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Original Research

Phosphatase of Regenerating Liver-1 Regulates Wing Vein Formation through TGF-β Pathway in *Drosophila melanogaster*

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Abstract

Background: *Drosophila* Phosphatase of Regenerating Liver-1 (PRL-1) is the only homolog of the mammalian PRLs with which it shares high sequence and structural similarities. Whilst PRLs are most notable for their high expression in malignant cancers and related promotion of cancer progression, the specific biological functions of the PRLs remain largely elusive. **Methods**: Here, using a gain-of-function approach, we found that PRL-1 functions during wing vein development in *Drosophila melanogaster* (*Drosophila*). Overexpression of *Drosophila* PRL-1 caused dose-dependent wing vein proliferation. **Results**: Genetic screening of the main TGF-β signaling factors, Mad and Smox, showed that the RNAi-mediated knockdown of Mad could alleviate the extra vein phenotype caused by overexpressed PRL-1 and lead to loss of the posterior section of longitudinal veins. However, knockdown of Smox resulted in an identical phenotype with or without the overexpression of *Drosophila* PRL-1. Clonal analyses revealed that overexpression of PRL-1 led to decreased expressions of activated phospho-Mad protein, as measured by immunostaining. Real-time PCR showed that the transcriptional levels of *Smox* were significantly increased upon overexpression of the *Drosophila* PRL-1 in wing discs, with a dose dependent effect. **Conclusions**: We propose that the main function of *Drosophila* PRL-1 in wing development is to affect the phospho-Mad levels and *Smox* transcriptional levels, therefore influencing the competitive balance for Medea between Mad and Smox. Our study demonstrates the novel role for *Drosophila* PRL-1 in regulating TGF-β signaling to influence wing vein formation which may also provide insight into the understanding of the relationship between PRLs and TGF-β signaling in mammals.

Keywords: PRL-1; Smox; TGF-β; wing vein; *Drosophila*

1. Introduction

Drosophila PRL-1 is the only homolog for the three human PRL proteins, PRL1, PRL2, and PRL3. The PRLs belong to a family of dual specificity phosphatases (DSP) classified as a subgroup of protein tyrosine phosphatases (PTP). They were originally discovered as immediate-early gene products in the regenerating liver [1–3]. Mammalian PRLs commonly possess a tyrosine phosphatase domain and a CAAX prenylation motif (where C is the cysteine, A is an aliphatic amino acid and X is any amino acid) which is located next to a polybasic region in the C-terminal for membrane anchoring [3]. Many studies on clinical samples have demonstrated the connection between PRL overexpression and the malignancy of various types of cancers such as colorectal, breast, and gastric carcinomas [4], and the implantation of cultured cancer cells, PRL1 or PRL3-overexpressed cells into nude mice, resulted in metastatic tumors [5].

After the recognition of its intrinsic involvement in metastatic cancers and in actively driving malignant progression, various animal models such as *Drosophila* [6], *Mus musculus* [7,8], and *Rattus norvegicus* [9] were then used to gain insight into the biological function and the underling molecular mechanisms of the PRLs. *Drosophila* PRL-1 was identified to be a growth inhibitor that could counteract the oncogenic activity of Src. *Drosophila* PRL-1 localizes to the lateral membrane of the wing discs and has also been reported as ubiquitously expressed and localized to both the cytoplasm and the plasma membrane in various tissues including embryo epidermis, larval midgut, gastric caecum, developing eye and in the wing discs themselves. The ability of *Drosophila* PRL-1 to inhibit growth was confirmed to be dependent on the CAAX motif that is required to localize *Drosophila* PRL-1 to the apical edge of the lateral membrane in the wing discs [6]. Several other recent studies have showed that *Drosophila* PRL-1 functions in the central nervous system. The absence of *Drosophila PRL-1*, for example, specifically reduces synapse organization in the terminal arbor of one target area of mechanosensory neurons. It has also been reported that untranslated

Drosophila PRL-1 mRNA (the long UTRs sequences of *Drosophila* PRL-1 mRNA) mediate the local translation of *Drosophila* PRL-1 in local synaptic arbor formation and that *Drosophila* PRL-1 provides a specificity factor to restrict InR-Akt signaling and synapse formation in a subcellular compartment of neurons [10]. Notably, knockdown of *Drosophila* PRL-1 in PDF clock neurons dramatically lengthens the circadian period. *Drosophila* PRL-1 could also mediate the dephosphorylation of TIM to set period length and behavioral phase in darkness, enabling behavioral adjustment to changes in day-night cycles [11]. Our previous study found that *Drosophila* PRL-1 is involved in protecting the nervous system against olfactory CO_2 stimulation. Mutation of *Drosophila PRL-1* flies exhibit a permanently held-up wing phenotype upon CO_2 exposure due to a neural dysfunction. We then showed that *Drosophila PRL-1* could interact with its downstream gene *Unextended wing* (*Uex*) and control Uex expression to maintain neuroprotective functions [12].

Despite there being a rapidly growing amount of literature on PRLs, our knowledge of the specific biological function of the PRL family of proteins remains limited. Our study used the *Drosophila* wing to examine the overexpression of PRL-1 in genetically controlled models. This system reveals that *Drosophila* PRL-1 functions as a newly discovered regulator of the transforming growth factor-β (TGF-β) signaling pathway to control wing vein pattern.

The adult wing of *Drosophila* has a stereotypical pattern of five longitudinal veins (LVs, L1–L5) and two crossveins (ACV, PCV) to act as the rigid supports necessary for flight [13]. Wing veins consist of rows of cells that differentiate into heavily pigmented cuticles and smaller apical sized cells. These are formed through specification of cell fate, not cell migration or cell shape change [14,15]. During metamorphosis several signaling pathways, including those mediated by EGF, BMP/Dpp, Hedgehog, Notch, and Wnt, define the expression of many transcription factors involved in the partition of the wing disc epithelium into proveins and interveins [14]. For example, in the third instar larvae imaginal wing disc, EGFR signaling is activated within the proveins to direct the subdivision of each provein in a central region that will then differentiate as a vein [16,17]; Notch signaling is involved in establishing the correct width of various wing veins [18]; and BMP signaling is required for the specification of both longitudinal proveins and intervein regions in the imaginal disc at the larval stage, also later required for crossvein formation during pupariation [14,17].

BMP (Bone Morphogenetic Protein) is another member of the TGF-β superfamily. BMP signaling in *Drosophila* includes the ligands Decapentaplegic (Dpp) and Glass bottom boat (Gbb), the type I receptor Thickveins (Tkv), the type II receptor Punt, and the transcription factor mothers against Dpp (Mad) [19,20]. Dpp is secreted either as a homodimer or a heterodimer with Gbb. These ligands bind to Tkv and Punt receptors to phosphorylate the C-terminal of Mad. Phosphorylated Mad (pMad) is then, together with co-Smad Medea, translocated into the nucleus for the transcriptional regulation of various downstream target genes such as *spalt-major* (*salm*), *spalt-related* (*salr*), *optomotor-blind* (*omb*), *spalt* (*sal*), *vestigial* (*vg*), *brinker* (*brk*), and *Ultrabithorax* (*Ubx*) [19,21–23]. Other signals mediated by TGF-β ligands include those of the TGF-β/Activin pathways. In *Drosophila*, Activin signaling is mediated by the secreted ligands Activin (dAct) and Dawdle (Daw), the type I receptor Baboon (Babo), the type II receptor Punt, and Smox (Smad on X, *Drosophila* Smad2). Activin signals are transduced in a similar fashion as in BMP signaling via their binding to type I and II receptors, the C-terminal phosphorylation of Smox, followed by heterotrimeric complex formation with Medea and then transcriptional activation of target genes in the nucleus [24]. It should be noted that the dAct/Smox signal pathway shares several components with the Dpp/Mad branch including Punt and Medea [25]. Both dAct/Smox and Dpp/Mad pathways play important roles in fly wing vein development [22,23,26,27]. It has been reported that Medea functions as the limiting factor between Mad and Smox signaling pathways. The phosphorylated transcription factors Mad and Smox compete for association with rate-limiting quantities of Medea to control vein formation [26].

In this study, we used a gain-of-function approach to investigate *Drosophila* PRL-1 functions during wing development. Overexpression of *Drosophila* PRL-1 caused the formation of extra vein tissue at the posterior end of the PCV along the longitudinal vein 5 (L5), with a dose dependent effect. Genetic screening found that RNAi-mediated knockdown of Mad could alleviate the extra vein phenotype caused by overexpressed *Drosophila* PRL-1 and lead to loss of the posterior of longitudinal vein 4. Knockdown of Smox on the background of overexpressing *Drosophila PRL-1* in the wing disc showed a phenotype similar to the knockdown of Smox alone. Clonal analyses revealed that overexpression of *Drosophila* PRL-1 resulted in lower expression of the activated phospho-Mad protein as measured by immunostaining. Real-time PCR showed that the transcriptional levels of *Smox* were significantly increased upon the overexpression of *Drosophila* PRL-1 in the wing disc, with a dose dependent effect. We propose that the main function of PRL-1 in *Drosophila* wing development is to affect the phospho-Mad levels and Smox transcriptional levels, therefore potentially influencing the competitive balance for Medea between Mad and Smox. Our study demonstrates a novel role for PRL-1 in regulating TGF-β signaling during wing vein formation. This may provide additional valuable insight into the understanding of the relationship between PRLs and TGF-β signaling in mammals.

2. Materials and Methods

2.1 Comparison of PRL Sequences

The *Drosophila* PRL-1 amino acid sequence was downloaded from Flybase (http://flybase.org/) and human PRL1-3 amino acid sequences were downloaded from the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/). The Clustal X 2.0 software (European Bioinformatics Institute, Hinxton, Cambridgeshire, CB10 1SD, UK) was used for the multiple amino acid sequence alignments of *Drosophila* PRL-1 and human PRL1-3. Results were presented using the DNAman software (version 9.0, Lynnon Corporation, 116 RUE DU MILICIEN, VAUDREUIL-DORION QC J7V 9M5, Canada).

2.2 Fly Strains and Genetics

Flies were reared on a standard cornmeal medium at 25 °C. The following strains were used: w^{1118}, Engrailed-GAL4 (Chao Tong, Zhejiang University, Hangzhou, China), UAS-PRL-1 (generated by our lab, Guo *et al*. [12]), hsFlp; Act5C>y^+>Gal4 UAS-green fluorescent protein (GFP)/CyO (from J.C. Pastor-Pareja, Tsinghua University, Beijing, China); *PRL-1*^{16} (as in Guo *et al*. [12]); and UAS-Mad RNAi (THU4727, THU5761, THU5760, THU4801), UAS-Smox RNAi (THU2384), UAS-Med RNAi (THU2306, THU4652) (all from the Tsinghua Fly Center, Beijing, China).

2.3 Clonal Analysis

We used the flip-out system to generate overexpression clones in the brain. To induce ectopic expression of *Drosophila* PRL-1, we crossed hsFlp;Act5C>y^+>Gal4 UAS- GFP/CyO flies with UAS-PRL-1 flies or wild type (as the control) and the larvae were heat shocked for 1 hr at 24–48 hr after egg laying (AEL). The larvae were then incubated at 25 °C until 120 hr AEL at which time they were dissected. Overexpression clones with the genotype hsFLP/+;Act5C>y^+>Gal4 UAS-GFP/UAS-PRL-1 were marked by the presence of GFP.

2.4 Adult Wing Analysis

Adult female or male wings (N = 20 for each genotype, repeated three times) were removed and mounted in 80% glycerol mounting medium. Images of wings were obtained on an Eclipse 80i microscope (Nikon, Tokyo, Japan). Wing posterior and total areas were measured using ImageJ (version 1.51, LOCI, University of Wisconsin, Madison, Wisconsin, USA). Statistical analysis was performed using a student's *t*-test.

2.5 Staining Procedures and Microscopy

Drosophila brains from third instar larvae were dissected in ice cold 1×PBS (10 mM NaH_2PO_4/Na_2HPO_4, 175 mM NaCl, pH 7.4) and fixed for 20 min in PBS with 4% paraformaldehyde. Fixed brains were washed three times with 0.1% Triton X-100 in PBS (PBT) and blocked in PBT containing 3% BSA for 0.5 h at room temperature. Brains were then incubated with the primary antibodies overnight at 4 °C, followed by three washes with PBT before incubating with secondary antibodies for 2 h. DAPI was added for the last 20 min. After three further washes with PBT, brains were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). The primary antibody used was rabbit anti-phospho-Mad [1:800, Phospho-Smad1/5 (Ser463/465) (41D10) Rabbit mAb, GST, #9516]. The secondary antibody was Alexa Fluor 555 conjugated (1:250 or 1:500; Thermo Fisher Scientific, Waltham, MA, USA). DAPI (1 g/mL; Sigma-Aldrich, St. Louis, MO, USA) was used to stain for nuclei. GFP was directly observed under a confocal microscope. Images were taken using an Olympus FV1000 confocal microscope (Olympus, Tokyo, Japan) and processed using Adobe Photoshop CS6 (Adobe, San Jose, CA, USA). ImageJ was used to generate a sum-intensity z stack projection and a measurement of the total fluorescence intensity.

2.6 Quantitative Real-Time PCR

Total RNA was isolated from dissected disc cells using Trizol® Reagent (Invitrogen, Carlsbad, CA, USA, #15596018). A 1 μg RNA and First-Strand cDNA synthesis kit (Invitrogen) was used to produce single-stranded cDNA. Real-time PCR was performed using a Power SYBR Green PCR Master Mix (ABI) and an ABI 7900HT Fast real-time PCR system. All experiments were performed in triplicate.

Rp49 was used as an endogenous control. The following PCR primers (5′-3′) were used; *rp49* Forward, GCTAAGCTGTCGCACAAA, *rp49* Reverse, TCCGGTGGGCAGCATGTG; *Snoo* Forward, ACCAGATCATCCTCGATCAG, *Snoo* Reverse, TTGTGGCTGCTTCATTGGC; *Smox* Forward, ACCGTTACAGATCAGAAGTG, *Smox* Reverse, GGTGAATGGCAACATCTC; *myo* Forward, ACGATGAGGAGTACGAGAG, *myo* Reverse, TGATGAGCACTTATCCAATCC; *EcR* Forward,ACAGGACGTAGATGCACAG, and *EcR* Reverse, CATATGCGTCGATATAGTGCG.

2.7 Statistical Analysis

Data shown in the Figs. 2B,2B',2C,4E,5A,5B are reported as mean ± SD. Statistical analysis was performed using the paired, two-tailed Student's *t*-test contained in the Microsoft-Excel program. Significance levels are indicated in the figure legends.

3. Results

3.1 Structural Characteristics of Drosophila PRL-1

The *Drosophila PRL-1* gene (CG4993), encoding the single *Drosophila* PRL protein (*Drosophila* PRL-1), is located on the left arm of the second chromosome. It consists of 176 amino acids in approximately 20-kDa protein (http:

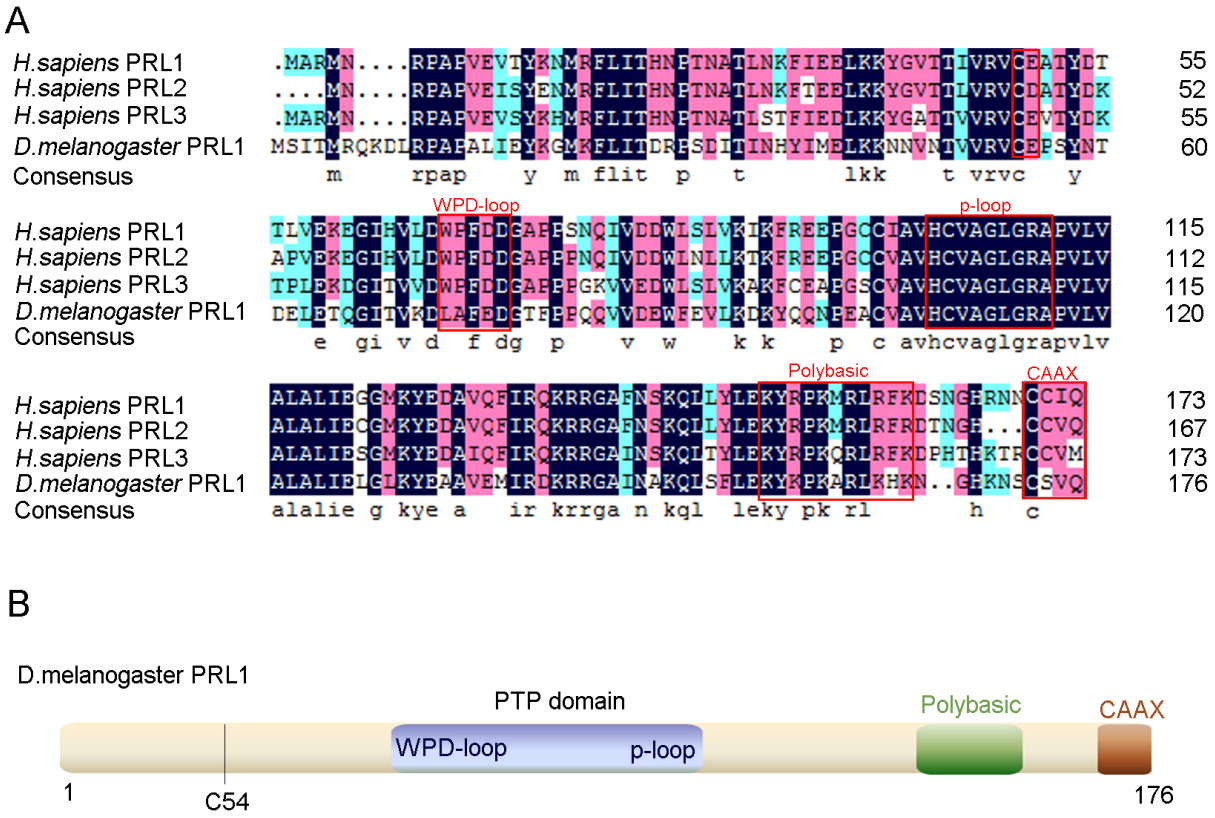

Fig. 1. Structural characteristics of PRLs. (A) Multiple sequence alignments of PRLs from *Homo sapiens* and PRL-1 from *Drosophila melanogaster*. Identical residues are marked with a black background, conserved residues with a blue background, and similar residues with a salmon background. The putative 'CXnE' motif, WPD-loop, P-Loop, a polybasic region and a CAAX prenylation motif are indicated with red squares. (B) Schematic diagrams of *Drosophila* PRL-1. The putative 'CXnE' motif begins at C54.

//flybase.org/). To evaluate the conservative properties of PRL phosphatases between humans and *Drosophila*, we aligned their amino acid sequences using public databases. Results showed that *Drosophila* PRL-1 shares an overall 75.7% similarity in comparison to all three human PRLs (Fig. 1A). More specifically, the amino acid similarity of *Drosophila* PRL-1 shows a high positive comparison with each of the human PRLs individually, with a 73% similarity between *Drosophila* PRL-1 and human PRL-1, 75% for human PRL-2, and 74% for human PRL-3. We then performed bioinformatics analysis on the structural characteristics of *Drosophila* PRL-1. *Drosophila* PRL-1 contains several domains also characteristic of mammals including the putative 'CXnE' motif [3], the conserved catalytic PTP domain that is critical to phosphatase activity [28], the polybasic region, and the CAAX prenylation motif for targeting to the plasma membrane [29] (Fig. 1A,B, red rectangles), showing that that *Drosophila* PRL-1 shares high sequence and structural similarities with human PRLs.

3.2 Overexpressing Drosophila PRL-1 Increases Wing Vein Formation

We had previously generated a *Drosophila PRL-1* deletion mutant which showed a wing hold-up phenotype when stimulated under a high concentration of CO_2 treatment. After inhibition of the CO_2 neuron receptor Gr63a or Gr21a, the wind hold-up phenotype could be rescued [12]. We did not notice any morphological changes in the wings of these *Drosophila PRL-1* mutants. Using transgenic flies containing the full-length of *Drosophila PRL-1* [12], we then performed experiments by crossing UAS-PRL-1 flies to several lines that expressed the transcriptional activator GAL4 in a tissue-specific manner. Among these, the overexpression of PRL-1 in developing larval wing discs, as driven by engrailed-Gal4 (En-Gal4) expressed in the posterior compartment of the wing, led to reduction of adult wings size (Fig. 2A). This had also been noted in the Pagarigan *et al.* [6] study. Here, the ratio of posterior compartment to the entire wing showed a 2.7% reduction ($p = 5.79475 \times 10^{-7}$) in the single copy overexpressed PRL-1 male, a 6.7% reduction ($p = 4.88867 \times 10^{-9}$) in the double copy overexpressed PRL-1 male, a 1.9% reduction ($p = 6.75841 \times 10^{-5}$) in single copy overexpressed PRL-1 female, and a 4.6% reduction ($p = 8.18251 \times 10^{-7}$) in double copy overexpressed PRL-1 female (Fig. 2A,B'). This indicates that *Drosophila* PRL-1 has dose dependent effect on growth inhibition.

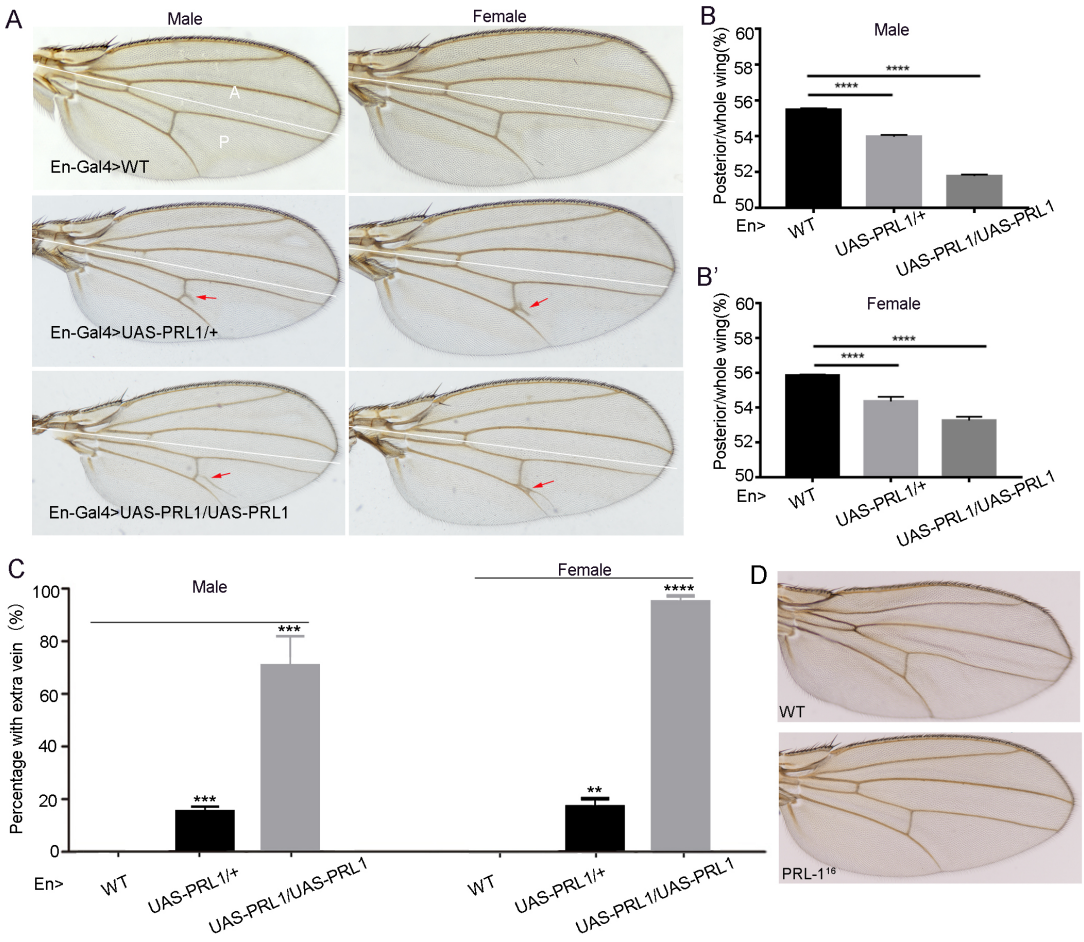

Fig. 2. Overexpression of Drosophila PRL-1 caused dose-dependent wing vein proliferation. (A) Adult wings from flies expressing the following transgenes under the control of En-Gal4; Left panes are wings from males; Right panes are wings from females. The lines divide wings into anterior (A) and posterior (P) parts. (B,B') The ratio of posterior compartment (P) to the entire wing (A+P) (n = 20 in each genotype, and with experiments repeated three times). (B) Male, (B') female. (C) The percentage of adult wings with ectopic vein branches; n = 20, repeated three times. (D) Wing vein pattern in the *PRL-1* mutants. Data are presented as means ± SD. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Interestingly, we observed that the posterior wing compartment of both male and female showed an extra branch at the posterior end of the PCV along the longitudinal vein 5 (L5) in En-Gal4>UAS-PRL-1 fly wings, as compared to an adult wild-type wings (Fig. 2A, red arrows). It was notable that the ectopic vein branch of the wings of flies with double copy PRL-1 overexpression was longer than that in those with a single copy (Fig. 2A). In addition, the percentage of flies with extra wing veins was higher upon double PRL-1 overexpression than that upon single PRL-1 overexpression (Fig. 2C), with 70% and 95.4% upon double overexpression, and 15% and 17.3% upon single overexpression, in males and females, respectively. We also analyzed the wing vein pattern in the *PRL-1* mutants and failed to notice any abnormal morphological or wing vein phenotypes (Fig. 2D). These results suggest that *Drosophila* PRL-1 is a dosage-dependent regulator of wing vein formation.

3.3 Drosophila PRL-1 is a Novel Regulator of TGF-β Pathway in Wing Vein Development

To further investigate the functions and molecular mechanism of *Drosophila* PRL-1 in wing vein development, we obtained several RNA interference (RNAi) transgenic fly lines that were known to be involved in the pathways that control vein formation. We performed screening experiments using the flies with a background of PRL-1 overexpression driven by En-Gal4. When the antisense RNA of the target gene is expressed by the En-Gal4 driver, the RNA transcribed from the target DNA forms double-stranded RNA with the antisense RNA that is disrupted by the RNAi machinery and depleted from the target gene product at the posterior of wing. Using RNAi, we could therefore compare the phenotype resulting from a specific gene knockdown in the posterior of wing tissue and attempt to clarify the relationship between the specific gene and PRL-1.

We found that RNAi-mediated knockdown of Mad could alleviate the extra vein phenotype caused by overexpressed PRL-1 (Fig. 3A–D, yellow arrows), but this also led to a loss of the posterior portion of longitudinal vein 4 (Fig. 3D, red arrows), which was an identical phenotype seen in some Dpp partial loss-of-function mutants [26,30]. Notably, downregulating Mad only in the posterior portion of wing tissue did not result in any obvious phenotype (Fig. 3C), while knock down of Mad together with simultaneous overexpression of PRL-1 could induce vein loss. This demonstrates that the efficiency of the knockdown of Mad alone is not sufficient to lead vein loss, and that this only occurs in combination with PRL-1. In this way the data clearly indicates a *Drosophila* PRL-1 genetic interaction with Mad. We then wondered whether overexpressing PRL-1 has any effect on Mad levels. We carried out a flip-out method using heat shock induced FLP (hs-FLP); Act5C>y^{+}>Gal4 UAS-GFP flies and then generated overexpression PRL-1 clones. We failed to find any ideal wing imaginal disc clones, but found mitotic clones in the brain. Therefore, we continued to analyze the correlation between PRL-1 and phospho-Mad in the brain. We used an anti-phospho-Mad antibody for the GFP detection of the phosphorylated levels of Mad in the PRL-1 overexpression cells and found that the activated phospho-Mad levels were down-regulated by almost 50% in overexpression PRL-1 cells as measured by immunostaining (Fig. 4). This may explain the observation of the combination of the overexpression of PRL-1 together with Mad RNAi down-regulating Dpp signaling to a certain degree, and that this is sufficient to induce the loss of wing veins.

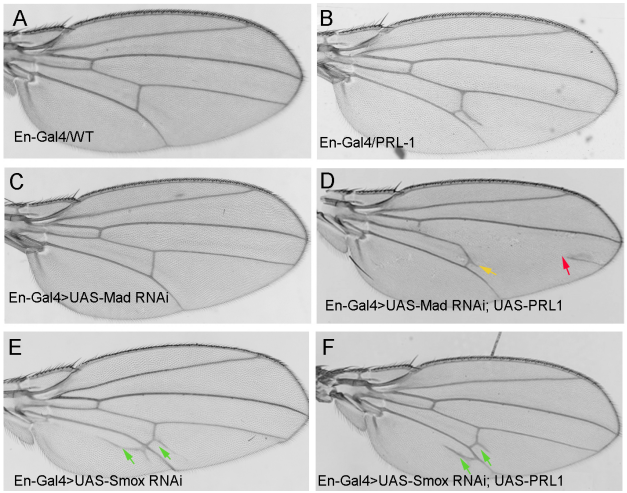

Fig. 3. Adult wings from flies expressing the following transgenes under the control of En-Gal4. (A) En-Gal4>WT; n = 20. (B) En-Gal4>UAS-PRL-1; n = 20. (C) En-Gal4>UAS-Mad RNAi (THU4727); n = 20. (D) En-Gal4>UAS-PRL-1; UAS-Mad RNAi; n = 14. (E) En-Gal4>UAS-Smox RNAi; n = 20. (F) En-Gal4>UAS-PRL-1; UAS-Smox RNAi; n = 16.

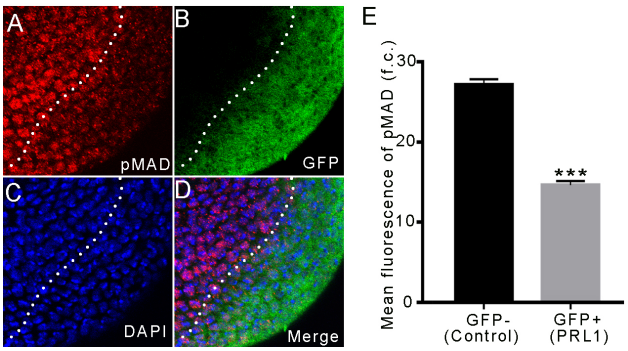

Fig. 4. Overexpressing PRL-1 led to decreased expressions of activated phospho-Mad protein. (A–D) Overexpression of PRL-1 marked by GFP in the brain. Lower expression of phospho-Mad is observed in overexpressing PRL-1 clone cells as compared with the wild-type cells around the clone. (A) Phospho-Mad (red). (B) GFP (green). (C) DAPI (blue). (D) Merge. (E) Mean fluorescence of phospho-Mad; n = 6. Data are presented as means ± SD. *** $p < 0.001$.

As both Dpp/Mad and dAct/Smox pathways both play important roles in fly wing vein development [26,27] and share several components [25], we therefore performed RNAi-mediated knockdown of Smox to confirm the phenotype. We found that down-regulating the *Smox* gene led to ectopic vein tissue formation in the vicinity of L5 and an extra branch at the posterior end of the PCV (Fig. 3E, green arrows), which showed a similar phenotype as noted in Sander *et al.* [26]. When we knocked down Smox on the background of overexpressing PRL-1, the ectopic vein phenotype was almost same as that in the down-regulation of Smox alone (Fig. 3F, green arrows). This may suggest that PRL-1 functions upstream of Smox. To further confirm this hypothesis, we carried out Real-time PCR experiments to check *Smox* levels. Results showed that, except for *Smox*, the levels of genes such as *Sno oncogene* (*Snoo*), *Myoglianin* (*Myo*), and *EcR*, that are also involved in Activin signaling pathway, remained unchanged upon PRL-1 overexpression (Fig. 5A). Additionally, the transcriptional levels of *Smox* were shown to be increased in a dose-dependent manner in the PRL-1-overexpressed wing discs (Fig. 5B). The overexpression of PRL-1 triggering the up-regulation of *Smox* probably acts to compensate for decreased Activin signaling. These results suggest that *Drosophila* PRL-1 is a regulator of TGF-β pathway and genetically interacts with Mad and Smox, affecting the phosphorylation of Mad, as well as the transcriptional levels of *Smox*, and therefore influencing the competitive balance for Medea between Mad and Smox during wing vein development.

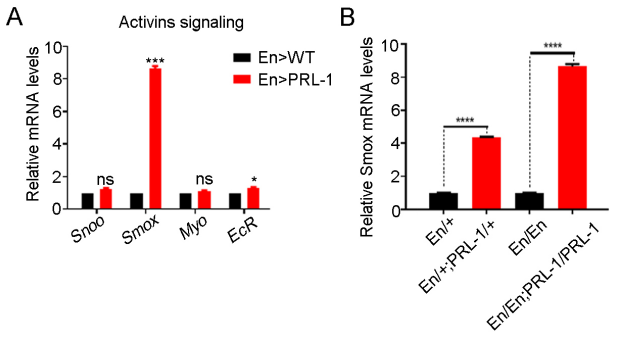

Fig. 5. Overexpressing PRL-1 increases the transcriptional levels of *Smox*. (A) Real-time PCR analysis of the relative mRNA levels of genes involved in the Activin signaling pathway showing that only *Smox* is obviously increased in overexpressing PRL-1 wing discs; n = 3. (B) The transcriptional levels of *Smox* in the discs from different copy numbers of En-gal4 and UAS-PRL-1 animals indicate that the transcriptional levels of *Smox* is a dose dependent effect in the overexpression of PRL-1 wing disc s; n = 3. Data are presented as means ± SD. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

4. Discussion

Drosophila PRL-1 belongs to the Phosphatase of Regenerating Liver (PRL) family, also known as protein tyrosine phosphatase 4A (PTP4A). In mammals it consists of three members, PRL-1, 2 and 3. Numerous studies using cultured cells have demonstrated that the PRLs can induce cell proliferation, migration, and invasiveness in cancers [29,31]. Using the *Drosophila* wing as a model system, here we report that the homolog of mammalian PRLs, *Drosophila* PRL-1, functions during wing vein development through its regulation of the TGF-β signaling pathway. By gain of function analysis, overexpression of *Drosophila* PRL-1 in the wing was noted to result in extra wing veins. By contrast, loss of function experiments involving *PRL-1* mutant lines failed to yield any wing vein deficiencies (Fig. 2D). This indicates that whilst the loss of PRL-1 may fail to negatively affect cell proliferation, that its overexpression positively promotes it in certain circumstance. This is reminiscent of the role of its mammalian counterparts PRL-1, PRL-2 and PRL-3 which reportedly have high expressions in a several human cancers [4]. Here, in particular, we reveal a new mechanism of PRL-1 and its involvement in regulating the TGF-β pathway.

Using bioinformatics methods to analyze the conservative properties of PRL phosphatases between human and *Drosophila* we found that *Drosophila* PRL-1 shares a 75.7% similarity between all three human PRLs and with many specific structural similarities noted (Fig. 1). This is consistent with Zeng *et al.* [32] and Lin *et al.* [33] who found that *Drosophila* PRL-1 shares high similarities (74%–76%) to all three mammalian PRLs. In our previous study, we found that a holding-up of wings phenotype in the PRL-1 deletion mutant flies could be rescued by overexpressing human PRL-1 or human PRL-2, which implies that human PRL phosphatases may function in a similar neuroprotective capacity [12]. However, in the present study, overexpressing human PRL-1 or human PRL-2 by En-gal4 in the wing failed to result in any similar ectopic wing vein anomalies as seen upon *Drosophila* PRL-1 overexpression (data not shown). This could be because the expression level is not sufficient, or that some of signal transduction members for human PRLs are absent in *Drosophila*. Pagarigan *et al.* [6] reported that overexpressing *Drosophila* PRL-1 could inhibit growth in a manner dependent on the CAAX motif that is integral to the localization of *Drosophila* PRL-1 to the apical edge of the lateral membrane.

The *Drosophila* adult wing has a stereotypical pattern of longitudinal veins and crossveins which are constructed mainly of two cell types, vein cells and intervein cells. The specification of vein territories and the differentiation of wing veins and intervein regions are regulated by a high number of complex signaling pathways including those of EGFR, transforming growth factor-β, Hedgehog, Notch, Wnt, and JAK-STAT [18,26,34–39]. The various roles and interrelationships among these are far from completely understood. In *Drosophila*, the TGF-β signaling pathway can be divided into two broad signaling families, those of the Dpp and those of the Activin pathways, to control various cellular processes including those of cell proliferation, differentiation, apoptosis, cell plasticity, and migration [20,26].

In this scenario, ectopic PRL-1 lowers the expression of the activated phospho-Mad protein, in which a decrease in Dpp signaling and elevation of the transcriptional levels of Smox may act to downregulate the Activin pathway. It has been previously reported that RNAi-mediated Babo knockdown also resulted in decreased Activin signaling and led to a significant increase in the transcription of Smox [40]. These increased transcriptional levels of Smox could lead to a compensation role upon decreased Babo expression. The Activin/Smox signal pathway shares its component Medea with the Dpp/Mad pathway, and Medea has been reported to function as a limiting factor between the Mad and Smox signaling pathways [26]. Overexpressing PRL-1 could potentially cause downregulation of both the Dpp pathway and Activin pathway, therefore, influencing the competitive balance for Medea between Mad and Smox during wing vein pattern formation, as shown in the schematic of Fig. 6.

Our genetic experiments show that the knockdown of Mad on the background of ectopic PRL-1 leads to the loss of the extra vein, thus reversing the phenotype. This suggests that PRL-1 and Mad may function in parallel pathways to regulate vein formation. However, the detailed molecular mechanism of how PRL-1 affects the phosphorylation of Mad awaits further investigation. PRL-1 and

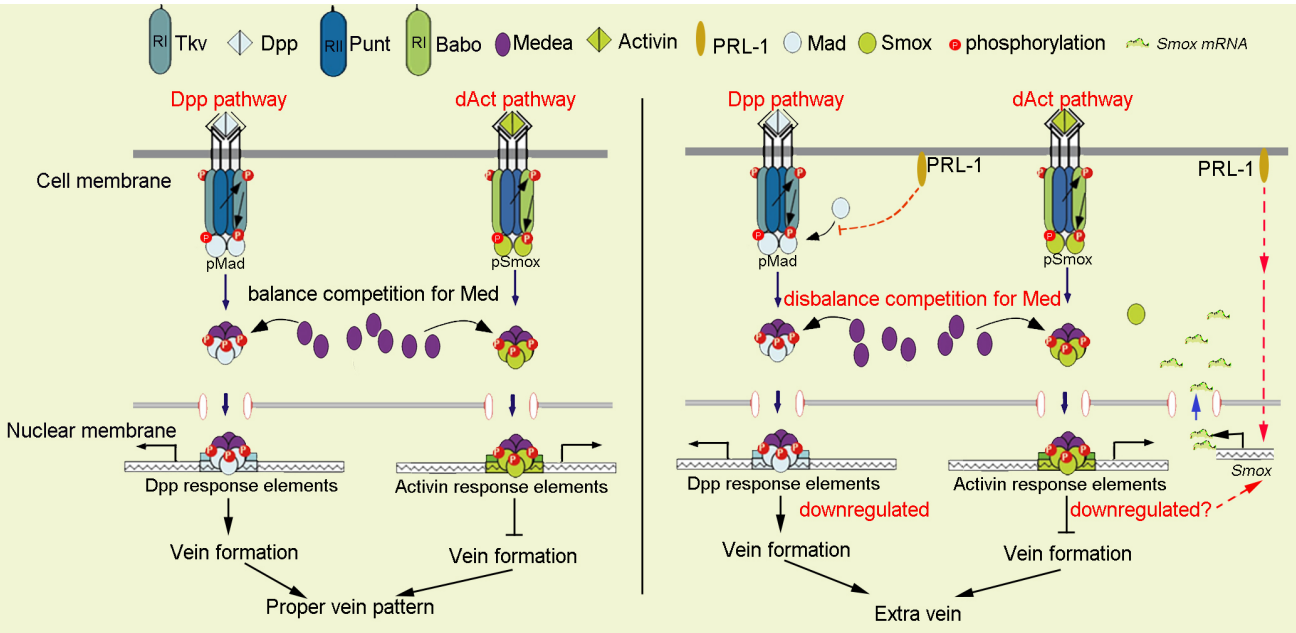

Fig. 6. Schematic representation of the putative mechanism by which PRL-1 could regulate the TGF-β pathway. Overexpression of PRL-1 affects the phosphorylation of Mad as well as the transcriptional levels of Smox. This may therefore influence the competitive balance for Medea between Mad and Smox during wing vein development and lead to extra wing veins.

Smox may act in a linear pathway in the regulation of vein formation. The ectopic vein phenotype caused by knocking down Smox was nearly identical to that of the combination of Smox knockdown and overexpression of PRL-1 in the posterior of the wing (Fig. 3E,F). Together, these experiments suggest that *d*PRL-1 acts, at least in part, upstream of Smox.

5. Conclusions

Our study identified a novel function of PRL-1 in wing vein formation. We propose that the main function of PRL-1 in *Drosophila* wing development is to affect the phospho-Mad levels and *Smox* transcriptional levels, therefore influencing the competitive balance for Medea between Mad and Smox. This study demonstrates a novel role for PRL-1 in regulating TGF-β signaling to influence wing vein formation. It could also lead to valuable clarification of the relationship between PRLs and TGF-β signaling in mammals.

Author Contributions

YX, HZ and ZL designed the experiments and interpreted the data. HZ, ZL, XY (Xin Yuan) and HW contributed to experiment completion and data analysis. HZ, ZL and YX wrote the initial manuscript. YX and XY (Xiaohang Yang) revised the manuscript. All authors contributed helpful suggestions for this manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

References

[1] Kitamura K, Mizuno Y, Sasaki A, Yasui A, Tsuiki S, Kikuchi K. Tsuiki and K. Kikuchi: Molecular-Cloning and Sequence-Analysis of Cdna for the Catalytic Subunit 1-Alpha of Rat-Kidney Type-1 Protein Phosphatase, and Detection of the Gene-Expression at High-Levels in Hepatoma-Cells and Regenerating Livers as Compared to Rat Livers. Journal of Biochemistry. 1991; 109: 307–310.

[2] Kitamura T, Nakamura K, Mizuno Y, Kikuchi K. Gene Expressions of Protein-Tyrosine Phosphatases in Regenerating Rat-Liver and Rat Ascites Hepatoma-Cells. Japanese Journal of Cancer Research. 1995; 86: 811–818.

[3] Rios P, Li X, Köhn M. Molecular mechanisms of the PRL phosphatases. FEBS Journal. 2013; 280: 505–524.

[4] Bessette DC, Qiu D, Pallen CJ. PRL PTPs: mediators and markers of cancer progression. Cancer and Metastasis Reviews. 2008; 27: 231–252.

[5] Zeng Q, Dong JM, Guo K, Li J, Tan HX, Koh V, *et al*. PRL-3 and

PRL-1 promote cell migration, invasion, and metastasis. Cancer Research. 2003; 63: 2716–2722.
[6] Pagarigan KT, Bunn BW, Goodchild J, Rahe TK, Weis JF, Saucedo LJ. Drosophila PRL-1 Is a Growth Inhibitor That Counteracts the Function of the Src Oncogene. PLoS ONE. 2013; 8: e61084.
[7] Jiao Y, Ye DZ, Li Z, Teta-Bissett M, Peng Y, Taub R, *et al*. Protein tyrosine phosphatase of liver regeneration-1 is required for normal timing of cell cycle progression during liver regeneration. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2015; 308: G85–G91.
[8] Cramer JM, Zimmerman MW, Thompson T, Homanics GE, Lazo JS, Lagasse E. Deletion of Ptp4a3 reduces clonogenicity and tumor-initiation ability of colitis-associated cancer cells in mice. Stem Cell Research. 2014; 13: 164–171.
[9] Schmidt J, de Avila J, McLean D. Regulation of protein tyrosine phosphatase 4a1, B-cell translocation gene 2, nuclear receptor subfamily 4a1 and diacylglycerol O-acyltransferase 1 by follicle stimulating hormone in the rat ovary. Reproduction, Fertility and Development. 2006; 18: 757.
[10] Urwyler O, Izadifar A, Vandenbogaerde S, Sachse S, Misbaer A, Schmucker D. Branch-restricted localization of phosphatase Prl-1 specifies axonal synaptogenesis domains. Science. 2019; 364: eaau9952.
[11] Kula-Eversole E, Lee DH, Samba I, Yildirim E, Levine DC, Hong H, *et al*. Phosphatase of Regenerating Liver-1 Selectively Times Circadian Behavior in Darkness via Function in PDF Neurons and Dephosphorylation of TIMELESS. Current Biology. 2021; 31: 138–149.e5.
[12] Guo P, Xu X, Wang F, Yuan X, Tu Y, Zhang B, *et al*. A Novel Neuroprotective Role of Phosphatase of Regenerating Liver-1 against CO2 Stimulation in Drosophila. IScience. 2019; 19: 291–302.
[13] Diaz-Benjumea FJ, García-Bellido A. Genetic analysis of the wing vein pattern of Drosophila. Roux's Archives of Developmental Biology. 1990; 198: 336–354.
[14] Blair SS. Wing vein Patterning in Drosophila and the analysis of intercellular signaling. Annual Review of Cell and Developmental Biology. 2007; 23: 293–319.
[15] Bier E. Drawing lines in the Drosophila wing: initiation of wing vein development. Current Opinion in Genetics and Development. 2000; 10: 393–398.
[16] Sturtevant MA, Roark M, Bier E. The Drosophila rhomboid gene mediates the localized formation of wing veins and interacts genetically with components of the EGF-R signaling pathway. Genes and Development. 1993; 7: 961–973.
[17] Sotillos S, De Celis JF. Interactions between the Notch, EGFR, and decapentaplegic signaling pathways regulate vein differentiation during Drosophila pupal wing development. Developmental Dynamics. 2005; 232: 738–752.
[18] de Celis JF, Bray S, Garcia-Bellido A. Notch signalling regulates veinlet expression and establishes boundaries between veins and interveins in the Drosophila wing. Development. 1997; 124: 1919–1928.
[19] Moustakas A, Heldin CH. The regulation of TGF beta signal transduction. Development. 2009; 136: 3699–3714.
[20] Zhao B, Chen YG. Regulation of TGF-beta Signal Transduction. Scientifica. 2014; 2014: 874065.
[21] Cook O, Biehs B, Bier E. Brinker and optomotor-blind act coordinately to initiate development of the L5 wing vein primordium in Drosophila. Development. 2004; 131: 2113–2124.
[22] Matsuda S, Shimmi O. Directional transport and active retention of Dpp/BMP create wing vein patterns in Drosophila. Developmental Biology. 2012; 366: 153–162.
[23] Winter SE, Campbell G. Repression of Dpp targets in the Drosophila wing by Brinker. Development. 2004; 131: 6071–6081.
[24] Shi Y, Massagué J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell. 2003; 113: 685–700.
[25] Zheng X, Wang J, Haerry TE, Wu AY, Martin J, O'Connor MB, *et al*. TGF-beta signaling activates steroid hormone receptor expression during neuronal remodeling in the Drosophila brain. Cell. 2003; 112: 303–315.
[26] Sander V, Eivers E, Choi RH, De Robertis EM. Drosophila Smad2 opposes Mad signaling during wing vein development. PLoS ONE. 2010; 5: e10383.
[27] Peterson AJ, O'Connor MB. Activin receptor inhibition by Smad2 regulates Drosophila wing disc patterning through BMP-response elements. Development. 2013; 140: 649–659.
[28] Hardy S, Kostantin E, Hatzihristidis T, Zolotarov Y, Uetani N, Tremblay ML. Physiological and oncogenic roles of the PRL phosphatases. The FEBS Journal. 2018; 285: 3886–3908.
[29] Wei M, Korotkov KV, Blackburn JS. Targeting phosphatases of regenerating liver (PRLs) in cancer. Pharmacology and Therapeutics. 2018; 190: 128–138.
[30] Nicholls RE, Gelbart WM. Identification of Chromosomal Regions Involved in decapentaplegic Function in Drosophila. Genetics. 1998; 149: 203–215.
[31] Yoshida A, Funato Y, Miki H. Phosphatase of regenerating liver maintains cellular magnesium homeostasis. Biochemical Journal. 2018; 475: 1129–1139.
[32] Zeng Q, Si X, Horstmann H, Xu Y, Hong W, Pallen CJ. Prenylation-dependent Association of Protein-tyrosine Phosphatases PRL-1, -2, and -3 with the Plasma Membrane and the Early Endosome. Journal of Biological Chemistry. 2000; 275: 21444–21452.
[33] Lin M, Lee H, Wang S, Li H, Hsien H, Cheng K, *et al*. Expression of phosphatase of regenerating liver family genes during embryogenesis: an evolutionary developmental analysis among Drosophila, amphioxus, and zebrafish. BMC Developmental Biology. 2013; 13: 18.
[34] Yan R, Luo H, Darnell JE, Dearolf CR. A JAK-STAT pathway regulates wing vein formation in Drosophila. Proceedings of the National Academy of Sciences. 1996; 93: 5842–5847.
[35] Huppert SS, Jacobsen TL, Muskavitch MA. Feedback regulation is central to Delta-Notch signalling required for Drosophila wing vein morphogenesis. Development. 1997; 124: 3283–3291.
[36] Guichard A, Biehs B, Sturtevant MA, Wickline L, Chacko J, Howard K, *et al*. Rhomboid and Star interact synergistically to promote EGFR/MAPK signaling during Drosophila wing vein development. Development. 1999; 126: 2663–2676.
[37] Matakatsu H, Lee YS, Kim J, Blair SS. Involvement of the MADS domain transcription factor Mef2 in vein formation in the Drosophila wing. Developmental Biology. 2002; 247: 490.
[38] Roch F, Jiménez G, Casanova J. EGFR signalling inhibits Capicua-dependent repression during specification of Drosophila wing veins. Development. 2022; 129: 993–1002.
[39] Prasad N, Shashidhara LS. Gliolectin positively regulates Notch signalling during wing-vein specification in Drosophila. The International Journal of Developmental Biology. 2015; 59: 187–194.
[40] Shinji J, Gotoh H, Miyanishi H, Lavine MD, Lavine LC. The activin signaling transcription factor Smox is an essential regulator of appendage size during regeneration after autotomy in the crayfish. Evolution & Development. 2019; 21: 44–55.