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Engrailed homeoprotein acts as a signaling molecule in the developing fly

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SUMMARY

Homeodomain transcription factors classically exert their morphogenetic activities through the cell-autonomous regulation of developmental programs. In vertebrates, several homeoproteins have also been shown to have direct non-cell-autonomous activities in the developing nervous system. We present the first in vivo evidence for homeoprotein signaling in *Drosophila*. Focusing on wing development as a model, we first demonstrate that the homeoprotein Engrailed (En) is secreted. Using single-chain anti-En antibodies expressed under the control of a variety of promoters, we delineate the wing territories in which secreted En acts. We show that En is a short-range signaling molecule that participates in anterior crossvein development, interacting with the Dpp signaling pathway. This report thus suggests that direct signaling with homeoproteins is an evolutionarily conserved phenomenon that is not restricted to neural tissues and involves interactions with bona fide signal transduction pathways.

KEY WORDS: *Drosophila*, Engrailed, Signaling molecule

INTRODUCTION

Homeoproteins are best known for their well-established cell-autonomous transcription factor activity. However, the identification of two highly conserved sequences within their DNA-binding domains (homeodomains) that drive their secretion and internalization (Chatelin et al., 1996; Joliot et al., 1998; Joliot and Prochiantz, 2004) has led to the proposal that several homeoproteins also serve as signaling molecules. Indeed, signaling activity has been demonstrated in the vertebrate visual system for Pax6 (Lesaffre et al., 2007) and Otx2 (Sugiyama et al., 2008). A third example concerns Engrailed 1 and 2 (En1/2), in which extracellular En1/2 were found to influence growth cone turning decisions (Brunet et al., 2005) and to regulate retinotectal patterning (Wizenmann et al., 2009). The present study aimed to test whether such a mechanism also exists in flies by examining the *Drosophila* Engrailed homeoprotein (En) during wing development.

In *Drosophila*, En is an important transcription factor in anterior-posterior (A/P) wing patterning (Morata and Lawrence, 1975; Lawrence and Morata, 1976) and in neurodevelopment (Bhat and Schedl, 1997; Colomb et al., 2008). The larval wing imaginal disc, which gives rise to the adult wing, is subdivided into non-intermingling anterior (A) and posterior (P) compartments. En is present in the P compartment, where it acts as a transcription factor to establish and maintain P cell identity (Lawrence and Morata, 1976; Hidalgo, 1994; Zecca et al., 1995; Chanas et al., 2004). En is also known to be expressed (Blair, 1992) and to function in a few cell rows in the A compartment that abut the A/P boundary (Hidalgo, 1994). P compartment cells synthesize Hedgehog (Hh), which diffuses over a few cell rows into the A compartment where it induces, according to its concentration, the expression of several target genes, including its receptor *patched* (*ptc*), the *Drosophila* Bmp2/4 ortholog *decapentaplegic* (*dpp*) and *en* (Tabata and Kornberg, 1994; Guillen et al., 1995; Tabata et al., 1995; Strigini and Cohen, 1997). En has been described as a transcription factor (Solano et al., 2003) that is able, for example, to repress *dpp* expression (Hidalgo, 1994; Sanicola et al., 1995; Maschat et al., 1998) or to activate *hh* expression (Zecca et al., 1995; Tabata et al., 1995; Alexandre and Vincent, 2003).

dpp is first expressed just anterior to the A/P boundary in the wing imaginal disc and finally appears along the veins in the pupal disc (de Celis, 1997; Conley et al., 2000). BMPs (Dpp or Gbb) signal through the Thickveins (Tkv) receptor to phosphorylate Mothers against dpp (Mad) (Conley et al., 2000). Reducing BMP signaling selectively disrupts the development of veins and crossveins, which can be monitored by the activity of phosphorylated Mad (pMad) (Segal and Gelbart, 1985; Bangi and Wharton, 2006; Blair, 2007). In addition, the secreted Crossveinless 2 (Cv-2) and Tkv proteins both help to localize or stabilize Dpp signaling in the crossveins. Proper Dpp signaling thus involves a delicate balance between the activities of Dpp itself, Tkv and Cv-2 (Serpe et al., 2008).

Here, we introduce En as a new component in this signaling pathway. Indeed, although the anterior crossvein (ACV) lies partly outside of the *en* expression domain, we show that its formation requires En function. We find that an extracellular pool of En protein is present in the wing disc that diffuses beyond its expression domain. In order to specifically block this extracellular En signal in vivo, we developed a genetic approach based on single-chain anti-En antibodies. Using a secreted form of the single-chain anti-En antibody, we demonstrate that extracellular En

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is required for the formation of the ACV. First, we delineated the source of En signal that is responsible for ACV formation within a thin territory anterior to the A/P boundary, and show that the cells that respond to the En signal are located in a larger domain that extends more anteriorly. Second, we show that to form the ACV, the En signal acts on the different components of the Dpp signaling pathway, i.e. Dpp itself, Tkv and Cv-2. In particular, blocking En secretion lowers the pMad level independently of the endogenous level of nuclear En. Finally, we provide evidence for distinct activities of nuclear En and the extracellular En signal in vivo through two different, but complementary, mechanisms of action.

MATERIALS AND METHODS

Fly strains

UAS-*en*RNAi includes a 649 bp genomic fragment covering the first exon (nucleotides 526 to 1174 of the *en* cDNA sequence; FlyBase annotation CG9015). Note that the RNAi has been constructed against a specific region of En that is not conserved in its sister Invected (Inv). This genomic fragment was cloned as inverted repeats, with the 280 bp *en* intron 2 intervening, into the pUASt vector. *en* but not *inv* RNAs are affected by UAS-*en*RNAi (see Fig. S1 in the supplementary material). The level of En protein was found to be substantially reduced in the wing disc by immunodetection (see Fig. S2 in the supplementary material). Note that this *en*RNAi behaves as a hypomorphic mutation of En, as the *en*RNAi-induced phenotypes are enhanced when tested over other *en* amorphic mutations.

UAS-En-GFP was constructed by insertion of the *en* cDNA into the pTWG 1076 Gateway vector (*Drosophila* Genomics Resource Center) using the Gateway recombination protocol (Invitrogen).

Mutations used in this study were en^{X31}, which corresponds to a deficiency covering both *en* and *inv* (Gustavson et al., 1996), and the null alleles dpp^{D6} and tkv^{a12} (Tanimoto et al., 2000), which were kindly provided by T. Tabata. The deficiency covering *cv-2*, *Df(2R)Pu-D1* [referred to as *Df(cv-2)*] and the *ptc*-lacZ flies (with a *lacZ* enhancer trap) were obtained from the Bloomington Stock Center. We used the following Gal4 drivers: *MS1096*-Gal4, *en*-Gal4 (Tabata et al., 1995), *ptc*-Gal4 (Brand and Perrimon, 1993), *hh*-Gal4 (Tanimoto et al., 2000) and *dpp*-Gal4 (Tanimoto et al., 2000), which were kindly provided by T. Tabata.

Flies were grown at 25°C or 29°C as indicated.

Immunocytochemistry

For standard immunostaining, imaginal discs were dissected in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (PFA) for 20 minutes and blocked in PBS containing 1% BSA and 0.1% Triton X-100 (PBT) for several hours. They were incubated overnight at 4°C with primary antibodies in PBT, washed for 1 hour, and then incubated for 2 hours with secondary antibodies. DNA was stained with DAPI (1 μM) for 20 minutes. After washing, tissues were mounted in anti-fade ProLong Gold (Invitrogen).

Detergent-free immunostaining was performed as described (Furrer et al., 2007). To detect intracellular antigens in the same tissues, we performed a post-fixation step in 4% PFA for 10 minutes after the secondary antibody wash, followed by classical immunostaining.

Fluorescence images were acquired using Zeiss LSM 510 Meta and LSM 780 confocal laser-scanning microscopes (40× and 63× objectives) at the IGH RIO Imaging platform.

The following antibodies were used: mouse anti-En 4F11 (1/100) (Patel et al., 1989), rabbit anti-En (1/200; Santa Cruz), rabbit anti-pMad (1/1000; kindly provided by Ed Laufer, Columbia University, New York, USA), rabbit anti-Myc (1/500; A-14, Santa Cruz Biotechnology), rabbit anti-β-galactosidase (1/1000; Cappel), rabbit anti-GFP (1/400; Molecular Probes) and Alexa-conjugated secondary antibodies (1/800; Alexa 488, 546, 633; Molecular Probes).

All experiments were performed on at least 20 discs (ten larvae) and performed at least three times.

Flp-out clones

The *hs*-Flp/Act5C>CD2>Gal4, UAS-GFP system (Britton and Edgar, 1998) was used to induce random *en* expressing clones. Clones were induced by subjecting yw *hs*-Flp122/+; Act5C>CD2>Gal4, UAS-GFP/UAS-En second instar larvae at 48 hours after egg laying (AEL) to a heat shock at 37°C for 20 minutes. Clones are visualized by GFP.

Construction of single-chain anti-En antibodies

Single-chain recombinant antibodies (ScFvs) were prepared from total RNA of anti-En 4F11 hybridoma cells provided by Nipam Patel (Patel et al., 1989). Cloning of ScFvs was achieved as described (Lesaffre et al., 2007) into a pGem vector and a pSecB vector (Invitrogen), which contains a signal peptide. Each ScFv insert (with or without the secretion signal peptide) was inserted into a pDonR-21 Gateway vector (Invitrogen) and further inserted into appropriate vectors by recombination. From these vectors, the pUASt-4F11-6xmyc-tag and the pUASt-SP4F11-6xmyc-tag vectors were constructed by the Gateway method, using the Gateway pTWM 1108 (*Drosophila* Genomics Resource Center). *Drosophila* transgenic lines were obtained by injection (performed by the Best-Gene Corporation).

Co-immunoprecipitation experiments

Protein extracts were prepared from dechorionated *Drosophila* embryos, which were crushed in two volumes of DXB buffer (25 mM Hepes pH 6.8, 250 mM sucrose, 1 mM $MgCl_2$, 1 mM DTT, 150 mM KCl, 0.1% Triton X-100, protease inhibitors), centrifuged for 5 minutes at 3000 rpm (1000 ***g***) and the supernatants recovered. Co-immunoprecipitations were performed using Seize Classic G (Pierce). For each sample, 50 μl protein G beads were first incubated overnight at 4°C with a rabbit anti-Myc antibody (Santa Cruz sc789) and then incubated for 90 minutes at 4°C with 500 μg (~400 μl) of embryonic protein extract containing En-GFP, En-GFP and 4F11-myc, or En-GFP and SP4F11-myc. Beads were washed with binding buffer and eluted directly in loading buffer before 10% SDS-PAGE and western blot using a monoclonal mouse anti-GFP antibody (1/1000; Invitrogen A11120) to detect the En protein. The tagged En-GFP protein was estimated at 87 kDa. The same membrane was used to detect the ScFvs with the monoclonal mouse anti-Myc antibody (1/1000; Abcam 9E10). The 4F11-myc protein was estimated at 24.7 kDa and SP4F11-myc at 37.5 kDa.

ScFv expression in mammalian cells

HEK293 cells were transfected by lipofection (Lipofectamine 2000, Invitrogen) with pUASt-4F11-6xmyc-tag or the pUASt-SP4F11-6xmyc-tag vector together with Gal4-VP16 constructs. After 24 hours, cells were fixed (4% PFA, 10 minutes) and permeabilized (0.3% Triton X-100, 5 minutes) for intracellular detection of ScFvs with #9E10 anti-Myc antibody (1/500) and Alexa 488-conjugated secondary antibody (1/800) together with Alexa 546 WGA (1 μg/ml; Molecular Probes), or the conditioned media was purified on nickel beads (Dynal) and the accumulation of ScFvs analyzed by western blot (9E10, 1/1000).

RESULTS

ACV formation requires an En signal in the wing discs

Using the UAS/Gal4 system (Brand and Perrimon, 1993), we specifically reduced En protein synthesis by RNA interference (*en*RNAi), which is equivalent to a hypomorphic mutation that specifically reduces *en* mRNA (see Fig. S1 in the supplementary material) and protein (see Fig. S2 in the supplementary material) levels. Lowering En in the entire wing with the *MS1096*-Gal4 driver resulted in the absence of the anterior (ACV) and posterior (PCV) crossveins (Fig. 1A). It is noteworthy that the ACV, which is formed during pupal development, is in the A compartment, with half of the crossvein falling within the late En expression domain in the A compartment (denoted the Ptc/En domain; see Fig. 5I) (Blair, 1992); the other half being situated in a more anterior position, clearly outside the domain of En expression (see Fig. S3 in the supplementary material).

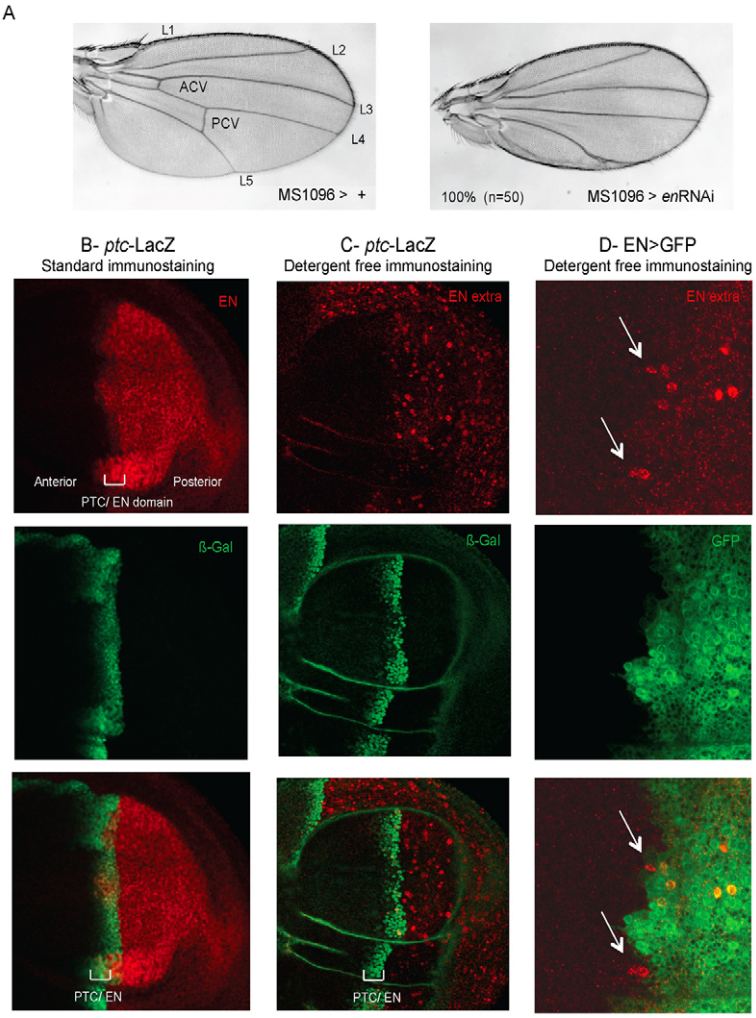

Fig. 1. En expression and crossvein formation. (**A**) *MS1096*-Gal4;+ adult *Drosophila* wings (left) show a normal pattern of veins, with longitudinal veins (L1 to L5) and anterior (ACV) and posterior (PCV) crossveins. In *en* loss-of-function wings obtained by RNA interference (UAS-*en*RNAi under the *MS1096*-Gal4 driver), ACV and PCV are absent (right). *n*, the number of wings examined. (**B**) Wing imaginal disc from *ptc*-lacZ L3 larva labeled with anti-En (red) and anti-β-galactosidase (green) antibodies by standard immunostaining; the bottom image is a merge. Intracellular En is detected in the posterior cells and overlaps the Ptc domain. A *z*-projection of all focal planes is shown. (**C**) Wing imaginal disc from *ptc*-lacZ L3 larva labeled with anti-En by detergent-free immunostaining. Following En detection, intracellular β-galactosidase expression was detected. Extracellular En is present in the posterior compartment and the Ptc/En domain (see merged image). A *z*-projection of all focal planes is shown. (**D**) Wing imaginal disc from *en*-Gal4; UAS-GFP L3 larva labeled with anti-En by detergent-free immunostaining. Extracellular En is present outside (arrows) the normal domain of En expression as identified by GFP fluorescence (green) (see merged image). Images in D are magnified 3× compared with B and C. One focal plan is shown.

In light of the previously described non-cell-autonomous En1/2 activity in vertebrates (Wizenmann et al., 2009), and because the *Drosophila* En ortholog also contains sequences for secretion and import (see Fig. S4 in the supplementary material), we envisaged that En might act directly as a signaling molecule in the formation of the ACV. To investigate this possibility, we first examined whether En is present in the extracellular matrix in late L3 wing imaginal discs. Using standard immunocytochemistry protocols with detergent treatment, En was detected in all posterior cells and, in lower amounts, in the anterior cells overlapping the *ptc* expression domain (the Ptc/En domain, Fig. 1B; see Fig. 5I). To determine whether En protein is also present in the extracellular space, we performed detergent-free immunocytochemistry on wing discs (Furrer et al., 2007). Using this method, we detected unambiguous staining in all En-expressing domains (posterior cells and the Ptc/En anterior domain) (Fig. 1C). This labeling was specific for En (it was decreased by *en*RNAi, see Fig. S5 in the supplementary material) and heterogeneous, with immunoreactive patches of uneven size dispersed throughout the En expression domain. The distinction between intracellular and extracellular staining was further analyzed by ectopic expression of an En-GFP fusion protein in the larval eye disc, which has larger cells than the wing disc. In these cells, En extracellular staining never overlapped with the predominant intracellular nuclear GFP staining (see Fig. S6 in the supplementary material). As additional controls, we also verified that non-secreted proteins, such as β-galactosidase or the D-SRF (Blistered – FlyBase) MADS-box transcription factor, did not stain under detergent-free conditions (see Fig. S7 in the supplementary material).

Extracellular En diffusion is a key requirement for the signaling hypothesis. In *en*-Gal4; UAS-GFP wing discs, we could detect some extracellular En outside of its normal domain of expression (Fig. 1D). This was confirmed by producing En gain-of-function clones within the wing disc, in which we were able to detect extracellular En up to three cell rows away from the producing clones (Fig. 2). These findings indicate that En protein is indeed able to diffuse in vivo and might be a short-range signaling molecule. Finally, we verified the full-length status of the secreted protein by two complementary approaches. Extracellular En expressed in the wing disc could be visualized by the immunodetection of a Myc tag added at either end of the En protein, and the En protein secreted by HEK293 cells was full length (see Fig. S8 in the supplementary material).

Taken together, these results strongly suggest that En homeoprotein secretion and diffusion take place in the *Drosophila* wing disc.

Engineering tools to block extracellular En

To investigate the function of secreted En, we developed a strategy based on the design of specific anti-En single-chain (ScFv) antibodies. Although previous work suggested that 4F11 anti-En antibody can bind both En and the related transcription factor Inv

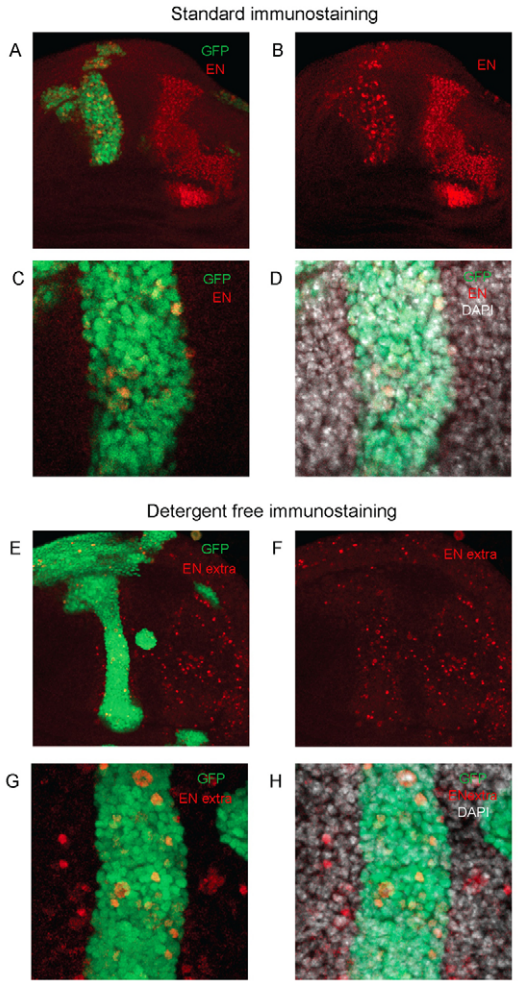

Fig. 2. Analysis of En gain-of-function clones. Large En-expressing clones in the anterior compartment were obtained using the *flp*-out method. Clones are marked by GFP expression (green) and are labeled with anti-En (red). (**A-D**) En detected by standard immunostaining is visualized within the clone. (A) Detection of an anterior clone. (B) En expression in the clones is not as homogeneous as it is in the posterior compartment. (C) Magnification of the clone visualized in A. (D) Clones marked by GFP expression are labeled with anti-En and stained with DAPI (gray). (**E-H**) Extracellular En is detected by detergent-free immunostaining. (E) Detection of an anterior clone by GFP. (F) Patchy extracellular En staining is observed in the posterior compartment, as well as within the clone marked by GFP. (G) Magnification of the clone allows better visualization of extracellular En outside of the GFP-marked clone. (H) Clones marked by GFP expression are labeled with anti-En and with DAPI. Note that En protein can be detected up to three cell rows away from the GFP boundary.

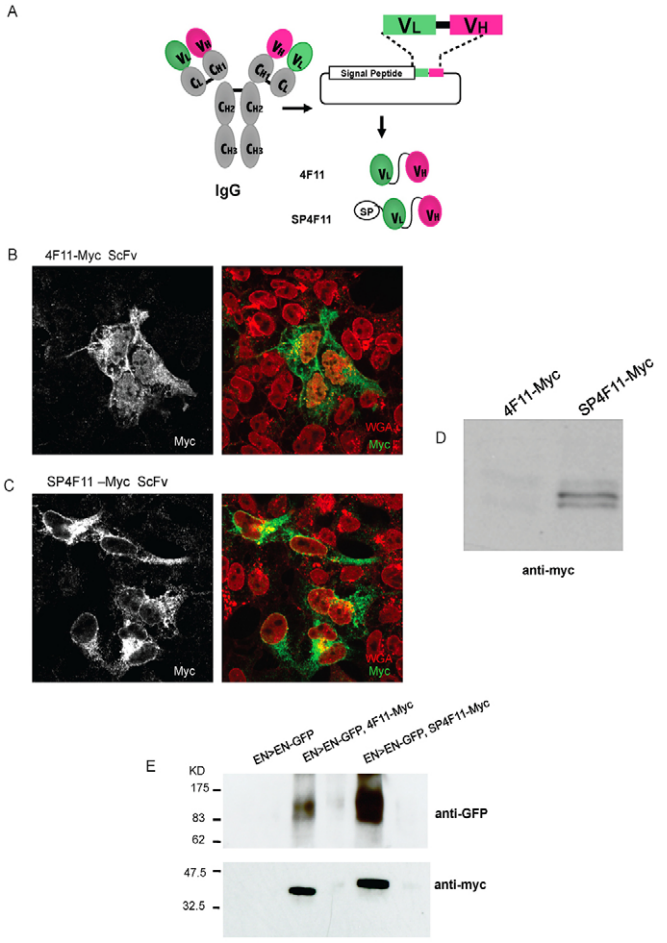

Fig. 3. Construction and characterization of anti-En single-chain antibodies. (**A**) Diagram of the single-chain antibodies (ScFvs) constructed from anti-En 4F11 hybridoma cells (Patel et al., 1989). A single minigene containing coding sequences for the heavy and light variable domains, with an intervening linker, was cloned downstream of the *UAS* sequences in a Myc-tagged vector. SP4F11 differs from 4F11 by the presence of an upstream secretion signal peptide (SP). (**B**,**C**) Intracellular localization of 4F11 (B) or SP4F11 (C) ScFvs expressed in HEK293 cells, visualized by anti-Myc (green). SP4F11 is only detected in the secretion compartment and is excluded from the nucleus (WGA staining in red). (**D**) Conditioned media from HEK293 cells expressing 4F11 or SP4F11 ScFvs were analyzed by western blot using anti-Myc antibody, demonstrating that only SP4F11 is detectable in the medium. (**E**) Co-immunoprecipitation experiments were performed to verify that 4F11-Myc and SP4F11-Myc recognize En in vivo. Protein extracts from *en*-Gal4/UAS-En-GFP (EN>EN-GFP), *en*-Gal4/UAS-En-GFP; UAS-4F11-Myc (EN>EN-GFP, 4F11-Myc) or *en*-Gal4/UAS-En-GFP; UAS-SP4F11-Myc (EN>EN-GFP, SP4F11-Myc) embryos were used. Polyclonal anti-Myc antibody was used for the immunoprecipitation. Immunoprecipitated En protein is detected with anti-GFP (upper panel) and the ScFvs with anti-Myc (lower panel) antibody. No En-GFP protein was retained on the anti-Myc-bound resin in the absence of the ScFvs.

(Patel et al., 1989), we observed that 4F11 preferentially recognizes En (see Fig. S2 in the supplementary material). Therefore, we concentrated on the construction of 4F11 ScFvs. This approach has the advantage of blocking En functions without modifying the En sequence. Most importantly, the antibody can be targeted to the extracellular or the intracellular compartment depending on the presence of a signal peptide for secretion (Cardinale et al., 2004; Lesaffre et al., 2007).

We used the immunoglobulin light and heavy chains expressed by 4F11 anti-En hybridoma cells (Patel et al., 1989) to construct expression vectors for secreted and non-secreted anti-En antibodies. These ScFv antibodies comprise a single polypeptide chain that is composed of both light and heavy chain variable domains separated by a linker domain and fused to Myc tags (Fig. 3A). Two versions were made that differ by the presence (SP4F11) or absence (4F11) of a signal peptide for secretion. When transfected into HEK293 cells, 4F11 (Fig. 3B) and SP4F11 are

differentially distributed within the cells, with SP4F11 staining only seen in the secretion pathway (Fig. 3C). Accordingly, only SP4F11 was recovered in the cell culture medium (Fig. 3D). Even when strongly co-expressed, En and SP4F11 proteins never colocalized within expressing cells (see Fig. S9 in the supplementary material).

We next constructed transgenic flies expressing each ScFv under the control of the *UAS* promoter, allowing their expression through the UAS/Gal4 system. First, we tested the ability of the 4F11 and SP4F11 antibodies to recognize En protein in vivo by co-immunoprecipitation experiments on protein extracts from embryos expressing En-GFP alone or En-GFP together with the 4F11 or SP4F11 ScFvs (Fig. 3E). The antibodies were pulled down using an anti-Myc antibody bound to a resin. The En-GFP protein was specifically retained with both 4F11 and SP4F11 and did not bind the resin in the absence of antibodies. It is noteworthy that 4F11 bound En-GFP less efficiently than did SP4F11, possibly because intracellular single-chain antibodies are partially misfolded owing to the reduction of their disulfide bonds in the reductive intracellular milieu (Cardinale et al., 2004).

Finally, when expressed in the Ptc domain of larval wing imaginal discs, we detected both 4F11 and SP4F11 under permeabilizing conditions (Fig. 4A,C). By contrast, when the detergent-free protocol was applied to the wing disc, we detected extracellular SP4F11 (Fig. 4D) but not 4F11 (Fig. 4B). We further observed that extracellular SP4F11 did not diffuse far from its domain of expression (compare Fig. 4D with 4C).

Based on its subcellular localization, SP4F11 should thus only interfere with extracellular En and inhibit its putative signaling activities. By contrast, 4F11 (unless fully denatured by the reductive intracellular milieu) should block intracellular En activity and show an effect similar to that of *en*RNAi. The effects of the two antibodies were tested on wing morphology. 4F11 expression in the entire wing pouch using the *MS1096*-Gal4 driver led to the loss of both the ACV and all or part of the PCV (Fig. 4E), thereby reproducing the *en* loss-of-function phenotype induced by RNAi (Fig. 1A), albeit with lower efficiency (80% missing the ACV and 51% missing the PCV with 4F11 versus 100% missing the ACV and PCV with *en*RNAi). By contrast, SP4F11 expression throughout the wing pouch only affected the formation of the ACV, with 95% of wings missing ACVs but always with normal PCVs (Fig. 4F).

In conclusion, the formation of the PCV depends mainly on intracellular En activity (it is blocked specifically by 4F11 or *en*RNAi but not SP4F11; Fig. 4E and Fig. 1A). By contrast, ACV development requires extracellular En, as demonstrated by its absence upon SP4F11 expression (Fig. 4F). Notably, morphogenesis of the entire ACV depends on extracellular En activity even though its anterior half lies in the En-negative domain (see Fig. S3 in the supplementary material).

Identification of En-secreting and En-responding cells

As the extracellular pool of En protein was detected throughout the P compartment, as well as at the A/P boundary, in the Ptc/En domain (Fig. 1C), we investigated which wing domains provide the extracellular En signal required for ACV development. We used different Gal4 drivers expressed in distinct patterns (Fig. 5I). The adult wing phenotypes obtained when *en* function was inhibited with *en*RNAi were then compared with those resulting from the neutralization of secreted En by SP4F11. To fully appreciate this comparison, one must consider that in a context of *en* loss of function (*en*RNAi), both the intracellular (see Fig. S2 in the supplementary material) and extracellular (see Fig. S5 in the supplementary material) pools of En are decreased. By contrast, SP4F11 does not affect En synthesis or secretion (see Fig. S5 in the supplementary material) and can only interfere with the En signaling functions by blocking En diffusion and/or internalization. Indeed, we verified that SP4F11 expressed in the wing disc did not affect the transcription of the different components of the Dpp signaling pathway (*dpp*, *cv-2* and *tkv*) that play important roles in crossvein formation (see Fig. S10 in the supplementary material). Therefore, we reasoned that comparing the effects of *en*RNAi and SP4F11 expression on ACV formation would shed light on the source of extracellular En that is involved in ACV development and on the population of cells that responds to this signal.

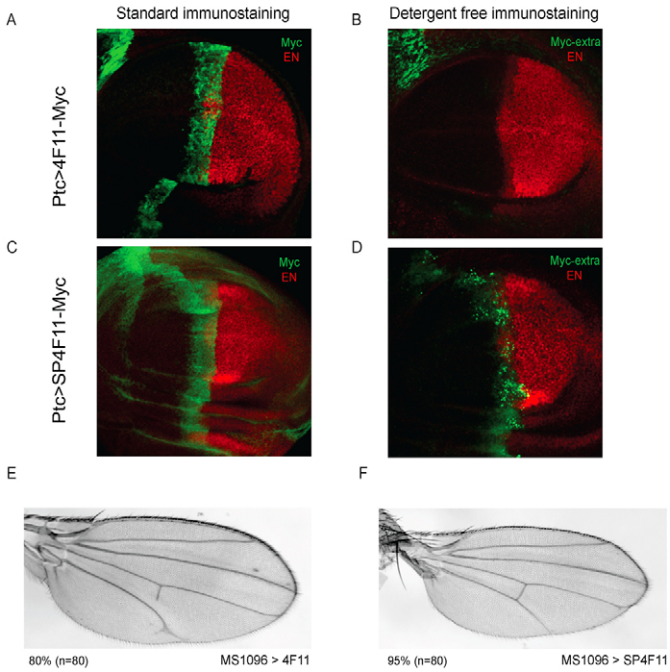

Fig. 4. Expression and action of anti-En single-chain antibodies. (**A-D**) Detection with an anti-Myc antibody (green) of the 4F11 (A,C) and SP4F11 (B,D) ScFvs expressed in *Drosophila* wing discs with the *ptc*-Gal4 driver, using standard (A,C) or detergent-free (B,D) conditions. En expression is visualized using standard immunostaining (red). Note that only SP4F11 (D) and not 4F11 (B) is extracellular. (**E**,**F**) Adult wing phenotypes obtained with the expression of 4F11 (E) or SP4F11 (F) ScFv, expressed in the entire wing using the *MS1096*-Gal4 driver. The penetrance of the phenotypes is noted by percentage; *n*, the number of wings analyzed. Note that, in contrast to 4F11, SP4F11 only antagonizes ACV morphogenesis.

Expressing *en*RNAi in the En domain (Fig. 5A), i.e. in the P compartment plus the Ptc/En anterior cells (see Fig. 5I), reproduced the phenotypes obtained when En was reduced in whole wings (Fig. 1A). However, expressing *en*RNAi exclusively in the P compartment with the *hh*-Gal4 driver had no effect on ACV formation (Fig. 5B). This indicates that ACV formation depends specifically on En protein synthesis in the Ptc/En anterior cells. We confirmed this by expressing *en*RNAi specifically in these cells using the *ptc*-Gal4 driver. ACVs were absent in 88% of the wings (Fig. 5C), demonstrating that the formation of the ACV indeed requires En protein produced from the Ptc/En domain and not from the P compartment.

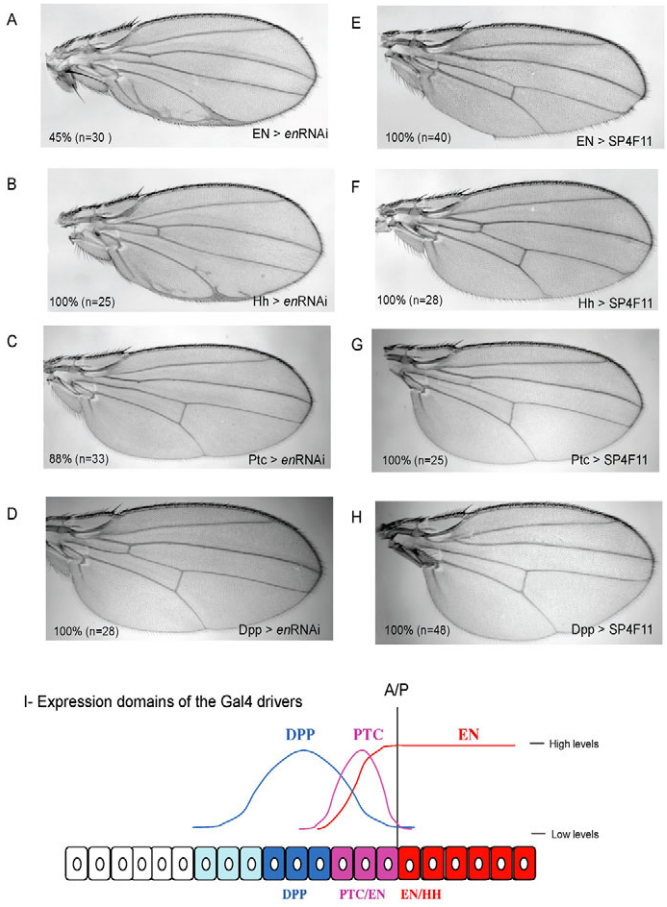

Fig. 5. Identification of the wing domains that require En signal. (**A-H**) ACV formation was analyzed following expression of (A-D) *en*RNAi or (E-H) SP4F11 ScFv with the indicated Gal4 drivers (see I). The penetrance of the loss of ACV phenotype is noted by percentage; *n*, the number of wings analyzed. (**I**) The domains and levels of expression of the different Gal4 drivers used in this study: *en*-Gal4 (EN), *hh*-Gal4 (HH), *ptc*-Gal4 (PTC), *dpp*-Gal4 (DPP). A/P indicates the boundary between the anterior and posterior compartments.

We then interfered with extracellular En by expressing SP4F11 using the same drivers. Similar to the *en*RNAi results, ACVs were always missing with *en*-Gal4 (Fig. 5E) and *ptc*-Gal4 (Fig. 5G), but we saw no effect on ACV formation with *hh*-Gal4 (Fig. 5F). These results suggest that En secreted from the Ptc/En domain is responsible for ACV formation and that extracellular En in the P compartment plays no major role in this process. Expression of *en*RNAi under the control of the *dpp*-Gal4 driver, which is mainly active in more anterior non-En-expressing domains, had no effect on ACV formation (Fig. 5D), whereas expression of SP4F11 led to the loss of ACVs in 100% of the wings (Fig. 5H). This establishes that neutralizing extracellular En in a domain anterior to its site of production affects ACV morphogenesis.

From this series of experiments, we conclude that, apart from its function as a nuclear transcription factor, En also acts as a signaling molecule. Indeed, we have shown that En is secreted by cells from the Ptc/En domain and is received by anterior adjacent cells (Dpp domain) to act in the specification of the ACV.

En and Dpp signaling cooperate in ACV formation

Dpp signaling participates in the patterning of the central domain of the wing during larval development and in vein and crossvein morphogenesis during pupal development (de Celis, 1997; Blair, 2007). Thus, reduction of *dpp* function affects the differentiation of all veins and crossveins (Segal and Gelbart, 1985).

Expression of SP4F11 in the Ptc or Dpp domain led to a loss of the ACV (Fig. 5G,H), a phenotype that mimics the loss of function of *dpp*. This suggests that extracellular En positively 'interacts' with Dpp signaling. We tested this hypothesis by first examining possible genetic interactions between *en* and *dpp* using double heterozygotes with mutations affecting both *en* and *dpp*. Heterozygous mutation in either *dpp* or *en* produced no wing phenotype (Fig. 6A). However, in double heterozygotes for both *en* and *dpp* the ACVs were missing (Fig. 6B and see Fig. S11 in the supplementary material). Genetic interactions were also found between *en* and *cv-2* or *tkv*, both of which participate in the Dpp signaling pathway leading to ACV development, with *cv-2* and *tkv* having antagonistic roles in ACV formation (Fig. 6C,D), in agreement with their functions (Serpe et al., 2008).

The observation that *en* 'interacts' with *dpp* to form the ACV is seemingly at odds with the known intracellular role of En in repressing *dpp* expression (Hidalgo, 1994; Sanicola et al., 1995; Maschat et al., 1998). In other words, could En inhibit *dpp* expression and, at the same time, synergize with Dpp signaling to construct the ACV? To address this, we tested the impact of En inactivation (using either *en*RNAi or SP4F11) on the efficiency of the Dpp signaling pathway as revealed by the phosphorylation of Mad, detected with a phospho-specific antibody (anti-pMad) (Conley et al., 2000; Tanimoto et al., 2000) (Fig. 7; see Fig. 6E).

In wild-type third instar wing discs, high levels of Dpp signaling were observed in the posterior region abutting the A/P boundary, whereas a slightly lower intensity was observed in the anterior domain (Fig. 7A,D), which covers the six cell rows included within the expression domains of the Ptc and Dpp drivers (see Fig. S12 in the supplementary material; see Fig. 5I).

We tested whether the En protein produced by the Ptc/En cells and required for the construction of the ACV (Fig. 5C) potentiates Dpp signaling. Inhibiting En expression using RNAi with the *ptc*-Gal4 driver led to a clear loss of Dpp signaling in the anterior six cell row domain (Fig. 7B and see quantification in Fig. S13 in the supplementary material). This shows that *en* enhances Dpp signaling efficiency not only in cells in which it is expressed, but also in the abutting anterior domain that does not express *en*.

We next tested whether this En interaction with Dpp signaling (as revealed by pMad staining) takes place via En signaling by analyzing the consequences for Dpp signaling of blocking extracellular En with SP4F11 in the Ptc/En domain. In *ptc*-Gal4/UAS-SP4F11 larvae, we observed a reduction in pMad staining in the six cell row anterior domain abutting the A/P boundary (Fig. 7C), although nuclear En was still present within the Ptc/En domain (see quantification in Fig. S14 in the supplementary material). To further distinguish between En extracellular and intracellular activities, we used the more discriminative *dpp*-Gal4 driver (Fig. 7D and see Fig. S12 in the supplementary material). Inactivation of En synthesis by RNAi with the *dpp*-Gal4 driver did not modify pMad staining (Fig. 7E and see quantification in Fig. S13 in the supplementary material), consistent with the absence of defects in ACV formation (Fig. 5D). In sharp contrast, SP4F11 expression using the same driver fully antagonized pMad staining in the anterior domain (Fig. 7F and see quantification in Fig. S13 in the supplementary material).

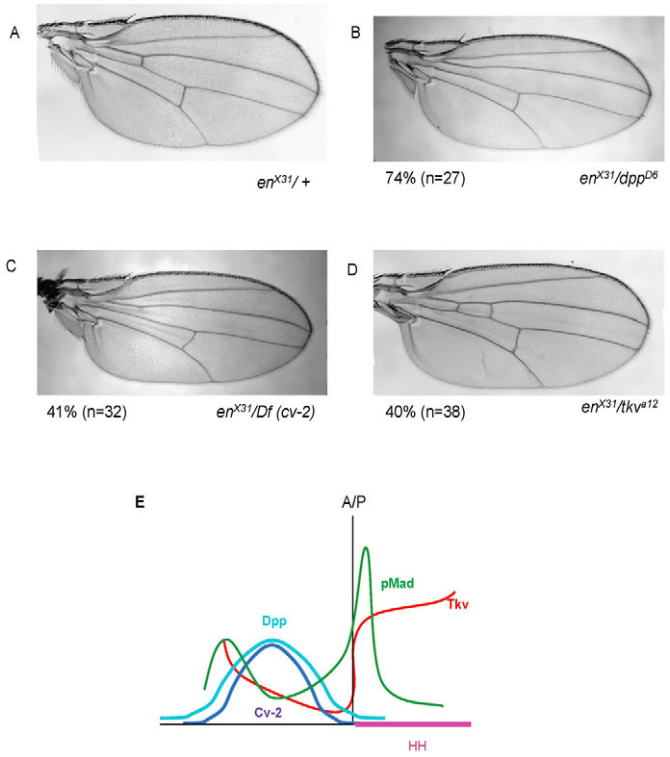

Fig. 6. Genetic interactions between *en* and different components of the Dpp signaling pathway. Interactions were tested between a deficiency uncovering *en* (en^{X31}) and mutations affecting genes involved in crossvein formation. (**A**) en^{X31}/+ adult *Drosophila* wings as control. (**B**) en^{X31}/dpp^{D6} adult wings, with 74% showing a missing ACV phenotype. An amorphic *en* mutation (en^{B86}) tested for interaction with dpp^{D6} gave the same adult wing phenotype and penetrance (see Fig. S11 in the supplementary material). (**C**) en^{X31}/*Df(2R)Pu-D17* adult wings [*Df(2R)Pu-D17* deletes the *cv-2* locus], of which 41% display a missing ACV phenotype. (**D**) en^{X31}/tkv^{a12} adult wings, of which 40% display two ACVs. *n*, the number of wings analyzed. (**E**) Patterns of Dpp signaling partner expression in the L3 wing disc that lead to pMad activation. A/P indicates the boundary between the anterior and posterior compartments.

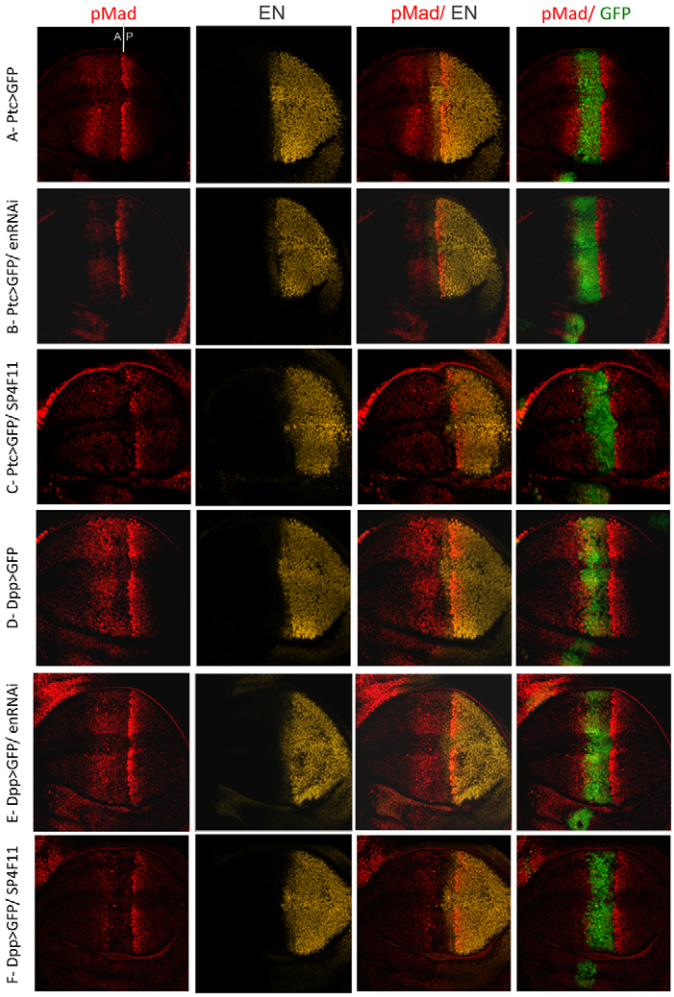

Fig. 7. En action on Dpp signaling in L3 larval wing imaginal discs. *Drosophila* L3 larval wing imaginal discs were analyzed for pMad (red) and En (yellow) expression in different genetic backgrounds. (**A**) *ptc*-Gal4; UAS-GFP. (**B**) *ptc*-Gal4; UAS-GFP/UAS-*en*RNAi. (**C**) *ptc*-Gal4; UAS-GFP/UAS-SP4F11. (**D**) *dpp*-Gal4; UAS-GFP. (**E**) *dpp*-Gal4; UAS-GFP/UAS-*en*RNAi and (**F**) *dpp*-Gal4; UAS-GFP/UAS-SP4F11. pMad/En and pMad/GFP merged images are shown in columns 3 and 4, respectively. GFP marks either the Ptc domain (A-C) or the Dpp domain (D-F), which correspond to the anterior six cell row domain that abuts the A/P boundary and covers three cell rows of the Ptc/En domain plus three cell rows of the Dpp domain that do not express En. Note that the high-level expression of pMad in the posterior cells abutting the A/P boundary is conserved in the different genetic backgrounds, in contrast to the pMad expression in the anterior cells (for quantification see Fig. S13 in the supplementary material).

These results establish that extracellular En enhances Dpp signaling, not only through a paracrine mode of action illustrated by its activity on the three anterior-most rows of Dpp-positive cells, but also in an autocrine manner on cells of the Ptc/En domain that secrete En protein. Notably, although the ACV is only formed during pupal development, we observed positive regulation of Dpp signaling by extracellular En as early as late L3. We thus propose that early activation by the En signal of Dpp signaling in the six cell row region where the presumptive ACV lies (see Fig. S3 in the supplementary material) is part of the molecular pathway that initiates ACV formation.

DISCUSSION

Direct, non-cell-autonomous homeoprotein activity has been previously reported in vivo for Pax6 (Lesaffre et al., 2007), Otx2 (Sugiyama et al., 2008) and En1/2 (Wizenmann et al., 2009). However, these reports only concern the developing vertebrate nervous system, raising the issue of the phylogenetic conservation and tissue specificity of this mode of signaling. Here, we show for the first time in vivo that homeoprotein signaling occurs outside of the nervous system and in an invertebrate. Specifically, using a series of biochemical and genetic tools we have demonstrated that En is secreted in *Drosophila* and participates in the morphogenesis of anterior structures of the wing, in particular in the formation of the ACV.

Performing detergent-free immunostaining, we first identified an extracellular pool of En protein that is produced both within the P compartment (En/Hh domain) and in the anterior-most region of the En domain (Ptc/En domain) (Fig. 5I). We also developed a tool

(SP4F11 secreted single-chain antibody) to interfere specifically with the extracellular En signal. Using this antibody, we found that whereas the formation of both the PCV and the ACV depends on En expression (Fig. 1A), the formation of the ACV requires extracellular En. Further, using particular Gal4 drivers, we showed that the pool of En protein secreted from the En/Hh P compartment is dispensable for ACV formation. By contrast, ACV formation requires an En signal that originates from the Ptc/En domain. We also found that En secreted from this Ptc/En territory acts both cell-autonomously in the same Ptc/En domain and non-cell-autonomously in territories in which *en* is never expressed (the Dpp domain) (Figs 5 and 7).

When expressed with the *dpp* driver, SP4F11 and *en*RNAi show different effects on ACV formation, ruling out an effect on En synthesis in this context. The fact that different effects are also observed on pMad levels strongly supports a role of extracellular En in ACV morphogenesis. Indeed, inhibition of Mad phosphorylation following SP4F11 expression does not correlate with a decrease in intracellular En levels (see Fig. S14 in the supplementary material). In view of all of these results, we propose that En should be considered not only as a nuclear transcription factor, but also as a short-range signaling molecule with morphogenetic activities.

Although our results demonstrate a function for secreted En, they do not establish that the protein is transferred into recipient cells, even though we favor this hypothesis on the basis of the presence of the internalization sequence in the En homeodomain (see Fig. S4 in the supplementary material) and by analogy with previous studies (Brunet et al., 2005; Tassetto et al., 2005). Technical limitations might derive from the assertion that, if En secretion is similar in flies and chick, its secretion would be limited to 5% of the intracellular content (Maizel et al., 1999; Joliot et al., 1997; Wizenmann et al., 2009).

Our results show that the extracellular and intracellular En pools have distinct activities. Indeed, based on genetic interactions between *en* and *dpp*, *tkv* or *cv-2* (Fig. 6) and on analysis of Mad phosphorylation (Fig. 7), we have established that extracellular En activity enhances Dpp signaling. This positive action of extracellular En on Dpp signaling contrasts with the known repressive role that En has on *dpp* expression when it acts as a transcription factor (Hidalgo, 1994; Sanicola et al., 1995; Maschat et al., 1998). In other words, a reduction of nuclear En might lead on the one hand to *dpp* activation, but on the other hand to reduced Dpp signaling. Considering that the initiation of ACV formation in the presumptive six cell row anterior domain requires Dpp signaling, the action of nuclear En on *dpp* expression must be compensated by an independent action of the En signal, which together with Cv-2 and Tkv modulates Dpp signaling.

How the En signal acts during wing morphogenesis remains an open question. A first observation is that the En signal does not cross the A/P boundary. Indeed, blocking the En signal from the posterior cells (in Hh>SP4F11 flies, for instance) does not modify the formation of the ACV, in sharp contrast with the absence of the ACV in Ptc>SP4F11 flies. This suggests that the extracellular En posterior pool, which is intact in this genetic background, is not able to compensate for this loss of En signal from the anterior cells. We found that the En signal from the posterior cells might have a function in the formation of the posterior margin that is completely independent of its role in the anterior part (see Fig. S15 in the supplementary material). Previous work in an in vitro model for axonal guidance showed that extracellular En must be internalized in order to exert its guidance activity, and that it regulates local protein synthesis within the growth cone (Brunet et al., 2005). A similar mechanism might be at work with En signaling in *Drosophila*. Indeed, we have identified an extracellular pool of En protein in the embryonic ventral nerve cord (S.L. and F.M., unpublished). This suggests that En secretion might be a general feature in *Drosophila*. In addition, we found that other homeoproteins, such as Ubx, are also potentially secreted, suggesting that this property of En might be extended to other homeoproteins in *Drosophila* (see Fig. S16 in the supplementary material).

Our observation that En signaling cooperates with the Dpp signaling pathway is reminiscent of recent observations made in the vertebrate nervous system that En, following internalization by growth cones, reduces the threshold of activation for the Ephrin signaling pathway (Wizenmann et al., 2009). The fact that En cooperates with the Ephrin and Dpp signaling pathways, as well as the existence of homeoprotein signaling in plants (Lucas et al., 1995; Tassetto et al., 2005), support the notion that homeoproteins are very ancient signaling entities and that distinct classical transduction mechanisms have been recruited more recently, possibly because they increase developmental and physiological robustness.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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