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Jumu is required for the activation of JAK/STAT in *Drosophila* lymph gland development and epidermal wounds

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ABSTRACT

The regulatory mechanism of hematopoiesis and innate immunity in *Drosophila* is highly similar to that in mammals, and *Drosophila* has become a suitable model to understand vertebrate hematopoiesis and the immune response. JAK-STAT signaling pathway components are widely conserved during evolution, and contribute to hematopoiesis and multiple tissue damage and immune responses. Here, we demonstrate that Stat92E is widely expressed in the lymph gland, and the loss of *jumu* inhibits the maintenance of the JAK/STAT pathway in the CZ and MZ but not in the PSC of the lymph gland. Furthermore, we found that clean puncture wounding of the larval epidermis can lead to the activation of JAK/STAT signaling and the generation of lamellocytes, and Jumu is required for the activation of JAK/STAT in response to epidermal wounds.

1. Introduction

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is a conserved metazoan signaling system that controls multiple biological processes in development and tissue homoeostasis. In mammals, the JAK/STAT pathway is comprised of four JAK and seven STAT genes, and mainly regulates embryonic development, hematopoiesis and immunity, and stem cell maintenance [1,2]. In *Drosophila*, the known JAK/STAT pathway ligands consist of only three related interleukin-6 (IL-6)-like cytokine proteins called Unpaired (Upd), Upd2 and Upd3, which activate one receptor, called Domeless (Dome). Moreover, *Drosophila* has a single JAK, Hopscotch (Hop), and one STAT transcription factor, Stat92E [2]. Activated Stat92E dimers induce the expression of target genes including SOCS36E, which encodes a negative regulator [3]. JAK-STAT pathway regulates the development and function of both germline stem cells (GSCs) and somatic cyst stem cells (CySCs) in the adult testis, and controls the response to tissue ablation [2].

JAK/STAT signaling is also associated with several aspects of hematopoiesis and the innate immune system in *Drosophila*. A gain-of-function mutation in the JAK hop can cause an increased number of plasmatocytes and the massive differentiation of lamellocytes and lead to the formation of melanotic tumors [4,5]. During *Drosophila* larval development, the JAK/STAT signaling plays important roles in lymph gland hematopoiesis. Upd3 is expressed and activates the JAK-STAT pathway in the medullary zone (MZ), which is required to maintain a pool of prohemocytes [6]. In response to wasp parasitism, the expression of Upd3 and Dome is decreased and lat expression is increased in the MZ, leading to complete switching off of the JAK-STAT pathway, thus inducing the massive differentiation of lamellocytes [6]. Moreover, Pvf1 produced by posterior signaling center (PSC) cells activates Pvr on differentiating cells in the cortical zone (CZ), and the Stat92E-dependent Pvr/STAT/Adgf-A cascade in CZ cells regulates the quiescence of prohemocytes in the MZ [7]. In addition, activation of JAK/STAT signaling in hemocytes is required for their increased proliferation in response to both tumors and tissue damage [8–11]. The secretion of JAK-STAT-activating cytokines by hemocytes also regulates the humoral systemic response following septic injury [12].

Jumu is a member of the *Drosophila* Forkhead transcription factor family, which is a homolog of mouse FOXN1. Jumu is involved in the growth, development and morphogenesis of *Drosophila* [13]. Jumu can also inhibit the expression of the Nidogen enhancer with its homolog CHES-1, as in *Drosophila* hearts [14]. Our previous

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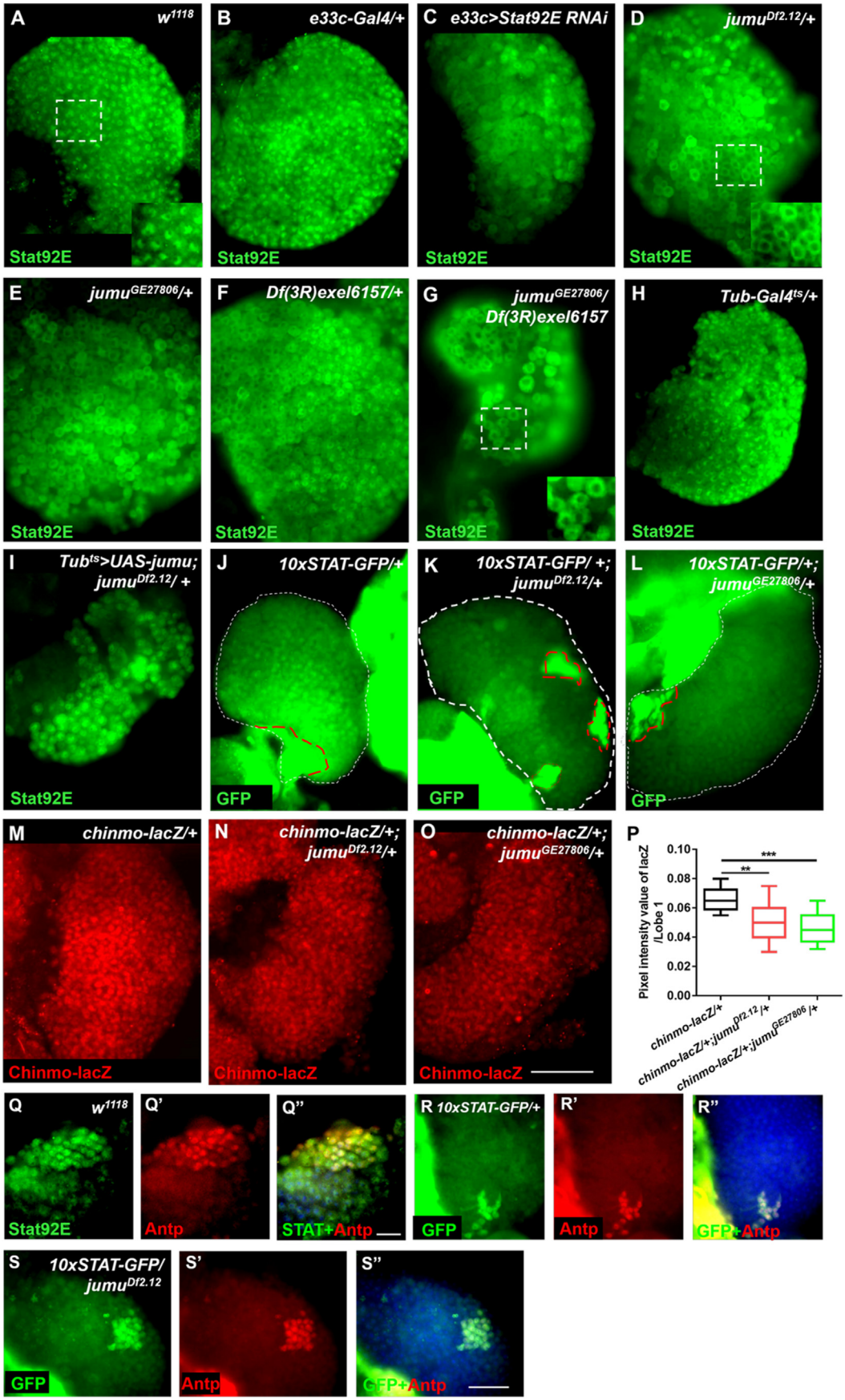
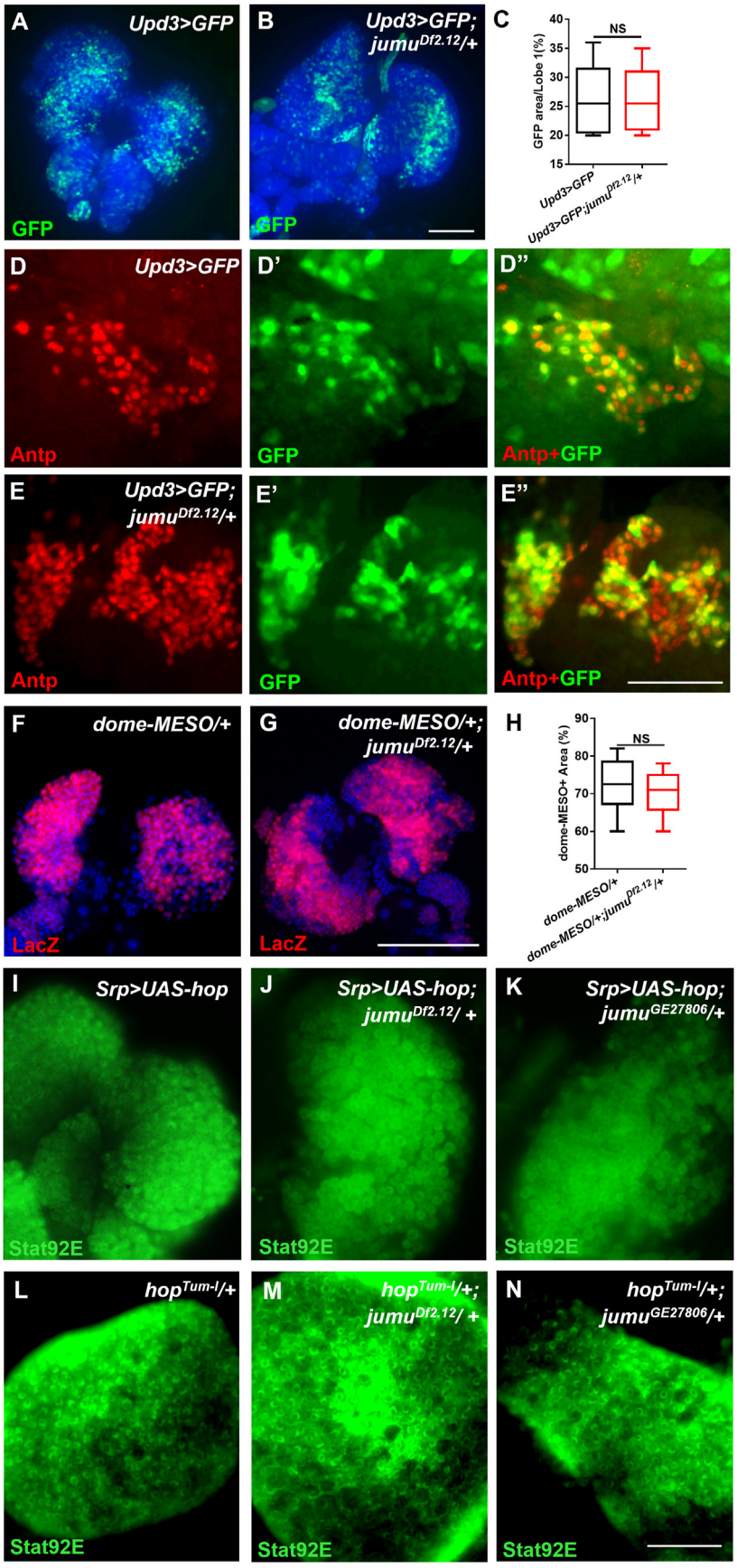


Fig. 1. Loss of *jumu* affects the maintenance of the JAK/STAT pathway in the lymph gland. (A–I) Immunostaining against Stat92E in the lymph glands of third-instar larvae. (J–L) The 10 × STAT-GFP reporter in the lymph glands of third-instar larvae was detected with GFP antibodies. (M–O) The Chinmo-lacZ reporter in the lymph glands of third-instar larvae was detected with β-gal antibodies. (P) Quantification of the pixel intensity of the Chinmo-lacZ reporter. (Q-Q") Immunostaining against Stat92E and the PSC cell marker Antp. (R–S") Immunostaining against GFP and the PSC cell marker Antp. Dashed white and red lines outline the edges of the primary and PSC zones, respectively, in Figures J–L. Scale bars: 50 μm (A–O), 20 μm (Q-S"). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)


A
Upd3>GFP
GFP
B
Upd3>GFP;
jumuDf2.12/+
GFP
C
NS
GFP area/Lobe 1(%)
40
35
30
25
20
15
Upd3>GFP
Upd3>GFP;jumuDf2.12/+
D
Upd3>GFP
Antp
D'
GFP
D''
Antp+GFP
E
Upd3>GFP;
jumuDf2.12/+
Antp
E'
GFP
E''
Antp+GFP
F
dome-MESO/+
LacZ
G
dome-MESO/+;
jumuDf2.12/+
LacZ
H
NS
dome-MESO+ Area (%)
90
80
70
60
50
dome-MESO/+
dome-MESO/+;jumuDf2.12/+
I
Srp>UAS-hop
Stat92E
J
Srp>UAS-hop;
jumuDf2.12/ +
Stat92E
K
Srp>UAS-hop;
jumuGE27806/+
Stat92E
L
hopTum-l/+
Stat92E
M
hopTum-l/+;
jumuDf2.12/ +
Stat92E
N
hopTum-l/+;
jumuGE27806/+
Stat92E

studies have shown that Jumu maintains the proper differentiation of prohemocytes by cell-autonomously regulating the expression of Col in MZ and by noncell-autonomously preventing the generation of expanded PSC cells, and a deficiency of *jumu* throughout the lymph gland can induce the differentiation of lamellocytes by activating Toll signaling [15]. Moreover, we found that Jumu also regulates the development, differentiation and phagocytosis of circulating hemocytes in *Drosophila* [16].

In this study, we investigated the role of Jumu in the activation of the JAK/STAT signaling pathway in the *Drosophila* lymph gland and response to epidermal wounds. Loss of *jumu* affects the localization of Stat92E and the maintenance of the JAK/STAT pathway in the lymph gland. Moreover, we found that epidermal wounds can induce the activation of the JAK/STAT signaling pathway in circulating hemocytes and lymph glands, and *jumu* deficiency can inhibit the systemic activation of JAK/STAT signaling in the immune response to epidermal wounds.

2. Materials and methods

2.1. Fly stocks and culture

We used the following lines in this study: w^{1118}, *chinmo-lacZ* and hop^{Tum-l} were obtained from Bloomington; *P{en2.4- GAL4}e33C (e33C-Gal4)* was a gift from Dominique Ferrandon; $jumu^{GE27806}$ was purchased from GenExel (Daejeon, South Korea); $jumu^{Df2.12}$ and *Df(3R)Exel6157* were gifts from Alan M. Michelson;*10×STAT-GFP* and *Tub-Gal4*^{ts} (*Tub-Gal4, Tub-Gal 80*^{ts}) were obtained from the Tsinghua Fly Center; *dome-MESO-lacZ* was obtained from Jiwon Shim [17]; *Stat92E RNAi* (*v43866*), *upd3-Gal4-UAS-GFP* and *Srp-Gal4* were gifts from Li Hua Jin. *UAS-hop* was gifts from Rong wen Xi. The crosses involving RNAi lines or *Tub-Gal4*^{ts} were reared at 29 °C, and the other strains and crosses were reared at 25 °C on standard cornmeal-yeast medium.

2.2. Immunohistochemistry

For antibody staining, the lymph glands and hemocytes of third-instar larvae were fixed and stained as described previously [15,16]. The following primary antibodies were used: rat anti-Stat92E (gifts from Jin Li Hua), rabbit anti-GFP (1:100, Thermo Fisher Scientific), mouse anti-β-galactosidase (Promega), mouse anti-Antp (Developmental Studies Hybridoma Bank), and mouse anti-L1 (gifts from I. Ando). Alexa Fluor 488-, Alexa Fluor 568- and Alexa Fluor 594-conjugated secondary antibodies (Molecular Probes) were used.

2.3. Image analysis and quantification

All images used for quantification were captured with a Leica or a Zeiss Axioplan 2 microscope, and all quantification analyses were performed using ImageJ as described previously [15,16]. The pixel intensity values of Chinmo-lacZ and 10 × STAT-GFP were defined as the average pixel intensity values in each primary lobe (integrated intensity in one primary lobe/area of the primary lobe). The proportions of Upd3-GFP and domeMESO in the primary lobes were defined as the average area of Upd3-GFP or domeMESO + cells in all optical sections of a primary lobe compared with the area of the primary lobe.

2.4. Clean puncture wounding

The third instar larvae were placed on a slide and washed with PBS, and then, the third instar larval cuticle was pierced with a 0.15 mm steel needle at the dorsal midline. For injury of larvae, the needle was sterilized by flaming. All control and mechanically stressed larvae were left on fly food and dissected 4 h after treatment.

2.5. Quantitative real-time PCR

Total RNA from entire third-instar larvae was isolated with TRIzol (Invitrogen). The obtained total RNA was used to generate cDNA with M-MLV (H-) Reverse Transcriptase (Vazyme). Real-time PCR amplification was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme) on an ABI7300 real-time PCR system. The primer sequences used are shown in supplementary file 1.

2.6. Statistical analysis

Statistical analyses were performed with two-tailed unpaired Student's *t*-tests or one-way ANOVAs using GraphPad Prism software. The thresholds for statistical significance were established as $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

3. Result

3.1. Jumu is required for the maintenance of the JAK/STAT pathway in the lymph gland

In previous studies, we showed that a deficit in *jumu* expression can enlarge the area of the MZ-specific markers domeless-Gal4, UAS-2 × EGFP (dome > GFP) in the lymph gland; however, the transcription levels of the JAK/STAT target genes hop and Stat92E are reduced in *jumu*-deficient circulating hemocytes [15,16]. These results led us to ask whether Jumu regulates the activation of JAK/STAT. We first detected the expression of Stat92E in the lymph gland through anti-Stat92E antibody staining. In wild-type larvae, Stat92E was expressed in the entire lymph gland and was mainly located in the nuclei (Fig. 1A). Next, we knocked down *Stat92E* in the entire lymph gland using *e33c-Gal4*, and found that the expression level of Stat92E was clearly reduced in the *e33c > Stat92E RNAi* mutant (Fig. 1B and C). Moreover, we found that Stat92E was mainly located in the cytoplasm of lymph gland cells of *jumu* mutants (Fig. 1D–G), and the overexpression of *jumu* in the entire lymph gland using the ubiquitous driver *Tub-Gal4*^{ts} rescued the nuclear localization of Stat92E in the $jumu^{Df2.12}$ mutant (Fig. 1H and I). Next, we detected STAT reporter 10×STAT-GFP, which expresses GFP under the control of binding sites for the STAT transcription factor. In wild-type larvae, the 10×STAT-GFP reporter was mainly expressed in the MZ of the lymph gland (Fig. 1J). Moreover, the expression of the 10×STAT-GFP reporter was clearly reduced in the MZ of the lymph gland of *jumu* mutants (Fig. 1K and L). Chinmo is a well-established important downstream mediator of the JAK/STAT pathway and is also expressed in the larval lymph gland [18]. Therefore, we used *chinmo-lacZ*-expressing flies to detect the activation of JAK/STAT signaling. As expected, *jumu* mutants displayed a reduced Chinmo-lacZ expression signal in the lymph gland (Fig. 1M–P).

Fig. 2. Hyperactive JAK can recover the nuclear localization of Stat92E in the lymph glands of *jumu* mutants. (A, B) Upd3>GFP was used to mark the expression of Upd3 in the lymph glands of third-instar larvae. (C) Quantification of the proportions of the primary lobes occupied by Upd3>GFP + area. (D-E") Immunostaining against GFP and the PSC cell marker Antp in the lymph glands of third-instar larvae. (F, G) The dome-MESO-lacZ reporter in the lymph glands of third-instar larvae was detected with β-gal antibodies. (H) Quantification of the proportions of the primary lobes occupied by the dome-MESO-lacZ + area. (I–N) Immunostaining against Stat92E in the lymph glands of third-instar larvae. Scale bars: 50 μm (A–G), 100 μm (I–N).

It should be noted that the 10×STAT-GFP reporter also likely showed a strong fluorescent signal in the PSC domain in addition to the MZ of the lymph gland (Fig. 1J). To further determine whether Stat92E is located in the PSC, we stained the lymph gland with the anti-Stat92E antibody and anti-Antp antibody. Antp is a PSC marker, and we found that Stat92E colocalized with Antp in most PSC cells (Fig. 1Q–Q''). These results suggested that Stat92E is also expressed in the PSC in addition to the CZ and MZ. Similar to Stat92E, the 10×STAT-GFP reporter also colocalized with Antp (Fig. 1R–R''). Moreover, we found that the decreased expression of *jumu* did not affect the expression of the 10×STAT-GFP reporter in the PSC, especially the ectopic PSC cells in *jumu*$^{Df2.12}$ mutant, which maintained the normal fluorescent signal of the 10×STAT-GFP reporter (Fig. 1J-L, Fig. 1S-S'') (the ectopic PSC cells in *jumu*$^{Df2.12}$ were described in our previous study [15]). Taken together, these results suggested that Jumu is required for the maintenance of the JAK/STAT pathway in the CZ and MZ but not in the PSC of the lymph gland.

3.2. Hyperactive JAK can rescue the Stat92E localization defect detected in the lymph gland of jumu mutants

To further investigate how Jumu regulates the localization of Stat92E, we first detected the expression of upstream cytokines of JAK/STAT signaling. We found that the expression of Upd3 was not reduced in the *jumu* mutant lymph gland (Fig. 2A–C). Moreover, the lack of *jumu* did not significantly affect the expression of Upd3 in the PSC (Fig. 2D-E''). Dome is a functional receptor in JAK/STAT signaling, and our previous study showed that the absence of *jumu* did not cause a decrease in the expression of dome > GFP [15]. Next, we also used *dome-lacZ* expressing flies to detect the expression of Dome. Similarly, we found that the expression of dome-MESO-lacZ was not obviously decreased in the *jumu* mutant (Fig. 2F–H). Stat92E is phosphorylated on a critical tyrosine residue by the activated JAK Hopscotch (Hop), generating an active Stat92E dimer that translocates to the nucleus. Thus, we asked whether Jumu affects Stat92E localization by regulating the expression of JAK. We

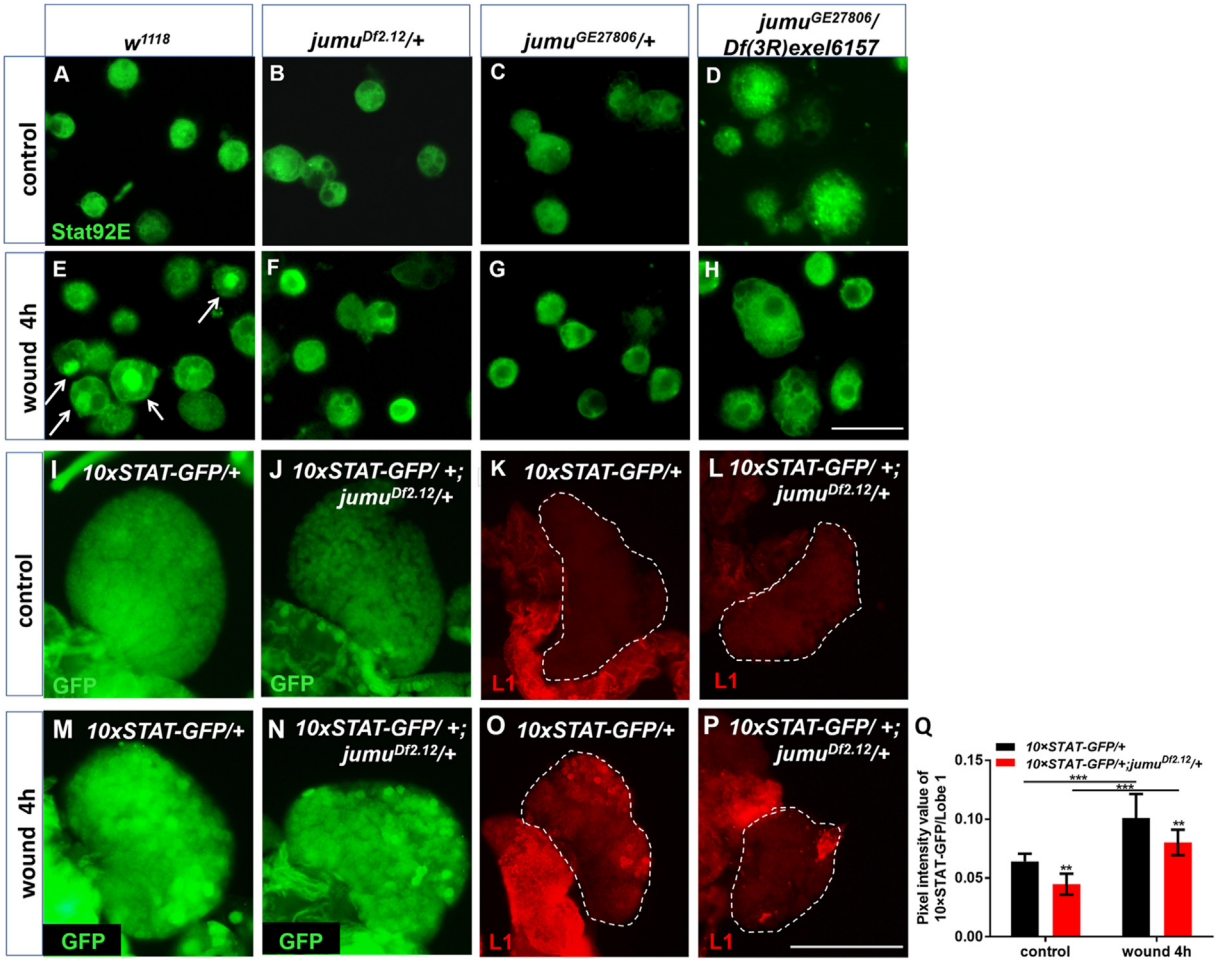


Fig. 3. Jumu is required for epidermal wound-induced activation of the JAK/STAT pathway in circulating hemocytes and lymph glands. (A–H) Immunostaining against Stat92E in circulating hemocytes isolated from third-instar larvae under normal conditions (A–D) and 4 h after epidermal wounding (E–H). The white arrows in E indicate activated Stat92E. (I, J, M, N) A 10 × STAT-GFP reporter of JAK/STAT pathway target in the lymph glands of third-instar larvae was detected with GFP antibodies under normal conditions (I, J) and 4 h after epidermal wounding (M, N). (K, L, O, P) Immunostaining against lamellocyte marker L1 in the lymph glands of third-instar larvae under normal conditions (I, J) and 4 h after epidermal wounding (M, N). Dashed white lines outline the edges of the primary lobes. (Q) Quantification of the pixel intensity of the 10 × STAT-GFP reporter. Scale bars: 20 μm (A–H), 100 μm (I–P).

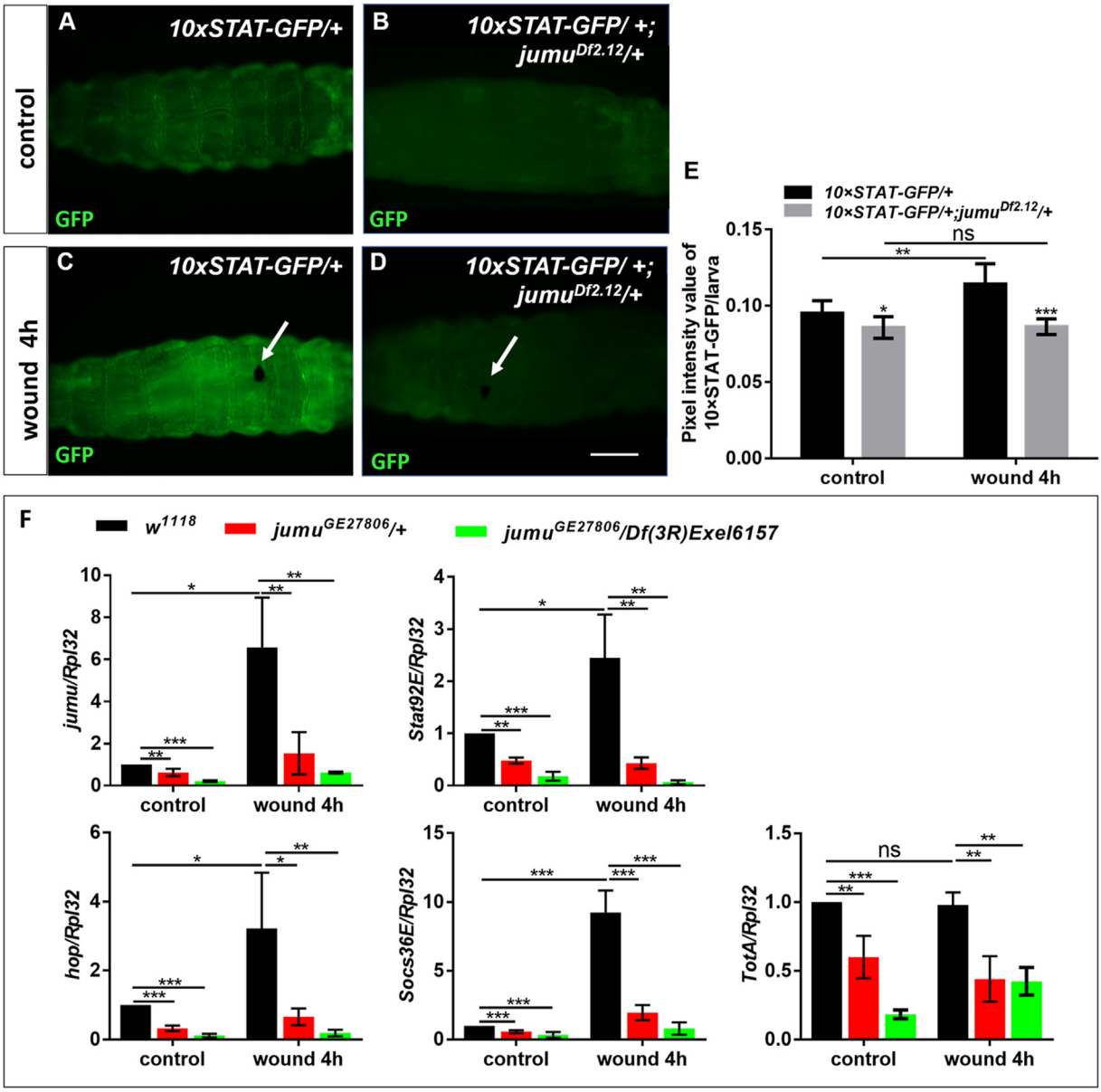


Fig. 4. Loss of *jumu* affects systemic activation of the JAK/STAT pathway in epidermal wounds. (A–D) Fluorescence microscopy of unwounded (A, B) and wounded (C, D)10 × STAT-GFP in third-instar larvae. The arrows in C and D mark puncture wound site. (E) Quantification of the pixel intensity of the 10 × STAT-GFP reporter. (F) Real-time PCR analysis of JAK/STAT pathway target gene transcription levels in unwounded and wounded larvae. Scale bars: 200 μm (A–D).

found that the overexpression of wild-type *hop* in the entire lymph gland did not obviously rescue the nuclear localization of Stat92E in *jumu* mutants (Fig. 2I–K). hop^{Tuml} is a constitutively activated JAK mutant, and we found that Stat92E is mainly located in the nuclei of lymph gland cells of the hop^{Tuml} mutant (Fig. 2L). Moreover, the activation of JAK rescued the Stat92E localization defect detected in the lymph gland of *jumu* mutants, and most Stat92E moved to the nucleus (Fig. 2M, N).

3.3. *Jumu is required for epidermal wound-induced activation of the JAK/STAT pathway*

It has been suggested that tissue damage and mechanical stress can stimulate the cellular immune response through the JAK/STAT signaling pathway [19]. Here, we asked whether the loss of *jumu* also affects the activation of the JAK/STAT signaling pathway associated with immune stress. We used a steel needle to prick the dorsal side epidermis of third-instar larvae, and then detected the activation of the JAK/STAT signaling pathway. We noticed that Stat92E widely resides in the cytoplasm and nucleus of circulating hemocytes under normal conditions (Fig. 3A), but 4 h after epidermal wounding, Stat92E was enriched in the nuclei of most circulating hemocytes in wild-type w^{1118} (Fig. 3E). The decreased expression of *jumu* did not obviously affect the localization of STAT92E in circulating hemocytes before wounding (Fig. 3B–D) but led to a defect in the nuclear enrichment of Stat92E after 4 h of wounding (Fig. 3F–H). Moreover, we found that wild-type larval lymph glands exhibited enhanced 10×STAT-GFP reporter activity and increased lamellocytes after wounding (Fig. 3I, K, M, O, Q). The epidermal wound also resulted in the enhancement of the 10×STAT-GFP reporter signal and the generation of lamellocytes in the lymph glands of *jumu* mutants, but the level of the reporter signal and the number of lamellocytes were significantly less than those in the lymph glands of wild-type larvae (Fig. 3J, L, N, P, Q).

Taken together, these results suggest that epidermal wound can induce the activation of the JAK/STAT signaling pathway in circulating hemocytes and lymph glands, and deficiency of the *jumu* gene can inhibit the activation of JAK/STAT signaling.

To further determine whether the JAK/STAT pathway can be systematically activated by epidermal wounds, we first detected the fluorescence of 10×STAT-GFP in whole larvae after 4 h of wounding. There was a stronger 10×STAT-GFP fluorescence signal at 4 h in wounded wild-type larvae (Fig. 4A, C, E); however, the GFP signal was not increased in wounded *jumu* mutant larvae (Fig. 4B, D, E). Moreover, the GFP reporter signal intensity in *jumu* mutant larvae was obviously lower than that in the wild-type larvae before and after epidermal wounding (Fig. 4A–E). Next, we further detected activation of the JAK/STAT pathway by assessing the transcription levels of target genes. The mRNA levels of the JAK/STAT target genes *Stat92E, hop* and *Socs36E* were significantly increased in wild-type larvae once wounded for 4 h, but the transcription level of *TotA*, which is regulated by the JAK/STAT pathway in response to septic injury [12], was not increased (Fig. 4F). Similar to the 10×STAT-GFP fluorescence signal results, the transcription levels of JAK/STAT target genes were not obviously increased in wounded *jumu* mutant larvae (Fig. 4F). Moreover, the mRNA levels of JAK/STAT target genes in both unwounded and wounded *jumu* mutant larvae were significantly lower than those in wild-type larvae (Fig. 4F). To further investigate whether Jumu regulates the immune response to epidermal wounds, we detected the transcription level of *jumu* in wounded wild-type larvae. Surprisingly, the mRNA level of *jumu* increased nearly six times in wild-type larvae once wounded for 4 h (Fig. 4F). These findings demonstrated that Jumu positively regulates the systemic activation of the JAK/STAT pathway in the immune response to epidermal wounds.

4. Discussion

Several studies have revealed the function of Stat92E in lymph gland hematopoiesis: it is required cell-autonomously for plasmatocyte differentiation; moreover, it also contributes to the maintenance of prohemocytes in a noncell autonomous manner in the CZ [7,20]. Here, we found that Stat92E is expressed in the whole anterior lobes of the lymph gland, especially in the PSC; however, the role of Stat92E in the PSC has not been reported. Furthermore, we showed that the distributions of Stat92E and 10×STAT-GFP in the lymph gland are different. The expression level of Stat92E in the whole lymph gland was relatively uniform; however, 10×STAT-GFP displayed a higher fluorescence signal in the PSC than in the MZ and the CZ. The other JAK/STAT target genes such as Dome, Upd3 and Chinmo also display different expression patterns in the lymph gland [6,18]. Accordingly, the differential expression patterns of the JAK-STAT pathway target genes in the lymph gland indicate the complex spatial-temporal regulatory relationship among those genes of this signaling.

Notably, our results show that Stat92E displays different subcellular localizations in the lymph gland and circulating hemocytes: Stat92E is obviously located in the nuclei of lymph glands but uniformly expressed in circulating hemocytes. Stat92E can be tyrosine phosphorylated by JAK and then allows them to form dimers and translocate into the nucleus, and activated Stat92E promotes the transcription of their target genes [21]. The strong nuclear localization signal of Sat92E gains further evidence for the activation and regulation role of the JAK/STAT pathway in maintaining the homoeostasis of lymph gland hematopoiesis. Although the constitutive activation of Stat92E in circulating hemocytes is relatively weak compared with that in the lymph gland, the maintenance of low activation of the JAK/STAT pathway is required for the normal proliferation and differentiation of circulating hemocytes [4,5]. Our previous studies have shown that Jumu is required for *Drosophila* hematopoiesis and cellular immunity [15,16]. Here, we further found that Jumu regulates the activation of JAK/STAT in the *Drosophila* lymph gland. Moreover, we found that the regulation of Jumu on Stat92E is not dependent on Upd3 and Dome. We analyzed the 2000 bp promoter sequence of the hop and Stat92E genes using Ensemble, a web system for predicting promoters, and found 19 and 9 putative FKH transcription factor binding sites on the promoter sequence, respectively, using JASPAR (data not shown). Because Jumu contains a conserved FKH DNA-binding domain, we speculate that Jumu may regulate the expression of hop and Stat92E at the transcriptional level by directly binding to its promoter via the FKH domain. Consistently, our results also show that Jumu regulates the transcription of *hop* and *Stat92E*.

It has been suggested that the JAK/STAT pathway directly contributes to multiple immune and stress-related processes via complex communication and cascades among multiple tissues, and its proper function and regulation are crucial for the host [22]. For instance, the JAK-STAT cytokine Upd3 secreted by hemocytes regulates the humoral systemic response following septic injury by activating the JAK/STAT pathway in fat body cells, and *totA*, which is a target gene of the AK/STAT pathway, can be induced [12]. However, in our results, the expression of *totA* was not increased after epidermal wounding, suggesting that totA might not participate in epidermal wounding. JAK/STAT signaling in the lymph gland controls the induction of lamellocyte differentiation in response to wasp infection, and lamellocytes can initiate the encapsulation reaction [6,23]. Moreover, a recent study suggests that JAK/STAT signaling in muscles is required for the cellular immune response against wasp infection [24]. In addition, forceps squeezing of *Drosophila* larvae can stimulate lamellocyte generation of the lymph gland through the JAK/STAT signaling pathway [19]. Similarly, our studies show that the epidermal wound of *Drosophila* larvae can induce the systemic activation of JAK/STAT. Consistent with our research, a recent study also showed that the JAK/STAT and Toll pathways are activated upon wounding in adult hemocytes [25]. Previous studies have shown that clean puncture wounding results in the transcriptional activation of the *dorsal* and *spz* genes in epidermal cells adjacent to wounds in late-stage embryos, and the Toll pathway is involved in epidermal wound healing by regulating the expression of barrier repair genes [26,27]. Here, we did not find an obvious 10×STAT-GFP signal accumulating in the epidermal cells around the wound. Thus, whether the JAK/STAT pathway directly contributes to wound repair and how this pathway participates in the immune response to epidermal wounds of larvae remain to be investigated. Moreover, we found that the loss of *jumu* did not affect the healing of wounds, which might be because the activation of the Toll pathway was caused by the severe deficiency of *jumu* contribute to wound repair [15,16]. Moreover, the significant increase in the transcription level of *jumu* upon puncture wounding suggests the important positive regulatory role of Jumu in the immune response to epidermal wounds. However, the regulatory mechanisms of Jumu in wound repair and epidermal wound-induced activation of the JAK/STAT pathway remain to be elucidated.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.12.115.

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