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Notch signaling downstream target *E(spl)mbeta* is dispensable for adult midgut homeostasis in *Drosophila*

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ABSTRACT

Adult tissue homeostasis is maintained by residential stem cells through the proper balance of stem cell self-renewal and differentiation. The adult midgut of *Drosophila* contains multipotent intestinal stem cells (ISCs), and Notch signaling plays critical roles in the proliferation and differentiation of these ISCs. However, how Notch signaling downstream targets regulate ISC proliferation and differentiation still remains unclear. Here we find that Notch signaling downstream targets *E(spl)mbeta* and *E(spl)malpha* are differentially expressed in ISCs and their progeny. Interestingly, we find that midgut homeostasis is not affected in *E(spl)mbeta* null mutant. No obvious defects are observed in the intestines ectopically expressing *E(spl)mbeta* or *E(spl)malpha*. Importantly, we find that the defects in ISC proliferation and differentiation observed in *Notch* mutant cannot be rescued by ectopic expression of *E(spl)mbeta* or *E(spl)malpha*. Together, these data indicate that the proliferation and differentiation of ISCs are not regulated by individual Notch downstream target, but by different downstream targets collectively.

1. Introduction

Adult stem cells maintain tissue homeostasis by the renewal of differentiated cells. A good example is the intestinal tract, in which the frequently lost cells are constantly replenished by the progeny of ISCs. The self-renewal and differentiation of adult stem cells must be tightly controlled. Disruption of this proper balance leads to depletion or excessive proliferation of stem cells, eventually resulting in severe diseases such as cancer (Xie and Spradling, 1998; Radtke and Clevers, 2005; Lin, 2008; Morrison and Spradling, 2008). Therefore, understanding of the underlying regulatory mechanisms controlling adult stem cell proliferation and differentiation will provide insight into the potential development of therapeutic applications for human diseases.

The posterior midgut of the adult *Drosophila* intestine has proven to be an excellent model system to study how adult stem cell proliferation and differentiation are regulated. Mammalian and *Drosophila* intestines share marked similarities in terms of development, cellular make-up and genetic control (Stainier, 2005; Casali and Batlle, 2009; Wang and Hou, 2010). Previous studies demonstrated that the *Drosophila* adult midgut is maintained by ISCs interspersed along the base membrane (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). ISCs constantly undergo asymmetric self-renewing divisions and produce non-dividing, undifferentiated daughter cells, termed enteroblast (EB) (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Notch signaling plays important roles in various developmental contexts in both *Drosophila* and mammals (Artavanis-Tsakonas et al., 1999; Bray, 2000; Lai, 2004; Mathur et al., 2010; Guruharsha et al., 2012; Penton et al., 2012; Perrimon et al., 2012; Noah and Shroyer, 2013). The ligand of the Notch pathway, Delta (Dl), is specifically expressed in the ISCs, while the Notch receptor is expressed in both ISCs and EBs (Ohlstein and Spradling, 2006, 2007). ISCs signal via Dl to activate Notch signaling in their EB daughters (Ohlstein and Spradling, 2007). EB will terminally differentiate into either an absorptive enterocyte (EC) or a secretory enteroendocrine (ee) cell determined by the level of Notch signaling it received: high levels of Notch signaling promote EBs to become ECs, while low Notch signaling specifies EBs into ee cells (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2007; Perdigoto et al., 2011). In addition to specifying the fate of the progeny, Notch signaling also plays a critical role in ISC proliferation. In the absence of Notch signaling, as in *Notch* or *Su(H)* mutant, cells downstream of the ISC continue to divide, rather than to exit the cell cycle or to differentiate into ECs (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Previous study demonstrated that the Notch downstream target genes of the *Enhancer of split complex* [*E(spl)-C*] play an important role in ISC proliferation and differentiation (Bardin et al., 2010). However, the role(s) of

Abbreviations: ISCs, intestinal stem cells; EB, enteroblast; Dl, Delta; ee, enteroendocrine cells; *E(spl)-C*, *Enhancer of split complex*; ACI, after clone induction; MARCM, mosaic analysis with a repressible cell marker; *esg*, *escargot*; *N*, Notch.

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individual Notch downstream target in ISC proliferation and differentiation remains unexplored.

In this study, we investigate the functions of Notch downstream targets *E(spl)mbeta* and *E(spl)malpha* in ISC proliferation and differentiation. Interestingly, although *E(spl)mbeta* is differentially expressed in ISCs and EBs, no defects in ISC proliferation and differentiation are observed in *E(spl)mbeta* null mutant. Moreover, tissue homeostasis is not affected by ectopic expression of *E(spl)mbeta* or *E(spl)malpha*. Furthermore, defective ISC proliferation and differentiation observed in *Notch* mutant cannot be restored by the expression of *E(spl)mbeta* or *E(spl)malpha*. We propose that the proliferation and differentiation of ISCs are not regulated by individual Notch downstream target, but by different downstream targets collectively.

2. Materials and methods

2.1. Fly lines and husbandry

Flies were maintained on standard media at 25 °C. 2–3 day old flies were picked and transferred to a 29 °C incubator, unless otherwise specified. Flies were transferred to new vials with fresh food every day, and dissected at time points specified. In all experiments, only the female posterior midgut was analyzed. Information for alleles and transgenes used in this study can be found either in FlyBase or as noted: *FRT19A-N$^{264\text{-}39}$*, *esgGal4*, *UAS-GFP*, *tubGal80^{ts}* (*esg^{ts}*, gift from N. Perrimon), *E(spl)mbeta-lacZ* (gift from S. Bray), *E(spl)malpha-lacZ* (gift from J.W. Posakony), *P{EP}G8712* (BL30183), *UAS-mbeta* (BL26675), *UAS-malpha* (BL27179), *P{Δ2-3}* (BL3664), *w(white)^{RNAi}* (BL33613) (from TRiP at Harvard Medical School).

2.2. Generation of E(spl)mbeta$^{10\text{-}1}$ mutant

A viable P-element line *P{EP}G8712* is inserted 3 bp upstream of *E(spl)mbeta* transcription initiation site. *E(spl)mbeta$^{10\text{-}1}$* was generated by P-element-mediated imprecise excision. The mutant was determined by genomic PCR amplification of a fragment spanning the P-element insertion site using a Forward primer (5′-GACCAGACGATAGTCCGAGTG-3′) and a Reverse primer (5′-CAGAAGCCACCACCACCACTAC-3′) (Fig. 2A). The *E(spl)mbeta$^{10\text{-}1}$*, carrying a 404 bp deletion from the 5′ UTR to the middle of the coding exon, is likely a functionally null mutant.

2.3. Immunostaining and fluorescence microscopy

For standard immunostaining, intestines were dissected in 1× PBS (10 mM NaH_2PO_4/Na_2HPO_4, 175 mM NaCl, pH 7.4), and fixed in 4% paraformaldehyde for 25 min at room temperature. Samples were rinsed, washed with 1× PBT (0.1% Triton X-100 in 1× PBS) and blocked in 3% BSA in 1× PBT for 45 min. Primary antibodies were added to the samples and incubated at 4 °C overnight. The following primary antibodies were used: mouse mAb anti-Dl (C594.9B, 1:50), mouse mAb anti-Prospero (MR1A, 1:100) obtained from Developmental Studies Hybridoma Bank (DSHB), rabbit anti-β-galactosidase (Cappel, 1:5000), and mouse anti-β-galactosidase (Cell Signaling, 1:1000). The primary antibodies were detected by fluorescent-conjugated secondary antibodies from Jackson ImmunoResearch Laboratories. Secondary antibodies were incubated for 2 h at room temperature. DAPI (Sigma; 0.1 μg/ml) was added after secondary antibody staining. The samples were mounted in a mounting medium (70% glycerol containing 2.5% DABCO). All images were captured by a Zeiss inverted confocal microscope and were processed in Adobe Photoshop and Illustrator.

2.4. RT-qPCR

RNA was extracted from 20 midguts using TRIzol (Invitrogen). RNA was cleaned using RNAeasy (QIAGEN), and cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). qPCR was performed using the GoTaq qPCR Master Mix kit (Promega). Data were acquired using an iQ5 System (Bio-Rad). RT-qPCR was performed in duplicate on each of 3 independent biological replicates. All results are presented as Mean ± SEM of the biological replicates. Ribosomal gene *RpL11* was used as normalization control.

2.5. Construction of Su(H)Gal4 and E(spl)malphaGal4

The primer pair (ERI-5: CACCGAATTCTCTAGACTCGCATAACCGG and ERI-3: GAATTCGGATCCCCCGGTACCTAG) was used to amplify the *GBE + Su(H)* binding site from genomic DNA extracted from the *GBE + Su(H)-lacZ* line (gift from S. Bray). The primer pair (ERI-5: CACCGAATTCATCGAGTGTGCCCC and ERI-3: GAATTCAGAGTGCGTGTT GCTTGAAAGGC) was used to amplify a 2.9 kb *E(spl)malpha* regulatory region from genomic DNA. They were sub-cloned into *pChs-GAl4* vector (Apitz, 2002). The transgenic lines were obtained by standard P-element-mediated germline transformation. The drivers were further recombined with *UAS-GFP*.

2.6. MARCM ISC mosaic analysis

The clonal analyses were achieved using the MARCM system. The ISC clones were induced by heat shocking 3–5 day-old adult flies at 37 °C for 60 min. The flies were maintained at a 25 °C incubator and transferred to new vials with fresh food every day. The sizes of the marked clones were assayed at 6 days after clone induction (6D ACI, clones from 10 midguts for each genotype were assayed).

2.7. Data analysis

The number of intestines scored is indicated in the text. The number of *esg$^+$* cells was determined in at least five different guts. To determine the relative number of *esg$^+$* and ee cells (by Pros), confocal images of 40× lens/1.0 zoom from the posterior midgut of different genotypes indicated were acquired. The relative number of *esg$^+$* and ee cells was determined using Image-Pro Plus software from each confocal image. At least five different guts were observed for each set. Statistical analysis was done using the Student's *t*-test. PEMS 3.1 software was used for SEM analyses and Sigma plot software for graph generation. The graphs were further modified using Adobe Photoshop and Illustrator.

3. Results

3.1. Expression pattern of Notch downstream targets E(spl)mbeta and E(spl)malpha in Drosophila adult midgut

Notch downstream targets *E(spl)-Complex* have been demonstrated to promote differentiation and thus be repressed in ISCs (Bardin et al., 2010), indicating that most, if not all, of Notch downstream targets should be repressed in ISCs. However, the role of individual target

Fig. 1. Expression pattern of Notch downstream targets *E(spl)mbeta* and *E(spl)malpha* in *Drosophila* adult midgut. (A–B″) *E(spl)mbeta* (by lacZ, red) is expressed in the progenitors (by *esgGal4*, *UAS-GFP*, green). Note that the expression level of *E(spl)mbeta* is different in the paired progenitors (white and yellow arrowheads). lacZ and GFP are shown separately in black-white, respectively (A′, A″, B′ and B″). (C–D″) *E(spl)mbeta* (by lacZ, red) is expressed in both ISCs (by Dl, green, white arrowheads) and EBs (yellow arrowheads). Note that the expression of *E(spl)mbeta* is weaker in ISCs, but stronger in EBs. lacZ and Dl stainings are shown separately in black-white, respectively(C′, C″, D′, and D″). (E) *E(spl)malpha* (by lacZ, red) is mainly expressed in EBs (yellow arrowheads), and also in ISCs (by Dl, green, white arrowheads) occasionally, but the level is much weaker. lacZ and Dl stainings are shown separately in black-white, respectively (E′ and E″). (F) *E(spl)malphaGal4* (by *UAS-GFP*, green) is mainly expressed in EBs (yellow arrowhead), but also weaker in ISCs (by Dl, red, white arrowhead) occasionally. GFP and Dl staining are shown separately in black-white, respectively (F′ and F″). Blue indicates DAPI staining for DNA. Scale bars, 10 μm.