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Numb provides a fail-safe mechanism for intestinal stem cell self-renewal in adult *Drosophila* midgut

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eLife Assessment

The authors examine the role of Numb, a Notch inhibitor, in intestinal stem cell self-renewal in Drosophila during homeostasis and regeneration. The significance is **important** as the authors demonstrate the ISC maintenance is reduced when both BMP signaling and Numb expression is reduced. The strength of evidence is **convincing** as large sample sizes and statistical analyses are provided.

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Abstract

Stem cell self-renewal often relies on asymmetric fate determination governed by niche signals and/or cell-intrinsic factors but how these regulatory mechanisms cooperate to promote asymmetric fate decision remains poorly understood. In adult *Drosophila* midgut, asymmetric Notch (N) signaling inhibits intestinal stem cell (ISC) self-renewal by promoting ISC differentiation into enteroblast (EB). We have previously shown that epithelium-derived BMP promotes ISC self-renewal by antagonizing N pathway activity (Tian and Jiang, 2014). Here we show that loss of BMP signaling results in ectopic N pathway activity even when the N ligand Delta (Dl) is depleted, and that the N inhibitor Numb acts in parallel with BMP signaling to ensure a robust ISC self-renewal program. Although Numb is asymmetrically segregated in about 80% of dividing ISCs, its activity is largely dispensable for ISC fate determination under normal homeostasis. However, Numb becomes crucial for ISC self-renewal when BMP signaling is compromised. Whereas neither Mad RNAi nor its hypomorphic mutation led to ISC loss, inactivation of Numb in these backgrounds resulted in stem cell loss due to precocious ISC-to-EB differentiation. Furthermore, we find that *numb* mutations resulted in stem cell loss during midgut regeneration in response to epithelial damage that causes fluctuation in BMP pathway activity, suggesting that the asymmetrical segregation of Numb into the future ISC may provide a fail-save mechanism for ISC self-renewal by offsetting BMP pathway fluctuation, which is important for ISC maintenance in regenerative guts.

Introduction

Adult organs such as intestine rely on resident stem cells to replenish damaged tissues during homeostasis and regeneration (Biteau et al. 2011; Jiang and Edgar 2012). *Drosophila* midgut, an equivalent of mammalian small intestine, has emerged as a powerful system to study stem cell biology in adult tissue homeostasis and regeneration (Casali and Batlle 2009; Jiang and Edgar 2011; Jiang et al. 2016). Intestine stem cells (ISCs) in adult midguts are localized at the basal side of the gut epithelium where they can undergo asymmetric cell division to produce renewed ISCs and enteroblasts (EBs) that differentiate into enterocytes (ECs) (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006). At low frequency, an ISC daughter is fated to preEE/EEP that differentiates into two enteroendocrine cells (EEs) after another round of cell division (Biteau and Jasper 2014; Beehler-Evans and Micchelli 2015; Zeng and Hou 2015; Chen et al. 2018). About 20% ISCs undergo symmetric cell division to produce two ISCs or two EBs (O'Brien et al. 2011; Goulas et al. 2012; Tian and Jiang 2014). The decision between ISC self-renewal and differentiation into EB lineage is controlled by Notch (N) signaling whereby N activation drives ISC differentiation into EB (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006; Ohlstein and Spradling 2007; Bardin et al. 2010). The asymmetric N signaling between ISC and EB is influenced by Par/integrins-directed asymmetric cell division and differential BMP signaling (Goulas et al. 2012; Tian and Jiang 2014). EC-produced BMP ligands containing Decapentaplegic (Dpp) and Glass bottom boat (Gbb) heterodimers are secreted basally and concentrated on the basement membrane aligning the basal side of the gut epithelium (Tian and Jiang 2014). After asymmetric cell division of ISCs, basally localized daughter cells transduce higher levels of BMP signaling activity than the apically localized daughter cells and the differential BMP signaling promotes ISC self-renewal by antagonizing N pathway activity through an unknown mechanism (Tian and Jiang 2014; Tian and Jiang 2017).

The N pathway inhibitor Numb plays a decisive role in asymmetric cell fate determination in *Drosophila* peripheral and central nervous systems whereby Numb segregates asymmetrically into one daughter during division of a neuronal precursor cell and confers distinct fates to the two daughter cells (Uemura et al. 1989; Rhyu et al. 1994; Spana et al. 1995). The mammalian homologs of Numb are also critical for asymmetric fate determination during neurogenesis and myogenesis (Zhong et al. 1996; Conboy and Rando 2002; Petersen et al. 2002; Shen et al. 2002; Petersen et al. 2004). Previous studies showed that, during asymmetric division of an ISC in *Drosophila* adult midgut, Numb was preferentially segregated into the basally localized daughter that becomes future ISC (Goulas et al. 2012; Salle et al. 2017); however, many *numb* mutant clones retained ISC after many rounds of cell division although they failed to produce EE (Bardin et al. 2010; Salle et al. 2017). Given the well-established role of Numb in blocking N pathway activity and the observation that Numb is asymmetrically segregated into the future ISC, it is surprising and puzzling that loss of Numb does not lead to ectopic N pathway activation that drives ISC-to-EB differentiation. We speculate that BMP signaling may play a more dominant role in ISC self-renewal than Numb and that the BMP signaling gradient could generate differential N signaling between the apical and basal pair of ISC daughters to generate ISC/EB binary fates even when Numb is depleted (Tian and Jiang 2017). Accordingly, attenuating BMP signaling may unmask the role of Numb in ISC self-renewal.

To test this hypothesis, we employed RNA interference (RNAi) and genetic mutations to inactivate Numb in otherwise wild type background or in midguts defective in BMP signaling due to RNAi or genetic mutation of *mothers against decapentaplegic* (*mad*), which encodes a signal transducer in BMP signaling pathway (Sekelsky et al. 1995; Newfeld et al. 1997). Consistent with our previous findings (Tian and Jiang 2014), neither *mad* RNAi nor its hypomorphic mutation led to ISC loss. However, inactivation of Numb in these backgrounds resulted in stem cell loss due to precocious ISC-to-EB differentiation. By carefully examining mutant clones for multiple *numb*

alleles, we also observed an increased number of *numb* clones that lack ISCs compared with wild type control clones. Interestingly, the stem cell loss phenotype was exacerbated by feeding flies with bleomycin, which resulted in EC damage and fluctuation of BMP signaling (Amcheslavsky et al. 2009; Tian et al. 2017), underscoring an important role of Numb in ISC maintenance during gut regeneration.

Results

Loss of BMP signaling results in ectopic N pathway activity even when Dl is depleted

Our previous study showed that depleting the type II receptor Punt (Put) for BMP in progenitor cells (ISC/EB) resulted in precocious ISC-to-EB differentiation, leading to stem cell loss (Tian and Jiang 2014). In Put deficient progenitor cells, the N pathway was activated in the absence of detectable N ligand Delta (Dl). Progenitor cells deficient for both Put and N failed to differentiate to EBs and formed stem cell-like tumors (Tian and Jiang 2014). These observations imply that inactivation of Put may unleash a ligand-independent N pathway activity that drives precocious ISC-to-EB differentiation, leading to stem cell depletion. However, it remains possible that a trace amount of Dl that is beyond the detection by immunostaining might activate N in Put deficient progenitor cells.

To further explore the relationship between the BMP and N pathways, we carried out genetic epistatic experiments to determine the functional relationship between Put and Dl. We depleted Put and Dl either individually or in combination via RNAi in midgut progenitor cells using the *esg-Gal4 tub-Gal80^{ts}* (*esg^{ts}*) system, in which Gal4 is under the control of a temperature sensitive Gal80 (McGuire et al. 2004). *UAS-GFP* was included in the *esg^{ts}* system to mark all precursor cells whereas *Su(H)-lacZ* (also called *Su(H)GBE-lacZ*), a transcriptional reporter of N signaling, was used to monitor N pathway activity and mark the EBs. 3- to 5-day-old adult females expressing *UAS-Put-RNAi* and *UAS-Dl-RNAi* individually or in combination via *esg^{ts}* were shifted to 29 ^{0}C for 10 days prior to dissection, followed by immunostained with the corresponding antibodies. For quantification, we focused on the posterior region (R4) of the midguts to minimize the variation (Buchon et al. 2013). In control guts, most pairs of *esg>GFP^{+}* precursor cells, which may represent two recently divided ISC daughters, contained only one *Su(H)-lacZ* positive cell, indicating that these ISCs divided asymmetrically to produce one ISC and one EB (**Figure1A, B-B''** and **F**). In line with our previous findings, most *esg>GFP^{+}*precursor pairs from the Put RNAi guts expressed *Su(H)-lacZ* in both ISC daughters, suggesting that ISCs underwent symmetric cell division to produce two EBs when Put was depleted (**Figure1C-C''** and **F**). By contrast, *esg>GFP^{+}*cells in Dl RNAi guts formed ISC-like tumor clusters that were negative for *Su(H)-lacZ* (**Figure1D-D''** and **F**), similar to the ISC-like tumor clusters in guts containing *Dl* or *N* mutant clones (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006; Ohlstein and Spradling 2007; Siudeja et al. 2015), suggesting that in Dl RNAi guts, N pathway activity was diminished. Put and Dl double RNAi suppressed the formation of ISC-like clusters (**Figure1E**). In these guts, *esg-GFP^{+}* cells exhibited *Su(H)-lacZ* expression (**Figure1E-E''** and **F**), suggest that they activate the N pathway and differentiate into EBs. Hence, loss of BMP signaling resulted in ectopic N pathway activity that drives ISC-to-EB differentiation even when Dl was depleted.

Numb is important for ISC maintenance when BMP pathway activity is attenuated

Our previous study showed that partial loss of BMP pathway activity in several genetic background including Mad RNAi and *mad$^{1-2}$*, a hypomorphic allele of *mad*, did not lead to ISC loss whereas more complete loss of BMP signaling in Put RNAi guts or *put* mutant ISC lineage clones

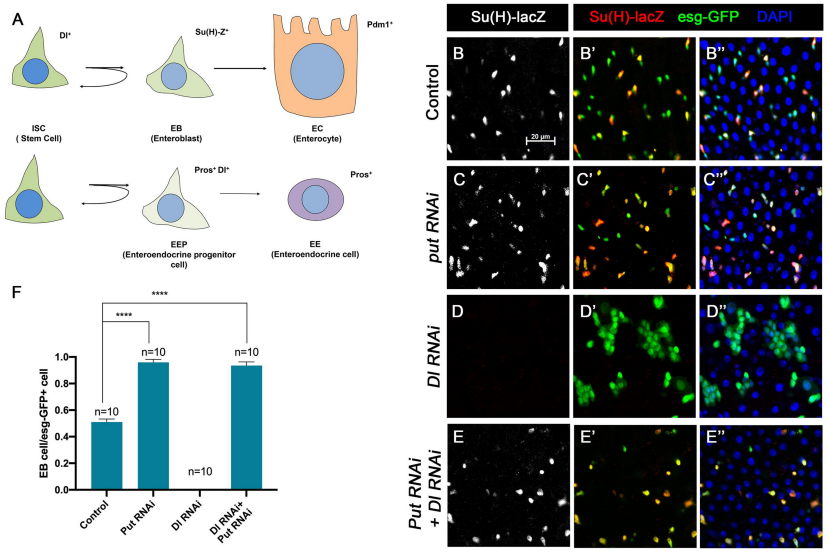


Figure 1.

BMP signaling inhibits Dl-independent N pathway activity to promote ISC self-renewal

(A) A scheme for the ISC lineage in *Drosophila* midgut. (B-E'') Representative images of Control guts (B-B''), midguts expressing *UAS-Put-RNAi* (C-C''), *UAS-Dl-RNAi* (D-D''), or *UAS-Put-RNAi* + *UAS-Dl-RNAi* (E-E'') with *esg-Gal4*^{ts}, *UAS-GFP* at 29°C for 10 days and immunostained for Su(H)-lacZ (grey or red) and GFP (green). Su(H)-lacZ is used as a marker for EB. DAPI (blue) staining indicates nuclei. Compared with control guts (B-B''), Put knockdown (C-C'') in precursor cells (green) caused an increase of EB pairs. Dl knockdown induced stem cell-like tumor. Put and Dl double knockdown induced a dramatic increase of EBs. (F) Quantification of percentage of EB cells of each genotype. Data are mean ± SD from three independent experiments. One-way ANOVA was performed for statistical comparisons. Scale bar (20 μm) is shown in B.

resulted in ISC loss. It is possible that a backup mechanism for ISC self-renewal may exist, which could compensate for the partial loss of BMP signaling to prevent ectopic N pathway activation that drives differentiation. During an asymmetric ISC division, the N inhibitor Numb is segregated into the basally localized daughter that becomes the future ISC (Goulas et al. 2012; Salle et al. 2017). We hypothesized that the asymmetric distribution of Numb may provide such a backup mechanism to ensure that the basally localized ISC daughter has lower N pathway activity than the apically localized one when differential BMP signaling is compromised so that the differential N signaling between the apical and basal is still sufficient to drive asymmetric fate determination. To test this hypothesis, we inactivated Numb and Mad either individually or in combination using two independent approaches: 1) RNAi and 2) genetic mutations. For the RNAi experiments, 3~5-day old females expressing *UAS-Numb-RNAi, UAS-Mad-RNAi, or UAS-Numb-RNAi + UAS-Mad-RNAi* under the control of esg^{ts}were transferred to 29°C for 14 days. The guts were then dissected out for immunostaining to detect the expression of *esg>GFP*, Dl-lacZ (ISC marker), E(spl)mβ-CD2 (EB marker) and Pros (EE marker). Because preEE expressed both Dl-lacZ and Pros and Dl-lacZ signals could be found in some EBs due to its perdurance, we counted Dl-lacZ$^+$ mβ-CD2$^-$ Pros$^-$ cells as ISCs and mβ-CD2$^+$ cells as EBs. Compared with control guts (**Figure 2A-A''**), Mad (**Figure 2B-B''**) or Numb (**Figure 2C-C''**) single RNAi guts contained comparable number of Dl-lacZ$^+$ mβ-CD2$^-$ Pros$^-$ cells and E(spl)mβ-CD2$^+$ cells (**Figure 2E-G**). By contrast, in Numb and Mad double RNAi guts (**Figure 2D-D''**), there was a significant decrease in the number of precursor cells (**Figure 2E**) and Dl-lacZ$^+$ mβ-CD2$^-$ Pros$^-$ cells (**Figure 2F**), and a simultaneous increase in the number of E(spl)mβ-CD2$^+$ cells (**Figure 2G**), suggesting that inactivation of both Mad and Numb results in stem cell loss, likely due to precocious ISC to EB differentiation.

In the second approach, we generated guts that carried mad^{1-2}or $numb^4$ single mutant clones or mad^{1-2}, $numb^4$ double mutant clones using the MARCM system that positively mark the clones with GFP expression. 3~5-days-old females of appropriate genotypes were heat-shocked for 1 hr for clonal induction and kept at 18°C for 14 days prior to dissection. ISCs were identified as Dl$^+$ cells or mβ-CD2$^-$ Pros$^-$ cells containing small nuclei. ISC-containing clones (ISC$^+$) and clones without ISCs (ISC$^-$) were quantified for each genotype. We also quantified the size of ISC lineage clones for each genotype by counting GFP$^+$ cells in individual clones. Consistent with previous findings (Tian and Jiang 2014; Salle et al. 2017), the average size of mad^{1-2} clones is significantly larger than the control clones (**Figure 3A-A', B-B', E-E', F-F', K**) whereas $numb^4$ clones had similar clone size distribution compared with control clones (**Figure 3C-C', G-G', K**). In addition, most of mad^{1-2} or $numb^4$ clones contained at least one ISC similar to control clones (**Figure 3K**). However, the average size of mad^{1-2} $numb^4$ clones is significantly smaller than that of control clones (**Figure 3D-D', H-H', K**). More importantly, a much larger fraction of mad^{1-2} $numb^4$ clones (~40%; n=252) did not contain ISC (**Figure 3L**), many of which only contained ECs with large nuclei and stained positive for Pdm1(**Figure 3I-J'**). Taken together, these results suggest mad^{1-2} $numb^4$ double mutation leads to ISC loss.

Inactivation of Numb and Mad leads to precocious ISC-to-EB differentiation

We employed a two-color lineage tracing system called RGT (Tian and Jiang 2014; Tian et al. 2017) to determine whether simultaneous inactivation of Numb and Mad would change the outcome of an ISC division. In this system, FLP/FRT-mediated mitotic recombination in individual dividing ISCs will generate two distinctly labelled clones that express either RFP (red) or GFP (green) (**Figure 4A**). As shown schematically in Figure4B, asymmetric ISC division (ISC/EB) will generate one clone with multiple cells and a twin spot that contains only one EC. Symmetric self-renewing division (ISC/ISC) will produce two multiple-cell clones whereas symmetric differentiation division (EB/EB) will produce two clones each of which contains one EC. Control or RNAi expressing adult flies containing *hs-FLP FRT19A ubi-GFPnls/FRT19A ubi-mRFPnls;* esg^{ts} were grown at 29 °C for 8 days (for *Mad-RNAi* only) or 14 days (for control, *Numb-RNAi*, and *Numb-RNAi*

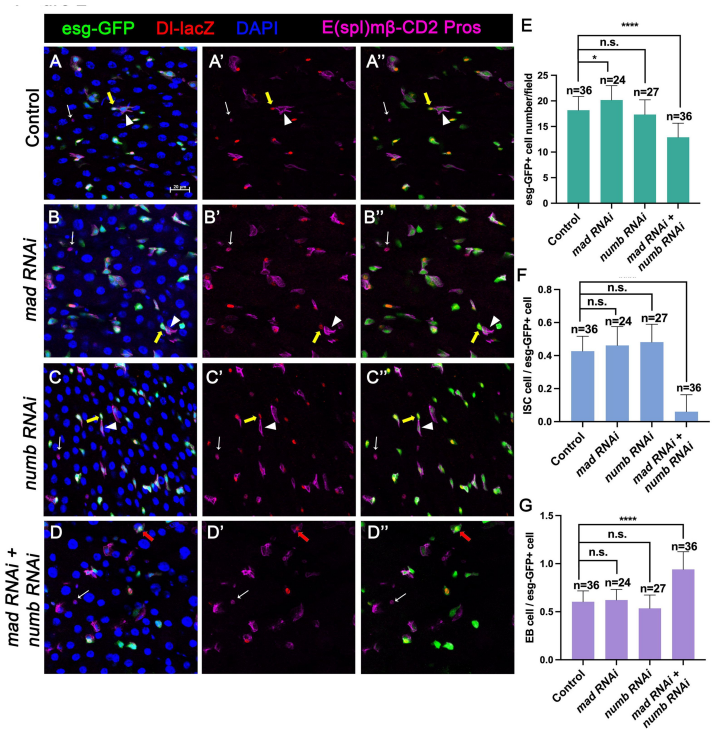


Figure 2.

Numb is important for ISC maintenance when BMP pathway activity is attenuated

(A-D'') Representative images of adult midguts expressing *UAS-mCherry-RNAi* (Control) (A-A''), *UAS-Mad-RNAi* (B-B''), *UAS-Numb-RNAi* (C-C'') and *UAS-Mad-RNAi* + *UAS-Numb-RNAi* (D-D'') with *esg-Gal4^{ts}, UAS-GFP* at 30°C for 14 days and immunostained for Dl-lacZ (red), E(spl)mβ-CD2 (cytoplasmic magenta) and Pros (nuclear magenta), which are markers for ISC, EB and EE, respectively. DAPI (blue) staining indicates nuclei. Yellow arrows indicate ISCs (Dl-lacZ^{+} E(spl)mβ-CD2^{-} Pros^{-}),white arrowheads indicate EBs (E(spl)mβ-CD2^{+}), and white arrows indicate EEs (Pros^{+}) in Control, Mad, or Numb single knockdown guts. Red arrow indicated a Dl-lacZ^{+}, E(spl) mβ-CD2^{+} cells in Mad and Numb double knockdown guts. Scale bar (20 μm) is presented in (A). (E-G) Quantification of number of precursor cells (E), percentage of ISC cells (F) and percentage of EB cells (G) of each genotype. Data are mean ± SD from three independent experiments. ****, $p < 0.0001$. One-way ANOVA was performed for statistical comparisons.

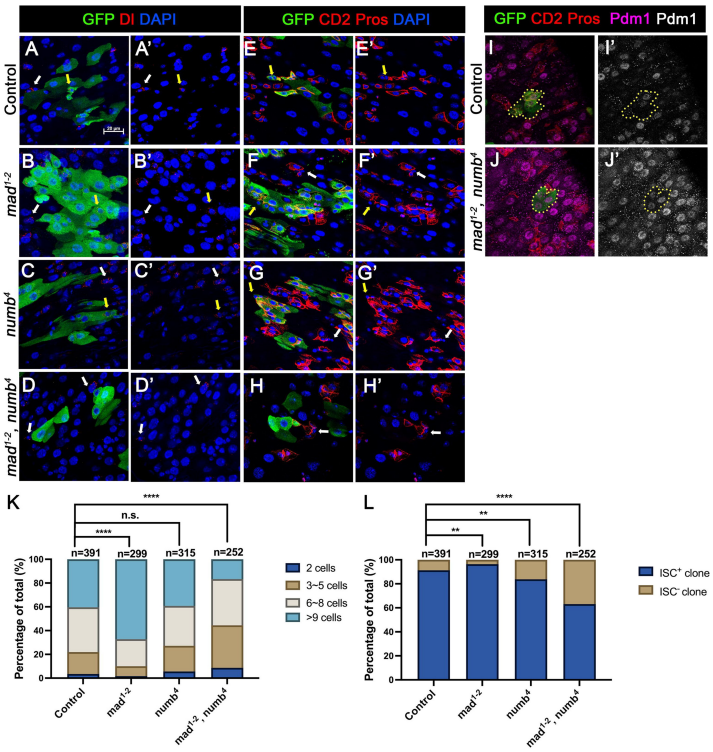


Figure 3.

Loss of ISC in Numb and Mad depleted guts is due to ISC-to-EB differentiation

(A-H') Representative images of adult midguts containing MARCM clones (green) of *FRT40* (Control) (A, A', E, E'), *mad*$^{1-2}$ (B, B', F, F'), *numb*^{4} (C, C', G, G') and *mad*$^{1-2}$, *numb*^{4} (D, D', H, H') and immunostained for GFP (green) and Dl (red and grey in A-D') or E(spl)mβ-CD2 and Pros (red in A-D' and grey in E-H') at 14 days (grown at 18°C) after clone induction. GFP marks the clones. DAPI (blue) staining indicates nuclei. ISCs inside and outside the clones are indicated by yellow and white arrows, respectively. (I) Representative images of adult midguts containing MARCM clones (green) of control (I, I') or *mad*$^{1-2}$, *numb*^{4} (J, J') immunostained for GFP (green), E(spl)mβ-CD2 and Pros (red), and Pdm1 (magenta and grey). Scale bar (20 μm) is presented in (A). (K) Quantification of clone size for the indicated genotypes 14 days after clone induction. (L) Quantification of numbers of clones with or without ISCs. Data are mean ± SD from three independent experiments. **, $p < 0.01$, ****, $p < 0.0001$. L^2 test was performed for statistical comparisons.

+ *Mad-RNAi*) before clone induction by heat shock at 37 °C for 1 hr. After clone induction, the flies were incubated at 18 °C for another 4 days before guts were dissected out for analysis (**Figure 4C**). The frequencies of ISC/EB, ISC/ISC and EB/EB divisions in control guts were 69%, 15% and 16% respectively (n=119) (**Figure 4D-F, P**). Mad RNAi guts had higher frequency of ISC/ISC (30%), and lower frequency of EB/EB (5%) division compared to control guts (n=73) (**Figure 4G-I, P**). The increase in symmetric self-renewing division in Mad RNAi guts is likely due to an increase in BMP ligand production in these guts because BMP signaling in EC inhibits BMP ligand expression (Tian et al. 2017). The frequencies of different ISC division classes in Numb RNAi guts are comparable to those of control guts (ISC/EB: 73%, ISC/ISC: 18%, EB/EB: 9%, n=127) (**Figure 4J-L, P**). By contrast, Mad and Numb double RNAi guts had lower frequency of ISC/ISC division (11%) and much higher EB/EB division (37%) than control guts (n=54) (**Figure 4M-O, P**). Thus, inactivation of Numb in backgrounds where BMP signaling was compromised altered the ISC division outcome that favors symmetric differentiation division leading to ISC loss.

Numb mutant clones exhibit weak ISC loss phenotype

When we examined adult midguts containing $numb^4$ clones, we noticed a slight increase in the frequency of ISC^- clones compared to the control guts even though the average clone size of $numb^4$clones was comparable to that of the control clones (**Figure 3K, L**), suggesting that *numb* mutation may result in a mild stem cell loss phenotype. To verify this result, we examined another *numb* allele, $numb^{15}$. By immunostaining for Dl expression that marks ISC, we found that both $numb^4$ *and* $numb^{15}$ clones contained similarly higher frequence of Dl^- clones than the control clones (**Figure 5 A-C', E**). Consistent with previous findings (Bardin et al. 2010; Salle et al. 2017), most $numb^{15}$ clones grew into large size similar to the control clones (**Figure 5D**), suggesting that many ISC^- *numb* clones lost ISC at late stages during their clonal growth. We also examined Pros expression and found that *numb* mutant clones did not contain $Pros^+$ cells (Figure5-figure supplement 1A-C'), which is consistent with a previous study showing that *numb* is required for EE fate regulation (Salle et al. 2017).

Numb is important for ISC maintenance during regeneration

The weak ISC loss phenotype associated with *numb* mutant clones could be due to fluctuation in BMP pathway activity under normal homeostasis because the expression of two BMP ligands Dpp and Gbb is uneven in homeostatic guts (Tian and Jiang 2014). If so, the stem cell loss phenotype caused by *numb* mutations could be enhanced under conditions where tissue damage causes more dramatic and widespread fluctuation in BMP signaling activity in regenerative guts. To test this possibility, we fed adult female flies carrying either control or *numb* clones in the guts with sucrose (mock), bleomycin, or dextran sodium sulfate (DSS). In mock-treated control guts, approximately 12% (n=178) of the clones did not contain Dl^+ cell, while 21% (n=216) of the $numb^4$and 24% (n=219) of the $numb^{15}$ clones were void of stem cells (**Figure 6A-A', D-D', G-G', J**). Previous studies showed that bleomycin treatment caused EC damage and enhanced the fluctuation in BMP ligand production whereas DSS affected basement membrane organization but did not increase the fluctuation in BMP ligand production (Amcheslavsky et al. 2009; Tian et al. 2017). In guts treated with bleomycin, 12% (n=160) of control clones did not contain Dl^+ cells (**Figure 6B-B', J**). However, bleomycin feeding resulted in a dramatic increase of Dl^- ISC-lineage clones in guts containing *numb* mutant clones as Dl^+ cells were absent in 43% (n=149) of $numb^4$ and 45% (n=213) of $numb^{15}$clones (**Figure 6E-E', H-H', J**). By contrast, DSS feeding did not increase the frequency of Dl^- clones in guts containing *numb* mutant clones, as the frequencies of Dl^- clones in control, $numb^4$and $numb^{15}$ clonal guts were 10% (n=167), 24% (n=165) and 21% (n=141), respectively (**Figure 6C-C', F-F', I-I', J**). Bleomycin also resulted in a reduction in *numb* mutant clone size, as compared with the mock-treatment (**Figure 6K**). Taken together, these results suggest that Numb plays an important role in ISC maintenance in regenerative guts in response to bleomycin-induced tissue damage.

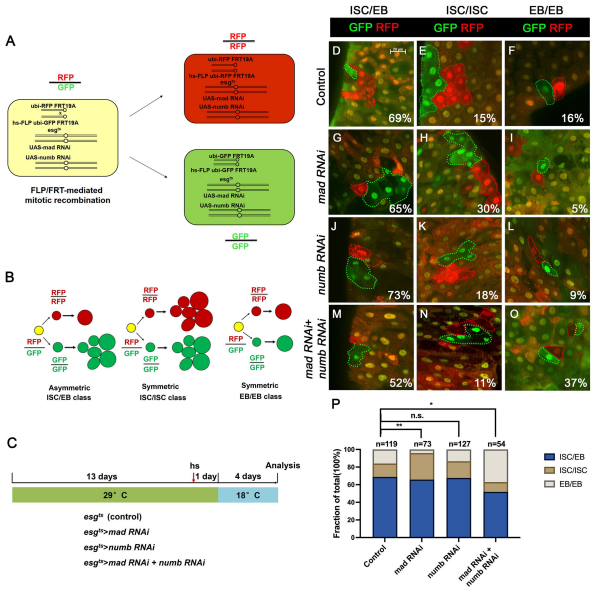


Figure 4.

Depletion of both Numb and Mad leads to more EB/EB division

(A) Scheme of an ISC division that produces differentially labeled daughter cells (RFP^+ GFP^- and RFP^- GFP^+) through FRT-mediated mitotic recombination. Adapted from (Tian and Jiang 2014).
(B) Scheme of differentially labeled twin clones generated by FLP/FRT-mediated mitotic recombination of dividing ISCs. Adapted from (Tian and Jiang 2014).
(C) Scheme of twin-spot experiments. 3~5-day-old adult flies of indicated genotype are grown at 29°C for 14 days before heat shock to induce clones. After one-day recovery at 29°C, the flies are raised at 18°C for 4 days prior to analysis.
(D-O) Representative images of twin-spot clones from adult midguts of the indicated genotypes. Scale bar 20 μm is shown in (D).
(P) Quantification of twin spots of different classes from guts of the indicated genotypes Data are mean ± SD from three independent experiments. *, $p < 0.05$, **, $p < 0.01$. L^2 test was performed for statistical comparisons.

Figure 5.

numb mutant clones exhibit weak stem cell loss phenotype

(A-C') Representative images of adult midguts containing MARCM clone (green) of *FRT40* (Control) (A, A'), *numb*^{4} (B, B'), and *numb*^{15} (C, C') and immunostained for Dl (red), GFP (green) and DAPI (blue) at 14 days after clone induction. GFP marks the clones. ISCs inside and outside the clones are indicated by yellow and white arrows, respectively. Scale bar (20 μm) is shown in (A).
(D) Quantification of clone size distribution for the indicated genotypes at 14 days after clone induction.
(E) Quantification of numbers of clones with or without ISC.
Data are mean ± SD from three independent experiments.*, $p < 0.05$, **, $p < 0.01$. L^2 test was performed for statistical comparisons.

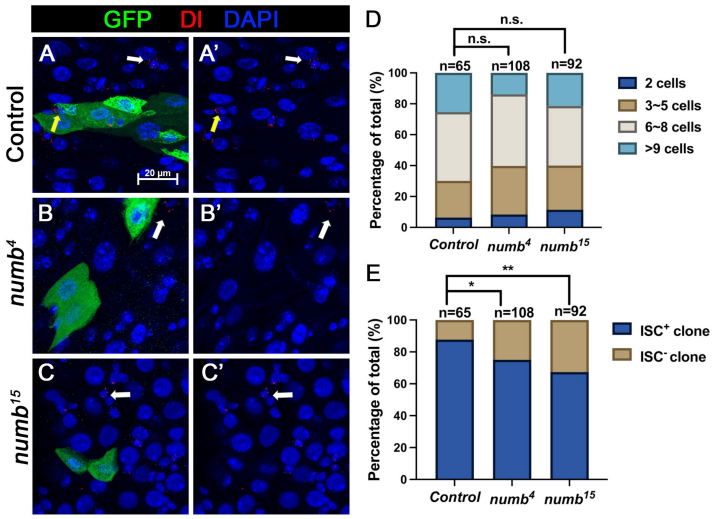
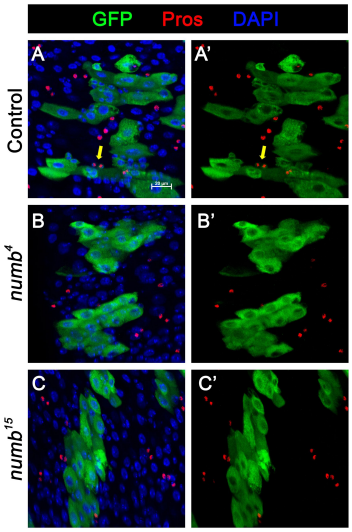


Figure 5-figure supplement 1.

Numb is required for EE fate determination.

(A-C') ISC MARCM clone (green) of control (A, A'), *numb*^{4} (B, B'), and *numb*^{15} (C, C') are stained for Pros (red) at 14 days after clone induction. Representative clone in control guts (A, A') contains EE cells (Pros positive), as indicated with yellow arrows. Representative clones in *numb*^{4} (B, B') and *numb*^{15} (C, C') guts do not contain any EE cells. Scale bar (20 μm) is presented in (A).



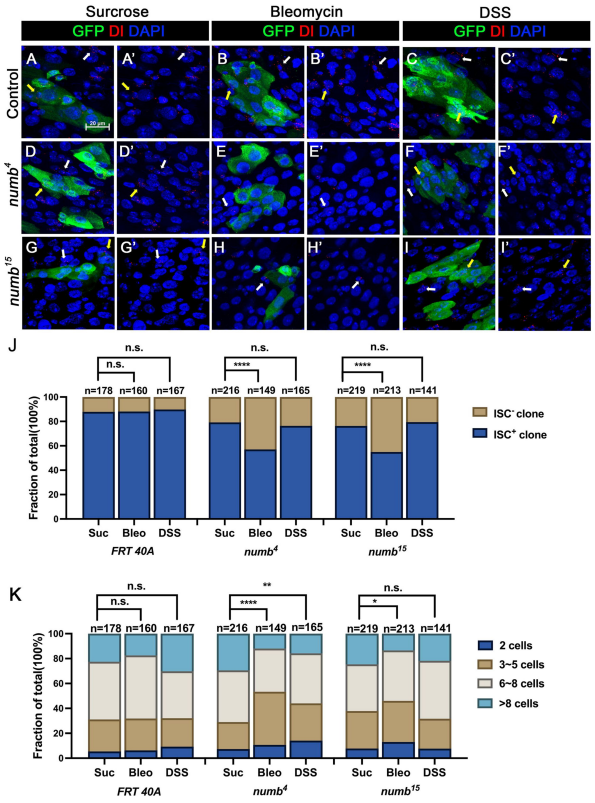


Figure 6.

Numb is critical for ISC maintenance during regeneration

(A-I') Adult flies of indicated genotype were treated with sucrose, bleomycin or DSS for 24h at 14 days after clone induction and recovered for another 4 days before dissection. Guts containing MARCM clones of the indicated genotype were stained for GFP (green) and Dl (red and white). GFP marks the clones. DAPI (blue) staining indicates the nuclei. Stem cells inside and outside the clones are indicated by yellow and white arrows, respectively. Scale bar (20 μm) is shown in (A).
(J) Quantification of the percentage of clones with or without ISCs.
(K) Quantification of clone size distribution for the indicated genotypes.
Data are mean ± SD from three independent experiments.*, $p < 0.05$, **, $p < 0.01$ ****, $p < 0.0001$. L^2 test was performed for statistical comparisons.

Discussion

Despite the prominent role of Numb in asymmetric cell fate decision in the nervous system and the observation that Numb is asymmetrically segregated in dividing ISCs, whether Numb plays any role in ISC fate determination has remained a mystery. Here we demonstrated that Numb is important for ISC self-renewal during regeneration. We found that Numb is largely dispensable for homeostatic ISC self-renewal due to the predominant role of BMP signaling in this context. Indeed, we found that Numb becomes critical for ISC self-renewal under conditions where BMP signaling is compromised.

Previous studies did not score a stem cell loss phenotype associated with *numb* mutant clones because the majority of *numb* mutant ISC lineage clones could grow into large size comparable to control clones (**Figs. 3** and **5**)(Bardin et al. 2010; Goulas et al. 2012; Salle et al. 2017). Instead, Salle et al showed that *numb* mutant clones lacked EEs, suggesting that Numb is important for EE fate determination (Salle et al. 2017), which we confirmed in this study (Figure S1). However, by carefully examining ISC/EB markers associated with *numb* mutant clones, we noticed an increase in the fraction of *numb* mutant clones that lack ISCs compared with the control clones (**Figures 3** and **5**). By introducing *mad* mutation (mad^{1-2}) into the *numb* mutant background, we found that $numb^{4}$ mad^{1-2} double mutant clones had a much higher frequence to lose ISCs than $numb^{4}$clones even though mad^{1-2} single mutant clones showed no ISC loss phenotype compared with control clones (**Figure 3**).

Our previous study showed that immediately after bleomycin treatment, there was an increase in the ISC population size due to a transient surge in BMP ligand production (Tian et al. 2017). However, during regeneration, BMP ligand production was downregulated due to the autoinhibition of BMP ligand expression by BMP signaling in ECs (Tian et al. 2017). The reduction in BMP ligand production promoted ISC-to-EB differentiation to reset the ISC population size back to the homeostatic level after regeneration (Tian et al. 2017). It is likely that asymmetric distribution of Numb in dividing ISCs may prevent excessive ISC-to-EB differentiation that could otherwise lead to a decreased ISC population during regeneration. Indeed, we observed an increase in the frequency of *numb* mutant clones lacking ISCs in response to bleomycin treatment, suggesting that Numb becomes critical for ISC maintenance during gut regeneration in response to tissue damage.

Based on our findings in current and previous studies, we propose the following working model to account for the cooperation between Numb and BMP signaling in the regulation of ISC self-renewal under homeostatic and tissue regeneration (**Figure 7**). Under homeostatic conditions, most ISCs divide basally so that the basally localized ISC daughters inherit Numb and transducing higher levels of BMP signaling activity than the apically situated daughter cells, the combined differential activities in BMP signaling and Numb drive robust asymmetric division outcomes to produce ISC/EB pairs (**Figure 7A**). In the *numb* mutant background, differential BMP signaling activities between the apical and basal daughter cells suffice to drive asymmetric division outcomes in most cases (**Figure 7B**). In the *mad* mutant background, the BMP signaling activity gradient becomes shallower but differential Numb activity between the apical and basal daughter cells can compensate for the compromised BMP signaling gradient to drive asymmetric division outcomes (**Figure 7C**). However, in *numb mad* double mutant background or in *numb* mutant guts damaged by bleomycin feeding, the compromised BMP signaling activity gradient alone is often insufficient to drive asymmetric division outcomes, leading to ISC loss due to symmetric EB/EB division outcomes (**Figure 7D**). One interesting question is why asymmetric Numb activity is unable to drive asymmetric ISC division outcome in the absence of BMP signaling as seen in *put* mutant background. One possibility is that the Numb level is too low in midgut ISCs so that the asymmetric Numb inheritance during ISC division is not robust enough to ensure

asymmetric N signaling in the absence of BMP signaling. Indeed, previous studies indicate that endogenous Numb was undetectable using Numb antibodies (Goulas et al. 2012; Couturier et al. 2013; Salle et al. 2017). Another non-mutually exclusive possibility is that Numb may not be able to counter the ligand-independent N pathway activity unleashed in *put* mutant backgrounds. Future study is needed to test these possibilities and to determine the precise mechanism by which BMP signaling inhibits N pathway activity.

Materials and methods

Drosophila genetics and transgenes

Flies were maintained on cornmeal at 25°C. Transgenic lines and mutants include: *UAS-Put-RNAi* (VDRC #107071); *UAS-Dl-RNAi* (BL#28032); *UAS-Mad-RNAi (*VDRC #12635); *UAS-Numb-RNAi* (BL #35045); *UAS-mCherry-RNAi* (BL #35785); *tub-Gal80^{ts}*, *esg-Gal4*, *Su(H) Gbe-lacZ (Su(H)-lacZ)*; *E(spl)mβ-CD2* (BL#83353); *numb^{4}* is a strong allele, *numb^{15}* is a null allele, and *mad$^{1-2}$* is a hypomorphic allele (Flybase). *yw hs-FLP UAS-GFP; tub-Gal80 FRT40A* was used for MARCM clonal analysis. *yw hs-FLP FRT19A ubi-GFPnls* and *yw FRT19A ubi-mRFPnls* were used for twin spot clone analysis. For experiments involving *tubGal80^{ts}*, crosses were set up and cultured at 18°C to restrict Gal4 activity. 2 to 3-day-old progenies were shifted to 29°C for the indicated periods of time to inactivate *Gal80^{ts}*, allowing Gal4 to activate *UAS* transgenes in all experiments, only the female posterior midguts were analyzed.

Genotypes for Drosophila used in each figure

- Figure 1
    - (B-B”) *Su(H)-lacZ/+; esg-Gal4 tub-Gal80^{ts}/+*
    - (C-C”) *Su(H)-lacZ/+; esg-Gal4 tub-Gal80^{ts}/UAS-Put-RNAi*
    - (D-D”) *Su(H)-lacZ/+; esg-Gal4 tub-Gal80^{ts}/+; UAS-Dl-RNAi/+*
    - (E-E”) *Su(H)-lacZ/+; esg-Gal4 tub-Gal80^{ts}/ UAS-Put-RNAi; UAS-Dl-RNAi/+*
- Figure 2
    - (A-A”) *yw/+; esg-Gal4 tub-Gal80^{ts}/+; UAS-mCherry-RNAi, E(spl)mβ-CD2/Dl-lacZ*
    - (B-B”) *yw/+; esg-Gal4 tub-Gal80^{ts}/UAS-Mad-RNAi; E(spl)mβ-CD2/Dl-lacZ*
    - (C-C”) *yw/+; esg-Gal4 tub-Gal80^{ts}/+; UAS-Numb-RNAi, E(spl)mβ-CD2/Dl-lacZ*
    - (D-D”) *yw/+; esg-Gal4 tub-Gal80^{ts}/ UAS-Mad-RNAi; UAS-Numb-RNAi, E(spl)mβ-CD2/Dl-lacZ*
- Figure 3
    - (A-A’) *yw,hs-FLP/+; FRT40A tub-GAL80/ FRT40A; UAS-GFP/+*
    - (B-B’) *yw,hs-FLP/+; FRT40A tub-GAL80/ FRT40A mad$^{1-2}$; UAS-GFP/+*
    - (C-C’) *yw,hs-FLP/+; FRT40A tub-GAL80/ FRT40A numb^{4}; UAS-GFP/+*
    - (D-D’) *yw,hs-FLP/+; FRT40 tub-GAL80/ FRT40A mad$^{1-2}$, numb^{4}; UAS-GFP/+*
    - (E-E’, I-I’) *yw,hs-FLP/+; FRT40A tub-GAL80/ FRT40A; UAS-GFP/ E(spl)mβ-CD2*
    - (F-F’) *yw,hs-FLP/+; FRT40A tub-GAL80/FRT40A mad1-2; UAS-GFP/ E(spl)mβ-CD2*
    - (G-G’) *yw,hs-FLP/+; FRT40A tub-GAL80/ FRT40A numb; UAS-GFP/ E(spl)mβ-CD2*
    - (H-H’, J-J’) *yw,hs-FLP/+; FRT40A tub-GAL80/ FRT40A mad$^{1-2}$, numb^{4}; UAS-GFP/E(spl)mβ-CD2*
- Figure 4

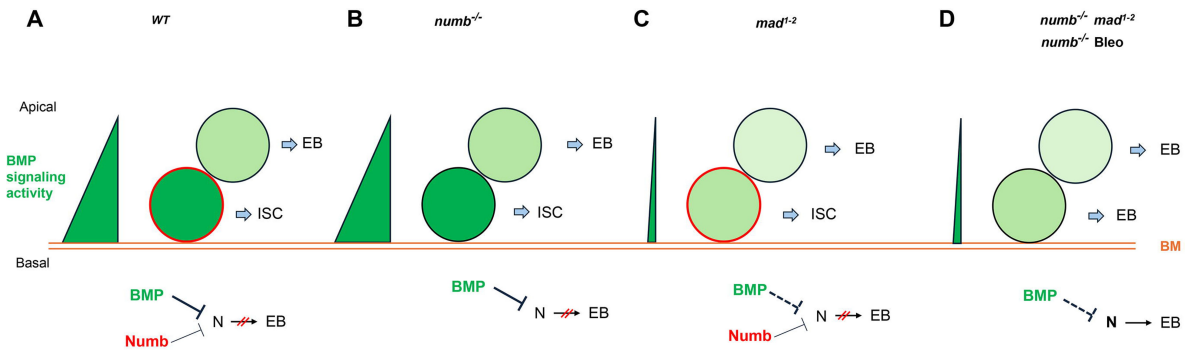


Figure 7.

Model for Numb and BMP signaling in ISC/EB fate decision.

(A) During asymmetric ISC division, the basal ISC daughter transduces higher level of BMP signaling and inherits higher level of Numb activity than the apical one. Inhibition of N by BMP signaling and Numb promotes ISC fate.
(B) In *numb* mutant background, differential BMP signaling between the basal and apical ISC daughters is sufficient to generate differential N pathway activities to drive asymmetric fate decision.
(C) In *mad* mutant background, the shallow BMB activity gradient acts in conjunction with the asymmetric Numb activity to generate differential N pathway activities between the basal and apical ISC daughters to drive asymmetric fate decision.
(D) In *numb mad* double mutant background or in guts containing *numb* mutant clones and injured by bleomycin (Bleo) feeding, the shallow BMB activity gradient is often insufficient to generate asymmetric N pathway activation, leading to precocious ISC-to-EB differentiation.
BM: basement membrane. Bleo: Bleomycin; BM: basement membrane; thin line and dashed line indicate weak inhibition.

- (D-F) *yw hs-FLP, FRT19A ubi-GFPnls/FRT19A ubi-mRFPnls; esg-Gal4 tub-Gal80^{ts}/+*
- (G-I) yw hs-FLP, FRT19A ubi-GFPnls/FRT19A ubi-mRFPnls; esg-Gal4 tub-Gal80^{ts}/ *UAS-Mad-RNAi*
- (J-L) *yw hs-FLP, FRT19A ubi-GFPnls/FRT19A ubi-mRFPnls; esg-Gal4 tub-Gal80^{ts}/+; UAS-Numb-RNAi/+*
- (M-O) *yw hs-FLP, FRT19A ubi-GFPnls/FRT19A ubi-mRFPnls; esg-Gal4 tub-Gal80^{ts}/UAS-Mad-RNAi; UAS-Numb-RNAi/+*

- Figure 5 and Figure 5-figure supplement 1

  - (A-A') *yw hs-FLP/+; FRT40A tub-GAL80/ FRT40A; UAS-GFP/+*
  - (B-B') *yw hs-FLP/+; FRT40A tub-GAL80/ FRT40A numb^{4}; UAS-GFP/+*
  - (C-C') *yw hs-FLP/+; FRT40A tub-GAL80/ FRT40A numb^{15}; UAS-GFP/+*

- Figure 6

  - (A-C') *yw hs-FLP/+; FRT40A tub-GAL80/ FRT40A; UAS-GFP/+*
  - (D-F') *yw hs-FLP/+; FRT40A tub-GAL80/ FRT40A numb^{4}; UAS-GFP/+*
  - (G-I') *yw hs-FLP/+; FRT40A tub-GAL80/ FRT40A numb^{15}; UAS-GFP/+*

MARCM clone analysis

For MARCM clone induction, crosses were set up and cultured at 18°C to avoid spontaneous clones. 2-to-3-day-old females were subjected to heat shock at 37°C for 1 hr and then kept at 18°C for another 14 days before dissection. Flies were transferred to new vials with fresh food every 2 days. The sizes of the clones were quantified from at least 10 midguts for each genotype.

Twin spot clone analysis

For twin spot clone generation, 2-to-3-day-old flies were kept at 29°C for 14 days and heat-shocked at 37°C for 1 hr and then raised at 29°C for another 4 days before dissection. Flies were transferred to new vials with fresh food every 2 days.

Feeding experiments

Flies were cultured in an empty vial containing a piece of 2.5 × 3.75-cm chromatography paper (Fisher) wet with 5% sucrose (MP Biomedicals) solution as feeding medium (mock treatment) or with 25 μg/mL bleomycin (Sigma-Aldrich) or 5% DSS (40 kDa; MP Biomedicals) for one day at 30°C. After treatment, flies were recovered on normal food at 18°C for another 4 days before dissection.

Immunostaining

Female flies were used for gut immunostaining in all experiments. The entire gastrointestinal tract was taken and fixed in 1 X PBS plus 8% EM grade formaldehyde (Polysciences) for 2 hours. Samples were washed and incubated with primary and secondary antibodies in a solution containing 1 X PBS, 0.5% goat serum (Thermos Fisher), and 0.1% Triton X-100 (Bio-rad). The following primary antibodies were used: mouse anti-Delta (DSHB), 1:10; rabbit anti-LacZ (MP Biomedicals), 1:1,000; mouse anti-CD2 (Thermos Fisher), 1:1000; chicken anti-GFP (Abcam), 1:1000; mouse anti-Pros (DSHB), 1:10; rabbit anti-Pdm1 (from Dr. Xiaohang Yang), 1:1000; Alexa Fluor-conjugated secondary antibodies were used at 1:1000 (Invitrogen). DAPI (4',6-Diamidino-2-Phenylindole) is a nuclear dye (Thermos Fisher). Guts were mounted in 70% glycerol and imaged

with a Zeiss confocal microscope (Zeiss LSM 710 inverted confocal) using 40X oil objectives (imaging medium: Zeiss Immersol 518F). The acquisition and processing software was Zeiss LSM Image Browser, and image processing was done in Adobe Photoshop.

Quantification and Statistical analysis

For Figures and 2, cell number of the indicated cell types were counted per ROI (region of interest) on images taken using LEICA DFC345 FX camera on a LEICA DMI 400 B microscope, equipped with a 40 × objective lens. For each genotype, 8~12 guts were analyzed. In each gut, three ROI were randomly selected in R4 of midguts for quantification. One-way ANOVA was performed for statistical comparisons. For **Figures 3** - **6**, all GFP^+ clone cells (≥2) in midguts were counted individually. For each genotype, at least 10 guts were calculated. L^2 test was performed for statistical comparisons. All statistical significances were calculated in Prism 10 (GraphPad Software, Inc). *, p <0.05, **, p <0.01, ***, p <0.001, ****, p <0.0001; n.s., not significant.

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We thank Bing Wang for technical assistance, Vienna Drosophila Resource Center and Bloomington Drosophila Stock Center for fly stocks, and Developmental Studies Hybridoma Bank for antibodies. This work is supported National Institutes of Health Grant GM118063 and Welch Foundation Grant I-1603 to J.J.

Additional information

Author Contributions

J.J. designed research; M.L. and A.T. performed research; M.L., A.T. and J.J. analyzed data; and M.L. and J.J. wrote the manuscript.

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Reviewer #1 (Public review):

Summary:

By way of background, the Jiang lab has previously shown that loss of the type II BMP receptor Punt (Put) from intestinal progenitors (ISCs and EBs) caused them to differentiate

into EBs, with a concomitant loss of ISCs (Tian and Jiang, eLife 2014). The mechanism by which this occurs was activation of Notch in Put-deficient progenitors. How Notch was upregulated in Put-deficient ISCs was not established in this prior work. In the current study, the authors test whether a very low level of Dl was responsible. But co-depletion of Dl and Put led to a similar phenotype as depletion of Put alone. This result suggested that Dl was not the mechanism. They next investigate genetic interactions between BMP signaling and Numb, an inhibitor of Notch signaling. Prior work from Bardin, Schweisguth and other labs has shown that Numb is not required for ISC self-renewal. But the authors wanted to know whether loss of both the BMP signal transducer Mad and Numb would cause ISC loss. This result was observed for RNAi depletion from progenitors and for mad, numb double mutant clones. Of note, ISC loss was observed in 40% of mad, numb double mutant clones, whereas 60% of these clones had an ISC. They then employed a two-color tracing system called RGT to look at the outcome of ISC divisions (asymmetric (ISC/EB) or symmetric (ISC/ISC or EB/EB)). Control clones had 69%, 15% and 16%, respectively, whereas mad, numb double mutant clones had much lower ISC/ISC (11%) and much higher EB/EB (37%). They conclude that loss of Numb in moderate BMP loss of function mutants increased symmetric differentiation which lead caused ISC loss. They also reported that numb15 and numb4 clones had a moderate but significant increase in ISC-lacking clones compared to control clones, supporting the model that Numb plays a role in ISC maintenance. Finally, they investigated the relevance of these observation during regeneration. After bleomycin treatment, there was a significant increase in ISC-lacking clones and a significant decrease in clone size in numb4 and numb15 clones compared to control clones. Because bleomycin treatment has been shown to cause variation in BMP ligand production, the authors interpret the numb clone under bleomycin results as demonstrating an essential role of Numb in ISC maintenance during regeneration.

Strengths

i. Data are quantified with statistical analysis
ii. Experiments have appropriate controls and large numbers of samples
iii. Results demonstrate an important role of Numb in maintaining ISC number during regeneration and a genetic interaction between Mad and Numb during homeostasis.

Weaknesses

None noted in the revised manuscript.

https://doi.org/10.7554/eLife.104723.2.sa3

Reviewer #2 (Public review):

Summary:

This work assesses the genetic interaction between the Bmp signaling pathway and the factor Numb, which can inhibit Notch signalling. It follows up on the previous studies of the group (Tian, eLife, 2014; Tian, PNAS, 2014) regarding BMP signaling in controlling stem cell fate decision as well as on the work of another group (Sallé, EMBO, 2017) that investigated the function of Numb on enteroendocrine fate in the midgut. This is an important study providing evidence of a Numb-mediated back up mechanism for stem cell maintenance.

Strengths:

(1) Experiments are consistent with these previous publications while also extending our understanding of how Numb functions in the ISC.
(2) Provides an interesting model of a "back up" protection mechanism for ISC maintenance.

Weaknesses:
(1) Aspects of the experiments could be better controlled or annotated:
(a) As they "randomly chose" the regions analyzed, it would be better to have all from a defined region (R4 or R2, for example) or to at least note the region as there are important regional differences for some aspects of midgut biology.
(b) It is not clear to me why MARCM clones were induced and then flies grown at 18{degree sign}C? It would help to explain why they used this unconventional protocol.

(2) There are technical limitations with trying to conclude from double-knockdown experiments in the ISC lineage, such as those in Figure 1 where Dl and put are both being knocked down: depending on how fast both proteins are depleted, it may be that only one of them (put, for example) is inactivated and affects the fate decision prior to the other one (Dl) being depleted. Therefore, it is difficult to definitively conclude that the decision is independent of Dl ligand.

(3) Additional quantification of many phenotypes would be desired.
(a) It would be useful to see esg-GFP cells/total cells and not just field as the density might change (2E for example).
(b) Similarly, for 2F and 2G, it would be nice to see the % of ISC/ total cell and EB/total cell and not only per esgGFP+ cell.
(c) Fig1: There is no quantification - specifically it would be interesting to know how many esg+ are su(H)lacZ positive in Put- Dl- condition compared to WT or Put- alone. What is the n?
(d) Fig2: Pros + cells are not seen in the image? Are they all DllacZ+?
(e) Fig3: it would be nice to have the size clone quantification instead of the distribution between groups of 2 cell 3 cells 4 cell clones.
(f) How many times were experiments performed?

(4) The authors do not comment on the reduction of clone size in DSS treatment in Figure 6K. How do they interpret this? Does it conflict with their model of Bleo vs DSS?

(5) There is probably a mistake on sentence line 314 -316 "Indeed, previous studies indicate that endogenous Numb was not undetectable by Numb antibodies that could detect Numb expression in the nervous system".

Comments on revisions:

The authors have by and large addressed my main points.

https://doi.org/10.7554/eLife.104723.2.sa2

Reviewer #3 (Public review):

Summary:

The authors provide an in-depth analysis of the function of Numb in adult Drosophila midgut. Based on RNAi combinations and double mutant clonal analyses, they propose that Numb has a function in inhibiting Notch pathway to maintain intestinal stem cells, and is a backup mechanism with BMP pathway in maintaining midgut stem cell mediated homeostasis.

Strengths:

Overall, this is a carefully constructed series of experiments, and the results and statistical analyses provides believable evidence that Numb has a role, albeit weak compared to other pathways, in sustaining ISC and in promoting regeneration especially after damage by bleomycin, which may damage enterocytes and therefore disrupt BMP pathway more. The results overall support their claim.

The data are highly coherent, and support a genetic function of Numb, in collaborating with BMP signaling, to maintain the number and proliferative function of ISCs in adult midguts. The authors used appropriate and sophisticated genetic tools of double RNAi, mutant clonal analysis and dual marker stem cell tracing approaches to ensure the results are reproducible and consistent. The statistical analyses provide confidence that the phenotypic changes are reliable albeit weaker than many other mutants previously studied.

Weaknesses:

In the absence of Numb itself, the midgut has a weak reduction of ISC number (Fig. 3 and 5), as well as weak albeit not statistically significant reduction of ISC clone size/proliferation. I think the authors published similar experiments with BMP pathway mutants. The mad1-2 allele used here as stated below may not be very representative of other BMP pathway mutants. Therefore, it could be beneficial to compare the number of ISC number and clone sizes between other BMP experiments to provide the readers a clearer picture how these two pathways individually contribute (stronger/weaker effects) to the ISC number and gut homeostasis.

The main weakness of this manuscript is the analysis of the BMP pathway components, especially the mad1-2 allele. The mad RNAi and mad1-2 alleles (P insertion) are supposed to be weak alleles and that might be suitable for genetic enhancement assays here together with numb RNAi. However, the mad1-2 allele, and sometime the mad RNAi, showed weakly increased ISC clone size. This is kind of counter-intuitive that they should have a similar ISC loss and ISC clone size reduction.

A much stronger phenotype was observed when numb mutants were subject to treatment of tissue damaging agents Bleomycin, which causes damage in different ways than DSS. Bleomycin as previously shown to be causing mainly enterocyte damage, and therefore disrupt BMP signaling from ECs more likely. Therefore, this treatment together with loss of numb led to highly significant reduction of ISC in clones and reduction of clone size/proliferation. One improvement is that it is not clear whether the authors discussed the nature of the two numb mutant alleles used in this study and the comparison to the strength of the RNAi allele. Because the phenotypes are weak, and more variable, the use of specific reagents is important.

Furthermore, the use of possible activating alleles of either or both pathways to test genetic enhancement or synergistic activation will provide strong support for the claims.

For the revision, the authors have provided detailed responses, comments, and a revised manuscript that together satisfactorily answer all my questions. The manuscript read well and the flow of information is quite clear. I do not have further concerns and support the manuscript moving forward.

https://doi.org/10.7554/eLife.104723.2.sa1

Author response:

The following is the authors' response to the original reviews

> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *By way of background, the Jiang lab has previously shown that loss of the type II BMP receptor Punt (Put) from intestinal progenitors (ISCs and EBs) caused them to*

> *differentiate into EBs, with a concomitant loss of ISCs (Tian and Jiang, eLife 2014). The mechanism by which this occurs was activation of Notch in Put-deficient progenitors. How Notch was upregulated in Put-deficient ISCs was not established in this prior work. In the current study, the authors test whether a very low level of Dl was responsible. But co-depletion of Dl and Put led to a similar phenotype as depletion of Put alone. This result suggested that Dl was not the mechanism. They next investigate genetic interactions between BMP signaling and Numb, an inhibitor of Notch signaling. Prior work from Bardin, Schweisguth and other labs has shown that Numb is not required for ISC self-renewal. However the authors wanted to know whether loss of both the BMP signal transducer Mad and Numb would cause ISC loss. This result was observed for RNAi depletion from progenitors and for mad, numb double mutant clones. Of note, ISC loss was observed in 40% of mad, numb double mutant clones, whereas 60% of these clones had an ISC. They then employed a two-color tracing system called RGT to look at the outcome of ISC divisions (asymmetric (ISC/EB) or symmetric (ISC/ISC or EB/EB)). Control clones had 69%, 15% and 16%, respectively, whereas mad, numb double mutant clones had much lower ISC/ISC (11%) and much higher EB/EB (37%). They conclude that loss of Numb in moderate BMP loss of function mutants increased symmetric differentiation which lead caused ISC loss. They also reported that $numb^{15}$ and $numb^{4}$ clones had a moderate but significant increase in ISC-lacking clones compared to control clones, supporting the model that Numb plays a role in ISC maintenance. Finally, they investigated the relevance of these observation during regeneration. After bleomycin treatment, there was a significant increase in ISC-lacking clones and a significant decrease in clone size in $numb^{4}$ and $numb^{15}$ clones compared to control clones. Because bleomycin treatment has been shown to cause variation in BMP ligand production, the authors interpret the numb clone under bleomycin results as demonstrating an essential role of Numb in ISC maintenance during regeneration.*
>
> *Strengths:*
>
> *(i) Most data is quantified with statistical analysis*
>
> *(ii) Experiments have appropriate controls and large numbers of samples*
>
> *(iii) Results demonstrate an important role of Numb in maintaining ISC number during regeneration and a genetic interaction between Mad and Numb during homeostasis.*
>
> *Weaknesses:*
>
> *(i) No quantification for Fig. 1*

Quantification of Fig.1 has been added.

> *(ii) The premise is a bit unclear. Under homeostasis, strong loss of BMP (Put) leads to loss of ISCs, presumably regardless of Numb level (which was not tested). But moderate loss of BMP (Mad) does not show ISC loss unless Numb is also reduced. I am confused as to why numb does not play a role in Put mutants. Did the authors test whether concomitant loss of Put and Numb leads to even more ISC loss than Put-mutation alone.*

We have tested the genetic interaction between put and numb using Put RNAi and Numb RNAi driven by esg^{ts}. According to the results in this study and our previously published data, *put* mutant clone or *esg^{ts} > Put-RNAi* induced a rapid loss of ISC (whin 8 days). We did not observe further enhancement of stem cell loss phenotype in Put and Numb double RNAi guts.

> *(iii) I think that the use of the word "essential" is a bit strong here. Numb plays an important role but in either during homeostasis or regeneration, most numb clones or*

> *mad, numb double mutant clones still have ISCs. Therefore, I think that the authors should temper their language about the role of Numb in ISC maintenance.*

We have revised the language and changed “essential” to important”.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *This work assesses the genetic interaction between the Bmp signaling pathway and the factor Numb, which can inhibit Notch signalling. It follows up on the previous studies of the group (Tian, Elife, 2014; Tian, PNAS, 2014) regarding BMP signaling in controlling stem cell fate decision as well as on the work of another group (Sallé, EMBO, 2017) that investigated the function of Numb on enteroendocrine fate in the midgut. This is an important study providing evidence of a Numb-mediated back up mechanism for stem cell maintenance.*
>
> *Strengths:*
>
> *(1) Experiments are consistent with these previous publications while also extending our understanding of how Numb functions in the ISC.*
>
> *(2) Provides an interesting model of a "back up" protection mechanism for ISC maintenance.*
>
> *Weaknesses:*
>
> *(1) Aspects of the experiments could be better controlled or annotated:*
>
> *(a) As they "randomly chose" the regions analyzed, it would be better to have all from a defined region (R4 or R2, for example) or to at least note the region as there are important regional differences for some aspects of midgut biology.*

Thank you for the suggestion. In fact, we conducted all the analyses in region 4, we have added statement to clarify this in the revised manuscript.

> *(b) It is not clear to me why MARCM clones were induced and then flies grown at 18{degree sign}C? It would help to explain why they used this unconventional protocol.*

We kept the flies at 18°C to avoid spontaneous clone.

> *(2) There are technical limitations with trying to conclude from double-knockdown experiments in the ISC lineage, such as those in Figure 1 where Dl and put are both being knocked down: depending on how fast both proteins are depleted, it may be that only one of them (put, for example) is inactivated and affects the fate decision prior to the other one (Dl) being depleted. Therefore, it is difficult to definitively conclude that the decision is independent of Dl ligand.*

In our hand, Dl-RNAi is very effective and exhibited loss of N pathway activity (as determined by the N pathway reporter Su(H)-lacZ) after RNAi for 8 days (Fig. 1D). Therefore, the ectopic Su(H)-lacZ expression in Punt Dl double RNAi (fig. 1E) is unlikely due to residual Dl expression. Nevertheless, we have changed the statement “BMP signaling blocks ligand-independent N activity” to” Loss of BMP signaling results in ectopic N pathway activity even when Dl is depleted”

> *(3) Additional quantification of many phenotypes would be desired.*

> *(a) It would be useful to see esg-GFP cells/total cells and not just field as the density might change (2E for example).*

We focused on R4 region for quantification where the cell density did not exhibit apparent change in different experimental groups. In addition, we have examined many guts for quantification. It is very unlikely that the difference in the esg-GFP+ cell number is caused by change in cell density.

> *(b) Similarly, for 2F and 2G, it would be nice to see the % of ISC/ total cell and EB/total cell and not only per esgGFP+ cell.*

Unfortunately, we didn't have the suggested quantification. However, we believe that quantification of the percentage of ISC or EB among all progenitor cells, as we did here, provides a meaningful measurement of the self-renewal status of each experimental group.

> *(c) Fig1: There is no quantification - specifically it would be interesting to know how many esg+ are su(H)lacZ positive in Put- Dl- condition compared to WT or Put- alone. What is the n?*

Quantification of Fig.1 has been added.

> *(d) Fig2: Pros + cells are not seen in the image? Are they all DllacZ+?*

Anti-Pros and anti-E(spl)mβ-CD2 were stained in the same channel (magenta). Pros+ exhibited "dot-like" nuclear staining while CD2 staining outlined the cell membrane of EBs. We have clarified this in the revised figure legend.

> *(e) Fig3: it would be nice to have the size clone quantification instead of the distribution between groups of 2 cell 3 cells 4 cell clones.*

Because of the heterogeneity of clone size for each genotype, we chose to group clones based on their sizes (2, 3-6, 6-8, >8 cells) and quantified the distribution of individual groups for each genotype, which clearly showed an overall reduction in clone size for mad numb double mutant clones. We and others have used the same clone size analysis in previous studies (e.g., Tian and Jiang, eLife 2014).

> *(f) How many times were experiments performed?*

All experiments were performed at least 3 times.

> *(4) The authors do not comment on the reduction of clone size in DSS treatment in Figure 6K. How do they interpret this? Does it conflict with their model of Bleo vs DSS?*

Guts containing $numb^4$ clones treated with DSS exhibited a slight reduction of clone size, evident by a higher percentage of 2-cell clones and lower percentage of > 8 cell clones. This reduction is less significant in guts containing $numb^{15}$ clones. However, the percentage of Dl^+-containing clones is similar between DSS and mock-treated guts. It is possible that ISC proliferation is lightly reduced due to $numb^4$ mutation or the genetic background of this stock.

> *(5) There is probably a mistake on sentence line 314 -316 "Indeed, previous studies indicate that endogenous Numb was not undetectable by Numb antibodies that could detect Numb expression in the nervous system".*

We have modified the sentence.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *The authors provide an in-depth analysis of the function of Numb in adult Drosophila midgut. Based on RNAi combinations and double mutant clonal analyses, they propose that Numb has a function in inhibiting Notch pathway to maintain intestinal stem cells, and is a backup mechanism with BMP pathway in maintaining midgut stem cell mediated homeostasis.*
>
> *Strengths:*
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> *Overall, this is a carefully constructed series of experiments, and the results and statistical analyses provides believable evidence that Numb has a role, albeit weak compared to other pathways, in sustaining ISC and in promoting regeneration especially after damage by bleomycin, which may damage enterocytes and therefore disrupt BMP pathway more. The results overall support their claim.*
>
> *The data are highly coherent, and support a genetic function of Numb, in collaborating with BMP signaling, to maintain the number and proliferative function of ISCs in adult midguts. The authors used appropriate and sophisticated genetic tools of double RNAi, mutant clonal analysis and dual marker stem cell tracing approaches to ensure the results are reproducible and consistent. The statistical analyses provide confidence that the phenotypic changes are reliable albeit weaker than many other mutants previously studied.*
>
> *Weaknesses:*
>
> *In the absence of Numb itself, the midgut has a weak reduction of ISC number (Fig. 3 and 5), as well as weak albeit not statistically significant reduction of ISC clone size/proliferation. I think the authors published similar experiments with BMP pathway mutants. The mad^{1-2} allele used here as stated below may not be very representative of other BMP pathway mutants. Therefore, it could be beneficial to compare the number of ISC number and clone sizes between other BMP experiments to provide the readers with a clearer picture of how these two pathways individually contribute (stronger/weaker effects) to the ISC number and gut homeostasis.*

Thanks for the comment. We have tested other components of BMP pathway in our previously study (Tian et al., 2014). More complete loss of BMP signaling (for example, Put clones, Put RNAi, Tkv/Sax double mutant clones or double RNAi) resulted in ISC loss regardless the status of numb, suggesting a more predominant role of BMP signaling in ISC self-renewal compared with Numb. We speculate that the weak stem cell loss phenotype associated with numb mutant clones in otherwise wild type background could be due to fluctuation of BMP signaling in homeostatic guts.

> *The main weakness of this manuscript is the analysis of the BMP pathway components, especially the mad^{1-2} allele. The mad RNAi and mad^{1-2} alleles (P insertion) are supposed to be weak alleles and that might be suitable for genetic enhancement assays here together with numb RNAi. However, the mad^{1-2} allele, and sometimes the mad RNAi, showed weakly increased ISC clone size. This is kind of counter-intuitive that they should have a similar ISC loss and ISC clone size reduction.*

We used mad^{1-2} and mad RNAi here to test the genetic interaction with numb because our previous studies showed that partial loss of BMP signaling under these conditions did not

cause stem cell loss, therefore, may provide a sensitized background to determine the role of Numb in ISC self-renewal. The increased proliferation of ISC/ clone size associated with mad^{1-2} and mad RNAi is due to the fact that reduction of BMP signaling in either EC or EB non-autonomously induces stem cell proliferation. However, in mad numb double mutant clones, there was a reduction in clone size due to loss of ISC in many clones.

> *A much stronger phenotype was observed when numb mutants were subject to treatment of tissue damaging agents Bleomycin, which causes damage in different ways than DSS. Bleomycin as previously shown to be causing mainly enterocyte damage, and therefore disrupt BMP signaling from ECs more likely. Therefore, this treatment together with loss of numb led to a highly significant reduction of ISC in clones and reduction of clone size/proliferation. One improvement is that it is not clear whether the authors discussed the nature of the two numb mutant alleles used in this study and the comparison to the strength of the RNAi allele. Because the phenotypes are weak and more variable, the use of specific reagents is important.*

We have included information about the two numb alleles in the "Materials and Methods". $numb^{15}$ is a null allele, and the nature of $numb^{4}$ has not been elucidated. According to Domingos, P.M. et al., $numb^{15}$ induced a more severe phenotype than $numb^{4}$ did. Consistently, we also found that more $numb^{15}$ mutant clones were void of stem cell than $numb^{4}$ mutant clones.

> *Furthermore, the use of possible activating alleles of either or both pathways to test genetic enhancement or synergistic activation will provide strong support for the claims.*

Activation of BMP (esgts>Tkv^{CA}) alone induced stem cell tumor (Tian et al., 2014) whereas overexpression of Numb did not induce increase stem cell number although overexpression of Numb in wing discs produced phenotypes indictive of inhibition of N (our unpublished observation), making it difficult to test the synergistic effect of activating both BMP and Numb.

> ***Reviewer #1 (Recommendations for the authors):***
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> *- Cartoon of RGT in Fig 4 needs to be improved. We need to know what chromosome harbors the esgts. It is not sufficient to simply put the location of the ubi-GFP and ubi-RFP (on 19A) and not show the location of other components of the RGT system.*

Thank you for the suggestion. We have revised the cartoon in Fig. 4 to include all three pairs of chromosomes and indicate where the esgts driver and UAS-RNAi are located. In addition, we have included the genotypes for all the genetic experiments in the Method section.

> *- Quantification of the results in Fig. 1*

Quantification of Fig.1 has been added.

> *- The authors need to explain the premise more carefully (see above) and explain whether or not they tested put, numb double knockdowns.*

We have explained why not testing put numb double RNAi (see above).

> ***Reviewer #2 (Recommendations for the authors):***
>
> *The number of times the experiments have been performed would be useful to include.*

This information has been added in the figure legends.

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