Ttk69 acts as a master repressor of enteroendocrine cell specification in Drosophila intestinal stem cell lineages

Chenhui Wang, Xingting Guo, Kun Dou, Hongyan Chen and Rongwen Xi*

ABSTRACT
In adult Drosophila midgut, intestinal stem cells (ISCs) periodically produce progenitor cells that undergo a binary fate choice determined primarily by the levels of Notch activity that they receive, before terminally differentiating into enterocytes (ECs) or enteroendocrine (EE) cells. Here we identified Ttk69, a BTB domain-containing transcriptional repressor, as a master repressor of EE cell specification in the ISC lineages. Depletion of ttk69 in progenitor cells induced ISC proliferation and caused all committed progenitor cells to adopt EE fate, leading to the production of supernumerary EE cells in the intestinal epithelium. Conversely, forced expression of Ttk69 in progenitor cells was sufficient to prevent EE cell specification. The expression of Ttk69 was not regulated by Notch signaling, and forced activation of Notch, which is sufficient to induce EC specification of normal progenitor cells, failed to prevent EE cell specification of ttk69-depleted progenitors. Loss of Ttk69 led to derepression of the acheate-scute complex (AS-C) genes scute and asense, which then induced prospero expression to promote EE cell specification. These studies suggest that Ttk69 functions in parallel with Notch signaling and acts as a master repressor of EE cell specification in Drosophila ISC lineages primarily by suppressing AS-C genes.

KEY WORDS: Intestinal stem cell, Drosophila midgut, Tramtrack, Ttk69, Enteroendocrine cell, Notch, acheate-scute complex, Prospero

INTRODUCTION
Enteroendocrine (EE) cells in the intestinal epithelium regulate a number of physiological functions, including intestinal motility, appetite, food digestion and immunity, and their dysregulation has been linked to various diseases, such as inflammatory bowel disease, enteric anendocrinosis and neuroendocrine tumor (Harrison et al., 2013; van der Flier and Clevers, 2009). Therefore, understanding how EE cells are coordinately specified along with other cell lineages from local intestinal stem cells (ISCs) might not only contribute to our understanding of tissue homeostasis and regeneration, but also help to illuminate disease mechanisms.

The Drosophila midgut has emerged as an attractive system with which to study the process of multiple cell lineage differentiation from ISCs as it is simpler, yet similar in cellular composition and regulatory mechanisms to the mammalian intestine, along with advantages in terms of the capacity for genetic manipulation (Casali and Batte, 2009; Jiang and Edgar, 2011; Takashima et al., 2013). In the posterior midgut, the multipotent ISCs periodically produce committed progenitor cells termed enteroblasts (EBs), which exit the cell cycle and are subjected to a binary fate choice to differentiate into either absorptive enterocytes (ECs) or secretory EE cells (Fig. 1A). The level of Notch activity is critical in directing ISC differentiation and determining the binary fate choice of EBs, as loss of Notch leads to the accumulation of ISCs and EE-like cells, whereas forced Notch activation is able to deplete ISCs by forcing differentiation into ECs (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). The specific, but not static, expression of the ligand Delta (Dl) in ISCs has led to the model that ISCs control the fate of their own daughter cells via differential levels of Notch activation: Dlhigh ISC-derived EBs receive strong Notch activation and consequently commit differentiation towards EC fate, whereas Dllow ISC-derived EBs receive weak or no Notch activation and commit differentiation towards the EE fate (Ohlstein and Spradling, 2007).

Transcriptional activation of EE cell differentiation is mediated by the acheate-scute complex (AS-C) proneural genes, which include the homologous genes acheate (ac), scute (sc), lethal of scute [l(3)sc, or l(l)sc] and asense (ase), which are localized in a single cluster in the genome. Functional analyses suggest that the AS-C genes, and sc in particular, are necessary and sufficient for EE cell specification (Amcheslavsky et al., 2014; Bardin et al., 2010). In some, but not all, tumor cells caused by the loss of Notch, sc and ase are derepressed, indicating that Notch may participate in the suppression of AS-C genes to control EE cell differentiation (Zeng et al., 2013). EE cell differentiation also requires autonomous JAK/STAT signaling activity, and genetic analyses suggest that JAK/STAT is epistatic to Notch signaling in EE cell specification (Beebe et al., 2010; Jiang et al., 2009; Lin et al., 2010). Similarly, the chromatin regulator Osa is required for the differentiation of ISC progeny into EE cells by regulating ase (Zeng et al., 2013). Other factors known to be required for EE cell specification include Tsc1 and Tsc2 (Gigas – FlyBase), the loss of function of which induces hyperactivation of Target of rapamycin (Tor) and, consequently, failure of EE cell specification (Kapuria et al., 2012; Quan et al., 2013). A recent study revealed that differentiating EE cells also provide a negative feedback via Slit-Robo signaling to restrict EE cell production from ISCs (Biteau and Jasper, 2014).

The Drosophila tramtrack (ttk) locus encodes two proteins, Ttk69 and Ttk88, via alternative splicing (Read and Manley, 1992). These isoforms possess divergent C-terminal zinc-finger domains for DNA binding, but share common N-terminal sequences containing a conserved BTB/POZ domain. The different DNA-binding domains result in different DNA binding specificities between Ttk69 and Ttk88 and thus, conceivably, different functions (Read and Manley, 1992). Previous studies have demonstrated that Ttk is a transcriptional repressor and is involved in regulating cell fate specification during many aspects of Drosophila development. For instance, Ttk69 acts as a regulator of cell fate choice between neural and non-neural identity (Badenhorst, 2001; Badenhorst et al.,

Received 11 February 2015; Accepted 10 August 2015
More recent studies reveal that Ttk69 also functions in non-neural tissues, such as during follicle cell differentiation (Boyle and Berg, 2009; Sun et al., 2008) and tracheal development (Araujo et al., 2007; Rotstein et al., 2012), and is also involved in cell cycle regulation (Baonza et al., 2002). But whether Ttk has a function in the adult midgut has not been reported.

Here, from a genetic screen, we identified Ttk69 as a key regulator of cell fate specification in ISC lineages of adult Drosophila midgut. Loss of Ttk69 causes all EBs to adopt EE cell specification. Our studies suggest that Ttk69 functions in parallel with Notch signaling and acts as a master repressor of EE cell specification by suppressing AS-C complex genes.

RESULTS

Loss of ttk causes the production of supernumerary EE cells

To further understand the fate choice mechanisms in the ISC lineages of the adult Drosophila midgut, we performed a GAL4/UAS system-based screen with a UAS-transgenic RNAi library from VDRC and Harvard (Brand and Perrimon, 1993; Dietzl et al., 2007; McGuire et al., 2004; Ni et al., 2011). Individual UAS-RNAi lines were crossed to flies carrying esg-Gal4, UAS-GFP; Tub-Gal80ts (referred to as esgGal4ts), which allows temperature-controlled RNAi induction in progenitor cells (ISCs and EBs). Crosses were conducted at permissive temperature (18°C) and 3- to 5-day-old esgGal4ts>UAS-RNAi flies were shifted to the restrictive temperature (29°C) for 1 week prior to dissection and analysis. After screening ∼2000 midgut-expressed genes, we identified one that when knocked down gave rise to a unique phenotype: the formation of supernumerary EE cells surrounding GFP+ cells in the epithelium.

EE cells, which can be specifically marked by Prospero (Pros), are normally sparsely distributed in the midgut epithelium (Fig. 1B). Under normal conditions, ∼80-90% of EBs adopt EC specification and ∼10-20% adopt EE cell specification. As a consequence, EE cells represent only 10-20% of ISC progeny in the midgut epithelium (Biteau and Jasper, 2014; Ohlstein and Spradling, 2007). However, knockdown of ttk by RNAi (v10855) caused the production of supernumerary EE cells along the entire midgut (Fig. 1C), including the copper cell region (not shown). Similar results were obtained by expressing another RNAi transgene with different targeting sequences (TRIP#36748) (Fig. 1D).

Ttk69 regulates EE/EC specification

Because ttk is essential for animal viability, to confirm that depleting ttk is responsible for the phenotype we generated ttk mutant cells in the gut epithelium by induced mitotic
recombination using the MARCM system (Lee and Luo, 1999), and analyzed cellular behavior in the GFP-marked mutant cell clones, as previously described (Lin et al., 2008, 2010; Wang et al., 2013; Xu et al., 2011). The ttk locus encodes two proteins, Ttk69 and Ttk88, via alternative splicing, and, in contrast to GFP-marked wild-type clones (Fig. 2A,A′), clones on day 7 after clone induction (ACI) that are homozygous for ttkD2-50, an amorphic allele that affects both Ttk69 and Ttk88 function (Giesen et al., 1997), contained supernumerary Pros+ EE cells and occasional polyploid ECs (Fig. 2B,B′). Clones homozygous for ttk1e11, an allele that specifically disrupts Ttk69 (Lai and Li, 1999), displayed a very similar phenotype to ttkD2-50 (Fig. 2C,C′). The formation of some ECs in the mutant clone, which can be marked by Pdm1 (Nubbin – FlyBase) expression (Fig. 2E,E′), could be due to residual activity of Ttk products in the mutant cells. By contrast, clones homozygous for ttk1, which disrupts Ttk88 only (Xiong and Montell, 1993), displayed no obvious difference to the wild-type clones (Fig. 2D,D′).

The excessive Pros+ cells in ttk69 mutant clones were able to express neuropeptides, such as Tachykinin (Tk) and Allatostatin A (AstA) (supplementary material Fig. S1), indicating that they are able to differentiate into mature EE cells. The mutant clones also contained many Dllow cells with marginal detection of Dl expression (Fig. 2F,F′; supplementary material Fig. S2). Quantitative analysis of the cell population in ttk69 mutant clones on day 7 ACI revealed that ~62% of cells were Pros+ and ~16% were Dllow (Fig. 2G). Interestingly, many Dl low cells co-expressed Pros (Fig. 2F″). These cells are probably progenitor cells committed to, or in the process of, EE cell specification (Fig. 2G). Therefore, the vast majority of cells in ttk69 mutant clones are EE cells and EE-committed progenitor cells.

In addition to changes in cellular composition, the ttk69 mutant clones grew much faster than wild-type clones, as evidenced by significantly larger clone sizes and increased mitotic figures of the mutant clones (Fig. 2H,H′ compared with 2I,I′). The Dl+ cells in the mutant clones are probably ISC-committed progenitor cells that are mitotically active and responsible for clonal outgrowth of the
mutant cells, as cells with mitotic figures were only found within these cell populations (supplementary material Fig. S2). These observations indicate that Ttk69 also negatively regulates ISC proliferation, which is consistent with the observation that the esg>GFp+ cells are moderately increased in esg>ttk-RNAi intestines (Fig. 1C,D). Ttk69 is known to be a negative regulator of cell proliferation in neural and eye disc development by suppressing the cell cycle regulators cyclin E and String (Badenhorst, 2001; Baonza et al., 2002). Consistent with this role, Cyclin E and String were significantly upregulated in esg>ttk-RNAi intestines (supplementary material Fig. S3).

Taken together, these data suggest that Ttk69, but not Ttk88, regulates the proliferation of ISCs and the binary fate choice of EBs, which, when disrupted, leads to the increased production of EBs, followed by unambiguous commitment to EE specification, leading to excessive EE cell production and EE tumors.

**Ttk69 is required in both ISCs and EBs for proper cell fate specification**

Because esg>Gal4 is expressed in both ISCs and EBs, and the MARCM clones contain both ISCs and the derived progenies, the above experiments could not distinguish whether Ttk69 functions in ISCs or EBs to repress EE cell specification. We found that knocking down ttk using Dl-Gal4, an ISC-specific Gal4 (Zeng et al., 2010), produced a similar supernumerary EE cell phenotype (Fig. 3B), suggesting that Ttk69 is required in ISCs to prevent excessive EE commitment. Strikingly, knocking down ttk using Su(H)GBE-Gal4 also produced the extra EE cell phenotype (Fig. 3C,D). However, the number of Dl+ cells in the intestinal epithelium remained largely unchanged (Fig. 3E,F,G), indicating that the EBs with ttk69-RNAi are probably not dedifferentiated into ISCs. Interestingly, some EBs with ttk69-RNAi re-entered mitosis (Fig. 3I), which is probably due to derepression of expression of Cyclin E and String.

To determine whether the extra EE cells were indeed derived from Su(H)GBE-Gal4+ EBs, we conducted a directed cell lineage-tracing analysis of Su(H)GBE-Gal4+ EBs with ttk-RNAi by generating flies carrying Su(H)GBE-Gal4, Tub-Gal80Δ [Su(H) GBE-Gal4Δ], UAS-flp, and the FLP-out cassette (Act<stop<lacZ). These elements allow conditional activation of Flapase in Su(H)GBE-Gal4+ cells and therefore all descendants of Su(H)GBE-Gal4+ cells will be marked by lacZ expression (Fig. 3I). The cell lineage-tracing results revealed that the extra EE cells were lacZ+ and were therefore derived from Su(H)GBE-Gal4+ EBs (Fig. 3K,L). Because Su(H) GBE-Gal4+ cells are EC-committed EBs (Biteau and Jasper, 2014), these results indicate that depletion of Ttk69 is sufficient to induce mitosis of EBs and override their previous cell commitment, and force these EC-committed EBs to adopt EE cell specification instead. It is conceivable that some degree of dedifferentiation might have occurred in Ttk69-depleted EBs to erase EC commitment and to re-enter the cell cycle. Taken together, these observations suggest that Ttk69 functions in both ISCs and EBs to prevent excessive cell division and EE cell specification.

**Overexpression of Ttk69 is sufficient to repress EE cell specification**

The above data imply that the level of Ttk69 expression could be a determining factor in the cell fate decision of progenitor cells. To determine its expression pattern, we generated a polyclonal antibody against Ttk69. The anti-Ttk69 signal was virtually undetectable in ttk69 mutant ISCs or ovarian follicle cell clones, and displayed significantly high levels in MARCM clones overexpressing ttk69 (supplementary material Fig. S4), demonstrating that this antibody is highly specific. Using this antibody, we found that Ttk69 was generally expressed in all epithelial cells in the midgut, but with different levels in different cell types. The lowest level was found in ISCs and EE cells, a moderate level in Su(H)-lacZ+ EBs, and highest levels in ECs (Fig. 3B,C). The expression pattern is largely consistent with its role as a repressor of EE cell specification in the ISC lineages.

To test whether elevation of Ttk69 expression in ISCs and EBs is sufficient to prevent EE cell specification, we generated ISC clones with forced Ttk69 expression by the MARCM system and examined the consequences. As a control, ∼45% of wild-type ISC clones at 7 days contained at least one EE cell (Fig. 3C,G). By contrast, ISC-containing clones with forced Ttk69 expression were much smaller and consisted of only 2-5 cells, the non-stem cells in the clones were virtually all ECs, and many clones no longer contained ISCs but only differentiating or differentiated ECs (Fig. 3D,G). Therefore, Ttk69 overexpression is sufficient to prevent EE cell production from ISCs. The maintenance of ISC identity in some Ttk69-overexpressing clones indicates that, unlike Notch activation, forced Ttk69 expression does not seem to promote differentiation of ISC into EC fate.

To further test whether Ttk69 has an instructive role for EC differentiation, we examined whether Ttk69 is able to induce EC specification of Notch+ progenitor cells. As a control, Notch−/− ISC clones generated both Dl− ISC-like cell clusters and Pros+ EE cell clusters (Fig. 4E,E′). Co-expressing Ttk69 in Notch−/− ISC clones prevented tumor development and EE cell specification, but failed to induce EC differentiation, as these mutant cells remained small and likely diploid, with strong DI expression (Fig. 4F,F′). These observations suggest that Ttk69 acts more like a barrier for EE cell specification, rather than a promoter for EC specification. Therefore, Ttk69 negatively regulates ISC proliferation and its expression is necessary and sufficient to repress EE cell specification from progenitors.

**The relationship between Ttk69 and Notch signaling in cell fate regulation**

Because Ttk is known to interact with Notch in controlling cell fate decisions in a number of developmental processes (Boyle and Berg, 2009; Giesen et al., 1997; Guo et al., 1995; Xiong and Montell, 1993), and Notch signaling plays a central role in controlling the choice of stem cells in the digestive tract, including gastric stem cells in the copper cell region where depletion of Ttk69 also causes the extra EE cell phenotype (data not shown), Ttk69 might have a functional or regulatory relationship with Notch signaling in the control of EE cell specification.

We first determined whether the loss of Ttk69 affects Notch signaling. GBE-Su(H)m8-lacZ [referred to hereafter as Su(H)-lacZ] is a Notch activation reporter, which is specifically expressed in EBs in which Notch is activated. As expected, this marker was no longer detectable in Notch-RNAi clones (Fig. 5A,A′). However, its scattered expression pattern was retained within ttk69−/− clones (Fig. 5B,B′), indicating that depletion of Ttk69 does not autonomously affect Notch signaling activation. Conversely, although forced activation of Notch by expressing an intracelullar domain of Notch (NΔI) is sufficient to induce ISC differentiation toward ECs, it failed to induce differentiation of ttk−/− cells, as the vast majority of mutant cells in the clones remained small and likely diploid, and did not turn on the EC marker Pdm1 (Fig. 5D,D′). These cells also displayed partial impairment in differentiation toward EE cells, as most cells failed to turn on Pros or Tk expression (Fig. 5Ea-d). It was previously shown that Ttk is required for the Notch-dependent mitotic-to-endocycle
switch in *Drosophila* follicle cells (Jordan et al., 2006), and this function could potentially explain why the endocycle does not occur in intestinal cells when Ttk is ablated, even though Notch is activated. Alternatively, conflicts in differentiation programs could cause delay or improper cell lineage differentiation, leading to the generation of aberrant cells. However, loss of Notch did not affect the expression of Ttk69, as the level of Ttk69 protein in Notch mutant clones remained similar to that in wild-type ISCs (Fig. 5C,C‘). Taken together, these data suggest that the expression and function of Ttk69 are independent of Notch signaling, and vice versa, indicating that Ttk69 and Notch signaling act in parallel in controlling the binary fate decision of EBs.

**Ttk69 is epistatic to multiple cell fate regulators in repressing EE cell specification**

The ability to override the fate of EC-committed progenitor cells following the depletion of Ttk69 indicates that Ttk69 could be a master repressor of EE cell fate. Previous studies revealed that the
process of EB to EE cell differentiation can be blocked by mutations in several regulatory pathways. Loss of JAK/STAT signaling activity causes EBs to arrest at undifferentiated states, as they are unable to differentiate further into ECs or EE cells (Beebe et al., 2010; Jiang et al., 2009; Lin et al., 2010). Depletion of the chromatin remodeling factor Osa by RNAi produces a similar phenotype (Zeng et al., 2013). Loss of Tsc1/2 leads to hyperactivation of Tor, which prevents EB differentiation into EE cells, but leaves EC differentiation unaffected (Kapuria et al., 2012; Quan et al., 2013).

To understand the epistatic relationships between Ttk69 and these regulators in EE cell specification, we generated and analyzed double-mutant clones. Strikingly, co-depleting Ttk69 allowed all mutant progenitor cells, including JAK/STAT-deficient cells (Fig. 6A), Tsc1Q87X mutant cells (Fig. 6C) and Osa-depleted progenitors (Fig. 6E) to unidirectionally differentiate into EE cells (Fig. 6B,D,F-H), indicating that Ttk69 is epistatic to multiple cell fate regulators as a master gatekeeper for EC specification by repressing EE fate. In dome, Tsc1 or osa-RNAi mutant cells, Ttk69 expression was not obviously upregulated (supplementary material Fig. S5), indicating that these pathways do not seem to directly regulate the level of Ttk69 expression, although the possibility of post-translational regulation of Ttk69 cannot be excluded.

Fig. 4. Overexpression of Ttk69 is sufficient to repress EE cell specification. (A-B) The expression pattern of Ttk69 in the midgut. Ttk69 (immunostaining, red) (A,A') was expressed in Dl+ ISCs (yellow arrowhead), Pros+ EE cells (white arrows), EBs (black arrowhead) as well as polyploid ECs, but at different levels. (B) The relative expression levels of Ttk69 among the different cell types. *N*act indicates activation of Notch signaling. (C-D') Pros+ EE cells were rarely observed in MARCM clones overexpressing Ttk69 (D,D'), as compared with the control (C,C'). (E-F') In comparison to N55e11 clones (E,E'), which contained both ISC-like and EE-like cell clusters, Ttk69 overexpression prevented the formation of large cell clusters and inhibited the formation of EE cells (F,F', arrow). (G) Quantification of the percentage of clones containing EE cells in C-F'. n=15-25 intestines. Error bars indicate s.e.m. ***P<0.001 (Student’s t-test). Scale bars: 20 μm.

Fig. 5. The relationship between ttk69 and Notch signaling in cell fate regulation. (A,A') Su(H)-lacZ was significantly downregulated in clones expressing Notch-RNAi. (B,B') Su(H)-lacZ was largely unaffected in clones mutant for ttk1e11. (C,C') The Ttk69 expression level was unaffected in clones mutant for N55e11. (D,D') Overexpression of an active form of Notch (Nicd) in ttk1e11 clones failed to induce differentiation towards ECs. (E) Reduced production of Pros+ (a,b) or Tk+ (c,d) EE cells in ttk1e11 clones expressing Nicd. GFP, green; DAPI, blue. Scale bars: 20 μm.
Ttk69 functions upstream of AS-C genes in EE cell specification

Previous studies have demonstrated an essential role for the AS-C complex genes in EE cell specification (Bardin et al., 2010). The excessive EE cell formation following the depletion of ttk69 was reminiscent of the phenotype caused by forced expression of AS-C complex genes. We therefore performed an epistasis analysis between ttk69 and AS-C genes.

Df(1)scB57 is a chromosome deficiency allele, in which the coding regions for all AS-C genes are deleted. In Df(1)scB57 clones, Pros+ or Tk+ cells failed to develop (Fig. 7A, A'), as observed previously (Bardin et al., 2010). As a control, expression of a sc transgene in Df(1)scB57 clones allowed the formation of EE cells (Fig. 7B, B'). However, knocking down ttk69 in Df(1)scB57 clones did not rescue the failure of EE cell formation (Fig. 7C-D), suggesting that AS-C genes are epistatic to ttk69 in EE cell specification.

By real-time quantitative PCR (RT-qPCR), we found that sc and ase, but not ac or lsc, are significantly upregulated in esg>tk-RNAi compared with wild-type intestines (Fig. 7E), and antibody staining of Sc and Ase revealed that both proteins are upregulated in ttk69 mutant clones (Fig. 7F-G'). Consistent with this observation, the supernumerary EE cell phenotype following ttk69 depletion could be effectively suppressed by simultaneous knockdown of both sc and ase, but not either alone (Fig. 7H-L).

Taken together, these data suggest that Ttk69 controls EE cell specification by suppressing the expression of both sc and ase genes.

Relationships among ttk69, AS-C genes and Pros in EE cell specification

Pros, a common EE cell marker, is a transcription factor previously best known as a cell fate determinant in ganglion mother cells of the Drosophila central nervous system (Hirata et al., 1995; Spana and Doe, 1995). Consistent with its role in cell fate regulation, recent studies suggest that Pros regulates EE cell specification since depleting Pros in ISCs leads to a reduction of EE cells in the midgut epithelium (Biteau and Jasper, 2014). To directly test whether Pros is autonomously required for EE cell specification, we generated MARCM clones mutant for the loss-of-function allele pros17. None of the cells in the mutant clones (n=50 clones examined) expressed either Tk or AstA, two EE cell-specific peptide hormones (Fig. 8A). As Tk+ and AstA+ EE cells make up the majority of the EE population in the posterior midgut, it can be deduced that Pros is cell-autonomously required for EE cell specification and/or maturation. We found that Pros may have an instructive role for EE cell specification, as overexpression of Pros in ISC clones was sufficient to deplete ISCs by inducing their differentiation into EE cells (Fig. 8B, C).
Next, we performed epistasis studies to investigate the regulatory relationships among pros, ttk69 and AS-C genes. In Ttk69-depleted clones, co-depleting Pros completely suppressed the supernumerary EE cell phenotype, and virtually all mutant cells within the clones remained undifferentiated, as they failed to turn on the expression of Tk (Fig. 8G) and Pdm1 (Fig. 8F) and retained relatively high levels of Dl expression (Fig. 8G). In sc overexpression clones, co-depleting Pros also completely suppressed the supernumerary EE cell phenotype and, similarly, virtually all mutant cells within the clones remained undifferentiated (Fig. 8I; data not shown).

Conversely, although Ttk69 overexpression is sufficient to prevent EE cell specification, co-expression of Pros allowed the production of hormone-producing EE cells (Fig. 8J). These data demonstrate that pros is epistatic to ttk69 and sc in EE cell specification, and suggest a Ttk69–AS-C–Pros regulatory axis in controlling the EE specification of progenitor cells and in mediating excessive EE cell production caused by the loss of Ttk69: following Ttk69 depletion, the AS-C genes are derepressed, followed by the induction of Pros. Pros then acts as an EE-determining factor to promote EE cell specification, leading to excessive EE cell production (Fig. 8K).

**DISCUSSION**

In the *Drosophila* midgut we identified that Ttk69, a BTB domain-containing transcriptional repressor, acts as a master repressor of EE cell specification. Because many aspects of cell fate regulation in the intestinal epithelium are similar between *Drosophila* and mammals, and BTB domain-containing proteins are found throughout eukaryotes (Perez-Torrado et al., 2006), it is possible that a functional counterpart of Ttk69 might also exist in mammalian intestine and regulate key cell fate decisions.

We consider Ttk69 as a master repressor of EE cell specification in *Drosophila* midgut for a number of reasons. First, loss of Ttk69 causes the committed progenitor cells to unidirectionally adopt EE cell specification, leading to a dramatic supernumerary EE cell phenotype. Second, Ttk69 is not only required to repress EE cell specification, but also its ectopic expression is sufficient to prevent progenitor cells from adopting EE fate. Moreover, loss of Ttk69 is
sufficient to prevent Notch activation-induced EC specification and induce EE cell specification instead. That is to say, depletion of Ttk69 is able to override EC commitment of Notch-activated progenitor cells and turn them instead to EE cell commitment. In addition, loss of Ttk69 is able to induce EE cell specification of differentiation-defective progenitors caused by various mutations, such as in JAK/STAT, Tsc1 or Osa, indicating that Ttk69 acts as a final gatekeeper downstream of multiple regulators to prevent excessive EE cell specification. Lastly, our mechanistic studies demonstrate that Ttk69 suppresses EE cell specification by preventing Pros expression via suppressing AS-C genes (sc and ase). Therefore, the Ttk69–Sc/Ase–Pros regulatory axis controls the specification of EE cells from ISCs (Fig. 8K).

It is yet to be determined whether Ttk69 suppresses AS-C genes directly or indirectly. In the Drosophila embryo, sc seems to be directly suppressed by Ttk69, as its expression is significantly upregulated in ttk mutant embryos and significantly decreased in ttk overexpression embryos (Rotstein et al., 2012). In addition, analysis of Ttk69 ChIP-seq results from modENCODE data reveals potential binding activity of Ttk69 in the regulatory regions of sc (The modENCODE Consortium et al., 2010; Rotstein et al., 2012). These observations indicate that Ttk69 could directly regulate the AS-C genes to control cellular fate in many developmental processes, including EE cell specification in ISC lineages—a hypothesis that warrants further investigation. In addition to the master function of Ttk69, our studies also suggest that Pros can be considered as a master EE determinant, as its expression is both necessary and sufficient for EE cell specification. We propose that Ttk69 and Pros act as a master repressor and a master activator, respectively, of EE cell specification in the ISC lineages of the adult Drosophila midgut.

Prior to this study, Notch signaling was known to be a key cell fate regulator in the ISC lineages and different levels of Notch activation determine the binary fate of EBs, with high Notch activation favoring EC differentiation and low or no Notch activation favoring EE cell differentiation (Ohlstein and Spradling, 2007). Largely consistent with this hypothesis, cell lineage-tracing studies demonstrate that the Notch-activated committed progenitors, namely Su(H)-Gal4+ EBs, are unipotent EC-committed progenitors (Biteau and Jasper, 2014). Our studies suggest that Ttk69 does not function through regulating Notch signaling. As reflected by the Notch activation reporter, Notch is properly activated in ttk69 mutant clones. In addition, our functional and cell lineage-tracing studies demonstrate that the EC-committed EBs will adopt EE cell specification upon depletion of Ttk69. Moreover, forced Notch activation could not initiate EC differentiation and prevent EE cell specification when Ttk69 was depleted. On the other hand, Notch signaling does not appear to be upstream of Ttk69 in controlling the EC versus EE decision, as the expression of Ttk69 is not regulated by Notch activity. Therefore, Ttk69 and Notch are likely to function in two parallel pathways to control the binary fate decision of EBs (Fig. 8K).
Although DI/Notch is a major signaling pathway controlling cell fate decisions in the midgut, how it is regulated is poorly understood. The DI expression level in ISCs seems not to be static, and this property could be essential for the generation of EBs with differential levels of Notch activity and, consequently, alternative cell fates. But how DI expression is regulated in ISCs is unknown. Similarly, it remains to be determined how the expression or function of Ttk69 is regulated in the context of cell fate decisions. Ttk69 protein is expressed in all epithelial cells in the midgut but at different levels. The lowest level is found in ISCs and EE cells, a pattern that is consistent with its role as an EE fate suppressor. In addition, transcriptional depletion or overexpression of Ttk69 is sufficient to allow or prevent, respectively, EE cell specification. These observations indicate that transcriptional regulation of Ttk69 might hold the key in controlling Ttk69 function, although regulation at the post-transcriptional level, which occurs in other developmental processes (Li et al., 1997; Okabe et al., 2001), could provide additional layers of functional control.

It has recently been proposed that EE cells could differentiate directly from ISCs rather than indirectly from EE-committed EBs (Biteau and Jasper, 2014; Zeng and Hou, 2015). Because Ttk69 depletion does not compromise ISC self-renewal, this indicates that the extra EE cells are differentiated from EBs rather than ISCs. Therefore, a more plausible explanation for the supernumerary EE cell phenotype following Ttk69 depletion in progenitor cells would be as follows: depletion of Ttk69 in ISCs will cause ISCs to produce EE-committed progenitor cells only; it will also cause ISCs to divide more rapidly, thereby generating more EE-committed progenitor cells; depletion of Ttk69 in EC-committed EBs will cause a certain degree of dedifferentiation to dividing progenitor cells that can only give rise to EE cells. These effects together lead to the production of supernumerary EE cells. Further elucidation of the regulatory mechanisms of Ttk69 and DI/Notch signaling will be the next steps towards a complete understanding of cell fate decisions in the midgut stem cell lineage.

MATERIALS AND METHODS

Fly strains

The following stocks were used in this study: UAS-ttk-RNAi#1 (VDRC, v10855); UAS-ttk-RNAi#2 (BDSC, 36748); FRT82B-ttkD2-50 (gift from Marta Limargas, Molecular Biology Institute of Barcelona, Spain); esg-Gal4, UAS-GFP (gift from Shigeo Hayashi, RIKEN Center for Developmental Biology, Japan); Gbe-Su(H)m8-lacZ (gift from Sarah Forciniti, Tsinghua Fly Center, Beijing); UAS-Flp, FRT82B-pro17 (Tsinghua Fly Center, Beijing); UAS-Notch-RNAi, FRT82B-N55e11 (BDSC, 36748); UAS-ttk-RNAi#1 (BDSC, 36748); FRT19A-N55e11 (gift from Ting Xie, Stockholm University, Sweden); 1:3000); rabbit polyclonal anti-Allatostatin A (DSHB; 1:100); mouse anti-Pros (DSHB; 1:300); mouse anti-Allatostatin A (DSHB; 1:300); rabbit anti-phospho-Histone H3 (Upstate, 6570; 1:1000); rabbit anti-Tachykinin (a gift from Dick Nassel, Stockholm University, Sweden; 1:3000); rabbit polyclonal anti-β-galactosidase (Cappell, 0855976; 1:6000); rat anti-Sc (gift from Steve Crews, University of North Carolina at Chapel Hill, USA; 1:1000); rabbit anti-FGFR (Invitrogen, A11122; 1:2000); rabbit anti-Ase (gift from Yuh-Nung Jan, UCSC, USA; 1:1000); rabbit anti-Pros (gift from Yuh-Nung Jan; 1:1500); rabbit anti-Sc (gift from Yuh-Nung Jan, UCSC, USA; 1:400); and rabbit anti-Pdm1 (gift from Xiaohang Yang, Zhejiang University, China; 1:1000). Secondary antibodies were goat anti-rabbit, anti-mouse or anti-rat IgGs conjugated to Alexa Fluor 488, Alexa Fluor 568 or Cy5 (Molecular Probes, A11034-A11036, A10524; 1:300). Images were captured using a Zeiss LSM510 confocal microscope. All images were adjusted and assembled in Adobe Photoshop and Illustrator.

RT-qPCR

Total RNA from 30-50 adult intestines was isolated using TRIzol (Invitrogen) following the manufacturer’s instructions. cDNAs were synthesized using the High-Fidelity cDNA Synthesis Kit (Roche). RT-qPCR was performed using the SYBR PrimeScript RT-PCR Kit (Takara) on an ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems). RT-qPCR was repeated for three independent biological replicates. Expression of each gene was normalized to Gapdh, and relative levels were calculated using the 2−ΔΔCT method. Primer sequences were (S3’ forward and reverse): Gapdh, GAAATTAAGGCCAAGGTTCAGG and GTACCAAAGAGATACGGCTTCA; ac, TTTCACAAGCAGCAGAGGAG and ACCATCGTTAATCGCGTCGACA; sc, AATGTAGACCAAATCCAGTCG and CACCCACCTTTGCAAATCC; x/c, TCAAACTGTGATTAATCTC- GCTTGC and TCGCCGGAATTTGAGATGTTG; gse, GCAACACCGCAGAATTCAC and AGGCGAACCTTTTCTCCAG.

Acknowledgements

We thank the members of the fly community as cited in the Materials and Methods for generously providing fly stocks and antibodies; the Bloomington Drosophila Stock Center (BDSC), Tsinghua Fly Center, Vienna Drosophila RNAi Center (VDRC) and Developmental Studies Hybridoma Bank (DSHB) for reagents; and members of the R.X. laboratory for helpful comments.

Competing interests

The authors declare no competing or financial interests.

Author contributions

C.W. and R.X. conceived and designed the experiments, analyzed the data and wrote the manuscript. C.W., X.G., K.D. and H.C. performed the experiments.

Funding

This work was supported by National Basic Science 973 grants [2011CB812700 and 2014CB849700] from the Chinese Ministry of Science and Technology.

Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl?doi:10.1242/dev.123208/DC1
References

Amcheslavsky, A., Song, W., Li, Q., Nie, Y., Bragatto, I., Ferrand, D.,
Perrimon, N. and Ip, Y. T. (2014). Enteroendocrine cells support intestinal stem-


morphogenetic events during Drosophila tracheal development. Development

134, 3665-3676.

Badenhorst, P. (2001). Tramtrack controls glial number and identity in the

Drosophila embryonic CNS. Development 128, 4093-4101.


Baonza, A., Murawsky, C. M., Travers, A. A. and Freeman, M. (2002). Pointed and

Tramtrack69 establish an EGRF-dependent transcriptional switch to regulate


(2001). Tramtrack controls glial number and identity in the

Drosophila PNS. Development 128, 2307-2316.


transgenic RNAi library for conditional gene inactivation in Drosophila.

Nature 448, 401-415.


