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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.

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), $N^{264\text{-}39}$ (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.