− and Vangl2Lp+/− mice revealed a genetic interaction between Atmin and Vangl2. Thus we show for the first time that Atmin is critical for normal kidney development and we present evidence that mechanistically, Atmin modifies Wnt signaling pathways, specifically placing it as a novel effector molecule in the non-canonical Wnt/PCP pathway. The identification of a novel modulator of Wnt signaling has important implications for understanding the pathobiology of renal disease.

INTRODUCTION

Tissue morphogenesis is a critical component of all stages of kidney development and disruption of any of these steps can lead to a variety of developmental defects that impact on kidney function (1,2). Metanephric kidney development initiates at E10.5 in mice when the ureteric bud evaginates from the Wolffian duct and invades the metanephric mesenchyme. Reciprocal signaling between mesenchymal and epithelial cells promotes branching morphogenesis of the ureteric bud, eventually leading to the establishment of nephrogenic progenitors and their subsequent differentiation into mature nephrons containing glomeruli (3,4).

The planar cell polarity (PCP) signaling pathway has emerged as a major and evolutionarily conserved regulator of morphogenetic processes including gastrulation, neurulation and lung branching morphogenesis (5–8). A key function of the PCP pathway is modification of the actin-myosin cytoskeleton to enable morphogenetic movement of tissue, shaping of cells and/or directed cell migration, all of which are critical for normal development and optimum organ function (9–13). In the kidney, perturbation of directed cell movements during tubule morphogenesis, podocyte development and the orientation of cell division and cell adhesion have all been related to defective PCP (14,15). Mutations in the PCP-associated genes Wnt9b and Fat4 have been shown to lead to cyst formation in
postnatal kidneys (16,17) while other murine PCP gene mutations are homozygous lethal, like Vangl2, show early hallmarks of cyst formation such as dilated tubules (9).

The PCP pathway is one of several that can be activated by Wnt ligands. These Wnt-associated pathways have been broadly grouped into the canonical pathway, which is mediated via β-catenin, and two β-catenin independent pathways; the PCP pathway and the Calcium pathway (18). Research has focused on these pathways because both hyper- and hypo-activation of Wnt signaling pathways has been linked to various genetic defects and adult diseases, including renal hypodysplasia, Alzheimer’s disease, osteoporosis and cystic kidney disease (18–21). Although the pathways diverge downstream, more proximally, a number of components, including Wnts, Frizzleds and Dishevelleds, are common to more than one pathway (22). While some Wnts appear to predominantly signal via one downstream pathway, e.g. Wnt11 signaling via the PCP pathway, other Wnts important in kidney development, such as Wnt4 and Wnt9b, have been shown to operate through more than one downstream pathway, signaling via both canonical and non-canonical branches (23,24). Ultimately, the intracellular response to a Wnt ligand is determined by precise control of the timing and expression of particular combinations of Wnt ligands, their receptors and the dynamics of these associations.

ATMIN is an essential Zn$^{+2}$ finger protein and was initially identified as a DNA damage response protein (25) involved in the base excision repair pathway and the in vivo oxidative stress response (26). Atmin has also been found to regulate ciliogenesis in the node by acting as a transcription factor to initiate Dynll1 gene expression (27). Similarly, an interaction between ATMIN and DYNLL1 has also been observed in several other contexts (28,29). Interestingly, Jurado and colleagues recently established that Atmin is a transcriptional regulator of embryonic lung development; acting via its SQ/TQ cluster domain, independently of its role in the DNA damage response. However, the molecular mechanisms by which Atmin modulates lung development and its transcriptional targets are not yet known (30). Because of the many links between cilia and cystic kidney disease, as well as the previously established role in lung organogenesis, we wished to investigate whether ATMIN was required for kidney development.

Here we show that ATMIN is critical for normal kidney organogenesis and we reveal a novel role for Atmin in modulating Wnt signaling pathways and more specifically, as an effector of the Wnt/PCP pathway.

RESULTS

Atmin<sup>Gpg6/Gpg6</sup> embryos display abnormal kidney morphology

Studies have shown that Atmin<sup>Gpg6/Gpg6</sup> embryos die around E14 and display a phenotype indicative of a complex ciliopathy including gross edema, exencephaly and left-right patterning defects (31). These mice carry a point mutation (T to A) in the third zinc finger domain of Atmin, which causes a serine to cysteine amino acid substitution and results in loss of function (27,31). As a first step in elucidating the potential role of Atmin in kidney development, quantitative real-time PCR (qRT–PCR) analysis of Atmin expression in kidneys was conducted. Atmin expression was detected in cDNA obtained from whole mouse kidney at postnatal days 7, 10, 14 and 21 (Fig. 1A). Atmin was also detected in cDNA from mouse glomerular isolates and in differentiated mouse podocytes (glomerular epithelial cells) (Fig. 1A). In addition, Atmin<sup>Gpg6/Gpg6</sup> embryos were examined at E13.5, an early stage of metanephric development, to determine whether kidney development was affected by the Atmin mutation in Gpg6. Atmin<sup>Gpg6/Gpg6</sup> kidneys were consistently smaller than wild-type (WT) and morphologically distinct (Fig. 1B), displaying an increased length to width ratio (Fig. 1C). Wholemount immunostaining with pan-cytokeratin, to highlight the ureteric bud tree, revealed that branching morphogenesis was severely disrupted in E13.5 Atmin<sup>Gpg6/Gpg6</sup> kidneys (Fig. 1E compared with D). Histological examination of E13.5 WT and Atmin<sup>Gpg6/Gpg6</sup> littermate kidneys stained with periodic acid-Schiff (PAS) also highlighted the early branching morphogenesis defects in Atmin<sup>Gpg6/Gpg6</sup> (Fig. 1G) compared with WT (Fig. 1F). Quantification of this defect from wholemount pan-cytokeratin immunostained kidneys revealed a mean of 22 ureteric bud tips in homozygous kidneys compared with a mean of 40 bud tips in heterozygotes or WT kidneys (Fig. 1H).

More detailed examination of E13.5 kidney sections immunostained with pan-cytokeratin revealed cellular disorganization in Atmin<sup>Gpg6/Gpg6</sup>, where the epithelial cells were randomly orientated with respect to each other, rather than the uniform alignment typical of epithelial cells during tubulogenesis. Compared with the well-ordered and neatly arranged epithelial cells in WT sections (Fig. 2A–C), the extent of cellular disorganization in homozygous kidney sections (Fig. 2D–F) meant that epithelial cells were difficult to distinguish from the surrounding mesenchyme by 4',6-diamidino-2-phenylindole (DAPI) staining alone (compare Fig. 2B with E), without the use of an epithelial cell marker (cytokeratin in Fig. 2A, C, D and F). Since significant disorganization and misalignment of epithelia was observed in Atmin<sup>Gpg6/Gpg6</sup>, it was hence important to determine whether apical-basal polarity was disturbed. Despite the overall disruption to epithelial organization in the developing tubules, immunolabelling with the apical membrane marker aPKCζ showed normal apical localization of this protein on the luminal side of tubules in both WT (Fig. 2G) and homozygous kidneys (Fig. 2H and I). The normal localization of aPKCζ indicated there was no overt disruption to apical-basal polarity in Atmin<sup>Gpg6/Gpg6</sup> kidneys.

Cilia architecture and hedgehog signaling are not disturbed in Atmin<sup>Gpg6/Gpg6</sup> kidneys

Recent studies have shown a link between ATMIN and ciliogenesis in the node that leads to perturbation of left-right asymmetry (27). Despite the overall disruption to epithelial organization in the developing kidneys, examination of cilia highlighted by immunofluorescence with acetylated tubulin revealed no obvious difference in cilia morphology between WT and Atmin<sup>Gpg6/Gpg6</sup> (Fig. 3A and B). Quantification showed no significant difference in the number of cilia between WT and Atmin<sup>Gpg6/Gpg6</sup> whole kidneys, or within ureteric buds or renal vesicles alone (Fig. 3C). Cilia length was also unaltered in Atmin<sup>Gpg6/Gpg6</sup> kidneys (Fig. 3D).
Although we did not detect disruption of ciliogenesis, primary cilia are the site for other important cellular functions, including Hedgehog signaling and trafficking of intraflagellar transport molecules. Gene expression studies on E13.5 WT and Atmin\textsuperscript{Gpg6/Gpg6} kidneys for genes crucial to these functions were also conducted. The levels of \textit{Ihh}, \textit{Shh}, \textit{Ptch1}, \textit{Gli1} and \textit{Ift88} were not significantly different between WT and Atmin\textsuperscript{Gpg6/Gpg6} kidneys (Fig. 3E). These data show that Atmin is likely not required for either ciliogenesis or key cilia-associated functions in the kidney. Nevertheless, consistent with published results \textit{Dynll1} expression was decreased by 80% in Atmin\textsuperscript{Gpg6/Gpg6} kidneys, whereas \textit{Dynll2} expression remained unchanged (Supplementary Material, Fig. S1).

\textit{Atmin\textsuperscript{Gpg6/Gpg6}} kidneys exhibit phenotypes consistent with Wnt/PCP pathway disruption

To investigate the underlying cause for the reduced number of epithelial structures in Atmin\textsuperscript{Gpg6/Gpg6} kidneys, potential alterations in proliferation or apoptosis were investigated. No significant difference was detected in the percentage of proliferating cells in Atmin\textsuperscript{Gpg6/Gpg6} (Supplementary Material, Fig. S2B, D and I) kidneys compared with WT (Supplementary Material, Fig. S2A, C and I) either in whole kidney sections or when calculating proliferation in epithelial and mesenchymal cells separately (Supplementary Material, Fig. S2J). Similarly, no change in the level of apoptosis was observed in homozygous mutant kidneys (Supplementary Material, Fig. S2E, G and K) compared with WT (Supplementary Material, Fig. S2F, H and K). In addition, investigation of key genes required for kidney branching morphogenesis, \textit{Gdnf} and \textit{Ret}, revealed no change in expression between WT and Atmin\textsuperscript{Gpg6/Gpg6} kidneys (Supplementary Material, Fig. S2L). The lack of change in cell proliferation, apoptosis or early differentiation markers suggested that the reduced number of epithelial structures and cellular disorganization observed in Atmin\textsuperscript{Gpg6/Gpg6} kidneys could be due to defective tissue morphogenesis rather than a cell division or apoptosis defect.

Previous studies have shown that the PCP signaling pathway is important for precisely shaping epithelial branches (morphogenesis) during lung and kidney development by affecting cytoskeletal organization (9,10). In addition, the PCP pathway has been linked to regulating cytoskeleton distribution in podocytes (9,11). Moreover, mouse mutants with defective PCP signaling

**Figure 1.** Atmin is expressed in the kidneys and its disruption results in kidney morphogenesis defects. (A) Atmin is expressed in whole mouse kidneys at postnatal day P7, P10, P14 and P21, in isolated mouse glomeruli and in differentiated mouse podocytes. (B) Atmin\textsuperscript{Gpg6/Gpg6} E13.5 kidneys are smaller than WT. (C) An increased length to width ratio was observed in Atmin\textsuperscript{Gpg6/Gpg6} kidneys compared with WT (\(n = 6\) per genotype, \(P < 0.01\)). Wholemount cytokeratin immunostaining of kidneys (D and E) revealed a reduced number of ureteric bud tips in Atmin\textsuperscript{Gpg6/Gpg6} (E) compared with WT (D) or heterozygous littersmates. Images shown are maximum intensity projections following \(z\)-stacks through whole kidneys. PAS staining of E13.5 kidneys (F and G) highlighted histological abnormalities in Atmin\textsuperscript{Gpg6/Gpg6} (G) compared with WT animals (F). (H) Quantification showed an average of 50% fewer bud tips in Atmin\textsuperscript{Gpg6/Gpg6} kidneys (\(n = 12\)) compared with WT or heterozygous littersmates (\(n = 18\); \(P < 0.0001\)).
do not show changes in levels of cell proliferation or apoptosis and instead show altered cytoskeleton distribution (10). To investigate whether perturbation of Atmin was associated with defective PCP signaling, actin cytoskeletal distribution was compared in E13.5 WT and Atmin<sup>Gpg6/Gpg6</sup> kidneys. Phalloidin staining of F-actin revealed diffuse cortical actin in Atmin<sup>Gpg6/Gpg6</sup> kidney epithelial cells, whereas these areas of substantially expanded actin distribution were never observed in WT kidneys (compare Fig. 4B with D). These changes in actin cytoskeleton distribution are indicative of a disrupted cytoskeleton network. To further investigate whether perturbation of Atmin was associated with defective PCP signaling, the average length:width ratio of E13.5 embryos

Figure 2. Cytoskeletal differences are observed in Atmin<sup>Gpg6/Gpg6</sup> embryos. In z-stack images of WT (A) and Atmin<sup>Gpg6/Gpg6</sup> (D) kidney sections immunostained for cytokeratin (green), epithelial cells appear highly disorganized and randomly orientated. (B and E) Epithelial cells are difficult to distinguish from surrounding mesenchyme by DAPI in Atmin<sup>Gpg6/Gpg6</sup> (compare E with B). Cytokeratin stained renal vesicles, with disorganized epithelial cells that are frequently multi-layered and misaligned with respect to one another are observed in the mutant (F) compared with WT, where the cells show a regular order and are uniformly aligned with each other (C). Normal apical-basal polarity was observed in E13.5 Atmin<sup>Gpg6/Gpg6</sup> kidneys (H and I) and their WT littermates (G) as determined by aPKC<sub>z</sub> staining of E13.5 whole z-stack sections. Images are representative of at least four animals in each category.
was calculated. A significantly lower length:width ratio was observed in homozygous mutant embryos compared with WT (Fig. 4E; Atmin<sup>Gpg6/Gpg6</sup> 2.6, WT 2.8; n = 8, P < 0.05). A reduced length:width ratio is frequently observed in mouse mutants of PCP-associated genes as a result of convergent extension defects; therefore these results were consistent with Atmin<sup>Gpg6/Gpg6</sup> mice manifesting a defective PCP pathway.

The orientation of cell division is perturbed in Atmin<sup>Gpg6/Gpg6</sup> homozygous kidneys

The orientation of cell division is commonly used as a read-out of PCP signaling. It is usual that cell division within kidney tubules takes place parallel to the basal membrane in the x-axis, i.e. perpendicular to the tubular longitudinal y-axis and it is possible to...
visualize this either by examining whole tubules or by looking at transverse sections through tubules, where cell division primarily occurs at the luminal surface (32,33). The orientation of epithelial cell division relative to the basal membrane was therefore examined in transverse sections of E13.5 WT and Atmin<sup>Gpg6/Gpg6</sup> kidneys. The normal bias toward a uniform orientation of cell division, parallel to the basal membrane, was present in WT kidney sections (Fig. 4F), whereas in Atmin<sup>Gpg6/Gpg6</sup> kidneys, a much more random cell division orientation was observed (Fig. 4G). Quantification of the angle of division showed 100% of WT cells undergoing cell division at an angle between 0 and 20° from the basal membrane axis (Fig. 4H). In Atmin<sup>Gpg6/Gpg6</sup> kidney sections, the angle of cell division was randomly distributed within each of the 10° brackets between 0 and 90° (Fig. 4H).

The Atmin<sup>Gpg6</sup> mutation affects transcription of key genes in the non-canonical Wnt/PCP pathway

Because the morphological defects observed in Atmin<sup>Gpg6/Gpg6</sup> kidneys were consistent with perturbation of the Wnt/PCP pathway, expression of genes relevant to canonical Wnt and non-canonical Wnt/PCP signaling were examined. No difference in expression was observed for Wnt4, a gene most frequently associated with the canonical Wnt pathway, whereas the genes Wnt1b and Wnt11 that are usually linked to the non-canonical Wnt/PCP pathway showed decreased expression in Atmin<sup>Gpg6/Gpg6</sup> compared with WT (Fig. 5A). Furthermore, a 0.6-fold decrease in the expression of Dvl1 was observed, whereas the levels of Dvl2 and Dvl3 remained unchanged (Fig. 5B). Interestingly, no difference was detected in other PCP pathway genes; Fuzzy and Daam1, but there was an almost 2-fold increase in Daam2 expression in Atmin<sup>Gpg6/Gpg6</sup>, further emphasizing the involvement of Atmin in the PCP/non-canonical Wnt pathway (Fig. 5C).

β-Catenin and Vangl2 proteins are altered in Atmin<sup>Gpg6/Gpg6</sup> kidneys

Since transcriptional differences relevant to the Wnt signaling pathway were observed between the Atmin<sup>Gpg6/Gpg6</sup> and WT embryonic kidneys, the levels of β-catenin and Vangl2, two key proteins for the canonical and noncanonical/PCP Wnt signaling pathways, respectively, were also examined. A striking decrease in β-catenin was seen in Atmin<sup>Gpg6/Gpg6</sup> by immunofluorescence (Fig. 6B) compared with WT (Fig. 6A) and western blotting confirmed a 40% reduction in both total and active β-catenin (Fig. 6E, F and I). An overall decrease in Vangl2 immunostaining was also observed in Atmin<sup>Gpg6/Gpg6</sup> kidney sections (Fig. 6D) compared with WT (Fig. 6C) and significantly, apical enrichment of Vangl2, which is required for its proper function, appeared considerably reduced in Atmin<sup>Gpg6/Gpg6</sup> (compare insets in Fig. 6C and D). Intriguingly, similar levels of both β-catenin and Vangl2 transcripts were observed in Atmin<sup>Gpg6/Gpg6</sup> and WT kidneys (Fig. 6J), indicating that Atmin does not directly act to alter these molecules at the transcriptional level. Similarly, no change was detected in expression levels of Axin2, another critical gene in the canonical Wnt pathway, between WT and Atmin<sup>Gpg6/Gpg6</sup> kidneys (Fig. 6J). Nevertheless, loss of Atmin function specifically affects both β-catenin and Vangl2 proteins.

Atmin genetically interacts with Vangl2

Taken together, our results showed that the phenotype observed in the Atmin<sup>Gpg6/Gpg6</sup> kidneys most closely resembled that of other PCP pathway mutants. Furthermore, our data suggested that Atmin and Vangl2 might genetically interact; this possibility was tested by conducting intercrosses between Atmin<sup>Gpg6/Gpg6</sup> and Vangl2<sup>2/2</sup> heterozygotes (Fig. 7A–D and I). Fifteen percent of double heterozygotes exhibited craniorachischisis, a similar level to that previously observed between Vangl2 and another PCP associated factor Ptk7 (34) (Fig. 7I). In contrast, mild architectural kidney defects were observed even in Atmin<sup>Gpg6/Gpg6</sup>; Vangl2<sup>2/2</sup> embryos that genotyped as double heterozygotes but did not show craniorachischisis. Specifically, WT kidneys contained clearly distinguishable cortex and medullary regions (Fig. 7E). Atmin<sup>Gpg6/Gpg6</sup> (Fig. 7F) and Vangl2<sup>2/2</sup> (Fig. 7G) single heterozygote kidneys also showed distinct cortex and medullary regions; however, this distinction was not visible in Atmin<sup>Gpg6/Gpg6</sup>; Vangl2<sup>2/2</sup> (Fig. 7H). This phenotype is indicative of a differentiation defect and a similar lack of cortico-medullary definition is observed in kidneys homozygous for the Lp mutation in Vangl2 (9). Our data show that Atmin and Vangl2 genetically interact; moreover the disparity in the number of double heterozygous embryos exhibiting craniorachischisis and kidney defects suggests that the Atmin-Vangl2 interaction may be more important in some specific aspects of embryo development, such as kidney formation, than in others, e.g. neural tube closure. The genetic interaction between Atmin and Vangl2 revealed in these experiments provides additional evidence that Atmin is important for modulating the Wnt/PCP pathway in normal kidney development.

DISCUSSION

Atmin and kidney development

The molecular and phenotypic analyses of Atmin<sup>Gpg6/Gpg6</sup> embryos presented in this manuscript reveal that ATMIN is...
required for normal kidney organogenesis, with homozygous mutants displaying defective branching morphogenesis leading to formation of fewer epithelial bud tips. Furthermore, we observe disrupted cytoskeleton organization in $Atmin^{Gpg6/Gpg6}$ kidneys, as has previously been shown for other PCP pathway mutants (9,12,35). ATMIN is also required for lung organogenesis, another organ that undergoes branching morphogenesis to form the tubular network of airways (30). As in the lungs, factors affecting epithelial tube formation can impact on organ function; for example, in the kidneys, there is a direct relationship between impaired development, resulting in generation of fewer glomeruli, and reduced kidney function (36–38).

**Atmin regulates Wnt signaling pathways**

Wnts are required for many aspects of kidney development and homeostasis; the PCP pathway branch regulates morphogenesis of metanephric mesenchyme and ureteric bud-derived structures, including podocytes, through cytoskeleton reorganization/rearrangement (9,11,12). Our investigation reveals that $Atmin$ plays a role in modifying Wnt signaling pathways; in particular, $Atmin^{Gpg6/Gpg6}$ embryos exhibit defects associated with PCP pathway disruption including a shortened rostral-caudal axis, disrupted epithelial organization, altered cytoskeleton distribution and mis-orientation of cell division.

Although several aspects of $Atmin^{Gpg6/Gpg6}$ embryos resemble those of other PCP pathway-associated mutants such as Vangl2 and Wnt5a, there are also some distinct differences in the phenotypes. For example, homozygous core PCP pathway mutations like Vangl2 or Celsr1 usually result in the severe neural tube defect, craniorachischisis, whereas the $Atmin$ homozygotes show exencephaly, a different neural tube closure defect (30,31,39). Moreover the genetic interaction that we observed between $Atmin$ and Vangl2 is not as frequent as for some other core PCP pathway genes, where the protein products have been shown to bind directly to each other. Instead, the percentage of embryos exhibiting a genetic interaction indicates that ATMIN may function as an effector molecule in the kidney, in a similar manner to other PCP effectors important in some, but not all, contexts, e.g. PTK7 (34). A similarly low percentage of Vangl2/PTK7 double heterozygotes exhibit craniorachischisis (34) but nevertheless, there is ample evidence that PTK7 makes an important contribution to the PCP signaling pathway (40,41). Interestingly, our analysis of Wnt pathway genes showed altered gene expression of key Wnts important for kidney development, as well as a specific reduction in $Dvl1$ but not in $Dvl2$ or $Dvl3$. Wnt signaling pathways are activated by the binding of a Wnt ligand to a membrane bound, usually Frizzled, receptor. Subsequently, recruitment of one of the $Dvl$ genes to the Wnt/frizzled complex occurs prior to recruitment of additional proteins more specific to a particular downstream signaling branch; for example the canonical or PCP pathway. Since a dramatic decrease was detected in both $\beta$-catenin (canonical Wnt pathway-specific) and Vangl2 (PCP pathway-specific) proteins, this indicates that the transcriptional changes seen in $Atmin$ homozygous kidneys impact on more than one downstream pathway. Notably, most of the transcriptional changes that we do see are in upstream components of Wnt signaling pathways, i.e. selected Wnt ligands and $Dvl1$. Downstream of this, we see effects on both canonical and non-canonical pathway components; we therefore propose that ATMIN likely influences Wnt signaling pathways somewhere upstream, at the level of one or more of the common Wnt pathway components (Fig. 7). However, whether or not Atmin is a direct transcriptional regulator of one or more common Wnt signaling pathway components is yet to be determined.
Figure 6. Decreased β-catenin and Vangl2 levels are observed in Atmin<sup>Gpg6/Gpg6</sup> E13.5 embryonic kidneys compared with WT littermates. Kidney sections from WT (A and C) and Atmin<sup>Gpg6/Gpg6</sup> (B and D) embryos were immunostained for β-catenin (A and B) and Vangl2 (C and D). β-Catenin immunostaining was significantly reduced in Atmin<sup>Gpg6/Gpg6</sup> (B) compared with WT (A). Vangl2 immunostaining was also dramatically decreased and apical enrichment was altered in Atmin<sup>Gpg6/Gpg6</sup> (D) compared with WT (C). Overlays of DAPI and Vangl2 localization emphasize the loss of apical enrichment of Vangl2 protein in Atmin<sup>Gpg6/Gpg6</sup> (D inset) versus WT (C inset; n = 4). All images are maximum projections of confocal z stacks taken at 0.4 μm intervals. Images were taken in identical conditions and post-acquisition manipulations were identical. Western blotting revealed a 0.4-fold decrease (I) in β-catenin (P < 0.0001) (E) and active β-catenin (P = 0.03) (F) protein levels compared with WT. GAPDH was used as a loading control (G and H). β-catenin, Axin2 and Vangl2 mRNA expression remained unaltered in E13.5 Atmin<sup>Gpg6/Gpg6</sup> embryos compared with WT littermates (J, n = 4).
Atmin\(^{Gpg6/+}\) mutants genetically interact with Vangl2\(^{Lp/+}\). Intercrosses of Atmin\(^{Gpg6/+}\) with Vangl2\(^{Lp/+}\) generated double heterozygous embryos, with a percentage of double heterozygotes displaying craniorachischisis (D), a phenotype that was never observed in WT (A). Atmin\(^{Gpg6/+}\) Vangl2\(^{Lp/+}\) (B) or Atmin\(^{+/+}\) Vangl2\(^{Lp/+}\) (C) E13.5 embryos. The number of double heterozygotes displaying craniorachischisis compared with other genotypes is statistically significant (P < 0.05). Atmin\(^{Gpg6/+}\) Vangl2\(^{Lp/+}\) E13.5 kidneys (H) appeared delayed without clear demarcation between the cortical and medullary regions that was evident in the other three genotypes (E–G). (I) Graphical representation of the percentage distribution of embryonic phenotypes collected from intercrosses. The number of embryos examined per genotype is indicated at the top of each column. A small decrease in kidney size was observed in 20% of Atmin\(^{Gpg6/+}\) mice. (J) A schematic diagram representing potential modes of interaction between Atmin and Wnt signaling.
Although an exhaustive study of the expression and function of all the individual components of canonical and non-canonical Wnt signaling is beyond the scope of the current manuscript, our results have revealed some interesting findings about the effect of the Atmin<sup>Gpg6</sup> mutation on components of Wnt signaling pathways. For example, a number of studies have highlighted mutual antagonism between different downstream Wnt signaling pathways within the same tissue/cell-type. In this model, canonical and non-canonical Wnts activate one downstream pathway, while simultaneously repressing the other due to competition for Frizzled binding. This is thought to be achieved by Wnts associating with pathway-specific co-receptors such as Lrp6 and Ror2 (42). Consequently, the finding that both β-catenin and Vangl2 proteins were perturbed in Atmin<sup>Gpg6/Gpg6</sup> kidneys was somewhat unexpected; however, much of the work showing mutual antagonism of Wnt signaling pathways has been conducted in vitro and it is now becoming clear that at the level of tissues or whole organs, the picture is much more complex. In vivo, rather than distinct signaling pathways operating separately, interactions between pathways appear much more intricate with integration occurring at many different levels (43). This complexity is evident when looking at Wnt pathway regulation in human disease; for example, studies of Meckel-Gruber syndrome associated proteins have revealed defects in either canonical or non-canonical Wnt signaling depending on the particular mouse model (44). In chronic obstructive pulmonary disease (COPD) both over- and under-expression of β-catenin has been associated with disease (45,46). Moreover, a recent study looking at global gene expression changes in COPD patients showing mutual antagonism of Wnt signaling pathways has been conducted in vivo and it is now becoming clear that at the level of tissues or whole organs, the picture is much more complex. In vivo, rather than distinct signaling pathways operating separately, interactions between pathways appear much more intricate with integration occurring at many different levels (43).

This complexity is evident when looking at Wnt pathway regulation in human disease; for example, studies of Meckel-Gruber syndrome associated proteins have revealed defects in either canonical or non-canonical Wnt signaling depending on the particular mouse model (44). In chronic obstructive pulmonary disease (COPD) both over- and under-expression of β-catenin has been associated with disease (45,46). Moreover, a recent study looking at global gene expression changes in COPD noted an enrichment of Wnt pathway genes; importantly, the majority of both canonical and non-canonical Wnt signaling components were simultaneously downregulated (47). This study further emphasizes the complexities of Wnt signaling in organogenesis.

**Daam2 is specifically upregulated in Atmin<sup>Gpg6/Gpg6</sup> kidneys**

The functions of the Dishevelled associated activator of morphogenesis (Daam) genes 1 and 2 are still not well understood but, as inferred by their name, they associate with DvlS and usually operate in the PCP pathway to regulate cytoskeleton dynamics (see schematic in Fig. 7J). Work in Xenopus showed that Daam1 knockdown alters morphogenesis of the pronephric tubules and collecting duct but does not result in an obvious cystic phenotype (7). In chick, Daam2 has been suggested to modulate formation and stabilization of Wnt/receptor complexes (48). Previous mouse studies have identified a critical role for Daam1 in heart morphogenesis and for Daam2 in the developing gut (49,50) and in vitro, addition of exogenous Wnt5a led to re-distribution of Daam1 concomitant with increased stress fiber formation in both mouse and human podocyte cells (11). Collectively, these investigations highlight the importance of the Daam genes in cell and tissue morphogenesis during organ development. Interestingly, the expression of Daam1 and 2 in most organs is highly restricted and non-overlapping. For example in the lungs, Daam2 is restricted to the mesenchyme, whereas Daam1 is epithelial specific (51). According to the Genitourinary Development Molecular Anatomy Project, GUDMAP database, Daam1 shows little if any expression in kidneys, while Daam2 is present at much higher levels and is restricted to the stroma and vasculature. The differing patterns and levels of Daam1 and 2 in the kidneys indicate that here, Daam2 is the more important family member and it is therefore unsurprising that in Atmin<sup>Gpg6/Gpg6</sup> kidneys a specific change in Daam2 alone is observed.

**ATMIN, DYNLL1 and cilia interactions**

The Atmin<sup>Gpg6</sup> mutation was recently associated with ciliogenesis, specifically in the node, where reduced cilia length is thought to be the cause of the left-right patterning defects present in the embryos (27). However, in the same manuscript cilia in the limbs and neural tube revealed mild structural and no functional defects. Similarly, we did not observe any difference in cilia length or number in Atmin<sup>Gpg6/Gpg6</sup> kidneys and consistent with this data, levels of the key cilia gene Ifb88 were unaltered in the homozygotes. Despite the absence of changes in cilia structure, the possibility remained that signaling pathways associated with primary cilia might be affected. However, our investigation of one such signaling pathway, the Hh pathway, did not show any difference between Atmin<sup>Gpg6/Gpg6</sup> and WT kidneys. In fact, investigations have suggested an association between Wnt signaling and primary cilia structure and function, though this remains a controversial area that has not yet been definitively proven (14,21,52–55).

Another key protein interaction that has been shown in several contexts is that between ATMIN and the dynein motor protein, DYNLL1 (27–29). Consistent with previous studies, downregulation of Dynll1 but not Dynll2 was detected in Atmin<sup>Gpg6/Gpg6</sup> kidneys. DYNLL1 can function in cilia but also as part of the cytoplasmic dynein complex. In the cytoplasm, DYNLL1 is important for transport of cargo, carrying them toward the minus ends of microtubules and can also be found at the cortex, where it pulls on microtubules attached to the spindle poles during mitosis (56). Although DYNLL1 is both axonemal and cytoplasmic, the fact that no defects were observed in the Hedgehog pathway or in cilia size suggests that in the kidneys, the functions of DYNLL1 and thereby disrupt planar polarity pathway functions (either protein trafficking and/or orientation of mitosis). However, an alternative possibility is that mutations in Atmin may lead to direct disruption of the actin-myosin cytoskeleton, since abnormal cytoskeletal organization was observed in the homozygous kidneys. In support of this idea, another PCP effector molecule, Wdpcp, was recently shown to be required for ciliogenesis but interestingly, Cui <em>et al.</em> (13) established that the PCR defects in Wdpcp mutants are not due to abnormal ciliogenesis; rather they result from direct perturbation of the actin cytoskeleton, as we have observed in Atmin<sup>Gpg6/Gpg6</sup> kidneys.

Taken together, the molecular and phenotypic analysis of Atmin<sup>Gpg6/Gpg6</sup> embryos presented in this manuscript reveals that ATMIN is an important regulator of Wnt signaling pathways during kidney development. Given the critical role of Wnt
signaling in kidney development and diseases, the identification of a novel modulator has important implications both for further studies of Wnt signaling pathways and for the design of potential therapeutic strategies.

MATERIALS AND METHODS

Mice
The ENU-derived AtminGpg6 mice were identified in an ENU mutagenesis screen at MRC Harwell, as previously described (31). Gpg6 mice show a T to A transversion in exon 3 of Atmin, correlating with the third Zinc Finger. This results in a cysteine to serine substitution in the fourth canonical residue (27). Looptail mice (Jackson Laboratories, Bar Harbour, ME, USA) carry a point mutation at position 464 that results in a serine to asparagine transition (57,58). Congenic lines of Vangl2fl/fl and AtminGpg6 mice were maintained on a C3H/HeH background. Looptail homozygotes were identified by craniorachischisis and heterozygotes by the presence of a looped tail. Genotyping was carried out using pyrosequencing assays to directly amplify the mutations; WT littermates were used as controls (primers available on request).

Histology and immunostaining
Kidneys were fixed in 4% paraformaldehyde, dehydrated, wax-embedded and sectioned at 5 µm. In some experiments, PAS staining was used to help distinguish epithelial and mesenchymal compartments. Immunohistochemistry was performed using 10 µm cryosections for the following antibodies: phosphohistone H3 (pH3; Millipore, Dundee, UK), Cleaved caspase-3, β-histone H3 (pH3; Millipore, Dundee, UK), phospho- and total β-catenin (Cell Signalling Technology, Danvers, MA, USA), Vangl2 (raised in rabbit against the following Vangl2 specific peptide: CLAKKVSGFKVYSLGEENST, 21st Century Biochemicals, MA, USA). Histology and immunostaining was performed using DAPI. As negative controls, primary antibodies were omitted. Bound antibodies were detected with either Alexa Fluor 488 or Cy3 (Santa Cruz Biotechnology, Dallas, TX, USA), rhodamine-conjugated phalloidin (Invitrogen, Paisley, UK), and biotinylated secondary antibodies (Invitrogen). Cell nuclei were visualized by staining with DAPI. As negative controls, primary antibodies were omitted.

Quantification of kidney cilia numbers and length
Representative, comparative E13.5 z-stack images of kidney sections immunostained for acetylated tubulin from six WT and six AtminGpg6/Gpg6 embryos were counted for total, ureteric bud and renal vesicle cilia in Adobe Photoshop. The average cilium length was estimated from the same images as described above, for 50 cilia for each genotype using the line segment tool in Adobe Illustrator. The average (mean) number of cilia or the average cilium length for each genotype was calculated in Microsoft Excel.

Western blotting
Immunoblotting was carried out using 10 µg/lane of E13.5 WT and AtminGpg6/Gpg6 whole kidney protein extract (n = at least 9 per genotype run on 3 independent blots); using total β-catenin antibody (Sigma), 1:4000; or non-phospho β-catenin antibody (Cell Signaling Technology), 1:1000. GAPDH (Abcam), 1:5000 was used for loading control.

Proliferation and apoptosis analysis
Proliferation and apoptotic indices were calculated by counting the numbers of phospho histone-H3+, or cleaved caspase-3-expressing cells, respectively, in kidney sections from three individuals of each genotype, as a percentage of total (DAPI-stained) nuclei. Ki67 staining was used for assessment of percentage proliferation in epithelium and mesenchyme compartments separately. Images are representative of at least three animals for each genotype.

Quantitative RT–PCR
To measure Atmin expression levels, 500 ng of RNA was isolated from either whole CD1 mouse kidneys at P7, P10, P14 or P21, isolated glomeruli or differentiated podocytes using TRI Reagent and cDNA prepared for PCR using the iScript cDNA synthesis kit (Biorad) as per manufacturer’s instructions. Atmin levels are shown relative to the levels of the housekeeping gene β-actin and all assays were performed in triplicate. Glomerular isolations were performed using magnetic beads as previously described (38). Differentiated mouse podocyte cells (gift from Peter Mundel, Harvard Medical School, USA) were cultured on tissue culture plastic coated with 1% Matrigel substrate. For on-going proliferation, cells were cultured at 33°C in a 5% CO2 incubator in RPMI (Invitrogen) supplemented with 10% FCS (Invitrogen), antibiotics and IFN-γ (10 U/mL). To induce differentiation, cells were thermoshifted to 37°C and cultured in RPMI in the absence of IFN-γ for 14 days.

To compare gene expression levels in WT and AtminGpg6/Gpg6 one µg RNA was isolated from WT and AtminGpg6/Gpg6 E13.5 littermate kidneys using the RNeasy mini kit (QIAGEN) and cDNA was prepared for qRT–PCR using the High Capacity cDNA Reverse transcription kit (ABI). Quantitative real-time PCR was performed for the genes Ihh, Shh, Patch1, Gli1, Ift88, Dynll1, Dynll2, Gdnf, Ret, Wnt4, Wnt9b, Wnt11, Dvl1, Dvl2, Dvl3, Daam1, Daam2, Fuzzy, Vangl2, Axin2 and β-catenin. All assays were provided by ABI. Alterations in gene expression in AtminGpg6/Gpg6 were expressed relative to the mean intensity in WT embryos over β-actin expression, which was given a standardized value of 1. Negative controls of reactions without cDNA template were included. All qRT–PCR assays were performed in triplicate on at least four different embryos of each genotype. Primer details are available upon request.

Statistical methods
Data were analyzed using unpaired two-tailed t-tests, unless otherwise stated. Significance was accepted at P < 0.05; error bars in all data represent standard error mean.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.
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Conflict of Interest statement. None declared.

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