Scribble participates in Hippo signaling and is required for normal zebrafish pronephros development

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Spatial organization of cells and their appendages is controlled by the planar cell polarity pathway, a signaling cascade initiated by the protocadherin Fat in *Drosophila*. Vertebrates express 4 Fat molecules, Fat1–4. We found that depletion of Fat1 caused cyst formation in the zebrafish pronephros. Knockdown of the PDZ domain containing the adaptor protein Scribble intensified the cyst-promoting phenotype of Fat1 depletion, suggesting that Fat1 and Scribble act in overlapping signaling cascades during zebrafish pronephros development. Supporting the genetic interaction with Fat1, Scribble recognized the PDZ-binding site of Fat1. Depletion of Yes-associated protein 1 (YAP1), a transcriptional co-activator inhibited by Hippo signaling, ameliorated the cyst formation in Fat1-deficient zebrafish, whereas Scribble inhibited the YAP1-induced cyst formation. Thus, reduced Hippo signaling and subsequent YAP1 disinhibition seem to play a role in the development of pronephric cysts after depletion of Fat1 or Scribble. We hypothesize that Hippo signaling is required for normal pronephros development in zebrafish and that Scribble is a candidate link between Fat and the Hippo signaling cascade in vertebrates.

Fat | planar cell polarity | polycystic kidney disease

Mutations in more than 20 seemingly unrelated human genes cause polycystic kidney disease. In many patients, mutations of these genes are associated with a plethora of extrarenal abnormalities, ranging from polydactyly and CNS defects to obesity and blindness (1). Virtually all gene products localize to the primary, non-motile cilium, a microtubular organelle attached to most body cells. Hence, it has been postulated that a dysfunction of the cilium is responsible for the diverse manifestations, including kidney cysts. Multiple pathways use the cilium as a signaling platform or are modulated by ciliary signals (2). One such pathway, the Wnt signaling cascade, plays an important role in kidney development (3). Although the β -catenin–dependent branch of this pathway is required to convert metanephric mesenchyme into tubular epithelium, the β -catenin–independent planar cell polarity (PCP) pathway seems to promote nephron differentiation and maturation during later developmental stages (4, 5). The PCP pathway originally was identified as a signaling cascade that organizes cells and their appendages in the *Drosophila* wing and eye but is increasingly recognized as playing a critical role in vertebrate organogenesis (6). *Drosophila* genetics has delineated 3 classes of PCP proteins, the upstream PCP proteins (Fat, Dachous, 4-jointed), the PCP core proteins (Frizzled, Dishevelled, Flamingo, Strabismus, Prickle, Diego), and downstream PCP effector proteins (Inturned, Fuzzy, RhoA). Mutations of *Drosophila* Fat are associated with reversed dorsal–ventral polarity, abnormal distal-to-proximal wing development, and hyperplastic overgrowth of all larval imaginal discs [reviewed in (7)]. These phenotypes define 2 distinct branches of *Drosophila* Fat signaling: the Fat polarity pathway and the Fat tumor suppressor/Hippo pathway. In *Drosophila* Hippo signaling

[reviewed in (8)], Fat is required to recruit Expanded to the plasma membrane, facilitating the assembly of a protein complex that contains the scaffold protein Salvador and the Ste20 family kinase Hippo. Hippo phosphorylates and activates the Dbf2-related kinase Warts; Warts in turn, complexed with Mob1-related protein Mats, phosphorylates Yorkie. Phosphorylation of Yorkie, a transcriptional co-activator, prevents its nuclear translocation and activation of Yorkie target genes such as *cyclin E* and *diap1* (9, 10). Upstream signals that activate Fat and the molecular links between Fat and the Expanded/Hippo/Warts cascade remain ill defined. Localization and stabilization of Expanded at apical cell junctions depends on Fat, because loss of Fat leads to reduced Expanded levels and mislocalization (9, 11). However, studies have failed to demonstrate a direct interaction between Expanded and Fat or between Expanded and other components of the Hippo pathway.

Based on sequence similarities and domain architecture, *Drosophila* Fat is related most closely to vertebrate *Fat4*. *Fat4* deficiency in mice causes developmental abnormalities of the inner ear, neural tube, and kidney (12). Disruption of oriented cell division and defective elongation of kidney tubules result in cystic kidneys, suggesting that *Fat4* controls PCP signaling during renal development. Because the zebrafish embryo is amenable to rapid genetic manipulation, we targeted zebrafish Fat1 (zFat1) and zebrafish Fat4 (zFat4) by antisense morpholino oligonucleotides (MO) to compare their roles during zebrafish pronephros development. Here we show that knockdown of zFat1, but not of zFat4, caused extensive pronephric cysts. Epistasis assays revealed a strong genetic interaction between Fat1 and Scribble. Scribble recognized the PDZ-binding site that decorates vertebrate Fat1 but is absent in Fat4. Surprisingly, depletion of zebrafish Yes-associated protein 1 (zYAP1) ameliorated the changes caused by Fat1 and Scribble knockdown, indicating that a dysregulation of Hippo signaling contributes to the formation of pronephric cysts caused by the absence of Fat1 and Scribble.

Results

Knockdown of Zebrafish Fat1, but Not of Zebrafish Fat4, Promotes Profound Cystogenesis. Because knockout of *Fat4* in mice results in cystic kidney disease (12), we targeted zFat4 with MO. Substantial (> 10%) cyst formation was detectable only at 3.75 pmol zFat4 MO (Fig. S1). At comparable efficacy (Fig. S2),

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Fig. 1. Zebrafish Fat1 genetically interacts with zebrafish Scribble. (A) MO-induced knockdown of zFat1 was compared with the knockdown of zebrafish Prickle 2 (zPk2), zebrafish Daam1 (zDaam1), zebrafish Protocadherin 8 (zPcdh8), zebrafish Fuzzy (zfuzzy), and zScrib. White arrowheads indicate examples of cyst formation. (B) Pronephric cyst formation, caused by the depletion of these molecules, was scored at 55 h post fertilization (h.p.f.), using the transgenic zebrafish line Wt1b:gfp. A reproducible degree of cyst formation (30%–40%) was noted in zFat1-depleted embryos without significant reduction in larval survival. (C) Epistasis assays between components of the PCP pathway revealed a strong synergism between zFat1 MO (0.125 pmol) and zScrib MO (0.125 pmol) injections. Transverse sections at the level of glomerulus and proximal tubules revealed bilateral pronephric cyst formation adjacent to the glomerulus in combined knockdown of Fat1 and Scribble in zebrafish embryos. (D) The presence of pronephric cysts was scored at 50–55 h.p.f. in zebrafish embryos injected with low MO concentrations. Note that pronephric cysts are hardly detectable after single injections of either 0.125 pmol zFat1 MO or 0.125 pmol zScrib MO, whereas the combination causes pronephric cysts in > 10% of microinjected embryos. (*, $P < 0.05$; **, $P < 0.0001$).

knockdown of zFat1 was strongly cystogenic: more than 50% of the microinjected zebrafish embryos developed pronephric cysts (Fig. S1). Co-injection of 0.5 pmol zFat4 MO slightly augmented zFat1 MO-induced cyst formation (Fig. S1), indicating that the 2 molecules, both expressed in the zebrafish pronephros (Fig. S2), may act in different signaling cascades. To identify downstream components of the zFat1, we performed epistasis assays with several candidate proteins implicated in vertebrate PCP signaling. Knockdown of zebrafish Prickle 2, Protocadherin 8, Daam1, Scribble (zScrib), and Dapper 2 resulted in pronephric cysts (Fig. 1A and B). Combined knockdown of zFat1 and different PCP proteins defined several molecules that intensified the pronephric cyst formation induced by zFat1 depletion (Fig. 1C and D). Marked synergy occurred between zFat1 and zScrib. Although minute amounts of zFat1 MO and zScrib MO (13) were not cystogenic when injected singly (0.125 pmol), together they increased the rate of pronephric cyst formation to $\approx 10\%$. This observation suggests that zFat1 and zScrib participate in overlapping signaling pathways to maintain tubular geometry.

Physical Interaction Between Zebrafish Fat1 and Scribble. Scribble is a cytoplasmic protein that contains 4 PDZ domains. The 4

C-terminal amino acids of both zFat1 and mouse Fat1 (mFat1) constitute a class I PDZ-binding site (HTEV). Accordingly, we tested whether the cytoplasmic tail of Fat1 interacts with Scribble by co-expressing full-length Scribble with the C-terminal cytoplasmic (cyt) domain of mFat1 fused to a secreted Ig truncation and the transmembrane domain of CD7 (slg.7). Scribble was immunoprecipitated by the slg7.mFat1-cyt construct (Fig. 2A). Deletional analysis further delineated the PDZ domains of Scribble and the PDZ-binding site of mFat1 as essential interaction domains (Fig. 2B and C). To provide further evidence of a direct interaction between Fat and Scribble, we generated recombinant GST-fusion proteins of the Scribble PDZ domains and precipitated slg7.mFat1 from lysates of transiently transfected HEK 293T cells. slg7.mFat1-cyt was precipitated by the PDZ2–4 and PDZ3–4 of Scribble but not by the PDZ domains of Par-3 or GST alone (Fig. 2D). These results demonstrate that mFat1 interacts with the PDZ domains of Scribble, presumably PDZ domains 3 and/or 4. Unlike *Drosophila* Fat-like protein, *Drosophila* Fat also contains a C-terminal PDZ-binding site (class III; EEYV). To assess whether the PDZ-binding site of *Drosophila* Fat interacts with Scribble, we replaced the cytoplasmic and transmembrane domain of slg7.mFat1-cyt with the corresponding *Drosophila* sequences (slg.TM.dFat). This construct, which partially rescued the cystic phenotype caused by the knockdown of zFat1 (Fig. S3), immunoprecipitated co-transfected human Scribble, confirming that both *Drosophila* Fat and mammalian Fat1 interact with Scribble (Fig. 2E). However, the interaction between Scribble and *Drosophila* Fat was not contingent on the C-terminal PDZ-binding site (Fig. S4), suggesting that Scribble recognizes internal ligands of *Drosophila* Fat instead of or in addition to the C-terminal PDZ-binding site. A similar binding mode has been described recently for the interaction between the PDZ protein Par-6 and the integral membrane protein Pals1 (14). In contrast to Fat1, Fat4 did not interact with Scribble (Fig. 3F). Fat1 co-localized with Scribble in NRK-52E cells (Fig. 2G and H).

Scribble Links Fat1 to the Hippo Pathway. The up-regulation of the YAP1 target gene *Survivin* by depletion of either Fat1 or Scribble (Fig. S5) prompted us to test the hypothesis that cyst formation in zFat1-deficient zebrafish is caused by defective Hippo signaling. MO-mediated depletion of zYAP1 (Fig. S6), the zebrafish homologue of *Drosophila* Yorkie, promoted pronephric cyst formation; remarkably, similar effects were observed with modest overexpression of YAP1 (Fig. 3A and C). This finding suggests that abnormalities of the Hippo pathway disrupt the development of the zebrafish pronephros. If cyst formation consequent to zFat1 deficiency results from an overactive Hippo pathway, then depletion of zYAP1 might oppose cystogenesis. As shown in Fig. 3B, cyst formation caused by knockdown of zFat1 was partially rescued by knockdown of zYAP1, suggesting that defective Hippo signaling contributes to the pronephric cyst formation in zebrafish embryos. Conversely, co-expression of 0.025 and 0.1 μg *Scribble* mRNA partially rescued the cystic phenotype caused by overexpression of YAP1; 0.2 μg *Scribble* mRNA was less effective, indicating that a precise balance between YAP1 and Hippo signaling is required to prevent cyst formation (Fig. 3D). We examined the functional effect of Scribble on Hippo pathway in HEK 293T cells by using the YAP1-dependent Gal4-TEAD4/5xUAS luciferase reporter (15). Scribble inhibited YAP1-dependent luciferase expression as effectively as Lats2 (Fig. 4A). Co-expression of the YAP1^{S127A} mutant, which lacks the Lats2-dependent 14–3-3 binding site, restored the Gal4-TEAD4-mediated luciferase expression, supporting our hypothesis that Scribble participates in canonical Hippo signaling (Fig. 4A). To evaluate further the function of Scribble in Hippo signaling, we compared the effect of zFat1 and zScrib on the YAP1 target gene *Survivin* in zebrafish embryos (10). Control, zScrib, or zFat1 MO was co-injected with a luciferase construct containing the

Fig. 2. Molecular interaction between Fat1 and Scribble. (A) Human full-length Scribble was co-expressed with the cytoplasmic tail of mFat1 fused to the slg7 tag (slg7.mFat1-cyt) or with control proteins (slg7.TRPC4 C-terminal and slg7.nephrin-cyt) in HEK 293T cells. After immunoprecipitation with protein A-Sepharose beads, eGFP-tagged human Scribble was present in immunoprecipitates immobilized by slg7.mFat1-cyt but not by slg7.TRPC4 C-terminal or slg7.nephrin-cyt. Equal expression of Scribble in cellular lysates was confirmed by antibody against GFP. (B) Scribble interacts with the C-terminal PDZ-binding site of Fat1. Wild-type Fat1, but not a truncated version lacking the last 3 amino acids (amino acids 4596–4598), interacted with Scribble. Expression levels of Scribble and the slg7-tagged proteins are shown in the lower panels. (C) Fat1 interacts with the PDZ domain containing the C-terminal part of Scribble. HEK293T cells were transfected with slg7.Fat1 and a Flag-tagged Scribble truncation containing either the amino-terminal half (hScrib N), the C-terminal half (hScrib C), or the 4 PDZ domains of Scribble (hScrib PDZ). Fat1 immobilized C-terminal half and the PDZ domain-containing part but not the amino-terminal half of Scribble. The middle panel shows the expression of the Scribble truncations. The bottom panel shows the precipitated slg7.mFat1-cyt. (D) The C-terminal domain of Fat1 interacted with a recombinant GST fusion protein containing the third and fourth or the second, third, and fourth PDZ domain of Scribble. Fat1 (slg7.mFat1-cyt) expressed in HEK 293T cells was incubated with GST or GST fusion proteins as indicated. The Par3 PDZ domains 1 and 2, used as a control, did not bind to Fat1. (E) *Drosophila* Fat interacts with Scribble. An mFat1 fusion protein containing the slg tag, a short part of extracellular domain, and the transmembrane and C-terminal domain of Fat1 and an identical *Drosophila* Fat fusion protein precipitated Scribble but not the control protein slgTM.nephrin. (F) Fat1, but not Fat4, interacts with Scribble. mFat4 cytoplasmic tail (mFat4-cyt) was fused to the slg7 tag. Neither slg7.Fat4-cyt nor the control protein slg7.nephrin-cyt interacted with Scribble. (G) Endogenous Fat and Scribble co-localize in NRK-52E cells. Confluent NRK-52E cells were labeled with rabbit anti-Fat1 antiserum and goat anti-Scribble antiserum. Both Fat1 and Scribble co-localized at the plasma membrane of NRK-52E cells. (H) Co-localization of endogenous Fat1 and Scribble was confirmed by confocal microscopy. Both Fat1 and Scribble co-localized at cell–cell contacts of NRK-52E cells. The red line depicts the site of z-section (Top Row). Z-reconstruction (x-z direction) of a z-stack (15 planes, z-distance 0.2 μ m), showing co-localization of Fat1 and Scribble (Bottom row). (Scale bars: G, 20 μ m; H, 5 μ m.)

Fig. 3. Control of YAP1 expression is essential for normal zebrafish pronephros development. (A) Knockdown of zYAP1 by MO (zYAP1 MO) results in pronephric cysts. Lateral view of embryos injected with zYAP1 MO (0.5 pmol, 1.25 pmol, and 2.5 pmol) at 55 h.p.f. A curled tail and shortening of the body (arrows) are observed in the zYAP1 morphants (Upper). At higher concentration, zYAP1 MO injections led to cyst formation (Lower). (B) Cyst formation caused by zFat1 MO was partially rescued by the simultaneous knockdown of zYAP1. (C) Overexpression of human YAP1 (hYAP1) recapitulated the loss-of-function phenotypes (cyst formation) caused by zFat1 knockdown (zFat1 MO). (D) Over-expression of human YAP1 resulted in pronephric cysts in zebrafish embryos. This result was reversed by co-injection of human Scribble RNA (hScrib). (Numbers in parentheses indicate the total number of embryos used in each experiment.)

Survivin promoter (1430 bp) (16). Although the depletion of zScrib and zFat1 alone had only a modest effect, the combined knockdown of zScrib and zFat1 activated the *Survivin* reporter (Fig. 4B). To support further the role of Scribble in canonical Hippo signaling, we determined the nuclear fraction of YAP1 in the presence of Scribble and compared the effect with that of Lats2. Scribble reduced the nuclear fraction of YAP1 as efficiently as Lats2 (Fig. 4C and D). These results were confirmed, demonstrating the nuclear exclusion of YAP1 in the presence of Scribble or Lats2 by immunofluorescence (Fig. 4F). Warts phosphorylates Yorkie to generate a 14–3–3 binding site. In *Drosophila*, a C-terminal Fat truncation activates Hippo signaling even though most of the extracellular domain is lacking. We found that a similar mFat1 truncation (slgTM.mFat1) could mediate 14–3–3 binding to YAP1. Furthermore, YAP1 immobilized 14–3–3 in the presence of slgTM.mFat1 or Scribble but not in the absence of these proteins (Fig. 4E), supporting our hypothesis that Scribble can participate in Hippo signaling.

Discussion

Distinct signaling cascades control organ growth, shape, and size. In *Drosophila*, Fat and Fat-Like molecules function in an

Fig. 4. Scribble inhibits YAP1 activation. (A) Wild-type YAP1 activity (Black Bars) is repressed by Scribble (Scrib), whereas the YAP1^{S127A} phosphorylation mutant (Gray Bars) reverses the Scribble-mediated inhibition. The 5xUAS-luciferase reporter, GAL4-TEAD4, β -galactosidase, and V5.YAP1 were simultaneously transfected into HEK 293T cells with plasmids as indicated. Luciferase activity was measured and normalized to β -galactosidase activity. Lats2 was used as a positive control for active Hippo signaling. (B) The combined knockdown of zScrib and zFat1 activated the YAP1-dependent *Survivin* reporter construct (pLuc-Survivin 1430) in zebrafish embryos when compared with single MO injections combined with a control MO. (C) HEK 293T cells expressing V5-YAP1 (Left), V5-YAP1 plus Scribble (Middle), or Lats2 (Right) were analyzed by subcellular fractionation. Nuclear YAP1 decreased in response to active Hippo signaling. Nuclear Lamin and cytosolic tubulin were used to control for the quality of the fractionation. C, cytoplasmic; N, nuclear. (D) Scribble and Lats2 are present in the cytosolic fraction. (E) HEK 293T cells were transfected with GFP-YAP1, HA-14-3-3 β , Fat1, or Scribble, as indicated. GFP-YAP1 was immunoprecipitated with anti-GFP, and immobilized 14-3-3 β was stained with anti-HA antibodies. Expression of the 2 proteins mFat1 and Scribble led to the interaction of YAP1 with 14-3-3 β . (F) HeLa cells were transfected with GFP-YAP1 or GFP-YAP1 plus V5-Scribble or Flag-Lats2 and were stained with anti-V5, anti-Scribble and anti-Flag antibodies. Nuclear YAP1 signal is detectable in cells expressing GFP-YAP1 alone, whereas cells expressing Scribble or Lats2 show a predominant cytoplasmic staining of YAP1.

interconnected signaling network that regulates spatial orientation of cells and their appendages, as well as organ growth and size (8). We found that *Drosophila* Fat and mFat1 interact with Scribble, but Fat4 does not, suggesting that *Drosophila* Fat signaling resembles a mix of Fat1 and Fat4 functions. Scribble is a cytoplasmic protein that contains 4 PDZ domains and several amino-terminal leucine-rich repeats. Like Fat, *Drosophila* Scribble regulates the growth of imaginal disc epithelia, but only Scribble mutations cause neoplastic overgrowth that disrupts overall tissue organization [reviewed in (7)], probably involving additional signaling pathways. Scribble forms a polarity complex with Discs Large and Large giant larvae. This complex is enriched at the basolateral septate junction adjacent to the adherens junction and controls the specification of the basolateral plasma membrane by antagonizing the Par polarity complex [reviewed in (17)]. Scribble function markedly differs among organisms. In *Drosophila*, the Scribble complex is required for asymmetric division of neuroblasts; loss-of-function mutations lead to disrupted apical-basal polarity, impaired cell cycle exit, and tissue overgrowth. Scribble is required for convergent extension movements in zebrafish (13) and causes severe neural tube defects when deleted in

mice (18, 19); both findings support a role of Scribble in PCP signaling. In addition, Scribble regulates cell protrusions and actin organization through regulation of Rac activity and localization, exerting essential functions in cell migration (20–22). These divergent phenotypes indicate that vertebrate Fat and Scribble modified their functions during evolution. We postulate that subtle changes in PDZ-binding affinities and/or mutation of PDZ-binding sites allowed Scribble to gain access to alternative signaling pathways. The *Drosophila* Fat PDZ-binding site differs from the vertebrate Fat1 PDZ-binding site and is not required for the interaction with Scribble, providing further evidence for a diversification of Fat functions during evolution. *Drosophila* Fat is a well-characterized cell surface receptor that regulates tissue and organ growth, but it remains unclear how Fat controls Hippo signaling. Fat promotes the recruitment of *Expanded* to the plasma membrane to activate Hippo. However, the concept of a simple linear pathway is challenged by genetic evidence that Fat acts parallel to *Expanded* (23) and by the finding that Dachs, an unconventional myosin, seems to control the abundance of Warts downstream of Fat (24). Our findings support the involvement of additional components in Fat-induced signaling. Although *Drosophila*

Fat couples to both PCP and Hippo signaling, in vertebrates 4 Fat molecules seem to partition off different signaling pathways. Interestingly, vertebrate Scribble seems to promote both PCP and Hippo signaling pathways, functioning as a molecular coordinator that controls spatial orientation of cells through PCP signaling while inhibiting their growth through activation of the Hippo complex. Directed cell migration, proliferation, and polarity must be synchronized precisely to achieve tissues and organs of defined size and structure. The Fat/Scribble complex may be one of the organizers that facilitate the necessary cross-talk between different signaling cascades. Significantly, cyst formation in zebrafish results from either YAP1 knockdown or overexpression, suggesting that Hippo signaling is essential for normal zebrafish pronephros development. The role of Hippo signaling in renal development also is supported by the observation that the targeted deletion of TAZ/Wwtr1 in mice is associated with cystic kidney disease (25, 26). The transcriptional co-activator TAZ shows homology and shares the domain architecture with YAP1 (27) and was recently identified as a target of Hippo signaling (28). Continuous proliferation with the inability to differentiate terminally is one of the hallmarks of the neoplastic overgrowth caused by Scribble mutations in *Drosophila* (7). Curiously, less-than-terminal differentiation of tubular epithelial cells in combination with their persistent proliferation has been postulated to cause polycystic kidney disease for many years (29). Further studies will need to determine whether components of the Fat/Hippo signaling pathways play a role in this common hereditary disorder.

Materials and Methods

Plasmids and Reagents. The intracellular domains of mFat1 and mFat4 were isolated by RT-PCR from mouse kidney RNA. Membrane-bound fusion proteins of the C-terminal cyt domain of mFat1 and mFat4 were generated using a pCDM8 cassette that contained the leader sequence of CD5 fused to the CH2 and CH3 domain of human IgG1 followed by the transmembrane region of CD7. A *Drosophila* Fat construct containing the membrane-proximal part of the extracellular domain, the transmembrane domain, and the cyt domain was synthesized by Geneart. A full-length cDNA clone of human Scribble was kindly provided by P.O. Humbert (Peter MacCallum Cancer Center, East Melbourne, Australia). Truncations of Fat1-cyt, Scribble, and full-length human YAP1 cDNA were generated by PCR and standard cloning procedures. The 3xFLAG-FRMD6 plasmid (BC020521) was provided by GeneCopoeia. The p-Luc 1430bp construct was obtained from S. Liu (Schering-Plough Research Institute, Kenilworth, NJ). The 5xUAS-luciferase reporter and the Gal4-TEAD4 were a gift from Kun-Liang Guan (University of California, La Jolla, CA).

Co-Immunoprecipitation. Co-immunoprecipitations were performed as described previously (4). Briefly, HEK 293T cells were transiently transfected by the calcium phosphate method. After incubation for 24 h, cells were washed and lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton-X 100, 25 mM NaF, 12.5 mM Na₄P₂O₇, 0.1 mM EDTA, 50 mM NaCl, 2 mM Na₃VO₄, and protease inhibitors. After centrifugation (15,000 × g, 15 min, 4 °C) and ultracentrifugation (100,000 × g, 30 min, 4 °C), cell lysates containing equal amounts of total protein were incubated for 1 h at 4 °C with the appropriate antibody followed by incubation with 50 μL of protein G or A-Sepharose beads for ≈3 h. The beads were washed extensively with lysis buffer, and bound proteins were resolved by SDS/PAGE. Antibodies anti-HA (1:1000) (Covance, Sigma), anti-M2 (1:3000) (Sigma), mouse monoclonal anti-GFP 1:1000 (Santa

Cruz), mouse monoclonal anti-V5 (1:5000) (Serotec), rabbit polyclonal, goat polyclonal anti-Scribble antiserum (1:500) (Santa Cruz K-21), and HRP anti-human IgG (1:3000) (Sigma) were used for Western blot analysis. Cell fractionation was performed in subconfluent cells grown on 10-cm plates for 24 h. Cells were mechanically disrupted in hypotonic buffer (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and protease inhibitors). The cytoplasmic fraction represented the supernatant after centrifugation at 3000 × g for 10 min. The pellet, washed in hypotonic buffer, centrifuged at 15,000 × g for 20 min, and extracted with hypertonic buffer (20 mM Hepes, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, and protease inhibitors), yielded the nuclear fraction. Both cytoplasmic and nuclear fractions were analyzed by Western blotting.

Immunofluorescence. NRK-52E cells were fixed in a 50% solution of methanol-acetone for 10 min, permeabilized with 0.2% Triton X-100, and blocked in PBS containing 0.2% horse serum. Immunostainings for Fat1 and Scribble were performed with a rabbit polyclonal anti-Fat1 antiserum (1:300) (30) and a goat polyclonal anti-Scribble antiserum (1:150) (Santa Cruz), in combination with fluorescently labeled secondary antibodies. Images were obtained with an LSM 510 confocal microscope (Zeiss). Image analysis and 3D reconstruction were performed using Imaris Software (Bitplane). Z-stacks were performed with the pinhole set to a 1.0-μm optical slice thickness and a z-distance of 0.2 μm.

Pull-Down Assay. HEK 293T cells were transiently transfected with plasmid DNA as indicated. Cells were lysed in 1% Triton X-100, 20 mM Tris-HCl (pH 7.5), 50 mM NaF, 15 mM Na₄P₂O₇, 1 mM EDTA, 50 mM NaCl, 2 mM Na₃VO₄, and protease inhibitors for 15 min on ice. Following centrifugation the supernatant was incubated for 1 h at 4 °C with 4–8 μg recombinant purified GST or GST-PDZ domain fusion protein prebound to glutathione-Sepharose beads (Amersham Biosciences). Bound proteins were separated by 10% SDS/PAGE, and precipitated proteins were visualized with anti-FLAG antibody (Sigma). Equal loading of recombinant proteins was confirmed by Coomassie blue staining of the gels.

Luciferase Assay. HEK 293T cells seeded in 12-well plates were transiently transfected with a luciferase reporter construct, a β-galactosidase expression vector (provided by C. Cepko, Harvard Medical School, Boston, Massachusetts), and vectors directing the expression of proteins as indicated. The total amount of DNA was 1 μg per well. Cells were serum-starved for 12 h, collected in cold PBS, and lysed in 100 μL of reporter lysis buffer (Applied Biosystems) for 10–15 min at 25 °C, followed by centrifugation at 15,000 × g for 5 min to remove insoluble material. Luciferase activity was determined using a commercial assay system (Applied Biosystems) and was normalized to β-galactosidase activity to correct for transfection efficiency.

Zebrafish Embryo Manipulation. The transgenic zebrafish line Wt1b::GFP was described recently (31). Antisense MO were designed by Gene Tools to target either the translation start or an exon-splice donor site causing splicing defects of the mRNA. The sequences are available in the SI text. All MOs were diluted in 200 mM KCl, 10 mM Hepes, and 0.1% phenol red (Sigma). The injection amounts varied between 0.125 and 3.75 pmol (as indicated); the injection volume was 4.6 nL/embryo. At the single-cell stage, embryos were DNA injected with 500 pg pLuc-Survivin 1430. Rescue experiments were performed by co-injecting 780 pg of in vitro-transcribed *Drosophila* Fat1 mRNA with zFat1 MO or by co-injecting 100 ng of human Scribble mRNA with 200 ng of human YAP1 mRNA. The single mRNA concentrations used for rescue experiments were determined in dose-response curves to minimize effects on cyst formation.

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1. Fliegau M, Benzing T, Omran H (2007) When cilia go bad: Cilia defects and ciliopathies. *Nat Rev Mol Cell Biol* 8:880–893.
2. Eggenchwil JT, Anderson KV (2007) Cilia and developmental signaling. *Annu Rev Cell Dev Biol* 23:345–373.
3. Benzing T, Simons M, Walz G (2007) Wnt signaling in polycystic kidney disease. *J Am Soc Nephrol* 18:1389–1398.
4. Simons M, et al. (2005) Inversin, the gene product mutated in nephronophthisis type II, functions as a molecular switch between Wnt signaling pathways. *Nat Genet* 37:537–543.
5. Fischer E, et al. (2006) Defective planar cell polarity in polycystic kidney disease. *Nat Genet* 38:21–23.
6. Klein TJ, Mlodzik M (2005) Planar cell polarization: An emerging model points in the right direction. *Annu Rev Cell Dev Biol* 21:155–176.
7. Hariharan IK, Bilder D (2006) Regulation of imaginal disc growth by tumor-suppressor genes in *Drosophila*. *Annual Review of Genetics* 40:335–361.
8. Reddy BV, Irvine KD (2008) The Fat and Warts signaling pathways: New insights into their regulation, mechanism and conservation. *Development (Cambridge, UK)* 135:2827–2838.
9. Willecke M, et al. (2006) The fat cadherin acts through the hippo tumor-suppressor pathway to regulate tissue size. *Curr Biol* 16:2090–2100.
10. Dong J, et al. (2007) Elucidation of a universal size-control mechanism in *Drosophila* and mammals. *Cell* 130:1120–1133.

11. Silva E, Tsatskis Y, Gardano L, Tapon N, McNeill H (2006) The tumor-suppressor gene *Fat* controls tissue growth upstream of expanded in the Hippo signaling pathway. *Curr Biol* 16:2081–2089.
12. Saburi S, et al. (2008) Loss of *Fat4* disrupts PCP signaling and oriented cell division and leads to cystic kidney disease. *Nat Genet* 40:1010–1015.
13. Wada H, et al. (2005) Dual roles of zygotic and maternal *Scribble1* in neural migration and convergent extension movements in zebrafish embryos. *Development (Cambridge, UK)* 132:2273–2285.
14. Penkert RR, DiVittorio HM, Prehoda KE (2004) Internal recognition through PDZ domain plasticity in the Par-6-Pals1 complex. *Natural Structure & Molecular Biology* 11:1122–1127.
15. Zhao B, et al. (2008) TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev* 22:1962–1971.
16. Li F, Altieri DC (1999) Transcriptional analysis of human survivin gene expression. *Biochem J* 344 Pt 2:305–311.
17. Humbert PO, Dow LE, Russell SM (2006) The *Scribble* and *Par* complexes in polarity and migration: Friends or foes? *Trends Cell Biol* 16:622–630.
18. Murdoch JN, et al. (2003) Disruption of *scribble* (*Scrb1*) causes severe neural tube defects in the circletail mouse. *Hum Mol Genet* 12:87–98.
19. Zarbalis K, et al. (2004) A focused and efficient genetic screening strategy in the mouse: Identification of mutations that disrupt cortical development. *PLoS Biol* 2:E219.
20. Dow LE, et al. (2007) The tumour-suppressor *Scribble* dictates cell polarity during directed epithelial migration: Regulation of Rho GTPase recruitment to the leading edge. *Oncogene* 26:2272–2282.
21. Qin Y, Capaldo C, Gumbiner BM, Macara IG (2005) The mammalian *Scribble* polarity protein regulates epithelial cell adhesion and migration through E-cadherin. *J Cell Biol* 171:1061–1071.
22. Osmani N, Vitale N, Borg JP, Etienne-Manneville S (2006) *Scrib* controls *Cdc42* localization and activity to promote cell polarization during astrocyte migration. *Curr Biol* 16:2395–2405.
23. Feng Y, Irvine KD (2007) *Fat* and *expanded* act in parallel to regulate growth through warts. *Proc Natl Acad Sci USA* 104:20362–20367.
24. Cho E, et al. (2006) Delineation of a *Fat* tumor suppressor pathway. *Nat Genet* 38:1142–1150.
25. Hossain Z, et al. (2007) Glomerulocystic kidney disease in mice with a targeted inactivation of *Wwtr1*. *Proc Natl Acad Sci USA* 104:1631–1636.
26. Makita R, et al. (2008) Multiple renal cysts, urinary concentration defects, and pulmonary emphysematous changes in mice lacking *TAZ*. *American Journal of Physiology. Renal Physiology* 294:F542–553.
27. Kanai F, et al. (2000) *TAZ*: A novel transcriptional co-activator regulated by interactions with 14-3-3 and PDZ domain proteins. *EMBO J* 19:6778–6791.
28. Lei QY, et al. (2008) *TAZ* promotes cell proliferation and epithelial-mesenchymal transition and is inhibited by the hippo pathway. *Mol Cell Biol* 28:2426–2436.
29. Grantham JJ (1990) Polycystic kidney disease: Neoplasia in disguise. *Am J Kidney Dis* 15:110–116.
30. Moeller MJ, et al. (2004) Protocadherin *FAT1* binds *Ena/VASP* proteins and is necessary for actin dynamics and cell polarization. *EMBO J* 23:3769–3779.
31. Perner B, Englert C, Bollig F (2007) The Wilms tumor genes *wt1a* and *wt1b* control different steps during formation of the zebrafish pronephros. *Dev Biol* 309:87–96.