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RESEARCH ARTICLE

STEM CELLS AND REGENERATION

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 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 Batlle, 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: Dl^{high} ISC-derived EBs receive strong Notch activation and consequently commit differentiation towards EC fate, whereas Dl^{low} 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 *achaete* (*ac*), *scute* (*sc*), *lethal of scute* [*l'sc*, or *l(1)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 differentiated 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.,

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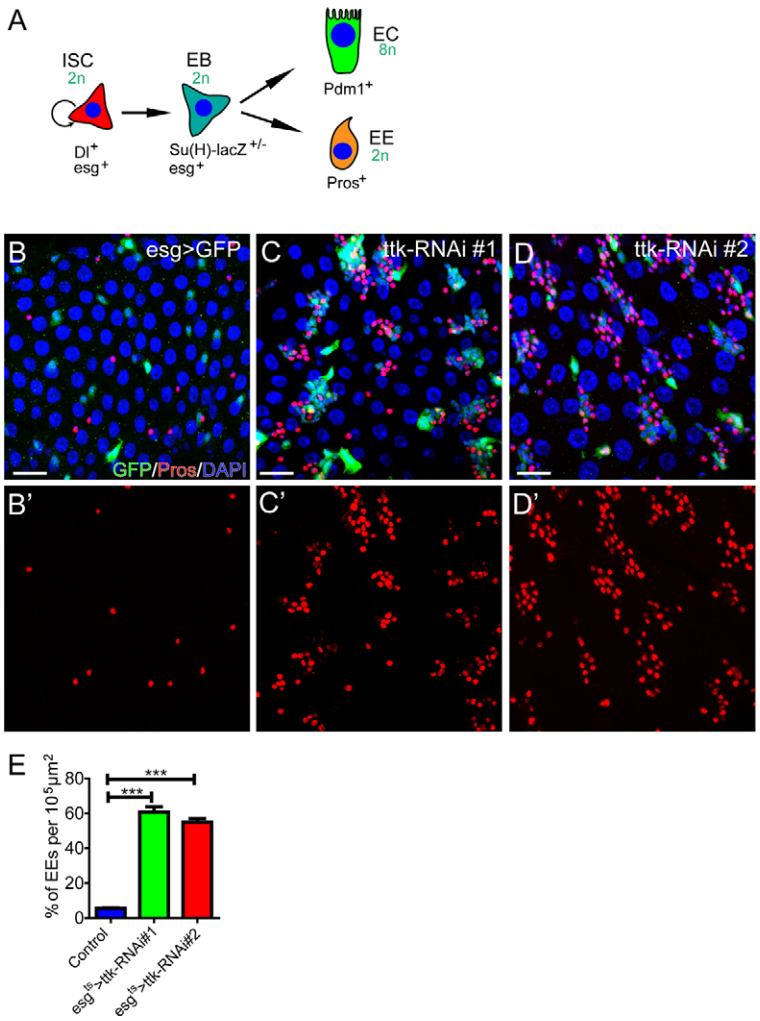

Fig. 1. Depletion of *tramtrack* (*ttk*) causes the production of supernumerary EE cells. (A) The ISC lineages in the *Drosophila* posterior midgut. ISC, intestinal stem cell; EB, enteroblast; EC, enterocyte; EE, enteroendocrine cell. Markers and ploidy for each of the cell types are shown. (B-D′) Compared with the wild-type control (B,B′), knockdown of *ttk* by expressing either *ttk-RNAi#1* (C,C′) or *ttk-RNAi#2* (D,D′) in *esg*$^{+}$ progenitor cells resulted in a significant increase in the number of EE cells (marked by anti-Pros, red) in the epithelium. Scale bars: 20 µm. (E) Quantification of the percentage of EE cells in the posterior midgut. Mean±s.e.m. *n*=20-30 intestines. ***P<0.001 (Student's *t*-test).

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 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-Gal80*^{ts} (referred to as *esgGal4*^{ts}), 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 *esgGal4*^{ts}*>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). *ttk-RNAi* did not eliminate *esg*$^{+}$ cells, and the extra EE cells surrounded each GFP^{+} cell or cell cluster (Fig. 1C,D), implying that depletion of Ttk does not affect ISC fate but causes their committed daughters to preferably adopt EE cell 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

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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 $ttk^{D2\text{-}50}$, 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 ttk^{1e11}, an allele that specifically disrupts Ttk69 (Lai and Li, 1999), displayed a very similar phenotype to $ttk^{D2\text{-}50}$ (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 ttk^{1}, 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 Dl^{low} 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 Dl^{low} (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. 2I,I′ compared with 2H,H′). The Dl^{+} cells in the mutant clones are probably ISCs/uncommitted progenitor cells that are mitotically active and responsible for clonal outgrowth of the

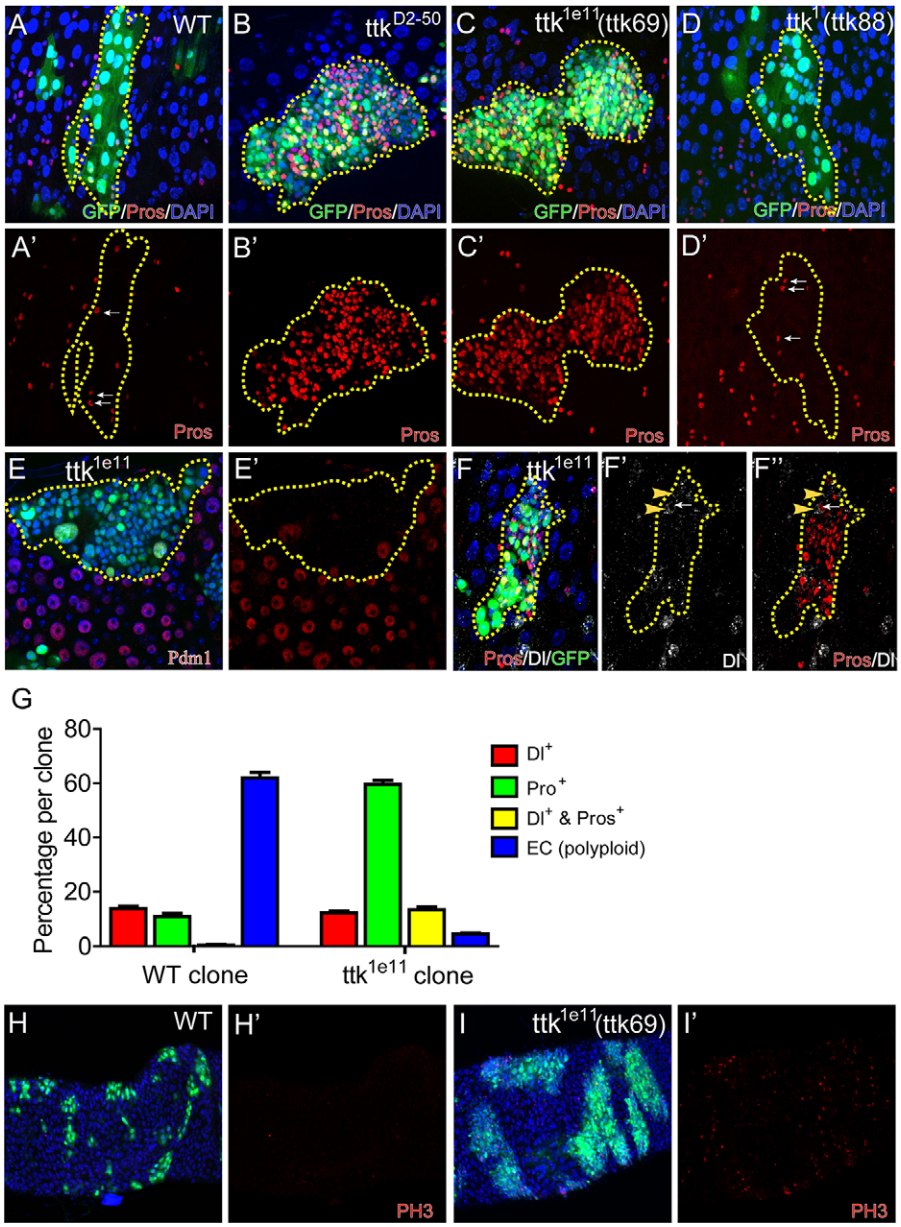

Fig. 2. Ttk69 regulates EE/EC specification. GFP-marked clones were generated using the MARCM system and were examined on day 7 after clone induction (ACI). (A-D′) In a typical wild-type clone (A,A′) the majority cell type was polyploid EC, whereas $Pros^{+}$ EE cells (red, arrows) were much less abundant. By contrast, the major cell type was EE in clones mutant for $ttk^{D2\text{-}50}$ (B,B′) and ttk^{1e11} (C,C′). The cell composition in ttk^{1} clones (D,D′) was similar to that of wild-type clones (arrows indicate EE cells). (E,E′) A ttk^{1e11} clone that contains a few ECs as marked by anti-Pdm1. (F-F″) A ttk^{1e11} clone that contains 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 and ECs in wild-type and ttk^{1e1} clones on day 7 ACI. Mean±s.e.m. n=42 for wild-type clones, n=37 for *ttk* clones. (H-I′) Compared with wild-type clones (H,H′), there were significantly more mitotic cells (marked by anti-PH3) in ttk^{1e11} clones (I,I′). GFP, green; DAPI, blue.

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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 *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, which, when disrupted, leads to the increased production of EBs, followed by unanimous 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 derepressed 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-Gal80^{ts}* [*Su(H)GBE-Gal4^{ts}*]; *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 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. 4A-B). 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. 4C,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. 4D,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 Dl 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 intracellular domain of Notch (N^{icd}) 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

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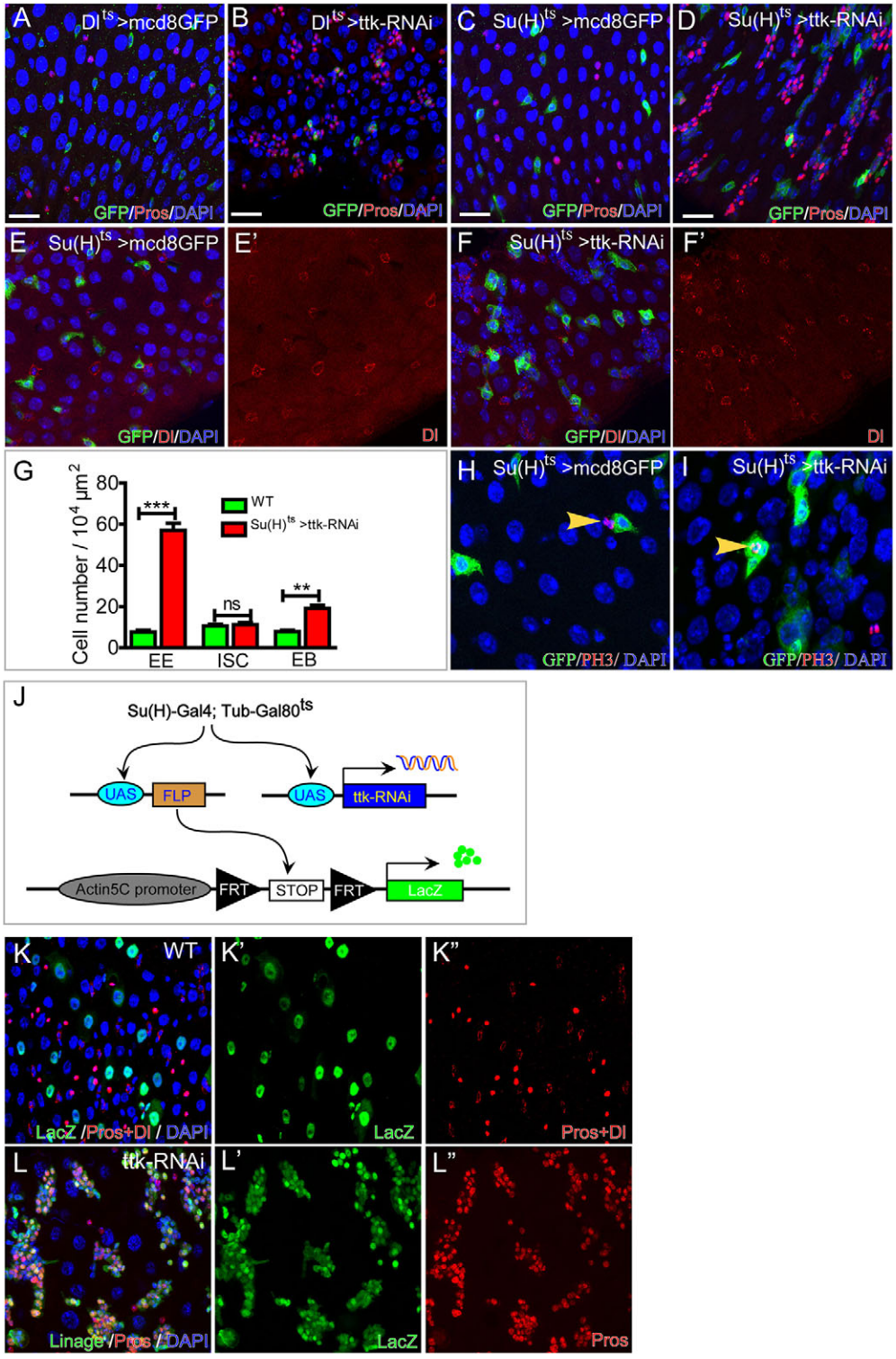

Fig. 3. Ttk69 is required in both ISCs and EBs for proper cell fate specification. (A,B) Knockdown of *ttk* by expression of *ttk-RNAi* driven by *Dl-Gal4^{ts}* for 7 days at 29°C resulted in excess EE cells (B), as compared with the wild-type control (A). (C,D) Knockdown of *ttk* by expression of *ttk-RNAi* driven by *Su(H)-Gal4^{ts}* for 7 days at 29°C resulted in excess EE cells (D), as compared with the wild-type control (C). Scale bars: 20 µm. (E-F′) Knockdown of *ttk* by expression of *ttk-RNAi* driven by *Su(H)-Gal4^{ts}* for 7 days at 29°C did not cause any significant increase in the number of ISCs (F,F′), as compared with the wild-type control (E,E′). (G) Quantification of the number of EE cells, ISCs and Su(H)-Gal4^{+} EBs in wild-type and *Su(H)>ttk-RNAi* intestines. Mean±s.e.m. *n*=20-30 guts. ***P<0.001, **P<0.01 (Student's *t*-test); ns, no statistically significant difference. (H,I) Mitotic cells, as indicated by phospho-Histone H3 (PH3) staining (red, arrowheads), were not present in Su(H)>GFP^{+} cells in wild-type midgut (H). By contrast, knockdown of *ttk* driven by *Su(H)-Gal4^{ts}* caused some Su(H)>GFP^{+} cells to re-enter mitosis (I). (J-L″) Cell lineage-tracing analysis of EBs expressing *ttk-RNAi*. (J) The directed cell lineage-tracing strategy. Flies of genotype *Su(H)-Gal4/ttk-RNAi; UAS-FLP/Act<stop<lacZ, Tub-Gal80^{ts}* 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 expression of the lineage marker *lacZ*. (K-K″) After shifting to the restrictive temperature for 7 days, no Pros^{+} EE cell was derived from Su(H)-Gal4^{+} EBs in wild-type intestines. (L-L″) By contrast, with *Su(H)-Gal4>ttk-RNAi*, the vast majority of the cells derived from Su(H)-Gal4^{+} EBs were Pros^{+} EE cells.

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

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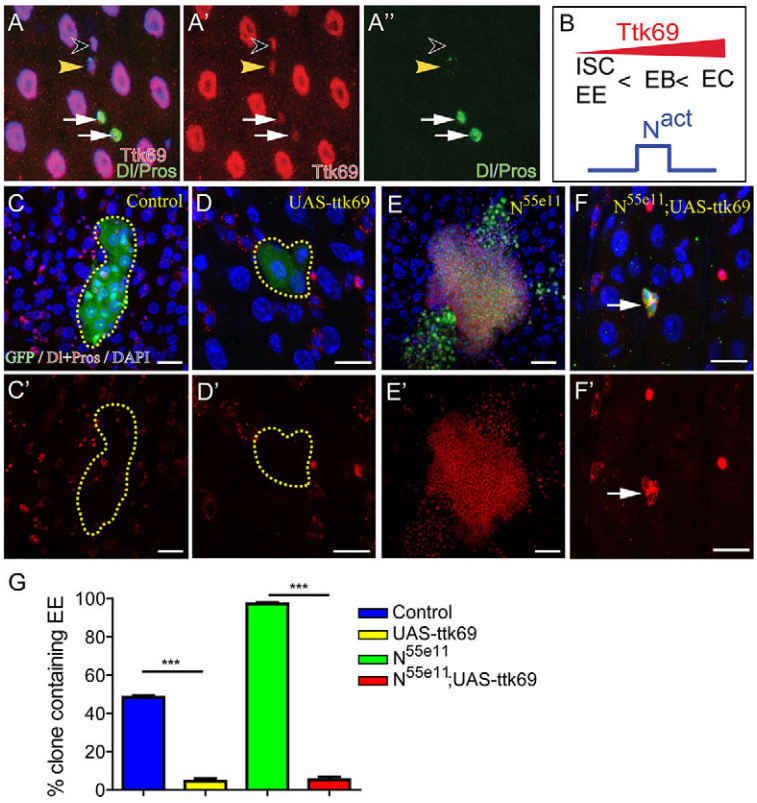

Fig. 4. Overexpression of Ttk69 is sufficient to repress EE cell specification. (A-B) The expression pattern of Ttk69 in the midgut. Ttk69 (immunostaining, red) (A,A′) was expressed in Dl^{+} ISCs (yellow arrowhead), Pros^{+} EE cells (white arrows), EBs (black arrowhead) as well as polyploid ECs, but 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 *N^{55e11}* 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.

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), *Tsc1^{Q87X}* 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.

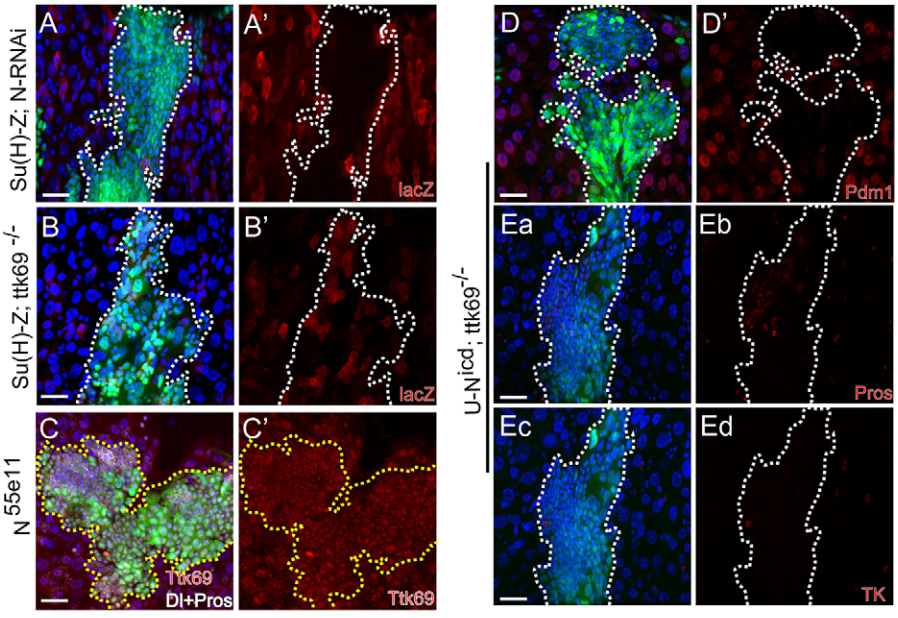

Fig. 5. The relationship between *ttk69* and Notch signaling in cell fate regulation. (A,A′) *Su(H)-lacZ* was significantly downregulated in clones expressing *Notch-RNAi*. (B,B′) *Su(H)-lacZ* was largely unaffected in clones mutant for *ttk^{1e11}*. (C,C′) The Ttk69 expression level was unaffected in clones mutant for *N^{55e11}*. (D,D′) Overexpression of an active form of Notch (N^{icd}) in *ttk^{1e11}* clones failed to induce differentiation towards ECs. (E) Reduced production of Pros^{+} (a,b) or Tk^{+} (c,d) EE cells in *ttk^{1e11}* clones expressing N^{icd}. GFP, green; DAPI, blue. Scale bars: 20 µm.

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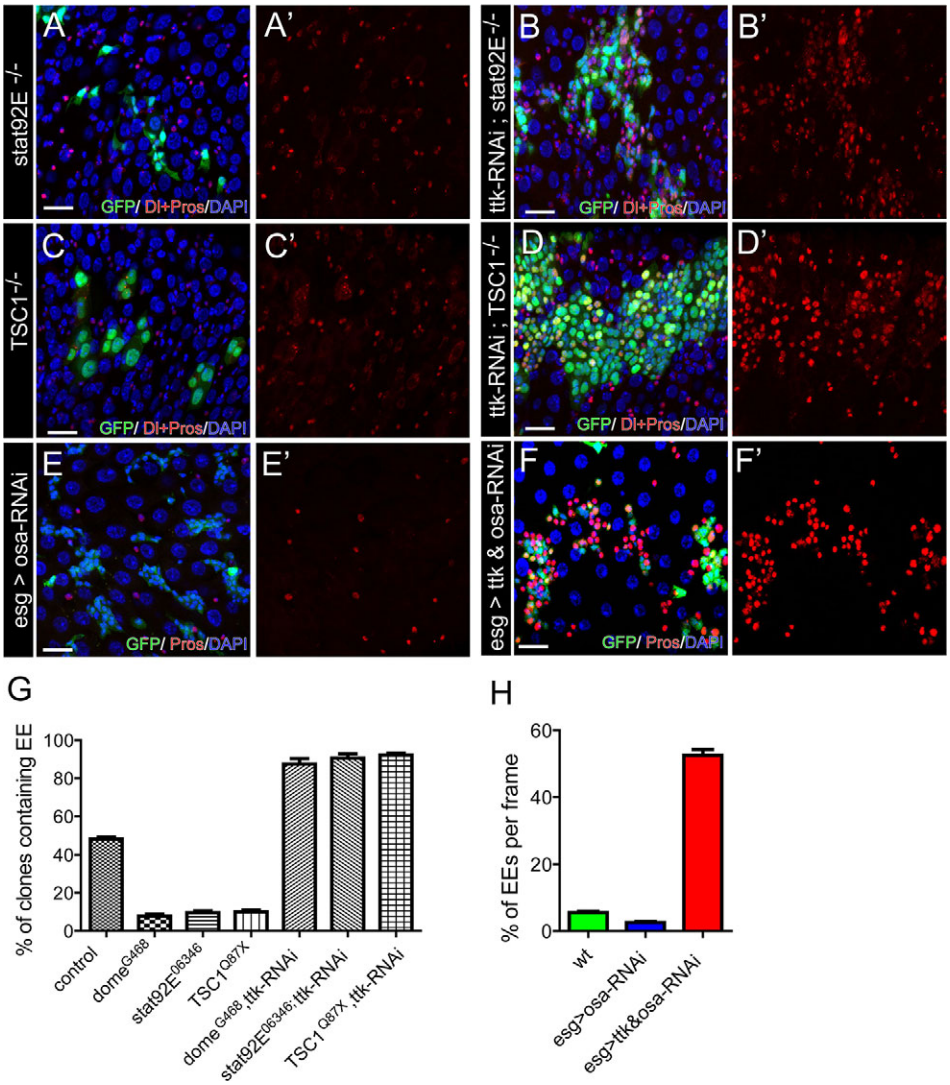

Fig. 6. Ttk69 is epistatic to multiple cell fate regulators in repressing EE cell specification. (A-B′) In clones mutant for *Stat92E* alone (A,A′), EE cells were rarely observed. By contrast, knockdown of *ttk* in *Stat92E* clones (B,B′) led to robust production of EE cells. (C-D′) In clones mutant for *Tsc1* alone (C,C′), EE cells were rarely observed, whereas 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-Gal4^{ts}* for 7 days at 29°C blocked the differentiation of progenitor cells. By contrast, simultaneous knockdown of *ttk* and *osa* (F,F′) driven by *esg-Gal4^{ts}* led to excess EE cell formation. (G) Quantification of the percentage of clones containing EE cells in A-D. (H) Quantification of the percentage of EE cells per frame. Error bars indicate s.e.m. *n*=10-15 intestines. 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)sc^{B57}* is a chromosome deficiency 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′), as observed previously (Bardin et al., 2010). As a control, expression of a *sc* transgene in *Df(1)sc^{B57}* clones allowed the formation of EE cells (Fig. 7B,B′). However, knocking down *ttk69* in *Df(1)sc^{B57}* 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 *l'sc*, are significantly upregulated in *esg^{ts}>ttk-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 *pros^{17}*. 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).

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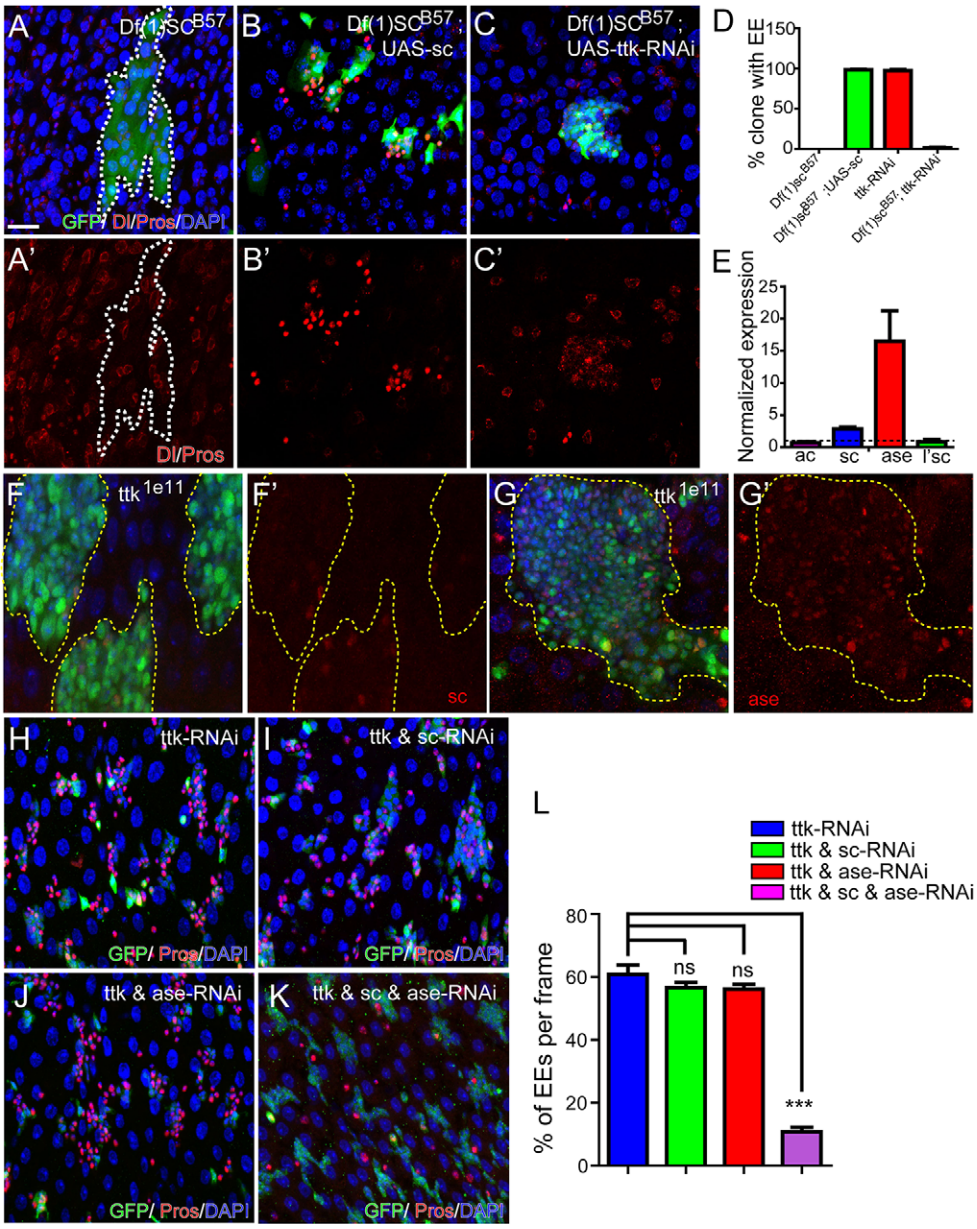

Fig. 7. Ttk69 inhibits EE cell specification through suppressing *sc* and *ase*. (A-C′) Epistasis analysis of *AS-C* and *ttk69* in EE cell specification. GFP-marked ISC clones were examined on day 7 ACI. In clones deficient for *AS-C* complex genes (A,A′), EE cells were not observed. Overexpression of *sc* in *Df(1)sc*^{B57} mutant clones allowed effective EE cell production (B,B′), whereas knockdown of *ttk69* in *Df(1)sc*^{B57} mutant clones did not allow EE cell production (C,C′). (D) Quantification of the percentage of ISC clones of the indicated genotypes that contained EE cells. (E) RT-qPCR analysis of *AS-C* gene transcription in *esg*^{ts}*>ttk-RNAi* (v10855) intestines (after 7 days at 29°C), as compared with that in wild-type intestines. *Gapdh* was used as a normalization control. Mean±s.d. (F-G′) Sc (F,F′) and Ase (G,G′) proteins were upregulated in *ttk69* mutant clones. (H-L) In comparison to knockdown of *ttk* alone driven by *esg-Gal4*^{ts} (H), additional knockdown of *sc* (I) or *ase* (J) could not prevent excessive EE cell production, whereas simultaneous knockdown of both *sc* and *ase* could (K). (L) Quantification of EE cell production in intestines of the indicated genotypes. Mean±s.e.m. *n*=10-15 guts. ***P<0.001 (*t*-test); ns, no significant difference. Scale bar: 20 µm.

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. 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 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

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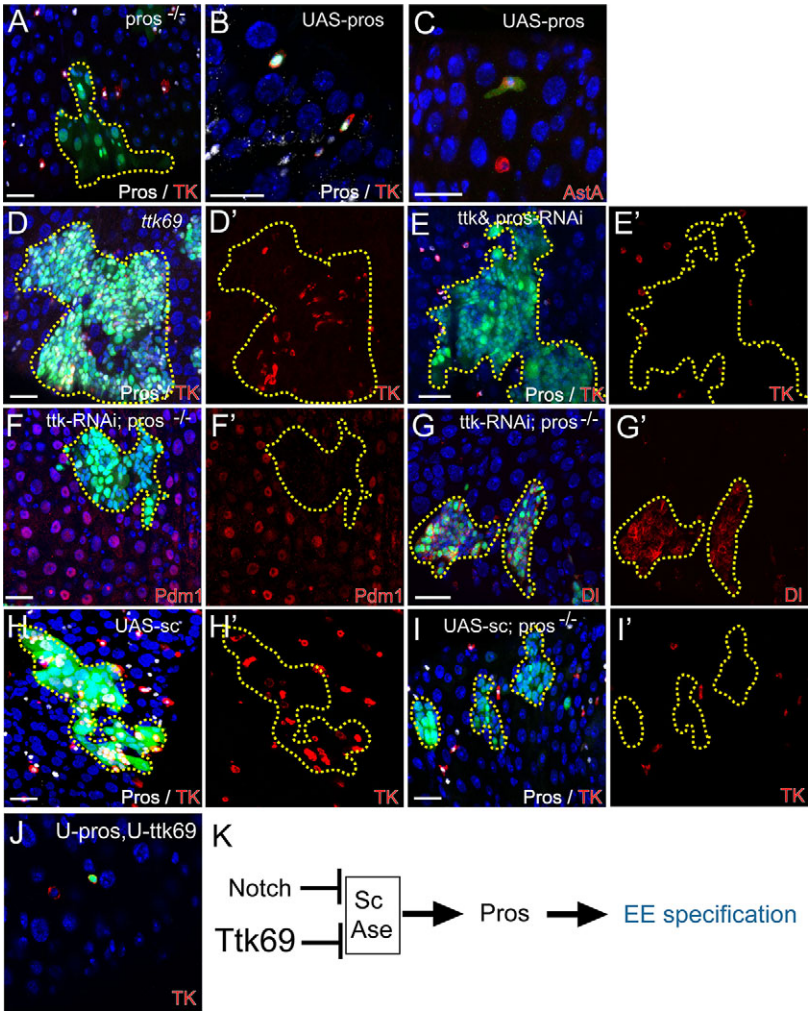

Fig. 8. Pros is epistatic to Ttk69 and AS-C in EE cell specification. GFP-marked ISC clones were generated either by the MARCM system or FLP-out technique and examined on day 7 ACI, except (D-E′) which were examined on day 14. (A) An ISC clone mutant for *pros*^{17}. No Tk^{+} EE cells were detected in *pros*^{17} clones. (B,C) Overexpression of Pros in MARCM clones led to EE cell specification. EE cells are marked by Tk (B) or AstA (C). (D,D′) A *ttk*^{1e11} clone that contains many Tk^{+} EE cells. (E,E′) Knockdown of Pros suppressed Tk^{+} EE cell production in clones expressing *ttk-RNAi*. (F,F′) Pdm1^{+} ECs were rarely present in *pros*^{17} clones expressing *ttk-RNAi* on day 7 ACI. (G,G′) Dl^{+} ISCs were present in *pros*^{17} clones expressing *ttk-RNAi* on day 7 ACI. (H,H′) An Sc-expressing clone that contained Tk^{+} EE cells. (I,I′) Overexpression of Sc in clones mutant for *pros*^{17} failed to produce Tk^{+} EE cells. (J) A single-cell clone with Ttk69 and Pros overexpression was positive for Tk expression. (K) Model for the role of Ttk69 in EE cell specification. Ttk69 acts in parallel with Notch in preventing EE cell specification by suppressing the expression of the *AS-C* genes *sc* and *ase*, the activation of which is sufficient to induce Pros, an EE cell fate determination factor. Scale bars: 20 µm.

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 *ttk69* 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).

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Although Dl/Notch is a major signaling pathway controlling cell fate decisions in the midgut, how it is regulated is poorly understood. The 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 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 Dl/Notch signaling will be the next steps towards a complete understanding of cell fate decisions in the midgut stem cell lineages.

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-ttk$^{D2\text{-}50}$* (gift from Marta Llimargas, 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 Bray, University of Cambridge, UK); *UAS-N^{icd}* (gift from Ting Xie, Stowers Institute for Medical Research, USA); *Su(H)-Gal4* and *Dl-Gal4* (gift from Xiankun Zeng and Steven Hou, National Cancer Institute, USA); *UAS-sc-RNAi* (Tsinghua Fly Center, #2205); *UAS-ase-RNAi* (Tsinghua Fly Center, #2271); *FRT82B*, *FRT82B-ttk^{1}*, *FRT82B-ttk^{1e11}*, *FRT19A*, *FRT19A-^{N55e11}* and *FRT19A-Df(1)scB57* were all obtained from BDSC; *UAS-pros-RNAi* (BDSC, #26745); *UAS-osa-RNAi* (Tsinghua Fly Center, #1909; or BDSC, #31266); *FRT82B-pro^{17}*, *UAS-Notch-RNAi*, *UAS-ttk69*, *Tub-Gal80^{ts}*, *UAS-Flp*, *Act<stop<lacZ*, *UAS-pros*, *Dl-lacZ* and *UAS-sc* were all obtained from BDSC.

Mosaic analysis

The MARCM system (Lee and Luo, 1999) or FLP-out technique (Struhl and Basler, 1993) was used to generate clones in intestines. Mosaic clones were induced by heat shocking 3- to 5-day-old females once for 1 h at 37°C in a 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^{ts}-mediated gene depletion

esg-Gal4, UAS-GFP; Tub-Gal80^{ts}; Su(H)-Gal4, UAS-GFP; Tub-Gal80^{ts} and *Tub-Gal80^{ts}; Dl-Gal4, UAS-GFP* flies were crossed to *UAS-RNAi* transgenic flies at 18°C. 3- to 5-day-old female flies of the 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 antibody

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

Immunostaining

Immunostaining of *Drosophila* midgut was performed as previously described (Lin et al., 2008). The following primary antibodies were used: mouse anti-Dl (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 (Cappel, 0855976; 1:6000); rat anti-Sc (gift from Steve Crews, University of North Carolina at Chapel Hill, USA; 1:300); rabbit anti-GFP (Invitrogen, A11122; 1:2000); rabbit anti-Ase (gift from Yuh-Nung Jan, UCSF, USA; 1:1000); rabbit anti-Pros (gift from Yuh-Nung Jan; 1:1500); rabbit anti-Ase (gift from Cheng-Yu Lee, UCSF, 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 $2^{-\Delta\Delta CT}$ method. Primer sequences were (5′-3′, forward and reverse): *Gapdh*, GAAATTAAGGCCAAGGTTCAGG and GTACCAAGAGATCAGCTTC; *ac*, TTTTCAACGACGACGAGGAG and ACCATTGCTTAAATCGGCTA; *sc*, AATGTAGACCAATCCCAGTCG and CACCACCCTTTGTCAAATCC; *l'sc*, TCAAACTGTGGTAAACTC-GCTGTC and TCGGCGGAATTGTAGATGTG; *ase*, GCACAACCAGC-AGAATCAAC and AGGCAAACCCTTTCTTCCAG.

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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.

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Supplementary material

Supplementary material available online at
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