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

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ABSTRACT

In adult *Drosophila* midgut, intestinal stem cells (ISCs) periodically produces progenitor cells that undergo a binary fate choice determined primarily by the levels of Notch activity they receive, before terminally differentiate into enterocytes (ECs) or enteroendocrine cells (EEs). 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 induces ISC proliferation and rendered all committed progenitor cells to adopt EE cell specification, 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 progenitor cells. We found that loss of Ttk69 led to derepression of *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.
INTRODUCTION

Enteroendocrine 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 enteroendocrine cells are coordinately specified along with other cell lineages from local intestinal stem cells may 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 to study the process of multiple cell lineage differentiation from intestinal stem cells (ISCs) due to simpler yet similar cellular composition and regulatory mechanisms to the mammalian intestine, along with advantages in genetic manipulation (Casali and Batlle, 2009; Jiang and Edgar, 2011; Takashima et al., 2013). In the posterior midgut, the multipotent ISCs periodically produce committed progenitor cells named enteroblasts (EBs), which exit cell cycle and are subjected to a binary fate choice to differentiate into either absorptive enterocytes (ECs) or secretory enteroendocrine cells (EEs) (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 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 leads to a model that ISCs control the fate of their own daughter cells by
differential levels of Notch activation: Dl\textsuperscript{high} ISC-derived EBs receive strong Notch activation and consequently commit differentiation towards EC fate, whereas Dl\textsuperscript{low} ISC-derived EBs receive weak or no Notch activation and consequently commit differentiation towards EE fate (Ohlstein and Spradling, 2007). Transcriptional activation of EE differentiation is mediated by the \textit{acheate-scute} complex (\textit{AS-C}) proneural genes, which includes homologous genes \textit{achaete} (\textit{ac}), \textit{scute} (\textit{sc}), \textit{lethal of scute (l'sc)}, and \textit{asense (ase)} that are localized in a single cluster in the genome. Functional analyses suggest that the \textit{AS-C} genes, \textit{sc} in particular, are necessary and sufficient for EE specification (Amcheslavsky et al., 2014; Bardin et al., 2010). In some but not all tumor cells caused by the loss of Notch, \textit{sc} and \textit{ase} are derepressed, indicating that Notch may participate in the suppression of \textit{AS-C} genes to control EE differentiation (Zeng et al., 2013). EE differentiation also requires autonomous Jak/Stat signaling activity, and genetic analyses suggest that Jak/Stat is epistatic to Notch signaling in EE specification (Beebe et al., 2010; Jiang et al., 2009; Lin et al., 2010). Similarly, a chromatin regulator Osa is shown to be required for the differentiation of ISC progeny into EEs by regulating \textit{ase} (Zeng et al., 2013). Other known factors required for EE specification include Tsc1/2, whose loss of function induces hyperactivation of TOR and consequently failed EE specification (Kapuria et al., 2012; Quan et al., 2013). A recent study reveals that differentiated EEs also provide a negative feedback via Slit-Robo signaling to restrict EE production from ISCs (Biteau and Jasper, 2014).

The \textit{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 a common N-terminal
sequences containing a conserved BTB/POZ domain. The different DNA-binding domains result in the different DNA-binding specificities between Ttk69 and Ttk88, thus conceivably different functions (Read and Manley, 1992). Previous studies have demonstrated that Ttk is a transcriptional repressor and in 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 cell identity (Badenhorst, 2001; Badenhorst et al., 2002; Giesen et al., 1997; Guo et al., 1995; Li et al., 1997; Okabe et al., 2001; Tang et al., 1997; Xiong and Montell, 1993). More recent studies reveal that Ttk69 also functions in non-neural tissues, such as 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 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 specification. Our studies suggest that Ttk69 functions in parallel with Notch signaling and acts as a master repressor of EE specification by suppressing AS-C complex genes.
RESULTS

Loss of tramtrack (ttk) causes production of supernumerary EEs

To further understand the fate choice mechanisms in the ISC lineages in 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-Gal80\textsuperscript{ts} (referred to as esgGal4\textsuperscript{ts}), which allows temperature-controlled RNAi induction in progenitor cells (ISCs and EBs). Crosses were conducted at permissive temperature (18°C) and 3-5 days old esgGal4\textsuperscript{ts}>UAS-RNAi flies were subsequently shifted to restrict temperature (29°C) for one week prior to dissection and analysis. After screening approximately 2000 midgut-expressed genes, we identified one gene whose knockdown gave rise to a unique phenotype: the formation of supernumerary EEs surrounding GFP\textsuperscript{+} cells in the epithelium. EEs, which can be specifically marked by Prospero (Pros), are normally sparsely distributed in the midgut epithelium (Fig. 1B). Under normal conditions, about 80-90% of EBs adopts EC specification and 10-20% of them adopts EE 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 production of supernumerary EEs 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). Ttk-RNAi did not eliminate esg\textsuperscript{+} cells, and the extra EEs were surrounding each GFP\textsuperscript{+} cell or cell clusters (Fig. 1C,D), implying that depletion of Ttk does not affect ISC fate but causes their committed
daughters to preferably adopt EE specification.

**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; Lin et al., 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 *ttk*<sup>D2-50</sup>, an amorphic allele that affects both Ttk69 and Ttk88 function (Giesen et al., 1997), contained supernumerary Pros<sup>+</sup> EEs and occasional polyploid ECs (Fig. 2B,B’). Clones homozygous for *ttk*<sup>lle11</sup>, an allele that specifically disrupts Ttk69 (Lai and Li, 1999), displayed a virtually similar phenotype to *ttk*<sup>D2-50</sup> (Fig. 2C,C’). The formation of some ECs in the mutant clone, which can be marked by Pdm1 expression (Fig. 2E,E’), could be due to residual activity of Ttk products in the mutant cells. By contrast, clones homozygous mutant for *ttk*<sup>1</sup>, which disrupts Ttk88 only (Xiong and Montell, 1993), displayed no obvious difference to the wild type clones (Fig. 2D,D’). The excessive Pros<sup>+</sup> cells in *Ttk69* mutant clones were able to express neuropeptide, such as Tachykinin (TK) or Allatostatin A (AstA) (Supplementary material Fig. S1), indicating that they are able to differentiate into mature EEs. The mutant clones also contained many Dl<sub>low</sub> cells with marginal detection of Dl expression (Fig. 2F, F’ and Supplementary material Fig. S2). Quantitative analysis of cell population in *ttk69* mutant clones on day 7 ACI revealed that approximately 62% of cells were Pros<sup>+</sup> cells, and approximately 16% of cells
were DI<sub>low</sub> cells (Fig. 2G). Interestingly, many DI<sub>low</sub> cells also co-expressed with Pros (Fig. 2F’’). These cells are probably progenitor cells committed to or in the process of EE specification (Fig. 2G). Therefore, the vast majority of cells in ttk69 mutant clones are EEs and EE-committed progenitor cells. In addition to changes in cellular composition, the ttk69 mutant clones grew much faster than wild type clones, evidenced by significantly larger clone sizes and increased mitotic figure of the mutant clones (Fig. 2I,I’ compared to 2H,H’). The DI<sup>+</sup> cells in the mutant clones are probably ISCs/uncommitted 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 population (Supplementary material Fig. S2). These observations indicate that Ttk69 also negatively regulates ISC proliferation, which is also consistent with the observation that the esg>GFP<sup>+</sup> 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 cell cycle regulator cyclin E and String (Badenhorst, 2001; Baonza et al., 2002). Consistent with this role, cyclin E and String were significantly up-regulated in Ttk69 mutant clones and/or 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 whose disruption causes increased production of EBs, followed by unanimous commitment to EE specification, leading to excessive EE 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 ISC or EB to repress EE specification. We found that knocking down ttk using Dl-Gal4, an ISC-specific Gal4 (Zeng et al., 2010), produced similar supernumerary EE phenotype (Fig. 3B,B’), 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 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 into mitosis (Fig. 3I), which is probably due to de-repressed expression of cyclin E and String. To determine whether the extra EEs 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-Gal80ts (Su(H)GBE-Gal4ts); UAS-flp, and the flp-out cassette (Act<stop<lacZ). These elements allow conditional activation of flpase in Su(H)GBE-Gal4+ cells and therefore all descendants of Su(H)GBE-Gal4+ cells will be marked by lacZ expression (Fig. 3J). The cell lineage tracing results revealed that the extra EEs were lacZ+, and therefore were 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 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 cell cycle. Taken together, these observations suggest that Ttk69 functions in both ISCs and EBs to prevent excessive cell division and EE specification.
Overexpression of Ttk69 is sufficient to repress EE specification

The above data imply that the level of Ttk69 expression could be a determining factor in cell fate decision of progenitor cells. To determine the expression pattern of Ttk69, we generated a polyclonal antibody against Ttk69. The anti-Ttk69 signal was virtually undetectable in ttk69 mutant ISC 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 EEs, moderate level in Su(H)-lacZ+ EBs, and highest level in ECs (Fig. 4A-A”,B). The expression pattern is largely consistent with its role as a repressor of EE specification in the ISC lineages.

To test whether elevation of Ttk69 expression in ISCs and EBs is sufficient to prevent EE specification, we generated ISC clones with forced Ttk69 expression by the MARCM system and examined the consequences. As a control, approximately 45% of wild type ISC clones of 7 days old contained at least one EE (Fig. 4C,G). By contrast, ISC-containing clones with forced Ttk69 expression was much smaller, and consisted of only 2-5 cells, and the non-stem cells in the clones were virtually all ECs, and many clones no longer contained ISCs but only contained differentiating or differentiated ECs (Fig. 4D, G). Therefore, Ttk69 overexpression is sufficient to prevent EE 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 asked whether Ttk69 was able
to induce EC specification of $N^{-/-}$ progenitor cells. As a control, $N^{-/-}$ ISC clones generated both DI$^{+}$ ISC-like cell clusters and Pros$^{+}$ EE clusters (Fig. 4E,E'). Co-expressing Ttk69 in $N^{-/-}$ ISC clones prevented tumor development and EE 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 specification, rather than a promoter for EC specification. Therefore, Ttk69 negatively regulates ISC proliferation and its expression is necessary and sufficient to repress EE specification from progenitor cells.

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 developing processes (Boyle and Berg, 2009; Giesen et al., 1997; Guo et al., 1995; Xiong and Montell, 1993), and Notch signaling has 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 extra EE phenotype (data not shown), Ttk69 might have a functional or regulatory relationship with Notch signaling in the control of EE specification. We first determined whether the loss of Ttk69 affects Notch signaling. GBE-Su(H)m8-lacZ (refer to as Su(H)-lacZ hereafter) is a Notch activation reporter, which is specifically expressed in EBs where 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 N signaling activation. Conversely, although forced activation of Notch by expressing an intracellular domain of Notch (N$^{icd}$) is sufficient to induce ISC differentiation
toward EC, 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 Pdm1, an EC marker (Fig. 5D,D’). These cells also displayed partial impairment in differentiation toward EEs, as most cells failed to turn on Pros or TK expression (Fig. 5E1-4). It was previously shown that Ttk is required for Notch dependent mitotic-to-endocycle switch in Drosophila follicle cells (Jordan et al., 2006), this function could potentially explain why endocycle is not occurring in intestinal cells when Ttk is ablated, even Notch is activated. Alternatively, conflicts in differentiation programs could cause delay or improper cell lineage differentiation, leading to the generation of aberrant cells. On the other hand, 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 is 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 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 fate. Previous studies have revealed that the process of EB differentiation to EE 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 EEs (Beebe et al., 2010; Jiang et al., 2009; Lin et al., 2010). Depletion of a chromatin remodeling factor Osa by RNAi produces a similar phenotype (Zeng et al., 2013). Loss of Tsc1/2 leads to hyperactivation of Target of
Rapamycin, which prevents EB differentiation into EE, but leaves EC differentiation occurring normally (Kapuria et al., 2012; Quan et al., 2013). To understand the epistatic relationships between Ttk69 and these regulators in EE specification, we generated double mutant clones, and studied the consequences. Strikingly, co-depleting Ttk69 allowed all mutant progenitor cells, including JAK/STAT deficient cells (Fig. 6A), TSCI^{Q87X} mutant cells (Fig. 6C) and Osa-depleted progenitor cells (Fig. 6E) to unidirectionally differentiate into EEs (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 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 is not excluded.

Ttk69 functions upstream of AS-C genes in EE specification

Previous studies have demonstrated an essential role for the AS-C complex genes in EE specification (Bardin et al., 2010). The excessive EE formation following the depletion of ttk69 was reminiscent of the phenotype caused by forced expression of AS-C complex genes. We therefore performed the epistatic analysis between ttk69 and AS-C genes. Df(1)sc^{B57} is a chromosome deficient allele, in which the coding regions for all AS-C genes are deleted. In Df(1)sc^{B57} clones, Pros^{+} or TK^{+} cells failed to develop (Fig. 7A,A’), similar to the previous observation (Bardin et al., 2010). As a control, expression of a sc transgene in Df(1)sc^{B57} clones allowed the formation of EEs (Fig. 7B,B’). However, knocking down ttk69 in Df(1)sc^{B57} clones did not rescue the failure of EE formation (Fig. 7C,C’,D), suggesting that AS-C genes are epistatic to ttk69 in EE specification. By real-time quantitative PCR (RT-qPCR), we found that sc
and *ase*, but not *ac* or *lsc* were significantly up-regulated in *esg<sup>ts</sup>*-*ttk-RNAi* intestines compared to wild type ones (Fig. 7E), and antibody staining to Sc and Ase revealed that both proteins were upregulated in *ttk<sup>69</sup>* mutant clones (Fig. 7F-G”). Consistent with this observation, the supernumerary EE phenotype following *ttk<sup>69</sup>* 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 specification by suppressing the expression of both *sc* and *ase* genes.

**Relationships among *Ttk<sup>69</sup>*, *AS-C* genes and *Pros* in EE specification**

Pros as a common EE marker is a transcriptional factor previously best known as a cell fate determinant in ganglion mother cells of *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 specification because depleting Pros in ISCs leads to reduction of EEs in the midgut epithelium (Biteau and Jasper, 2014). To directly test whether Pros is autonomously required for EE specification, we generated MARCM clones mutant for *pros<sup>17</sup>*, a loss of function allele of *pros*. None of the cells in the mutant clones (*n*=50 clones examined) expressed either Tk or AstA, two EE-specific peptide hormones (Fig. 8A). As Tk<sup>+</sup> and AstA<sup>+</sup> EEs make up majority of EE population in the posterior midgut, it can be deduced that Pros is cell-autonomously required for EE specification and/or maturation. We found that Pros may have an instructive role for EE specification, as overexpression of Pros in ISC clones was sufficient to deplete ISCs by inducing their differentiation into EEs (Fig. 8B,C).
Next we performed epistatic 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 phenotype, and virtually all mutant cells within the clones remained undifferentiated, as they failed to turn on the expression of TK (Fig. 8E) 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 phenotype, and similarly, virtually all mutant cells within the clones remained undifferentiated (Fig. 8I and data not shown). Conversely, although Ttk69 overexpression is sufficient to prevent EE specification, co-expression of Pros allowed the production of hormone-producing EEs (Fig. 8J). These data demonstrate that pros is epistatic to ttk69 and sc in EE specification, and suggest a Ttk69–AS-C–Pros regulatory axis in controlling EE specification of progenitor cells and in mediating excessive EE 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 specification, leading to excessive EE production (Fig. 8K).
DISCUSSION

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

We consider Ttk69 as a master repressor of EE specification in *Drosophila* midgut for a number of reasons. First, loss of Ttk69 causes the committed progenitor cells to unidirectionally adopt EE specification, leading to a dramatic supernumerary EE phenotype. Second, Ttk69 is not only required to repress EE specification, its ectopic expression is sufficient to prevent progenitor cells from adopting EE specification. Moreover, loss of Ttk69 is sufficient to prevent Notch-activation-induced EC specification and induce EE specification instead. That is to say, depletion of Ttk69 is able to override EC-commitment of Notch-activated progenitor cells and turn them into EE commitment. In addition, loss of Ttk69 is able to induce EE specification of differentiation-defective progenitors caused by various mutations, such as JAK/STAT, Tsc1, or Osa, indicating that Ttk69 acts as a final gatekeeper downstream of multiple regulators to prevent excessive EE specification. Lastly, our mechanistic studies demonstrate that Ttk69 suppresses EE 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 EEs from ISCs (Fig. 8K). It is yet to be determined whether Ttk69 directly suppresses *AS-C* genes or indirectly. In *Drosophila* embryo, *sc* seems to be directly suppressed by Ttk69, as its expression is significantly up-
regulated in ttk mutant embryos, and significantly decreased in ttk69 overexpressed embryos (Rotstein et al., 2012). In addition, analysis of Ttk69 ChIP-seq results from modeENCODE data reveals potential binding activity of Ttk69 on the regulatory regions of sc (mod 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 specification in ISC lineages, a hypothesis 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 specification. We propose that Ttk69 and Pros respectively act as a master repressor and a master activator of EE specification in the ISC lineages of 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 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 specification upon depletion of Ttk69. Moreover, forced Notch activation could not initiate EC differentiation and prevent EE specification when Ttk69 is depleted. On the other hand, Notch signaling appears not to be upstream of
Ttk69 in controlling EC versus EE decision, as the expression of Ttk69 is not regulated by Notch activity. Therefore, Ttk69 and Notch likely function in two parallel pathways to control the binary fate decision of EBs (Fig. 8K).

Although Dl/Notch is a major signaling pathway controlling cell fate decisions in the midgut, how it is regulated is poorly understood. Dl 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 Dl 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 with different levels. The lowest level is found in ISCs and EEs, 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 EE specification. These observations indicate that transcriptional regulation of Ttk69 may hold the key in controlling Ttk69 function, although post-transcriptional level of Ttk69, which occurs in other developmental processes (Li et al., 1997; Okabe et al., 2001), could provide additional layers of functional control. It have been proposed recently that EEs could be directly differentiated from ISCs rather than indirectly from EE-committed EBs (Biteau and Jasper, 2014; Zeng and Hou, 2015). Because Ttk69 depletion does not comprise ISC self-renewal, indicating that the extra EEs are differentiated from EBs, rather than ISCs. Therefore, a more plausible explanation for the supernumerary EE phenotype following Ttk69 depletion in progenitor cells would be like this: depletion of Ttk69 in ISCs will cause ISCs to produce EE-committed progenitor cells only; it will also cause ISCs to divide faster, thereby generating more EE-committed
progenitor cells; depletion of Ttk69 in EC-committed EBs will cause certain degree of dedifferentiation to become dividing progenitor cells that can only give rise to EEs. These effects together lead to the production of supernumerary EEs. Further elucidation of the regulatory mechanisms of Ttk69 and Dl/Notch signaling will be the next immediate steps towards complete understanding of cell fate decisions in the midgut stem cell lineages.
MATERIALS AND METHODS

Fly strains and culture

The following stocks were used in this study: UAS-ttk-RNAi#1 (VDRC, v10855); UAS-ttk-RNAi#2 (BDSC, 36748); FRT82B-ttk\(^{D2-50}\) (gift from Marta Llimargas); esg-Gal4, UAS-GFP (gift from Shigeo Hayashi); Gbe-Su(H)m8-lacZ (gift from Sarah Bray); UAS-N\(^{cd}\) (gift from Ting Xie); Su(H)-Gal4 and Dl-Gal4 (gift from Xiankun Zeng and Steven Hou); UAS-sc-RNAi (Tsinghua fly center, #2205); UAS-ase-RNAi (Tsinghua fly center, #2271); FRT82B; FRT82B-ttk\(^{1}\); FRT82B-ttk\(^{11}\); FRT19A; FRT19A-\(^{N55e11}\); FRT19A-Df (1)scB57; UAS-pros-RNAi (BDSC, #26745); UAS-osarRNAi (Tsinghua fly center #1909 or BDSC #31266); FRT82B-pro\(^{17}\); UAS-Notch-RNAi; UAS-ttk69; Tub-Gal80\(^{ps}\); UAS-Flp; Act<stop<lacZ; UAS-pros; Dl-lacZ and UAS-sc were obtained from Bloomington Stock Center.

Mosaic analysis

The MARCM system (Lee and Luo, 1999) or Flip-out technique (Struhl and Basler, 1993) were used to generate clones in intestines. Mosaic clones were induced by heat shocking 3-5 days old females once for an hour in 37°C water bath. Flies were subsequently fed with regular food supplied with yeast paste and transferred every 2 days prior to dissection and analysis.

Gal4/UAS/Gal80\(^{ps}\) mediated gene depletion

esg-Gal4, UAS-GFP; Tub-Gal80\(^{ps}\); Su(H)-Gal4, UAS-GFP; Tub-Gal80\(^{ps}\) and Tub-Gal80\(^{ps}\); Dl-Gal4, UAS-GFP flies were crossed to UAS-RNAi transgenic flies at 18°C. 3-5 day old female flies with desired genotype were shifted to 29°C and cultured on corn meal supplied with yeast paste for 7 days before dissection. The flies were
transferred every 2-3 days.

**Preparation of rabbit polyclonal anti-Ttk69**

Polyclonal antibody directed against Ttk69 was raised in rabbit using the following synthetic peptide: CTALATVAAANLAGQPLGV. The cysteine residual that was added at the N terminal end of the peptide was used to conjugate keyhole limpet hemocyanin (KLH). Serum obtained from immunized rabbit was purified by antigen affinity chromatography. Purified anti-serum at final dilution of 1:200 was used.

**Immunostaining**

Immunostaining of *Drosophila* midgut was performed as previously described (Lin et al., 2008). The following primary antibodies were used in this study: mouse anti-DI (Developmental Studies Hybridoma Bank (DSHB); 1:100); mouse anti-Pros (DSHB, 1:300); mouse anti-Allatostatin A (DSHB, 1:300); rabbit anti-phosphor-Histone H3 antibody (Upstate, 1:1000); rabbit anti-Tachykinin antibody (a gift from Dick Nassel, 1:3000); rabbit polyclonal anti-lacZ antibody (Cappel, 1:6000); rat anti-sc (1:300, gift from Steve Crews), rabbit anti-GFP (Invitrogen, 1:2000), rabbit anti-Ase (1:1000, gift from Yuh-Nung Jan), Rabbit anti-Pros (1:1500, gift from Yuh-Nung Jan) rabbit anti-Ase (1:400, gift from Cheng-Yu Lee) rabbit anti-Pdm1 (gift from Xiaohang Yang, 1:1000); Secondary antibodies used in this study: goat anti-rabbit, anti-mouse, or anti-rat IgGs-conjugated to Alexa (488, 568 or Cy5) (Molecular Probes, 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 manufacturer's instructions. cDNAs were synthesized using high fidelity cDNA Synthesis Kit (Roche). RT-qPCR was performed using SYBR PrimeScript RT-PCR kit (Takara) on an ABI PRISM 7500 fast Real-time PCR System (Applied Biosystems). Real-time PCR was repeated for three independent biological replicates. Expression of each gene was normalized to GAPDH, and relative levels were calculated using the $2^{\Delta\Delta CT}$ method. Primers sequences were shown below:

GAPDH forward: 5'-GAAATTAAGGCCAAGGTTCAAGG-3'

GAPDH reverse: 5'-GTACCAAGAGATCAGCTTC-3'

ac forward: 5'-TTTTCAACGACGACGAGGAG-3'

ac reverse: 5'-ACCATGGCTTAAATCGGCTA-3'

sc forward: 5'-AATGTAGACCAATCCCAGTCG-3'

sc reverse: 5'-CACCACCCTTTGTCAAATCC-3'

l'sc forward: 5'-TCAAACTGTGGTGAAACTCGCTGTC-3'

l'sc reverse: 5'-TCGGCGGAATTGTAGATGTG-3'

ase forward: 5'-GCACAACCAGCAGAATCAAC-3'

ase reverse: 5'-AGGCAAAACCCTTTTCTCCAG-3'
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AUTHOR CONTRIBUTIONS

CW and RX conceived and designed the experiments, analyzed the data and wrote the manuscript. CW, XG, KD and HC performed the experiments.
REFERENCES


Figures

A

B

esg>GFP

C

ttk-RNAi #1

D

ttk-RNAi #2

B'

C'

D'

E

% of EEs per 10^5 um^2
Fig. 1. Depletion of tramtrack (ttk) causes production of supernumerary EEs.

(A) The ISC lineages in *Drosophila* posterior midgut. ISC, intestinal stem cell; EB, enteroblast; EC, enterocyte cell; EE, enteroendocrine cell; cell markers and cell ploidy for each indicated cell types have been shown.

(B-D’) Compared to wild type control (B-B’), knockdown of *ttk* by expressing either ttk-RNAi#1 (VDRC, v10855) (C-C’) or ttk-RNAi#2 (BDSC, #36748) (D-D’) in esg’ progenitor cells resulted in significant increase in the number of EEs (marked by anti-Pros, in red) in the epithelium. Scale bars: 20 μm.

(E) Quantification of the percentage of EE cells in the posterior midgut. Bars represent mean ± s.e.m. n = 20-30 intestines. *** denotes student’s t test p<0.001.
Fig. 2. Ttk69 regulates EE/EC specification.

The GFP-marked clones were generated using MARCM system and were examined
on day 7 after clone induction.

(A-D’) A typical wild type clone (A-A’), the majority cell type was polyploid EC, and Pros+ EE (in red, arrows) was much less abundant. In contrast, the major cell type was EE in clones mutant for ttkD2-50 (B-B’) and ttklell (C-C’). The cell composition in ttkl clone (D-D’) was similar to wild type clone (arrows indicate EEs).

(E-E’) A ttklell clone that contained a few ECs marked by anti-Pdm1.

(F-F”) A ttklell clone that contained Dl+ cells (yellow arrowheads) as well as Dl+ Pros+ cells (white arrow).

(G) Quantification of the percentage of Dl+ cells, Pros+ cells, Dl+ Pros+ cells as well as ECs in wild type and ttklell clones on day 7 ACI. Bars represent mean ± s.e.m.

(H-I’) Compared to wild type clones (G-G’), there were significantly more mitotic cells (marked by anti-PH3) in ttklell clones(H-H’).
Fig. 3. Ttk69 is required in both ISCs and EBs for proper cell fate specification.

(A-B) Knockdown of ttk by expression of ttk-RNAi driven by Dl-Gal4ts for 7 days at 29°C resulted in excess EE cells (B). Wild type control was shown in A.

(C-D) Knockdown of ttk by expression of ttk-RNAi driven by Su(H)-Gal4ts for 7 days at 29°C resulted in excess EE cells(D). Wild type control was shown in C.

(E-F’) Knockdown of ttk by expression of ttk-RNAi driven by Su(H)-Gal4ts for 7 days at 29°C did not cause significant increase in the number of ISCs (F-F’). Wild type control was shown in E-E’.

(G) Quantification of the number of EE, ISC and Su(H)-Gal4+ EBs in wild type and Su(H) ttk-RNAi intestines. Bars represent mean ± s.e.m. n = 20-30 guts. *** denotes student’s t test p<0.001. ** denotes p<0.01. ns denotes no statistically significant difference.

(H-I) Mitotic cells as indicated by PH3 staining (red, indicated by arrowheads) were not present in Su(H)>GFP+ cells in wild type midgut (H). In contrast, knockdown of ttk driven by Su(H)-Gal4ts caused some Su(H)>GFP+ cells to re-enter mitosis.

(J-L) Cell lineage tracing analysis of EB cells expressing ttk-RNAi. (J) An illustrative diagram of directed cell lineage tracing strategy. The flies with the genotype Su(H)-Gal4/ttk-RNAi; UAS-FLP/Act<stop<lacZ, Tub-Gal80ts were generated by crosses at 18°C and were subsequently shifted to 29°C. At 29°C, Su(H)-Gal4 was activated and drove the expression of ttk-RNAi as well as FLP recombinase, which in turn caused the excision of the FLP-out cassette to initiate the expression of lineage marker lacZ.

(K-K”) After shifted to restrict temperature for 7 days, no Pros+ EE cell was derived from Su(H)-Gal4+ EBs in wild type intestines. (L-L”) With Su(H)-Gal4>ttk-RNAi, the vast majority of the cells derived from Su(H)-Gal4+ EBs were Pros+ EE cells.
Fig. 4. Overexpression of Ttk69 is sufficient to repress EE specification.

(A-B) The expression pattern of Ttk69 in the midgut. Ttk69 (anti-Ttk69, in red) (A and A’) was expressed in DI+ ISC (yellow arrowhead), Pros+ EEs (white arrows), EB (black arrowhead) as well as polyploid ECs, but at different levels. (B) A schematic diagram showing the relative expression levels of Ttk69 among different cell types.

(C-D) Pros+ EE cells were rarely observed in MARCM clones overexpressing Ttk69 (D-D’). Control in (C-C’).
(E-F”) In comparison to N55e11 clone (E-E’) which contained both ISC- and EE-like cell clusters, Ttk69 overexpression prevented the formation of large cell clusters and inhibited the formation of EEs (F-F’).

(G) Quantification of the percentage of clone containing EE cells in C-F’. n = 15-25 intestines. Error bars indicate SEM and *** denotes student t test p<0.001.
Fig. 5. The relationship between ttk69 and Notch signaling in cell fate regulation.

(A-A’) Su(H)-lacZ was significantly down-regulated in clones expressing Notch-RNAi.

(B-B’) Su(H)-lacZ was largely unaffected in clones mutant for ttk1e11.

(C-C’) Ttk69 expression level was unaffected in clones mutant for N55e11.

(D-D’) Overexpression of an active form of Notch (Nnicd) in ttk1e11 clones failed to induce differentiation towards ECs.

(E1-E4) Reduced production of Pros+ (E1-E2) or TK+ (E3-E4) EE cells in ttk1e11 clones expressing Nnicd.
Fig. 6. Ttk69 is epistatic to multiple cell fate regulators in repressing EE specification.

(A-B’) In clones mutant for stat93E alone (A-A’), EE cells were rarely observed. In contrast, knockdown of ttk in stat92E clones(B-B’) led to robust production of EE cells.

(C-D’) In clones mutant for Tsc1 alone (C-C’), EE cells were rarely observed. In contrast, knockdown of ttk in Tsc1 clones(D-D’) led to robust production of EE cells.
(E-F’) Knockdown of *osa* alone (E-E’) driven by *esg-Gal4ts* for 7 days at 29°C blocked the differentiation of progenitor cells. In contrast, simultaneous knockdown of *ttk* and *osa* (F-F’) driven by *esg-Gal4ts* led to excess EE formation.

(G) Quantification of the percentage of clones containing EE cells in A-D.

(H) Quantification of the percentage of EE cells per frame.
Fig. 7. Ttk69 inhibits EE specification through suppressing sc and ase.

(A-C) Epistatic analysis of AS-C and ttk69 in EE cell specification. GFP-marked ISC clones were examined 7 days after clone induction. In clones deficient for AS-C complex genes (A-A’), EE cells were not observed. Overexpression of sc in
Df(1)sc\textsuperscript{B57} mutant clones allowed effective EE production (B-B’), but knockdown of ttk69 in Df(1)sc\textsuperscript{B57} mutant clones did not allow EE production (C-C’).

(D) Quantification of the percentage of ISC clones of indicated genotypes that contained EEs.

(E) RT-qPCR analysis of A5-C gene transcription in esg\textsuperscript{ts}>ttk-RNAi (v10855) intestines (after 7 days at 29°C) compared to those in wild type intestines. GAPDH was used as a normalization control, means ± SD were shown.

(F-F’) Sc protein was upregulated in ttk69 mutant clones.

(G-G’) Ase protein was upregulated in ttk69 mutant clones.

(H-L) In comparison to knockdown of ttk alone driven by esg-Gal4\textsuperscript{ts} (H-H’), knockdown of sc (I-I’) or ase (J-J’) alone could not prevent excessive EE production followed by knockdown of ttk in progenitor cells, but simultaneous knockdown of both sc and ase (K-K’) could. (L) Quantification of EE production in intestines of indicated genotypes. Data were presented as mean ± s.e.m., ns denotes no significant difference, *** denotes t test p<0.001.
Fig. 8. Pros is epistatic to Ttk69 and AS-C in EE specification.

GFP-marked ISC clones were generated either by MARCM system or Flip-out technique and examined on day 7 after clone induction except (D-E) which were examined on day 14.
(A) An ISC clone mutant for pros\textsuperscript{17}. No TK\textsuperscript{+} EE cell was detected in pros\textsuperscript{17} clones.

(B-C) Overexpression of Pros in MARCM clones led to EE specification. EEs were marked by TK (B) or AstA (C).

(D-D’) A ttk\textsuperscript{le11} clone that contained many TK\textsuperscript{+} EE cells.

(E-E’) Knockdown of Pros suppressed TK\textsuperscript{+} EE production in clones expressing ttk-RNAi.

(F-F’) Pdm1\textsuperscript{+} ECs were rarely present in pros\textsuperscript{17} clones expressing ttk-RNAi on day 7 ACI.

(G-G’) Dl\textsuperscript{+} ISCs were present in pros\textsuperscript{17} clones expressing ttk-RNAi on day 7 ACI.

(H-H’) A Sc-expressing clone that contained TK\textsuperscript{+} EE cells. (I-I”) Overexpression of Sc in clones mutant for pros\textsuperscript{17} failed to produce TK\textsuperscript{+} EEs.

(J) A single cell clone with Ttk69 and Pros overexpression was positive for TK expression.

(K) A schematic model for the role of Ttk69 in EE specification.