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The *Drosophila* putative histone acetyltransferase Enok maintains female germline stem cells through regulating Bruno and the niche

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ABSTRACT

Maintenance of adult stem cells is largely dependent on the balance between their self-renewal and differentiation. The *Drosophila* ovarian germline stem cells (GSCs) provide a powerful *in vivo* system for studying stem cell fate regulation. It has been shown that maintaining the GSC population involves both genetic and epigenetic mechanisms. Although the role of epigenetic regulation in this process is evident, the underlying mechanisms remain to be further explored. In this study, we find that Enoki mushroom (Enok), a *Drosophila* putative MYST family histone acetyltransferase controls GSC maintenance in the ovary at multiple levels. Removal or knockdown of Enok in the germline causes a GSC maintenance defect. Further studies show that the cell-autonomous role of Enok in maintaining GSCs is not dependent on the BMP/Bam pathway. Interestingly, molecular studies reveal an ectopic expression of Bruno, an RNA binding protein, in the GSCs and their differentiating daughter cells elicited by the germline Enok deficiency. Misexpression of Bruno in GSCs and their immediate descendants results in a GSC loss that can be exacerbated by incorporating one copy of *enok* mutant allele. These data suggest a role for Bruno in Enok-controlled GSC maintenance. In addition, we observe that Enok is required for maintaining GSCs non-autonomously. Compromised expression of *enok* in the niche cells impairs the niche maintenance and BMP signal output, thereby causing defective GSC maintenance. This is the first demonstration that the niche size control requires an epigenetic mechanism. Taken together, studies in this paper provide new insights into the GSC fate regulation.
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Introduction

Adult stem cells have the capacity for continuously generating self-renewing and differentiating daughter cells, thus ensuring the tissue renewal and homeostasis throughout the life of an animal (Morrison and Spradling, 2008). Maintaining a stable population of stem cells is mainly dependent on how their self-renewal and differentiation are finely orchestrated. It has been well documented that failure to repress differentiation could lead to a defect in the stem cell maintenance (Xie, 2013). Therefore, the differentiation control is crucial for maintaining a stem cell population *in vivo*.

The *Drosophila* ovarian germline stem cells (GSCs) have been widely used as a working platform for addressing the regulatory mechanisms governing adult stem cell fate and behavior (Kirilly and Xie, 2007). In the anterior germarium of each ovariole, the basic structural unit of the *Drosophila* ovary, a group of two or three GSCs reside in a well-defined somatic niche that mainly contains cap cells (CpCs) and terminal filament cells (TFCs). The GSC constantly divide asymmetrically so that one daughter cell remaining in contact with the niche retains stem cell characteristic, whereas the other moves out of the niche, acquiring cystoblast (CB) cell fate. Numerous studies show that controlling GSC self-renewal involves both intrinsic and extrinsic mechanisms that repress differentiation (Xie, 2013). So far, at least three complex/pathways have been identified as key regulators for this process. Bone morphogenetic protein (BMP)/Decapentaplegic (Dpp) signals from the niche maintain the GSC fate through activating the BMP signaling pathway in GSCs (Xie and Spradling, 1998, 2000). This is achieved by repressing GSC differentiation via silencing the transcription of the differentiation promoting gene *bag-of-marbles* (*bam*) (Chen and McKearin, 2003; Song et al., 2004). Once the distal daughter cells of dividing GSCs are displaced away from the niche, however, they no longer receive BMP signals, relieving the repression of *bam* expression and differentiating into CBs. Although the role of BMP signaling-mediated *bam* silencing is certain, there still might be other unknown transcriptional targets of the BMP pathway linked to the GSC fate regulation. In addition to the BMP/Bam pathway, the Nanos/Pumilio (Nos/Pum) complex and the miRNA pathway are cell-autonomously required for GSC maintenance (Bhat, 1999; Forbes and Lehmann, 1998; Jin and Xie, 2007; Lin and Spradling, 1997; Park et al., 2007; Wang and Lin, 2004; Yang et al., 2007). In the case of Nos/Pum complex-dependent regulation, two translational repressors, Nos and Pum, prevent GSC differentiation by repressing the translation of a set of differentiation promoting genes in GSCs that are yet to be identified, except for *brain*

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tumor (*brat*) (Bhat, 1999; Forbes and Lehmann, 1998; Gilboa and Lehmann, 2004; Harris et al., 2011; Lin and Spradling, 1997; Wang and Lin, 2004). It is noteworthy that the expression pattern of Nos in GSCs and CBs is also subject to regulation by other differentiation regulatory factors such as Sex-lethal (Sxl) and Bam, indicating the importance of complex molecular circuits in the control of GSC/CB differentiation (Chau et al., 2012; Li et al., 2009). In parallel, genetic analyses have demonstrated that the miRNA pathway components including Dicer-1, Loquacious and Argonaute 1 are essential for maintaining GSCs cell-autonomously, though the miRNAs and their target mRNAs involved in the fate regulation are still elusive (Jin and Xie, 2007; Park et al., 2007; Yang et al., 2007). Given that known differentiation regulatory programs are still limited, and the molecular circuit connecting those complex/pathways is lacking, the question of how the GSC/CB differentiation is controlled remains to be addressed.

It has been reported that the niche size control is also important for sustaining the GSC population, and Notch signaling plays an instructive role in the formation and maintenance of the GSC niche (Hsu and Drummond-Barbosa, 2011; Song et al., 2007; Ward et al., 2006). More recently, systemic insulin signals were shown to regulate GSC maintenance through controlling the niche size via Notch signaling (Hsu and Drummond-Barbosa, 2009, 2011). Significantly, the age-dependent decline in the number of GSCs and CpCs is attributable to attenuated insulin signaling (Hsu and Drummond-Barbosa, 2009). Thus, Notch signaling-controlled maintenance of the GSC niche could potentially be an *in vivo* model system for investigating how regulation of adult stem cell aging related to tissue/organ aging occurs. Besides those cell-autonomous and non-autonomous genetic factors described above, the physical interaction between CpCs and GSCs is also indispensable for GSC maintenance. In this case, DE-Cadherin (DE-Cad) mediated adhesion of GSCs to CpCs anchors GSCs in the niche, ensuring their continuous self-renewal at adulthood (Song et al., 2002).

Increasing evidence has implicated epigenetic regulation in GSC maintenance. We and others have shown that a number of epigenetic factors involving chromatin remodeling or histone modification act in controlling GSC self-renewal presumably through preventing precocious differentiation in a BMP/Bam pathway-dependent or -independent manner (Buszczak et al., 2009; Eliazer et al., 2011; Maines et al., 2007; Wang et al., 2011; Xi and Xie, 2005; Xuan et al., 2013; Yin and Lin, 2007). Although it is evident that GSC fate could be regulated at the epigenetic level, the underlying mechanisms are not well understood. In the present study, we found that Enoki mushroom (Enok), a *Drosophila* putative histone acetyltransferase, has a cell-autonomous role in GSC self-renewal control independent of the BMP/Bam pathway. Further molecular and genetic analyses identified Bruno, an RNA-Recognition-Motifs-containing RNA binding protein with multiple functions in the ovary and early embryo (Filardo and Ephrussi, 2003; Kim-Ha et al., 1995; Moore et al., 2009; Parisi et al., 2001; Sugimura and Lilly, 2006; Wang and Lin, 2007; Webster et al., 1997), as an intermediate factor for Enok-controlled GSC maintenance. Meanwhile, we observed that Enok is also required for the control of GSC niche size, as well as niche signal output, and consequently for maintaining GSCs. This is the first demonstration that a putative epigenetic factor is involved in the GSC niche maintenance. Together, our studies reveal a novel mechanism that underlies the GSC fate regulation.

Material and methods

Fly strains and genetics

All *Drosophila* strains were maintained and crossed at 25 °C unless otherwise stated. The following fly stocks were used in this study:

Canton S (*CS*) strain was used as wild type.

Mutant alleles: $enok^1$ and $enok^2$ (Bloomington *Drosophila* Stock Center, BDSC) (Scott et al., 2001), $enok^{K1293}$ (from Takashi Suzuki) (Berger et al., 2008), Mad^{12} (from Yu Cai) (Sekelsky et al., 1995), N^{264-39} (BDSC) (Song et al., 2007).

enok RNAi: TH142 and TH150 (Tsinghua Fly Center, China), V37536 and V37527 (Vienna *Drosophila* RNAi Center, VDRC), B29518 (BDSC);

Gal4/UAS: *nos-Gal4.NGT* (Li and Gergen, 1999), *nos-Gal4.VP16* (Van Doren et al., 1998), *bab1-Gal4* (Bolivar et al., 2006), *Act-Gal4* and $tub\text{-}Gal80^{ts}$ (McGuire et al., 2003) (BDSC), *UASp-bruno* (from Anne Ephrussi) (Filardo and Ephrussi, 2003), *UAS-dpp* (from Ting Xie) (Nellen et al., 1996), *UAS-dally* (from Zhaohui Wang) (Jackson et al., 1997), *UAS-NICD* (Notch intracellular domain, from Marc Haenlin) (Neumann and Cohen, 1996);

Reporter lines: *Dad-lacZ* (from Yu Cai) (Tsuneizumi et al., 1997), *bamP-GFP* (from Lilach Gilboa) (Chen and McKearin, 2003), *m7-lacZ* (from Ting Xie) (Song et al., 2007), *Dl-lacZ* (BDSC) (de Celis et al., 1998), *Ser-lacZ* (from Daniela Drummond-Barbosa) (Bachmann and Knust, 1998);

UAS-enok was generated by cloning full-length *enok* cDNA (from Takashi Suzuki) (Scott et al., 2001) into pUAST vector and standard P element-mediated transformation (Rubin and Spradling, 1982; Spradling and Rubin, 1982).

Mosaic clones were generated by mitotic recombination using FLP/FRT system (Xu and Rubin, 1993). To generate GSC clones, *hsFLP*; *FRTG13 ubiGFP* was crossed to *FRTG13*, *FRTG13* $enok^1$ or *FRTG13* $enok^2$ or *hsFLP*; *FRT42D ubiGFP* was crossed to *FRT42D*, *FRT42D* $enok^{K1293}$. Two-day-old female adult progenies of appropriate genotype were heat-shocked at 37 °C twice a day on three consecutive days for one hour each time. Ovaries were then dissected at day 2, 7, 14 and 21 after the last heat-shock treatment for analysis. For analyzing *bamP-GFP*, GSC clones were generated by crossing *hsFLP*; *FRT42D arm-lacZ* to *FRT42D* $enok^{K1293}$. Specifically, we used *bab1-Gal4*, *UAS-FLP* or *hsFLP* for inducing CpC or TF clones. In the case of using *hsFLP*, we heat-shocked the third instar larvae at 37 °C twice a day for two consecutive days.

RNAi-based knockdown experiments and *bruno* misexpression were performed by Gal4/UAS binary system (Brand and Perrimon, 1993). Overexpression of *bruno* in adult germ cells and knocking down *enok* in niche cells were first set up at 25 °C and shifted to 29 °C after eclosion for stronger phenotypes.

Antibodies and immunofluorescence

Antibody staining was carried out as described previously (Li et al., 2008). The following primary antibodies were used: mouse anti-α-Spec (1:20, DSHB 3A9(323 or M10-2)), rabbit anti-Cleaved-Caspase-3 (1:500, Cell Signaling #9661), rabbit anti-pMad (1:1000, from Edward Laufer) (Gancz et al., 2011), mouse anti-β-galactosidase (1:100, DSHB 40-1a), mouse anti-Bam (1:5, DSHB Fly Bag-of -Marbles), rabbit anti-GFP (1:1000, Invitrogen A11122), rabbit anti-Nos (1:1000, from Akira Nakamura), mouse anti-Sxl (DSHB), guinea pig anti-A2BP1 (from Michael Buszczak) (Tastan et al., 2010), mouse anti-Orb (DSHB), rabbit anti-Bruno (1:1000, from Mary A. Lilly) (Sugimura and Lilly, 2006), rabbit anti-Vasa (1:200, Santa Cruz sc-30210), mouse anti-Lamin C (1:10, DSHB LC28.26), rat anti-DE-Cad (1:50, DSHB DCAD2), mouse anti-Arm (1:20, DSHB N2 7A1 ARMADILLO). Secondary antibodies conjugated with Alexa Fluor 488, 546, 647 (Invitrogen) were used at 1:1000 dilutions. DAPI (Invitrogen) was used to visualize nuclei. TUNEL assay was performed using *In Situ* Cell Death Detection Kit from Roche.

Confocal images were captured on Leica TCS SP5 laser confocal microscope.

GSC and CpC identification, counting and statistical analysis

GSCs were identified by the presence of a spectrosome anchored to the CpC contact site. CpCs were identified by positive Lamin C staining plus their position and morphology for distinguishing them from TFCs. Germline cells were marked by Vasa staining.

Student's *t* test and Mann–Whitney test were chosen to calculate *p*-values.

Quantitative RT-PCR

The relative expression level of *dpp* or *dally* in *enok* knockdown and control ovaries was determined by quantitative PCR using comparative C_T method. *rp49* was served as normalization control. To exclude the interference of the vitellarium region of ovariole, we used bam^{BG} mutant background for this assay. RNA was isolated using TRIzol (Invitrogen) and then subjected to DNase treatment and reverse transcription using FastQuant RT Kit (TIANGEN Biotech) for cDNA synthesis according to the manufactures' instructions. Quantitative real-time PCR was performed on the Mastercycler ep realplex[4] PCR system (Eppendorf). The primers for amplifying *dpp*, *dally* or *rp49* were described previously (Xuan et al., 2013).

Results

Enok is required intrinsically for GSC maintenance

Given that Enok plays an essential role in normal egg laying (Scott et al., 2001), we assumed that this *Drosophila* putative histone acetyltransferase is implicated in GSC-derived germ cell development in the ovary. Recently, it has been reported that GSC fate regulation including stem cell maintenance and differentiation involves epigenetic mechanisms (Buszczak et al., 2009; Eliazer et al., 2011; Maines et al., 2007; Wang et al., 2011; Xi and Xie, 2005; Xuan et al., 2013; Yin and Lin, 2007). This prompted us to investigate whether *enok* is required for maintaining GSCs during oogenesis. For this purpose, we first performed a clonal analysis. In the experiment, GSC clones homozygous for either the *enok* mutant allele or wild type control were induced by FLP/FRT-based mitotic recombination, and the frequency of the marked clones was documented at different time points after clonal induction (ACI). The GSC clones were marked by the absence of GFP expression and the presence of an anteriorly anchored spectrosome (α-Spectrin staining, α-Spec) (Fig. 1A-F). In contrast to that of the controls, the rate of GSC clones homozygous for the mutant allele $enok^1$, $enok^2$ or $enok^{K1293}$ showed a rapid and steady decline in a 3-week time course ACI respectively (Fig. 1I and Table S1), suggesting a cell-autonomous role of Enok in GSC maintenance. To validate this observation, we further knocked down *enok* in the germline by expressing *enok* RNAi transgenes in combination with *nos-Gal4* driver. Prior to this experiment, we tested five *enok* RNAi transgenes that were either generated at the Tsinghua Fly Center of China (TH142 and TH150) or ordered from the VDRC (V37526 and V37527) or the BDSC (B29518) for targeting specificity. RT-PCR-based semi-quantitative assay revealed that ubiquitous expression of each RNAi transgene can remarkably down-regulate *enok* transcription in the larval tissues (Fig. S1), indicating on-target effects of the transgenes on endogenous *enok*. Consistent with the clonal analysis described above, the cell counting showed that RNAi-based reduced expression of *enok* in the germline causes a significant decline in GSC number per germarium during day 2 to day 21 after eclosion, albeit decreased GSC number at eclosion was observed (Fig. 1J and Table S2). Collectively, these data clearly demonstrate that Enok functions in maintaining GSCs in a cell-autonomous manner.

Defective GSC maintenance in *enok* mutants could be elicited by cell death or precocious differentiation. We next performed a number of assays for differentiating these possibilities. Both anti-Cleaved-Caspase 3 and TUNEL labeling failed to detect any apoptotic signal in the marked *enok* mutant GSCs ($n=199$ and 65,

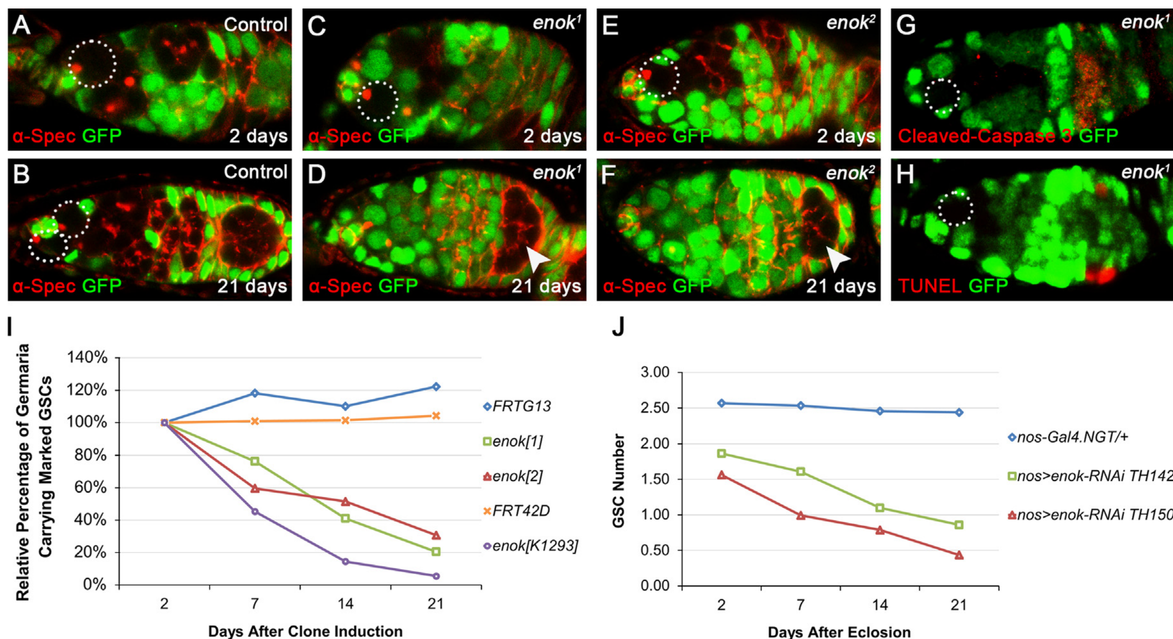

Fig. 1. *enok* is required intrinsically for GSC maintenance rather than survival. (A–F) Germaria with wild type control (A, B), $enok^1$ (C, D) or $enok^2$ (E, F) mutant GSC clones (broken circles) marked by the absence of GFP and the presence of an anteriorly anchored spectrosome (α-Spec staining). In the wild type control, marked GSCs are observed at 2 days and 21 days ACI (A, B). Conversely, marked *enok* mutant GSCs are only present at 2 days ACI (C, E), but absent at 21 days ACI (D, F) and differentiate into marked mutant cysts (arrowheads in D and F). (G, H) GSC clones homozygous for $enok^1$ (broken circles) do not display apoptotic signals as indicated by cleaved-Caspase 3 staining (G) and TUNEL assay (H). (I) Graph shows the relative percentage of germaria containing marked wild type control or *enok* mutant GSCs over a 3-week period ACI. The initial percentages at day 2 ACI are normalized to 100%. Note that *FRTG13* and *FRT42D* were used as the wild type controls for $enok^1$, $enok^2$ and $enok^{K1293}$, respectively. (J) GSC number counting-based graph indicates that expressing *enok* RNAi in the germline causes a gradual GSC loss.

respectively) (Fig. 1G and H). Combined with the evidence that lost *enok* mutant GSCs can develop into differentiated germline cysts (Fig. 1D and F), we argue that GSC loss induced by *enok* mutations results from aberrant stem cell differentiation, rather than defective cell survival albeit other forms of non-apoptotic cell death for the mutant GSCs cannot be ruled out. To further exclude the possibility that Enok functions in controlling GSC division, we measured the relative division rate for the mutant GSCs. As showed in Table S3, *enok* mutant GSCs and wild type controls divide at similar rates.

BMP signaling plays an instructive role in controlling GSC self-renewal. To determine if removal of *enok* function from GSCs impairs the response of the stem cells to BMP signals emitted from the niche cells, we analyzed the expression of phosphorylated Mothers against dpp (pMad) and the reporter gene *Daughters against dpp-lacZ* (*Dad-lacZ*), respectively. As indicators of active BMP signaling pathway, high levels of pMad or *Dad-lacZ* expression are restricted to the two to three GSCs in wild type germaria (Fig. 2A and C). In our experiments, expression of pMad in marked $enok^{1}$ mutant GSCs was as high as that in the neighboring control ones of the same germarium (92.3%, $n=78$) (Fig. 2B). By contrast, *Dad-lacZ* expression in the majority of the mutant GSCs was significantly reduced, compared with that in the unmarked control GSCs ($enok^{1}$: 56.0%, $n=134$; $enok^{2}$: 70.3%, $n=74$; $enok^{K1293}$: 58.8%, $n=68$) (Fig. 2D). The discordant data on pMad and *Dad-lacZ* expression imply that *enok* mutant GSCs are capable of responding to the BMP signals at least at the level of Mad activation, but fail to activate *Dad* transcription. Based on these preliminary results, we further tested *enok* for genetic interactions with BMP signaling pathway in GSC maintenance. As depicted in Fig. S2, heterozygosity for Mad^{12} allele did not exacerbate GSC loss induced by *enok* knockdown in the germline. All these data suggest that the cell-autonomous role of Enok in GSC maintenance is independent of BMP signal-mediated Mad activation.

It is known that BMP signaling promotes GSC self-renewal through repressing the expression of *bam*, a differentiation-promoting gene in GSCs. We, therefore, further characterized the intrinsic role of Enok in GSC maintenance with regard to BMP signaling by examining *bam* expression in the mutant GSCs. Consistent with pMad staining, *bam* silencing was still present in the mutant GSCs, as indicated by the expression pattern of both *bamP-GFP* and endogenous Bam ($n=66$ and 51, respectively) (Fig. 2E and F). Thus, these results provide more evidence suggestive of a BMP/Bam pathway-independent role of Enok in maintaining GSCs intrinsically.

Ectopic expression of Bruno contributes to the GSC loss elicited by enok mutations in the germline

To further explore the mechanisms underlying the cell-autonomous role of Enok in GSC maintenance, we examined if *enok* mutations disrupt the expression pattern of several key genes such as *nos*, *sxl*, *bruno* (also known as *arrest*), *A2BP1 and orb* which are involved in GSC-derived germ cell differentiation and cyst formation. The studies showed that the expression patterns of all examined genes except for *bruno* remain unchanged in *enok* mutant GSCs (Fig. S3). In the wild type germaria, Bruno expression is present at high levels in late germline cysts starting from 16-cell cysts, and barely detectable in GSCs, CBs and early cysts (Fig. 3A and E). Strikingly, high levels of Bruno were evident in the cytoplasm of the marked *enok* mutant GSCs and CBs ($enok^{1}$: 100%, $n=59$; $enok^{2}$: 100%, $n=121$; $enok^{K1293}$: 100%, $n=63$) (Fig. 3B-D), indicating an ectopic expression of *bruno* caused by loss of *enok*. Likewise, we found that RNAi-based down-regulation of *enok* expression in the germline leads to an up-regulation of Bruno in the GSCs and their differentiating progenies (Fig. 3F), reminiscent of the *enok* loss-of-function phenotype. To functionally characterize the misexpression of *bruno* in GSCs and early germ cells, we next analyzed how GSCs are maintained in the germaria expressing *UASp-bruno* under the control of *nos-Gal4* driver. As shown in Fig. 3G, misexpression of *bruno* in GSCs and their immediate descendants induced a stepwise decline in GSC numbers per germarium within the 2-week time course after fly eclosion. Moreover, heterozygosity for $enok^{K1293}$ exacerbated the GSC loss elicited by *bruno* overexpression (Fig. 3H). These molecular and genetic studies suggest that ectopic expression of Bruno in the GSCs links *enok* mutations to the cell-autonomous defects in GSC maintenance.

Based on a number of observations presented above, we proposed that Enok intrinsically maintains GSCs presumably by controlling the stem cell differentiation. To obtain more evidence, we then investigated whether ectopic Bruno expression in the germline could disturb the differentiation pattern of the primordial germ cells (PGCs), the GSC precursors, which keep proliferating in the developing larval gonads

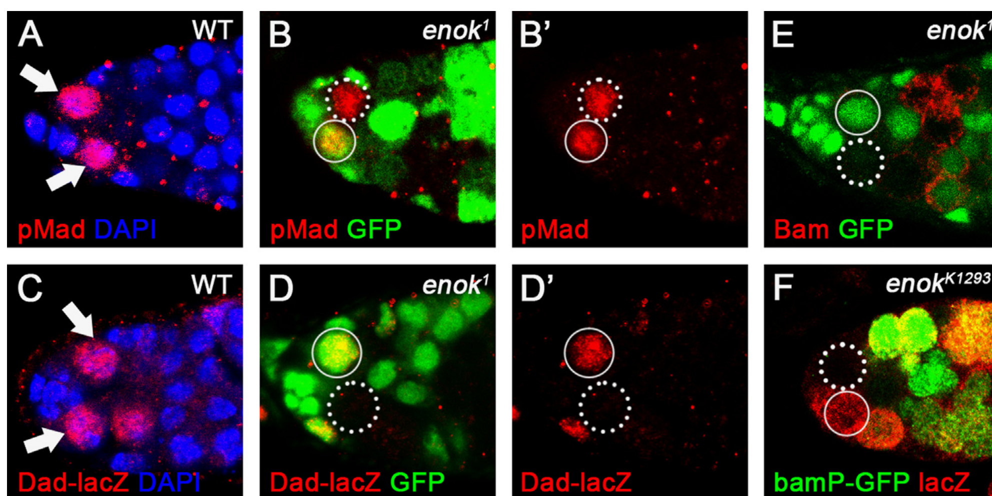

Fig. 2. Enok maintains GSC intrinsically in a BMP/Bam independent manner. Marked GSC clones are GFP (B, D, and E) or *arm-lacZ* (F) negative and indicated by broken circles. The neighboring unmarked ones are indicated by solid circles. (A, B') In the wild type, high level pMad is present in GSCs (arrows in A). Clearly, the marked mutant GSC has an indistinguishable pMad signal compared with the neighboring unmarked GSC (B, B'). (C, D') Staining intensity for *Dad-lacZ* is significantly diminished in $enok^{1}$ GSC clone (D, D'). Note that *Dad-lacZ* is highly expressed in the wild type GSCs (arrows in C). (E, F) Repression of Bam expression is still present in *enok* mutant GSC clones, as evidenced by staining for Bam protein (E) and *bamP*-GFP (F).

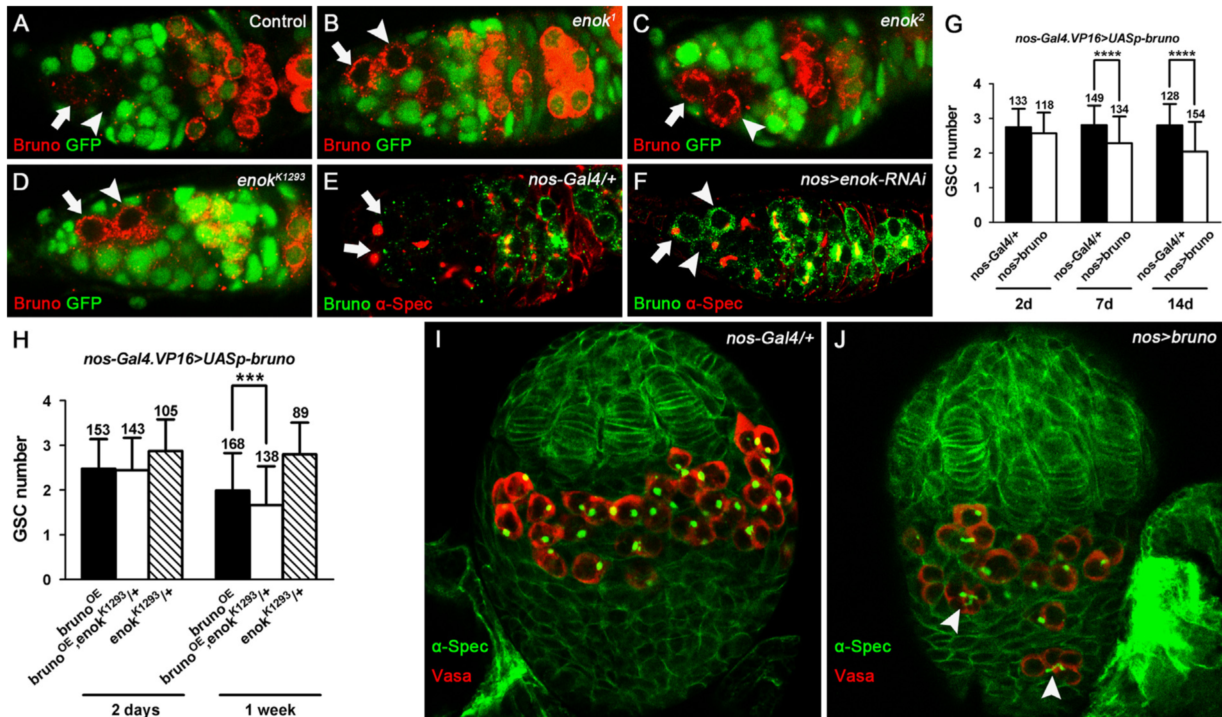

Fig. 3. *enok* mutations in the germline cause ectopic expression of Bruno, contributing to defective GSC maintenance. Germline mutant clones are GFP negative. GSCs and CBs are indicated by arrows or arrowheads, respectively. (A) Bruno is endogenously expressed at high levels from late germline cysts within wild type germaria. (B–D) Ectopic expression of Bruno is evident in mutant GSC and CB clones homozygous for $enok^{1}$ (B), $enok^{2}$ (C) or $enok^{K1293}$ (D). (E, F) Germline expression of *enok* RNAi leads to up-regulated Bruno expression in GSCs and CBs (F), compared with those in *nos-Gal4*/+ control (E). (G) Graph shows the GSC number change in the control or germline-specific *bruno* overexpression germaria over a 14-day time course after eclosion. Overexpression of *bruno* causes a gradual GSC loss. The number of analyzed germaria is shown above each bar. $^{****}p<0.0001$. (H) GSC number counting-based graph shows that $enok^{K1293}$ heterozygosity significantly enhances the GSC number reduction in *bruno* overexpression germaria 1 week after eclosion. Note that females with one copy of $enok^{K1293}$ alone have no defect in GSC maintenance. In this experiment, flies were maintained at 18 °C during the larval and pupal stages and shifted to 29 °C after eclosion to exclude early development defects. The number of analyzed germaria is shown above each bar. OE: overexpression. $^{***}p<0.001$. (I, J) Germline-specific *bruno* overexpression (J) induces precocious PGC differentiation at late third instar larval stage evidenced by branched fusomes (arrowheads in J). Note that there are only spherical spectrosomes in control PGCs (I).

without further differentiation until onset of the pupal development (Gilboa and Lehmann, 2004; Wang and Lin, 2004; Zhu and Xie, 2003). For that purpose, we stained the gonads with germline-specific *bruno* overexpression using the anti-α-Spec antibody. In the wild type, all PGCs carried a single spherical fusome (spectrosome) or two dividing spherical fusomes at late third instar (Fig. 3I). However, we observed that all the *bruno* overexpression gonads (100%, $n=21$) contained PGCs with branched fusomes indicative of a precocious cell differentiation (Fig. 3J). Taking this observation together with molecular and genetic data described above, we propose that Enok deficiency-induced ectopic expression of Bruno contributes to the GSC loss probably through promoting cell differentiation.

enok Knockdown in the niche impairs BMP signaling and GSC maintenance

GSC maintenance involves both intrinsic and extrinsic mechanisms. To discern the possibility that Enok also has a non-cell autonomous role in controlling GSC self-renewal, we tested if *enok* knockdown in the niche cells causes a defect in GSC maintenance. To this end, three independent *enok* RNAi transgenic lines (V37526, V37527 and B29518) were combined with the *bab1-Gal4* driver respectively for an RNAi-based knockdown approach. Consistently, niche-specific expression of each RNAi transgene resulted in a significant decrease of GSC numbers per germarium during a 2-week period after fly eclosion (Fig. 4A–C and Table S4). Since *bab1-Gal4* driver is expressed in most of the somatic precursor cells of the developing gonads at larval stage, as well as in CpCs, TFCs and escort cells of adult ovaries (Bolivar et al., 2006), we do not preclude the possibility that the GSC loss observed in *bab1-Gal4*-mediated *enok* knockdown ovaries could be a secondary consequence resulting from altered cell specification and differentiation involving CpC fate induction during ovary morphogenesis. To determine if Enok in the niche has a role in maintaining GSCs non-cell autonomously, we then combined $tubP\text{-}Gal80^{ts}$ with the RNAi transgene and *bab1-Gal4*, and performed a temperature shift assay. In the experiments, the females were raised at 18 °C until eclosion and then shifted to 29 °C for a number of days. The subsequent cell number counting revealed that knocking down *enok* in the niche only at adulthood causes a similar gradual GSC loss with that elicited by reduced expression of Enok in the niche throughout development (Fig. 6F and data not shown). These results demonstrate that Enok in the niche plays a non-cell autonomous role in maintaining GSCs independent of its possible involvement into the cell fate determination in development. Further studies showed that the expression pattern of both Lamin C and Engrailed, the cell fate specific markers for CpCs remains normal in the germaria expressing *enok* RNAi under the control of *bab1-Gal4* (Fig. 5B and data not shown). Thus, these data not only indicate that *enok* knockdown does not switch the niche cell fate in ovary morphogenesis, but also support the notion that Enok in the niche functions in GSC maintenance.

The niche keeps GSCs in an undifferentiated and self-renewing state by producing BMP signals. To analyze if *enok* knockdown in the niche perturbs the niche signaling output that is essential for controlling GSC self-renewal, we next examined the expression levels of pMad in GSCs within the germaria expressing *enok* RNAi under the control of *bab1-Gal4*. Reduced levels of pMad expression were present in a significantly higher percentage of the mutant

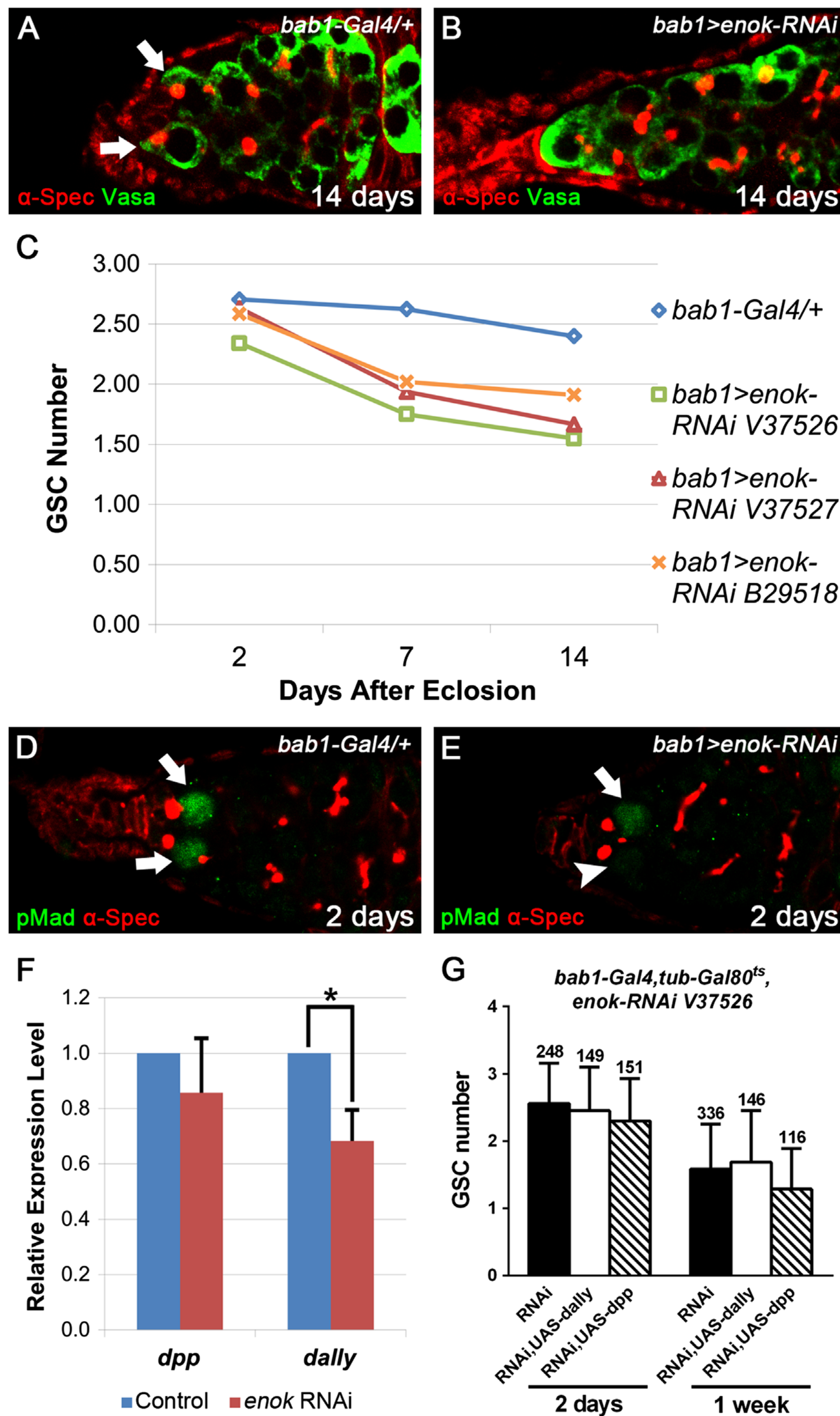

Fig. 4. GSC maintenance and BMP signaling are compromised when reducing *enok* expression in the niche. (A, B) No GSC is detected in a 14-day-old germarium with *enok* knockdown in the niche (B), while the control germarium contains two GSCs (arrow in A). (C) Graph shows the GSC number change in *enok* knockdown with three independent RNAi transgenes or control germaria over a 2-week time course after eclosion. Niche-specific expression of *enok* RNAi causes a gradual GSC loss. (D) In a 2-day-old *bab1-Gal4*/+ control germarium, high level pMad is evident in two GSCs (arrow). (E) One GSC (arrowhead) has a significant reduction of pMad signal compared with the other (arrow) in a germarium with *enok* knockdown in the niche 2 days after eclosion. (F) Quantitative RT-PCR data shows that RNAi-based knockdown of *enok* in the niche causes a significant reduction in the expression of *dally*, but not *dpp* in ovary. $^{*}p<0.05$. (G) Overexpressing *dally* or *dpp* by *bab1-Gal4* cannot impede the GSC decline induced by *enok* knockdown in the niche. The number of analyzed germaria is shown above each bar.

GSCs than that of the control ones (23.2% *vs.* 4.0%, $n=92$ for both) (Fig. 4D and E), indicating that *enok* mutations in the niche compromise the niche-derived BMP signaling activities. Given that the compromised BMP signaling output in the niche could result from a defect in *dpp* expression and/or Dpp morphogen diffusion, we further distinguished these possibilities by quantifying the expression of *dpp* and *dally*, a glypican-encoding gene that facilitates BMP ligands diffusion, in the niche-specific *enok* knockdown ovaries. As depicted in Fig. 4F, *dally*, but not *dpp* mRNA levels were significantly decreased in the mutant ovaries, suggesting that

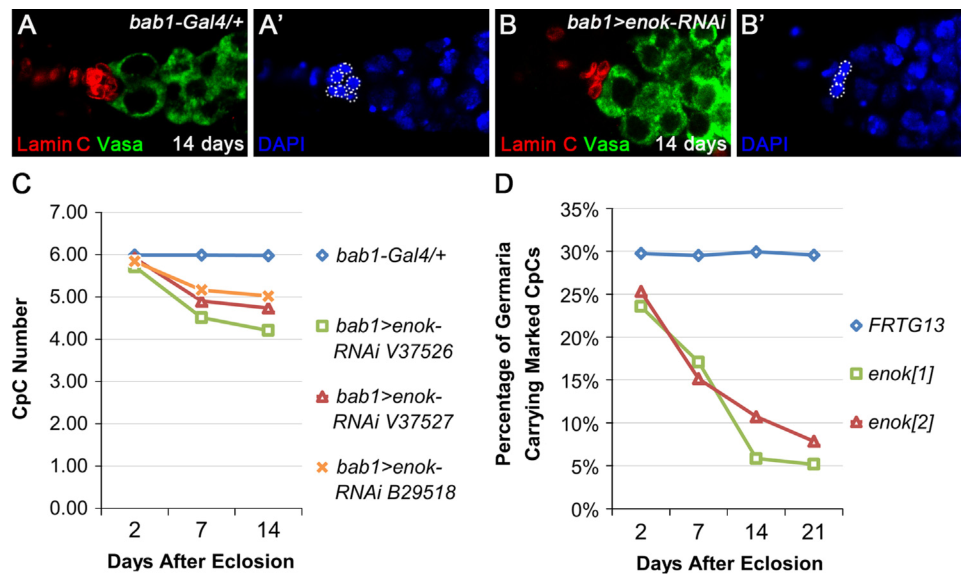

Fig. 5. *enok* is cell autonomously required for CpC maintenance. (A, B′) A 14-day-old *bab1-Gal4/+* control germarium contains six CpCs (A, A′), while only three CpCs are detected in the germarium with *enok* knockdown in the niche (B, B′). CpCs are Lamin C staining positive and indicated by broken circles in A′ and B′. (C) Graph shows the CpC number change in the control or *enok* knockdown germaria over a 14-day time course after eclosion. Niche-specific expression of *enok* RNAi causes a gradual and steady decline in the number of CpCs. (D) FRT clone counting-based Graph shows the percentage of germaria containing either marked wild type control or *enok* mutant CpCs at different time points over a 3-week period after eclosion. Compared with that in the wild type control, the frequency of mutant CpC clones dramatically decreases in the time course.

reduced *dally* expression accounts for the BMP signaling defects, presumably contributing to the GSC loss phenotype. To clarify if impaired niche signaling underlies defective GSC maintenance, we targeted *UAS-dally* or *UAS-dpp* for expression in *enok* knockdown niche cells, and then checked how the GSCs are maintained. Clearly, overexpression of *dally* or *dpp* alone cannot attenuate the GSC decline induced by *enok* knockdown in the niche (Fig. 4G). These results led us to propose that niche signaling restoration may not be sufficient for rescuing the GSC loss phenotype.

Enok is essential for maintaining the niche size via Notch signaling

As described above, increasing BMP signaling output alone by overexpressing *dally* or *dpp* failed to restore the defective GSC maintenance induced by *enok* knockdown in the niche. This observation led us to hypothesize that the non-cell autonomous role of Enok in GSC maintenance may involve more mechanisms besides the niche signaling output control. Given the findings that the niche size control is directly correlated with GSC maintenance in the adult ovary (Hsu and Drummond-Barbosa, 2009, 2011; Song et al., 2007), we asked if Enok is required for maintaining the niche and, indirectly, GSCs. To test this possibility, we first knocked down *enok* in the niche cells, and counted CpC numbers at different time points within a 2-week period after fly eclosion. Remarkably, each of the three independent RNAi lines (V37526, V37527 and B29518) gave a similar result showing defective CpC maintenance (Fig. 5A–C and Table S5). To validate those RNAi transgenic lines for the on-target effects, we further co-expressed wild type *enok* with RNAi transgene V37526 under the control of *bab1-Gal4*. The rescue experiment showed that the CpC loss phenotype is elicited by RNAi-based targeting for *enok* in the niche cells (Fig. S1). We next determined if Enok intrinsically controls CpC maintenance by generating CpC clones homozygous for mutant *enok* alleles in the mosaic germaria and tracing them within 21 days after eclosion. As revealed in Fig. 5D, the rate of *bab1*-Flpase-induced *enok* mutant CpC clones dropped dramatically over the time course, while the percentage of the control ones remained steady (Table S6). Hence, the clonal analysis not only provides more evidence indicative of a role of Enok in maintaining CpCs, but also indicates that Enok acts in this process cell autonomously.

A number of studies have shown that Notch signaling controls the formation and maintenance of the GSC niche during development, and insulin signals are required for CpCs maintenance via modulating Notch signaling (Hsu and Drummond-Barbosa, 2009, 2011; Song et al., 2007; Ward et al., 2006). We, therefore, tested whether Enok is essential for activation of Notch signaling pathway in the niche. For this experiment, the *E(spl)m7-lacZ* (*m7-lacZ*) reporter was used to monitor Notch signaling activities. As shown in Fig. 6A, marked expression of the *m7-lacZ* was evident in both GFP-positive and negative CpCs of the mosaic germaria harboring CpC clones from the control FRT chromosome. By contrast, *m7-lacZ* expression was dramatically reduced in marked *enok* mutant CpCs ($enok^1$: 97.0%, $n=67$; $enok^2$: 97.3%, $n=110$; $enok^{K1293}$: 96.6%, $n=116$) (Fig. 6B), indicating that CpCs lacking *enok* function fail to activate Notch signaling. To rule out the possibility that Enok is also involved in the Notch signal production, we analyzed the expression of two Notch ligands, Delta (Dl) and Serrate (Ser) in the TFCs. Clearly, expression of both *Dl-lacZ* and *Ser-lacZ* was present in the mutant TFCs homozygous for $enok^1$ (100%, $n=56$ and 53, respectively), as well as in the neighboring wild type ones in the mosaic germaria (Fig. 6C and D), further showing that Enok in the niche is required intrinsically for the response of CpCs to Notch pathway signals.

We next sought to functionally link impaired Notch signaling to defective CpC maintenance induced by niche-specific *enok* knockdown. For this purpose, we first studied genetic interactions of *enok* with Notch signaling in the niche size control. In our experiments (Fig. S4), females heterozygous for $Notch^{264-39}$ had a reduction in CpC numbers at 7 days after eclosion. This reduction was significantly enhanced by introducing one copy of *enok* mutant allele into $Notch^{264-39}$ heterozygotes ($N^{264-39}/+$; $enok^1/+$). Since *enok* heterozygotes had a normal CpC number, the studies above suggest that *enok* genetically interacts with *Notch* in controlling CpC number. To test if activation of Notch signaling could rescue the CpC loss phenotype, we then co-expressed *enok* RNAi transgene with *NICD*, a constitutively active form of Notch, specifically in the niche at adulthood by using both *babl-Gal4* and $tubP\text{-}Gal80^{ts}$, and determined how the CpC maintenance is controlled. As predicted, the CpC loss can be fully restored by forced expression of an activated Notch in the *enok* mutants (Fig. 6E). Collectively, these molecular

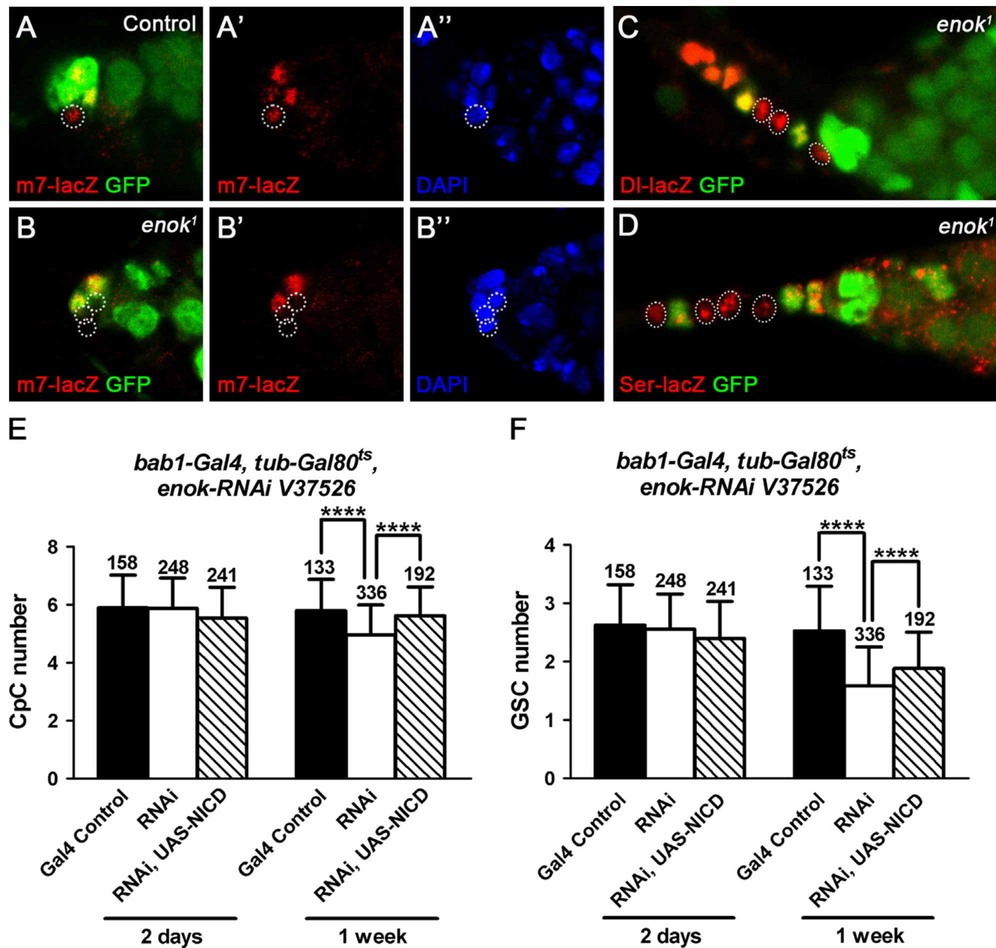

Fig. 6. Enok-controlled CpC maintenance is dependent on Notch signaling. Marked CpC or TFC clones are GFP negative and indicated by broken circles. (A, B′) In the wild type control, Notch signaling reporter *m7-lacZ* is normally expressed in both marked and unmarked CpC cells (A, A′). Conversely, loss of *m7-lacZ* expression is evident in marked *enok* mutant CpCs (B, B′). (C, D) The expression level of Notch ligands *Dl* (C) and *Ser* (D) is unchanged in TFC clones mutant for *enok*. (E) Quantification of the CpCs in germaria with *bab1-Gal4* only, *bab1-Gal4* driven expression of *enok* RNAi and co-expression of *UAS-NICD* with *enok* RNAi under the control of $Gal80^{ts}$ at two different time points over an 1-week period after eclosion. Significantly, *enok* knockdown-induced decrease in CpC number is rescued by expressing a constitutively active form of Notch. (F) Quantification of GSCs in the same germaria described in (E). Co-expression of NICD with *enok* RNAi significantly attenuate the GSC number decline caused by *enok* knockdown in the niche. Note that the number of germaria analyzed is shown above each bar (E, F). ****$p < 0.0001$.

and genetic studies demonstrate that Enok is required for maintaining CpC numbers via Notch signaling.

Given that *enok* knockdown in the niche impairs the maintenance of CpCs and GSCs simultaneously, we reasoned that the GSC loss in niche-specific *enok* mutants is attributable to defective CpC maintenance. To test this assumption, we further examined how the GSCs are maintained in the germaria co-expressing *enok* RNAi with *NICD*. As described above, forced activation of Notch signaling can sufficiently restore the CpC decline elicited by reduced expression of *enok* in the niche (Fig. 6E). Further, this restoration resulted in a significant suppression of the GSC loss, albeit reduced GSC number was still there (Fig. 6F). Altogether, these studies demonstrate that Enok in the niche functions in maintaining CpCs via Notch signaling, thereby contributing to GSC maintenance.

Loss of enok function does not perturb the accumulation of DE-cad complex at the GSC–niche junction

Previous studies have identified DE-Cad complex-mediated adhesion between CpCs and GSCs as a key process for anchoring GSCs in the niche for their self-renewing capacities (Song et al., 2002). We sought to determine if Enok is also required for this process. To this end, we generated the GSC or CpC clones mutant for *enok* using FLP/FRT technique and analyzed the expression levels of both DE-Cad and Armadillo (Arm, *Drosophila* β-catenin) at the GSC–niche junction. In mosaic germaria bearing either *enok* mutant GSCs or CpCs, the accumulation of DE-Cad complex at the GSC–CpC junction was similar between the marked mutant cells and unmarked control ones (GSC clones: 100%, $n=80$ for DE-Cad and 100%, $n=60$ for Arm; CpC clones: 93.7%, $n=63$ for DE-Cad and 91.5%, $n=71$ for Arm) (Fig. 7). Thus, we exclude the possibility that Enok controls GSC self-renewal through regulating DE-Cad or Arm accumulation at the junction.

Discussion

As a *Drosophila* putative histone acetyltransferase of the MYST family, Enok has been shown to be essential for neuroblast proliferation in the mushroom body (Scott et al., 2001). In this paper, we present evidence that Enok is required intrinsically and extrinsically for maintaining GSCs in the ovary. In the case of intrinsic mechanisms, we identified Bruno as an intermediate factor for Enok-controlled GSC maintenance. Molecular and genetic studies revealed that *enok* mutations in the germline lead to ectopic expression of Bruno in the GSCs, thereby inducing GSC loss probably via promoting cell differentiation. Meanwhile, we show that Enok also has a non-cell autonomous role in controlling GSC self-renewal through

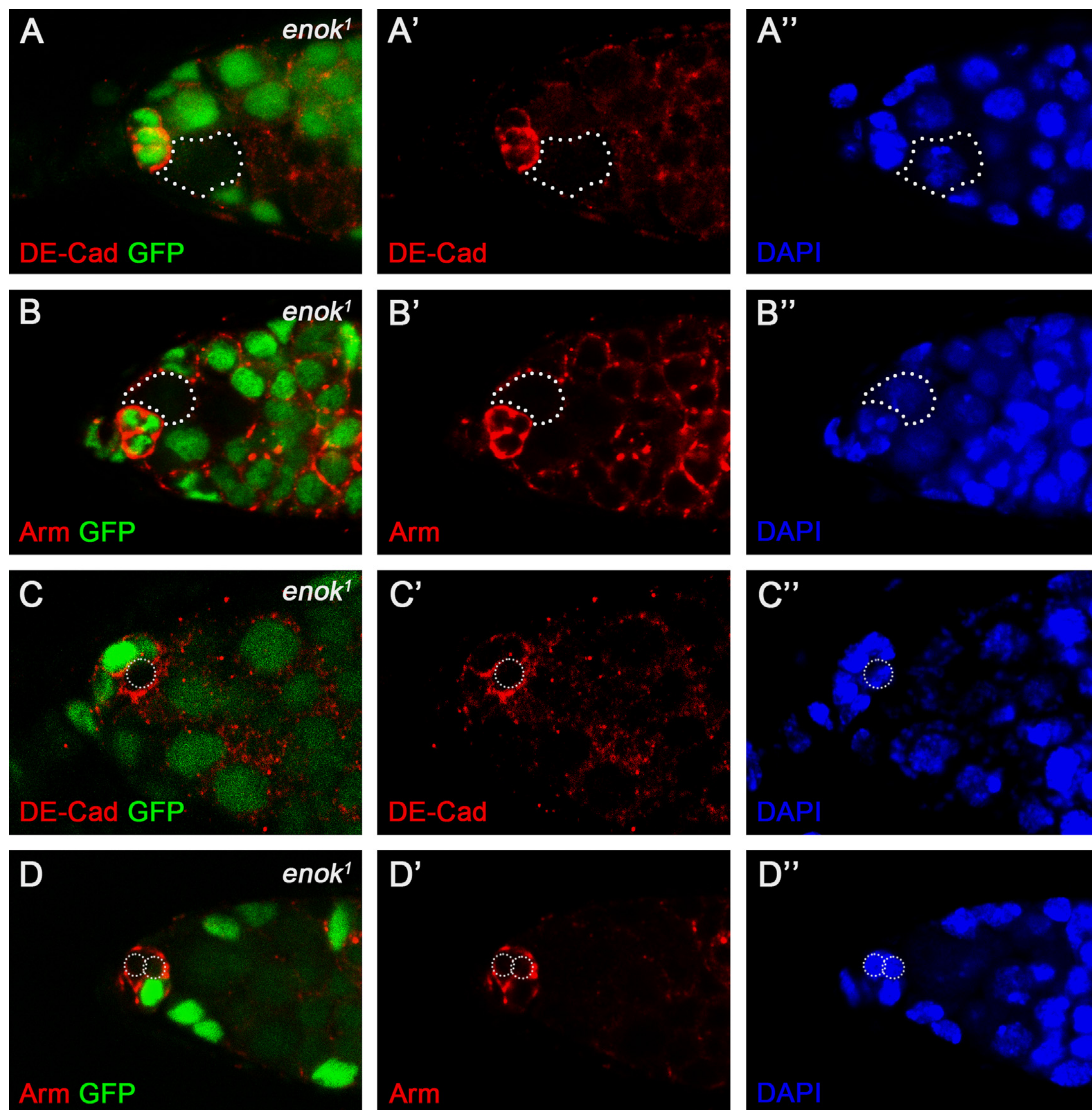

Fig. 7. *enok* is not essential for the accumulation of DE-Cad complex at the interface between GSC and CpC. GSC or CpC clones are GFP negative and indicated by broken lines. DE-Cad (A, A′, C, C′) and Arm (B, B′, D, D′) accumulate properly at the junction between the wild type CpC and *enok* mutant GSC (A, B) or between the wild type GSC and *enok* mutant CpC (C, D).

regulating the niche maintenance and niche-derived BMP signaling output. Thus, this study unraveled a novel regulatory mechanism governing the GSC maintenance mediated by a putative epigenetic regulator in *Drosophila* (Fig. 8). Since Moz and Qkf, the mammalian homologs of Enok, are involved in controlling self-renewal of adult stem cells such as hematopoietic and neural stem cells (Katsumoto et al., 2006; Merson et al., 2006; Thomas et al., 2006), the new findings in this paper will help to address how the adult stem cell fate regulation occurs in higher organisms.

A role for Bruno in Enok-mediated control of GSC self-renewal

Numerous studies have shown that GSC maintenance in the *Drosophila* ovary depends on at least three intrinsic machineries: the BMP/Bam pathway, the Nos/Pum complex and the miRNA pathway (Bhat, 1999; Chen and McKearin, 2003; Forbes and Lehmann, 1998; Gilboa and Lehmann, 2004; Jin and Xie, 2007; Lin and Spradling, 1997; Park et al., 2007; Song et al., 2004; Wang and Lin, 2004; Xie and Spradling, 1998, 2000; Yang et al., 2007). In the present study, we observed that Enok in the germline controls GSC self-renewal independently of BMP/Bam pathway. In the meantime, we found that loss of *enok* function does not intrinsically alter the expression pattern of either Nos or Pum in the GSCs, and that *enok* displays no genetic interactions with either *nos* or *pum* in GSCs maintenance (Fig. S3 and data not shown). Hence, the results exclude the possibility that the Nos/Pum complex is implicated in Enok-controlled GSC maintenance. Intriguingly, the molecular studies identified Bruno as a potential target of Enok involved in the GSC maintenance. Further genetic analyses suggest that increased expression of Bruno in the GSCs mutant for *enok* contributes to the GSC loss. *bruno* encodes an RNA-Recognition-Motifs-containing RNA binding protein which targets a number of mRNAs for their translational repression in the ovary and early embryo (Filardo and Ephrussi, 2003; Kim-Ha et al., 1995; Moore et al., 2009; Sugimura and Lilly, 2006; Wang and Lin, 2007; Webster et al., 1997). Early on, Bruno was shown to function in patterning the embryo along the AP and DV axis by regulating the translation of *oskar* and *gurken* mRNA during late oogenesis (Filardo and Ephrussi, 2003; Kim-Ha et al., 1995). Later, it was reported that Bruno plays a pivotal role in CB differentiation and germline cyst formation at early oogenesis via targeting the *Sex-lethal* (*Sxl*) gene (Wang and Lin, 2007). Here, we defined a novel function for Bruno in mediating the intrinsic requirements of Enok for maintaining GSCs.

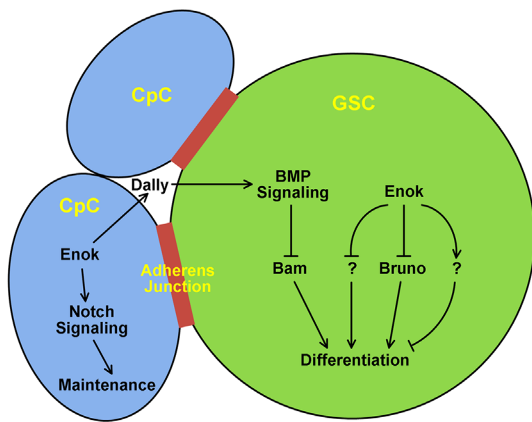

Fig. 8. A working model for Enok's roles in regulating GSC fate and the niche. For controlling GSC self-renewal, Enok in the CpCs maintains the niche size via Notch signaling and modulates the niche-derived BMP signaling through regulating *dally* expression. In parallel, Enok is required intrinsically for sustaining the GSC population presumably by controlling the stem cell differentiation. In this context, Bruno has been identified as an intermediate factor mediating Enok-controlled GSC maintenance. In addition, we hypothesize that Enok functions in regulating GSC differentiation through other unknown pathways.

We showed that misexpression of Bruno in the germline caused a derepression of PGC differentiation in the gonads from the late third instar larvae. This precocious differentiation phenotype further suggests that *bruno* gain-of-function in the *enok* mutants promotes GSC differentiation, thereby eliciting a stem cell loss.

To better understand how *enok* mutation-induced ectopic expression of Bruno promotes the GSC differentiation, we need to identify the potential mRNA target(s) of this RNA-binding protein in the GSCs and their immediate descendants that may function as the differentiation-inhibiting factor in this context. Of all known target genes of Bruno, only *Sxl* is dynamically expressed in early germ cells including GSCs and CBs, and essential for the GSC/CB fate switch (Chau et al., 2012). Preliminary data in our laboratory show that the expression pattern of *Sxl* remains unchanged in the mutant GSC or CB clones homozygous for the *enok* allele, ruling out a possible role of *Sxl* in Enok/Bruno-mediated differentiation control process (Fig. S3). Given that the Bruno Response Element (BRE) consensus sequences located in the 3′-UTR of the target mRNAs is important for Bruno binding, we will be searching for and characterizing the target candidates from the ovarian mRNAs that contain putative BRE sequences, based on bioinformatics approaches. However, it is noteworthy that Bruno can also regulate the expression of its target mRNA in a BRE-independent manner (Moore et al., 2009; Webster et al., 1997). Thus, high-throughput screens such as microarray analysis for differentially expressed genes in the *enok* mutant ovaries may give more clues for unraveling the mystery.

It has been described that Moz can acetylate histones H3 and H4 at a number of specific lysine residues both *in vitro* and *in vivo* (Doyon et al., 2006; Fraga et al., 2005; Kitabayashi et al., 2001; Voss et al., 2009). In particular, this MYST family histone acetyltransferase is found to be required for H3K9 acetylation at Hox gene clusters, thus for correct body segment patterning in mice (Voss et al., 2009). As the *Drosophila* homolog of Moz, Enok possesses a conserved MYST histone acetyltransferase (HAT) domain, as well as two PHD fingers and a shared N-terminal domain. Previous studies showed that a point mutation in the MYST HAT domain of Enok causes an arrest in neuroblast proliferation of mushroom body as a null allele (Scott et al., 2001). Combined with the observation in this paper that the same mutation (*enok*^{2}) gives defective GSC maintenance phenotype (Fig. 1F, I), we propose that the HAT activity is implicated in Enok's function during the indicated developmental processes. To further test this scenario, we will determine if the expression of Bruno in the early germ cells could be under the epigenetic control of Enok by examining a possible binding of Enok to *bruno* gene using chromatin immunoprecipitation (ChIP). In this case, high-throughput screens based on a combination of ChIP-seq and microarray analysis may lead us to identify more target genes of Enok that could mediate the GSC fate regulation controlled by this putative epigenetic factor.

Enok as a regulator for the niche maintenance

The GSC niche plays a key role in controlling GSC self-renewal in the ovary. Although the niche regulation itself is less understood, recent studies showed that systemic factors such as insulin signaling control the niche size, and consequently GSC maintenance at adulthood (Hsu and Drummond-Barbosa, 2009, 2011). Specifically, systemic insulin-like signals maintain the CpC population via modulating Notch signaling (Hsu and Drummond-Barbosa, 2009, 2011). In the present study, we provide the first evidence that the niche maintenance also requires a putative epigenetic factor, and that decrease in the CpC number induced by *enok* knockdown in the niche is attributable to impaired Notch signaling. Thus, identification and functional characterization of the targets of Enok in controlling the niche size would provide more insights towards understanding how the niche is maintained. Given that insulin signaling is required for controlling the normal decline of both CpCs and GSCs in the aging process (Hsu and Drummond-Barbosa, 2009), and that epigenetic regulation is important for aging stem cells in mammals (Pollina and Brunet, 2011), we assume that Enok-mediated niche maintenance via Notch signaling has implications in both niche and GSC aging. If this is the case, Enok activity in the niche should display an age-dependent decline. Furthermore, increasing Enok activity could significantly attenuate the age-dependent decrease in the number of both CpCs and GSCs.

In conclusion, we show in this paper that Enok controls GSC maintenance in the *Drosophila* ovary at multiple levels. In the case of a cell-autonomous control of GSC self-renewal, Enok acts in a BMP/Bam-independent manner. Instead, activation of Bruno expression in the GSCs and their differentiating progeny links *enok* mutations in the germline to the GSC loss. In parallel, Enok plays a non-autonomous role in maintaining the GSC population via regulating the niche size and niche-derived BMP signal output. Collectively, our results reveal a novel mechanism underlying a putative epigenetic factor-controlled GSC fate regulation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.10.001.

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