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Engrailed acts with Nejire to control *decapentaplegic* expression in the *Drosophila* ovarian stem cell niche

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ABSTRACT

Homeostasis of adult tissues is maintained by a small number of stem cells, which are sustained by their niches. In the *Drosophila* female germline stem cell (GSC) niche, Decapentaplegic (Dpp) is the primary factor that promotes GSC self-renewal. However, the mechanism regulating *dpp* expression in the niche is largely unknown. Here, we identify a 2.0 kb fragment located in a 5′ *cis*-regulatory region of the *dpp* locus containing enhancer activity that drives its expression in the niche. This region is distinct from a previously characterized 3′ *cis*-regulatory enhancer responsible for *dpp* expression in imaginal discs. Our data demonstrate that Engrailed, a homeodomain-containing transcription factor that serves as a cap cell marker, binds to this region and regulates *dpp* expression in cap cells. Further data suggest that En forms a complex with Nejire (Nej), the *Drosophila* ortholog of histone acetyltransferase CBP/p300, and directs Nej to this *cis*-regulatory region where Nej functions as the co-activator for *dpp* expression. Therefore, our study defines the molecular pathway controlling *dpp* expression in the *Drosophila* ovarian stem cell niche.

KEY WORDS: Germline stem cells, Niche, Decapentaplegic, Engrailed, Nejire, CBP/p300

INTRODUCTION

In adult organisms, stem cells undergo asymmetric divisions to self-renew and produce differentiated cells to maintain tissue homeostasis. The decision between self-renewal and differentiation of stem cells is strongly influenced by niche-derived signals (Hsu and Fuchs, 2012; Morrison and Spradling, 2008; Scadden, 2014).

The *Drosophila* ovary provides an ideal system to study how niche-associated signals regulate germline stem cell (GSC) self-renewal versus differentiation (Fuller and Spradling, 2007; Harris and Ashe, 2011; Lin, 2002; Losick et al., 2011; Xie, 2013). At the anterior tip of the ovary, the GSC niche is formed by several types of stromal cells, including terminal filament (TF) cells, cap cells and escort cells (ECs, previously known as inner germarial sheath cells). Besides providing a physical location to house GSCs, the niche also produces a range of signaling molecules, including Decapentaplegic (Dpp; the fly ortholog of BMP2/4), Hedgehog (Hh), Wnt factors and Unpaired (Upd), which act in concert to control GSC activity (Losick et al., 2011). Among these signaling molecules, Dpp is the primary factor that promotes GSC self-renewal by repressing the transcription of differentiation-promoting factor *bag of marbles* (*bam*) (Chen and McKearin, 2003a; McKearin and Spradling, 1990; Song et al., 2004). *dpp* is transcribed mainly in cap cells and, to a lesser extent, in ECs (Liu et al., 2015; Xie and Spradling, 2000). However, it remains largely unknown how its expression in these niche cells is regulated.

Engrailed (En) is a multifaceted homeodomain-containing transcription factor that plays an essential role in the development of *Drosophila* appendages and segments, and is also involved in the development of the nervous system (Morgan, 2006). En binds directly to specific DNA sequence and can function as a transcriptional activator or a transcriptional repressor depending on its associated co-factors (Alexandre and Vincent, 2003). In the germarium, En is expressed in both TF and cap cells and is used as a cap cell marker (Forbes et al., 1996; Ward et al., 2006). Removing En function from TFs leads to disorganization of TF stacks during ovary development, indicating a role of En in the proper organization of the TFs (Bolívar et al., 2006). Interestingly, one previous publication showed that germaria bearing cap cells mutant for En exhibited a GSC loss phenotype (Rojas-Rios et al., 2012), indicating a role for cap cell-expressed En in maintaining GSCs non-cell-autonomously. Although this report also suggested that En maintains GSCs indirectly by promoting *dpp* expression in ECs via Hh signaling (Rojas-Rios et al., 2012), several recent studies showed that Hh signaling in ECs instead promotes GSC differentiation (Li et al., 2015a; Liu et al., 2015; Lu et al., 2015). It was also reported that ectopic expression of En, but not Hh, in ECs results in an expansion of GSC-like cells with expanded Dpp signaling activation outside the niche (Eliazer et al., 2014), suggesting that En can promote Dpp signaling independently of Hh. Thus, the mechanism underlying En-mediated GSC maintenance remains elusive.

Nejire (Nej) is the *Drosophila* ortholog of vertebrate CREB-binding protein CBP/p300 (Chan and La Thangue, 2001). The CBP/p300 proteins contain multiple protein-protein interaction domains, including three cysteine-histidine (CH)-rich domains, one kinase-inducible domain interacting (KIX) domain, one bromodomain (Br), one SRC1-interacting domain (SID), and one histone acetyltransferase (HAT) domain. They function as versatile transcriptional co-activators with the ability to interact with a myriad of partners, including transcription factors and signaling molecules (Vo and Goodman, 2001).

In this study, we show that En controls *dpp* expression in cap cells by binding to a 5′ enhancer region, which drives heterologous reporter expression in the niche in a manner similar to endogenous *dpp*. Compromising En activity in cap cells leads to downregulation of *dpp* expression in the niche, while ectopic En expression in ECs results in ectopic *dpp* transcription. We further provide evidence showing that En forms complexes with Nej, directs Nej to this enhancer region, and that the En activity required to turn on *dpp* expression in cap cells is dependent on Nej function.

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RESULTS

Identification of a *cis*-regulatory region directing *dpp* expression in niche cells

Previous studies showed that *dpp* transcripts are expressed in niche cells, with high expression levels detected in cap cells and low expression levels in ECs (Liu et al., 2010, 2015; Song et al., 2004; Wang et al., 2008; Xie and Spradling, 2000). To investigate how *dpp* expression is regulated in these niche cells, we first examined the available *dpp* transcriptional reporters, including *BS1.0-lacZ* that recapitulates the *dpp* expression pattern in larval imaginal discs (Blackman et al., 1991). None of these lines, under the control of enhancer elements located in the 3′ *cis*-regulatory region, exhibited reporter activity in the ovarian niche (not shown).

We then focused on the 5′ *cis*-regulatory region and generated nine transgenic reporter lines (D1-D9) covering a span of ∼36 kb 5′ to the *dpp* coding region (Fig. S1A, Table S1). *lacZ* reporter activity was detected using an antibody against β-galactosidase (β-gal). Two lines exhibited reporter activity in niche cells, with *D3-lacZ* showing β-gal expression mainly in TF cells and *D6-lacZ* exhibiting reporter activity in cap cells and one to two adjacent TF cells (Fig. S1B,C). Further dissection of the D6 region identified a 4 kb fragment (D6.3) that drove strong reporter activity in all cap cells and several ECs (Fig. S1A,D-F), indicating that this region contains a cap cell-associated enhancer. Other regions (D6.1 and D6.2) exhibited weak enhancer activity in cap cells. Fine mapping (together with ChIP, see below, Fig. 2C) led to the identification of a 2.0 kb fragment located at the 3′ end of this region that drove strong β-gal expression in virtually all cap cells (Fig. 1A,B); we named this reporter line *dpp2.0-lacZ*. About 15.2% of the germaria examined (n=730) also showed weak β-gal expression in some ECs (Fig. 1C), a pattern reminiscent of endogenous *dpp* expression as detected by FISH (Liu et al., 2015).

In line with the notion that Notch signaling is required for the maintenance of cap cells in adult ovary (Song et al., 2007), β-gal expression was strongly reduced in cap cells with compromised Notch signaling activity (Fig. 1D, Fig. S1G). We further examined whether this reporter also reflects *dpp* transcriptional activation in ectopic cap cells induced by ectopic Notch signaling during niche formation (Song et al., 2007; Ward et al., 2006), and found β-gal expression in these cap cells located outside the normal niche position (Fig. 1E). Furthermore, *dpp2.0-lacZ* activity was upregulated in ECs with compromised Hh signaling (Fig. S1H), consistent with our previous study showing that Hh signaling in ECs dampens *dpp* expression (Liu et al., 2015). Together, these data show that *dpp2.0-lacZ* faithfully recapitulates *dpp* expression in niche cells.

En regulates *dpp2.0-lacZ* reporter activity in cap cells

We next sought to investigate the mechanism regulating *dpp2.0-lacZ* in the niche. Through bioinformatics analysis using PROMO (alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3), we found that this region contains consensus binding sequences for several well-characterized transcription factors (Table S2), including Engrailed (En), a homeodomain-containing factor that is generally used as a cap cell marker (Fig. 2A). To test whether En regulates *dpp2.0-lacZ* activity we used the *bab1-gal4* driver, which is expressed in TF and cap cells, in combination with an RNAi construct against *en*, and found that germaria with compromised En function (referred to as en^i germaria) failed to express this reporter (Fig. 2B).

Since En is a DNA-binding protein, we examined whether En binds directly to the 2.0 kb enhancer region. We ectopically expressed En with an epitope tag in S2 cells, which do not express En, and conducted ChIP experiments followed by real-time PCR. En was found to be highly enriched in this 2.0 kb region, as compared with other genomic regions (Fig. 2C).

To further define the region responsible for the cap cell-associated enhancer activity, we generated a series of small deletions (∼100 bp) across the 2.0 kb region and identified a 300 bp region essential for driving β-gal expression in cap cells (Table S3). Interestingly, four putative En binding sites (Kassis et al., 1989; Sanicola et al., 1995; Serrano and Maschat, 1998) were

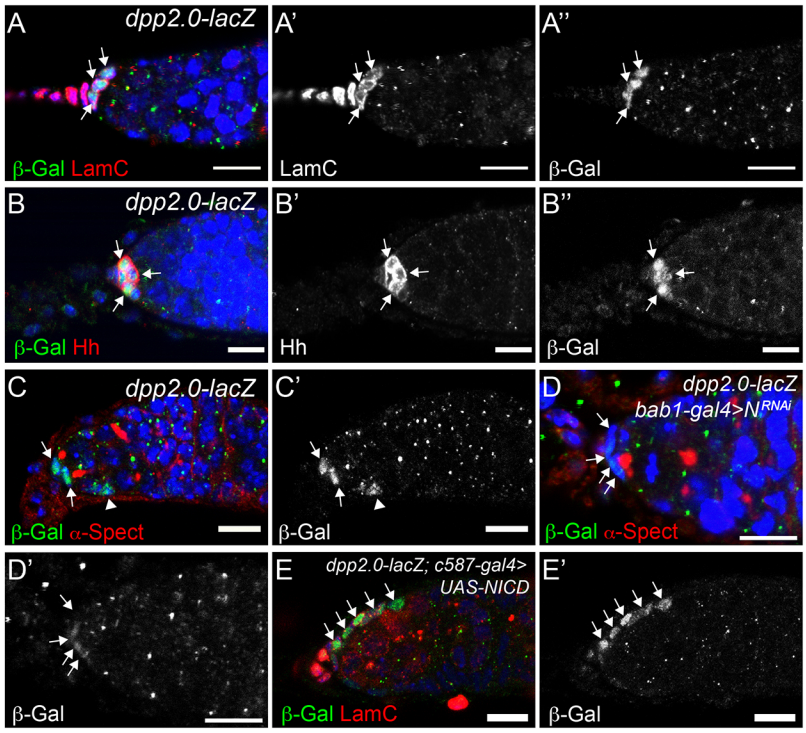

Fig. 1. *dpp2.0-lacZ* reporter expression in the niche. DNA is in blue (TO-PRO-3) and cap cells are indicated by arrows. (A-B″) A *dpp2.0-lacZ* germarium showing β-gal expression in cap cells labeled by LamC (A) or Hh (B). (C,C′) A *dpp2.0-lacZ* germarium showing β-gal expression in cap cells and one EC (arrowhead). (D,D′) A *bab1-gal4>*N^{RNAi} germarium exhibiting weak β-gal expression in cap cells. (E,E′) β-gal is detected in ectopic cap cells induced by Notch intracellular domain (NICD) overexpression. Scale bars: 10 µm.

Development (2017) 144, 3224-3231 doi:10.1242/dev.145474

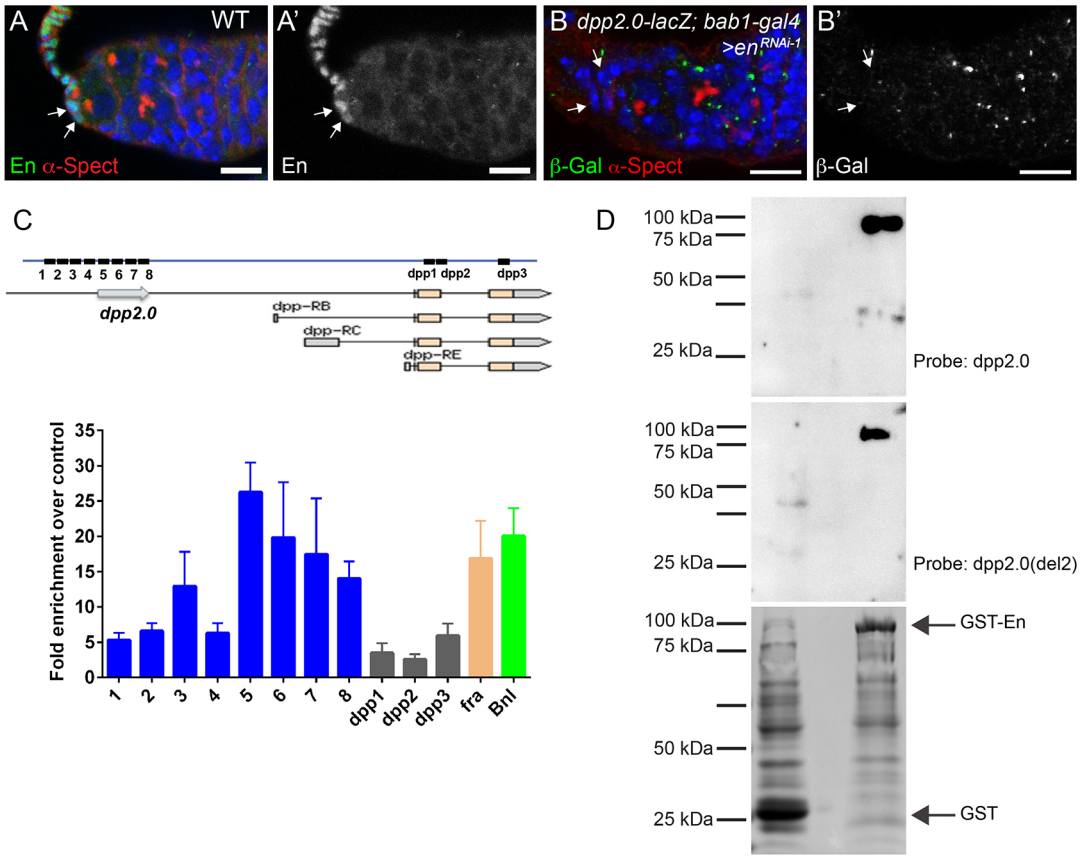

Fig. 2. En binds to the *dpp2.0* fragment. (A,A′) A WT germarium showing En expression in TF and cap cells. (B,B′) A *bab1-gal4>en*^{RNAi} germarium showing loss of *dpp2.0-lacZ* reporter activity. DNA is in blue (TO-PRO-3) and cap cells are indicated by arrows. Scale bars: 10 µm. (C) ChIP results showing fold enrichment of En on *dpp2.0* and flanking regions. Top panel depicts the regions detected; 1-4 are located 5′ to *dpp2.0*, while 5-8 are within *dpp2.0*; dpp1, dpp2 and dpp3 are located in the *dpp* coding region. dpp-RB, dpp-RC and dpp-RE are three annotated *dpp* isoforms (see Fig. S1). Bottom panel shows the relative enrichment of En in these regions as determined by real-time PCR. *fra* and *bnl*, two known targets of En, are used as positive controls. Each experiment was performed in triplicate. (D) Far-western blot showing binding of GST-En to *dpp2.0* and *dpp2.0* (Δ2) regions. Top, probe *dpp2.0*; middle, probe *dpp2.0* (Δ2); bottom, Coomassie Blue-stained gel that provides a loading control. Equal amounts of GST or GST-En were used for these experiments.

identified in this 300 bp region (Fig. S2A). To test the functional importance of these potential En binding sites, we generated transgenic reporter lines bearing single or double deletion of these sites and examined their enhancer activity in the niche. A small deletion that removes two adjacent putative binding sites [*dpp2.0-lacZ* (Δ2)] abolished enhancer activity in cap cells (Fig. S2A-D). We further investigated whether these two putative sites are involved in En binding and conducted far-western blot analysis. Whereas bacterially expressed GST-tagged En bound strongly to the 2.0 kb fragment, its binding to the *dpp2.0-lacZ* (Δ2) derivative was clearly compromised (Fig. 2D). *In vivo*, whereas *dpp2.0-lacZ* reporter activity was ectopically activated in ECs upon En overexpression, *dpp2.0-lacZ* (Δ2) did not respond to ectopic En expression (see below, Fig. 4B, Fig. S3K). Taken together, these data suggest that En acts through these sites to regulate enhancer activity in cap cells.

En maintains GSCs non-cell-autonomously

In addition to loss of β-gal reporter expression, *en*^{i} germaria also exhibited a GSC loss phenotype, which was observed using two independent RNAi constructs (Fig. S3A-C). In wild-type (WT) germarium, each niche houses two or three GSCs that are in contact with cap cells and undergo asymmetric divisions to produce a self-renewing GSC daughter that remains within the niche and a differentiating cystoblast (CB) daughter that moves away from the niche. GSCs possess an anteriorly positioned spectrosome, which is a spherical intracellular structure enriched in cytoskeletal proteins such as α-Spectrin, whereas CBs contain a randomly positioned spectrosome and differentiating cysts possess a branched fusome interconnecting individual cystocytes. Each control germarium contained 2.3±0.05 (n=124) GSCs, whereas each *en*^{i} germarium harbored only 0.9±0.08 (n=126) GSCs (P<0.001). To exclude potential off-target effects of RNAi, we generated cap cells mutant for En using a null allele [*en*^{E} (Gustavson et al., 1996)]. As reported previously (Rojas-Rios et al., 2012), these germaria exhibited a GSC loss phenotype (Fig. S3D,E), demonstrating a role of En in maintaining GSCs non-cell-autonomously.

Since En is expressed in TF and cap cells during their formation and plays a role in TF organization (Bolívar et al., 2006), disrupting En expression could potentially affect niche organization or function. We performed temperature-shift experiments for *en* knockdown to bypass its requirement during development and to check its function at the adult stage. These *en*^{i} germaria gradually exhibited a GSC loss phenotype (Fig. S3F), although their niches contained a similar number of cap cells as control counterparts (Fig. S3G), excluding the possibility that GSC loss is a result of defective niche formation.

En controls *dpp* expression in cap cells

We then investigated how En maintains GSCs in the niche. Our data indicate that the GSC loss observed in *en*^{i} germaria is not a result of germ cell death (Fig. S3H,I) but a consequence of precocious

Development (2017) 144, 3224-3231 doi:10.1242/dev.145474

differentiation, as those spectrosome-containing cells within the niche expressed P*bam-gfp*, a transcriptional reporter of *bam* (Chen and McKearin, 2003b). In control germaria, P*bam-gfp* was activated in CBs and germline cysts outside the niche (Fig. 3A). However, its activity was detected in the spectrosome-containing cells in the niche of *en*^{i} germaria (Fig. 3B).

Given earlier findings showing that Dpp signaling activation in GSCs directly represses the transcription of *bam* (Chen and McKearin, 2003a; Song et al., 2004), we examined phosphorylated Mad (pMad), a real-time reporter for Dpp signaling activation. Compared with the high levels present in control GSCs, pMad levels were significantly reduced in GSCs of *en*^{i} germaria (Fig. 3C,D), indicating defective signaling activation. Considering our results above showing that En binds the *cis*-regulatory region of *dpp* and regulates *dpp2.0-lacZ* reporter activity in cap cells, we hypothesized that En might directly regulate *dpp* expression in cap cells and therefore performed FISH experiments to detect *dpp* transcripts. As reported previously, *dpp* transcripts were mainly detected in cap cells of control germaria; however, *dpp* expression was strongly reduced in cap cells of *en*^{i} germaria (Fig. 3E,F). Together, these data show that En regulates *dpp* expression in cap cells and thereby maintains GSCs non-cell-autonomously.

To test whether ectopic En expression is sufficient to turn on *dpp* expression, we forced En expression in ECs, which are believed to share a common precursor pool with cap cells (Song et al., 2007). We employed *c587-gal4* in combination with a temperature-sensitive version of Gal80 (Gal80^{ts}) to drive En expression in ECs and prefollicular cells. As reported previously (Eliazer et al., 2014), these germaria contain ectopic spectrosome-containing cells outside the niche (Fig. S3J). Many of these ectopic spectrosome-containing cells also exhibited pMad (Fig. 4A), indicating ectopic Dpp signaling activation. In line with this, *dpp2.0-lacZ* reporter activity was also ectopically activated in these En-expressing ECs (Fig. 4B). Furthermore, elevated *dpp* transcript levels were detected in the En-expressing ECs by FISH (Fig. 4C compared with Fig. 3E). We also noted that *dpp2.0-lacZ* (Δ2) (Fig. S2A), which exhibits defective En binding activity (Fig. 2D), was not activated in these En-expressing ECs (Fig. S3K), supporting the notion that these two putative En binding sites are important for *dpp* activation *in vivo*. It is also worth noting that these ectopic En-expressing ECs did not express cap cell markers such as Hh (Fig. S3L), indicating that ectopic En does not transform ECs into cap cells. To examine the causal relationship between ectopic expression of En and the *dpp* transcripts observed in ECs, we tested genetic interaction by knocking down *dpp* in these En-expressing ECs. Our results show that formation of the ectopic spectrosome-containing cells, as induced by ectopic En expression, was strongly suppressed when *dpp* was knocked down in those cells (Fig. 4D, Fig. S3M-P).

Rojas-Rios et al. (2012) showed previously that cap cell-expressed En maintains GSCs indirectly by regulating *hh* expression in cap cells, which in turn promotes *dpp* expression in ECs (Rojas-Rios et al., 2012). To address whether En regulates cap cell-expressed *dpp* via *hh* expression, we examined *hh* expression in cap cells with compromised En function. Using a different source of anti-Hh antibody to that of Rojas-Rios et al. (2012) (see Materials and Methods), we found that Hh expression was still detectable (although at a lower level) in 55% (n=49) of *en*^{i} germaria, including those exhibiting GSC loss (Fig. S4A,B). Furthermore, in cap cells mutant for *en*^{E}, Hh was also detected at a reduced level (Fig. S4C). We next conducted FISH experiments to detect *hh* transcripts in these backgrounds. In control germaria, *hh* transcripts were detected in cap cells as well as in some ECs (Fig. S4D). Although the expression level was reduced, *hh* mRNA was still detected in 67% (n=21) of *en*^{i} germaria (Fig. S4E). In line with this, recent studies have shown that knocking down *hh* in the niche by *bab1-gal4* does not result in GSC loss, in contrast to the GSC loss phenotype observed in *en*^{i} germaria (Li et al., 2015a; Liu et al., 2015; Lu et al., 2015). We further conducted genetic rescue experiments by ectopically expressing *hh* in cap cells of the *en*^{i} germarium. When ectopically expressed in a WT background, these Hh-expressing germaria contained fewer spectrosome-containing cells (Fig. S4F, H). Similarly, ectopic Hh expression in cap cells of the *en*^{i} germaria did not rescue the GSC loss phenotype (Fig. S4G,H). Taken together with earlier results showing direct binding of En to the enhancer region of the *dpp* locus, these data support the notion that En directly regulates *dpp* expression in cap cells.

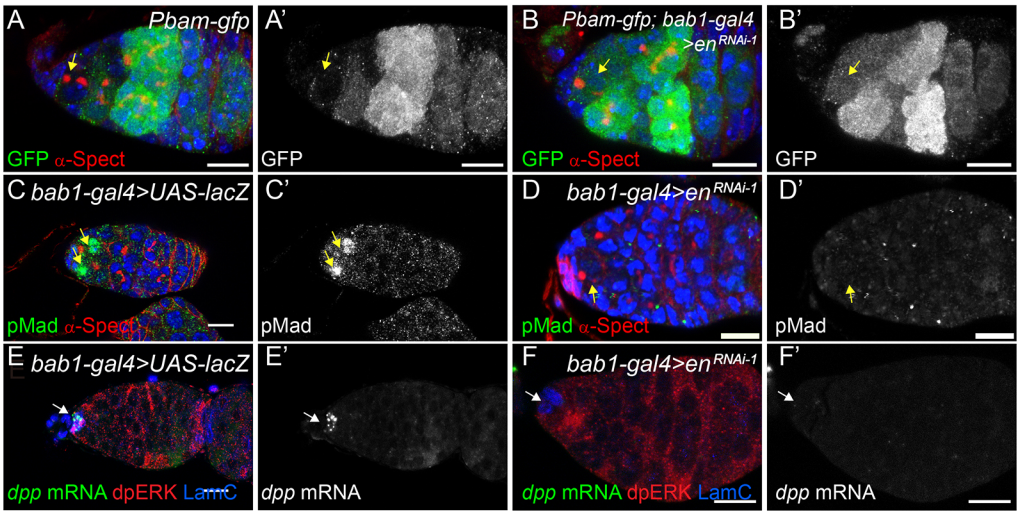

Fig. 3. En regulates *dpp* expression in the germarium. (A,A′) A P*bam-gfp* germarium showing that GFP expression is repressed in GSCs (yellow arrow). (B,B′) A *bab1-gal4>en*$^{RNAi-1}$ (VDRC #35697) germarium showing that P*bam-gfp* activity is activated in spectrosome-containing cells in the niche (yellow arrow). (C,C′) A control germarium showing pMad in GSCs (yellow arrows). (D,D′) A *bab1-gal4>en*$^{RNAi-1}$ (VDRC #35697) germarium exhibiting loss of pMad in the niche (yellow arrow). DNA is in blue (TO-PRO-3). (E-F′) *dpp* transcripts are detected in cap cells (labeled by LamC in blue) of a control germarium (E,E′) but not a *bab1-gal4>en*$^{RNAi-1}$ (v35697) germarium (F,F′). White arrows indicate cap cells; dpERK outlines ECs. Scale bars: 10 µm.

Development (2017) 144, 3224-3231 doi:10.1242/dev.145474

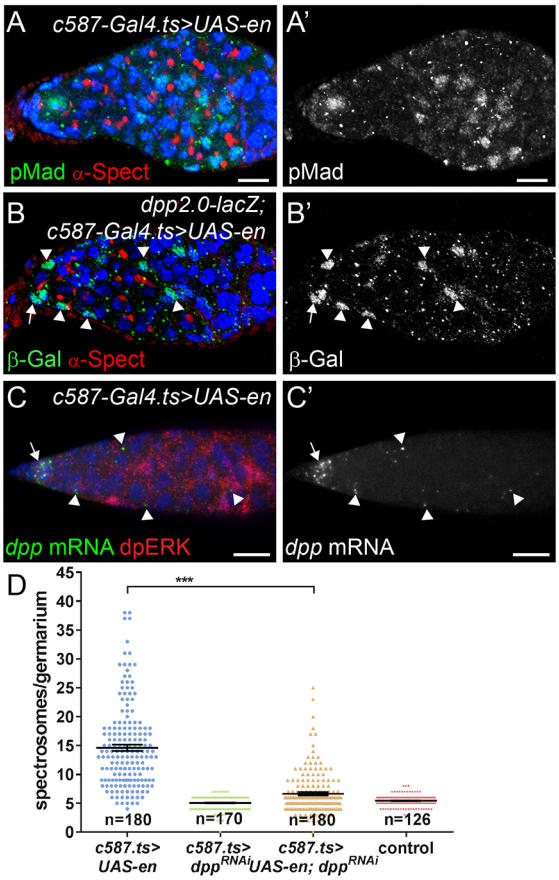

Fig. 4. Forced En expression in ECs activates *dpp* expression. (A,A′) A *c587-gal4.ts*/*UAS-en* germarium showing ectopic spectrosome-containing cells with pMad outside the niche. (B-C′) Cap cells are indicated by white arrows. A *c587-gal4.ts>UAS-en* germarium exhibiting ectopic β-gal expression (arrowheads in B,B′) and ectopic *dpp* transcripts (arrowheads in C,C′). DNA is in blue (TO-PRO-3). Scale bars: 10 µm. (D) Statistical data showing that knockdown of *dpp* in En-expressing ECs strongly suppresses the formation of the ectopic spectrosome-containing cells. *c587-gal4.ts>+* is used as control. Error bars indicate s.e.m. ***P<0.001.

Nej acts as a co-activator of *dpp* expression

As a sequence-specific DNA-binding protein, the ability of En to activate target gene expression depends on the co-activator with which it associates. To determine how En activates *dpp* transcription in cap cells we searched for its transcriptional co-activator. We tested Nejire (Nej), the fly ortholog of CBP/p300, a well-established transcriptional co-activator that can link sequence-specific transcription factors with the transcriptional machinery by serving as a protein bridge or by nucleating a multicomponent transcriptional regulator complex (Goodman and Smolik, 2000).

We first tested whether *nej* interacts genetically with *en* in the ovary. Whereas germaria singly heterozygous for either en^{E} or nej^{S342}, an EMS-induced hypomorphic allele (Florence and McGinnis, 1998), contained GSCs, germaria double heterozygous for en^{E} and nej^{S342} exhibited a GSC loss phenotype (Fig. 5A,B), suggesting that En and Nej function in the same genetic pathway for GSC maintenance. Since Nej was detected in all cells in the germarium (Fig. S5A), we tested whether the synergistic relation between Nej and En in maintaining GSCs reflects Nej function in the niche cells. We knocked down *nej* using *bab1-gal4* and found that these germaria exhibited premature GSC loss and reduced pMad levels (Fig. 5C,F). Furthermore, these germaria exhibited a strong reduction in *dpp2.0-lacZ* activity in cap cells (Fig. 5D), similar to that observed in en^{i} germaria. These data support a role of Nej in regulating *dpp* expression in the niche. Consistently, further knocking down Nej activity in cap cells of en^{i} germaria enhanced the GSC loss phenotype (Fig. 5E,F). Lastly, compromising Nej activity in En-expressing ECs strongly suppressed the formation of ectopic spectrosome-containing cells, prevented ectopic pMad presence outside the niche and, importantly, suppressed ectopic *dpp* transcription in the En-expressing ECs (Fig. S5B-F). These data suggest that Nej is required for En to activate *dpp* expression in cap cells.

To test whether En forms a complex with Nej we performed co-IP in S2 cells. Our results showed that the N-terminal portion of Nej including the KIX domain can interact with En, whereas other parts of Nej, including the Br, HAT and SID domains, are not essential for En binding (Fig. 5G,H). Additional results showed that this N-terminal region of Nej forms a complex with the C-terminal portion of En. Interestingly, this N-terminal portion also interacted with En[Act], a truncated variant of En that lacks the repressive domains and functions as a transcriptional activator *in vivo* (Fig. 5G,I) (Alexandre and Vincent, 2003). Together, these results support the notion that Nej is a transcriptional co-activator for En.

Finally, we investigated whether, like En, Nej occupies the 2.0 kb *dpp* enhancer region. However, we were not able to detect occupancy for endogenous Nej at this region in S2 cells (Fig. 5J). We reasoned that this could be due to lack of En expression in S2 cells and performed ChIP experiments in S2 cells with ectopic En expression. Indeed, in these conditions Nej was found to be enriched at the 2.0 kb enhancer region (Fig. 5J). These data support a role for En (as a sequence-specific transcription factor) as a link to bring Nej to this enhancer region.

DISCUSSION

The niche plays a vital role in maintaining stem cell function throughout the lifespan of an organism. The *Drosophila* ovary was one of the first *in vivo* systems shown to demonstrate the niche concept at the structural and molecular level, with Dpp being the primary niche-associated signal promoting GSC self-renewal (Xie and Spradling, 2000). In the GSC niche, *dpp* mRNA is mainly detected in cap cells and is weakly expressed in ECs. Several players, including epigenetic regulators such as the histone demethylase Lsd1 [Su(var)3-3], the histone H3K9 methyltransferase Eggless, H3K4 methyltransferase Set1, Piwi, and the Polycomb complex subunits, repress *dpp* expression in ECs (Eliazer et al., 2014; Jin et al., 2013; Li et al., 2016; Ma et al., 2014; Wang et al., 2011; Xuan et al., 2013). Knocking down the function of these genes in ECs activates ectopic Dpp signaling outside the niche, although the detailed mechanisms await further investigation. By contrast, the regulation of *dpp* in cap cells is less understood. JAK/STAT signaling plays a role in promoting *dpp* expression in cap cells via a mechanism that has yet to be identified (Lopez-Onieva et al., 2008; Wang et al., 2008). In this study, we show that the cap cell-expressed transcription factor En binds to a 2.0 kb fragment in the 5′ *cis*-regulatory region of *dpp* and controls its expression in cap cells. Our data further suggest that Nej is recruited by En to this *cis*-regulatory region and serves as the transcriptional co-activator for En to regulate *dpp* expression (Fig. S5G). Thus, this study unveils the underlying mechanism controlling *dpp* expression in cap cells.

Dpp, a member of the transforming growth factor β (TGFβ) superfamily, is widely employed during fly development, including in dorsal/ventral patterning of the embryo, morphogenesis of the larval gut, and anterior/posterior patterning of wing imaginal discs, as well as in stem cell regulation in the adult (Morata and

Development (2017) 144, 3224-3231 doi:10.1242/dev.145474

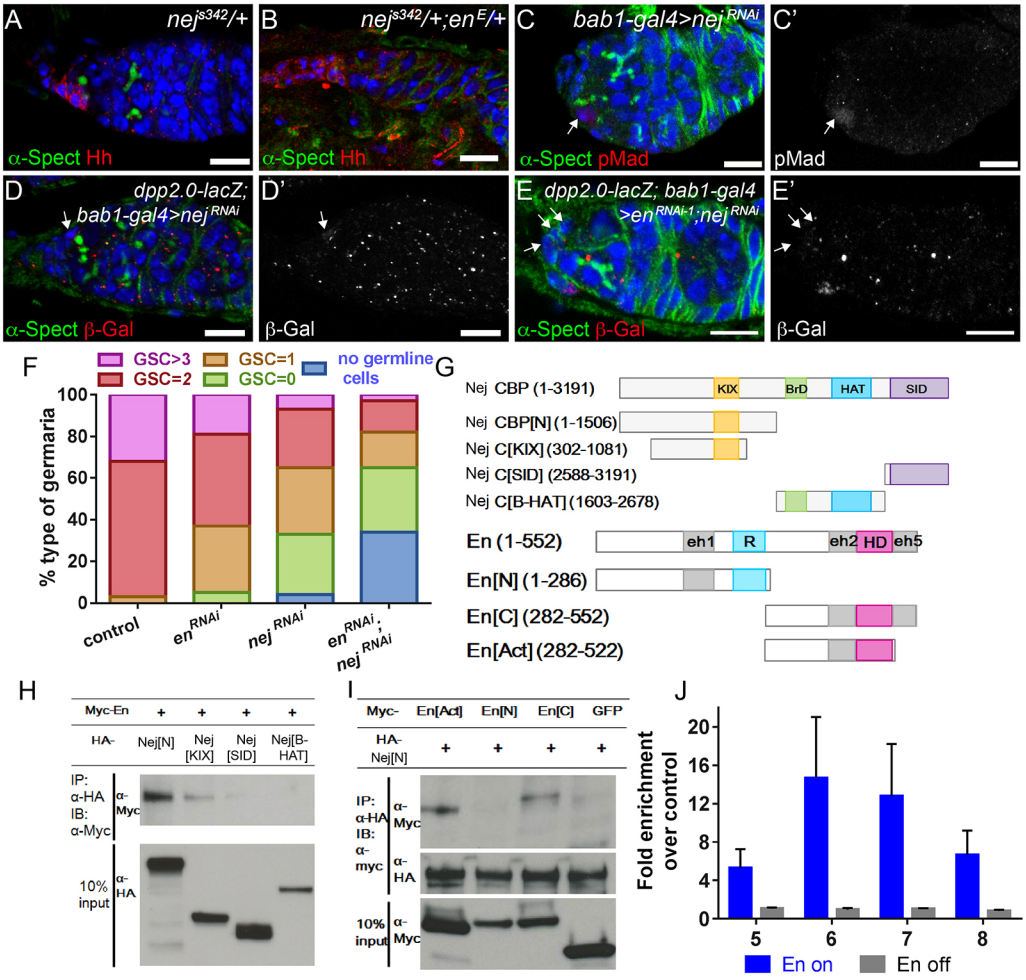

Fig. 5. En cooperates with Nej. (A,B) An nej^{s342}/+ germarium contains GSCs (A), whereas an nej^{s342}/+;en^{E}/+ germarium does not (B). (C,C′) A *bab1-gal4>*nej^{RNAi} germarium exhibiting reduced pMad levels. (D,D′) A *bab1-gal4>*nej^{RNAi} germarium showing loss of β-gal expression in cap cells (arrow). (E,E′) A *bab1-gal4>*en^{RNAi-1};nej^{RNAi} double-knockdown germarium exhibiting loss of GSCs as well as loss of β-gal expression. DNA is in blue (TO-PRO-3). Scale bars: 10 µm. (F) Statistical analysis showing the number of GSCs per germarium in *bab1-gal4>*en^{RNAi-1} (en^{RNAi}), *bab1-gal4>*nej^{RNAi} (nej^{RNAi}), and *bab1-gal4>*en^{RNAi-1};nej^{RNAi}. *n*=200 germaria for each genotype. (G) Domain structure of Nej and En. eh, Engrailed homologous domain; R, repressive domain; HD, homeodomain. (H) En interacts with the N-terminal region of Nej. (I) The N-terminus of Nej interacts with the C-terminus of En and En[Act]. (J) ChIP results showing enrichment of Nej on the *dpp2.0* region when En is expressed in S2 cells. 5-8 refer to the genomic regions indicated in Fig. 2C. Each experiment was performed in triplicate.

Lawrence, 1975; O'Connor et al., 2006). This suggests a complex regulation of *dpp* expression during development and, indeed, multiple enhancers have been identified to direct *dpp* expression in different developmental contexts (Blackman et al., 1991; St Johnston et al., 1990). Adding to this complexity, our study shows that one previously uncharacterized 2.0 kb fragment in the 5′ *cis*-regulatory region directs *dpp* expression in cap cells. This *cis*-regulatory region is distinct from the well-characterized 'disc enhancer' located at the 3′ UTR (Blackman et al., 1991; Masucci et al., 1990). Consistent with their cellular context-dependent enhancer activities, this 2.0 kb enhancer does not drive β-gal expression in a pattern similar to endogenous *dpp* expression in imaginal discs (not shown) and, likewise, the disc enhancer does not exhibit activity in niche cells. We also note that *dpp2.0-lacZ* reporter activity does not respond to ectopic Upd expression in ECs (not shown), a condition shown to activate ectopic *dpp* expression in ECs (Decotto and Spradling, 2005; Lopez-Onieva et al., 2008; Wang et al., 2008), thus indicating an intricate and complex regulation of *dpp* expression in the niche.

We further identify several En consensus binding sequences within this 2.0 kb fragment and our results show that removing two of these sites (region 10, Table S3) abolishes its enhancer activity in cap cells. We also note that deleting two adjacent regions (regions 9 and 11, Table S3) also abolishes reporter activity in the niche, suggesting that the proper organization of this genomic region is important for reporter activity. Furthermore, compromising En activity in cap cells abolishes the enhancer activity of the 2.0 kb fragment, diminishes *dpp* expression in cap cells and consequently leads to GSC loss. Conversely, ectopic expression of En in ECs results in ectopic *dpp* expression, ectopic activation of Dpp signaling outside the niche and the formation of ectopic pMad-positive GSC-like cells, which is suppressed by knocking down *dpp* in these cells. These data support a role of En in directly regulating *dpp* expression in the GSC niche via this 2.0 kb enhancer.

Our study also unveils distinct roles of En in controlling *dpp* expression in different developmental contexts. While early studies showed that, in imaginal discs, En directly associates with and acts through the disc enhancer in the 3′ UTR to suppress *dpp* expression in the En-expressing posterior compartment (Sanicola et al., 1995), our results show that En acts as a transcriptional activator to turn on *dpp* expression in the niche. It has been shown that En, similar to other homeobox genes, can act as a transcriptional activator or repressor depending on the co-factors that it associates with (McGinnis and Krumlauf, 1992). Thus, the distinct activity of En towards *dpp* expression in the imaginal disc versus the GSC niche is likely to be due to the different co-factor associated with En. Although the co-factor required for En-mediated suppression of *dpp* in imaginal discs remains elusive, our data support a role for Nej as the co-activator for En in the niche. Knocking down Nej activity in the niche compromises 2.0 kb enhancer activity in cap cells and leads to GSC loss as a consequence of reduced Dpp signaling. Furthermore, Nej also binds to the 2.0 kb fragment in an En-dependent manner in S2 cells. Consistent with Nej being the co-activator, the formation of ectopic spectrosome-containing cells

Development (2017) 144, 3224-3231 doi:10.1242/dev.145474

induced by ectopic En expression in ECs is strongly suppressed by compromising Nej function in these cells. Nej contains one HAT domain that can acetylate histone H3 lysine 27 (H3K27ac) to antagonize Polycomb complex-mediated transcriptional suppression via histone H3 lysine 27 trimethylation (H3K27me3) (Goodman and Smolik, 2000). A recent paper showed that Polycomb group genes function in ECs to suppress *dpp* expression and promote germ cell development (Li et al., 2016). Thus, it would be interesting to investigate whether the role of Nej as a co-activator for En-mediated *dpp* expression in cap cells encompasses its role in epigenetic regulation by antagonizing Polycomb group activity.

MATERIALS AND METHODS

Fly stocks and experimental conditions

The following lines were used in this study. y^1w^{1118} (as WT control), *c587-gal4* (or *c587*; from T. Kai, Temasek Life Sciences Laboratory, Singapore), P*bam-gfp* (from D. Chen and D. McKearin, UT Southwestern Medical Center, USA), UAS-*hh* (from J. Jiang, UT Southwestern Medical Center, USA), en^E (from K. Basler, University of Zurich, Switzerland), *Dad-lacZ* (from T. Tabata, University of Tokyo, Japan) and *bab1-gal4.UAS-flp* (from A. Gonzalez-Reyes, Centro Andaluz de Biologia del Desarrollo-CABD, Spain). Other stocks were obtained from Bloomington Drosophila Stock Center (BDSC), Kyoto Stock Center, NIG-Fly or Vienna *Drosophila* RNAi Center (VDRC): dpp^{dsRNA} (BDSC #25782), en^{dsRNA} (VDRC #35697 [#1], VDRC #105678 [#2]), nej^{dsRNA} (BDSC #27724), *bab1-gal4* (BDSC #6802). For overexpression or knockdown experiments, crosses were raised at 18°C and progeny of correct genotype were collected upon eclosure and fattened with freshly prepared wet yeast paste at 31°C before dissection. For crosses using *bab1-gal4*, progeny were fattened for 3-7 days. For crosses using *c587-gal4*, progeny were fattened for 9 days.

For enhancer/promoter dissection experiments, fragments of the *dpp* genomic region were amplified using the primers listed in Table S1 and inserted into the pattBLacZ vector (from K. Basler). The injection of these constructs and transgene generation were carried out by BestGene.

Immunostaining and fluorescent *in situ* hybridization (FISH)

Immunostaining and fluorescent *in situ* hybridization were performed as described previously (Li et al., 2014, 2015b). The following primers were used to generate FISH probes: *dpp*, 5′-AGGACGATCTGGATCTAGAT-CGGT-3′ and 5′-ACTTTGGTCGTTGAGATAGAGCAT-3′; *hh*, 5′-ATT-CGTCGATCAGTTCCCACGTGC-3′ and 5′-GATGGAATCCTGGAAG-AGCGATCC-3′. Primary antibodies were: rabbit anti-α-Spectrin (1:3000; generated in-house), rabbit anti-pMad (1:500; Cell Signaling, 9516S), rabbit anti-Hh (1:3000; from P. Beachy, Stanford University School of Medicine, USA), guinea pig anti-Vasa (1:6000; from T. Kai), guinea pig anti-Nej (1:1000; from F. Yu, Temasek Life Sciences Laboratory, Singapore), mouse anti-α-Spectrin [3A9, 1:100; Developmental Studies Hybridoma Bank (DSHB)], mouse anti-Bam (1:5; DSHB) and chicken anti-GFP (1:5000; Abcam, AB13970). Fluorescein (FITC)-, CyTM3- and Alexa Fluor 647-conjugated goat secondary antibodies against rabbit, mouse, chicken and guinea pig primary antibodies were purchased from Jackson ImmunoResearch Laboratories. The DNA dyes used were TO-PRO-3 (1:5000; Invitrogen) or Hoechst 33258 (1:5000; Invitrogen). Samples were analyzed using a Zeiss LSM510 Meta upright or Leica SP8 inverted confocal microscope and compiled by LSM image browser or LAS X. Images were processed in Adobe Photoshop CS6 and Illustrator CS6.

Cell culture, co-immunoprecipitation (co-IP) and chromatin immunoprecipitation (ChIP)

Drosophila S2 cell line was obtained from the Drosophila Genomics Resource Center and cultured in Shields and Sang M3 Drosophila Insect Medium (Sigma-Aldrich) at 25°C without CO_2.

Co-IP and ChIP were performed according to previous protocols (Luo et al., 2015). For each experiment, 1×10^7 to 2×10^7 cells were used. The following antibodies were used: mouse anti-Flag (Sigma, F3165-1MG; 1.5 µl undiluted), rat anti-HA (Roche, 11867423001; 1.5 µl undiluted) and guinea pig anti-Nej (from F. Yu; 1.5 µl undiluted). Primers for real-time PCR are listed in Table S4. *Rp49* (*RpL32*) was used as control for normalization.

Far-western blot

GST and GST-En were expressed in BL21(DE3) bacterial cells, which were then lysed with lysis buffer (PBS, 50 mM Tris pH 8.0, 0.1% Triton X-100, 0.5 mM $MgCl_2$, 1 mg/ml lysozyme) and placed on ice for 15 min. Lysed cell suspension was then mixed with 6× loading dye at a ratio of 1:1 and incubated at 97°C for 10 min. Lysate containing equal amounts of GST or GST-En protein (quantified by Coomassie Brilliant Blue R250 staining on a gel before running the actual experiment) were then loaded onto three separate polyacrylamide gels. Protein lysate was separated by SDS-PAGE and two gels were transferred onto PVDF membrane, while the third gel was stained with Coomassie Blue as a loading control. Membranes with proteins were incubated in 8 M urea at room temperature for 15 min on an orbital shaker to denature the proteins. Proteins were then renatured using ten different concentrations of urea, which were prepared by serially diluting 8 M urea in TBS at a volume ratio of 2:3, at 4°C for 15 min each. Membrane was then rinsed in TBS and blocked at 4°C for 2 h in blocking buffer (25 mM NaCl, 10 mM $MgCl_2$, 10 mM Hepes pH 8.0, 0.1 mM EDTA, 1 mM DTT, 5% skimmed milk). 1 µg/ml biotin-labeled *dpp2.0* or *dpp2.0* (Δ2) DNA probe was added to the hybridization buffer (25 mM NaCl, 10 mM $MgCl_2$, 10 mM Hepes pH 8.0, 0.1 mM EDTA, 1 mM DTT, 2.5% skimmed milk) and hybridized with membrane at 4°C overnight. Membranes were then washed three times with hybridization buffer at 4°C for 15 min. Biotin-labeled DNA probes were then detected using Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific) and membranes were exposed and imaged using the ChemiDoc Imaging System (Bio-Rad). The following 5′-biotin-conjugated primers were used to amplify the DNA probe from *dpp2.0-lacZ* or *dpp2.0-lacZ* (Δ2) template for far-western blotting: 5′-biotin-ATATTTCGCTACTCTTAATAGACCT-3′ and 5′-biotin-CAATCCGTTGACTGCACATCAAA-3′.

Statistical data

Spectrosomes were counted using a Nikon Eclipse 80i microscope with HBO mercury lamp. Results of real-time PCR were exported from ProteinAssist (Applied Biosystems). All statistical data were plotted using GraphPad Prism 7.0. *P*-values were calculated by unpaired *t*-tests in GraphPad Prism. $P<0.05$ was considered statistically significant. Error bars represent s.e.m.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: Y.C.; Methodology: L.L., C.K.S.; Validation: L.L.; Formal analysis: L.L., C.K.S., Y.C.; Investigation: L.L.; Data curation: L.L.; Writing - original draft: Y.C.; Writing - review & editing: L.L., C.K.S.; Supervision: Y.C.; Project administration: Y.C.; Funding acquisition: Y.C.

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Supplementary information

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