**spalt** is functionally conserved in *Locusta* and *Drosophila* to promote wing growth

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*Locusta* has strong fly wings to ensure its long distance migration, but the molecular mechanism that regulates the *Locusta* wing development is poorly understood. To address the developmental mechanism of the *Locusta* flying wing, we cloned the Dpp target gene *spalt* (*sal*) and analyzed its function in wing growth in the *Locusta*. The *Locusta* wing size is apparently reduced with vein defects when *sal* is interfered by injection of dsRNA, indicating that *sal* is required for locust wing growth and vein formation. This function is conserved during the *Drosophila* wing development. To better understand *sal*’s function in wing growth, we then used *Drosophila* wing disc as a model for further study. We found that *sal* promotes cell proliferation in the whole wing disc via positive regulation of a microRNA *bantam*. Our results firstly unravel *sal*’s function in the *Locusta* wing growth and confirm a highly conserved function of *sal* in *Locusta* and *Drosophila*.

*Locusta migratoria* is one of the global destructive migratory agricultural pests. Swarm formation and long-distance flight behavior are the two major reasons why *Locusta* plagues are so destructive even today. During the migratory stage, these insects can keep flying for hours over hundreds of kilometers. Therefore, besides gene expansion associated sufficient energy supply, well developed and functional wings are required to adapt their migratory behavior.

*Locusta* is an incomplete metamorphosis insect whose young nymph resembles the adult with visible developing forewings and hindwings. The wing shape and size are varied among geographical populations. Based on forewing shape, grasshopper *Trilophidia annulata* can be divided into geographical groups and microRNAs are associated with the polymorphism of adult wings. Biomechanical study shows that the fan-like distribution of veins improve fracture toughness in *Locusta*. However, little is known about the molecular mechanism of how the *Locusta* wing develops into such delicate structure.

Current understanding of insect wing development mechanism is mainly from the fruitfly *Drosophila melanogaster*. In comparison, the fruitfly, a Diptera insect, undergoes complete metamorphosis. The adult wings are developed from larval wing imaginal discs which are formed from late embryogenesis and undergo intricate cell proliferation, differentiation and morphogenesis during larval and pupal stage inside the body. The pattern formation is delicately regulated by organizers located in the anterior/posterior (A/P) and dorsal/ventral (D/V) boundaries which secrete signal molecules including the long-range morphogens Decapentaplegic (Dpp) and Wingless (Wg), and short-range morphogen Hedgehog (Hh). These morphogens form gradients to regulate the expression of their target genes and control almost all aspects of wing development.

The Dpp morphogen gradients control cell growth through negatively regulating the transcriptional suppressor *brinker* (*brk*), which downregulates *bantam* (*ban*), a developmentally regulated microRNA. *ban* promotes cell proliferation autonomously and suppresses proliferation-induced apoptosis. Various Dpp targets identified, such as *optomotor-blind* (*omb*), *spalt* (*sal*), and *vestigial* (*vg*), are repressed by *brk*. Among them, *omb* represses *ban* expression in the medial regions of the wing disc to promote cell proliferation while playing an opposite role in the lateral regions. The two *Drosophila* *sal* homologues *spalt major* (*salm*) and *spalt related* (*salr*) are also highly expressed in the central region of the wing pouch and mediate Dpp signaling pathway by promoting cell proliferation. *salm* and *salr* have similar function but are not fully redundant. For example, *salm*, but not *salr*, is evolutionally conserved in insects as a main regulator of fibrillar flight muscle fate. Downstream the Salm/Salr complex, the vein-specific expression of the *knirps* and *iroquois* gene complexes and a serious of candidate genes are regulated for wing vein patterning and growth. *sal* also participates in defining fly notum.

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and wing hinge\textsuperscript{27} and the melanin pigmentation of wing eyespots in butterflies\textsuperscript{28–30}. Therefore, sal may play a conserved role in insect wing development to generate the fast oscillating wings and whether sal functions through ban remains to be illustrated.

Figure 1. Identification of sal in Locusta. Two unigenes named Lmsal411 and Lmsal468 are found in locusts. Protein sequence alignment of Locusta and Drosophila Sal shows highly conserved sequences in the ZnF-C2H2 domains. Stars indicate the identical amino acids. Underlines show the putative Drosophila Salm ZnF-C2H2 domains.

Figure 2. Phylogenic tree of sal in insects. The amino acid sequences of L. migratoria a12971 and a6922, and potential Sal from other insects are used to build the neighbor-joining tree.
To better understand the developmental mechanism of *Locusta* flying wings, we cloned *Locusta sal* cDNAs and investigated the role of *sal* during wing blade and vein formation. By RNA interference (RNAi), we found that *sal* is required for wing growth and vein formation, which is conserved between the *Locusta* and *Drosophila*. Then, using the *Drosophila* wing disc model we dissected *sal* function in wing growth and found that *sal* promotes cell proliferation rate during larval wing development. Furthermore, mircoRNA *bantam* mediates the role of *sal* in cell proliferation regulation.

**Materials and Methods**

**Drosophila strains and Locusta.** All fruit flies used were *Drosophila melanogaster*. Flies were raised at 25 °C. Three transgenes UAS-salm\(^{27}\), UAS-salmRNAi (Tsinghua Fly Center THU3581) and UAS-salrRNAi (THU3147) were used to manipulate the expression level of *sal*. The efficiency of these RNAi lines has been recently verified by Tang et al.\(^{31}\). The Gal4 lines used were ci-Gal4\(^{32}\), sal-Gal4\(^{33}\), and ap-Gal4\(^{34}\) to drive the expression of the UAS transgene. The *bantam* enhancer reporter line was br-C12-lacZ\(^{35}\).

*Locusta migratoria* was raised at 28 °C with 60% relative humidity.

**Identification and sequence analysis of spalt from *L. migratoria* transcriptome.** The available *salm* and *salr* gene sequences from *Drosophila* were used as references to screen *Locusta migratoria* transcriptome database we previously established\(^{8}\). The potential candidates of *L. migratoria sal* genes were confirmed by searching the BLASTX algorithm against the non-redundant NCBI nucleotide database using a cut-off E-value of 10\(^{-5}\).

Based on the *Drosophila* Spalt protein sequences, the deduced *Locusta* Spalt protein domains were determined by using DNAMAN software (Lynnon Biosoft). Other insect Spalt and Spalt-like proteins are obtained from...
NCBI (https://www.ncbi.nlm.nih.gov/protein). Global protein alignment and neighbor-joining phylogenetic tree were performed by Geneious R9.

RNA interference. The primers to make the double-strand RNA probes were as follows:

- Lmsal411-F 5′-ATGTTGCAGCGGCGTGCACAAGAGG-3′
- Lmsal411-R 5′-TTGTCCCAGTTGTGCCAGCAGTGGA-3′
- Lmsal468-F 5′-GCCATAGACCCTGCTAAGGACCCAG-3′
- Lmsal468-R 5′-GTCCTTCACCTCTGCAGCTTGTATC-3′
- GFP-F 5′-CACAAGTTCAGCGTGTCCG-3′
- GFP-R 5′-GTTCACCTTGATGCCGTTC-3′

GFP-dsRNA, Lmsal411-dsRNA, and Lmsal468-dsRNA were injected into the 4th instar nymphs using 20 μg dsRNAs per insect. Nymphs were raised at 28 °C for 10 days until they grew up to the 5th instar. Imaging the whole body and wing discs using SONY DSC-HX1 camera before the wing discs were lysed.

Quantitative Real-Time RT-PCR Analysis. Total RNAs were extracted with Trizol reagent from the 5th nymph wing discs, digested with DNase I, and reverse transcribed using the FastQuant cDNA Synthesis kit (Tiangen Biotech), then quantitative real-time RT-PCR was performed using Go Taq qPCR Master Mix (Promega). The primers for quantitative RT-PCR were as follows:

- Lmsal411q-F 5′-GAGAATGCCAGGCCGCCACAGGG-3′
- Lmsal411q-R 5′-GGATCGCTTTGAAGAAGGGGT-3′
- Lmsal468q-F 5′-ACAAACATCGGAAGACGGA-3′
- Lmsal468q-R 5′-CTAGCTCTTTCATGCGGCA-3′

Relative transcript levels were assessed using the Comparative CT method. β-actin was used as an internal control.

BrdU staining. BrdU staining was performed as previously described20,36. Dissected third instar wing discs were co-cultured with BrdU (1:100) in Schneider’s medium for 40–50 min at 25 °C. Then, samples were fixed in 4% formaldehyde and washed in PBT before immunostaining.

Immunohistochemistry. The primary antibodies used were mouse anti-BrdU, 1:100 (MBL) and mouse anti-β-galactosidase 1:2000 (Promega Z3783), and the secondary antibody was anti-mouse DyLight 549 (1:200,
Figure 5. *sal* promotes cell proliferation in the *Drosophila* wing disc. (A) The cell proliferation rate is increased when *salm* is overexpressed in ci-Gal4 region. (B) The cell proliferation rate is increased when *salm* is overexpressed in ap-Gal4 region. (C and D) The cell proliferation rate is repressed when *salr* is knocked-down. (E) The cell proliferation rate is repressed when *salm* is knocked-down. GFP shows the Gal4 expressing domain. White boxes define the area of fluorescence quantification of BrdU staining.
Agrisera). Images were obtained using an Olympus FV10-ASW laser scanning confocal microscope and processed with Adobe Photoshop 8.0.

**Wing size measurement.** Image-J program was used to measure the wing areas and vein distances. High resolution images were opened in Image-J program, a straight line was drawn at the wanted sites between L2 and L4 veins to calculate the distance; and a wanted region as outlined along the L2 and L4 veins (for *Drosophila* wing) or the wing margin (for *Locusta* wing) to calculate the areas.

**Results and Discussion**

**Identification of *Locusta sal* genes.** Based on the transcriptome of the *Locusta* wing disc in our previous study, we blasted the cDNA sequences with salm and salr in *Drosophila* and identified two orthologous unigenes a12971 and a6922, which are 2087 bp and 1073 bp, respectively, in the *Locusta* (Sup. Fig. 1). We named these two putative genes as Lmsal411 and Lmsal468, respectively. Although these two unigenes were incomplete *spalt* gene sequences, both putative translations were predicted (Sup. Fig. 1). Protein sequence alignment showed that these two putative translations were 23.8% identity. Lmsal411, which was aligned with the anterior part of *Drosophila Sal*, was 33.1% identical to both Salm and Salr, while Lmsal468, aligned with the posterior part, showed a little higher identity (42.1% and 35.5%, respectively) (Fig. 1). The ZnF-C2H2 motifs showed a highly conservation in Lmsal411 (87.0% identity) and Lmsal468 (72.4% identity), indicating a conservative DNA binding motif of transcription factors. In addition, the phylogenetic analysis from different insect species revealed that Lmsal411 clustered with *Drosophila sal*, while Lmsal468 with bees (Fig. 2). Therefore, due to the less identity between these two translations and higher similarity to *Drosophila salm*, we speculate that these two unigenes may be different loci of the same *Locusta spalt*.

Above analysis implies that *Locusta sal* may share partial functions with that of *Drosophila*. In the *Drosophila* wing, the transcription factors Salm and Salr participate in wing growth and vein formation. However, the regional growth effects of Sal on wing growth and cell proliferation rate have not yet intensively investigated.

**Locusta sal regulates wing growth.** To test the functional conservation of sal, we performed RNA interference experiments in *Locusta* using Lmsal genes we identified. After 10 days of injection, the mRNA levels of each gene were measured using quantitative real-time RT-PCR. The mRNA expression levels of Lmsal411 and Lmsal468 were significantly reduced to 62.7 ± 9.1% (p < 0.05) and 36.1 ± 8.5% (p < 0.01), respectively, when the corresponding gene was knocked-down (Fig. 3A and B). We noted that Lmsal411 mRNA was reduced to 30.3 ± 21.8% when interfering with Lmsal468, and vise versa, Lmsal468 mRNA was reduced to 33.5 ± 11.3% when interfering with another (Fig. 3A and B). Thus, the expression of these two unigenes are dependent on each other, implying that they belong to either the same gene or one gene complex. As when knocking-down either gene, the efficiency of interference of both genes were similar and had no statistic difference (Fig. 3A and B), and
taken together with the above analysis of sequence identity, it is more likely that they are belonging to the same gene. When Lmsal411 was interfered in 4th instar, the wings of early 5th instar nymphs were remarkably reduced to 45% of the control (15.4 vs 27.5 mm²) (Fig. 3D and F). Consistently, interfering with Lmsal468 resulted in 69% less of wing area (8.6 vs 27.5 mm²) (Fig. 3E and F). The phenotypes of Lmsal468-RNAi were much more obvious than that of Lmsal411-RNAi (Fig. 3D and E) probably due to the higher RNAi efficiency of the former. Moreover, the hindwing concomitantly reduced with the forewings, while the body size was not apparently affected (Fig. 3D and E). The veins in the posterior part was slightly affected in Lmsal468-RNAi nymphs (arrow in Fig. 3E’). This result indicates that Locusta sal regulates wing growth and vein pattern formation.

**Drosophila sal promotes adult wing growth.** To further confirm sal is functionally conserved in other insects to promote wing growth, we regionally up- or down-regulated salm and salr in Drosophila wings and examined the adult wing size. Drosophila wing has five longitudinal veins, which are L1-L5 from the anterior to posterior part. Between L1 and L4 is the anterior part and other areas are the posterior wing. The distance from L2 to L4, which is indicated by a dashed line in Fig. 4A, was measured to monitor the size of the anterior part of the wing. The flies containing ci-Gal4, which is expressed in the anterior part of the wing, was used as a control and showed normal size and morphology. When salm was overexpressed, the relative distance was significantly increased (1.07 ± 0.01), while when knocked-down salm or salr, the distance was strikingly reduced (0.85 ± 0.01 and 0.94 ± 0.01, respectively) (Fig. 4B–D and I). To confirm this result, we used another driver sal-Gal4, which is expressed in the medial pouch to repeat the manipulation. Again, the relative area enclosed by L2 and L4 (sal-Gal4 expression region) was increased in salm overexpression wings and reduced in salm and salr knock-down wings (Fig. 4F–H and J). In addition, sal regulated the vein formation in all tested lines except ci-Gal4-salm which is consistent with previous studies21,25,26. These data confirm that Drosophila sal regulates wing growth and vein pattern formation. Thus, sal function in promoting wing growth is conserved in the fruitfly and locust.

**sal promotes cell proliferation in the wing disc.** To unravel the mechanism of wing growth regulated by sal, we use the Drosophila wing disc as the research model because it is easy of molecular labeling. We examined the cell proliferation rate in Drosophila wing discs by Bromodeoxyuridine (Brdu) incorporation which is widely used in cell proliferation studies. Brdu is a thymidine analog that can be incorporated into DNA during DNA synthesis in culture, and then be detected by specific anti-Brdu antibodies following with quantitative analysis of the anti-Brdu fluorescent intensities. In wild-type control, Brdu level is evenly distributed in the wing discs20. Elevating Salm level by expressing UAS-salm in the ci-Gal4 or ap-Gal4 domain, the level of Brdu level was increased (Fig. 5A and B). Within the pouch, the fluorescence intensity of Brdu was increased in the salm expressing regions, indicating an enhanced proliferation rate (Fig. 5A and B). Vice versa, compromising salm and salr by expressing UAS-salm-RNAi or UAS-salr-RNAi in the sal-Gal4 or ci-Gal4 domain, the level of Brdu staining was reduced which means that the cell proliferation rate was decreased. These data demonstrate that Drosophila sal promotes cell proliferation in the early wing developmental stages.

**sal promotes microRNA bantam expression.** We previously found that one of the Dpp target genes omb represses ban expression in medial regions of the Drosophila wing discs while plays an opposite role in lateral regions20. Subsequently we took the advantages of transgenic lines available for Drosophila to do further investigation, because we are lacking of genetic tools to do it on the Locusta wing to test whether and how sal regulates the expression of ban. ban transcription was monitored by br-C12-IacZ23 which was mainly expressed in the hinge/blade folds surrounding the wing pouch (Fig. 6A). Expression of UAS-salm in the medial region of the wing disc driven by dpp-Gal4 apparently upregulated ban transcription level (arrows in Fig. 6B and B’). To confirm this result, clones were generated in the wing disc. Consistently, ban was upregulated in both medial regions and lateral region (Fig. 6C–C”). Therefore, sal promotes ban expression in the wing disc in a non-regional specific manner, unlike the manner of omb.

Previous studies have illustrated several models to explain how Dpp gradient controls a uniform cell proliferation rate in the wing disc. Dpp restricts the transcription factor Brinker (Brk) domain to the lateral wing disc where suppresses the growth promoter ban15. While Brk activity is not essential for the medial wing disc growth38–40. Omb is a main mediator of Dpp signaling in the control of cell proliferation rate. Omb represses ban expression in medial regions of the wing discs20. Subsequently we took the advantages of transgenic lines available for Drosophila to do further investigation, because we are lacking of genetic tools to do it on the Locusta wing to test whether and how sal regulates the expression of ban. ban transcription was monitored by br-C12-IacZ23 which was mainly expressed in the hinge/blade folds surrounding the wing pouch (Fig. 6A). Expression of UAS-salm in the medial region of the wing disc driven by dpp-Gal4 apparently upregulated ban transcription level (arrows in Fig. 6B and B’). To confirm this result, clones were generated in the wing disc. Consistently, ban was upregulated in both medial regions and lateral region (Fig. 6C–C”). Therefore, sal promotes ban expression in the wing disc in a non-regional specific manner, unlike the manner of omb.

**References**

1. Eseren, M. Entomology: Can the war on locusts be won? Science 306, 1880–1882, doi: 10.1126/science.306.5703.1880 (2004).
2. Lovejoy, N. R., Mullen, S. P., Sword, G. A., Chapman, R. F. & Harrison, R. G. Ancient trans-Atlantic flight explains locust biogeography: molecular phylogenetics of Schistocerca. Proc Biol Sci 273, 767–774, doi: 10.1098/rspb.2005.3381 (2006).
3. Wang, X. et al. The locust genome provides insight into swarm formation and long-distance flight. Nat Commun 5, 2957, doi: 10.1038/ncomms3957 (2014).
4. Dirks, J. H., Parle, E. & Taylor, D. Fatigue of insect cuticle. J Exp Biol 192, 1924–1927, doi: 10.1242/jeb.083824 (2013).
5. Wootton, R. J., Herbert, R. C., Young, P. G. & Evans, K. E. Approaches to the structural modelling of insect wings. Philos Trans B Soc Lond B Biol Sci 358, 1577–1587, doi: 10.1098/rstb.2003.1351 (2003).
6. Liu, S. et al. De novo transcriptome analysis of wing development-related signaling pathways in Locusta migratoria manilensis and Ostrinia farnacalis (Guenée). PLoS One 9, e106770, doi: 10.1371/journal.pone.0106770 (2014).
7. Bai, Y., Dong, J. J., Guan, D. L., Xie, J. Y. & Xu, S. Q. Geographic variation in wing size and shape of the grasshopper Trilophidia annulata (Orthoptera: Oedipodidae): morphological trait variations follow an ecogeographical rule. Sci Rep 6, 32680, doi: 10.1038/rep32680 (2016).
8. Lovejoy, N. R., Mullen, S. P., Sword, G. A., Chapman, R. F. & Harrison, R. G. Ancient trans-Atlantic flight explains locust biogeography: molecular phylogenetics of Schistocerca. Proc Biol Sci 273, 767–774, doi: 10.1098/rspb.2005.3381 (2006).
9. Liu, S. et al. De novo transcriptome analysis of wing development-related signaling pathways in Locusta migratoria manilensis and Ostrinia farnacalis (Guenée). PLoS One 9, e106770, doi: 10.1371/journal.pone.0106770 (2014).
10. Bai, Y., Dong, J. J., Guan, D. L., Xie, J. Y. & Xu, S. Q. Geographic variation in wing size and shape of the grasshopper Trilophidia annulata (Orthoptera: Oedipodidae): morphological trait variations follow an ecogeographical rule. Sci Rep 6, 32680, doi: 10.1038/rep32680 (2016).
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Author Contributions
J.S. developed the concept and designed the experiments. D.W., J.L., S.L. and H.Z. performed the experiments. D.W., L.Z., W.S., and J.S. analyzed the data and wrote the manuscript.

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