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## The T-box transcription factor Midline regulates wing development by repressing *wingless* and *hedgehog* in *Drosophila*

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Chong-Lei Fu<sup>1</sup>, Xian-Feng Wang<sup>1</sup>, Qian Cheng<sup>1</sup>, Dan Wang<sup>1</sup>, Susumu Hirose<sup>2</sup> & Qing-Xin Liu<sup>1</sup>

Wingless (Wg) and Hedgehog (Hh) signaling pathways are key players in animal development. However, regulation of the expression of *wg* and *hh* are not well understood. Here, we show that Midline (Mid), an evolutionarily conserved transcription factor, expresses in the wing disc of *Drosophila* and plays a vital role in wing development. Loss or knock down of *mid* in the wing disc induced hyper-expression of *wingless* (*wg*) and yielded cocked and non-flat wings. Over-expression of *mid* in the wing disc markedly repressed the expression of *wg*, *DE-Cadherin* (*DE-Cad*) and *armadillo* (*arm*), and resulted in a small and blistered wing. In addition, a reduction in the dose of *mid* enhanced phenotypes of a gain-of-function mutant of *hedgehog* (*hh*). We also observed repression of *hh* upon overexpression of *mid* in the wing disc. Taken together, we propose that Mid regulates wing development by repressing *wg* and *hh* in *Drosophila*.

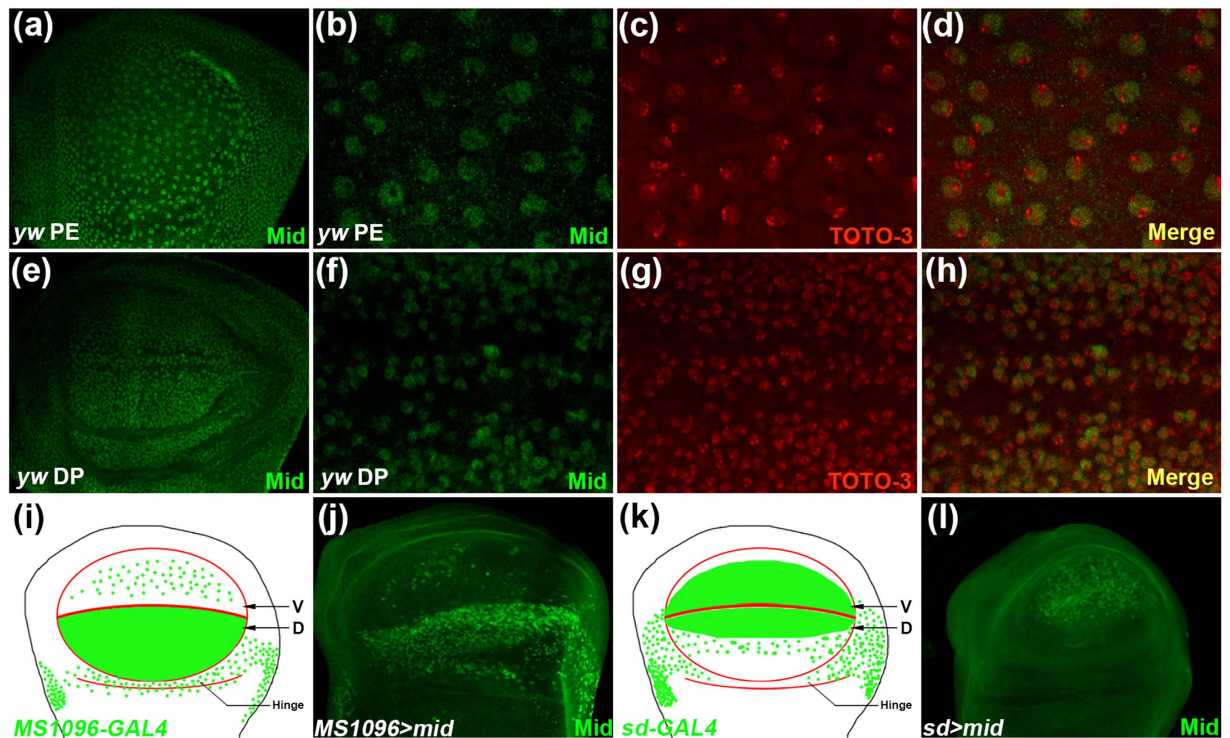
The development of multicellular organisms relies on participation and coordination of several signaling pathways that contribute to regulation of organ growth and pattern. Aberrant activity of these pathways can lead to many human diseases including cancers<sup>1–4</sup>.

The evolutionarily conserved Wg signaling pathway plays a crucial role in growth and pattern formation during development<sup>5–8</sup>. Signaling molecules of the Wg family are involved in a variety of cellular processes during development and mutations in components of Wg pathway are associated with many cancers<sup>1,9</sup>. In the *Drosophila* wing disc, as a gradient morphogen, Wg protein is secreted from the producing cells and acts at long range to activate target gene expression in surrounding cells<sup>10</sup>. Notch (N) induced the expression of *wg* in cells along the dorsal-ventral (DV) boundary<sup>11,12</sup>. The Wg/Wnt ligand sends a signal through binding to its receptor Frizzled (Fz) and co-receptor Arrow<sup>13,14</sup>. Then a multiprotein complex blocks degradation of Arm, a central component of the Wg pathway. As a consequence, free Arm enters into the nucleus and interacts with TCF/Lef1 to activate transcription of corresponding target genes in response to Wg signaling<sup>15–18</sup>. The expression of *wg* is activated by Dachs and repressed by Expanded and Hinge1<sup>19–21</sup>.

The Hh signaling pathway is evolutionarily conserved and plays an essential role in embryonic development and adult tissue patterning<sup>22–24</sup>. Deregulation of Hh signaling leads to various human diseases, including many cancers<sup>1,25,26</sup>. In *Drosophila* wing disc, Hh regulates cell proliferation and cell fate specification<sup>27</sup>. In the posterior (P) compartment, Hh is induced by Engrailed (En) and moves across the anterior-posterior (AP) boundary to form a concentration gradient<sup>25,28</sup>. When Hh binds to its receptor Patched (Ptc), Smoothed (Smo) is released from the unliganded Ptc-dependent inhibition<sup>29</sup>. Smo then modulates the activity of a transcription factor Ci<sup>30,31</sup>. Then, Ci translocates into the nucleus and activates the expression of target genes, such as *decapentaplegic* (*dpp*), *ptc* and *en*<sup>32,33</sup>. The expression of *hh* is activated by Ihog and repressed by Hyd<sup>34,35</sup>. Elucidation of the mechanism that regulates these pathways is essential to understanding the development of multicellular organisms.

<sup>1</sup>Laboratory of Developmental Genetics, Shandong Agricultural University, Tai'an, Shandong 271018, China.

<sup>2</sup>Department of Developmental Genetics, National Institute of Genetics, Mishima, Shizuoka 411-8540, Japan. Correspondence and requests for materials should be addressed to Q.-X.L. (email: liuqingxin@sdau.edu.cn)



**Figure 1. Mid is expressed in the PE and DP of wing discs.** (a–h) The expression pattern of Mid in the wing disc from a third instar larva of wild type stained with anti-Mid antibody. Optical sections at the plane of the PE (a–d) and DP (e–h). Mid was detected in the nucleus, labeled by TOTO-3, of both PE (b–d) and DP (f–h) cells. (i) Schematic representation of the *MS1096-GAL4* expression pattern. (j) The expression of Mid in the *MS1096-Gal4;UAS-mid* wing disc. (k) Schematic representation of the *sd-GAL4* expression pattern. (l) The expression of Mid in the *sd-Gal4;UAS-mid* wing disc.

In the nucleus, several common regulators are shared by more than one pathways, such as: Groucho (Gro), functions as a corepressor in the downstream of the N, Wnt, Hh, Dpp and EGFR pathways<sup>36,37</sup>; Lines (lin), serves as mediators of the Wg, N and Hh pathways<sup>38</sup> and *slimb* that negatively regulates both Arm and Ci<sup>39</sup>. This raises a possibility that still another factor can coordinate the Wg and Hh signaling pathways.

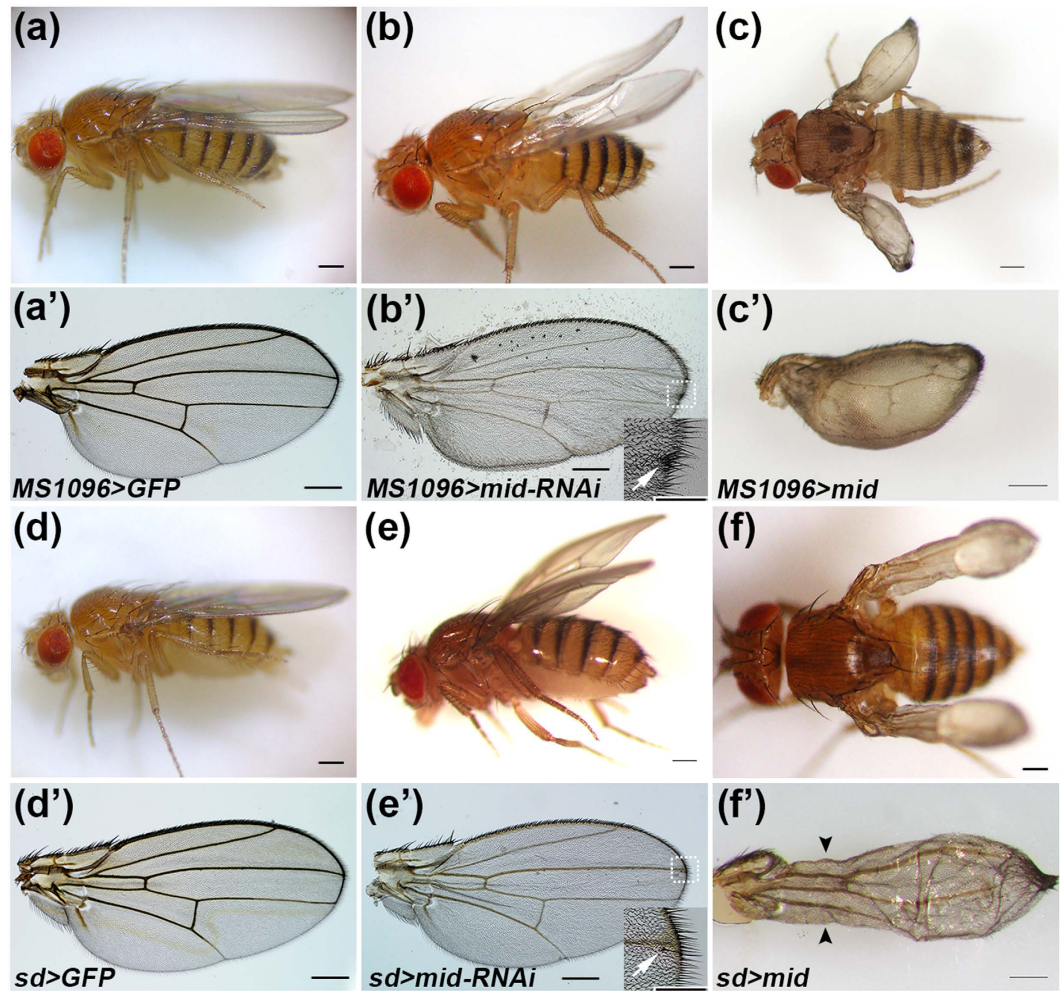
Mid is the *Drosophila* homolog of Tbx20 in vertebrates. As an evolutionarily conserved T-box family transcription factor, Mid plays a vital role in cell fate specification and tissue morphogenesis during a series of developmental processes<sup>40–45</sup>. During heart development, *mid* and its paralog *H15* are directly activated by Tinman (Tin) and required for the formation and specification of cardioblast<sup>46–49</sup>. In nervous system, Mid governs axon pathfinding through controlling the expression of multiple components of the two guidance systems, including Frazzled, Robo and Slit<sup>50</sup>. During development of imaginal disc, *mid* and *H15* function as selector genes to specify ventral fate in the leg disc<sup>51</sup>, and function within the Notch-Delta signaling pathway to specify SOP cell fate in the eye disc<sup>52</sup>. However, the function of Mid in wing development remains unclear.

In this study, we analyzed the function of Mid in the wing disc and its role during wing development. Our study reveals a novel role for Mid as an important regulator of the Wg and Hh signaling pathways.

## Results

**Mid expresses in the peripodial epithelium and disc proper cells of the wing disc.** To study the role of Mid in wing development, we first analyzed the expression pattern of Mid in the wing imaginal disc using immunostaining with anti-Mid antibody. In wild-type third instar larvae, Mid ubiquitously expressed in the nucleus marked by TOTO-3 in both peripodial epithelium (PE) (Fig. 1a–d) and the disc proper (DP) cells (Fig. 1e–h). The expression of Mid in the wing disc suggests that it possibly plays a role in wing development.

**Mid plays pivotal roles in wing development.** To gain more insight into the function of Mid in wing development, we analyzed phenotypes observed upon reduction in the *mid* function. Considering the lethality of *mid* null homozygotes during late embryogenesis (*mid*<sup>1</sup> and *mid*<sup>2</sup>), we expressed *mid* RNAi using *MS1096-GAL4* or *sd-GAL4* to knock down *mid* in pouch and margin cells of the wing disc. The expression patterns of *MS1096-GAL4* and *sd-GAL4* are schematically illustrated in Fig. 1(i,k), respectively. Compared with the control wings, knockdown of *mid* resulted in defect wings (Fig. 2a,a',b,b',d,d',e,e'). *MS1096 > UAS-mid-RNAi* adults exhibited cocked wings with a bowl-shaped depression (Fig. 2b). Likewise, inducing *UAS-mid-RNAi* with *sd-GAL4* resulted in cocked wings with a convex morphology (Fig. 2e). Knockdown of *mid* also induced ectopic wing hairs (Fig. 2b',e', Arrows and Figure S1b', Arrows). When *mid* RNAi was driven by a stronger Gal4 *sd(strong)-GAL4*, we observed a wider space between vein 3 and vein 4 (Figure S1a,b).

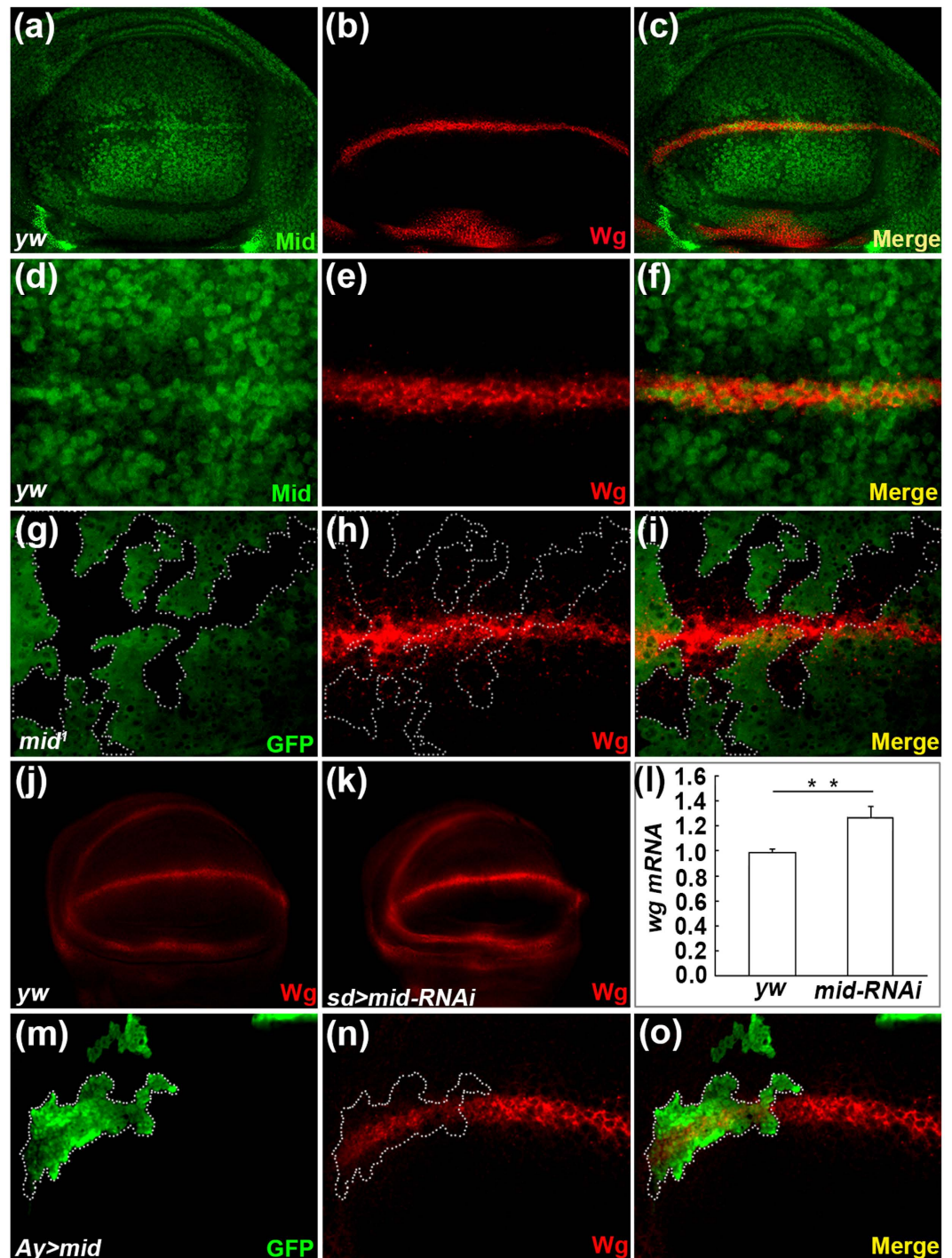


**Figure 2. Mid is required for wing development.** (a,a') Adult wings of *MS1096-Gal4; UAS-GFP* are shown as control. (b–c') Adult wings of *MS1096-Gal4;UAS-mid-RNAi* (b,b') and *MS1096-Gal4;UAS-mid* (c,c'). (d,d') Adult wings of *sd-Gal4;UAS-GFP* are shown as control. (e–f') Adult wings of *sd-Gal4;UAS-mid-RNAi* (e,e') and *sd-Gal4;UAS-mid* (f,f'). Insert of (b') or (e') represents magnification of the region marked by a white broken line. Arrows indicate ectopic wing hairs. Arrowheads indicate loss of tissue at the margin of wing blade. Scale bars: 500  $\mu$ m.

To investigate the effect when *mid* was overexpressed in the wing disc, we employed *UAS-mid* transgenic fly. Ectopic expression of *mid* by *MS1096-Gal4* (Fig. 1j) resulted a small and blistered wing with separated surfaces between dorsal and ventral, a phenotype that possibly caused by loss of adhesion between epidermal blades (Fig. 2c,c'). Furthermore, expression of *UAS-mid* with *sd-GAL4* (Fig. 1l) resulted in loss of tissue at the margin of wing blade, a phenotype typical for a reduction in the Wg signaling (Fig. 2f,f', Arrowheads). Taken together, loss-of-function and gain-of-function analyses demonstrate that Mid plays a indispensable role in the wing.

**Analysis of candidate downstream target genes of Mid.** Given that Mid is a T-box transcription factor<sup>50</sup>, it regulates wing development possibly through activating the expression of target genes. Therefore it will be fruitful to identify the target gene of Mid. To find targets of Mid, we carried out a microarray assay and compared expression profiles of wild-type and *mid* mutant embryos. Among genes that related to wing development, 104 genes were identified as significantly down-regulated and 102 genes were up-regulated in the *mid* mutant (Supplementary Tables S1 and S2). These genes were potential targets of Mid. Among these candidates, we found *wg* and *hh*, which are important for wing development. Given that the phenotypes induced by overexpressing *mid* were analogous to Wg pathway attenuation, and knockdown of *mid* mimicked Hh pathway hyper-activation, we should pay more attention on *hh* and *wg*.

**Mid represses the expression of *wg*.** To examine a possible role of Mid in the Wg pathway, we first compared the expression patterns of Mid and Wg in the wing disc from third instar larvae. We noticed that Mid and Wg were co-expressed in the DV boundary of DP cells (Fig. 3a–f). The co-localization of Wg and Mid suggests that they have some interaction in the wing disc.



**Figure 3. Mid represses the expression of *wg*.** (a–c) The expression of Mid (a) and Wg (b) in the wild-type wing disc. (c) Merged image of (a,b). (d–f) Magnified images of the partial region in (a–c) are shown. (g–i) Wg expression is activated in a *mid* mutant clone (mutant clone lack GFP signal and marked by a broken line) induced in the DP cells of the wing disc. The wing disc from a third instar larva of *hs-FLP; FRT42D, Ubi-GFP/FRT42D, mid<sup>1</sup>* is stained with anti-GFP antibody (g), anti-Wg antibody (h). (i) Merged image of (g,h). (j,k) Activation of the Wg expression in the wing disc of *sd-Gal4; UAS-mid-RNAi* (k) compared with that of *yw* (j). (l) qPCR analyses of the *wg* mRNA level between *yw* and *sd*>*mid-RNAi*. Error bar, SEM from three independent experiments. Student's tests, \*\*p < 0.01. (m–o) Somatic clone overexpressing Mid display downregulation of Wg (clone with GFP signal is marked by broken line). The wing disc from a third instar larva of *hs-FLP; UAS-mid/act > y<sup>+</sup> > gal4; UAS-GFP* is stained with anti-GFP antibody (m), anti-Wg antibody (n). (o) Merged image of (m,n).

To test whether Mid regulates the *wg* expression, we generated *mid* mutant clones, which effectively depletes the expression of Mid (Figure S2a–c), using FLP-FRT method in the wing disc (Fig. 3g). Compared with the neighboring control cells, *mid* mutant clones marked by loss of GFP showed increased Wg levels (Fig. 3g–i). In addition, we verify this result using RNAi-mediated *mid* knockdown. Knockdown of *mid* with *sd-Gal4* increased the expression of *wg* at the DV boundary (Fig. 3k). Furthermore, we detected the levels of *mid* and *wg* mRNA upon *mid* RNAi by qPCR analyses in the wing disc and found a significantly lower expression level of *mid* mRNA (Figure S3) and a higher expression level of *wg* mRNA (Fig. 3l). These results indicate that *mid* RNAi effectively knocked down *mid* mRNA and that Mid represses the expression of *wg*. We also generated *mid* FLP-out clones in wing disc by the FLP/FRT and the GAL4/UAS techniques. This resulted in reduction of Wg expression (Fig. 3m–o).

To investigate whether cell death increased upon ectopic expression of Mid, we expressed *UAS-mid* with *sd-GAL4* and monitored activation of apoptosis by cleaved Caspase-3 antibody. As shown in Figure S4(e), the activity was observed in a small region of the blade but not along the DV boundary, while Wg expression was repressed at the DV boundary (Figure S4d–f). This result suggests that the reduced expression of *wg* upon overexpression of Mid at the DV boundary is not due to increased cell death.

To analyze the genetic interaction between *mid* and *wg*, we induced *wg* RNAi driven by *sd-GAL4* and noted notches at the blade margin (Figure S5b) that are similar to Mid gain of function phenotype (Figure S5a). We then induced *UAS-mid;UAS-wg-RNAi* using *sd-GAL4* and noted a strongly enhanced phenotype, compared with Mid gain of function or Wg knock down alone. Sixty-five percent of the flies exhibit severe loss of wing veins and blistered wing (Figure S5c,  $n = 99$ ), and 35% of the flies even lead to loss of tissue in almost whole wing blade (Figure S5c',  $n = 54$ ). Moreover, an ectopic hair was also found in adults carrying the *mid* mutant clone (Figure S6a,b'), a phenotype analogous to *wg* ectopic expression.

In sum, our results demonstrate that Mid plays a negative role for *wg* expression in the wing disc.

**Mid negatively regulates the expression of *Arm*.** In order to test whether Mid affects the expression of *wg* downstream genes, we analyzed the expression of Arm/ $\beta$ -catenin. We observed repression of *arm* at the DV boundary when *mid* was overexpressed using the *sd-Gal4* driver (Fig. 4d–f). Furthermore, qPCR analyses revealed that overexpression of *mid* downregulated *wg* and *arm* mRNA levels (Fig. 4b,c). We next generated *mid* loss of function mutant clones (Fig. 4g) and FLP-out clones (Fig. 4j) in the DP cells of the wing disc. Arm expression is increased in *mid* mutant clone in the DV boundary (Fig. 4g–i) and decreased in *mid* overexpression FLP-out clone (Fig. 4j–l). Taken together, these results indicate that Mid negatively regulates the Arm expression in the wing disc.

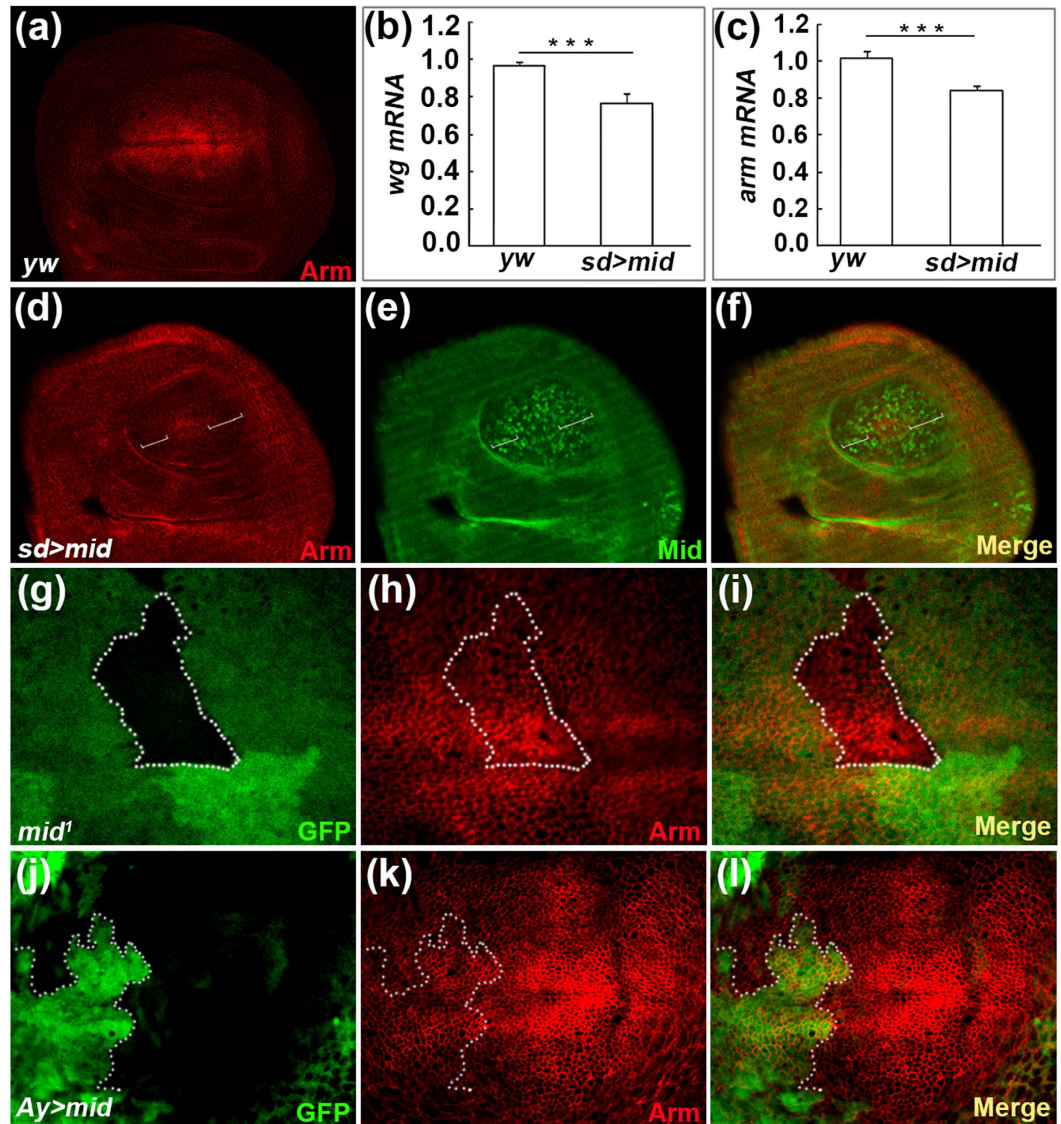
**Mid negatively regulates the expression of *DE-Cad*.** In response to the Wg signaling, DE-Cad regulates epithelial cell-cell adhesion at adherens junctions<sup>53</sup>. To test whether Mid regulates DE-Cad levels, we first induced *mid* loss of function mutant clones in the wing disc, which resulted in hyper-expression of DE-Cad (Fig. 5a–c and Figure S7). We next examined the expression of DE-Cad in wing discs of *yw* and *MS1096-GAL4;UAS-mid*. The control disc displayed a proximodistal (PD) gradient of DE-Cad and normal cell shape (Fig. 5d). However, *mid* overexpression abolished the PD gradient of DE-Cad and resulted in decreased apical cells and cell shape became slender, compared with the same region in the control wing discs (Fig. 5e). In addition, qPCR analyses showed reduced expression of *DE-Cad* when *mid* was overexpressed in wing discs (Fig. 5f). We also overexpressed *mid* in FLP-out clone and found downregulation of DE-Cad (Fig. 5g–i). These results indicate that Mid negatively regulates the expression of *DE-Cad*.

**Mid represses the expression of *hh*.** Our microarray data showed enhanced expression of *hh* in the *mid* mutant (Supplementary Table S1), suggesting that Mid is involved in regulation of Hh pathway. To address this possibility, we used *hh* mutant to test whether Hh and Mid have genetic interaction in wings. The *hh<sup>Mrt</sup>* mutant is a gain-of-function allele of *hh*, which leads to ectopic expression of Hh in the anterior compartment and exhibits a wing phenotype with an expanded anterior region and duplication of longitudinal L2 and L3 veins (compare Fig. 6b with 6a)<sup>28,54,55</sup>. Loss of one copy of *mid* apparently aggravated the phenotypes of *hh<sup>Mrt</sup>* heterozygous adult wing. Compared with the control counterparts, the anterior region of the wing is further enlarged and rounded when *mid* was mutant under *hh<sup>Mrt</sup>* background (Fig. 6c), and ectopic veins L2\* and L3\* were more obvious. The results were summarized in Fig. 6(d). Moreover, an ectopic vein L3 was also found in adults carrying the *mid* mutant clone (Figure S6a,b), a phenotype analogous to *hh* overexpression<sup>33</sup>. These results suggest that Mid acts as a negative modulator of Hh signaling.

Given that Mid negatively regulates Hh pathway and loss of *mid* increases *hh* mRNA levels, we investigated whether Mid regulates the expression of *hh*. We employed anti-Hh antibody and found that overexpression of *mid* decreased Hh protein levels (Fig. 6e–f). To further confirm that Mid regulates Hh at mRNA level, we used a *hh-lacZ* transgenic fly which could monitor the transcription of *hh*. Overexpression of *mid* repressed *hh-lacZ* expression in a non-cell autonomous manner (Fig. 6g–i). We also examined *hh-lacZ* expression in Mid FLP-out clones and found that reduction of *hh-lacZ* expression still occurs in a non-cell autonomous manner (Fig. 6j–l).

We next examined whether Mid negatively regulates Hh pathway activity. In the wing disc, Hh pathway activates the expression of target genes, including *dpp*. Overexpression of *mid* decreased both *hh* and *dpp* mRNA levels in wing discs (Fig. 6m,n), indicating that Mid represses Hh pathway activity. Taken together, our results demonstrate that Mid inhibits *hh* expression and compromise Hh signaling.

***mid* is not activated by Wg in the wing disc.** Previous studies have revealed that Mid regulates cell fate determination in leg discs, and the expression of *mid* is activated by Wg while repressed by Dpp<sup>51</sup>. It is interesting to examine whether the regulation of Wg upon Mid also occurs in the wing. We cannot analyze adult wings due



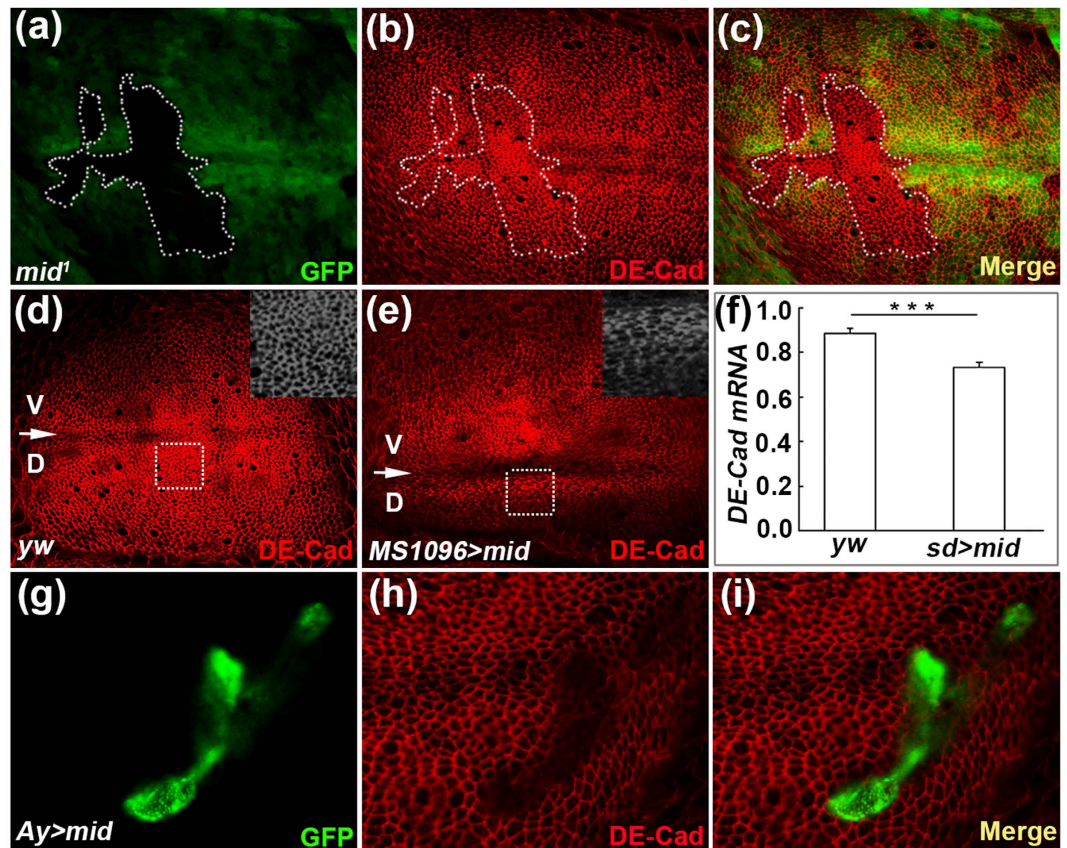
**Figure 4. Mid represses the expression of *arm*.** (a) The wing disc of wild type is stained with the anti-Arm antibody. (b,c) qPCR analyses of *wg* and *arm* mRNA level in *yw* and *sd > mid*. Error bar, SEM from three independent experiments. Student's tests, \*\*\* $p < 0.001$ . (d–f) The expression of Arm is repressed (marked by brackets) in the wing disc of *sd-Gal4; UAS-mid*. (g–i) Arm expression is activated in a *mid'* mutant clone (mutant clone lack GFP signal and is marked by broken line) induced in the DP cells of the wing disc. The wing disc from a third instar larva of *hs-FLP; FRT42D, Ubi-GFP/FRT42D, mid'* is stained with anti-GFP antibody (g), anti-Arm antibody (h). (i) Merged image of (g,h). (j–l) Somatic clone overexpressing Mid display downregulation of Arm (clone is marked with GFP signal and is marked by broken line). The wing disc is stained with anti-GFP antibody (j), anti-Arm antibody (k). (l) Merged image of (j,k).

to lethality caused by *wg* overexpression. Although overexpression of *wg* resulted in a larger wing disc, the level of Mid did not show any detectable changes (Figure S8a–c). We further verified this result using qPCR assays. Overexpression of *wg* apparently increased *wg* mRNA but did not affect *mid* and *hh* mRNA levels (Figure S8d–f). These observations demonstrate that although Mid plays important roles in different tissues, its regulation is distinct. It will be fruitful to investigate how *mid* is regulated in wings.

## Discussion

The Wnt and Hh signaling pathways are involved in many aspects of development and adult homeostasis. Members of the Wg and Hh families induce the expression of a series of target genes by activating conserved signaling cascades<sup>1,18,56</sup>. Although multiple signaling molecules of the Wg and Hh pathways have been identified, little is known about how these pathways are controlled and coordinated. Here we identified Mid as a common regulator of the Wg and Hh pathways.

We demonstrated that Mid acts as a negative regulator of the Wg pathway (Fig. 7) from three lines of evidence. Firstly, microarray analyses indicated that the level of *wg* mRNA was significantly increased in *mid* mutant



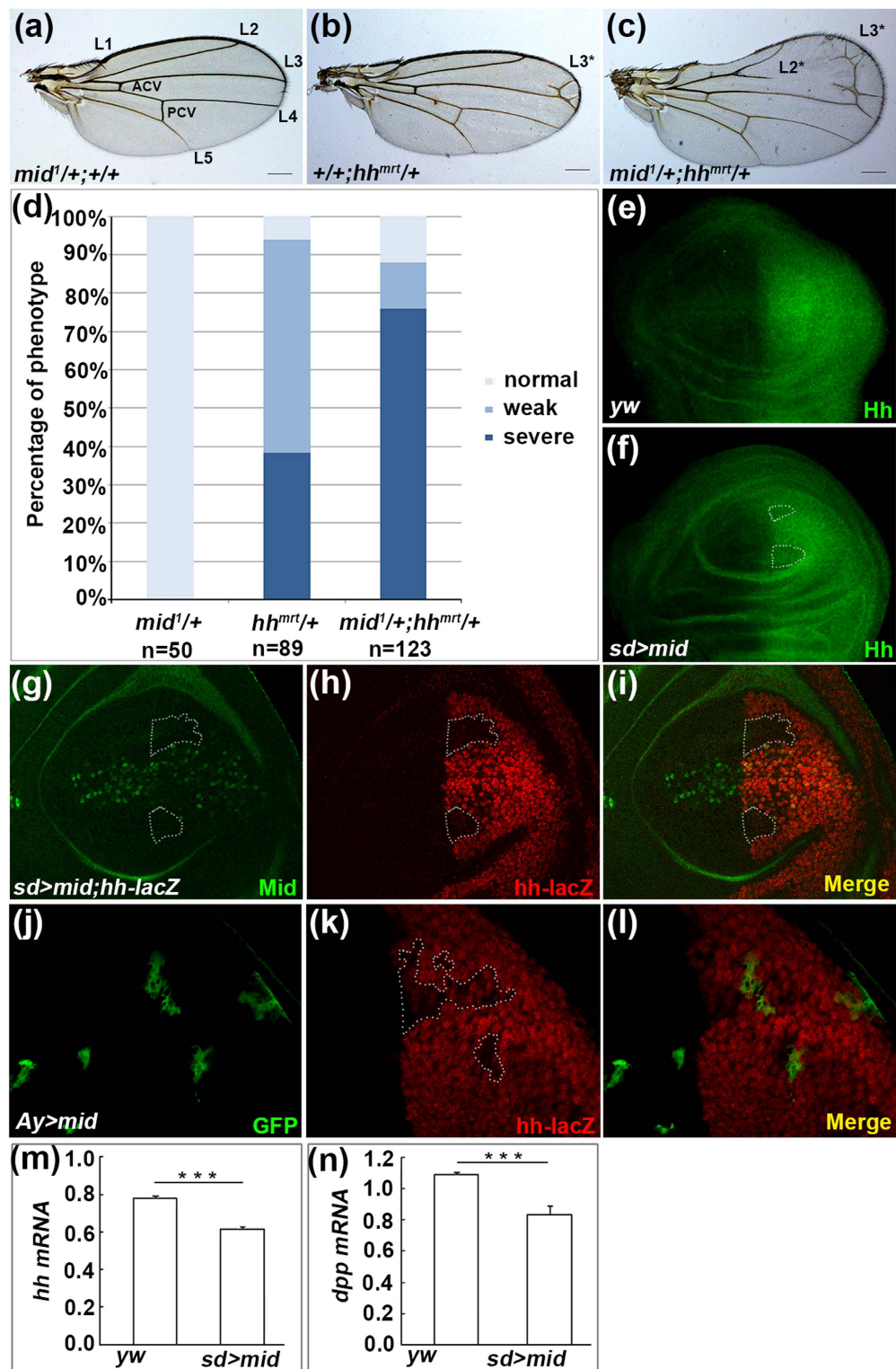
**Figure 5. Mid represses the expression of *DE-Cad*.** (a–c) *DE-Cad* expression is activated in a *mid* mutant clone (mutant clone lack GFP signal and is marked by a broken line) induced in the DP cells of the wing disc. The wing disc from a third instar larva of *hs-FLP; FRT42D, Ubi-GFP/FRT42D, mid<sup>1</sup>* is stained with anti-GFP antibody (a), anti-*DE-Cad* antibody (b). (c) Merged image of (a,b). (d,e) The expression of *DE-Cad* in the wing disc of wild type (d) and *sd-Gal4; UAS-mid* (e). The DV boundary is marked by an arrow. Insert in (d) is magnification of the region marked by a white broken line to show normal apical cell shape. Insert in (e) is magnification of the region marked by a white broken line to show slender apical cell shape. (f) qPCR analyses of *DE-Cad* mRNA level in *yw* and *sd > mid*. Error bar, SEM from three independent experiments. Student's tests, \*\*\**p* < 0.001. (g–i) Somatic clone overexpressing Mid display downregulation of *DE-Cad* (clone is marked with GFP signal). The wing disc is stained with anti-GFP antibody (g), anti-*DE-Cad* antibody (h). (i) Merged image of (g,h).

compared with the control counterpart. Secondly, loss of *mid* in the wing disc induced hyper-expression of *Wg*, whereas overexpression of *mid* reduced the expression of *Wg*. Lastly, Mid acts as a negative regulator of *arm* and *DE-Cad*.

What is the underlying mechanism of the Mid-mediated repression on *Wg*? Mid could directly repress *wg* together with Groucho as demonstrated in the embryonic ectoderm<sup>41</sup>. However, overexpression of *mid* in the wing disc decreased *Wg* even in regions where Mid is not overexpressed (our unpublished results), suggesting that Mid represses *Wg*, at least partly, in an indirect manner. Therefore, Mid possibly attenuates *Wg* through two mechanisms, direct and indirect. Further studies are necessary to dissect the detailed mechanisms.

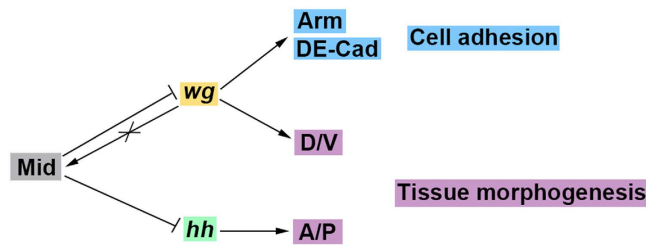
We also demonstrated that Mid acts as a negative regulator of the Hh pathway (Fig. 7) from four lines of evidence. Firstly, analyses using microarrays show that the level of *hh* mRNA was enhanced in the *mid* mutant. Secondly, using anti-Hh antibody and *hh-lacZ* reporter, we observed reduced expression of Hh upon overexpression of *mid* in the wing disc. Thirdly, *mid* reduction can enhance the wing phenotype of *hh<sup>Mrt</sup>*, a gain-of-function allele of *hh*. Lastly, we observed decrease in the expression of a *hh* target *dpp* in response to overexpression of *mid*. Overexpression of *mid* reduced the expression of *hh-lacZ* in a non-cell autonomous manner. Therefore, the repression of *hh* by Mid is not direct but mediated through an unknown mechanism.

Mid is a homolog of vertebrate Tbx20, an evolutionarily conserved transcription factor that is involved in many important events during development<sup>57,58</sup>. In both knock-down and overexpression experiments, we observed abnormalities of wing patterns in response to changes in the level of the *mid* function. Knock down of *mid* in the wing disc induced hyper-expression of *Wg* and its downstream *DE-Cad*, and yielded the cocked and non-flat wings. The hyper-expression of *DE-Cad* would lead to abnormally strong cell-cell adhesion and epithelial misfolding<sup>53</sup>. The cocked wing phenotype could be ascribed to the epithelial misfolding in the hinge region. As the *MS1096-gal4* driver is mainly expressed in the dorsal compartment (ref. 59, see also Fig. 1i), *mid*



**Figure 6. Mid represses the expression of *hh*.** (a–c) Genetic interaction between *mid* and *hh*. Adult wings of *mid*<sup>1/+</sup>; +/+ (a) +/+; *hh*<sup>mrt/+</sup> (b) and *mid*<sup>1/+</sup>; *hh*<sup>mrt/+</sup> (c). Note ectopic vein L3 (L3\*) in (b) and ectopic veins L2 (L2\*) and L3 (L3\*) with enlarged anterior compartment in (c). Scale bars: 200  $\mu$ m. (d) Percentage of the phenotype in (a–c). (e,f) The wing discs of wild type and *sd > mid* are stained with the anti-Hh antibody. The expression of Hh is repressed upon overexpression of *mid* (marked by broken lines in (f)). (g–i) The wing disc of *sd-Gal4; UAS-mid; hh-lacZ* is stained with the  $\beta$ -gal antibody. The expression of *hh-lacZ* reporter is repressed (marked by broken lines). (j–l) Somatic clone overexpressing Mid display downregulation of Hh (marked by broken lines). The wing disc from a third instar larva of *hs-FLP; UAS-mid; hh-lacZ/act > y<sup>+</sup> > gal4; UAS-GFP* is stained with anti-GFP antibody (j),  $\beta$ -gal antibody (k). (l) Merged image of (j,k). (m,n) qPCR analyses of the *hh* and *dpp* mRNA levels in *yw* and *sd > mid*.





**Figure 7. Model for Mid-mediated regulation of wing development.** Mid regulates wing development through repression of the Wg and Hh signaling in both tissue morphogenesis and cell adhesion. Mid represses the expression of *wg* to establish a proper expression level of Wg at the DV boundary. The repression of *wg* results in down-regulation of the downstream genes *arm* and *DE-Cad*, which affects the epithelial cell adhesion at adherens junction. By contrast, *wg* does not affect the expression of *mid*. Mid also represses the expression of *hh* to establish a proper expression level of Hh at the P compartment.

RNAi induced with this driver would lead to hyper-expression of DE-Cad mainly in the dorsal compartment. The resulting strong cell-cell adhesion would reduce the wing surface of the dorsal side. This could give rise to the concave wing. On the other hand, the *sd-gal4* driver mainly expresses in the ventral compartment (ref. 59, see also Fig. 1k) and hence, *mid* RNAi induced with this driver would give rise to the convex wing. Upon knock down of *mid* in the wing disc, we also observed ectopic wing hairs, a phenotype of enhanced *wg* function<sup>39</sup>, and the widening of the distance between L3 and L4 veins, a phenotype of enhanced *hh* function. This is consistent with our conclusion that *mid* regulates wing development by repressing *wg* and *hh*.

Overexpression of *mid* in the wing disc repressed the expression of *wg* and *hh*, which in turn resulted in the small wing phenotype. Overexpression of *mid* in the wing disc also repressed *arm* and *DE-Cad*. Reduced expression of Arm and DE-Cad weakens cell-cell adhesion. Therefore, the blistered wing phenotype upon overexpression of *mid* in the wing disc is most likely due to weakened cell-cell adhesion between the dorsal and the ventral sides.

In sum, we propose a model in which Mid coordinates the Wg and Hh signaling pathways and plays a vital role during wing development (Fig. 7).

## Methods

**Drosophila Stocks.** Following stocks were used: *yw, mid<sup>1</sup>/CyO, MS1096-GAL4, sd(strong)-GAL4, hh<sup>Mrt</sup>, UAS-mid, UAS-wg, UAS-GFP, UAS-wg-RNAi, hh-lacZ* were originally obtained from Bloomington Stock Center. *sd-GAL4* was obtained from *Drosophila* Genetic Resource Center. *UAS-mid-RNAi* was originally obtained from NIG-Fly. All fly stocks were grown at 25 °C. Mutant clonal analyses were done with *hs-FLP; FRT42D, Ubi-GFP/CyO* and *FRT42D, mid<sup>1</sup>/CyO*. FLP-out clonal analyses were done with *hs-FLP; UAS-mid/CyO* and *act > y<sup>+</sup> > gal4; UAS-GFP*.

**Antibodies and Immunohistochemistry.** The antibodies were used at the following dilutions: rabbit anti-Mid (1:500<sup>50</sup>), mouse anti-Wg (1:50 DSHB), mouse anti-Arm (1:200 DSHB), rabbit anti-Hh (1:800 gift of T. Tabata), mouse anti-40-1a (1:500 DSHB), 488 donkey anti-rabbit IgG conjugate (1:500, Alexa), Cy3-conjugated donkey anti-mouse IgG (1:500, Sigma), TOTO-3(1:200, Probes) and cleaved Caspase-3(1:200, Cell Signaling).

Images were acquired under Leica TCS SP5 confocal microscope, Olympus DP2-BSW microscope and Olympus cellSens, processed using Adobe Photoshop CS6.

**Microarray Analysis.** The *mid* heterozygote or mutant embryos were identified using CyOact-GFP balancer chromosome. Total RNA was extracted separately for each of the heterozygous and homozygous *mid* mutant embryos using the QIAGEN RNeasy isolation kit. The probes labeling and hybridization were performed as described previously<sup>60</sup>. Probes with fold-change >2.0 and P values <0.05 were considered to be differentially expressed. All the Raw and normalized microarray expression data have been deposited on the NCBI Gene Expression Omnibus (GEO) Web site ([www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo)) under accession number GSE73802.

**qPCR Analysis.** Total RNA was isolated from the wing disc of the third instar larvae using RNAPrep Pure Tissue kit (TIANGEN #DP431). Prime Script<sup>TM</sup> II 1<sup>st</sup> strand cDNA synthesis kit (TaKaRa #6210A) was used for cDNA synthesis. qPCR was performed in a 20 ul reaction containing 2 pmol of relevant primers (Supplementary Table S3) in Bio-Rad CFX96 real-time system using a SuperReal PreMix Plus (SYBR Green) Kit (TIANGEN #FP205).

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## Author Contributions

Conceived and designed the experiments: Q.-X.L., C.-L.F., X.-F.W. and S.H. Performed the experiments: C.-L.F. and X.-F.W. Analyzed the data: C.-L.F., Q.C. and D.W. Contributed reagents/materials/analysis tools: Q.-X.L. and C.-L.F. Wrote the paper: C.-L.F., S.H. and Q.-X.L.

## Additional Information

**Supplementary information** accompanies this paper at <http://www.nature.com/srep>

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