The tiptop/teashirt genes regulate cell differentiation and renal physiology in Drosophila

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SUMMARY
The physiological activities of organs are underpinned by an interplay between the distinct cell types that contain. However, little is known about the genetic control of patterned cell differentiation during organ development. We show that the conserved Teashirt transcription factors are decisive for the differentiation of a subset of secretory cells, stellate cells, in Drosophila melanogaster renal tubules. Teashirt controls the expression of the water channel Drip, the chloride conductance channel CLC-a and the Leukokinin receptor (LKR), all of which characterise differentiated stellate cells and are required for primary urine production and responsiveness to diuretic stimuli. Teashirt also controls a dramatic transformation in cell morphology, from cuboidal to the stella te shape, during metamorphosis. teashirt interacts with cut, which encodes a transcription factor that underlies the differentiation of the primary, principal secretory cells, establishing a reciprocal negative-feedback loop that ensures the full differentiation of both cell types. Loss of teashirt leads to ineffective urine production, failure of homeostasis and premature lethality. Stellate cell-specific expression of the teashirt paralogue tiptop, which is not normally expressed in larval or adult stellate cells, almost completely rescues teashirt loss of expression from stellate cells. We demonstrate conservation in the expression of the family of tiptop/teashirt genes in lower insects and establish conservation in the targets of Teashirt transcription factors in mouse embryonic kidney.

KEY WORDS: Cell differentiation, Drosophila, Kidney, Malpighian tubule, Organogenesis, Tiptop/Teashirt

INTRODUCTION
Organs are assemblies of differentiated cells, arranged into distinct configurations that allow them to carry out specialised functions. Specific tasks carried out by a particular organ are emergent properties that result from the coordinated interplay of the different specialised cell types they contain. Although we have extensive understanding of the physiological activities of organs at multiple levels, we know far less about the developmental mechanisms and genetic networks that bring about physiological maturation. For example, how are physiologically distinct cell types, which differ in gene expression, shape, location and function, established during organogenesis? These are important and fundamental questions, as these processes ultimately underpin integrated function for all organs. Here, we investigate the developmental genetic networks that establish secretory function in the Drosophila melanogaster renal tubule.

The role of the excretory system of an animal is to remove harmful substances from the body and to regulate ionic, acid-base and fluid balance. In insects, the renal or Malpighian tubules (MpTs) – a set of simple epithelial tubes – carry out these activities. Studies of renal tubule physiology have provided insight into the mechanisms underlying the clearance of toxins and regulation of urine production and modification (Beyenbach et al., 2010; Dow and Davies, 2001; Dow and Davies, 2003; Maddrell, 1981; Wessing and Eichelberg, 1978). Primary urine is secreted by main segment cells, driven by ion transport across the epithelium (Fig. 1). In Drosophila two physiologically distinctive cell types drive this process: principal cells (PCs or Type I cells), and stellate cells (SCs or Type II cells). PCs transport potassium ions, establishing a favourable electrochemical gradient that allows chloride ion movement through channels in SCs; water follows by osmosis facilitated by aquaporin water channels also in SCs (Fig. 1B) (Kaufmann et al., 2005; O’Donnell et al., 1998). The volume of urine production is regulated by internal physiology and environmental conditions through haemolymph-borne hormonal signals (Coast et al., 2001; Maddrell et al., 1991; Terhzaz et al., 1999). For example, PCs are activated by both cGMP- and cAMP-mediated pathways (Cabrero et al., 2002; Johnson et al., 2005; Kean et al., 2002), whereas in SCs leukokinin acts on its receptor (LKR), which elevates the chloride conductance of SCs (Fig. 1B) (reviewed by Beyenbach et al., 2010).

The developmental origins of PCs and Type II cells differ. PCs derive from epithelial primordia that bud out from the embryonic hindgut. Type II cells originate from mesenchymal cells that integrate into the tubule epithelium during mid-embryogenesis, differentiating as SCs in the secretory region and as bar cells in the initial and transitional segments (Denholm et al., 2003) (Fig. 1A,B). SCs become spaced in a regular pattern between PCs and can be distinguished by their smaller nuclear size. teashirt (tsh), which encodes a zinc-finger transcription factor, is expressed in SCs as they integrate and is an early marker that distinguishes them from the PCs. We therefore reasoned that tsh might regulate SC differentiation. Previous reports indicate that the tsh paralogue tiptop (tio) is also expressed in embryonic fly MpTs (Laugier et al., 2005) and that a tio/tsh orthologue is expressed in the developing MpTs of the beetle Tribolium castaneum (Shippy et al., 2008), further suggesting that these genes have important conserved roles in the development and/or function of insect MpTs.

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Drosophila tsh and tio are paralogous genes that encode zinc-finger transcription factors (Laugier et al., 2005). The gene pair is present in the genomes of all Drosophila species sequenced to date (Clark et al., 2007) but only as a single gene in other insects (including the closely related Diptera Anopheles gambiae and Aedes aegypti), suggesting a recent duplication event. Comparison of the genes among insects reveals that tio is the more ancestral gene, with tsh possessing more derived characteristics (Datta et al., 2011a; Datta et al., 2011b; Herke et al., 2005; Santos et al., 2010; Shippy et al., 2008). In the embryo, tsh promotes trunk segmental identities (Fasano et al., 1991) and contributes to the specification and patterning of other tissues, such as the salivary glands and midgut (Henderson et al., 1999; Mathies et al., 1994). Later, tsh contributes to the specification and patterning of adult appendages, including the leg, wing and eye (Bessa et al., 2009; Bessa and Casares, 2005; Bessa et al., 2002; Erkner et al., 1999; Singh et al., 2004; Singh et al., 2002; Soanes et al., 2001; Sun et al., 1995; Wu and Cohen, 2000; Wu and Cohen, 2002). In many tissues, tio acts redundantly with tsh so that flies lacking tio function are viable without obvious phenotypes.

Vertebrate teashirt family genes (Tshz) have been identified in humans, mice, chick, zebrafish and frog (Santos et al., 2010), where they act to pattern multiple tissues during embryogenesis (Caubit et al., 2008; Caubit et al., 2010; Erickson et al., 2011; Faralli et al., 2011; Koebertnick et al., 2006). Human TSHZ genes have proven or putative roles as disease loci, including juvenile angiofibroma, congenital aural atresia, congenital pelvi-ureteric junction obstruction, and breast and prostate cancers (Caubit et al., 2008; Feenstra et al., 2011; Jenkins et al., 2010; Schick et al., 2011; Yamamoto et al., 2011), although their precise roles in normal development and disease have not been established. Mouse Tshz genes are able to rescue the loss of trunk identity in Drosophila melanogaster (Manfroid et al., 2004), suggesting conservation over a wide evolutionary range.

Here, we characterise the genetic network that underpins physiological maturation in the MpTs. We find that tsh is a principal component of the SC differentiation hierarchy, controlling SC shape and the expression of genes required for terminal physiological differentiation. We show how tsh activity in SCs translates into integrated organ function and how this, in turn, influences the physiology of the whole animal, providing comprehensive insight into the function of Tsh-family transcription factors at multiple biological levels. We provide evidence that tio/tsh function in MpTs is conserved between insects separated in evolution by over 360 million years and, by extending our work to identify Tshz3 targets in the mouse kidney, reveal that some downstream components of the teashirt genetic network are shared between invertebrates and vertebrates.

**MATERIALS AND METHODS**

**Fly stocks**

Flies were cultured on standard media at 18°C or 25°C with ectopic expression at 25°C or 29°C. The following stocks were used: UAS-tsh-RNAi; P[TRIP1F02856]attP2 (Bloomington 28022); c724-Gal4 (Sözen et al., 1997); tsh; tio21; Df(2L)JF02856; UAS-CD8-GFP; LKR protein trap [FlyTrap YD0927 (Quiñones-Coello et al., 2007)]; Ctb-Gal4 (Sudarsan et al., 2002); UAS-tsh (Datta et al., 2009), original source A. Courey, UCLA, CA, USA. Embryos were collected on apple juice-agar plates.

**Generation of tsh tio double mutant**

We generated a 5 kb deficiency in tio (tio21) (supplementary material Fig. S1) by imprecise excision of the P-element tio-Gal444 (Tang and Sun, 2002). We then mobilised a w- P-element in a tio21 mutant background and screened for potential new insertions at the tsh locus using the characteristic ‘graded-eye’ phenotype (dark red fading to white along the anterior-posterior axis). P-element rescue confirmed that one insertion (tsh21) was in tsh (supplementary material Fig. S1). However, this line was homozygous viable with normal tsh expression. We therefore created an imprecise excision of this line to create a deficiency in tsh. Breakpoints were mapped by PCR from single homozygous mutant embryos using paired primers at ~10 kb intervals across the entire tsh-tio locus. This compound deficiency, Df(2L)JF02856 (supplementary material Fig. S1), removes seven protein-coding and one non-protein-coding genes between tsh and
tio. We confirmed that protein expression is abolished in Df(2L)tt by staining for anti-Tsh and anti-Tio (supplementary material Fig. S1).

**Tubule secretion assay**

Secretion assays were performed as described previously (Dow et al., 1994) at 23-25°C using 3- to 5-day-old adult flies. cAMP and LK (Sigma) were added at a final concentration of 1 mM and 100 μM, respectively, after ~30 and 60 minutes.

**Lethal phase analysis**
c724-Gal4 >tsh-RNAi (n=900) or control (c724-Gal4 alone, n=600) embryos were collected in batches of 100 and maintained at 25°C. Surviving animals were counted as: first instar hatchlings, pupae and eclosing adults.

**Fly weight measurements**

To measure wet-body weight, flies were anaesthetized with CO2, transferred to Eppendorf tubes on ice and weighed on a Mettler Toledo precision balance in triplicate. For dry-body weight, flies were sacrificed by freezing to −80°C and weighed on a Mettler Toledo precision balance.

**In situ hybridisation**

In situ hybridisation was carried out using digoxigenin-labelled RNA probes to embryonic and adult tissues as described previously (Denholm et al., 2005). To make the probes, we used RE60324 (Drip) and RE62514 (CIG-a). Embryos were dehydrated and mounted in Durcupan; adult tubules were mounted directly in 50% glycerol.

**Immunohistochemistry**

Antibody staining was carried out under standard conditions. Larval and adult tubules were dissected in ice-cold PBS and fixed (4%) paraformaldehyde/PBS on ice for up to 30 minutes, followed by 20 minutes at room temperature. The following antibodies were used: rabbit anti-Tsh (1:3000, S. Cohen, IMCB, Singapore), rat anti-Tio (1:500) (Laugier et al., 2005), mouse anti-Cut (1:100, DSHB), goat anti-GFP (1:500, Abcam) and mouse anti-Discs large (1:1000, DSHB). Appropriate biotinylated secondary antibodies were used with the Vector Elite ABC Kit (Vector Laboratories, CA, USA) for DAB staining. FITC- or Cy3-conjugated secondary antibodies were used with the Vector Elite ABC Kit (Vector Laboratories, CA, USA) for DAB staining. FITC- or Cy3-conjugated secondary antibodies were used with the Vector Elite ABC Kit (Vector Laboratories, CA, USA) for DAB staining. FITC- or Cy3-conjugated secondary antibodies were used with the Vector Elite ABC Kit (Vector Laboratories, CA, USA) for DAB staining. FITC- or Cy3-conjugated secondary antibodies were used with the Vector Elite ABC Kit (Vector Laboratories, CA, USA) for DAB staining. FITC- or Cy3-conjugated secondary antibodies were used with the Vector Elite ABC Kit (Vector Laboratories, CA, USA) for DAB staining. FITC- or Cy3-conjugated secondary antibodies were used with the Vector Elite ABC Kit (Vector Laboratories, CA, USA) for DAB staining. FITC- or Cy3-conjugated secondary antibodies were used with the Vector Elite ABC Kit (Vector Laboratories, CA, USA) for DAB staining. FITC- or Cy3-conjugated secondary antibodies were used with the Vector Elite ABC Kit (Vector Laboratories, CA, USA) for DAB staining. FITC- or Cy3-conjugated secondary antibodies were used with the Vector Elite ABC Kit (Vector Laboratories, CA, USA) for DAB staining. FITC- or Cy3-conjugated secondary antibodies were used with the Vector Elite ABC Kit (Vector Laboratories, CA, USA) for DAB staining. FITC- or Cy3-conjugated secondary antibodies were used with the Vector Elite ABC Kit (Vector Laboratories, CA, USA) for DAB staining. FITC- or Cy3-conjugated secondary antibodies were used with the Vector Elite ABC Kit (Vector Laboratories, CA, USA) for DAB staining.
RESULTS

Teashirt and Tiptop expression in Drosophila melanogaster Malpighian tubules

Tsh expression is first detected at low levels in a subpopulation of tubule cells at embryonic stage 13 (Fig. 2A,B). Based on their position on the posterior-facing side of the tubule (Denholm et al., 2003), we identify these cells as SCs. Tsh expression increases so that by stage 16, high levels are found in the entire SC population (Fig. 2C), which persists in larval and adult SCs (Fig. 2I,J). Tsh is not expressed in PCs at any stage (Fig. 2A-C,I,J). Expression of the tsh paralogue tio is found in a subset of PCs at stages 11 to 13 (Fig. 2D,E). Tio is first detected in SCs cells at stage 13 (Fig. 2F,G), overlapping completely with Tsh when its expression in PCs begins to fade. By stage 16, Tio is restricted to SCs (Fig. 2F), which persists in larval and adult SCs (Fig. 2I,J). Tsh is required in SCs for organ function because tsh mutants die as embryos (due to extra-renal defects) (Fasano et al., 1991), we used RNAi to knock down tsh in SCs using the tsh-GAL4, c724 (Sözen et al., 1997), which reflects a subset of tsh expression, including SCs from late embryonic stages throughout life (c724-GAL4>UAS-tshRNAi, termed tsh knockdown). Anti-Tsh staining of adult tubules reveals complete knock down using this approach (supplementary material Fig. S3).

We compared tubule secretion in control and tsh knockdown tubules using an established in vitro assay (Dow et al., 1994; Ramsay, 1954) that measured: (1) basal secretory rates and (2) secretory rates after the addition of cAMP, an agonist that stimulates cation transport through secretory PCs, and (3) after further addition of leucokinin, an agonist that stimulates chloride flux through SCs (Fig. 1B; Fig. 3A). Basal secretory rates were significantly lower for tsh knockdown tubules than for wild type [peak secretation rates of 0.21±0.03 (s.e.m.) nl min⁻¹, n=10 versus 0.48±0.06 nl min⁻¹, n=9]. Addition of cAMP increased secretory rates in knockdown tubules; however, peak rates were significantly lower than wild type [0.38±0.04 (s.e.m.) nl min⁻¹ versus 0.69±0.05 nl min⁻¹]. By contrast, leucokinin in addition to cAMP, which doubled secretory rate in wild-type tubules, had no further stimulatory effect in tsh knockdown adults. (Fig. 3.)

Fig. 3. Disrupted organ function and animal physiology in tsh knockdown flies. (A) Secretory rates (nl min⁻¹) in control (purple) and tsh knockdown (yellow) MtTs. Cyclic adenosine monophosphate (cAMP) and Leucokinin (LK) were added at ~30 and 60 minutes, respectively (arrows). Basal secretion rates are lower, and the response to LK is abolished in tsh knockdown tubules. (B) Uric acid crystals accumulate in the MtT lumen in the tsh knockdown. (C) Survival rates of control (purple, n=600) and tsh knockdown (yellow, n=900), nine replicates) animals from embryonic to pupal stages. The main lethal phase in tsh knockdown occurs during pupation. (D) Survival rates for control (purple, n=105) and tsh knockdown (yellow, n=140) adults. (E) Control (left) and tsh knockdown (right) 1-week-old adults. tsh knockdown adults have grossly distended abdomens. (F) Wet and dry weight measurements (mg; three flies/measurement) for adults: control (purple, n=13); tsh knockdown (yellow, n=26). Data are for females (equivalent results for males not shown).
knockdown tubules, with peak secretory rates remaining at 0.39±0.05 (s.e.m.) nl min⁻¹ compared with 1.2±0.05 nl min⁻² for wild-type (Fig. 3A). Compromised secretion for tsh knockdown tubules in intact animals is also suggested by the accumulation of high levels of luminal uric acid crystals that would normally be flushed away by urine flow (Fig. 3B). These data show that fluid secretion—a direct measure of tubule function—is significantly reduced in tsh knockdown tubules. We conclude that tsh activity in SCs is essential for the effective secretion of primary urine by MpTs.

**tsh is required in SCs for full viability**

To determine whether tsh knockdown in SCs affected whole-animal physiology, we compared viability between tsh knockdown and control (c724-Gal4 alone) flies. Embryonic and larval viability (number of eggs hatching and of larvae reaching pupation) is comparable for tsh knockdown and controls (73% versus 75% hatch and 66% versus 64% reach pupation, n=900 and 600, respectively). By contrast, only 12% of tsh knockdown animals emerge as adults compared with 56% of controls (Fig. 3C) and adult survival is strongly reduced (Fig. 3D). These data indicate that tsh in SCs is required for full viability during pupal and adult stages. As c724, although specific for tubules, is active in other tissues, such as the wing hinge (Soanes and Bell, 1999; Soanes et al., 2001), we cannot exclude the possibility that tsh knockdown elsewhere contributes to reduced viability.

**tsh activity in SCs is required for normal fluid balance**

Strikingly, tsh knockdown adults develop grossly distended abdomens within a few days of eclosion (Fig. 3E), a phenotype symptomatic of excess food intake (Al-Anzi et al., 2010), build up of internal gas or excess haemolymph volume, resulting from defective osmoregulation. We confirmed that the abdominal bloating was due to fluid retention in two ways. First, pricking submerged flies led to abdominal deflation without gas bubbles (data not shown). Second, wet weight measurements reveal that tsh knockdown adults are approximately twice as heavy as control flies, whereas dry weight measurements are equivalent, eliminating excess food intake as a cause of bloating (Fig. 3F). Together, these data show that fluid retention results from tsh activity in SCs. We conclude that tsh activity in SCs is essential for the effective secretion of primary urine by MpTs.

**Tsh regulates multiple features of the SC phenotype**

**Regulation of terminal gene expression**

Next, we explored the role of tsh in SCs at the cellular level. We dismissed the simple hypothesis that a reduction in SC numbers caused the physiological defects in tsh knockdown flies; on average, each adult anterior tubule contained 34±1.5 (s.e.m.) c724-positive cells (n=12) and each posterior tubule contained 20±1 (n=10), compared with 33±1 (n=7) in each anterior and 20±1 (n=6) in each posterior tubule in wild type. Furthermore, the spacing of SCs in knockdown tubules was indistinguishable from wild type (Fig. 4A,B).

As tsh encodes a transcription factor, it could regulate the expression of genes that define SCs. We chose three candidates known to be highly expressed and/or to have well-defined activities in SCs [Leucokinin receptor (Lkr) (Radford et al., 2002); Chloride channel-α (CIC-α) (Wang et al., 2004); and the aquaporin water channel (Drip) (Kaufmann et al., 2005)] (Fig. 1B). We compared expression in wild-type versus tsh knockdown adult tubules by in situ hybridisation (for Lkr, CIC-α and Drip) and immunostaining a protein-trap line (for LKR; http://flytrap.med.yale.edu/) (Quiñones-Coello et al., 2007). In agreement with previous reports, Lkr, Drip and CIC-α are expressed in adult SCs (Fig. 4C,E,G) (Kaufmann et al., 2005; Radford et al., 2002; Wang et al., 2004). By contrast, Lkr, Drip and CIC-α expression were completely abolished in tsh knockdown SCs. Arrowheads in E-G (L-L) tsh and tio regulate CIC-α expression redundantly in embryonic SCs. CIC-α expression in embryonic SCs is unaffected in either tsh or tio mutants (arrowheads, I-K), but completely abolished in tsh tio double mutants (arrowhead indicates tubule position, L). Scale bars: 30 μm in A-H; 50 μm in I-L.

**Fig. 4. tsh regulates terminal differentiation gene expression.**

(A,B) SC number and spacing is normal in control (A) and tsh knockdown (B) adult MpTs. SCs marked with c724>UAS-nlacz2 (red), tubules counterstained for DNA (blue). (C-H) tsh regulates SC gene expression inMpTs. Adult MpTs from control or wild-type (C,E,G), or tsh knockdown (D,F,H) animals. (C,D) LKR-GFP (LKR protein trap), (E,F) Drip and (G,H) CIC-α in situ hybridisation. LKR, Drip and CIC-α expression is completely abolished in tsh knockdown SCs. Arrowheads in E-G (L-L) tsh and tio regulate CIC-α expression redundantly in embryonic SCs. CIC-α expression (in situ hybridisation) in stage 17 embryos in wild type (I), tsh mutant (J), tio mutant (K) and tsh tio double mutant (L). CIC-α expression in embryonic SCs is unaffected in either tsh or tio mutants (arrowheads, I-K), but completely abolished in tsh tio double mutants (arrowhead indicates tubule position, L). Scale bars: 30 μm in A-H; 50 μm in I-L.
examined embryos mutant for the amorphic tsh⁸ allele. Interestingly, CIC-a expression in SCs is unaltered in tsh⁸ embryos (Fig. 4J). As tio is also expressed in SCs at this stage, it might contribute to CIC-a regulation in a redundant fashion with tsh. CIC-a is still expressed in SCs in amorphic tio⁴⁲⁷ embryos (Fig. 4K); however, in tsh tio double mutants, CIC-a expression is completely abolished (Fig. 4L), demonstrating that tsh and tio have overlapping activities in the control of gene expression in embryonic SCs.

Regulation of cell shape
In wild-type animals, stellate and bar-shaped cells undergo a dramatic morphogenetic transformation during pupation from the cuboidal shape in larvae (Fig. 2K) to either a stellate-shaped (main segment; Fig. 2J,L; Fig. 5A,E) or bar-shaped (initial and transitional segments; Fig. 5B) morphology in adult tubules. Strikingly, these morphogenetic transformations do not occur in tsh knockdown; cells remain cuboidal both in the main and initial-transitional regions of adult tubules (Fig. 5C,D,F). Three-dimensional surface rendering reveals the full extent of shape differences between control and tsh knockdown SCs (Fig. 5E,F). As SCs provide the main transcellular passage for chloride ions and water, their large surface area is needed to support plasma membrane-resident chloride and water channels. tsh knockdown SCs have less than half the surface area of control SCs (Fig. 5G). This reduction in SC surface area could contribute to the poor physiological performance of tsh knockdown tubules (Fig. 3A).

Together, these data show that tsh contributes to cell differentiation by regulating multiple and diverse aspects of the cell phenotype, including cell morphology and the expression of terminal differentiation genes that have crucial physiological activities in SCs.

SC expression of tio rescues tsh knockdown
We asked whether SC expression of tio rescues any of the tsh knockdown phenotypes. We analysed the expression of Drip, CIC-a and Lkr-GFP in adult tubules of animals in which tsh-RNAi and tio had been driven with c724Gal4. tio is expressed strongly in SCs of these animals whereas Tsh is undetectable (Fig. 6A). The expression of Lkr-GFP and Drip is fully restored (Fig. 6B,C), but CIC-a is only weakly expressed in a subset of SCs. (Fig. 6D). However, SCs expressing tio show a clear stellate morphology (Fig. 6A'), indicating that the cell shape change can be driven by either tio or tsh. Adults that emerge are not bloated (Fig. 6E) and viability is partially rescued; 22% of tio rescued animals eclose compared with 12% of tsh knockdown (Fig. 6F).

These results show that although tio is not required for SC differentiation, it is sufficient for multiple aspects of SC maturation. However, full differentiation of SCs, including robust expression of CIC-a, and complete rescue of lethality (Fig. 6F) is specifically dependent on tsh.

Tsh acts through a transcription factor network to control cell differentiation
In the adult tubules of wild-type flies, tsh expression is restricted to SCs, whereas cut expression is restricted to PCs, suggesting mutual antagonism between these transcription factors (Fig. 7). To test whether Tsh represses cut expression in SCs, we examined Cut expression in tsh-knockdown tubules. Although cut is not expressed in wild-type SCs (Fig. 7A,B), its expression is ectopically induced to a level found in PCs after tsh knockdown (Fig. 7C). Thus, one function of Tsh is to repress cut expression in SCs. Furthermore, ectopic expression of Tsh in PCs (C18-Gal4 > UAS-tsh, which induces a mosaic of tsh-expressing PCs) results in loss of Cut in cells expressing tsh (Fig. 7D), suggesting that Tsh represses cut expression. As tsh and tio act redundantly in the SCs of embryonic tubules, we used the same driver to express tio in PCs. Tio is also able to repress cut, and the degree of repression is proportional to the level of Tio induced (Fig. 7E).

We next asked whether Cut normally represses tsh expression in PCs. In embryos mutant for cut, SCs are not recruited to the developing tubules (Campbell et al., 2009) and morphogenesis is defective so that PCs form a blister associated with the hindgut (Hatton-Ellis et al., 2007). These mispositioned PCs do not express cut (Campbell et al., 2009); however, if we drive ectopic cut expression in SCs (c724 > UAS-cut) tsh is repressed (Fig. 7F). Thus, although the presence of Cut is sufficient to repress tsh expression in SCs, factors other than Cut prevent tsh expression in developing PCs. Together, these data establish a negative cross-regulatory network between cut and tsh, in which it is crucial to exclude the expression of cut in SCs and of tsh in PCs (Fig. 7K).

We asked whether the decisive role of Tsh in SC differentiation is mediated through its repression of cut. We therefore analysed whether PCs in cut mutants express SC differentiation genes and found that CIC-a is not expressed (compare Fig. 7G with Fig. 4I).

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**See more details in the image**
We then asked whether ectopic *tsh*, and the consequent repression of *cut*, is sufficient to induce SC differentiation in PCs. In some cases, PC shape is transformed by ectopic *tsh* expression, leading to a more bar-shaped morphology (Fig. 7H,I) but we found no upregulation of genes normally expressed in SCs, such as LKR (Fig. 6D). These data suggest that although ectopic *Tsh* represses the expression of *cut* in PCs, loss of *cut* alone is not sufficient to induce SC differentiation.

Taken together, our data reveal that Tsh is a key factor in SC differentiation, acting at multiple levels that include the repression of *cut* (and thereby PC differentiation) and, independently, the induction of SC gene expression and morphology (Fig. 7K).

**Conservation of tsh gene function**

Morphologically distinct Type I and II MpT cells are widespread in insects (reviewed by Dow, 2012), suggesting that the segregation of physiological activities between different cell types is an ancient and conserved feature. To determine whether *tio/tsh* is expressed in Type II cells in other insect species, we chose the beetle *Tribolium castaneum* as a coleopteran [in which the *tio/tsh* orthologue is expressed in embryonic tubules (Shippy et al., 2008)] and the cricket *Gryllus bimaculatus* as an orthopteran representative. These species are distantly related to *Drosophila melanogaster* (a dipteran), sharing a common ancestor ~280 (beetle) and 360 (cricket) million years ago. Using the *Drosophila* Tio antibody, we probed the pattern of expression in adult tubules and found that the *tio/tsh* orthologue is expressed in the tubules of both *Tribolium* and *Gryllus* in a subset of cells with smaller nuclei than their neighbours and spaced in the same way as SCs in *Drosophila* tubules (Fig. 8A,B). These results reveal the presence of two cell subtypes in *Tribolium* and *Gryllus* tubules, and suggest that the differentiation of one of them (putative Type II cells) is under the control of *tio/tsh*. Together, our data imply that the presence of physiologically distinctive Type II cells and the control of their differentiation by the *tio/tsh* gene family are ancestral features of insect MpTs.

We have already shown that one of the vertebrate orthologues of the *tio/tsh* genes, *Tshz3*, is expressed in the ureteric mesenchyme around the ureteric duct of embryonic mouse kidney, where it is required for the differentiation of smooth muscle (SM) cells (Caubit et al., 2008). At the onset of the myogenic programme [embryonic day (E) 14.5], *Tshz3* is required *in vivo* for the expression of myocardin (*Myocd*), which enhances transcription of genes coding for smooth muscle cell contractile proteins [e.g. Smooth muscle actin alpha (SMAA) (Caubit et al., 2008)]. To characterise further the role of *Tshz3* in ureteric mesenchyme cell differentiation, we performed microarray expression profiling experiments by directly comparing samples of wild-type and *Tshz3*−/− mutant ureters at E14.5. To identify transcripts downregulated in the SM compartment of *Tshz3* mutant ureters at the onset of the myogenic programme, transcripts were ranked according to their P-value and we selected those with a P-value lower or equal to that of *Sm22*. The data filtering resulted in a list of 676 genes, representing 4.6% of 14,579 validated transcripts. The selected genes were tabulated in a descending order of the fold-change values. Among the 20 most downregulated genes are *Myocd* and several genes induced during smooth muscle differentiation [Cnn1, *Myh11*, Actg2, Acta2 (*Sma-alpha*), Transgelin (*Sm22-alpha*)], indicating that this approach identifies a set of *Tshz3* target genes in the SM layer of the ureter (supplementary material Table S1). This set included the Aquaporin water conductance channel (AQ1P1) (supplementary material Table S1). As an independent experimental validation of the microarray analysis, we assessed the expression of AQ1P1 expression by immunofluorescence in E18.5 ureters in heterozygous control and *Tshz3* mutant mice. In wild-type or control ureters, AQ1P1 is expressed in the stromal layer, smooth muscle layer and outer mesenchymal layers (Fig. 8C,F). However, AQ1P1 expression in the smooth muscle layer, where *Tshz3* activity is known to be required for tissue differentiation (Caubit et al., 2008), is significantly reduced in *Tshz3* mutants compared with heterozygous controls (Fig. 8D,F). Thus, the regulatory activity of *Tshz3* in the mouse kidney shows parallels with the activity of *tsh* in insect MpTs in regulating specialised renal cell differentiation.

**DISCUSSION**

Together our results reveal a decisive role for *teashirt* in the differentiation of a physiologically distinctive cell type in the
secretory region of the MpTs. tsh activity is required in Type II cells for the expression of genes that are key to their function, including chloride conductance (ClC-a), water conductance (Drip) and diuretic ligand sensitivity (Lkr). In addition, the eponymous cell shape changes that occur during pupation are abolished in the absence of tsh; SCs remain cuboidal, failing to extend arms around their PC neighbours to produce their stellate/bar shapes. Collectively, these defects in SC differentiation lead to a substantial reduction in viability, which we suggest is caused by defective excretion, as animals become bloated with fluid and the tubules fail to respond to diuretic stimuli, producing only very low levels of secreted primary urine.

Despite this prominent role for tsh in SC differentiation, it is clear that the emergence of Type II cells in the evolution of renal tubules was not dependent on the gene duplication that gave rise to tsh. Many insects with Type II cells, such as Calliphora (Berridge and Oschman, 1969), Periplaneta (Wall et al., 1975), Carausius (Taylor, 1971) and Aedes (O’Connor and Beyenbach, 2001), have only one tio/tsh family member. Furthermore, these cells have, in some cases, been shown to develop a stellate morphology in adult tubules. The function of Type II cells is not well understood in many species (see Taylor, 1971; Wessing et al., 1999), but in the mosquitoes Aedes and Anopheles, Type II cells show functional parallels with Drosophila SCs (reviewed by Beyenbach et al., 2010). Thus, aspects of the SC phenotype can develop and differentiate without the duplicated tsh gene. Our demonstration that the tio/tsh gene is expressed in a regularly spaced subset of tubule cells with small nuclei in both Tribolium and Gryllus suggests an evolutionarily conserved function for tsh-family members in Type II cell differentiation. We propose that the ancestral insect MpT contained two physiologically distinct cell types, where the differentiation of cells conducting anions and water was regulated by a Tio/Tsh-like transcription factor. We suggest that this state has been maintained in the tubules of most insects today, but in Drosophila, where a recent duplication has taken a predominant role. It will be interesting to determine whether the single tio/tsh gene is expressed in the MpTs of a wider range of insects, whether the pattern of expression correlates with the differentiation of SC-like characteristics, and whether gene knockdown results in

Fig. 7. The tsh gene network in SCs. (A) Wild-type adult tubule showing mutually exclusive expression of Cut (green) in PCs (large nuclei) and Tsh (red) in SCs (small nuclei). (B,C) Adult tubules stained for Cut (red) and DNA (white). In wild type (B), Cut is expressed in PCs (large nuclei) but not in SCs (small nucleus, circled). In tsh knockdown (C), Cut expression is ectopically induced in SCs (small nucleus, circled). (D) Adult tube with induced ectopic expression of tsh in PCs (CtB>UAS-tsh results in mosaic tsh expression in PCs) stained for Cut (green, white in individual channels) and Tio (red, white in individual channels). Ectopic tio expression in PCs leads to repression of Cut. (E) Adult tubule with ectopic expression of tio in PCs (CtB>UAS-tio results in mosaic and variable levels of tio expression in PCs) stained for Cut (green, white in individual channels) and Tio (red, white in individual channels). Ectopic tio expression in PCs leads to repression of Cut in a dose-dependent manner; high (+++), medium (++) and low (+) levels of Tio are indicated. (F) Adult tubule with ectopic expression of cut in SCs (C724>UAS-cut). Ectopic Cut expression leads to repression of Tsh in SCs (circled). (G) ClC-a in situ hybridisation in a cut mutant embryo. ClC-a is not ectopically expressed in PCs in the absence of Cut. The blister-shaped MpTs are outlined. (H,I) Adult MpTs stained for Disc-large (Dlg, green) to reveal cell shape and Tsh (red). Tsh-expressing PCs are transformed to a bar-shaped morphology (I) compared with control PCs (arrowheads, H). (J) Adult tubule with ectopic expression of tio in PCs in the absence of Cut. The blister-shaped MpTs are outlined. (K) Schematic drawing of the gene network controlling PC and SC differentiation. Scale bars: 30 μm in A,H,I; 10 μm in B-F; 50 μm in G,J.
defective Type II cell differentiation and organ physiology in these species. Tsh acts as a transcriptional repressor in fly embryonic tissues (Alexandre et al., 1996; Andrew et al., 1994; de Zulueta et al., 1994; Fasano et al., 1991; Robertson et al., 2004; Röder et al., 1992) and in transfected mammalian cells (Waltzer et al., 2001). Correspondingly, we show that Tsh shuts down the expression of cut in SCs. Indeed, the expression of cut in neighbouring PCs depends on the exclusion of Tsh. In a reciprocal relationship, ectopic Cut represses tsh expression in SCs. Under normal conditions, this negative-feedback loop establishes two cell populations (Fig. 6A), with cut-expressing Type I cells and tsh-expressing Type II cells. Tio can also repress cut when ectopically expressed in PCs (Fig. 6E), indicating an ancestral repressive function for Tio/Tsh. We suggest that a genetic network centred around cut and tio/tsh leading to Type I and II cell differentiation is an ancient and widespread feature of insect MpT development.

However, neither transcription factor alone is sufficient for cell fate. cut acts together with Krüppel in wild-type PC differentiation (Hatton-Ellis et al., 2007) and the induction of cut in SCs does not transform them into PCs. Similarly, although ectopic Tsh in PCs represses cut expression and alters their shape, it does not produce transformation into the SC or bar-shaped cell architecture or the induction of SC-specific gene expression. Aspects of SC fate are controlled by factors other than tsh or tio; in tio tsh mutant embryos, cells integrate into the tubule epithelium (Campbell et al., 2010; Denholm et al., 2003) but simply fail to differentiate. Additionally, although all Type II cells express Tsh-driven Drip, Lkr and CIC-a (unpublished data), bar-shaped cells adopt different cell shapes and vary in their patterns of gene expression [such as c649-Gal4 in bar cells but not SCs (Sözen et al., 1997)]. These data indicate that additional regulators differentiate between bar and SCs. One candidate, Homothorax, is expressed exclusively in initial and transitional segments (unpublished data) (Kurant et al., 1998; Wang et al., 2004) and is known to act in concert with Tsh to pattern the developing adult eye, wing and leg (Azpiazu and Morata, 2000; Bessa et al., 2002; Casares and Mann, 2000).

In both mouse and possibly human kidney development tsh-family genes underlie the differentiation of ureteral smooth muscle (Caubit et al., 2008). Thus, in both vertebrate and Drosophila...
nephrogenesis, these genes act to direct the differentiation of recruited mesenchymal cells that contribute, albeit with different functions, to the physiological competence of the mature organ.

Here, we show that, like Drosophila Tsh, mouse Tshz3 regulates the expression of a key differentiation gene, an aquaporin channel, in a specific subset of renal cells. The kidney phenotype of Tshz3 mutant mice resembles congenital pelviureteric junction obstruction, a common human kidney malformation (Caubit et al., 2010; El et al., 2007; Gunn et al., 1995; Ismailei et al., 2006), suggesting that Tshz3 or its targets may contribute to the human disorder. Furthermore, Tsh-family members regulate cell differentiation in multiple contexts during vertebrate development and have roles in several human diseases (Feenstra et al., 2011; Jenkins et al., 2010; Schick et al., 2011; Yamamoto et al., 2011). Further analysis of Tsh and its target genes in Drosophila MpTs may identify effectors of physiological differentiation that are relevant to vertebrate nephrogenesis and organogenesis, as well as potential candidate human disease genes.

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Competing interests statement
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References