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Homothorax Plays Autonomous and Nonautonomous Roles in Proximodistal Axis Formation and Migration of the Drosophila Renal Tubules

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The *Drosophila* Malpighian tubules (MpTs) serve as a functional equivalent of the mammalian renal tubules. The MpTs are composed of two pairs of epithelial tubes that bud from the midgut–hindgut boundary during embryogenesis. The MpT primordia grow, elongate and migrate through the body cavity to assume their final position and shape. The stereotypic pattern of MpT migration is regulated by multiple intrinsic and extrinsic signals, many of which are still obscure. In this work, we implicate the TALE-class homeoprotein Homothorax (Hth) in MpT patterning. We show that in the absence of Hth the tubules fail to rearrange and migrate. Hth plays both autonomous and nonautonomous roles in this developmental process. Within the tubules Hth is required for convergent extension and for defining distal versus proximal cell identities. The difference between distal and proximal cell identities seems to be required for proper formation of the leading loop. Outside the tubules, wide-range mesodermal expression of Hth is required for directing anterior migration. The nonautonomous effects of Hth on MpT migration can be partially attributed to its effects on homeotic determination along the anterior posterior axis of the embryo and to its effects on stellate cell (SC) incorporation into the MpT. *Developmental Dynamics 243:132–144, 2014.* © 2013 Wiley Periodicals, Inc.

Key words: renal tubules; meis proteins; tubule migration; proximodistal axis; homothorax; extradenticle

Key findings:

- Homothorax is differentially expressed along the proximodistal axis of the Malpighian tubules and is required for defining distal versus proximal cell identities.
- In the absence of Homothorax or Extradenticle the tubules fail to rearrange and migrate anteriorly.
- Homothorax is required within the tubules for proper formation of the leading loop and in surrounding mesodermal tissues for directing anterior migration.

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INTRODUCTION

The building block of the mammalian kidney, the nephron, is a complex "mini-organ" in which different physiological activities are controlled by a linear array of functional domains organized along the proximodistal axis. The most proximal structure of the nephron, the glomerulus, filtrates

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the blood. More distal portions of the nephron, which constitute the renal tubule, are responsible for reabsorption and secretion. The renal tubule of the mature nephron is subdivided into several segments with distinct morphologies and functions. The molecular mechanisms responsible for subdividing the nephron into functional segments are not fully understood (Schedl, 2007; Costantini and Kopan, 2010).

In flies, the function of the kidney is divided between two discrete systems. The nephrocytes are podocyte-like cells that filter the hemolymph in a manner highly similar to the glomerular podocytes (Weavers et al., 2009). The Malpighian tubules (MpTs) function similarly to the remainder of the mammalian nephron and collecting duct. Although less elaborate than the mammalian nephron, the MpTs are also subdivided into structural domains with different physiological roles and different patterns of gene expression along their proximodistal axis (Sözen et al., 1997). The molecular determinants involved in subdividing the MpTs into distinct domains are not known (reviewed in Denholm, 2013).

The MpTs arise at stage 10 of embryogenesis from the junction between the hindgut and midgut, where crosstalk between these two neighboring tissues results in activation of the Wingless (Wg) pathway. Activation of the Wg pathway leads to expression up-regulation of the zinc-finger transcription factor Krüppel (Kr) and its target, the homeodomain-containing protein Cut (Liu and Jack, 1992). The expression of Krüppel and Cut defines the identity of MpTs cells that, as a consequence, undergo complex shape changes and bud out from the hindgut as four MpT primordia (Skaer and Martinez Arias, 1992; Ainsworth et al., 2000; Hatton-Ellis et al., 2007).

Following the eversion of the MpT primordia a small group of cells in each tubule start to express proneural genes and then, through a process of lateral inhibition, a single cell is singled out as a tip mother cell (Hoch et al., 1994; Wan et al., 2000). The tip mother cell divides once to produce two daughter cells and through a second set of inhibitory interactions one daughter cell adopts a tip cell-identity while the other becomes the sibling cell (Hoch et al., 1994). The tip cell, which is the distal-most cell of each tubule, secretes a ligand of the epidermal growth factor receptor thus providing a mitogenic cue to the neighboring cells (Skaer, 1989; Baumann and Skaer, 1993; Wan et al., 2000; Sudarsan et al., 2002). By the end of embryonic stage 13, each primordium contains the final number of tubules cells.

The transformation from short and thick primordia to elongated thin tubules is achieved through convergent-extension movements of the tubule cells (Bertet et al., 2004). The tubules increase considerably in length, and the number of cells surrounding the lumen drops from 6–10, to just 2 (reviewed in Ainsworth et al., 2000; Jung et al., 2005). While elongating, the tubules migrate through the body cavity, one pair projecting toward the head and the other pair toward the posterior-most segments of the embryo. After the tubules assume their final position, the MpT cells differentiate and begin to express a set of channel proteins essential for their excretion activity (reviewed in Denholm, 2013).

The mature tubules consist of two major cell types: the principal cells (PCs, or type I cells) and the stellate cells (SCs, or type II cells). The PCs transport cations and organic solutes, and constitute the major tubule cell type. The SCs transport water and chloride ions and are scattered evenly along the tubule between the PCs (Wessing and Eichelberg, 1978; O'Donnell et al., 1998; Kaufmann, 2005). The SCs originate in the caudal mesoderm, which overlies the hindgut in the vicinity of the budding tubules. During their proliferation the MpT primordia interact with a subpopulation of these mesodermal cells, thereby inducing the recruitment of mesodermal cells into the growing tubules, where they undergo a mesenchymal-to-epithelial transition and disperse between the PCs (Denholm et al., 2003; Campbell et al., 2010). The SCs can be selectively identified by the expression of the transcription factor Teashirt (Tsh), which is not expressed in PCs (Denholm et al., 2013).

The MpTs remain intact during metamorphosis but undergo some morphological changes in the larval and pupal stages. Each tubule divides into four distinct regions along the proximodistal axis, regions that show clear differences in cell morphology and express different repertoires of channel proteins. The most distal part of the tubule, which contains prominent white luminal concretion bodies, is called the initial segment. It is followed by the transitional segment, the main segment (secretory), and the proximal segment (reabsorptive), which can be further divided into lower tubule and ureter (Wessing and Eichelberg, 1978; O'Donnell and Maddrell, 1995; Sözen et al., 1997). The division of the tubules to distinct segments, each expressing a specific set of channels is essential for their proper function. However, while the molecular determinants involved in early steps of MpTs development have been studied, very little is known about the process of MpT proximodistal regionalization. Similarly, our understanding of the molecular mechanisms that regulate the stereotypic pattern of MpTs migration, which is important for correct positioning of the MpT within the body cavity and efficient sampling of the haemolymph, remains rudimentary.

In this work, we studied the role of the TALE-class homeoprotein Homothorax (Hth) in patterning of the MpTs. Hth plays multiple roles in embryonic and adult development of *Drosophila* and affects numerous processes of cell fate and homeotic determination, cell proliferation and axis formation (e.g., Rieckhof et al., 1997; Abu-Shaar and Mann, 1998; Casares and Mann, 1998; Kurant et al., 1998; Pai et al., 1998; Mercader et al., 1999; Henderson and Andrew, 2000; Wernet et al., 2003; Aldaz et al., 2005; Salvany et al., 2009; Lopes and Casares, 2010; Ando et al., 2011; Singh et al., 2011; Bryantsev et al., 2012; Baek et al., 2013). Hth is expressed in the developing MpTs as well as in MpT surrounding tissues. However, its role in MpTs patterning has not yet been addressed. Here, we show that Hth plays both autonomous and nonautonomous roles in the patterning of MpTs. Within the tubule, Hth is required for cell intercalation

and proper definition of distal versus proximal cell identities, which in turn may be required for proper bending and definition of the leading loop. Outside the tubule, Hth is involved in setting the normal pattern of MpT anterior migration, probably by affecting cell identities along the migratory path of the tubules, as well as by affecting SC incorporation into the tubules.

RESULTS

Hth is Expressed Differentially in Distal and Proximal Regions of the MpTs

Expression of the *hth* gene in the MpTs has been previously reported (Kurant et al., 1998), however the spatial and temporal distribution of Hth within the developing tubules and its function there have not been described. To better characterize the expression pattern of Hth within the developing MpTs we doubly stained *wild-type* (*wt*) embryos with anti-Cut and anti-Hth antibodies. This staining revealed that in embryos, Hth colocalizes with Cut only in the distal portion of each tubule and is excluded from the proximal region during all stages of embryonic development (with the exception of SCs as described below) (Fig. 1A–F). No overlap was seen between the distal domain of Hth expression and the proximal-most domain of Odd Skipped (Odd) expression (Fig. 1G–I). In third instar larvae, Hth expression was evident along the entire tubule, but with a clear border between distal cells that expressed high levels of Hth and proximal cells that expressed low levels of Hth (Fig. 6F,G).

To establish whether Hth is expressed in both PCs and SCs, we doubly stained *tsh-GAL4/UAS-GFP* embryos, which express GFP specifically in SCs, with anti-Cut and anti-Hth. From this staining, we could conclude that Hth is expressed in SCs at both the distal and proximal regions of the tubule (Fig. 1J–M).

One of the major roles of Hth is to regulate the subcellular localization of the homeotic cofactor Extradenticle (Exd) (Rieckhof et al., 1997; Kurant et al., 1998; Pai et al., 1998). The developing MpTs are no exception in this respect. Double staining of *FasII-lacZ* embryos (an enhancer-trap that drives *lacZ* expression in MpT cells) with anti-β-Gal and anti-Exd revealed that Exd is expressed in the entire tubule, but it accumulates in the nuclei of PCs only in the distal part of tubules, where Hth is expressed (Fig. 1N–Q). Unlike the PCs, SCs exhibited nuclear localization of Exd throughout the entire tubule (Fig. 1Q). Anti-Hth and anti-Exd staining demonstrate that these proteins are not expressed in the tip cells at early stages (Fig. 1B,C,O,P) but accumulate in low levels in the tip cells in late embryonic stages (data not shown).

Hth is Required for Normal Migration and Elongation of the Anterior MpTs

To learn about the role of *hth* in MpTs development we performed phenotypic analyses of embryos carrying strong loss-of-function alleles of *hth* ($hth^{64\text{-}1}$ or $hth^{K1\text{-}8}$). As shown in Figure 2, *hth* mutant embryos exhibited severe defects in MpT migration and organization. Their tubules were shorter and much less organized than the tubules of control *yw* embryos and did not reach the anterior region of the body cavity (Fig. 2A,B). In these mutant embryos, the process of convergent extension was incomplete, leaving certain segments of the tubules three- to four-cell-diameter wide, unlike normal tubules that are two-cell-wide throughout their entire length (Fig. 2C,D). The lumen of such tubules was wider than normal and irregular in shape (Fig. 2E,F). Embryos lacking maternal and zygotic expression of *exd* exhibited similar phenotypes (Fig. 2G). Embryos that lack the entire *Bithorax* complex, in which abdominal segments A2–A8 are transformed into T3 identity, presented similar but distinct MpT phenotypes. Similarly to *hth* and *exd* mutants, their MpTs did not migrate anteriorly beyond the A3 or A4 segment and the tubules were often thicker than normal. However, unlike *hth* or *exd* mutants, they maintained the ability to form loops (Fig. 2H).

Live imaging of normal embryos suggests that to migrate anteriorly the MpT needs to form a distinct loop structure and that the apex of this loop (or "kink") leads the migration (Bunt et al., 2010) (Supplementary Movie S1, which is available online).

The tip cell is very active during migration, extending long filopodia and interacting with other cells along the MpT's migratory path. The tip cell does not seem to lead the migration, but it is possible that interactions of the tip cell with other cells along the way serve as an anchor, which is required for tubule stretching and thinning. Live imaging of *hth* mutant embryos revealed that the leading loop was not generated properly. Although the MpT primordia bended backward generating a primary kink, this bending did not lead to the formation of a stable loop, and instead of extending anteriorly the kink region often projected dorsally or laterally (Supplementary Movie S2).

Loss of Hth Affects SC Number and Tsh Expression

Defects in cell rearrangement and tubule migration were previously associated with loss of the tip cell or its activity (Wan et al., 2000), reduction in cell number (Baumann and Skaer, 1993), loss of SCs (Pütz et al., 2005), defects in various signaling pathways, or inability of the MpTs to interact with circulating hemocytes (Bunt et al., 2010). We therefore examined whether the defects seen in *hth* mutant embryos can be attributed to defects in any of these processes.

Anti-Cut staining of homozygous $hth^{64\text{-}1}$ embryos revealed that the tip cell is present in MpTs of mutant embryos (Fig. 3A). Moreover, live imaging of developing MpTs that express moesin-GFP, demonstrated that the tip cells of mutant embryos extended long filopodia and seemed as active and motile as the tip cells of *wt* embryos (Fig. 3B and Supplementary Movie S2).

To test if the absence of *hth* expression affected MpT cell proliferation, we counted the number of Cut-positive cells in the anterior MpTs of normal and homozygous $hth^{64\text{-}1}$ embryos. This analysis revealed a small but consistent difference: the anterior MpTs of *wt* embryos contained on average 140.9± 5.7 Cut-

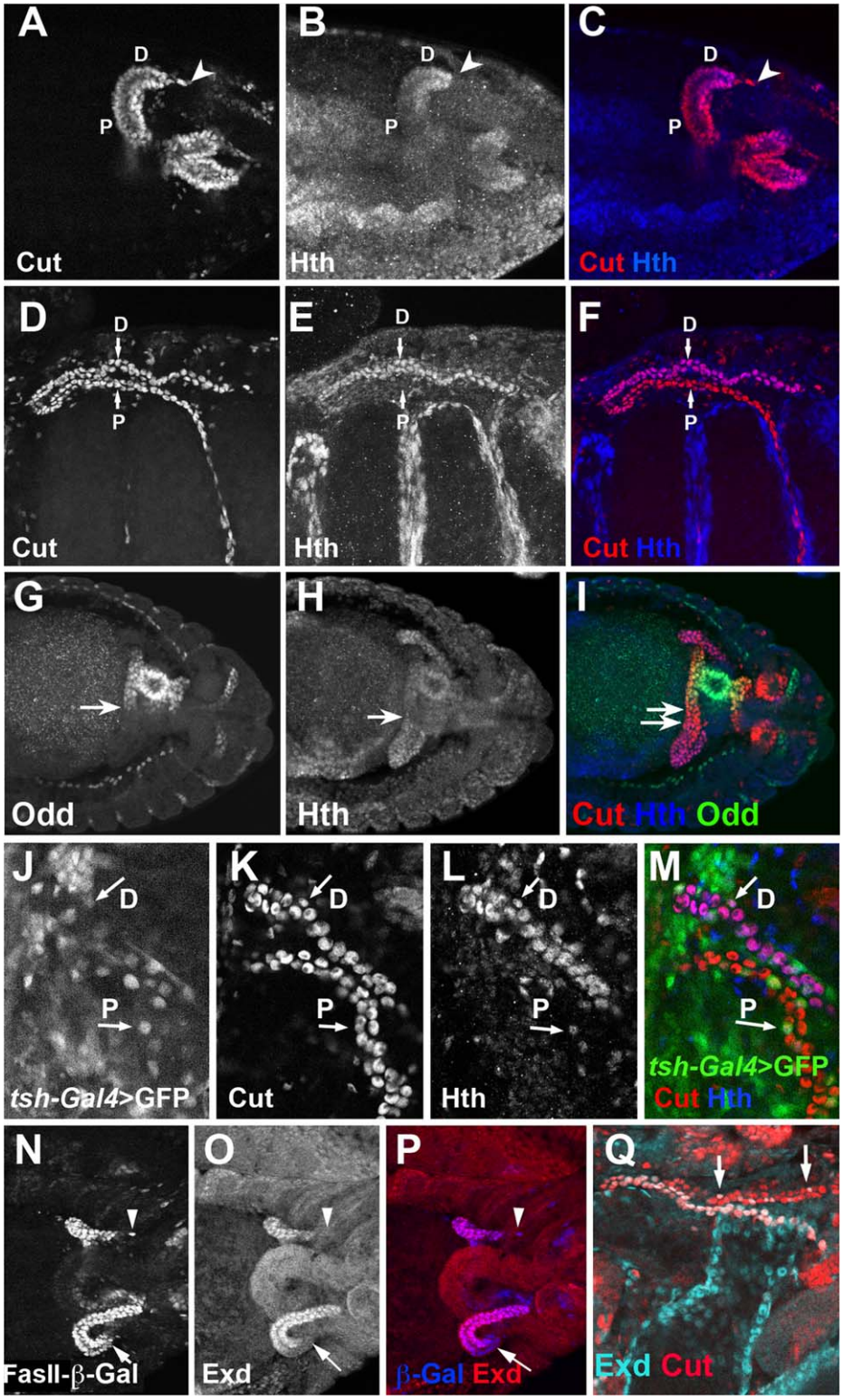

Fig. 1. Homothorax (Hth) is differentially expressed along the proximodistal axis of the MpTs. **A–F:** Malpighian tubules (MpTs) of stage 13 (A–C) and stage 16 (D–F) embryos stained with anti-Hth (blue) and anti-Cut (red). Hth expression is restricted to the distal (D) portion of the tubules. No expression is evident in the proximal region (P) or the tip cell (arrowhead). **G–I:** A stage 14 embryo stained with anti-Hth (blue), anti-Cut (red), and anti-Odd (green). The arrows mark the distal and proximal borders of Odd and Hth expression, respectively. **J–M:** MpTs of a *tsh-Gal4>UAS-GFP* stage 16 embryo stained with anti-Hth (blue) and anti-Cut (red). Hth is expressed in principal cells in the distal (D) portion of the tubules and in stellate cells (SC) in both distal (D) and proximal (P) portions of the tubule (the arrow points to proximally positioned SC). **N–Q:** MpTs of stage 13 (N–P) and stage 16 (Q) *FasII-lacZ* embryos stained with anti-β-galactosidase (blue) and anti-Exd (red or cyan). Nuclear localization of Exd is restricted to the distal portion of the tubule (arrows points to the proximal region where Exd is cytoplasmic). No expression is evident in the tip cell (arrowhead). **Q:** Nuclear localization of Exd is clearly evident in the SCs throughout the entire length of the tubule (arrows). In all images, embryos are oriented with their anterior side to the left.

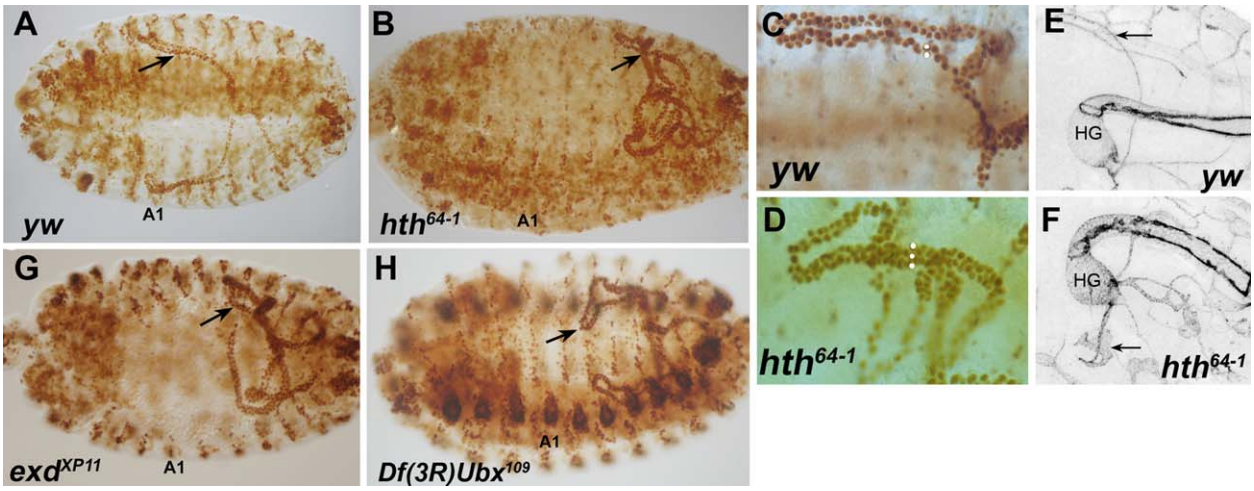

Fig. 2. Loss of *hth* leads to aberrant patterning of Malpighian tubules (MpTs). A,B: Dorsal view of stage 16 embryos stained with anti-Cut. Arrows point to the MpTs. **A:** A *yw* embryo. **B:** An $hth^{64\text{-}1}$ homozygous embryo. In normal embryos the anterior MpTs reach the A1 segment, whereas in *hth* mutants they fail to migrate anteriorly. **C,D:** A close-up view of MpTs of *yw* (C) and $hth^{64\text{-}1}$ homozygous (D) embryos. The MpTs of $hth^{64\text{-}1}$ embryo have larger circumferential cell number (dots mark cells surrounding the lumen). **E,F:** Dorsal view of MpTs of stage 16 *yw* (E) and $hth^{64\text{-}1}$ (F) embryos fluorescently stained with anti-Crumbs, which marks the apical membrane. The images were inverted for clarity using Adobe Photoshop. HG marks the hindgut and the arrows point to the lumen of the MpTs. **G,H:** Dorsal view of stage 16 embryos stained with anti-Cut. Arrows point to the MpTs. **G:** An exd^{XP11} embryo devoid of both maternal and zygotic contribution. **H:** A homozygous $Df(3R)Ubx^{109}$ embryo.

positive cells (n = 14), whereas MpTs of *hth* mutant embryos contained an average number of 125.7 ± 9.1 cells (n = 17; $P < 0.01$) (Fig. 3C). This relatively small effect on cell number suggests that the dramatic defects in MpT patterning in *hth* mutants cannot be attributed to defects in cell proliferation per-se. Because defects in MpT patterning could be caused by loss of SCs incorporation into the tubule (Pütz et al., 2005), we tested whether the observed 10% reduction in the total number of MpT cells reflects a more specific loss of SCs, which could contribute to the failure of tubule migration. Indeed, analysis of *wt* and *hth* mutant embryos immunostained with anti-Cut and anti-Tsh revealed that the MpTs of *hth* mutant embryos contained a smaller number of SCs as compared to normal embryos (Fig. 3C). We could identify 30.7 ± 3.7 SCs in each anterior tubule of normal embryos (n = 66), but only 11.4 ± 3.3 SCs in each anterior tubule of homozygous $hth^{64\text{-}1}$ embryos (n = 42; $p = 2.7E^{-38}$). In addition, the SCs that were incorporated into the tubules of *hth* mutant embryos were not uniformly dispersed between the PCs and the level of Tsh expression was lower as compared to SCs of *wt* embryos (Fig. 3D–G).

To test whether *hth* affects the interaction between the migrating MpTs and circulating hemocytes, we examined whether hemocytes physically interact with the MpTs in *hth* mutants, and whether this interaction leads to deposition of collagen IV around the tubules. The results of these experiments demonstrate that hemocytes do interact with the migrating tubules in the absence of Hth and that this interaction is probably functional as judged by the presence of Collagen IV around the tubules (Fig. 3H–S).

Autonomous Requirement for Hth Activity in the MpTs

hth expression is eliminated from all embryonic tissues of $hth^{64\text{-}1}$ homozygous embryos. Thus, we could not distinguish between autonomous and nonautonomous effects of *hth* loss-of-function on MpT patterning. To distinguish between the autonomous and nonautonomous effects of Hth, we performed rescue experiments in which Hth activity was restored either within the migrating tubules, or in various surrounding tissues in an *hth* mutant background.

Because the endogenous expression of Hth within the tubule is not uniform, we first needed to develop tools that would allow us to express the rescue transgene in a similar restricted pattern, only in the distal portion of the tubule. For that purpose, we searched the *hth* locus for regulatory regions that drive gene expression in the MpTs. Through these enhancer-bashing experiments we were able to identify a 4.1-kb fragment (hereafter referred to as hth^{MpT}), located upstream to exon 11, which drives gene expression specifically in the distal portion of each MpT (Fig. 4A–C). We used this fragment to construct an hth^{MpT}-*hth* rescue transgene (Fig. 4D) and an hth^{MpT}-*Gal4* transgene that allowed the restricted expression of any gene of interest in the distal portion of MpTs (Fig. 4E).

To quantify the results of the rescue experiments we measured two parameters: the percentage of MpTs that formed distinct leading loops, and the extent of anterior migration (a score of 1 reflects a MpT that reached the A1 segment, a MpT that reached A2 segment was scored 2, etc.). We first measured these parameters in *wt* embryos and then compared them with $hth^{64\text{-}1}$ mutants and hth^{MpT}-*Gal4*,$hth^{64\text{-}1}$/*UAS-hth*, $hth^{64\text{-}1}$ rescue embryos. In *wt* embryos, we could identify a distinct loop structure in 91 of 116 tubules examined (78%). In contrast, only 12 of 54 tubules of $hth^{64\text{-}1}$ mutant embryos formed loops correctly (22%). Expressing *hth* in the distal portion of the tubules restored the ability to form loops; normally

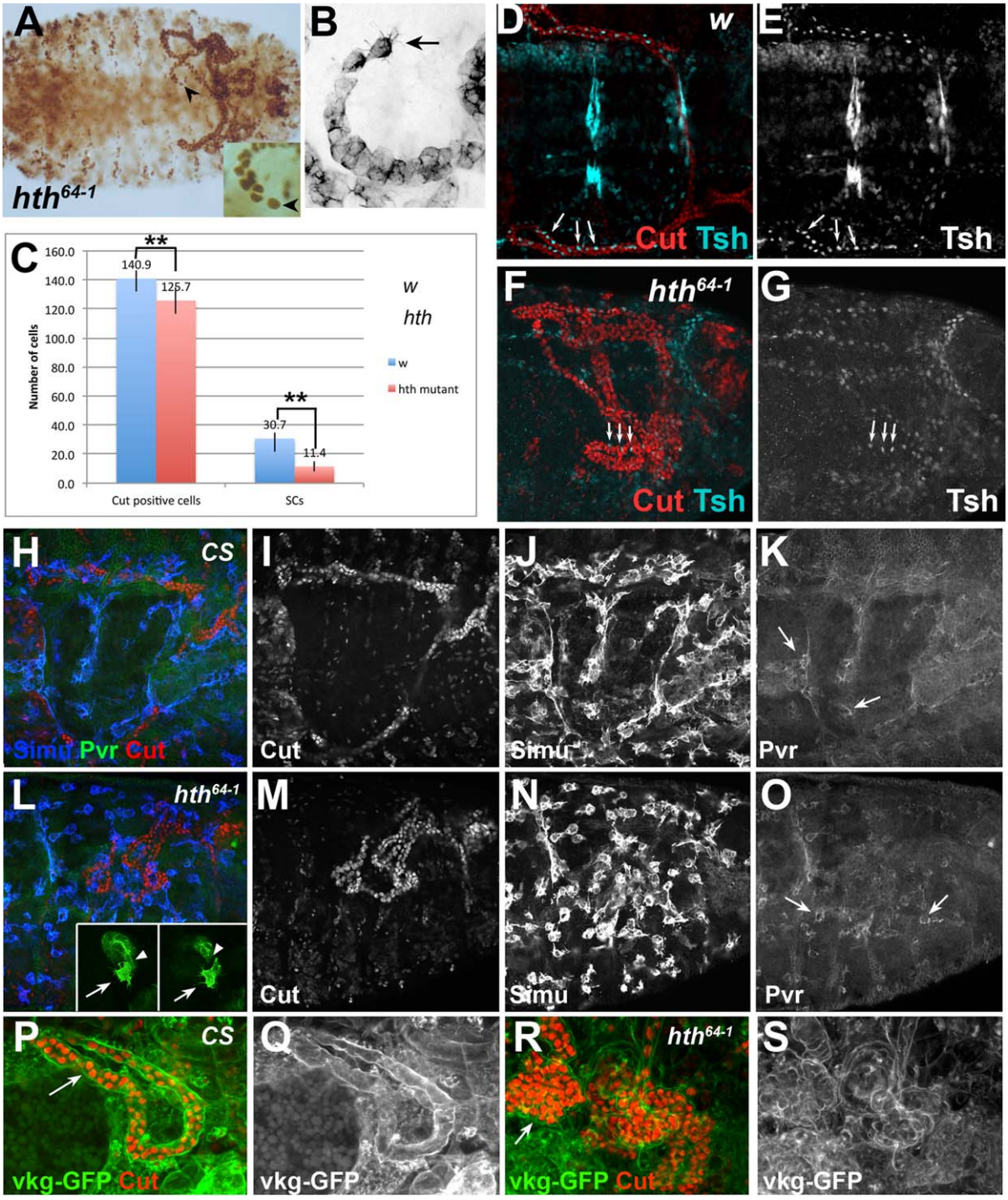

Fig. 3. Loss of *hth* leads to reduction in the number of Malpighian tubules' (MpTs) stellate cells (SCs), but has no major effect on the interactions between MpT cells and circulating hemocytes. **A,B:** $hth^{64\text{-}1}$ homozygous embryos stained with anti-Cut (A), or expressing Moesin-green fluorescent protein (GFP) under the regulation of *CutB-Gal4* (B). The tip cell is present in *hth* mutants (arrowhead in A) and it extends long filopodia during migration (arrow in B). **C:** The total number of Cut-positive cells and SCs in the anterior MpTs of *w* versus $hth^{64\text{-}1}$ embryos. MpTs of *w* embryos contained 140.9 ± 5.7 Cut-positive cells (n = 14), whereas MpTs of *hth* mutant embryos contained 125.7 ± 9.1 cells (n = 17; $P < 0.01$, **). 30.7 ± 3.7 SCs were identified in MpTs of *w* embryos (n = 66) as compared to 11.4 ± 3.3 SCs in MpTs of homozygous $hth^{64\text{-}1}$ embryos (n = 42; p = $2.7E^{-38}$). **D–G:** Dorsal view of stage 16 *wt* (D,E) and $hth^{64\text{-}1}$ (F,G) embryos stained with anti-Cut (red) and anti Tsh (cyan). Tsh expression (arrows) is lower in the mutants as compared to *wt* embryos (compare E and G). **H–O:** Stage 16 *CS* (H–K) or $hth^{64\text{-}1}$ (L–O) embryos stained with anti-Cut (red), anti Pvr (PDGF/VEGF-receptor related, green) and anti-Simu (marks the hemocytes in blue). The hemocytes express Pvr (arrows in K and O) and interact with the MpTs in both control and mutant embryos. The inset in L shows two MpTs of $hth^{64\text{-}1}$ embryos that express *CutB-Gal4>UAS-Moesin-GFP*. Note the two large hemocytes (arrows) in close proximity to the tip region of the MpT (arrowheads). **P–S:** Visualization of *vkg-GFP* (a collagen IV reporter) in *CS* (P–Q) and $hth^{64\text{-}1}$ (R,S) embryos. Collagen IV is deposited in the basement membrane ensheathing the MpTs in both normal and *hth* mutant embryos (arrows).

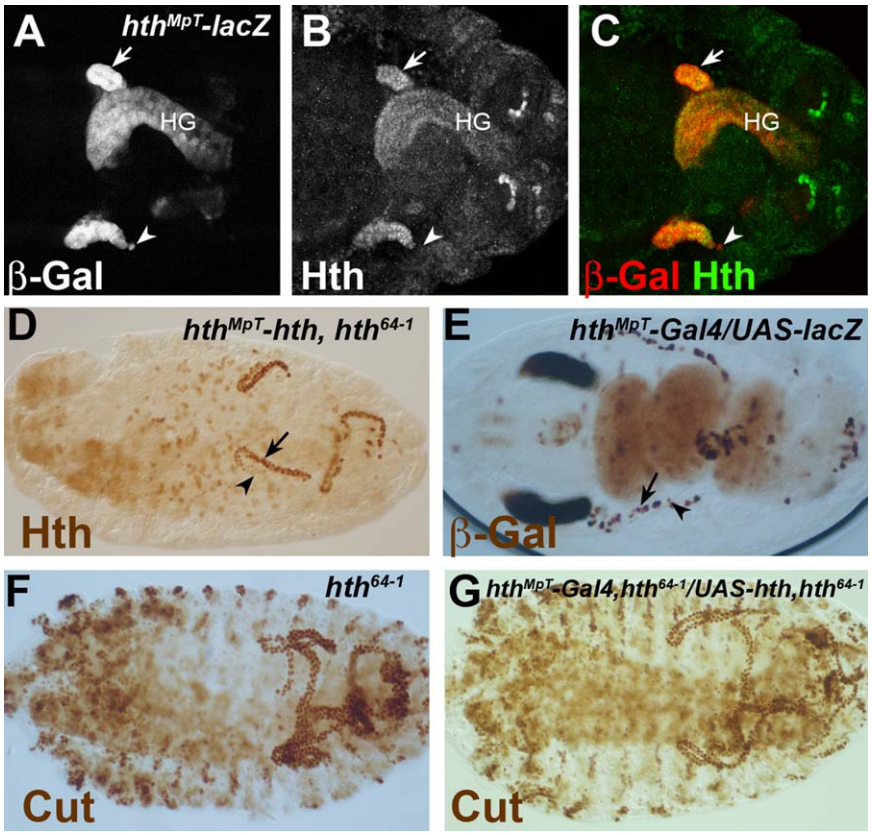

Fig. 4. Autonomous requirement for Homothorax (Hth) activity in the Malpighian tubules (MpTs). **A–C:** *hth^{MpT}-lacZ* embryo stained with anti-Hth (green) and anti-β-Gal (red). The *hth^{MpT}* regulatory element drives expression specifically in the hindgut (HG) and the distal portion of the MpTs, where Hth is expressed (arrows). Unlike the endogenous *hth* gene, the *hth^{MpT}-lacZ* reporter is expressed in the tip cell (arrowheads). **D:** A stage 16 *hth^{MpT}-hth*, *hth$^{64-1}$* embryo stained with anti-Hth. Hth expression is restored only in the distal (arrow), but not proximal (arrowhead), portion of the MpTs. **E:** A stage 16 *hth^{MpT}-Gal4/UAS-lacZ* embryo. The *hth^{MpT}-Gal4* driver drives nonuniform expression in principal cells only in the distal portion of the MpTs (arrow). Very weak β-Gal expression is evident in stellate cells in the distal and proximal portions of the tubule (arrowhead). **F,G:** *hth$^{64-1}$* and *hth^{MpT}-Gal4,hth$^{64-1}$/UAS-hth*, *hth$^{64-1}$* embryos stained with anti-Cut.

shaped loops were observed in 31 out of 39 of tubules of *hth-Gal4*, *hth$^{64-1}$/UAS-hth*, *hth$^{64-1}$* (79.5%) (Fig. 4F,G). Cell intercalation and reorganization into thin tubules was also improved dramatically in the "rescued" *hth^{MpT}-hth*, *hth$^{64-1}$* (Fig. 4D), or *hth^{MpT}-Gal4*, *hth$^{64-1}$/UAS-hth*, *hth$^{64-1}$* (Fig. 4G) embryos.

Whereas restoring *hth* expression in the distal segment was sufficient for rescuing the ability of the MpTs to form loops and for improving cell intercalation in distal regions of the MpTs, it was not sufficient for significantly rescuing anterior migration. In normal embryos, the tubules reached the A1-A2 segment (average score = 1.6; n = 114 tubules). In contrast, the anterior limit of MpT migration in *hth$^{64-1}$* embryos was between segments A3 to A4 (average score = 3.6; n = 54 tubules). The migration limit of tubules of the rescued embryos was also between segments A3 to A4 (average score = 3.2; n = 39 tubules) (Fig. 4F,G).

Hth Expression in the Mesoderm is Required for Proper Tubule Migration

Because *hth* expression within the distal region of the MpT was not sufficient to fully rescue MpT migration, we next tested the requirement for *hth* in other surrounding tissues. For these experiments, we used several Gal4 drivers that drive expression in the ectoderm (*69B-Gal4*), endoderm (*48Y-Gal4*), or mesoderm (*24B-Gal4*), to express *hth* in these tissues in an *hth$^{64-1}$* mutant background.

As shown in Figure 5, expression of *hth* in either the ectoderm or endoderm had no significant effect on tubule migration (Fig. 5A,B). However, restoring *hth* expression in the mesoderm was most effective and significantly improved tubule migration (Fig. 5C). The tubules of the rescued embryos reached the A1-A2 segments (average score = 1.7; n = 32 tubules) similarly to *wt* embryos (Fig. 5A–C). Because the MpTs normally migrate along the visceral mesoderm, which expresses *hth*, we used *bap-Gal4* to test whether restoring *hth* expression in the visceral mesoderm is sufficient for restoring MpT migration. The results of this rescue experiment demonstrated that restoring *hth* expression in the visceral mesoderm alone is not sufficient for rescuing MpTs migration (data not shown).

Restoring Hth Expression in the Mesoderm Partially Rescues SCs Incorporation

Because restoring *hth* expression in the mesoderm in *hth$^{64-1}$* background was the most effective in improving tubule migration, and because SCs

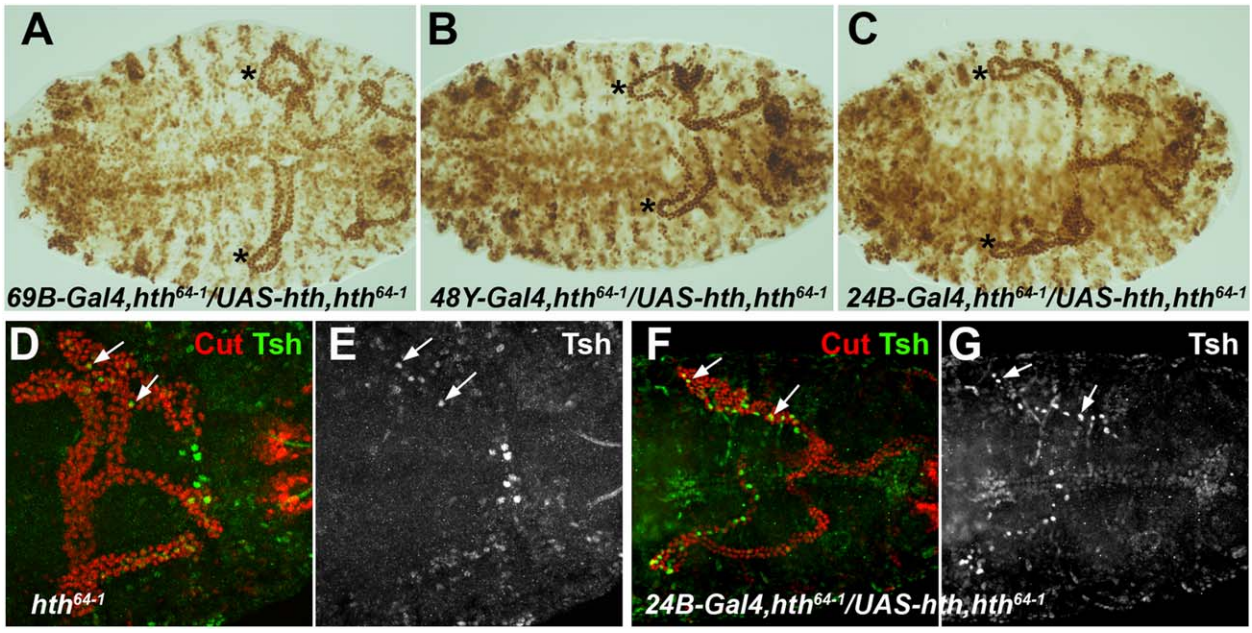

Fig. 5. Homothorax (Hth) expression in the mesoderm is required for anterior tubule migration. **A–C:** Dorsal view of stage 16 embryos stained with anti-Cut. **A:** A *69B-Gal4*, $hth^{64\text{-}1}$/*UAS-hth*, $hth^{64\text{-}1}$ embryo. **B:** A *48Y-Gal4*, $hth^{64\text{-}1}$/*UAS-hth*, $hth^{64\text{-}1}$ embryo. **C:** A *24B-Gal4*, $hth^{64\text{-}1}$/*UAS-hth*, $hth^{64\text{-}1}$ embryo. Expression of *hth* in either the ectoderm (under the regulation of 69B-Gal4) or endoderm (under the regulation of 48Y-Gal4) had no significant effect on tubule migration. In contrast, mesodermal expression (under the regulation of 24B-Gal4) was very effective and improved the migration significantly. Asterisks mark the anterior border of each tubule's migration. **D–G:** Malpighian tubules of $hth^{64\text{-}1}$ (D,E) and *24B-Gal4*, $hth^{64\text{-}1}$/*UAS-hth*, $hth^{64\text{-}1}$ (F,G) embryos stained with anti-Cut (red) and anti-Tsh (green). Whereas $hth^{64\text{-}1}$ embryos contained on average 11.4 ± 3.3 SCs in each anterior tubule, 16.7 ± 4.2 SCs were evident in each tubule of the rescue embryos (n = 37 tubules). This difference is statistically significant ($P < 0.0005$). The level of Tsh is higher in the rescued embryos.

originate in the mesoderm, we wanted to test whether the mesodermal expression of *hth* affected the number of SCs that were incorporated into the tubules. To address this question, we stained *hth* mutant embryos, in which the expression of Hth in the mesoderm was restored (*24B-Gal4*,$hth^{64\text{-}1}$/*UAS-hth*, $hth^{64\text{-}1}$), with anti-Cut and anti-Tsh and examined the number and distribution of SCs in their tubules as compared to *wt* and $hth^{64\text{-}1}$ embryos. A higher number of SCs, as compared to homozygous $hth^{64\text{-}1}$ embryos, was detected. The rescued embryos contained on average 16.7 ± 4.2 SCs in each anterior tubule (n = 37 tubules), as compared to 11.4 ± 3.3 SCs in tubules of $hth^{64\text{-}1}$ embryos. This difference is statistically significant ($P < 0.0005$). In comparison to normal embryos, these rescued embryos still lack almost half of the SCs ($P = 0.001$). However, the SCs that do integrate are more evenly dispersed between the PCs and the level of Tsh expression is higher than in mutant embryos (Fig. 5D–G). It is possible that the ~50% increase in number of SCs, from an average number of 11.4 to 16.7 cells, is sufficient for at least part of the evident improvement in organization and migration of the mutant tubules. We do not know why the rescue of SCs' incorporation is not more complete. A more detailed analysis of the temporal and spatial pattern of expression of the rescue transgene may help to address this question.

Taken together, the results of the rescue experiments suggest that Hth plays both autonomous and nonautonomous roles in MpT patterning. Within the tubule, Hth is required for loop formation and convergent extension, whereas wide-range mesodermal expression is required for proper anterior migration. Some of the nonautonomous functions may be mediated through the effect of Hth on SC incorporation into the developing MpT.

Restricted Expression of Hth Within the Tubule is Required to Define Distal vs. Proximal Cell Identities and Affects the Position of Loop Formation

Based on the pattern of Hth expression within the MpTs and the results of the hth^{MpT}-driven rescue, we made the following two assumptions: the restricted pattern of Hth expression within the tubules is required to define distal versus proximal identity. The difference between distal and proximal identities may define the position of the loop apex.

To test the suggested hypothesis, we performed a rescue experiment in which we induced *hth* expression in all Cut-positive cells in an $hth^{64\text{-}1}$ mutant background. In addition, we tested the effect of expressing *hth* ectopically throughout the entire tubule in otherwise normal embryos. If indeed *hth* defines distal versus proximal identity, and this in turn defines the position of the leading loop apex, then expressing *hth* throughout the tubule in a *wt* background is expected to lead to random and aberrant positioning of the kink. Additionally, if this is the case, then *cutB-Gal4* should be less efficient than hth^{MpT}-*Gal4* in rescuing the MpT phenotypes associated with *hth* loss-of-function.

Indeed, as shown in Figure 6A, uniform expression of *hth* throughout the entire length of the tubule, under the regulation of *cutB-Gal4*, could not

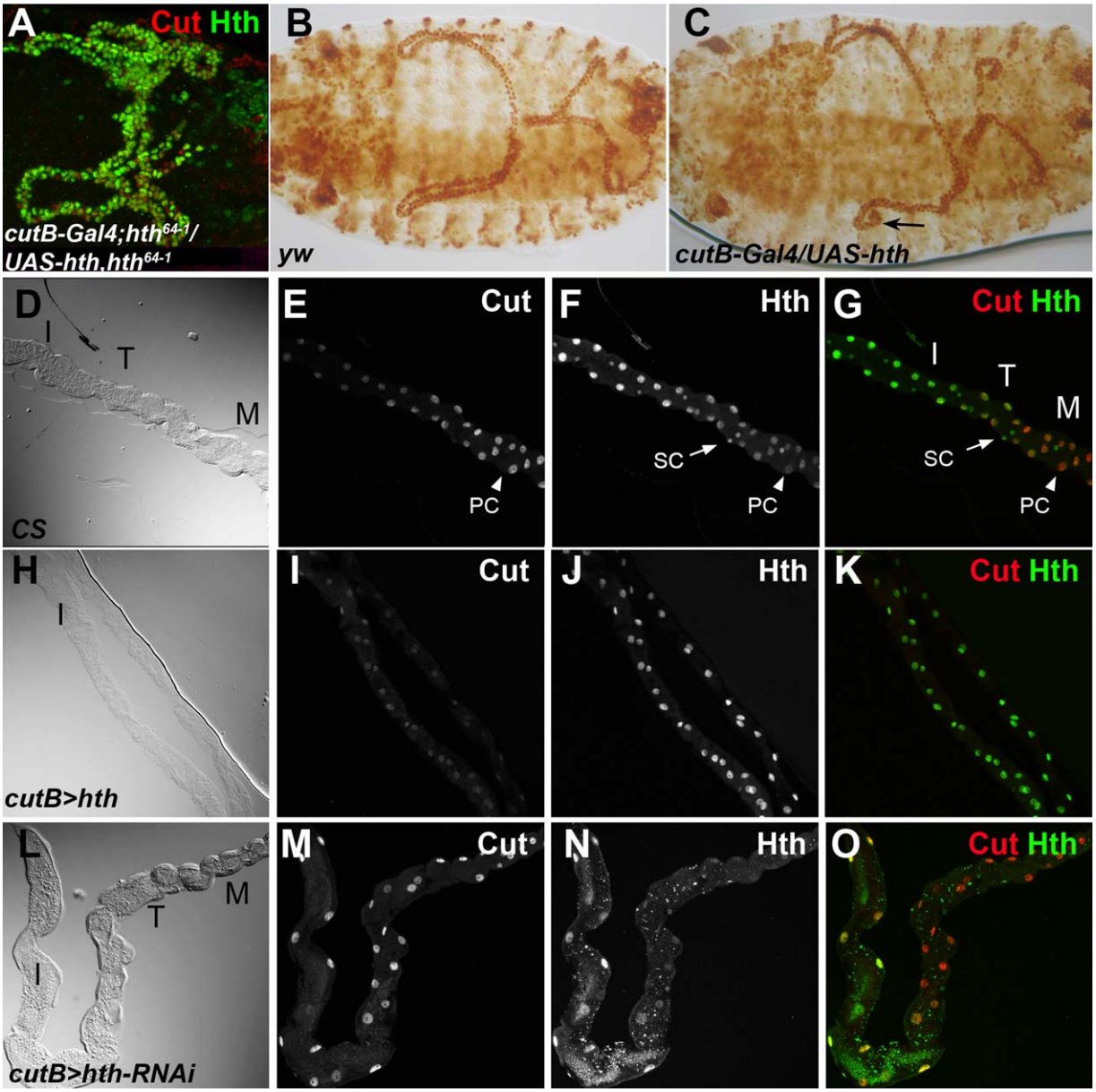

Fig. 6. Homothorax (Hth) is required for proximodistal regionalization of the Malpighian tubules (MpTs). **A:** The MpTs of *cutB-Gal4*, *hth*$^{64-1}$/*UAS-hth*, *hth*$^{64-1}$ embryo stained with anti-Hth (green) and anti-Cut (red). The MpTs reorganized into thin elongated tubes, but failed to migrate anteriorly. **B,C:** Dorsal view of stage 16 *yw* (B) and *cutB-Gal4*/*UAS-hth* (C) embryos stained with anti-Cut. Driving expression of *hth* along the entire tubule leads to abnormal pattern of bending, which generates abnormally short distal segments (arrow). **D–O:** MpTs of third instar larvae stained with anti-Hth (green) and anti-Cut (red). Distal is to the left. The initial (I), transitional (T) and main (M) segments are indicated. **D–G:** *CS* larvae. Reciprocal gradients of Hth and Cut expression are evident. **H–K:** In MpTs of *cutB-Gal4*/*UAS-hth* (*CutB>hth*) larvae the expression of Cut is reduced and the transitional and main segments exhibit morphological characteristics of the initial segment. **L–O:** Expression of *hth*-specific RNAi (*CutB>hth-RNAi*) transgene under the regulation of *cutB-Gal4* leads to down-regulation of Hth (N) and up-regulation of Cut (M) expression in distal portions of the MpTs.

rescue the mutant phenotype of the MpTs. The tubules did re-organized into two cell-diameter tube but the leading loop did not form correctly and the tubules did not migrate anteriorly. In addition, when the *cutB-Gal4* driver was used to force Hth expression along the entire tubule, effectively abolishing the hypothesized border between distal and proximal domains, we observed a variable pattern of loop formation. In 27 of 127 embryos examined (~21%), the kink formed in an abnormally distal position and the distal portion of the tubule (distal to the apex of the loop) was significantly shorter than normal (Fig. 6B,C). The results of these experiments support our working model and suggest that the spatially restricted expression of *hth* plays a role in defining proximal versus distal identities and that the difference between distal and proximal identities further define the position of the leading loop apex.

The differences between proximally and distally located cells become evident in post-embryonic stages, when different cells along the proximodistal

axis acquire distinguishable morphologies. To further test whether *hth* affects cell identity along the proximodistal axis, we used *cutB-Gal4* to drive expression of either *hth*, or *hth-RNAi*, and examined its influence on the morphology of MpTs in third instar larvae.

In MpTs of normal larvae, high levels of Hth are evident in the distal region (initial segment), whereas in more proximal regions the levels of expression are much lower (Fig. 6D–G). Surprisingly, when we examined the expression of Cut, we noticed that, unlike its uniform expression along the tubules during embryogenesis, its expression in larval MpTs is nonuniform showing an inverse gradient to that of Hth expression. In the distal segment where high levels of Hth are present, Cut expression seems to be repressed, whereas in proximal regions, where the level of Hth expression is very low, Cut is expressed in higher levels. (Fig. 6E,G).

When uniform expression of Hth was induced throughout the entire tubule, Cut expression was down-regulated in all tubule cells (Fig. 6H–K). In these MpTs it was difficult to distinguish between the cells of the three segments based on their morphology as all cells appeared similar and all of them contained some white luminal concretions (Fig. 6H). When, in contrast, the level of Hth was down-regulated by expressing an *hth*-RNAi transgene, a uniform expression of Cut was evident (Fig. 6L–O). In such larvae, the characteristic morphologies of distal and proximal cells were retained and appeared similar to *wt* larvae (Fig. 6L). These observations support our hypothesis that Hth defines distal cell identity and suggest that Hth down-regulates, either directly or indirectly, Cut expression in the distal portions of the tubules.

DISCUSSION

Cell-Fate Specification Along the MpT Proximodistal Axis

Similarly to the mammalian renal tubule, the fly MpT is divided into several distinct domains along its proximodistal axis that can be distinguished by their unique morphology, pattern of gene expression and function (Sözen et al., 1997).

The data presented here implicate the TALE-class homeoprotein Hth in proximodistal axis formation in the MpTs, where it is involved in defining distal cell identities. Previous studies have implicated Hth and its partner Exd in proximodistal axis formation in the adult appendages, where they define proximal cell-identities and create functional boundaries between proximal and distal regions of the appendage that are not otherwise separated by lineage restrictions (Abu-Shaar and Mann, 1998; Wu and Cohen, 1999, 2000; Azpiazu and Morata, 2000; Casares and Mann, 2000; Dong et al., 2001). Although a similar set of genes (e.g., *wg*, *Dpp*, *Dll*, *dac*, and *tsh*) plays a role in the process of regionalization and diversification along the proximodistal axis of different types of appendages (leg, antenna, wing), the network of genetic interactions between these genes is unique to each type of appendage and is sometimes responsible for the difference between them.

The proteins that work with, or against, Hth and Exd in regionalizing the MpT are not known. Odd is a transcriptional regulator that is expressed specifically in the proximal-most region of the tubule (Ward and Coulter, 2000; Tena et al., 2007) and may be required to define its unique identity and properties. The combined patterns of Hth and Odd expression divide the MpTs into three distinct domains of gene expression: a proximal Odd-positive/Hth-negative domain, a medial Odd-negative/Hth-negative domain and a distal Odd-negative/Hth-positive domain (Fig. 1G–I). This primary division can be later "translated" into differences in structure and physiological function through regulatory cascades of gene expression. However, the transcriptional targets of Hth/Exd in the MpTs and the other components of this genetic network remain to be identified. A new genetic tool that is described here, the hth^{MpT}-*Gal4* driver, can be used to manipulate gene expression specifically in the Hth-expressing distal cells and can thus help us, in combination with the available genomic and transcriptomic data (Wang et al., 2004; Dow, 2009) and other Hth-related tools (Inbal et al., 2001), to achieve this goal.

The data presented here suggest that one of the functions of Hth in determining distal identity may involve down-regulation of Cut expression in distal cells of larval tubules. Because during embryonic stages Cut is uniformly expressed along the entire MpT, the antagonistic relations between Cut and Hth are probably not involved in setting the primary border between distal and proximal territories, but may be required in later stages for the realization of the differences between distal and proximal cell identities.

Either the loss of Hth expression, or its uniform expression along the tubule, led to aberrant loop formation. We hypothesize that Hth defines distal identity, and that the difference between distal and proximal regions is required for proper loop formation. It is possible that the expression of some, yet unknown, factors is induced in cells at the border between distal and proximal segments endowing the expressing cells with the ability to lead the migration. Another possibility is that interactions between cells in the distal region and cells in the proximal region maintain the loop structure throughout migration.

How Does Hth Affect MpT Migration?

Hth affects MpT patterning in many different ways in both autonomous and nonautonomous manners. Within the tubules, Hth is required for the process of convergent extension that is critical for the tubule's elongation. It is not known what are the main molecular and cellular mechanisms that underlie convergent extension in the MpTs, but it was previously reported that this process is driven by internal forces and does not depend on other tissues (reviewed in Denholm, 2013). Because Hth is expressed differentially along the tubule, it may be involved in polarizing the tissue within the plane of the epithelium. Multiple genes were implicated in the process of convergent extension within the MpTs based on their mutant phenotypes; however, the genetic networks in which they function in the context of convergent extension are very roughly drawn (reviewed by Jung et al., 2005;

Denholm, 2013). The identification of Hth and Exd as new players in the convergent extension process may provide important entry points into the genetic network that controls this developmental plan.

In addition to its autonomous effects, Hth is required in other, mainly mesodermal, tissues for proper MpT migration. We do not know which molecules/pathways mediate Hth's effect on the MpTs. One potential candidate is the Dpp pathway. Dpp signaling is used as a guiding cue that directs the migrating tubules along their way (Bunt et al., 2010). Mutations in the genes encoding for the Dpp receptors *thick-vein* (*tkv*) or *punt* cause defects in tubule migration that resemble the *hth* phenotype (Bunt et al., 2010). Hth is required for correct pattern of Dpp expression in the visceral mesoderm (Stultz et al., 2006) and could thus affect MpT migration by affecting the map of guiding cues. Alternatively, Hth might modulate the levels of Dpp signal transduction, as was shown for example in the developing leg (Azpiazu and Morata, 2002), or affect the levels of Dpp receptor's expression within the tubules. Although our data suggest that MpTs that lack *hth* activity can still attract migrating hemocytes and that this interaction leads to deposition of Collagen IV, which allows the MpTs to respond properly to Dpp signaling, this analysis could not rule out subtler quantitative or qualitative effects on this complex interaction and its functional outcomes.

Another way by which Hth can affect MpT's migration nonautonomously is by determining homeotic identities along the anterior posterior axis of the embryo (Rieckhof et al., 1997; Kurant et al., 1998). When the anterior MpTs reach the first abdominal segment, A1, they stop migrating anteriorly and dip ventrally. The identity of the "stop sign" that prevent the MpTs from crossing the A1 border is not known. When we altered segmental identity by manipulating the expression of homeotic genes we could shift the anterior border of migration. For example, by ectopically expressing *abd-A* ubiquitously under the regulation of *da-Gal4* we could shift the A1 border anteriorly, and as a result, 80% (43 of 54) of the anterior tubules did not stop in A1 and reached the T3 segment (data not shown). In mutant embryos that lack the entire *Bithorax* complex, in which abdominal segments A2–A8 are transformed into T3 identity, the MpTs did not migrate anteriorly beyond the A3 or A4 segment (Fig. 2H).

These observations suggest that at least part of the nonautonomous effects of Hth on tubule migration are related to homeotic determination along the course of migration and determination of the anterior border, which serves as a stop sign to the migrating tubules.

Concluding Remarks

Drosophila is a powerful genetic tool for studying renal system development and function and is emerging as a translational model for human kidney pathologies (Cagan, 2003; Singh and Hou, 2008; Dow and Romero, 2010; Miller et al., 2013). The renal system is evolutionarily conserved and fly orthologues of numerous human genes involved in kidney pathologies are expressed specifically, or enriched in the MpTs (Wang et al., 2004; Chintapalli et al, 2007; Miller et al., 2013).

Here, we show for the first time that Hth and Exd are required for proper development of MpTs in *Drosophila*. Vertebrate homologues of Hth and Exd, namely proteins of the Meis and Pbx families and their homeotic counterparts (Hox proteins) are expressed in the kidney and their loss leads to aberrant phenotypes (Roberts et al., 1995; Schnabel et al., 2001, 2003; Di Giacomo et al., 2006; Wellik, 2011). In addition, *Meis1* and *Meis2* are significantly upregulated in kidney tumors, such as Wilms' tumor (Dekel et al., 2006). Given the relative simplicity of the fly MpTs, the remarkable similarity to mammalian renal tubules, the plethora of available genetic tools and the findings presented here, we believe that the fly MpTs can be used as a model for studying Hox and TALE-class homeoproteins-related aspects of kidney development and function.

EXPERIMENTAL PROCEDURES

Fly Strains

The following strains were used (described in FlyBase [Marygold et al., 2013], unless otherwise indicated): *Canton S* (*CS*), *yellow white* (*yw*), or *w* were used as *wt* controls. *Df(3R)Ubx^{109}*, *hth$^{64\text{-}1}$/TM6B*, *abd-A-lacZ*, *hth$^{K1\text{-}8}$/TM6B*, *abd-A-lacZ* (Kurant et al., 2001). Embryos lacking both maternal and zygotic expression of *exd* were produced as described in (Kurant et al., 1998). *P{A92}A31* (*FasII-LacZ*) was kindly provided by H. Skaer. The following Gal4 drivers and UAS strains were used: *24B-Gal4*, *69B-Gal4*, *48Y-Gal4*, *CutB-Gal4* (Jack and DeLotto, 1995), *bap-Gal4* (a gift of S. Merabet, Marseille, France). The *hth^{MpT}-Gal4* and *hth^{MpT}-hth* transgenes were generated in this work. *UAS-nls-GFP*, *UAS-GAS2-GFP* (Subramanian et al., 2003), *UAS-GFP-Moesin/CyO* (Edwards et al., 1997), *UAS-lacZ*, *UAS-abdA* (Capovilla and Botas, 1998), *vkg-GFP* (Buszczak et al., 2007), *UAS-hth^{12}* (Pai et al., 1998), *UAS-RNAi-hth/TM3* (*P{GD4741}v12764/TM3*, VDRC). The following strains were constructed by recombination for the rescue experiments: *UAS-hth^{12},hth$^{64\text{-}1}$/TM6*, *hth^{MpT}-Gal4,hth$^{64\text{-}1}$/TM6Z*, *24B-Gal4,hth$^{64\text{-}1}$/TM6Z*, *UAS-GFP-Moesin/CyO;hth$^{64\text{-}1}$/TM6Z*, *CutB-Gal4/Cy;hth$^{64\text{-}1}$/TM6Z*.

Generating the *hth^{MpT}-Gal4* and *hth^{MpT}-hth* Transgenes

The *hth^{MpT}* fragment is an *Eco*RI 4,152 bp-long genomic fragment that spans exon 11 and part of the intron upstream to it. We searched this region for regulatory elements (M. Mahroum, unpublished data), because this region is the insertion site of the lethal *P1-K1-8* insertion, which abolishes *hth* expression in homozygous embryos (Kurant et al., 2001). This genomic fragment was cloned into the *pChs-Gal4* vector (kindly provided by H. Apitz) for generating the *hth^{MpT}-Gal4* driver and was cloned upstream to a minimal *hs* promoter and full-length *hth* cDNA, or *lacZ*, in a *pCaSper* plasmid for generating the *hth^{MpT}-hth* rescue construct, or an *hth^{MpT}-lacZ* reporter construct.

Immunohistochemistry

Staining of whole-mount embryos was performed using standard techniques. The following primary antibodies were used: rabbit anti-Hth (1:500;

Kurant et al., 1998), rabbit anti-Tsh (1:3,000; Wu and Cohen, 2000), guinea pig anti-Odd (1:100, Kosman et al., 1998), rat anti-Pvr (1:100, Rosin et al., 2004), rabbit anti-Simu (1:5,000, Shklyar et al., 2013), mouse and rabbit anti-β-galactosidase (1:1,000, Promega and Cappel, respectively); mouse anti-Cut (mAb 2b10, 1:20), mouse anti-Exd (mAb B11M, 1:5) and mouse anti-Crumbs (mAb Cq4, 1:10) were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. Secondary antibodies for fluorescent staining were Cy3, or Cy5-conjugated anti-mouse/rabbit (Jackson Immunoresearch Laboratories). Stained embryos were mounted in Dako Fluorescent Mounting Medium (DakoCytomation, Glostrup, Denmark) and viewed using confocal microscopy (LSM 510, Zeiss). The secondary antibodies for non-fluorescent staining were biotinylated anti-mouse/rabbit detected with Vecta-Stain-Elite ABC-HRP kit (Vector Laboratories).

Live Imaging of Embryos

For imaging live embryos, embryos were collected, dechorionated in 50% bleach for 2 min, washed with water and quickly lined up on a 24 × 60 mm coverslip coated with scotch tape glue. Embryos were covered with 700-halocarbon oil and imaged using an inverted LSM510 Zeiss laser scanning confocal microscopy. Movies were generated by taking an image every 2–4 min over a 5- to 8-hr time period, using fast scanning and minimal laser intensity.

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REFERENCES

Abu-Shaar M, Mann RS. 1998. Generation of multiple antagonistic domains along the proximodistal axis during Drosophila leg development. Development 125:3821–3830.

Ainsworth C, Wan S, Skaer H. 2000. Coordinating cell fate and morphogenesis in Drosophila renal tubules. Philos Trans R Soc Lond B Biol Sci 355:931–937.

Aldaz S, Morata G, Azpiazu N. 2005. Patterning function of homothorax/extradenticle in the thorax of Drosophila. Development 132:439–446.

Ando M, Totani Y, Walldorf U, Furukubo-Tokunaga K. 2011. TALE-class homeodomain transcription factors, homothorax and extradenticle, control dendritic and axonal targeting of olfactory projection neurons in the Drosophila brain. Dev Biol 358:122–136.

Azpiazu N, Morata G. 2000. Function and regulation of homothorax in the wing imaginal disc of Drosophila. Development 127:2685–2693.

Azpiazu N, Morata G. 2002. Distinct functions of homothorax in leg development in Drosophila. Mech Dev 119:55–67.

Baek M, Enriquez J, Mann RS. 2013. Dual role for Hox genes and Hox cofactors in conferring leg motoneuron survival and identity in Drosophila. Development 140:2027–2038.

Baumann P, Skaer H. 1993. The Drosophila EGF receptor homologue (DER) is required for Malpighian tubule development. Dev Suppl 1993:65–75.

Bertet C, Sulak L, Lecuit T. 2004. Myosin-dependent junction remodelling controls planar cell intercalation and axis elongation. Nature 429:667–671.

Bryantsev AL, Duong S, Brunetti TM, Chechenova MB, Lovato TL, Nelson C, Shaw E, Uhl JD, Gebelein B, Cripps RM. 2012. Extradenticle and homothorax control adult muscle fiber identity in Drosophila. Dev Cell 23:664–673.

Bunt S, Hooley C, Hu N, Scahill C, Weavers H, Skaer H. 2010. Hemocyte-secreted type IV collagen enhances BMP signaling to guide renal tubule morphogenesis in Drosophila. Dev Cell 19:296–306.

Buszczak M, Paterno S, Lighthouse D, Bachman J, Planck J, Owen S, Skora AD, Nystul TG, Ohlstein B, Allen A, Wilhelm JE, Murphy TD, Levis RW, Matunis E, Srivali N, Hoskins RA, Spradling AC. 2007. The carnegie protein trap library: a versatile tool for Drosophila developmental studies. Genetics 175:1505–1531.

Cagan R. 2003. The signals that drive kidney development: a view from the fly eye. Curr Opin Nephrol Hypertens 12:11–17.

Campbell K, Casanova J, Skaer H. 2010. Mesenchymal-to-epithelial transition of intercalating cells in Drosophila renal tubules depends on polarity cues from epithelial neighbours. Mech Dev 127: 345–357.

Capovilla M, Botas J. 1998. Functional dominance among Hox genes: repression dominates activation in the regulation of Dpp. Development 125:4949–4957.

Casares F, Mann RS. 1998. Control of antennal versus leg development in Drosophila. Nature 392:723–726.

Casares F, Mann RS. 2000. A dual role for homothorax in inhibiting wing blade development and specifying proximal wing identities in Drosophila. Development 127:1499–1508.

Chintapalli VR, Wang J, Dow JA. 2007. Using FlyAtlas to identify better Drosophila melanogaster models of human disease. Nat Genet 39:715–720.

Costantini F, Kopan R. 2010. Patterning a complex organ: branching morphogenesis and nephron segmentation in kidney development. Dev Cell 18:698–712.

Dekel B, Metsuyanim S, Schmidt-Ott KM, Fridman E, Jacob-Hirsch J, Simon A, Pinthus J, Mor Y, Barasch J, Amariglio N, Reisner Y, Kaminski N, Rechavi G. 2006. Multiple imprinted and stemness genes provide a link between normal and tumor progenitor cells of the developing human kidney. Cancer Res 66:6040–6049.

Denholm B. 2013. Shaping up for action: the path to physiological maturation in the renal tubules of Drosophila. Organogenesis [Epub ahead of print].

Denholm B, Sudarsan V, Pasalodos-Sanchez S, Artero R, Lawrence P, Maddrell S, Baylies M, Skaer H. 2003. Dual origin of the renal tubules in Drosophila: mesodermal cells integrate and polarize to establish secretory function. Curr Biol 13:1052–1057.

Denholm B, Hu N, Fauquier T, Caubit X, Fasano L, Skaer H. 2013. The tiptop/teashirt genes regulate cell differentiation and renal physiology in Drosophila. Development 140:1100–1110.

Di Giacomo G, Koss M, Capellini TD, Brendolan A, Popperl H, Selleri L. 2006. Spatio-temporal expression of Pbx3 during mouse organogenesis. Gene Expr Patterns 6:747–757.

Dong PD, Chu J, Panganiban G. 2001. Proximodistal domain specification and interactions in developing Drosophila appendages. Development 128:2365–2372.

Dow JA. 2009. Insights into the Malpighian tubule from functional genomics. J Exp Biol 212:435–445.

Dow JA, Romero MF. 2010. Drosophila provides rapid modeling of renal development, function, and disease. Am J Physiol Renal Physiol 299:F1237–F1244.

Edwards KA, Demsky M, Montague RA, Weymouth N, Kiehart DP. 1997. GFP-moesin illuminates actin cytoskeleton dynamics in living tissue and demonstrates cell shape changes during morphogenesis in Drosophila. Dev Biol 191: 103–117.

Hatton-Ellis E, Ainsworth C, Sushama Y, Wan S, VijayRaghavan K, Skaer H. 2007. Genetic regulation of patterned tubular branching in Drosophila. Proc Natl Acad Sci U S A 104:169–174.

Henderson KD, Andrew DJ. 2000. Regulation and function of Scr, exd, and hth in the Drosophila salivary gland. Dev Biol 217:362–374.

Hoch M, Broadie K, Jackle H, Skaer H. 1994. Sequential fates in a single cell are established by the neurogenic cascade in the Malpighian tubules of Drosophila. Development 120:3439–3450.

Inbal A, Halachmi N, Dibner C, Frank D, Salzberg A. 2001. Genetic evidence for the transcriptional-activating function of Homothorax during adult fly development. Development 128:3405–3413.

Jack J, DeLotto Y. 1995. Structure and regulation of a complex locus: the cut gene of Drosophila. Genetics 139:1689–1700.

Jung AC, Denholm B, Skaer H, Affolter M. 2005. Renal tubule development in Drosophila: a closer look at the cellular level. J Am Soc Nephrol 16:322–328.

Kaufmann N, Mathai JC, Hill WG, Dow JA, Zeidel ML, Brodsky JL. 2005. Developmental expression and biophysical characterization of a Drosophila melanogaster aquaporin. Am J Physiol Cell Physiol 289:C397–C407.

Kosman D, Small S, Reinitz J. 1998. Rapid preparation of a panel of polyclonal antibodies to Drosophila segmentation proteins. Dev Genes Evol 208:290–294.

Kurant E, Eytan D, Salzberg A. 2001. Mutational analysis of the Drosophila homothorax gene. Genetics 157:689–698.

Kurant E, Pai CY, Sharf R, Halachmi N, Sun YH, Salzberg A. 1998. Dorsotonals/homothorax, the Drosophila homologue of meis1, interacts with extradenticle in patterning of the embryonic PNS. Development 125:1037–1048.

Liu S, Jack J. 1992. Regulatory interactions and role in cell type specification of the Malpighian tubules by the cut, Kruppel, and caudal genes of Drosophila. Dev Biol 150:133–143.

Lopes CS, Casares F. 2010. hth maintains the pool of eye progenitors and its downregulation by Dpp and Hh couples retinal fate acquisition with cell cycle exit. Dev Biol 339:78–88.

Marygold SJ, Leyland PC, Seal RL, Goodman JL, Thurmond J, Strelets VB, Wilson RJ,the FlyBase consortium. (2013). FlyBase: improvements to the bibliography. Nucleic Acids Res 41: D751–D757.

Mercader N, Leonardo E, Azpiazu N, Serrano A, Morata G, Martinez C, Torres M. 1999. Conserved regulation of proximodistal limb axis development by Meis1/Hth. Nature 402:425–429.

Miller J, Chi T, Kapahi P, Kahn AJ, Kim MS, Hirata T, Romero MF, Dow JA, Stoller ML. 2013. Drosophila Melanogaster as an Emerging Translational Model of Human Nephrolithiasis. J Urol [Epub ahead of print].

O'Donnell MJ, Maddrell SH. 1995. Fluid reabsorption and ion transport by the lower Malpighian tubules of adult female Drosophila. J Exp Biol 198: 1647–1653.

O'Donnell MJ, Rheault MR, Davies SA, Rosay P, Harvey BJ, Maddrell SH, Kaiser K, Dow JA. 1998. Hormonally controlled chloride movement across Drosophila tubules is via ion channels in stellate cells. Am J Physiol 274: R1039–R1049.

Pai CY, Kuo TS, Jaw TJ, Kurant E, Chen CT, Bessarab DA, Salzberg A, Sun YH. 1998. The Homothorax homeoprotein activates the nuclear localization of another homeoprotein, extradenticle, and suppresses eye development in Drosophila. Genes Dev 12:435–446.

Pütz M, Kesper DA, Buttgereit D, Renkawitz-Pohl R. 2005. In Drosophila melanogaster, the rolling pebbles isoform 6 (Rols6) is essential for proper Malpighian tubule morphology. Mech Dev 122:1206–1217.

Rieckhof GE, Casares F, Ryoo HD, Abu-Shaar M, Mann RS. 1997. Nuclear translocation of extradenticle requires homothorax, which encodes an extradenticle-related homeodomain protein. Cell 91:171–183.

Roberts VJ, van Dijk MA, Murre C. 1995. Localization of Pbx1 transcripts in developing rat embryos. Mech Dev 51: 193–198.

Rosin D, Schejter E, Volk T, Shilo BZ. 2004. Apical accumulation of the Drosophila PDGF/VEGF receptor ligands provides a mechanism for triggering localized actin polymerization. Development 131:1939–1948.

Salvany L, Aldaz S, Corsetti E, Azpiazu N. 2009. A new role for hth in the early pre-blastodermic divisions in drosophila. Cell Cycle 8:2748–2755.

Schedl A. 2007. Renal abnormalities and their developmental origin. Nat Rev Genet 8:791–802.

Schnabel CA, Selleri L, Jacobs Y, Warnke R, Cleary ML. 2001. Expression of Pbx1b during mammalian organogenesis. Mech Dev 100:131–135.

Schnabel CA, Godin RE, Cleary ML. 2003. Pbx1 regulates nephrogenesis and ureteric branching in the developing kidney. Dev Biol 254:262–276.

Shklyar B, Levy-Adam F, Mishnaevski K, Kurant E. 2013. Caspase activity is required for engulfment of apoptotic cells. Mol Cell Biol [Epub ahead of print].

Singh A, Tare M, Kango-Singh M, Son WS, Cho KO, Choi KW. 2011. Opposing interactions between homothorax and Lobe define the ventral eye margin of Drosophila eye. Dev Biol 359:199–208.

Singh SR, Hou SX. 2008. Lessons learned about adult kidney stem cells from the malpighian tubules of Drosophila. J Am Soc Nephrol 19:660–666.

Skaer H. 1989. Cell division in Malpighian tubule development in D. melanogaster is regulated by a single tip cell. Nature 342:566–569.

Skaer H, Martinez Arias A. 1992. The wingless product is required for cell proliferationin the Malpighian tubules anlage of Drosophila melanogaster. Development 116:745–754.

Sözen MA, Armstrong JD, Yang M, Kaiser K, Dow JA. 1997. Functional domains are specified to single-cell resolution in a Drosophila epithelium. Proc Natl Acad Sci U S A 94:5207–5212.

Stultz BG, Jackson DG, Mortin MA, Yang X, Beachy PA, Hursh DA. 2006. Transcriptional activation by extradenticle in the Drosophila visceral mesoderm. Dev Biol 290:482–494.

Subramanian A, Prokop A, Yamamoto M, Sugimura K, Uemura T, Betschinger J, Knoblich JA, Volk T. 2003. Shortstop recruits EB1/APC1 and promotes microtubule assembly at the muscle-tendon junction. Curr Biol 13:1086–1095.

Sudarsan V, Pasalodos-Sanchez S, Wan S, Gampel A, Skaer H. 2002. A genetic hierarchy establishes mitogenic signalling and mitotic competence in the renal tubules of Drosophila. Development 129:935–944.

Tena JJ, Neto A, de la Calle-Mustienes E, Bras-Pereira C, Casares F, Gomez-Skarmeta JL. 2007. Odd-skipped genes encode repressors that control kidney development. Dev Biol 301:518–531.

Wan S, Cato AM, Skaer H. 2000. Multiple signalling pathways establish cell fate and cell number in Drosophila malpighian tubules. Dev Biol 217:153–165.

Wang J, Kean L, Yang J, Allan AK, Davies SA, Herzyk P, Dow JA. 2004. Function-informed transcriptome analysis of Drosophila renal tubule. Genome Biol 5:R69.

Ward EJ, Coulter DE. 2000. odd-skipped is expressed in multiple tissues during Drosophila embryogenesis. Mech Dev 96:233–236.

Weavers H, Prieto-Sanchez S, Grawe F, Garcia-Lopez A, Artero R, Wilsch-Brauninger M, Ruiz-Gomez M, Skaer H, Denholm B. 2009. The insect nephrocyte is a podocyte-like cell with a filtration slit diaphragm. Nature 457:322–326.

Wellik DM. 2011. Hox genes and kidney development. Pediatr Nephrol 26:1559–1565.

Wernet MF, Labhart T, Baumann F, Mazzoni EO, Pichaud F, Desplan C. 2003. Homothorax switches function of Drosophila photoreceptors from color to polarized light sensors. Cell 115:267–279.

Wessing A, Eichelberg D. 1978. Malpighian tubules, rectal papillae and excretion. In: Ashburner, M, Wright, TRF, editors. The genetics and biology of Drosophila. London: Academic Press.

Wu J, Cohen SM. 1999. Proximodistal axis formation in the Drosophila leg: subdivision into proximal and distal domains by Homothorax and Distalless. Development 126:109–117.

Wu J, Cohen SM. 2000. Proximal distal axis formation in the Drosophila leg: distinct functions of teashirt and homothorax in the proximal leg. Mech Dev 94:47–56.