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RESEARCH ARTICLE

Novel roles for *GATAe* in growth, maintenance and proliferation of cell populations in the *Drosophila* renal tubule

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ABSTRACT

The GATA family of transcription factors is implicated in numerous developmental and physiological processes in metazoans. In *Drosophila melanogaster*, five different GATA factor genes (*pannier*, *serpent*, *grain*, *GATAd* and *GATAe*) have been reported as essential in the development and identity of multiple tissues, including the midgut, heart and brain. Here, we present a novel role for *GATAe* in the function and homeostasis of the *Drosophila* renal (Malpighian) tubule. We demonstrate that reduced levels of *GATAe* gene expression in tubule principal cells induce uncontrolled cell proliferation, resulting in tumorous growth with associated altered expression of apoptotic and carcinogenic key genes. Furthermore, we uncover the involvement of *GATAe* in the maintenance of stellate cells and migration of renal and nephritic stem cells into the tubule. Our findings of *GATAe* as a potential master regulator in the events of growth control and cell survival required for the maintenance of the *Drosophila* renal tubule could provide new insights into the molecular pathways involved in the formation and maintenance of a functional tissue and kidney disease.

KEY WORDS: *GATAe*, *Drosophila*, Malpighian tubules, Principal and stellate cells, Renal stem cells, Proliferation, Maintenance, Migration

INTRODUCTION

The formation and development of an organ is a complex process involving several programmed events, including changes in cell shape and adhesion, proliferation and differentiation (Hatton-Ellis et al., 2007; Wan et al., 2000; Ainsworth et al., 2000). Understanding the genetic pathways involved is crucial, as defects in any of these aforementioned processes can lead to malformations and lethality (Jung et al., 2005; Denholm, 2013; Ainsworth et al., 2000). These events can be readily studied in *Drosophila melanogaster* Malpighian tubules (MTs), a powerful model system for investigating mechanisms of cell differentiation, proliferation and development, as well as a model of kidney disease (Millet-Boureima et al., 2018; Dow and Romero, 2010).

Insect MTs perform functions equivalent to vertebrate kidney and liver, and share similar characteristics in terms of classes of genes expressed and cellular origins. Development of the MTs occurs during embryogenesis and they are completely functional by the first instar larval stage. The development and physiology of the fly renal tubule have been extensively investigated and reviewed in previous publications (Beyenbach et al., 2010; Denholm, 2013; Skaer, 1996; Dow, 2012; Beaven and Denholm, 2018; Bunt et al., 2010). Each tubule is composed of different segments (ureter, main, initial and transitional segments) that are functionally and genetically distinct (Chintapalli et al., 2012), and has two main epithelial cell types: the principal cell (PC) and the stellate cell (SC) (Dow, 2012; Denholm et al., 2003). In addition, the renal and nephritic stem cells (RNSCs, also known as tiny cells) may be observed in the ureters and lower tubules. It has been shown that these cells, which migrate from the midgut (MG) during metamorphosis, are able to act as stem cells in the MTs, and have a distinct gene expression profile from both the SC or PC (Takashima et al., 2013; Li et al., 2014, 2015; Singh et al., 2007; Sözen et al., 1997).

The GATA family of transcription factors (TFs) contain one or two zinc-finger domains that bind to the consensus DNA sequence A/T GATA A/G and is known to play crucial roles in the development and physiology of vertebrates, invertebrates, plants and fungi (Lentjes et al., 2016; Merika and Orkin, 1993). Aside from their developmental roles, vertebrate GATA factors are associated to diverse types of cancers, including intestinal tumors, leukemia, and breast cancer (Zheng and Blobel, 2010; Pihlajoki et al., 2016; Hellebrekers et al., 2009; Shaoxian et al., 2017). In humans, at least two GATA factors are expressed in the kidney (GATA3 and GATA5). Epigenetic alterations of these genes are associated with decreased survival in renal cell carcinomas (Peters et al., 2014a,b), and GATA3 itself is downregulated in this type of cancer (Yang et al., 2013). Moreover, GATA3 is required for the proper development of the human kidney, and haploinsufficiency of this gene induces hypoparathyroidism, sensorineural deafness and renal anomaly (HDR) syndrome (Van Esch et al., 2000; Ferraris et al., 2009). The GATA family is evolutionarily well known in both vertebrates and invertebrates, and although they perform similar functions across phyla, there are differences in the pattern of expression between all of them (Lentjes et al., 2016).

This study focused on the *Drosophila* TF *GATAe*, which is highly enriched in embryonic, larval and adult MTs (Wang et al., 2004; Okumura et al., 2005). Previous studies have demonstrated that *GATAe* is required for terminal differentiation of the embryonic MG (Okumura et al., 2005; Murakami et al., 2005; de Madrid and Casanova, 2018). In the adult MG, *GATAe* performs different functions depending on the cell type. First, it is necessary for the maintenance, differentiation and migration of the intestinal stem cells (Okumura et al., 2016; Zhai et al., 2017; Takashima et al., 2013; Dutta et al., 2015). In addition, *GATAe* is actively required to repress MG enterocyte shedding after bacterial infection (Zhai et al., 2018). More recently, *GATAe* has been shown to be involved in transcriptional regulatory changes associated with lifespan

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extension consequent to dietary restriction (Dobson et al., 2018). However, although *GATAe* expression in the MG has been well characterized, little is known about its possible MT functions, despite the abundance of *GATAe* in the MTs at all developmental stages.

Here, we demonstrate crucial roles for *GATAe* in determining and maintaining tubule morphology and functional capabilities by restrictively silencing *GATAe* expression to PC, SC and RNSC sub-populations in the tubule. Specific *GATAe* knockdown in PCs induced uncontrolled cell proliferation and tumorous growth throughout the MTs, impairing tubule function and significantly decreasing stress tolerance and lifespan. Silencing *GATAe* expression in SCs also affected cell survival, ultimately impacting on hormonal control of tubule osmoregulatory functions. Finally, *GATAe* is also required in RNSCs to ensure proper migration to the MT ureter. Taken together, these observations demonstrate novel requirements for *GATAe*, not only for the determination and maintenance of individual MT cell populations but as a crucial TF for tubule function and organismal viability.

RESULTS

GATAe is expressed in all cell types in the adult MTs

To characterize *GATAe* function in MTs, we employed the GAL4/UAS binary expression system (Brand and Perrimon, 1993; Duffy, 2002) to restrictively silence gene expression in the different MT cell populations. We first investigated the pattern of expression of *GATAe* larval and adult MTs, as FlyAtlas2 data indicated that *GATAe* is highly enriched in the larval and adult MTs (Leader et al., 2018). It has been shown that *GATAe*-Gal4 correlates the pattern of expression of *GATAe* in the adult MG (Zhai et al., 2018) and the embryonic MTs (Kvon et al., 2014). Using two different *GATAe*-Gal4 lines driving a GFP reporter, we found *GATAe* expression in larval stage 3 (L3) PCs but not in SCs (Fig. 1A), and that *GATAe* is present in all three types of cells (PCs, SCs and RNSCs, Fig. 1B,C) in the adult MTs.

We then silenced *GATAe* expression via RNAi using *GATAe*-Gal4. A flexible feature of the GAL4/UAS system is that the phenotypes induced are temperature sensitive (Duffy, 2002). At 29°C, *GATAe>GATAe* RNAi induced 100% lethality shortly after embryogenesis, phenocopying a *GATAe* null mutant (Okumura et al., 2016). Furthermore, qPCR experiments determined that *GATAe>GATAe* RNAi embryos exhibited significantly decreased expression levels of *GATAe* mRNA (~40%) compared with the control (Fig. S1A). At 26°C, *GATAe>GATAe* RNAi flies overcame embryonic lethality but died during metamorphosis. In addition, at this temperature, *GATAe>GATAe* RNAi pupae exhibited a smaller size compared with the controls (Fig. S1B). Only when *GATAe>GATAe* RNAi flies were allowed to develop at 18°C, did they survive until the adult stage, but none of them survived more than 7 days after adult eclosion.

We observed that the morphology and length of the *GATAe>GATAe* RNAi adult MGs (at 18°C) is drastically shorter compared with the parental controls (Fig. S1C,D), which correlates with previous studies that demonstrated the vital roles of *GATAe* for the terminal differentiation of the embryonic MG (Murakami et al., 2005; de Madrid and Casanova, 2018), and for its adult maintenance (Zhai et al., 2017, 2018; Okumura et al., 2016). Curiously, we found that the morphology of *GATAe>GATAe* RNAi adult MTs was also affected (Fig. 1D), an observation that has not been previously reported.

GATAe regulates PC morphology and is required for tubule function

We examined the effects of silencing *GATAe* in the MTs using the PC-specific Gal4 line *CapaR*-Gal4 (Terhzaz et al., 2015), and noticed that the *CapaR>GATAe* RNAi adult flies exhibited an inflated abdomen phenotype (Fig. 2A). We performed wet-dry weight measurements and confirmed that the increase in weight of *CapaR>GATAe* RNAi flies was indeed a consequence of increased water retention, presumably a consequence of impairment of fluid transport in the MTs (Fig. S1F). In addition, *CapaR>GATAe* RNAi flies exhibited significantly shorter lifespan compared with the controls (Fig. 2B). We then investigated whether the tubule-specific role of *GATAe* in fluid homeostasis could modulate desiccation and starvation stress responses, and survival of the whole organism. Indeed, their tolerance to both starvation and desiccation stresses were significantly reduced compared with the controls (Fig. S1E).

CapaR>GATAe RNAi MTs are shorter (less than half the length of wild-type parental MTs, Fig. S2B), thicker and with irregular shape compared with controls (Fig. 2D and Fig. S2C). Additionally, these MTs exhibited a significantly reduced number of cells (PCs

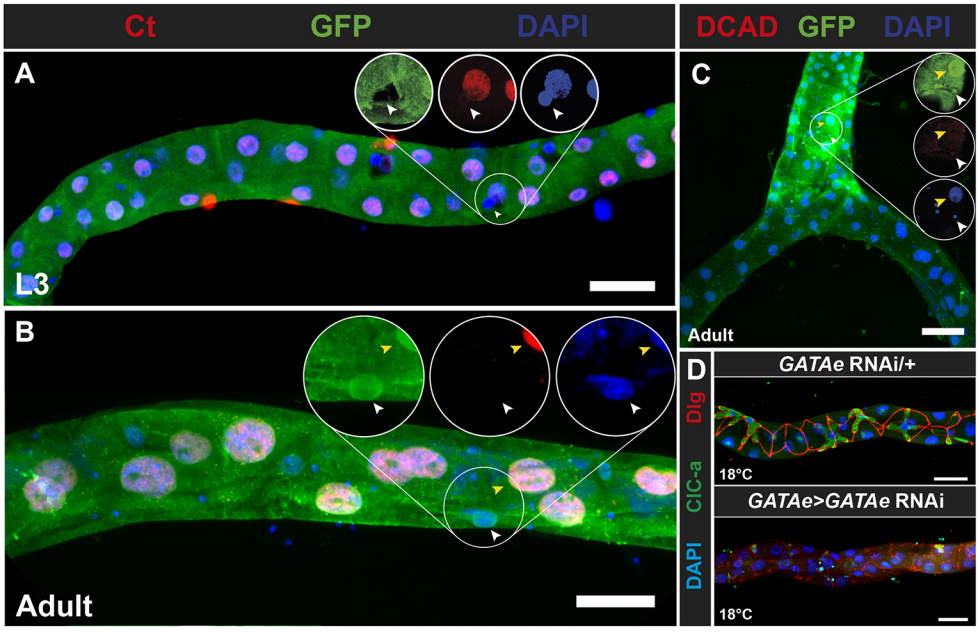

Fig. 1. The localization of *GATAe* in renal (Malpighian) tubules of *Drosophila*. (A,B) Expression of *GATAe*-driven GFP in (A) L3 and (B) adult MTs stained for Ct (red) and GFP (green), and with DAPI (blue). White arrowheads in A indicate a GFP^- SC. Yellow and white arrowheads in B indicate GFP^+ PCs and SCs, respectively. (C) Adult *GATAe*>GFP MTs stained for DCAD (red) and GFP (green), and with DAPI (blue). Yellow and white arrowheads indicate GFP^+ PCs and RNSCs, respectively. (D) Adult *GATAe>GATAe* RNAi and *GATAe* RNAi/+ MTs stained for Discs Large (Dlg, labeling septate junctions, red), ClC-a (SCs, green) and DAPI (blue). Scale bars: 50 µm.

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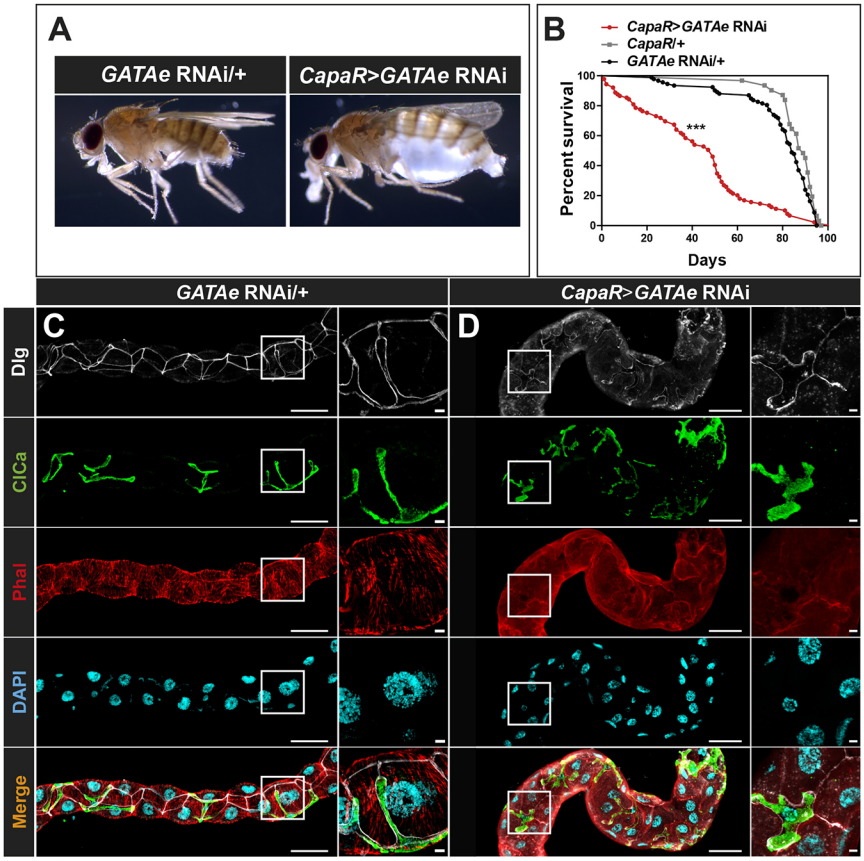

Fig. 2. Reduced *GATAe* levels in PCs affect water homeostasis, lifespan and tubule cell morphology. (A) *CapaR>GATAe* RNAi adult flies have bloated abdomens compared with controls. (B) Lifespans of *CapaR>GATAe* RNAi flies compared with both parental controls. Median survival times (days) are: *CapaR>GATAe* RNAi, 49; *CapaR*/+, 89; *GATAe* RNAi/+, 84. ***P<0.0001, n>100 flies. (C,D) Immunocytochemistry of control (C) and *GATAe* knockdown (D) using *CapaR*-Gal4. Merged images show the overlay of Dlg (white), ClC-a (green), phalloidin (F-actin, red) and DAPI (cyan). White boxes outline the regions shown at higher magnification on the right showing the shape of a SC. Scale bars: 50 µm and 5 µm (higher magnifications).

and SCs combined, Fig. S2A). This striking morphological phenotype was observed using three different UAS-*GATAe* RNAi lines (described in the Materials and Methods) crossed with PC-specific Gal4 drivers (*ctB*-Gal4 and *CapaR*-Gal4, Fig. S2A and C), and two different *GATAe*-Gal4 lines (Fig. 1D). Furthermore, *CapaR>GATAe* RNAi anterior MG regions did not show any detectable morphological defects (Fig. S3), indicating that the phenotype observed in their MT architecture is a consequence of *GATAe* knockdown specifically to the tubule PCs.

Cell-autonomous role of *GATAe* in principal cells

To determine a potential developmental role for *GATAe*, we silenced *GATAe* expression in the PCs from embryonic stage 9 using *ctB*-Gal4 (Saxena et al., 2014). No defects in morphology or migration of embryonic MTs were observed (Fig. 3B,B′). Interestingly, we obtained similar results using a null mutant line for *GATAe* (*GATAe*$^{-}$/*GATAe*$^{-}$) (Okumura et al., 2016), reinforcing that *GATAe* is not required for the embryonic development of the MTs (Fig. S2D). In order to delineate precisely the developmental window for *GATAe* function, we employed a conditional system using the temperature-sensitive Gal80 (Gal80^{ts}) construct (Pilauri et al., 2005), combined with the *CapaR*-Gal4 driver. *CapaR*^{ts}*>GATAe* RNAi flies were maintained at 18°C (where Gal4 expression is suppressed by Gal80^{ts}) until any of the larval stages (L1, L2 or L3 stage), and then switched to 29°C (where Gal4 is activated) until adult eclosion. The phenotype observed in these MTs phenocopied *CapaR>GATAe* RNAi MTs (Fig. 3D), indicating a crucial requirement for *GATAe* expression after the L3 stage. By contrast, *CapaR*^{ts}*>GATAe* RNAi flies raised at 29°C until L3 stage and then switched to 18°C until adult eclosion show similar MTs compared with controls (Fig. 3C). In addition, *CapaR>GATAe* RNAi flies exhibited normal MTs until L3 stage (Fig. S4A), after which stage MTs display strong morphological defects (Fig. S4B). These results demonstrate that *GATAe* is required from L3 stage onwards, and most probably during the pupal stage.

We then investigated whether reduced levels of *GATAe* in adult PCs could induce a tubule-specific phenotype over time in adult flies. As expected, *CapaR*^{ts}*>GATAe* RNAi flies raised at 18°C until the adult stage did not present any detectable structural alterations in their MTs (Fig. S5A). These flies were then switched to 29°C and MT morphology was examined at 7-day intervals over 28 days. Cell shape defects and disruption of the MT architecture were observed from 14 days after switching to 29°C (Fig. S5A), including abnormal proliferation of RNSCs, as revealed by the expression of the marker Hindsight (Bohère et al., 2018) (Hnt, Fig. S5B). These results indicate that, while *GATAe* does not appear to be necessary during embryogenesis, its expression in PCs is essential for maintaining proper cellular morphology and integrity of the MTs during the adult stage.

We next determined the potential cell-autonomous roles of *GATAe*. A widely used technique to study clonal analysis is the mosaic analysis with a repressible cell marker (MARCM) (Lee and Luo, 1999) technique. However, MARCM relies on mitotic recombination and, although it has been used to study RNSC behavior (Bohère et al., 2018; Singh et al., 2007; Zeng et al., 2010), this technique cannot be used in the PCs as these are a post-mitotic cell population. To overcome this issue and analyze the effects of clonal downregulation of *GATAe* in the PCs, we used the *Urate Oxidase* Gal4; *UAS-mCD8:GFP* fly line, which drives expression of membrane-bound GFP in a subset number of PCs in the tubule main segment (*UrO*-GFP) (Terhzaz et al., 2010), in conjunction with the *GATAe* RNAi. Remarkably, *UrO*-GFP*>GATAe* RNAi MTs

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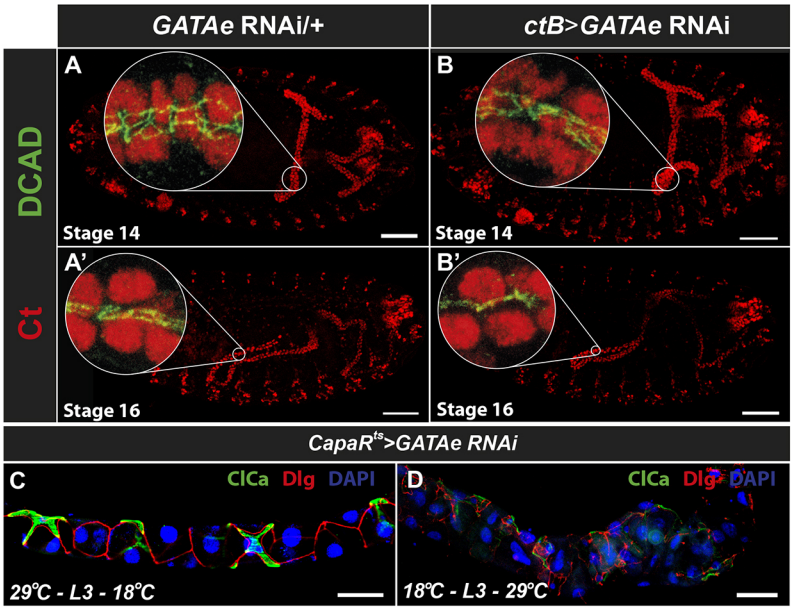

Fig. 3. *GATAe* is not required for embryonic development of the MTs. (A-B′) Embryos of stages 14 and 16 stained for Ct (red) and DCAD (green). Insets are optical projections of the circled areas. (C) Main segment of adult *CapaR*^{ts}*>GATAe* RNAi fly that has been raised at 29°C until L3 stage and then switched to 18°C. (D) Main segment of adult *CapaR*^{ts}*>GATAe* RNAi fly that has been raised at 18°C until L3 stage and then switched to 29°C. Scale bars: 50 µm.

display an expansion of tubule diameter exclusively surrounding the GFP^{+} cells (Fig. 4C,D), suggesting that *GATAe* is required cell specifically. In addition, we observed several bi-nucleated cells (Fig. S5C), suggesting an alteration in cell division.

Reduced levels of *GATAe* induce overproliferation of RNSCs

We also noticed that *CapaR>GATAe* RNAi MTs contain significantly more cells with smaller nuclei not only in the ureters but also in the tubule main segment compared with the controls (see Fig. 5H for quantifications). Interestingly, these cells are positive for Armadillo (Arm), D-Cadherin (DCAD) and Hnt (Fig. 5D,G), which are known markers for RNSCs (Bohère et al., 2018; Singh et al., 2012). In addition, these cells were also positive for Delta (Dl^{+}) (Fig. S5D), another known marker for RNSCs and intestinal stem cells (Li et al., 2014).

It is acknowledged that RNSCs of adult wild-type tubules are described as quiescent and rarely undergo division (Li et al., 2015; Zeng et al., 2010). However, using the cell division-specific marker phospho-histone H3 (PH3) (Micchelli and Perrimon, 2006), we confirmed that a number of potential RNSCs observed in adult *CapaR>GATAe* RNAi MTs were positive for PH3, indicating that these are proliferative cells (Fig. S5D). Furthermore, *CapaR>GATAe* RNAi MTs also contained cells that are DCAD^{-}, Arm^{-} and Dl^{-}, and possess smaller nuclei than PCs, which might be immature renal blasts (RBs, white arrows in Fig. 5G and Fig. S5D; previously described in by Singh et al., 2007). This suggests that these proliferative population of cells may undergo a partial differentiation process.

GATAe modulates cancer-related gene expression

Several cancers are caused by the failure to control proliferation and differentiation of stem cell populations (Reya et al., 2001; Parvy et al., 2018). Given the apparent increase in RNSCs and RBs in *CapaR>GATAe* RNAi MTs, we investigated the level of expression

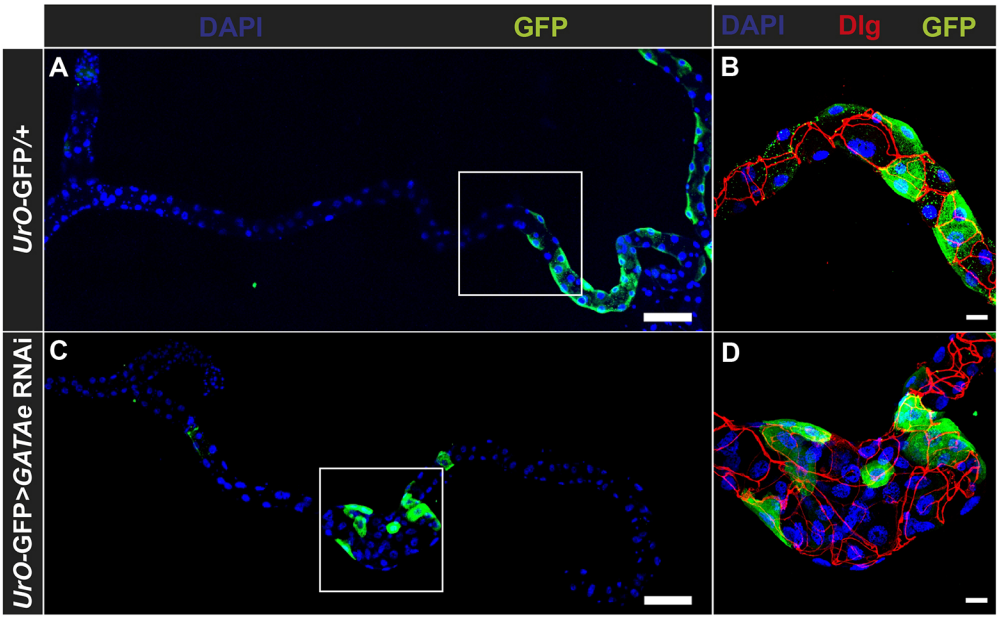

Fig. 4. Silencing *GATAe* in PCs using *UrO*-GFP. Comparison between control (A,B) and *GATAe* knockdown (C,D) adult female MTs stained with Dlg (red), GFP (green) and DAPI (blue). B and D are magnifications of the boxed regions in A and B, respectively. Scale bars: 100 µm in A and C; 20 µm in B and D.

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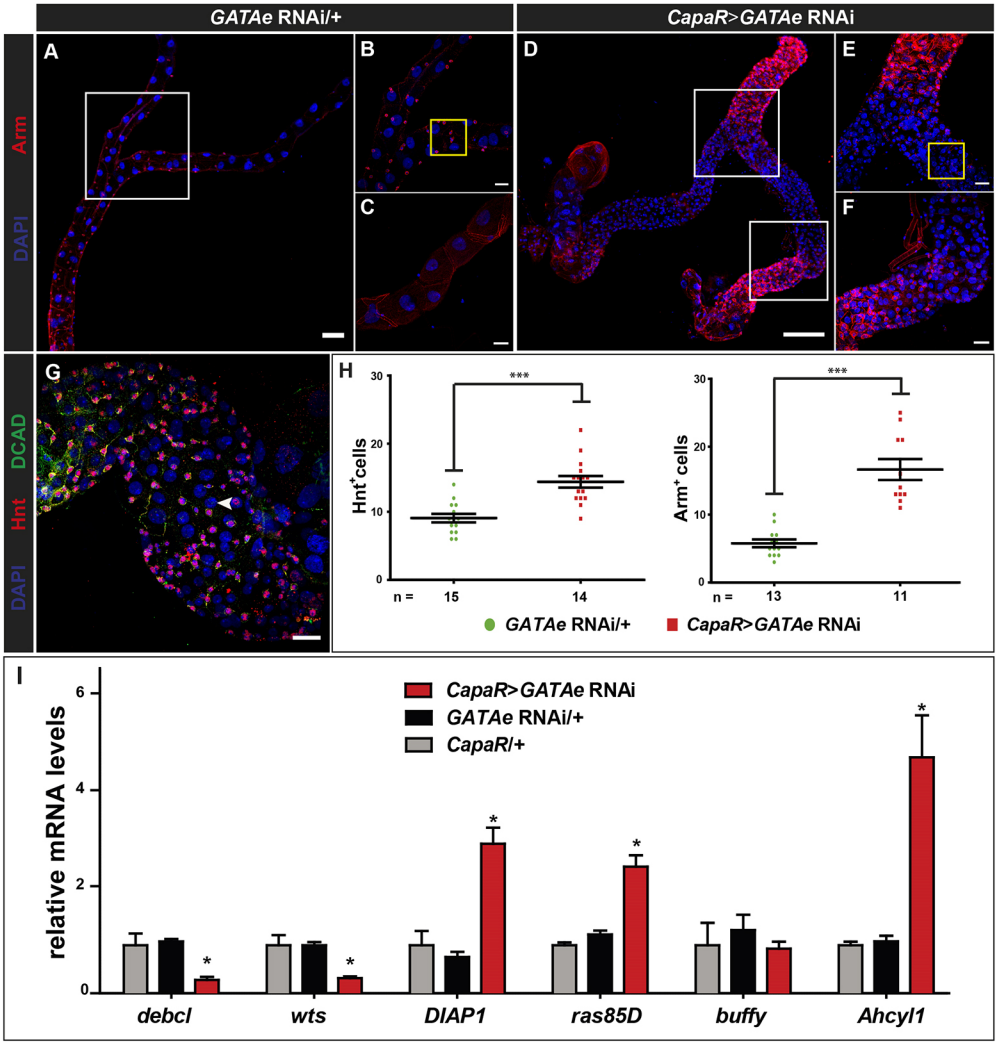

Fig. 5. *GATAe* knockdown induces proliferation of RNSCs. (A-F) Comparison of control (A-C) and *CapaR>GATAe* RNAi (D-F) adult MTs stained for Arm (red) and with DAPI (blue). B,E,F are magnifications of the boxed regions in A and D; C is a magnification of a region of the main segment that is not present in A. (G) Magnification of *CapaR>GATAe* RNAi adult main segment stained for DCAD (green) and Hnt (red), and with DAPI (blue). This region is filled with undifferentiated RNSCs, which are $DCAD^+$ and Hnt^+, and RBs, which are $DCAD^-$ and Hnt^- (white arrowhead). (H) Hnt^+ (left) and Arm^+ (right) cell number quantifications of *CapaR>GATAe* RNAi and control regions of lower tubules (representative 50 µm^2 regions are shown in yellow squares of B and E). *CapaR>GATAe* RNAi lower tubules exhibit a significant increase in the number of Arm^+ cells and Hnt^+ cells compared with controls. Data for Hnt^+ cells are: *GATAe* RNAi/+, 9.07±0.62, *n*=14; *CapaR>GATAe* RNAi, 14.4±0.84, *n*=15 (mean±s.e.m.). Data for Arm^+ cells are: *GATAe* RNAi/+, 5.71±0.56, *n*=13; *CapaR>GATAe* RNAi, 16.64±1.54, *n*=13 (mean±s.e.m.). ***P<0.001, Student's *t*-test, two-tailed. Scale bars: 50 µm for A and D; 20 µm for B,C,E-G. (I) Relative mRNA levels of different key genes involved in cancer and apoptosis. *P<0.05, Student's *t*-test, two-tailed.

of genes involved in cell proliferation and tumorigenesis: *Ras Oncogene at 85D* (*Ras85D*) and *warts* (*wts*) (Ren et al., 2010); and downstream apoptosis-related effectors: *death-associated Inhibitor of Apoptosis 1* (*Diap1*), *Buffy* and *death executioner bcl2* (*Debcl*) (Terhzaz et al., 2010; Goyal et al., 2000). *GATAe* knockdown MTs exhibit a significant upregulation of *Diap1* and the *Ras85D* expression (2.5- and 3-fold increase, respectively). By contrast, downregulation of *Debcl* and *wts* (0.7- and 0.6-fold decrease, respectively) were observed (Fig. 5I). However, no significant difference in *Buffy* expression levels was measured in tubules with reduced *GATAe* levels. Additionally, these MTs also exhibited a strong upregulation of *Adenosylhomocysteinase like 1* (*AhcyL1*) (~5-fold increase, Fig. 5I), a gene involved with methionine metabolism and recently shown to be involved in the control of *Drosophila* lifespan (Parkhitko et al., 2016).

We also found that the morphological phenotypes observed in *CapaR>GATAe* RNAi MTs are strikingly similar to previously reported defects induced by constitutive activation of *Ras* signaling (Zeng et al., 2010). Given that *CapaR>GATAe* RNAi MTs induced upregulation of *Ras85D*, we investigated whether activating *Ras* signaling in the PCs would induce similar morphological abnormalities. Interestingly, inducing a constitutively activated form of *Ras85D* (Wu et al., 2010) in the PCs (*CapaR>Ras^{V12}*) resulted in highly similar tubule defects to *CapaR>GATAe* RNAi flies (Fig. S6A). However, silencing both *GATAe* and *Ras* signaling [by the induction of *Ras85D* RNAi (Slack et al., 2015) or the dominant-negative form of *Ras85D*, *Ras^{N17}* (Lee et al., 1996)] did not rescue the tubule phenotype of *GATAe* knockdown (Fig. S6B), suggesting that other signaling pathways might be involved in the control of MT morphology. Altogether, these results indicate that *GATAe* directly or indirectly regulates the expression of several tumor-related genes in the MTs.

GATAe is required for stellate cell maintenance

To determine whether *GATAe* is necessary in the other secretory cells present in tubule main and initial segments, we silenced *GATAe* expression in SCs using a SC-specific GAL4 driver (*ClC-a*-Gal4; Cabrero et al., 2014). This resulted in a significant reduction in the number of SCs (~11 cells in *ClC-a>GATAe* RNAi compared with ~23 cells in control tubules, Fig. 6C). Surprisingly, these cells appeared localized to the initial segment of the MTs (Fig. 6B). We next investigated whether this reduction of SCs could impact on fluid secretion in isolated MTs (Dow et al., 1994). Data in Fig. 6D show that, although the basal secretion rates for *ClC-a>GATAe* RNAi MTs were not altered, fluid secretion rates were significantly reduced compared with the controls when MTs were stimulated with the diuretic neurohormone Kinin acting only in SCs (Terhzaz et al., 1999). These data demonstrate that the osmoregulatory MTs function is compromised in *GATAe* knockdown SCs.

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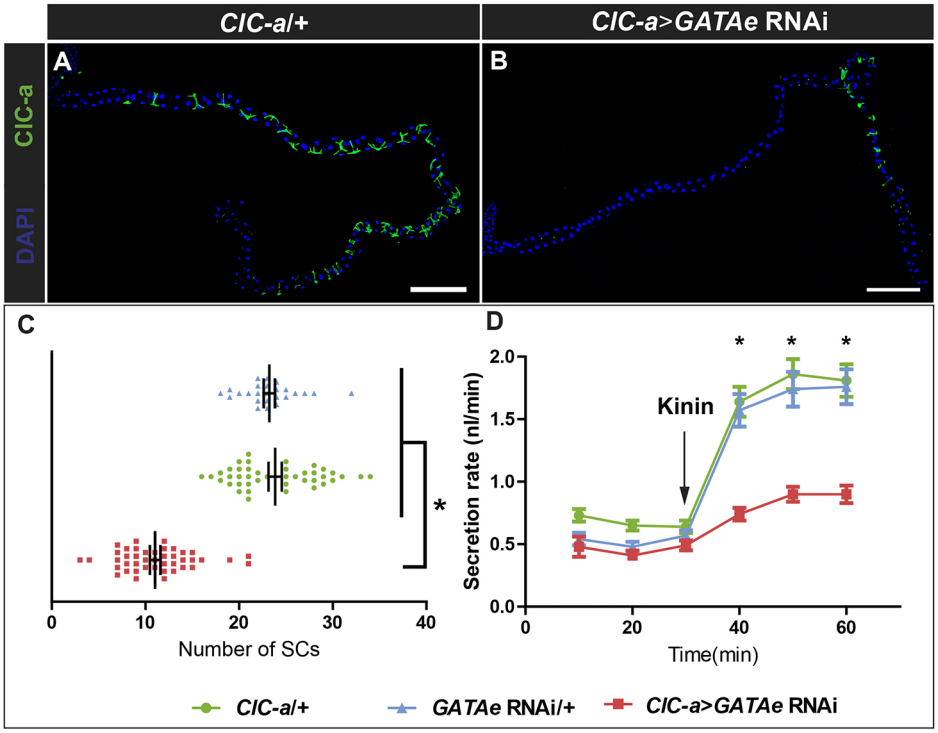

Fig. 6. Silencing *GATAe* in SCs induces reduced number of SCs and lower fluid secretion rates. (A,B) Immunocytochemistry of adult (A) control and (B) *ClC-a>GATAe* RNAi *GATAe* MTs stained for ClC-a (green) and DAPI (blue). Scale bars: 200 µm. (C) SC number quantifications of adult female MTs (mean ±s.e.m.) are: *ClC-a/+*, 23.86±4.6, *n*=42; *GATAe* RNAi/+, 23.24±3, *n*=25; *ClC-a>GATAe* RNAi, 11.04±3.8, *n*=45. **P*<0.05. (D) Fluid secretion rates (nl min^{-1}) of *GATAe* knockdown Malpighian tubules. *Drosophila* kinin (10^{-7} M) was added at 30 min (arrow). **P*<0.05, Student's *t*-test, two-tailed.

Curiously, this depletion of the SC population in *ClC-a>GATAe* RNAi MTs is apparently not a developmental defect; cell counts of SCs in *ClC-a>GATAe* RNAi MTs of L3 stage (Fig. S7E), although slightly reduced compared with controls, were significantly higher when compared with the number of SCs in the adult stage (Fig. 6C). Furthermore, SCs in *ClC-a>GATAe* RNAi L3 MTs stage were located in a regular pattern along the tubule (Fig. S7E), and do not mimic the phenotype of abnormal intercalation of SCs in the embryonic tubule (Campbell et al., 2010; Gautam and Tapadia, 2010; Denholm et al., 2003). Notably, the controls showed a low but still significant reduction of ClC-a^+ cells in the L3 stage when compared with the adult stage. ClC-a antibody also stains the cells in the enlarged initial segment known as bar cells in the adult stage (Fig. 6A). However, these cells do not seem to express ClC-a in the L3 stage (Fig. S7B,D). Thus, these data demonstrate that *GATAe* is required in the SCs for their survival and MT function at the adult stage, but it is not necessary for SC integration during embryogenesis.

Migration of the RNSCs to the MTs requires *GATAe*

Previous work has shown that the RNSCs migrate from the posterior MG and invade the ureters of MTs during metamorphosis, and that *GATAe* is required for the migration of these cells from the hindgut proliferation zone to the anterior MG (Takashima et al., 2013). Therefore, we employed the *esg*-Gal4 driver, which is known to be expressed in all the RNSCs before and during their migration to the MTs (Takashima et al., 2013), combined with the $Gal80^{ts}$ construct recombined with GFP (*esg-GFP*^{ts}) to assess whether *GATAe* is required for the migration of the RNSCs to the MTs. MTs from *esg-GFP*^{ts}*>GATAe* RNAi flies raised at 18°C and then transferred at 29°C at L3 stage were examined post-eclosion. Even though these MTs displayed a complete loss in the RNSC population (Fig. 7A′, D), no further morphological defects were seen in this organ. When *esg-GFP*^{ts}*>GATAe* RNAi flies were transferred from 18°C to 29°C 24 h or 48 h after puparium formation (APF) where the RNSCs are partially present in the ureter (Takashima et al., 2013), they displayed a normal localization of RNSCs in their MTs (Fig. 7B′), but were significantly reduced in number compared with the controls (Fig. 7D). Furthermore, *esg*-GFP^{ts}*>GATAe* RNAi switched at 29°C 1 day post-eclosion did not result in any significant alteration of the pattern of RNSCs up to 20 days at the permissive temperature (Fig. 7C′). Strikingly, cell quantifications confirmed that the number of RNSCs in the ureters and lower tubules is only significantly reduced when *GATAe* is silenced in this cell type during metamorphosis (Fig. 7D). Thus, these data indicate that *GATAe* is required for the migration or survival of this population of RNSCs during metamorphosis, but not for their maintenance in the adult stage.

DISCUSSION

In this study, novel functions for *GATAe* in *Drosophila* MTs have been identified. First, *GATAe* is crucial in the PCs from metamorphosis to maintain the correct architecture and cell proliferation in the adult tubule. Second, *GATAe* is required in the SCs for their survival, with loss of *GATAe* resulting in a reduction in SC population and MT secretory function, presumably owing to a reduction in the overall SC population. Finally, *GATAe* is necessary for the early migration of the RNSCs from the MG to the MTs during metamorphosis.

GATAe is required from the pupal stage in principal cells

GATAe loss in the PCs promotes morphological defects and uncontrolled proliferation and migration of RNSCs to more distal regions of the MTs. This organ persists from embryonic development throughout metamorphosis. However, during metamorphosis they still undergo tissue remodeling, initially shrinking by half their length and then elongating before eclosion (Denholm, 2013; Wessing and Eichelberg, 1979). Several factors have been shown to modulate changes in MT structure and function during this period, like the tumor suppressors *salvador* and *Scribbled*, which have been implicated in the regulation of the RNSCs (Zeng et al., 2010). In line with the hypothesis of metamorphosis as a critical juncture in

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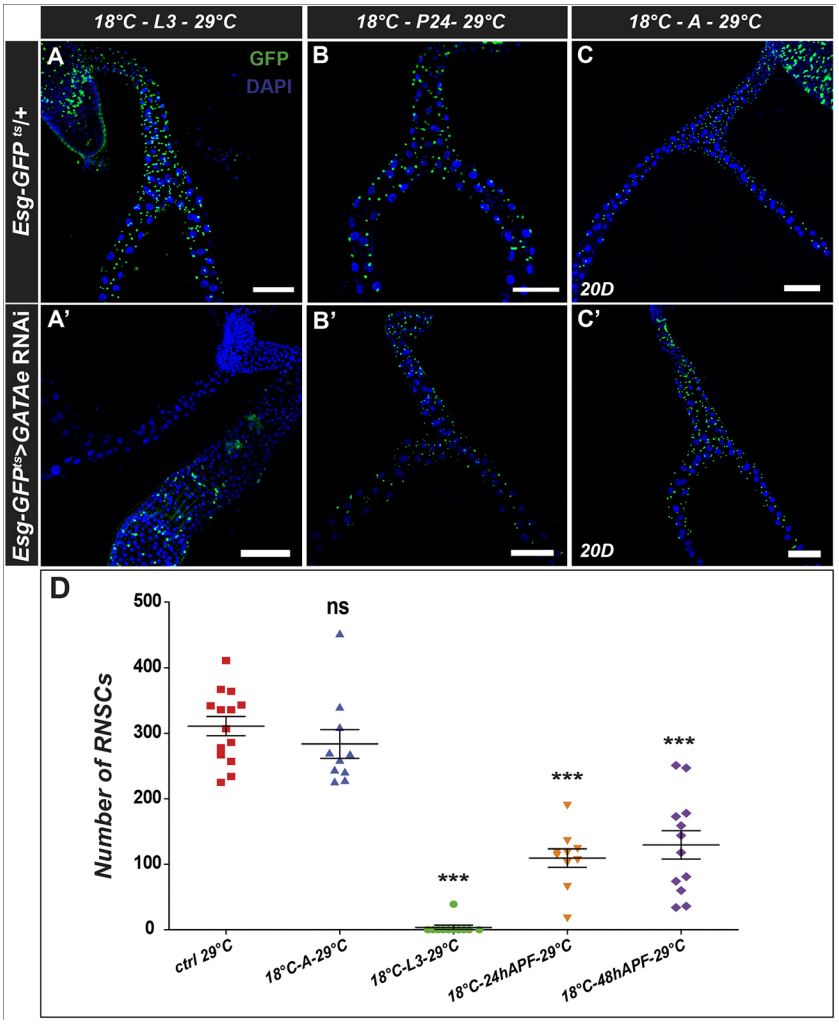

Fig. 7. *GATAe* knockdown in RNSCs induces migration defects. Immunocytochemistry of adult ureters in which *GATAe* was silenced using the esg-GFP^{ts} driver. (A,A′) Flies were raised at 18°C until L3 stage, and then switched to 29°C. (B,B′) Flies were raised at 18°C until 24 h APF and then transferred to 29°C. (C,C′) Esg-GFP^{ts}>*GATAe* RNAi flies that were raised at 18°C until adult eclosion and then switched to 29°C for 20 days. Scale bars: 100 µm. (D) Quantifications of RNSCs (Esg^{+}) in 7-day-old adult ureters of *esg*-GFP^{ts}>*GATAe* RNAi flies that have been transferred from 18°C to 29°C at L3 stage (green), 24 h APF (orange), 48 h APF (purple) or 1 day after adult eclosion (blue). Data for cells are (mean±s.e.m.): ctrl 29°C, 310.9±14.6, *n*=14; 18-A-29°C, 283.7±21.8, *n*=10; 18°C-L3-29°C, 3.54±3.5, *n*=11; 18°C-24 h APF-29°C, 109.5±14.1, *n*=10; and 18°C-48 h APF-29°C, 129.6±21.7, *n*=12. ***P<0.0001 versus the control (ctrl 29°C, which refers to 7-day-old female esg-GFP^{ts} flies raised at 29°C at all times; Student's *t-test*, two-tailed); ns=not significant (P>0.05).

the determination of the MT development, our data show that *GATAe* expression is required only from the pupal stage with no apparent requirement before this stage. In addition, the morphological defects of *GATAe* knockdown tubules phenocopied MTs with constitutive activation of *Ras* signaling either clonally (Zeng et al., 2010) or in the PCs (shown in this study). Upregulation of *Ras85D* gene expression in *GATAe* knockdown tubules indicates that *GATAe* could coordinate growth by regulating *Ras* pathway signaling. However, our data also suggest that *GATAe* could do so through alternative pathways, which deserve further investigation.

We demonstrated that *GATAe* requirement in the PCs is tissue-autonomous, as expression of *GATAe* RNAi driven by *UrO-Gal4: GFP* was enough to induce tumorous growth in the regions in and surrounding the GFP^{+} areas, indicating that *GATAe* can act autonomously and non-autonomously. Interestingly, these tubules did not show any obvious over-proliferation or presence of RNSCs, suggesting that tumorous growth is induced independently of any pathway that may produce RNSC overproliferation. Our data also show that *GATAe* appears necessary for regulation of factors such as *Debcl* or *Diap1*, which are known to regulate cell proliferation and homeostasis in a variety of tissues, including the MTs (Bohère et al., 2018; Ren et al., 2010; Terhzaz et al., 2010). A previous study has shown that *Diap1* is expressed in the RNSCs to ensure their survival, a process that is regulated by the Hippo pathway and *shavenbaby* (*ovo* – FlyBase) (Bohère et al., 2018). We hypothesize that signals from the PCs could be required for the control of this cell population by regulating *Diap1*, and that *GATAe* could be involved in this signaling from the PCs to the RNSCs. Finally, *GATAe* regulates adult maintenance of the MTs.

The implication of *GATAe* in the maintenance of SC and RNSC population

The SC population undergoes mesenchymal-to-epithelial transition and integrates into the MTs during stage 13 of embryonic development; by stage 15, they are completely intercalated in specific regions of both anterior and posterior tubules (Denholm et al., 2003; Sözen et al., 1997). This process is dependent on multiple factors, such as *hibris* and *serpent* (orthologs of human nephrin and GATA1, respectively; Artero et al., 2006), the activity of the *tiptop* and *teashirt* (Denholm et al., 2013), and ecdysone signaling (Gautam and Tapadia, 2010). In addition, we show that the SCs also require *GATAe* for their survival through metamorphosis, as the number and localization of SCs are significantly decreased in tubules with reduced *GATAe* levels. Curiously, the shape of the remaining SCs is not altered when compared with wild-type tubules. These findings suggest that *GATAe* performs a different function from *teashirt*, which determines the proper shape and physiology of the SCs during pupariation (Denholm et al., 2013).

The RNSC population migrates from the MG to the MTs during metamorphosis, and several factors are required for its migration and survival, such as *Rac1* or *shavenbaby*, or members of the Hippo

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pathway (Bohère et al., 2018; Takashima et al., 2013; Zeng et al., 2010). We show here that *GATAe* has a potential role in RNSC migration, as depletion of *GATAe* in this cell population induced a migration defect, leading to a few or no RNSCs in adult MTs. Strikingly, a similar phenotype was observed employing a dominant-negative form of *Rac1* (Takashima et al., 2013). This, together with our findings indicating no alteration in the RNSC population when *GATAe* was silenced from the adult stage, strongly suggest that *GATAe* could be required for RNSC migration rather than adult survival. The role of *GATAe* in the RNSCs contrasts with its function in the adult MG intestinal stem cells as *GATAe* is necessary for their maintenance (Okumura et al., 2016). This provides more evidence of the different functions of this TF, depending on its cellular context. However, unlike RNSCs, intestinal stem cells exhibit high division rates (Ohlstein and Spradling, 2007; Okumura et al., 2016), masking a possible subtle role for *GATAe* in regulating RNSC mitosis or proliferation. Therefore, it would be compelling for future investigations to explore whether *GATAe* could also be involved in regulating standard division rates and stemness of RNSCs.

The evolutionary conservation of GATA factors in relation to cancer

Our data indicate that knockdown of *GATAe* in PCs results in abnormal expression of different cancer and apoptosis-related genes in the adult *Drosophila* MTs. Our findings are in line with data from human GATA factors, which showed that GATA factors can act both as tumor suppressors or as oncogenes (Rodriguez-Bravo et al., 2017; Hellebrekers et al., 2009; Zheng and Blobel, 2010; Peters et al., 2014b).

We demonstrated that *GATAe* performs entirely different functions in each tubule cell-type, a behavior that has been previously reported for *GATAe* in the adult MG (as mentioned in the introduction). These divergent functions of one gene dependent on its cellular context also take place with human GATA factors; outside its hematopoietic functions, GATA3 has been linked to diverse types of human cancers. For example, GATA3 is a recognized indicator of breast cancer, and it has been shown that its expression is sufficient to stop tumor dissemination in breast cancers (Kouros-Mehr et al., 2008). However, GATA3 can also induce carcinogenesis in lymphoid precursor cells and converts double-positive thymocytes into a premalignant state (Nawijn et al., 2001; van Hamburg et al., 2008).

Further potential evolutionary conserved interactions in the GATA family can be concluded from our study. Our data demonstrate that low levels of *GATAe* reduce the expression of the proapoptotic gene *Debcl*, which is associated with the proliferation defects observed. Interestingly, interactions between GATA factors and members of the bcl-2 family have been reported. In vertebrates, GATA1 also interacts with Bcl-x to ensure the survival of erythroid cells (Gregory et al., 1999). In addition, GATA4 directly binds to another member of the Bcl family, Bcl2 (Kobayashi et al., 2006; Aries et al., 2004; Kobayashi et al., 2010), to induce cell survival due to drug-induced toxicity in the heart. We hypothesize that, in *Drosophila*, an interaction between GATA factors (*GATAe*) and bcl-2 members (*Debcl*) also occurs for MT homeostasis. Further experimentation would be required to elucidate the precise relationship between *GATAe* and *Debcl* in *Drosophila*.

Our findings demonstrate that MTs require expression of specific genes in all the three different cell types to ensure their correct architecture, maintenance and function in the adult stage. Our results reveal that *GATAe* plays a vital role in maintaining the adult homeostasis of the MTs, possibly acting as a tumor suppressor gene. They also show that the diverse roles of *GATAe* are highly dependent on the cellular context, in a similar way to its human counterparts. A model of the functions of *GATAe* in the three different cell types of the MTs is presented in Fig. 8. This model integrates the diverse roles of *GATAe* in all three MT cell types (PCs, SCs and RNSCs), although further investigation is required to identify those specific signaling pathways that interact with *GATAe* in these particular cellular contexts. To summarize, our findings provide a context for how abnormal cellular states may occur in different cell types of the same tissue and establish further homologies between insect and human GATA factors. Altogether, they provide evidence for the mechanisms of tissue homeostasis and tumor suppression that can be conserved throughout evolution.

MATERIALS AND METHODS

Fly stocks

Flies were maintained on a standard medium at 22°C, 55% humidity with a 12:12 h light:dark photoperiod. Unless otherwise specified, wild-type *Canton-S* (CS) flies crossed with both parental transgenic lines were used as controls. *GATAe*-Gal4 lines were obtained from Vienna *Drosophila* RNAi Centre [VDRC, #209818 and #205372 (Zhai et al., 2018; Kvon et al., 2014)] and both *GATAe*-Gal4 lines exhibited identical pattern of expression in MG and MTs. *GATAe* RNAi lines were obtained from VDRC (#10420, #10418; Takashima et al., 2013; Okumura et al., 2016), and the Bloomington *Drosophila* Stock Centre (BDSC, #33748; Okumura et al., 2016; Dutta et al., 2015; Dobson et al., 2018). All experiments using *GATAe* RNAi lines were performed using the construct #10420, except in Fig. S2, in which #33748 was employed. Lines used were: *CapaR*-Gal4, described by Terhzaz et al. (2012), *CapaR*-Gal4*;Tub-Gal80*^{TS}*(CapaR*^{ts}*)* and UAS-mCD8.GFP; *UrO*-Gal4, described by Terhzaz et al. (2010); *ClC-a*-Gal4 (VDRC, #31124); and *ctB*-Gal4, a gift from Dr Barry Denholm (University of Edinburgh, UK; Saxena et al., 2014). *Esg-Gal4.UAS:GFP/Cyo; TubGal80*^{TS}*/TM6B* (*Esg-GFP*^{ts}) (Micchelli and Perrimon, 2006) was a gift from Dr Julia Cordero (University of Glasgow, UK), and the mutant line *GATAe*$^{-}$/TM3,*Ser* (Okumura et al., 2016) was a gift from Prof. Takashi Adachi-Yamada (Gakushuin University, Tokyo, Japan).

Fixation and immunostaining

For embryo collection, adult females were allowed to lay eggs on grape juice agar plates for 9-24 h at 29°C. Embryos were collected and dechorionated in a fresh solution of 50:50 bleach and double-distilled H_2O for exactly 2 min and then washed thoroughly with double-distilled H_2O. Using a fine brush, the embryos were subsequently transferred to 2 ml of heptane and 2.25 ml of 4% formaldehyde in PBS (Thermo Fisher #12549079) was added. Embryos were left for 20 min on a shaker to allow fixation. The lower aqueous phase was then gently removed, and 2 ml of a fresh solution of methanol (100%) was added, and the sample gently shaken for 20 s. Devitellinized embryos, which appeared in the methanol phase, were collected and placed in methanol (100%) before being processed for immunocytochemistry.

Larval, pupal and adult (5-day-old adult female flies unless otherwise specified) tubules were dissected in PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. The following primary antibodies and dilutions were used: mouse anti-Ct (DSHB, 1/100), mouse anti-Dlg (DSHB, 1/500), mouse anti-Dl (DSHB, 1/50), mouse anti-Arm (DSHB, 1/100), rat anti-DCAD, (DSHB, 1:200), rabbit anti-ClC-a (Cabrero et al., 2014; 1/100), rabbit anti-GFP (Life Technologies, 1/1000) and rabbit anti-PH3 (Cell Signaling Technologies, *Ser10*, 1/1000). Incubations with the primary antibodies were performed overnight at 4°C. The following secondary antibodies were used: Alexa Fluor 546-conjugated goat anti-mouse (Thermo Fisher Scientific, 1/1000) and Alexa Fluor 488/633-conjugated goat anti-rabbit (Thermo Fisher Scientific, 1/1000). Other markers used included: DAPI (Sigma-Aldrich, 1 µg/ml) and rhodamine-coupled phalloidin (Thermo Fisher Scientific, 1/100). All samples were mounted in Vectashield (Vector Laboratories). Confocal images were captured using a Zeiss LSM 880 AxioObserver microscope (Zeiss,

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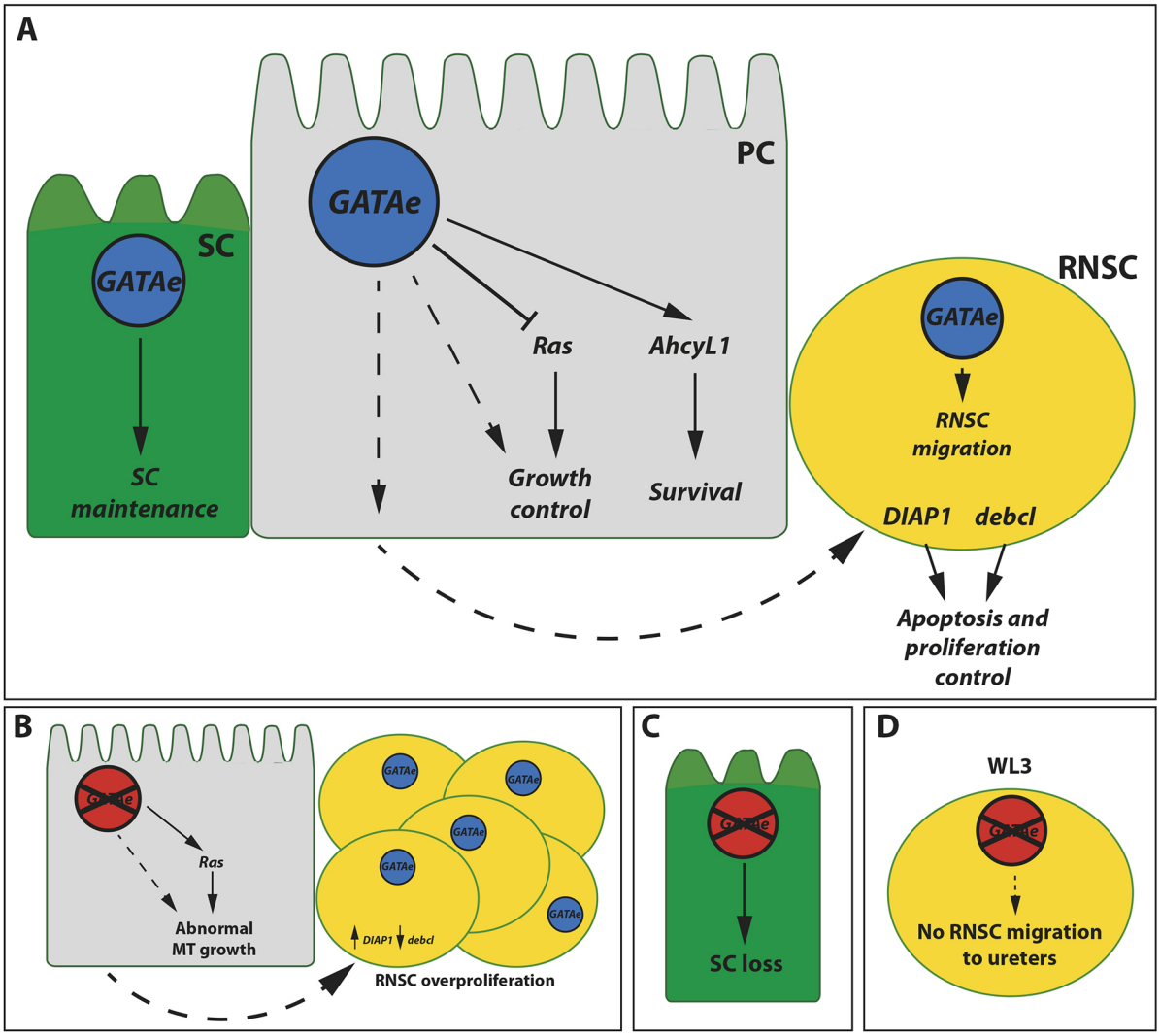

Fig. 8. Proposed model for the function of *GATAe* in the MTs. (A) Regulatory interactions of *GATAe* in the three different cell types of the MTs: PCs (gray), SCs (green) and RNSCs (yellow). T-bar indicates possible inhibition of gene expression. Dotted arrows indicate interaction through unknown pathways. In SCs, *GATAe* is required for their survival. In PCs, *GATAe* is required for the proper architecture of the MTs possibly through *Ras* signaling and other unknown pathways. *GATAe* is also necessary in the PCs to ensure normal proliferation of RNSCs through the activity of *Diap1* and *Debcl*. In addition, *GATAe* is required in RNSCs for their migration to the ureters during metamorphosis. (B) *GATAe* knockdown in the PCs impacts tubule growth, possibly through *Ras* signaling and other unknown pathways, and induces RNSCs proliferation. (C) Silencing *GATAe* in SCs is associated with a reduction of SC number. (D) *GATAe* is required in prospective RNSCs at L3 stage for their migration to the ureters. Reduced levels of *GATAe* in this larval stage induces a complete abolition of RNSCs in the adult ureters and lower tubule.

Oberkochen, Germany) and processed with Zeiss ZEN software and Adobe Illustrator CS5.1.

Quantitative RT-PCR

qRT-PCR amplifications were performed from adult female Malpighian tubules and embryos (12-14 h after egg laying). cDNA was synthesized from 500 ng total RNA using SuperScript II RT (Thermo Fisher Scientific), following the manufacturer's instructions. qRT-PCR was performed on an ABI StepOnePlus Detection System (Applied Biosystems) using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent), with the following primers: *GATAe* forward, 5′-ACCGCTGTCGATGAAGAAGG-3′ and reverse, 5′-GGACTGGAATTCTGCTGGCT-3′; *Diap1* forward, 5′-CGTGGTGCGATAAGAGGTGA-3′ and reverse, 5′-TTGAATAGCTGGGTCGCGTT-3′; *Ras85D* forward, 5′-GCAAGAGAGGTGGCCAAACA-3′ and reverse, 5′-TCGGCTTGTTCATTTTGCGG-3′; *wts* forward, 5′-AGCCGACAATAACTGGGTGG-3′ and reverse, 5′-CGAGTGATTGCCGTTCTCCT-3′; *RpL32* forward, 5′-TGACCATCCGCCCAGCATAC-3′ and reverse, 5′-ATCTCGCCGCAGTAAACG-3′, *Debcl* forward, 5′-TTTTTCGCTCCAGCATCACC-3′ and reverse, 5′-CGTCAATCCCAAGAACG-3′; *Ahcyl1* forward, 5′-GGCGAGACGGAAGAGGACT-3′ and reverse, 5′-AGAGAGCTGATAGAGACGGTG-3′; *Buffy* forward, 5′-GCCACACTACATTCCGCATCAC-3′ and reverse, 5′-ATTCATCGCCCAGCACTTC-3′. Data were normalized against the *rpl32* standard and expressed as fold change compared with controls±s.e.m. (n=3).

Lifespan and stress assays

For lifespan assays, adult female flies were kept on standard medium in groups of 30, transferred to fresh food every 2 days and were counted daily until no living flies remained. For starvation assays, 7-day-old female flies were anaesthetized on ice and placed in groups of 20 in 30 ml cotton-capped glass vials containing 1% aqueous agarose only. Vials were checked for dead flies every 4 h until no living flies remained. For desiccation experiments, the same protocol was followed, except that the vials were empty (no food or water), and the open end of the tube was sealed with parafilm (Bemis). Vials were checked every 2 h until no living flies remained. All experiments were run in triplicate with at least 30 flies in each run of specified genotype. All

vials were placed in an incubator at 22°C, 55% humidity with a 12:12 light: dark photoperiod. Survival data has been expressed as % survival±s.e.m.

Gravimetric estimates of body water

Body weight measurements of *CapaR>GATAe* RNAi flies were performed as described previously (Cabrero et al., 2014). Briefly, the weight of total body water was calculated by subtracting dry weight from wet weight. Experiments were run in triplicate with at least 30 flies of each genotype (n>90 flies for the three genotypes).

Malpighian tubule secretion assays

Secretion assays were performed as described previously (Dow et al., 1994). Basal and neuropeptide-stimulated secretion rates were measured every 10 min. After 30 min of baseline readings, the peptide *Drosophila* kinin was added at a concentration of 10^{-7} M, and secretion rates were measured every 10 min for 30 more min. At least seven tubules were used for each condition. Data were plotted as mean±s.e.m.

Data analysis

For mRNA level quantification or fluid secretion analysis, a two-tailed Student's *t-test*, taking P=0.05 as the critical value (for each independent group) were used. For wet- and dry-weight measurements, two-way ANOVA was used to compare each condition. P values were adjusted using the Sidak multiple comparisons test. For survival curves obtained in lifespan, starvation and desiccation assays, significance was assessed by the log-rank (Mantel-Cox) test. For cell counting experiments, significance was assessed comparing each column using a two-tailed Student's *t*-test, incorporating Welch's correction. All statistical analysis was performed using GraphPad Prism 7.0 software (GraphPad Software).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: G.M.-C., S.T., S.-A.D.; Methodology: G.M.-C., P.C., S.T.; Software: G.M.-C.; Validation: G.M.-C., P.C., S.T.; Formal analysis: G.M.-C.; Investigation: G.M.-C.; Resources: P.C., S.-A.D.; Data curation: G.M.-C.; Writing - original draft: G.M.-C., S.T.; Writing - review & editing: G.M.-C., P.C., J.A.T.D., S.T., S.-A.D.; Visualization: S.T., S.-A.D.; Supervision: J.A.T.D., S.T., S.-A.D.; Project administration: J.A.T.D., S.-A.D.; Funding acquisition: J.A.T.D., S.-A.D.

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Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.178087.supplemental

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