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Dual Origin of the Renal Tubules in *Drosophila*: Mesodermal Cells Integrate and Polarize to Establish Secretory Function

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Summary

Organs are made up of cells from separate origins, whose development and differentiation must be integrated to produce a physiologically coherent structure. For example, during the development of the kidney, a series of interactions between the epithelial mesonephric duct and the surrounding metanephric mesenchyme leads to the formation of renal tubules [1]. Cells of the metanephric mesenchyme first induce branching of the mesonephric duct to form the ureteric buds [2], and they then respond to signals derived from them. As a result, mesenchymal cells are recruited to the buds, where they undergo a mesenchymal-to-epithelial transition as they condense to form nephrons [3]. In contrast, the simple renal tubules of invertebrates, such as insect Malpighian tubules (MpTs), have always been thought to arise from single tissue primordia, epithelial buds that grow by cell division and enlargement and from which a range of specialized subtypes differentiate [4–6]. Here, we reveal unexpected parallels between the development of *Drosophila* MpTs and vertebrate nephrogenesis by showing that the MpTs also derive from two cell populations: ectodermal epithelial buds and the surrounding mesenchymal mesoderm. The mesenchymal cells are recruited to the growing tubules, where they undergo a mesenchymal-to-epithelial transition as they integrate and subsequently differentiate as a physiologically distinctive subset of tubule cells, the stellate cells. Strikingly, the normal incorporation of stellate cells and the later physiological activity of the mature tubules depend on the activity of *hibris*, an ortholog of mammalian NEPHRIN.

Results and Discussion

The four Malpighian tubules (MpTs) in *Drosophila* are derived from an ectodermal primordium at the junction between the hindgut and the midgut [6, 7]. Tubule buds evert from the gut and increase in size first by cell division and later by cell rearrangement and growth. The mature tubules are made up of 2 different epithelial cell types, an average of 484 principal MpT cells (PCs) and 110 $\pm$ 1 stellate cells (SCs), divided between the 4 tubules [8, 9] (Figures 1A and 1B). By midembryogenesis (stage 13), the PCs have been generated by division of the MpT primordial cells [8, 10], whereas SCs, marked out by *teashirt* (*tsh*) expression (Figure 1C), appear in the tubules later (stage 15), toward the end of embryogenesis. Since the PCs stop mitosis and start endoreplication during stage 13/14 [10], it is unlikely that the SCs are generated by further MpT cell divisions. Indeed, staining for DNA reveals that the SCs have smaller nuclei than PCs (Figure 1D), indicating that they undergo fewer rounds of endoreplication. Thus, PCs and SCs appear to arise from separate cell populations, and SCs are then integrated during tubule morphogenesis.

To test this, we screened adult flies derived from embryos into which a small number of genetically labeled nuclei had been injected at the early cleavage stage [11]. Cells derived from these nuclei were scored in adult flies; out of 128 tubules examined, 75 contained marked cells. Of these, 91% had either marked PCs (55/75; Figure 1E) or SCs (13/75; Figure 1F), and only 9% contained both marked PCs and SCs (7/75; Figure 1G). As an internal control, we analyzed marked PCs and hindgut cells, which derive from a single embryonic primordium [8]. Of 102 animals analyzed, 53 had marked cells in these tissues; 30% of these animals had marked cells in either PCs or hindgut, and 70% contained marked cells in both tissues. Comparison of these data argues that PCs and SCs derive from different populations of primordial cells and that SCs are recruited from outside the MpT primordium.

Because the MpT primordia arise as buds of the embryonic hindgut and maintain their epithelial organization throughout development, cells recruited from an external source must intercalate into the tubule epithelium and should at some time be found on the tubule surface. Sections of early stage-13 embryos do indeed reveal cells, stained for an early SC marker (see below), adhering to the growing MpT buds (Figure 2A). Sections of later embryos show cells initiating the expression of *tsh* and becoming integrated into the epithelium (Figures 2B and 2C). To assess whether SCs develop apicobasal polarity, we examined the distribution of the apical membrane markers Stranded-at-Second (SAS) and Crumbs

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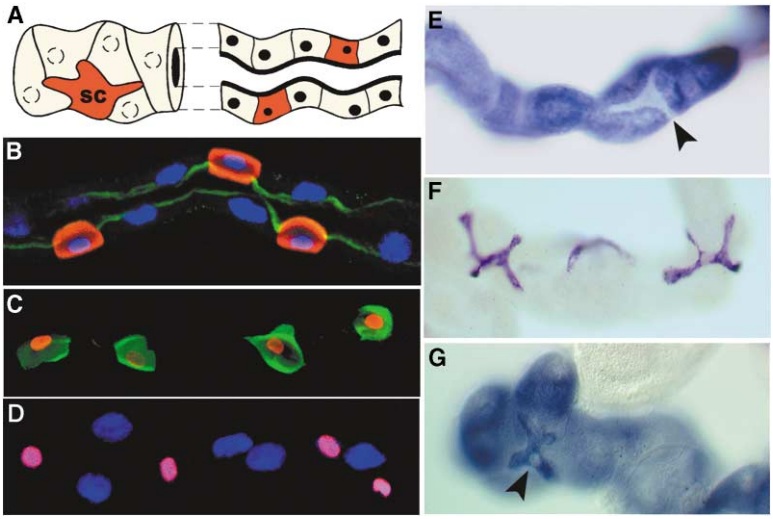

Figure 1. MpTs Contain Distinctive Cell Types from Different Origins

(A) A diagram of *Drosophila* MpT showing principal cells (yellow) and stellate cells (red) surrounding the lumen.

(B–D) Third instar MpTs stained to reveal SCs by C724(*tsh*)-GAL4 driving UAS-*lac*Z. (B) β-gal is shown in red, F-actin for lumenal actin is shown in green, and DNA is shown in blue. *tsh*GAL4-driven expression marks out regularly spaced SCs in the tubule epithelium. (C) β-gal is shown in green; anti-Tsh is shown in red. Tsh is expressed in a pattern identical to C724-driven expression of *lac*Z (B). (D) SCs have smaller nuclei than the PCs. Tsh is shown in red, DNA is shown in blue, and the overlap is shown in pink.

(E–G) Cells derived from injected, genetically marked nuclei (blue) are most commonly restricted to (E) PCs or (F) SCs. (G) MpTs with marked cells of both types are rare. The arrowheads indicate SCs.

(Crb) [12] as well as the organization of the actin cytoskeleton in SCs. As in PCs, SAS is restricted to the apical membrane of embryonic SCs (Figure 2D), and actin is concentrated in the lumenal brush border of both cell types (Figure 2E). These observations show that SCs undergo a mesenchymal-to-epithelial transition, becoming fully polarized. However, we find that Crb, an apical protein expressed in ectodermally derived epithelia, is not expressed in SCs (Figures 2F and 2F′). This finding underlines their distinct origin. Crb is not expressed in the mesoderm and, after initial expression in the endoderm and neurogenic ectoderm, is switched off as the primordial cells of the midgut and nervous system lose their epithelial character [13].

We argue that, as the SCs first appear as a nonepithelial cell population and do not express the ectodermal marker Crb, they are likely to derive from the mesoderm, endoderm, or delaminating neural precursors. To determine the origin of the SCs, we used tissue-specific GAL4 drivers and assessed whether they labeled SCs. In addition, to overcome the transient expression of some GAL4 lines, we permanently marked GAL4-expressing cells by FLP-mediated expression of the marker, β-galactosidase. Neither *serpent* nor *hindsight*, two genes that are expressed in the endoderm [14, 15], nor *elav*, which is expressed in neural cells from stage 12 [16], mark out the SCs (Figure 3A and data not shown). These data suggest that SCs do not originate from these tissues. In contrast, GAL4 driven by the predominantly mesodermal gene *twist* (*twi*) [17] does mark out SCs (Figure 3B). This finding indicates that SCs originate from a *twi*-expressing population of cells. We therefore assayed GAL4 drivers that are expressed in a subset of the embryonic mesoderm in the posterior terminal region, which gives rise to the caudal visceral mesoderm (CVM) [18, 19], and found that the T box gene *brachyenteron* (*byn*) and G447.2GAL4 positively label SCs. These results indicate that SCs might arise from the CVM primordium (Figures 3C and 3D). The CVM overlies the tubule primordia as they evert from the hindgut. While the majority of these cells migrate forward (Figure 3E) to invest the midgut and form longitudinal visceral muscles (and therefore defined as the CVM [18]), we find that a subset of labeled cells remains associated with the tubule primordia and later becomes incorporated into the tubules (Figures 3F–3H). These data reveal that SCs and CVM share a common origin in the posterior mesoderm.

To confirm this close relationship between the CVM and SCs, we checked for the presence of SCs in mutants affecting the mesoderm. SCs are still present in the tubules of embryos mutant for *twi*, which is expressed in the CVM but is not required for its specification or early development [18]. SCs are also present in embryos mutant for *bagpipe* and *tinman* (cf. Figures 3I and 3J), which are characteristic of the visceral mesoderm but are not expressed in the CVM [18, 20]. In *byn* mutants, the CVM and, consequently, the midgut longitudinal muscles are lost [18]. Correspondingly, we find that SCs are either absent or dramatically reduced in number (16 ± 3, $n = 19$ cf. 110 ± 1 in wild-type [9]) (Figure 3K). Surprisingly, in *snail* (*sna*), as well as in *twi,sna* double mutants, in which the CVM is reported to be completely lost [18], SCs are still present, although their numbers are strongly reduced (*sna*: 33 ± 3, $n = 12$, Figure 3L; *twi,sna*: 37 ± 4, $n = 10$). We therefore conclude that SCs arise from the group of posterior mesodermal cells that also gives rise to the CVM and that some of these cells move into the embryo during gastrulation, even in the absence of Twi and Sna. This distinction between CVM and SCs is underlined by the absence of other CVM markers [18] from SCs (Crocodile and bHLH54F, data not shown).

A group of *Drosophila* immunoglobulin-like proteins acts in cell-cell recognition and attraction during embryonic myogenesis [21–24]. One member of this family, *hibris* (*hbs*), an ortholog of vertebrate NEPHRIN, is expressed in the MpTs [23]. We find that *hbs* is expressed in a subset of MpT cells (Figure 4A) and that these cells express Tsh (Figure 4B), confirming that *hbs* is expressed in SCs. In the tubules of flies mutant for *hbs*, the number of SCs is significantly reduced (for hbs^{361}/*Df(2R)14*: 68 ± 4, $n = 11$ cf. 110 ± 1 in wild-type [9]), while the number of PCs is unaltered. hbs^{361} is not amorphic for the MpT phenotype, suggesting that a complete loss

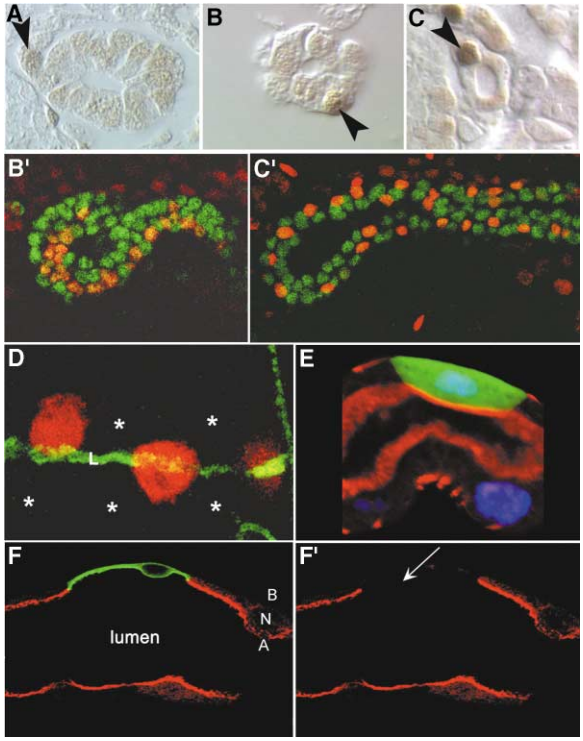

Figure 2. SCs Undergo a Mesenchymal-Epithelial Transition

(A–C) Wild-type embryonic tubules stained with antibodies for PCs (Cut; brown in [A]–[C]; green in [B′] and [C′]) and for SCs (either G447.2-driven β-gal [dark brown in (A)]) or Tsh (dark brown in [B] and [C]; red in [B′] and [C′]) to show (A) mesenchymal cells, not yet expressing Tsh, on the surface of a stage-12 MpT; (B and B′) Tsh-expressing SCs partially integrated into (B) stage-13 or (B′) stage-14 MpTs; and (C and C′) Tsh-expressing SCs now fully integrated into stage-15 elongated MpTs. The arrowheads indicate SCs.

(D) Embryonic MpTs stained for the apical membrane marker Stranded-at-Second (green); SCs around the narrow lumen (L) are labeled by *tsh*GAL4-driven *lac*Z (red). Asterisks mark the positions of PCs.

(E) The actin brush border is present in both cell types. Phalloidin-labeled actin is shown in red, SCs marked by β-gal are shown in green, and DNA is shown in blue.

(F and F′) The ectodermal apical membrane protein Crumbs is present only in PCs. SCs, indicated by the arrow in (F′) are marked by *tsh*GAL4-driven GFP (green). Crb is shown in red. B, basal side; A, apical side of epithelial cells; N, nucleus.

of Hbs might obliterate SCs from mutant MpTs. The strongest *hbs* allelic combinations are viable but die prematurely as adults with their MpTs laden with the excretory product, uric acid [23]. As uric acid is normally cleared from the tubule lumen by the secretion of urine [25], this phenotype could result from defects in the excretory function of the tubules. We therefore analyzed the physiological activity of the MpTs from *hibris* mutants.

Primary urine production in *Drosophila* depends on the combined activity of the PCs, which secrete K^+ into the lumen by a V^+-ATPase transporter [26], and the SCs, which express channels to permit the consequent flow of chloride ions and water [27]. Diuresis is regulated by a combination of pathways, one of which activates the PC V^+-ATPase transporter and can be triggered in vitro by cAMP [28], and a second, which activates SC chloride (and possibly water) transport and is triggered by leucokinin (LK-1) [27]. We tested the transport capacity of wild-type and *hbs* mutant MpTs taken from 1- to 3-day-old adults by first stimulating them with cAMP and then with LK-1. The results shown in Figure 4C indicate that, while the response to cAMP stimulation is not significantly altered in *hbs* mutants, the ability of their MpTs to meet the additional challenge imposed by LK-1 stimulation is markedly reduced. The residual responsiveness to LK-1 is likely to be due to the small number of SCs that are stably integrated into the mutant tubules. To investigate this possibility, we analyzed the relationship between SC number and physiological activity by assessing whether a tubule's response to LK-1 was significantly related to its complement of SCs. After physiological analysis, each tubule was fixed and stained for cell counting. A Y on X linear regression of the data was highly significant (Figure 4D; for analysis of variance, see the Experimental Procedures), revealing a close relation between the number of SCs in a tubule and its secretory capacity.

The recruitment of mesenchymal cells to epithelial tissues underpins the development of the vertebrate kidney, where the glomerulus and nephron derive from nephrogenic mesoderm that undergoes mesenchymal-epithelial transformation in response to signaling from the ureteric bud [1]. Failure in the process of recruitment leads to polycystic kidneys and, in the severest cases, to renal agenesis [29]. Here, we show, in a remarkable parallel, that the integration of mesenchymal cells with the developing epithelial tubule is also required for the physiological maturation of *Drosophila* excretory function. We demonstrate that SCs are marked out by the expression of the transcription factor Teashirt. Vertebrate orthologs of *tsh* have been identified in mouse [30], but it is not yet known whether these genes are expressed in the developing kidney. However, a mouse ortholog of a known target of *Drosophila tsh*, *dLarp*, is expressed at sites of epithelial/mesenchymal interaction, including the developing kidney [31].

We also show that the Nephrin-like immunoglobulin family member *hbs* is expressed in the SCs and is required for their proper acquisition by the MpT epithelium. Vertebrate NEPHRIN is expressed in the foot processes of glomerular podocytes, and patients carrying mutations in NEPHRIN (congenital nephrotic syndrome of the Finnish type-NPHS1) lack the slit diaphragm, exhibit "fused" podocyte foot processes, and suffer from massive proteinuria [32]. Consequently, it has been proposed that vertebrate NEPHRIN plays a structural role in the slit diaphragm [33]. Interestingly, we find that the point mutation in hbs^{361} results in the substitution of a highly conserved amino acid immediately adjacent to the defect in NPHS1 (Figure 4E). Using the system we describe, it will be possible, firstly, to establish whether the recruitment of SCs to *Drosophila* MpTs is truly analogous to the recruitment of nephrogenic mesenchyme in mechanistic terms and, secondly, to analyze the role of Hbs, and to identify its partners, in the integration of SCs into *Drosophila* MpTs.

Experimental Procedures

Immunostaining and Histochemistry

Immunostaining was performed by using standard techniques with antibodies against the following proteins: β-galactosidase

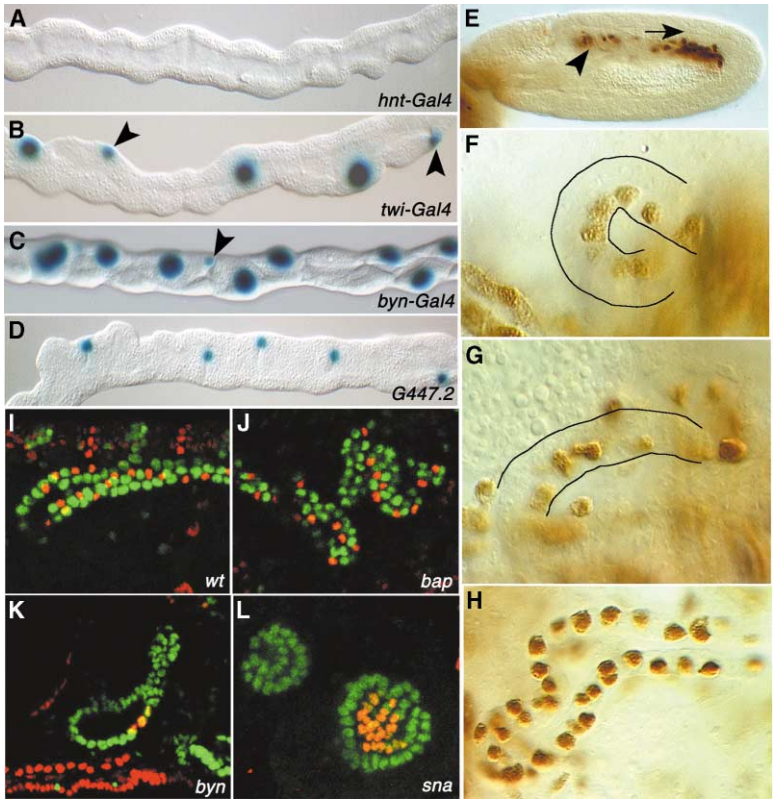

Figure 3. SCs Originate in a Subset of the Mesoderm

(A–D) Clones of cells expressing *lac*Z (blue) generated by tissue-specific GAL4 drivers. While (A) *hnt* generates no MpT clones, (B) *twi* and (C) *byn* generate clones in both PCs and SCs (PC clones arise through early hindgut expression of *twi* [12] and *byn* [14]). (D) The CVM GAL4 driver G447.2 [13] generates clones exclusively in SCs. The arrowheads indicate SCs. As a control, we used a PC GAL4 driver, which produced MpTs containing clones that were confined to PCs and did not overlap with independently labeled SCs (data not shown).

(E–H) Expression of G447.2GAL4 in cells of the caudal mesoderm overlying the embryonic hind- and posterior midgut. While the majority of these cells migrate forward (arrow) to invest the developing midgut, some remain associated with the MpT primordia (arrowhead in [E], stage 11) and later become incorporated into the MpT epithelium ([F] and [G], stage 13; [H], stage 16; dashed lines outline MpTs).

(I–L) Analysis of mutant embryos for the presence of SCs. MpT cells are labeled by Cut (green), and SCs are labeled by Tsh (red). In (J) *bap* mutant embryos, SCs are present in the MpTs, as in (I) wild-type. In (K) *byn* and (L) *sna* mutant MpTs, SCs are either absent or substantially reduced in number.

(1:10,000), Crb (1:10), Cut (1:200), Stranded-at-Second (1:500), and Teashirt (1:3000). F-actin was visualized by using Alexa488-conjugated phalloidin (Molecular probes), and nuclei were visualized by staining for DNA with TOTO3 (Molecular probes). For sectioning, embryos were embedded in Araldite after immunostaining and dehydration. Semithin sections were cut by using a Reichert Ultracut microtome and were mounted in Araldite for microscopy.

Lineage Analysis

The cell lineage of PCs and SCs was investigated by injecting a small number of *succinate dehydrogenase*$^{+}$ (*sdh*$^{+}$) nuclei into *sdh* temperature-sensitive (*sdh*^{8}) embryos at the early cleavage stage. Resulting adult flies were dissected, incubated at the restrictive temperature of 52°C in *Drosophila* Ringers solution for 15 min, and stained for succinate dehydrogenase for 2 days. The clones can be visualized because donor-derived *sdh*$^{+}$ cells stain blue, whereas the *sdh*^{8} host tissue remains unstained [11].

Clones expressing *lac*Z were induced by flipping out an interruption cassette from the line UAS-FLP;Act5C-FRT>S>FRT-*lac*Z. Flp-mediated recombination between the two FRT sites excises a transcriptional stop site and results in expression of *lac*Z under control of the Actin5C promoter. All descendants of the clone maintain *lac*Z expression. The following GAL4 lines were used to generate the clones: *hnt*-GAL4, *elav*-GAL4, *twi*-GAL4, *byn*-GAL4, *ctB*-GAL4, and GAL4$^{447.2}$. All crosses were maintained at 29°C. To reveal clones, third instar MpTs were dissected, fixed in 4% paraformaldehyde, and incubated in X-gal.

P Element Rescue

The position of the P{GAL4}C724 insert, which marks SCs [9], was determined by plasmid rescue by using standard procedures. A BLAST search of the *Drosophila* Genome Database with sequence flanking the P element revealed that it was inserted at position 309551 within contig AE003781, approximately 28 kb downstream of the *teashirt* (*tsh*) transcription unit (data not shown), in a region previously identified as containing *tsh* regulatory sequences [34, 35]. A P element insertion in the 5′ noncoding leader sequence of *tsh* (*tsh*^{04519}) was also shown to drive *lac*Z expression in the SCs (data not shown), and an antibody to Tsh revealed the protein in an identical MpT pattern to that driven by the P{GAL4}C724 insert (cf. Figures 1B and 1C). These findings indicate that the C724 expression pattern is driven by a *tsh* enhancer.

Fly Stocks

The following alleles were used in the mutant analysis: *bap*^{208}, *byn*^{5}, *twi*^{ID96}, *sna*^{IIG}, (*Df(2R) twi*^{10},*sna*^{16}), *Df(3R)e*^{D7} (which removes *bap* and *tin*), *Df(2R)14* (which removes *hbs*), *hbs*^{361}. The following *lac*Z enhancer trap lines were used: *hbs*$^{P36.1}$, *tsh*^{04319}, *croc-lac*Z.

Physiology

In vitro analysis of secretory rate was performed as previously described [27]. Tubules from adult flies were stimulated with cAMP and LK-1 (both from Sigma). To estimate SC number, tubules were stained with toluidine blue as previously described [10], and cells with small nuclei were counted in a blind test. Physiological data were subjected to a two-tailed heteroscedastic t test and were judged to be significantly different when a comparison between control and mutant gave $p < 0.05$. Y on X linear regression was carried out by using Systat 8.0 ($N = 39$, $F = 29.32$, d.f. $= 1.38$, $p < 0.000$).

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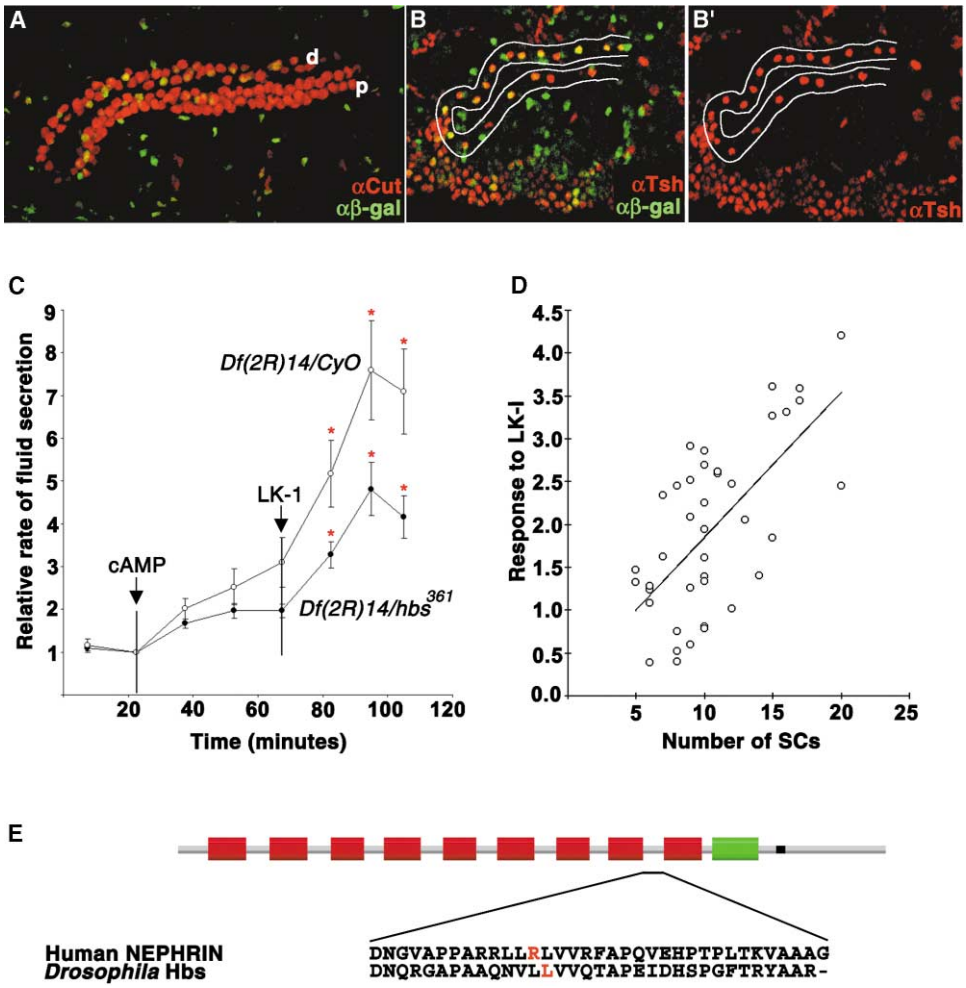

Figure 4. The *Drosophila* NEPHRIN Ortholog, *hibris*, Is Expressed in SCs and Is Required for Full MpT Physiological Activity

(A) *hibris* is expressed in the SCs of stage-15 embryonic tubules, as revealed by *hbs-lac*Z (β-gal, green; Cut, red; overlap, yellow; d, distal; p, proximal MpT).

(B and B′) MpTs stained for Tsh (red), which colocalizes with Hbs (green; the overlap is yellow in [B]).

(C and D) The physiological activity of the MpTs in the adults of *hbs* mutants is compromised. (C) A graph of the relative rates of fluid transport in mutant (*hbs*^{361}/*Df(2R)14*, n = 15) and control (*Df(2R)14/CyO*, n = 17) adult MpTs in vitro after stimulation with 20 μM cAMP and 100 μM LK-1. Values are means ± 1 SEM. Red asterisks indicate significantly different physiological performance between control and mutant MpTs ($p < 0.05$, see the Experimental Procedures). (D) X on Y linear regression analysis of SC number in a single MpT versus its stimulated fluid secretion rate measured in vitro (given as the relative increase in rate on stimulation with LK-1) in adult mutant (*hbs*^{361}/*Df(2R)14*) MpTs.

(E) A diagram showing the structure of Nephrin Ig domain subfamily proteins (transmembrane domain, black; fibronectin type III domain, green; Ig-C2 domain, red). Amino acid substitution (Leu to Arg) in *hbs*^{361} is adjacent to the amino acid altered in human NEPHRIN (Arg to Cys) in NPHS1 patients.

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