RESEARCH ARTICLE

Specific expression and function of the Six3 optix in Drosophila serially homologous organs

Amer Al Khatib1,2, Natalia Siomava3,*, Antonella Iannini1, Nico Posnien3 and Fernando Casares1,4

ABSTRACT

Organ size and pattern results from the integration of two positional information systems. One global information system, encoded by the Hox genes, links organ type with position along the main body axis. Within specific organs, local information is conveyed by signaling molecules that regulate organ growth and pattern. The mesothoracic (T2) wing and the metathoracic (T3) haltere of Drosophila represent a paradigmatic example of this coordination. The Hox gene Ultrabithorax (Ubx), expressed in the developing T3, selects haltere identity by, among other processes, modulating the production and signaling efficiency of Dpp, a BMP2-like molecule that acts as a major regulator of size and pattern. However, the mechanisms of the Hox signal integration in this well-studied system are incomplete. Here, we have investigated this issue by studying the expression and function of the Six3 transcription factor optix during Drosophila wing and haltere development. We find that in both organs, Dpp defines the expression domain of optix through repression, and that the specific position of this domain in wing and haltere seems to reflect the differential signaling profile among these organs. We show that optix expression in wing and haltere primordia is conserved beyond Drosophila in other higher diptera. In Drosophila, optix is necessary for the growth of wing and haltere. In the wing, optix is required for the growth of the most anterior/proximal region (the ‘marginal cell’) and for the correct formation of sensory structures along the proximal anterior wing margin; the halteres of optix mutants are also significantly reduced. In addition, in the haltere, optix is necessary for the suppression of sensory bristles.

KEY WORDS: Imaginal disc, Wing, Haltere, Dpp signaling, Six3, Optix, Drosophila

INTRODUCTION

During development, several positioning systems inform cells of their location. First, the Hox code defines position along the anterior-posterior axis. In insects, this system generates segmental diversity along this body axis. Next, in each segment, cells within an organ primordium obtain positional information from local signaling centers (Mann and Carroll, 2002). The Drosophila wing and haltere primordia constitute a paradigm in which the integration of these two positional systems has been intensively investigated. In the Drosophila thorax (T), the second and third segments develop two serially homologous organs: the wing (in T2) and the haltere (in T3). The specific expression of the Hox gene Ultrabithorax (Ubx) in T3 is responsible for the specific morphology of the haltere, a small modified wing that acts as a balancing organ during Drosophila flight. Mutants that cause the loss of Ubx during haltere development cause its transformation into wing (Lewis, 1978), while ectopic expression of Ubx in the developing wing results in wings transformed into haltere-like appendages (Gonzalez-Gaitan et al., 1990).

One of the major organ-positioning systems in the wing and haltere primordia, or ‘imaginal discs’, is a stripe of cells that bisects the disc and produces a BMP2 ligand, encoded by decapentaplegic (dpp). From this stripe, Dpp generates a signaling gradient that patterns the anterior-posterior axis of the appendage (Restrepo et al., 2014). In the wing, which is the ground state of the dorsal appendage, the Dpp gradient activates the nested expression of a number of target genes at different concentrations, such as spalt (sal), referring collectively to two highly related paralogous genes, sal-m and sal-r and optomotor blind (omb) (Nellen et al., 1996). The activation of sal and omb is indirect through Dpp signaling repressing brinker (brk), itself a repressor of the Dpp pathway (Campbell and Tomlinson, 1999; Winter and Campbell, 2004). Additionally, sal is also directly activated by the Dpp signaling cascade (Barrio and de Celis, 2004). In this way, Dpp controls the positioning of central pattern elements, such as the wing veins (de Celis et al., 1996; Sturtevant et al., 1997). In addition to patterning, Dpp signaling controls organ growth, so that mutants that lack Dpp signaling result in very reduced winglets (Posakony et al., 1990).

In the haltere, Ubx modifies the wing developmental program in two ways. First, as a transcription factor, Ubx regulates the expression of some targets. For example, Ubx represses sal expression (Weatherbee et al., 1998). Second, Ubx modifies the shape of the Dpp-generated signaling gradient indirectly, by controlling the expression of proteoglycans required for Dpp dispersion (Crickmore and Mann, 2006; de Navas et al., 2006b). Globally, these modifications of Dpp signaling and target gene activation by Ubx have been related to the size and patterning differences between halteres and wings.

Since Dpp signaling generates a signaling gradient that spans the whole wing pouch and its activity is required throughout the wing, it is expected to control the expression of target genes not only in central region of the pouch, but also in more lateral ones. The Six3-type transcription factor optix has been reported to be expressed in the lateral region of the wing pouch, as well as in the haltere (Seimiya and Gehrung, 2000). Functional studies show that optix is required for the normal patterning of the anterior portion of the wing.
and that its expression is negatively regulated by \textit{sal} genes (Organista et al., 2015). As \textit{sal} genes are Dpp signaling targets in the wing, this places \textit{optix} downstream of Dpp regulation. However, because \textit{sal} genes are not expressed in haltere discs (Weatherbee et al., 1998), the mechanism of \textit{optix} regulation in this organ is still unknown. Here, we analyze comparatively the expression, function and regulation of \textit{optix} in wing and haltere discs. In both discs, \textit{optix} expression is anteriorly restricted by Dpp signaling, although in the wing the precise expression boundary may be set with the collaboration of wing-specific Dpp targets, such as \textit{sal}. We show that \textit{optix} shows organ-specific functions: in the wing, we confirm previous results showing that it is necessary for the growth of the anterior/proximal wing (‘marginal cell’) and the development of wing margin sensory bristles. However, in the haltere, \textit{optix} is required for the suppression of sensory bristle formation. Overexpression of \textit{optix} in the entire wing pouch only affects anterior wing development, suggesting that other parts of the wing cannot integrate ectopic Optix input. This observation may provide a mechanistic explanation for a widespread re-deployment of \textit{optix} expression in wing spot formation in various butterfly species.

**RESULTS**

**The Six3 gene \textit{optix} is differentially expressed in the wing and haltere discs**

\textit{Optix} transcription, detected using RNA \textit{in situ} hybridization, is found in both the wing and haltere imaginal discs of late third instar (L3) larvae (Organista et al., 2015; Seimiya and Gehring, 2000) (Fig. 1A) in the pouch regions of both discs. These pouch regions give rise to the wing proper and the distal haltere’s article (capitellum), respectively (Fig. 1C) (Cohen, 1993). To examine the expression of \textit{optix} in detail, we used an Optix:GFP line (Sarov et al., 2016) that recapitulates \textit{optix} expression (Fig. 1B). We first mapped \textit{optix}-expressing domains in the wing and haltere discs relative to the anterior-posterior (AP) and dorso-ventral (DV) boundaries. We used \textit{apterous (ap; ap-Z)}, as a D marker, and \textit{hedgehog (hh; hh-Z)} as a P marker. Relative to the DV axis, \textit{optix} straddles symmetrically the DV boundary in both wing and haltere discs (Fig. 1D,E). \textit{optix} expression is restricted to the A compartment in both discs (Fig. 1F,G). However, the position that the \textit{optix} domain occupies along the AP axis is different: in the wing, \textit{optix} is restricted to the anteriormost region of the pouch, while in the haltere it occupies a more central position, closer to the AP border.

\textit{optix} expression in wing and haltere discs is conserved within higher diptera

\textit{Drosophila melanogaster} is a highly derived dipteran. To test whether the wing and haltere expression of \textit{optix} is conserved beyond \textit{Drosophila}, we analyzed the expression pattern of the \textit{optix} homologues in two Schizophoran fly species: \textit{Ceratitis capitata} (Tephritidae) and \textit{Musca domestica} (Muscidae). Using \textit{in situ} hybridization, \textit{optix} is detected in equivalent patterns in wing and haltere discs of these two species (Fig. 2A-D), indicating that \textit{optix}...
expression pattern is conserved during wing and haltere development within higher diptera.

**optix regulates size of wing and haltere**

In order to determine the role played by optix, we drove an optix-RNAi to the distal wing and haltere discs, using the nubbin-GAL4 (nub>) driver (Fig. 3A; Fig. S1). The adult wings of nub-GAL4:UAS-optixRNAi (’nub>optixRNAi’) flies were bent and smaller than those of their nub>+ siblings (Fig. 3B) (see similar results by Organista et al., 2015). This phenotype seemed to be mostly due to a much shorter longitudinal vein 2 (L2) and a reduction of the wing blade area anterior to this vein (the so-called ‘marginal cell’, in between veins 1 and 2 (Fig. 3C,D) to ~25% of the normal area. The density of trichomes in the wing tissue, which can be used as a proxy for cell size, is very similar in the marginal cell of nub>+ and nub>optixRNAi (density of trichomes in nub>optixRNAi is 0.93 times that of nub>+ controls). Therefore, the area reduction of the marginal cell is the result of reduced growth. This area reduction is accompanied by a loss of margin sensory bristles (Fig. S2). In the haltere, optix attenuation also resulted in significantly smaller organ size: the perimeter of nub>optixRNAi halteres is 75% that of controls (see Materials and Methods for statistical analysis). In addition, nub>optixRNAi halteres developed extra bristles in the capitellum, similar to those found in the halteres of Ubx+/− heterozygous individuals (Fig. 3E-G), which in this genotype is larger than wild type.

So far, our results indicated that optix is expressed at a different position along the AP axis of wing and haltere primordia, where it plays a role in organ size control. We decided to investigate next the mechanism responsible for the precise AP positioning of the optix domain.

**optix expression is complementary to high Dpp signaling levels**

In the wing and haltere primordial, AP information is conveyed by Decapentaplegic (Dpp), a BMP2-like molecule. Dpp is produced in a stripe along the AP axis from where it diffuses, creating a signaling gradient (Restrepo et al., 2014). Cells regulate gene expression according to the signaling levels they perceive, i.e. according to their position. The read-out of this Dpp signaling gradient is the phosphorylated form of the transcription factor Mad (pMad) (Sekelsky et al., 1995). However, the shape of the Dpp signaling

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Fig. 2. optix expression in other dipteran species. (A-D) In situ hybridization detection of optix in wing (A,C) and haltere (B,D) discs of third instar larvae from C. capitata and M. domestica. In both species, the expression pattern in wings and haltere discs is very similar to the one seen in D. melanogaster.

Fig. 3. optix knockdown affects wing and haltere development. (A) Expression of optix-RNAi is driven by the nubbin-GAL4 (nub>) driver. In wing and haltere discs, nub> drives expression in the central portion of the discs, including the wing and haltere pouches (outlined by the dashed white line), as observed by GFP expression in nub>GFP discs. Discs were counterstained with rhodamine-phalloidin, which marks filamentous actin (red, actin). Scale bar: 50 μm. (B) Male adult nub>optixRNAi. The inset shows a frontal view. Wings show a characteristic bend (arrows). (C,D) Wings from control (nub>+) (C) and nub>optixRNAi (D) males. In nub>optixRNAi, the L2 (arrow) is shorter and the wing area between the margin and L2 is severely reduced compared to the control. (E-G) Halteres from adult nub>+(E), nub>optixRNAi (F) and TM2, Ubx+/+(G) males. Red arrows point to ectopic bristles.
unambiguously assess the role of Dpp in the regulation of action of the Dpp pathway on wing disc (Fig. 5A,B). Although this result suggested a repressive blockade of the signaling. This was especially noticeable in the optix expression domain extended towards the disc center. In these regions of high and intermediate signal (100-600 arbitrary units) in the wing (Fig. 4B) as well as in the palate, where optix was displaced relatively to the pMad gradient, so that its expression was excluded from the peak pMad signal (100-150 arbitrary units) (Fig. 4A-D). This meant that the expression domain of optix relative to the Dpp signaling intensity was similar in both discs despite their optix domains being located far from (in the wing) or adjacent to (haltere) the AP border (Fig. 1). The complementarity of expression suggested that the positioning of optix expression was set by Dpp repressing optix.

Dpp signaling represses optix and sets the limits of its expression domain

Dpp signaling could be repressing optix directly or indirectly, through some of its targets. To test the hypothesis of a repressor role for the Dpp pathway, we first attenuated the expression of the Dpp type II receptor punt (Letou et al., 1995; Ruberte et al., 1995) using a punt-specific RNAi (Fig. 5). In nub>puntRNAi wing and palate discs the optix domain extended towards the disc center. In these discs, the pMad signal from the pouch is absent, confirming the blockade of the signaling. This was especially noticeable in the wing disc (Fig. 5A,B). Although this result suggested a repressive action of the Dpp pathway on optix, it could not rule out that low levels of Dpp signaling could be activating optix as, in our experiment, punt levels had been attenuated using an RNAi. To unambiguously assess the role of Dpp in the regulation of optix, we induced loss-of-function clones of tkv, the Dpp type I receptor (Brummel et al., 1994; Nellen et al., 1994). In these clones, which grow poorly and tend to extrude from the epithelium (Burke and Basler, 1996), we detected depression of optix:GFP in clones all along the AP axis of the wing (Fig. 5C). As these clones cannot transduce the Dpp signal, we conclude that Dpp signaling is a repressor of optix.

To examine the possibility that the repressive action was exerted through some of its targets, we analyzed the expression of optix in wing and palate discs relative to two known Dpp target transcription factors, spalt (sal) and optomotorblind (omb) (de Celis et al., 1996; Grimm and Pfullger, 1996; Kim et al., 1996; Nellen et al., 1996; Sturtevant et al., 1997). In the wing, the sal and optix domains are separated by an intermediate zone and do not overlap (Fig. 6A,A’). In the palate pouch, sal is not expressed (Fig. 6B,B’) and yet, as we showed above, optix expression is excluded from the regions of intermediate/high Dpp signal. These results do not rule out sal as an optix repressor in the wing, but suggest that it cannot be the sole repressor, as it is absent from the palate. Indeed, Organista et al. (2015) have shown that in sal-mutant wing discs, optix expression extends towards the disc’s center, but does not reach the AP boundary, indicating that additional Dpp-dependent mechanisms for optix repression must exist. Next, we analyzed the expression of optix relative to omb in omb-GAL4; UAS-cherry-RFP. While in the wing optix and omb were complementary to one another (Fig. 6C,C’), in the palate we detected significant overlap between both genes (Fig. 6D,D’). Therefore, omb does not seem to fulfill the repressor role either, because of its coexpression with optix in the palate.

optix seems to function parallel or downstream of Ubx

We found that there is a twofold relationship between optix and Ubx. On the one hand, Ubx is responsible for the modification of Dpp positional system in the palate, which then sets optix domains along the AP position. On the other, optix expression is required in the palate to suppress the formation of sensory bristles in this organ, a function known to be exerted by Ubx (Garcia-Bellido and Lewis, 1976; Weatherbee et al., 1998). In principle, this latter phenotype could be produced if optix were required for either Ubx expression or function. Alternatively, optix could be required for one Ubx activity: the suppression of bristle development. We tested the first possibility by examining Ubx expression in nub>optixRNAi palate discs, stained with an anti-Ubx antibody. In these discs we did not observe any change in Ubx protein levels relative to controls (Fig. 5A,B). This result was not unexpected, as a reduction in Ubx levels would have led, in addition to extra bristles, to an increase in palate size, something we do not observe in nub>optix RNAI individuals. Therefore, we favor the second alternative: that, in the palate, optix is necessary for bristle suppression parallel or downstream of Ubx.

Forced expression of optix throughout the wing disc results in extravenation in the anterior wing, but does not affect the rest of the organ

The fact that optix expression was restricted to the anteriormost region of the wing disc made us ask whether optix might affect wing development if ectopically expressed throughout the developing wing. We tested this by driving a UAS-optix transgene using nub-GAL4. Wings of nub>optix adults showed extravenation in the margin cell, the region in which optix is normally expressed and required (Fig. 7). However, the rest of the wing remained unaltered. This lack of effect was particularly unexpected: Optix is a transcription factor and we would have predicted that, similarly to what happens in the anteriormost wing, cells elsewhere in the pouch would have responded to its ectopic expression. This to us suggests that most parts of the wing pouch are “protected” from the action of Optix, either by lack of available DNA target sequences (an epigenetic effect) or the absence of a positive cofactor (or the presence of a repressor).

DISCUSSION

The Dpp signaling gradient is required for the patterning of the whole wing, from the center to its margin (Lecuit et al., 1996; Nellen et al., 1996; Posakony et al., 1990). This gradient is translated into a series of contiguous domains expressing distinct transcription factors, each required for the specification of specific features in the adult organ (Lawrence and Struhl, 1996; Restrepo et al., 2014). However, while the transcription factors acting in the central wing were known, the most anterior region of the wing, the region between the longitudinal vein 2 (L2) and the anterior margin (L1), lacked a specific transcription factor. In this paper, we show that this transcription factor, or at least one of them, is Optix.

Our results confirm previous findings (Organista et al., 2015) that optix is expressed in, and required for the growth of, this most
anterior sector of the wing, the so-called margin cell. We now show that optix is also required for the growth of the wing’s serially homologous organ, the haltere. This role is in agreement with previous results showing that Six3 regulates cell proliferation in vertebrate systems (Del Bene et al., 2004; Gestri et al., 2005). We further show that Dpp signaling plays a major role in setting the optix expression domain. Although it has previously been reported that sal genes are required to set the central limit of this domain, in discs lacking sal function, optix does not extend all the way to the AP border (Organista et al., 2015), suggesting additional mechanisms involved in optix repression. The fact that sal is not expressed in the haltere pouch and optix does not extend all the way to the AP border, the exclusion of optix expression from intermediate/high Dpp signaling in both wing and haltere, and the requirement of Dpp signaling to repress optix in any position of the anterior wing compartment globally suggested to us that either Dpp activates a different repressor closer to the AP border, or that Dpp signaling represses directly optix transcription. Our work cannot distinguish between these possibilities. Regarding another well-characterized Dpp target, omb, the extensive coexpression of omb

![Fig. 4. optix expression relative to the pMad signaling profile. (A-D) Optix:GFP wing (A) and haltere (C) discs stained for pMad (red), and counterstained with the nuclear marker DAPI (blue). GFP and pMad profiles for wing (B) and haltere (D) discs, across the solid white lines, are shown. Fluorescence intensity is provided in arbitrary units. A, anterior compartment; P, posterior compartment. The AP border is marked by the dashed white lines. Scale bars: 50 µm.](http://bio.biologists.org/)

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and optix in the haltere also seems to exclude omb as a repressor. Therefore, either another unknown repressor exists, or Dpp signaling acts as a direct optix repressor. While in the haltere, the domain of optix would be set directly by Dpp; in the wing, sal would be an additional repressor. By intercalating sal, the Dpp positioning system may be able to push the limit of optix expression farther away from the AP border of the wing. The Sal proteins have been previously shown to act as transcriptional repressors of knirps (kni) to position vein L2 (de Celis and Barrio, 2000). Thus, adding sal repression may help to align the optix domain with L2.

Fig. 5. Dpp signaling represses optix:GFP. (A,B) nub>optixRNAi;optix:GFP wing (A) and haltere (B) discs. The optix:GFP signal expands. In these discs, the pMad signal characteristic of the pouch is lost. Discs are counterstained with DAPI. Scale bars: 50 μm. (C) optix:GFP (green) wing disc containing tkv-mutant clones. The clones are marked by the absence of armZ (red). Anterior is left, dorsal up. (C’) A close-up of the boxed region in C. (C’’) An (x,z) confocal section along the dashed yellow line. The colored circles mark some clones in C’ and C’’. These clones, which are small and sort out basally, derepress optix:GFP cell autonomously.
additional repression would not be operating in the haltere, which lacks venation.

Interestingly, the logic of optix regulation by Dpp is different from that of other Dpp targets. The activation of the sal paralogs (sal-m and sal-r) and aristaless (al), another target required for vein L2 formation (Campbell and Tomlinson, 1998), proceeds through a double repression mechanism: in the absence of signal, the Brinker repressor keeps sal and al off. Activation of the pathway leads to the phosphorylation of the nuclear transducer Mad (pMad) which, in turn, relieves the repression on sal and al (Campbell and Tomlinson, 1999; Moser and Campbell, 2005). Therefore, optix regulation by Dpp signaling could be more direct, similar to that of brk.

One interesting aspect of optix function is that it plays an additional specific role in the haltere. In the wing, optix is required for the development of the anteriormost portion of the wing (including the margin bristles); in the haltere, optix serves to suppress the development of sensory bristles, a task known to be carried out by the Hox gene Ubx. We have ruled out a role for optix in regulating Ubx expression, at least when judged from Ubx protein levels (Fig. S3). Therefore, optix is required for a subset of the normal functions of Ubx. Since optix encodes a Six3-type transcription factor, this interaction could be happening at the level of target enhancers, where the combination of Ubx and Optix would allow the activation or repression of specific sets of genes.

Finally, we have observed that the expression of optix in wing and haltere primordia is conserved across higher Diptera (Fig. 2). Interestingly, optix is expressed in the developing wings of passion vine butterflies (genus Heliconius). In Heliconius species, optix has been co-opted for red color patterning in wings (Reed et al., 2011). However, the ancestral pattern found in basal Heliconini is in the proximal complex, a region that runs along the base of the forewing costa, the most anterior region of the forewing (Martin et al., 2014). This similarity between optix expression patterns in forewings of
Diptera and Lepidoptera make us hypothesize that an ancestral role of optix might have been 'structural', being required for the development of the anterior wing. Once expressed in the wings, recruitment of red pigmentation genes allowed optix co-option for color pattern diversification through regulatory evolution (Martin et al., 2014). We note that a prerequisite for this co-option in wing pigmentation patterning must have been that optix could not engage in promiscuous gene regulation, and that its function depends on other competence factors, which would limit its gene expression regulatory potential.

In situ hybridization

Images of optix mRNA expression in wing and haltere discs are unpublished data kindly shared by P. Tomancak (MPI-CBG, Dresden, Germany) and C. Dahmann (Technische Universität, Dresden, Germany), obtained using probes and methods described in Tomancak et al. (2007).

Orthologous optix sequences for C. capitata and M. domestica were obtained by NCBI BLAST starting with the Drosophila optix sequence. Fragments were amplified with generic-specific primers for C. capitata: forward, GACGCAGGAGGCGGAAACTCCTCC and reverse, GTTCAAGCTATGCGCCTGTGCCGGC; and for M. domestica: forward, GACGGACGGAGGGCAAACATCCTCC and reverse, CGGCCGCATCCAGTTTAAACGAAGGC. The digoxigenin (DIG)-labeled antisense RNA probes were synthesized from purified PCR products by using the DIG DNA Labeling Mix, T7-RNA Polymerase, and Protector RNase Inhibitor (Roche Applied Science, Mannheim, Germany) and fragmented to an average length of ~200 bp by adding an equal amount of sodium carbonate buffer (80 mM NaHCO₃, 120 mM Na₂CO₃, pH 10.2) and incubating at 60°C. Fragmented probes were diluted with HybeA buffer (0.5% formamide, 0.1 μg/ml sonicated salmon sperm DNA, 50 μg/ml Heparin, 5× SSC and 0.1% Triton X-100, in PBS) and used for in situ hybridization.

Wing and haltere imaginal discs were dissected from C. capitata and M. domestica third instars and fixed in 4% PFA for 30 min. After fixation, samples were washed three times with PBT for 20 min, rinsed once with 1:1 HybeA/PBT, and quickly washed three times with HybeA. Prehybridization was performed in HybeA at 65°C for 1 h. Preheated and chilled down probes were added to samples and hybridized overnight at 65°C. The next day, probes were discarded, samples were washed three times with preheated HybeA at 65°C for 20 min and one time with 1:1 HybeA/PBT, and incubated with 1 ml anti-DIG-AP antibody (Anti-Digoxigenin-AP, Fab fragments, Roche Applied Science, Mannheim, Germany, diluted 1:2000 in PBT) at room temperature for 1 h. Antibodies were removed, samples were washed three times with PBT for 20 min, and then washed three times with a freshly prepared detection NBT buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl₂, 0.1% Triton-X-100).
were expressed in arbitrary units. Constant so that intensity profiles would be comparable. Intensity profiles are considered. Within each experiment, confocal settings were maintained.


