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Genetic and epigenetic mechanisms regulating *hedgehog* expression in the *Drosophila* wing

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ABSTRACT

Stable subdivision of *Drosophila* limbs into Anterior (A) and Posterior (P) compartments is a consequence of asymmetric signaling by Hedgehog (Hh) from P to A cells. The activity of the homeodomain protein Engrailed (En) in P cells has been reported to help to generate this asymmetry by inducing the expression of *hedgehog* and simultaneously repressing the expression of the essential downstream component of the Hh signaling pathway Cubitus interruptus (Ci). In A cells, Ci has a major role in the repression of *hh*. Here we have revised the genetic and epigenetic mechanisms involved in the regulation of *hh* in the P compartment. First, we present evidence that *hh* expression in P cells is a consequence of the repression of *ci* by the activity of En. Thus, in the absence of Ci and En activities, cells do express *hh*. We also present data supporting the maintenance of *hh* expression in P cells through epigenetic mechanisms, and a permissive role of Notch signaling in this process. Notch and Trithorax (TrxG) group of proteins exert their action through a previously defined *hh* Polycomb Responsive Element (PRE).

Introduction

Drosophila limb primordia are subdivided into adjacent territories called compartments, cell populations that do not mix during development (García-Bellido et al., 1973). Short-range cell interactions between adjacent compartments lead to the restricted expression or activity of organizing molecules to the compartment boundaries (Basler and Struhl, 1994; Capdevila et al., 1994; Capdevila and Guerrero, 1994; de Celis et al., 1996; Diaz-Benjumea and Cohen, 1995). These organize the growth and pattern of the developing limb primordia.

Two orthogonal compartment boundaries behave as signaling centers and organize the growth and pattern of the developing wing primordium (Fig. 1A). Activation of the receptor Notch along the dorsal–ventral (DV) compartment boundary and the long range activity of the signaling molecule Dpp, a member of the BMP/TGF-β family, expressed along the anterior–posterior (AP) compartment boundary, execute the organizing activities of these signaling centers (Capdevila and Guerrero, 1994; de Celis et al., 1996; Diaz-Benjumea and Cohen, 1995; Zecca et al., 1995). The formation of the AP compartment boundary in *Drosophila* limbs and the restricted expression of Dpp along this boundary relies on asymmetric signaling of the secreted molecule Hedgehog (Hh) from P to A cells (Dominguez et al., 1996). This asymmetry is generated by the complementary activities of Engrailed/Invected and Cubitus interruptus (Ci) transcription factors in P and A cells, respectively (Fig. 1B). The homeodomain proteins Engrailed (En) and Invected induce *hh* and repress *ci* expression, the essential downstream component of the Hh signaling pathway, in P cells (Eaton and Kornberg, 1990; Tabata et al., 1995; Zecca et al., 1995). Ci represses *hh* expression in A cells (Apidianakis et al., 2001; Bejarano et al., 2007; Méthot and Basler, 1999) and, at the same time, confers the capacity to respond to Hh coming from P cells, thereby inducing Hh target gene expression. Thus, P cells express Hh while A cells respond to it.

The Polycomb (PcG) and the Trithorax (TrxG) group of proteins form the basis of a cellular memory system that maintains the transcriptional state of their target genes heritable during development (reviewed in Muller and Kassis, 2006; Schwartz and Pirrotta, 2007). The genes controlled by the PcG/TrxG system have PcG response elements (PREs), to which these proteins bind and either keep the gene permanently repressed (PcG) or active (TrxG). Some PREs have been shown to maintain the initial transcriptional state of a nearby reporter gene through several rounds of mitosis during development, and as such they have been termed Cellular Memory Modules (CMM, (Cavalli and Paro, 1998)). Interestingly, a 3.4-kb long fragment situated upstream of the *hh* transcription start site behaves as a PRE and exhibits CMM activity (Chanas and Maschat, 2005; Maurange and Paro, 2002). In A cells, PcG genes are involved in maintaining the repressive transcriptional state of *hh* (Randsholt et al., 2000), while TrxG genes maintain the active transcriptional state of *hh* once ectopically induced (Maurange and Paro, 2002). However, the role of the PcG and TrxG genes in maintaining *hh* expression in its endogenous expression domain, the P compartment, has not been studied to date.

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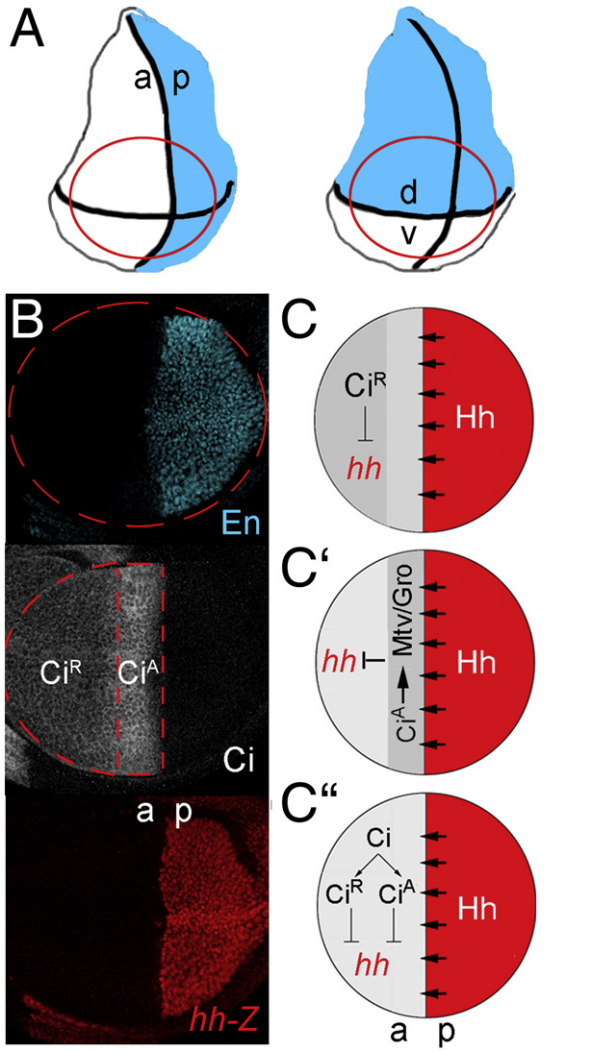

Fig. 1. Repression of *hh* in A cells: a central role of Ci. (A). The wing primordium is subdivided into anterior (a) and posterior (p) compartments, as well as into dorsal (d) and ventral (v) compartments. The contour of the wing pouch, which will give rise to the adult wing, is labeled with a red line. (B) Wild-type wing imaginal disc labeled to visualize Engrailed protein (En, blue), Cubitus Interruptus protein (Ci, white) and *hh-lacZ* (antibody to β-Gal, red) expression. The contour of the wing pouch is labeled with a dashed red line and the regions expressing the two distinct forms of Ci (Ci^A and Ci^R, see below) are also labeled. (C, C') Illustrations describing the two mechanisms involved in the repression of *hh* expression in the anterior compartment of the wing pouch. In anterior cells that do not receive the Hh signal, Ci is transformed to a transcriptional repressor (Ci^R), which represses *hh* expression (C). In anterior cells that do receive the Hh signal, the transcriptional activator form of Ci (Ci^A) is stabilized and induces the expression of Mtv, which, together with Gro, represses *hh* expression (C'). (C") Summary describing how the two forms of Ci repress *hh* expression in A cells. Anterior (a) and posterior (p) compartments are indicated.

Here we analyzed the contributions of En, Ci and the PcG/TrxG system in the regulation of *hh* expression in P cells of the *Drosophila* wing. First, we show that the initial transcriptional state of *hh* is a direct consequence of the presence or absence of Ci. It is well known that in A cells Ci represses *hh* expression (Apidianakis et al., 2001; Bejarano et al., 2007; Méthot and Basler, 1999), while En activity in P cells represses Ci expression (Eaton and Kornberg, 1990)(Dominguez et al., 1996). We show that in the absence of Ci and En activities, wing discs cells express *hh*. Thus, Engrailed is required in P cells to relieve Ci-mediated repression of *hh*. We also present evidence that TrxG genes are involved in the maintenance of *hh* expression in P cells. Interestingly, epigenetic maintenance of *hh* expression is positively regulated by the activity of Notch, whose activation is spatially restricted in the wing primordium. We show that Notch is required together with TrxG genes to maintain *hh* expression and they do so through the previously defined *hh* CMM (Maurange and Paro, 2002). To our knowledge, this is the first report in which Notch has been implicated in regulating the activity of a particular PRE.

Materials and methods

Drosophila strains

We used the following fly strains: *Df(2R)en[E]* deletes both *engrailed* and *invected* (Gustavson et al., 1996); *UAS-*N^{INTRA} (Struhl and Adachi, 1998); *UAS-*mam^{DN} (Helms et al., 1999);ci^{94} and *UAS-*Ci^{Cell} (Méthot and Basler, 1999); UAS-Ci^{3m} (a constitutively active activator form of Ci caused by mutated PKA phosphorylation sites (Price and Kalderon, 1999); vg^{Gal4} (the boundary enhancer of vestigial driving the expression of Gal4, (Williams et al., 1994); *hh-PRE-UAS-lacZ* (a 3.4-kb fragment containing the PRE, identified in the *hh* promoter region and linked to a GAL4/UAS-inducible *lacZ* gene (UAS-*lacZ*) (Maurange and Paro, 2002); *UAS-lacZ*, *UAS-GFP*, hh^{P30} (*hh-lacZ* in the text), *dpp-lacZ* (Flybase); ph^{504} (Randsholt et al., 2000); *UAS-*pho^{RNAi} and *UAS-*trx^{RNAi} (Vienna *Drosophila* RNAi Center); *UAS-*N^{dsRNA} (Presente et al., 2002); and *Actin>CD2>Gal4* (Pignoni and Zipursky, 1997).

Antibodies

Rat anti-Ci (Motzny and Holmgren, 1995), rabbit anti-βGal (Cappel), rabbit anti-GFP (Upstate), mouse anti-En (4D9), mouse anti-Wg (4D4) and rabbit anti-βGal (40-1A) are described in the Developmental Studies Hybridoma Bank.

Genetic mosaics

The following *Drosophila* genotypes were used to generate loss-of-function clones by the classic FLP/FRT system (Xu and Rubin, 1993):

hs-FLP; FRT42B Df(2R)en[E] / FRT42B Ubi.GFP; hh-lacZ/+

hs-FLP; FRT42B Df(2R)en[E] / FRT42B Ubi-GFP P(ci+); hh-lacZ/+ ; ci^{94}

(note in this case that the *P(ci+)* construct rescues the ci^{94} mutant background, and the FLP/FRT mediated recombination induces cells mutant for *en* and *ci* in an otherwise wild type background).

The following *Drosophila* genotypes were used to generate loss-of-function clones by the MARCM (mosaic analysis with a repressible cell marker) technique to simultaneously express diverse transgenes in the clones (Lee and Luo, 2001):

hs-FLP tub-Gal4 UAS-GFP; FRT42B Df(2R)en[E]/ FRT42B Gal80; UAS-N^{INTRA}*/ hh-lacZ*

hs-FLP tub-Gal4 UAS-GFP; FRT42B Df(2R)en[E]/ FRT42B Gal80; UAS-mam^{DN}*/ hh-lacZ*

hs-FLP tub-Gal4 UAS-GFP; FRT42B Df(2R)en[E]/ FRT42B Gal80; UAS-trx^{RNAi}*/ hh-lacZ*

To generate clones expressing Ci^{3m} or Ci^{Cell}, *Actin>CD2>Gal4; hh-Z* females were crossed with *hs-FLP; UAS-* Ci^{3m} or with *hs-FLP; UAS-*Ci^{Cell} males, respectively.

Clone induction by heat-shock was carried out 2–4 days before dissection of larval discs.

Spatiotemporal gene expression targeting in Drosophila

We used the TARGET system developed by (McGuire et al., 2004). Adult flies carrying a Gal4 driver, the Gal80ts construct and a UAS-transgene were allowed to lay eggs over a period of 24 h at 18 °C. The progeny was then raised at 18 °C to maintain the Gal4/UAS system inactivated and transferred to 29 °C for a period of 48 h before