Upregulation of the *Drosophila* Friend of GATA Gene *u-shaped* by JAK/STAT Signaling Maintains Lymph Gland Prohemocyte Potency[▽]

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Studies using *Drosophila melanogaster* have contributed significantly to our understanding of the interaction between stem cells and their protective microenvironments or stem cell niches. During lymph gland hematopoiesis, the *Drosophila* posterior signaling center functions as a stem cell niche to maintain prohemocyte multipotency through Hedgehog and JAK/STAT signaling. In this study, we provide evidence that the Friend of GATA protein U-shaped is an important regulator of lymph gland prohemocyte potency and differentiation. U-shaped expression was determined to be upregulated in third-instar lymph gland prohemocytes and downregulated in a subpopulation of differentiating blood cells. Genetic analyses indicated that U-shaped maintains the prohemocyte population by blocking differentiation. In addition, activated STAT directly regulated *ush* expression as evidenced by results from loss- and gain-of-function studies and from analyses of the *u-shaped* hematopoietic *cis*-regulatory module. Collectively, these findings identify U-shaped as a downstream effector of the posterior signaling center, establishing a novel link between the stem cell niche and the intrinsic regulation of potency and differentiation. Given the functional conservation of Friend of GATA proteins and the role that GATA factors play during cell fate choice, these factors may regulate essential functions of vertebrate hematopoietic stem cells, including processing signals from the stem cell niche.

Stem cells are essential for tissue regeneration and are defined by their capacity for self-renewal, potency, and differentiation. The choice between the maintenance of stem cell potency and differentiation is controlled by a complex combination of intrinsic factors and a protective stem cell niche (1, 29, 53, 63). Much of our current understanding about the regulation of these developmental processes comes from studies of hematopoietic stem cells (HSCs). In addition, cross-species conservation has shown that the genetically tractable *Drosophila* system can complement studies of vertebrate hematopoiesis (10, 11, 15, 23, 31, 41, 62).

Studies using *Drosophila melanogaster* have identified a class of blood cell progenitors (prohemocytes) with characteristics of HSCs, including quiescence, multipotency, and niche dependence (27, 32, 36, 40). The prohemocyte develops within a specialized organ called the lymph gland. The mature third-instar lymph gland consists of one pair of primary lobes and a series of secondary lobes (49). Within the primary lobe, three distinct domains or zones have been characterized based on their roles during hematopoiesis (27, 32, 40). The quiescent, stem cell-like prohemocytes reside within the medullary zone. During the process of differentiation, these cells migrate to the cortical zone. Here they become plasmatocytes and crystal cells, the primary blood cell lineages in the fly (27, 49). The third domain, the posterior signaling center (PSC), functions

as a stem cell niche by maintaining prohemocyte quiescence and potency through the Hedgehog and JAK/STAT signal transduction pathways (32, 37, 40). Because signaling pathways function throughout development, the regulation of stem cell potency and differentiation will be determined not only by the signaling molecules but also by the downstream effectors of these pathways. This underscores the importance of identifying the targets of niche-directed signals. Of particular interest are the key regulators and gene networks that control the choice between stem cell quiescence and proliferation and between the maintenance of potency and differentiation.

GATA factors are key regulators of HSC survival, proliferation, and differentiation (6, 59, 60). The *Drosophila* GATA factor Serpent (Srp) is required for the specification of prohemocytes but also acts later in hematopoiesis to drive plasmatocyte and crystal cell differentiation (18, 48, 52, 61). Given the extensive role of Srp in hematopoiesis, its activity must be regulated to prevent the depletion of the medullary zone prohemocyte pool. A likely candidate is U-shaped (Ush), a Friend of GATA (FOG) family member. These proteins are known to interact with GATA factors to modulate gene expression across taxa ranging from flies to humans (16). During embryonic crystal cell development, Ush converts Srp from an activator to a repressor of lineage commitment and differentiation by downregulating the crystal cell lineage activator, Lozenge (Lz) (18, 45). In addition, Ush and Srp are coexpressed in embryonic prohemocytes (17, 48, 52). In this study, we investigated the role of Ush during lymph gland hematopoiesis and whether *ush* expression is regulated by the PSC. Here we provide evidence that Ush acts as a key regulator of lymph gland prohemocyte potency. Our analyses indicate that Ush is required to preserve the prohemocyte pool by limiting differ-

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entiation and that *ush* expression requires activated STAT. The upregulation of *ush* expression by the JAK/STAT pathway positions Ush as a downstream target of the PSC and provides an important link between the stem cell niche and the intrinsic regulation of potency and differentiation. Our studies raise the possibility that these conserved factors interact to regulate vertebrate HSC biology.

MATERIALS AND METHODS

Fly strains. w^{1118} flies served as our wild-type stock. $y\ w^{67c23}$; *UAS-ush*, w^{1118}; $lz\ -1236/-737\ lacZ$ (*lz-lacZ*) and w^{1118}; *ush* $-174/-25\ lacZ$ were described elsewhere previously (17, 44, 45). The following strains were generous gifts from colleagues: $y^1\ w$; $ush^{vx22}/CyO, y^+$ and $y^1\ w$; $ush^{r24}/CyO, y^+$ (R. A. Schulz and R. P. Sorrentino, University of Notre Dame); domeMESO *lacZ* strains w; *domeMESO/CyO* and $w\ p\{w^+, dome\text{-}MESO\}BN1$ (M. P. Zeidler, University of Sheffield, and J. C. Hombria, Universidad Pablo de Olavide); and *FRT 82b*, e, ca $stat^{397\text{-}6}/TM3$ (D. J. Montell, Johns Hopkins School of Medicine). We obtained the following strains from the Bloomington stock center: $P\{Cg\text{-}GAL4.A\}$ (collagen-Gal4 [Cg-Gal4]) and $y^1\ v^1\ hop^{Tum\text{-}l}/Basc$ (*hop*$^{Tum\text{-}l}$).

Immunofluorescence. The dissection and fixation of larval lymph glands were preformed as previously described (45) except that lymph glands were fixed for 8 min at room temperature. Rabbit anti-U-shaped was used at a 1:1,000 dilution (17). The following mouse antibodies directed against specific hemocyte antigens were generous gifts from Istavan Ando, Biological Research Center of the Hungarian Academy of Sciences, and were used at the indicated dilutions: P1, 1:50 (34); L1, 1:200 (35), and anti-Hemese, 1:200 (33). Rabbit anti-prophenoloxidase A1 (anti-ProPO) was used at a 1:100 dilution and was a generous gift from F. C. Kafatos, EMBL (43). The following antibodies were obtained from the Developmental Studies Hybridoma Bank and were used at the indicated dilutions: mouse anti-Antennapedia (anti-Antp), 1:20; mouse anti-Patched (anti-Ptc), 1:60, and rat anti-DE-cadherin (anti-DE-cad), 1:20. Mouse anti-β-galactosidase was used at a 1:2,000 dilution (Promega). Alexafluor-555- or -488-conjugated secondary antibodies directed to rabbit, mouse, or rat (Invitrogen) were used at a 1:2,000 dilution. Ush localizes to the nucleus, and coexpression with cytosolic markers (*lz-lacZ* and domeMESO *lacZ*) or membrane-bound proteins (P1, DE-cad, and L1) was determined by observing Ush within the boundary of a single cell using confocal microscopy. In contrast, Antp is a nuclear protein, and the coexpression of Ush was assessed by determining the degree of colocalization between these proteins. Fluorescence was captured, analyzed, and recorded using Zeiss confocal microscopy or Zeiss Axioplan optics. Lymph gland size and densitometric mean values were determined using Zeiss Axiovision 4.6.3 quantitation software. Briefly, lymph glands were photographed using fluorescein isothiocyanate, Cy3, and differential interference contrast filters. At least 12 lymph glands from each genotype were outlined and sized using the outline spline interpolation feature. Axiovision software converted the RGB color image into gray and then calculated the densitometric mean for each color channel across the entire lymph gland. The statistical significances of the size and densitometric mean data were evaluated using the Student t test.

Gene expression analyses. $ush^{vx22}/CyO, y^+$ and $stat^{397\text{-}6}/TM3$ heterozygotes were tested over additional complementing chromosomes to verify that the observed phenotype was independent of the balancer chromosome. $y^1\ w$; ush^{vx22}/ush^{r24} *trans*-heterozygote larvae were generated by crossing $y^1\ w$; $ush^{vx22}/CyO, y^+$ and $y^1\ w$; $ush^{r24}/CyO, y^+$ adults and selecting larval progeny with yellow mouth hooks. *hop*$^{Tum\text{-}l}$ larvae and wild-type controls were cultured at three different temperatures, 23°C, 25°C, and 27°C, whereas all other larvae were cultured at 23°C. Cg-Gal4 flies were mated to either *UAS-ush* or w^{1118} strains, and progeny were evaluated to determine the effect of these transgenes on differentiating hemocytes. Fly strains carrying the wild-type *ush* $-174/-25$ *cis*-regulatory module (CRM) *lacZ* construct were crossed to either the $stat^{397\text{-}6}/TM3$ or w^{1118} strain to determine the effect of the reduction in *stat* activity on *ush* CRM *lacZ* expression. All experiments were done in triplicate at a minimum, with at least eight control and eight experimental lymph glands per experiment, unless otherwise indicated. Crystal cell counts were analyzed using the Student t test.

Generation of transgenic fly strains carrying a STAT site mutation in the *ush* $-174/-25$ CRM. The STAT site mutation was introduced into the *ush* $-174/-25$ CRM sequence using a method described previously by Barettino et al. (3) except that all of the targets were cloned into the pCRII-TOPO cloning vector (Invitrogen). The *ush* $-420/-25$ fragment (44) was used as a template with forward (CACACCCCTTTCTGTTTCTGCGATGTTATCTAAGCGC) and reverse (CGACTTCCTTCGCTCGCCTCGGAATTATT<u>TA</u>AAAC) oligonucleo-

tide primers to produce a fragment with two nucleotide base substitutions (C to T and G to A [underlined]) in the STAT binding site. The fragment was gel purified and used as a forward oligonucleotide primer with the reverse oligonucleotide primer GACGAGACGAGACCTCTTAGCCGAGACTCTCTG to produce the *ush* $-174/-25$ *lacZ* CRM carrying a mutated STAT binding site (*ush* $-175/-25$ mSTAT). This fragment was cloned into the pCRII-TOPO cloning vector and subsequently subcloned into the P-element CaSperR-Hsp43-AUG-β-gal germ line transformation vector (58). The sequence of the recombinant vector was verified prior to injection into w^{1118} embryos by Rainbow Transgenic Flies, Inc. Germ line transformants were established as previously described (20). Twenty independent lines were generated, and six were tested for *lacZ* activity in the larval lymph gland. In addition, males carrying the wild-type or mSTAT *lacZ* CRM were crossed to *hop*$^{Tum\text{-}l}$ or w^{1118} females to determine the effect of increased STAT activity on *ush* CRM *lacZ* expression.

RESULTS

Ush is differentially expressed in the third-instar larval lymph gland. Ush is expressed in third-instar primary and secondary lymph gland lobes (Fig. 1A) (17, 44). As a first step toward identifying the functions of Ush during lymph gland hematopoiesis, we determined the hemocyte-specific expression pattern of this transcriptional regulator. DE-cad is expressed in medullary zone prohemocytes, and this prohemocyte marker is later downregulated as these cells differentiate (27). Ush was coexpressed with DE-cad in these medullary zone cells (Fig. 1B and B′), demonstrating that Ush is expressed in prohemocytes.

In contrast, cortical zone cells exhibited a more complex Ush expression pattern. The pattern of expression was determined by measuring Ush coexpression with the plasmatocyte-specific
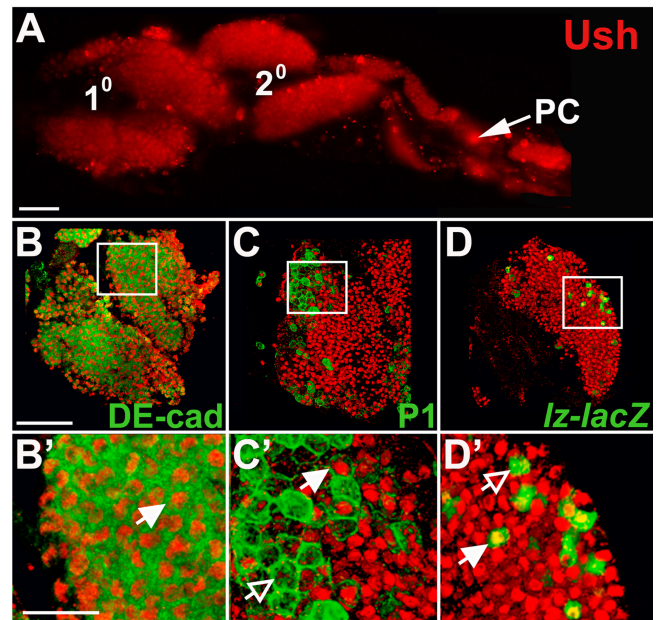

FIG. 1. Differential Ush expression in the third-instar larval lymph gland. (A) Ush expression in primary and secondary lymph gland lobes (fluorescence microscopy) (magnification, ×20). (B to D′) Ush coexpression with hemocyte-specific markers in the primary lymph gland lobe (confocal microscopy) (magnification, ×60). (B and B′) Ush and the medullary zone prohemocyte marker DE-cad. (C and C′) Ush and the plasmatocyte marker P1 (Nimrod). (D and D′) Ush and the crystal cell marker *lz-lacZ*. Closed arrows indicate coexpression, and open arrows indicate a lack of coexpression. PC, pericardial cells. Scale bars, 50 μm (A and B) and 15 μm (B′).

marker, P1 (Nimrod) (34), or the crystal cell-specific marker, *lz-lacZ* (45). While Ush was detected in both the plasmatocyte and crystal cell lineages, Ush was downregulated in cells from each lineage (Fig. 1C to D′). Previous work showed a similar expression pattern during embryonic crystal cell development (compare with insets in Fig. 1d and e in reference 17). Ush functions in these cells as part of a cross-regulatory subcircuit that blocks lineage commitment and differentiation by downregulating the crystal cell lineage activator, Lz, which in turn downregulates *ush* expression (45). Based on these studies, we asked if Ush maintains prohemocyte potency by blocking differentiation.

Ush limits prohemocyte differentiation in the lymph gland. To investigate the role of Ush in the maintenance of prohemocyte potency, we first determined the extent of blood cell differentiation in animals with altered levels of *ush* expression. *ush* is an essential gene, and homozygous null animals die during embryogenesis (46). Therefore, we utilized two fly strains with reduced *ush* expression levels that survive until adulthood: (i) *ush trans* heterozygotes (ush^{vx22}/ush^{r24}) that carry a null allele and a hypomorphic allele and (ii) *ush* heterozygotes ($ush^{vx22}/+$) that carry one null allele (21). We observed that late-third-instar *ush* heterozygous and *trans*-heterozygous lymph glands exhibited hyperplasia (Fig. 2A to C′), which is consistent with data from a previous report (56). In general, the lymph glands of *ush trans* heterozygotes were larger than those of heterozygotes, and heterozygous lymph glands were larger than those of wild-type controls. We measured the areas of at least 12 lymph gland images for each genotype. The size of *trans*-heterozygous lymph glands was $68,928 \pm 9,827$ μm². On average, this was twofold larger than heterozygous lymph glands ($32,782 \pm 9,746$ μm² [$P < 0.01$]) and threefold larger than wild-type lymph glands ($21,945 \pm 3,133$ μm² [$P < 0.01$]). These results showed that lymph gland size increased with decreasing levels of *ush* expression. We next investigated hemocyte differentiation in late-third-instar lymph glands of *ush trans* heterozygotes, heterozygotes, and wild-type controls.

Plasmatocytes comprise a major portion of the hemocyte population (36, 49, 57). As a result, unregulated plasmatocyte differentiation could dramatically deplete the prohemocyte pool. For this reason, we first asked if Ush blocks plasmatocyte differentiation to maintain the multipotent prohemocyte pool. Plasmatocyte numbers increased substantially in *ush* heterozygous lymph glands compared to wild-type controls (Fig. 2A to B′). In contrast, plasmatocyte numbers actually decreased in *ush trans*-heterozygous lymph glands. The number of plasmatocytes was often less than that observed in wild-type controls and considerably less than that in *ush* heterozygotes (Fig. 2A to C′). The observed increase in plasmatocyte numbers was not due solely to lymph gland size; otherwise, an increase in numbers of plasmatocytes would have been observed for both genotypes. Rather, the data suggest that Ush functions to restrict the number of prohemocytes that become plasmatocytes. The loss of one functional *ush* copy (heterozygote) appears to release the prohemocyte differentiation block, whereas the loss of more than one functional *ush* copy (*trans* heterozygote) limits plasmatocyte production.

The increase in plasmatocyte numbers suggested that *ush* overexpression would block plasmatocyte production. To test this hypothesis, we used the UAS/Gal4 system (5) with the *Cg-Gal4* (2) driver and *UAS-ush* to overexpress *ush* (Cg>Ush) in the plasmatocyte lineage. Cg>Ush produced almost a complete loss of plasmatocytes compared to *Cg-Gal4/+* and wild-type controls (Fig. 2A, A′, D, and E). In contrast, Cg>Ush did not alter the expression of the panhemocyte marker Hemese and did not alter lymph gland size (Fig. 2F and data not shown). These results indicate that the loss of plasmatocytes in the Cg>Ush lymph gland was due to a differentiation block rather than a reduction in the total number of hemocytes. Collectively, these *ush* loss- and gain-of-function studies indicate that a reduction in the level of Ush expression is required for differentiation to proceed.

Crystal cells and plasmatocytes constitute the primary blood cell lineages. In the lymph gland, both lineages are derived from medullary zone prohemocytes (27). Therefore, if Ush blocks prohemocyte differentiation, then it should block both crystal cell and plasmatocyte differentiation. Using the crystal cell-specific differentiation marker ProPO (43), we observed that the number of crystal cells, like plasmatocytes, increased in *ush* heterozygous lymph glands. The number of crystal cells increased by more than twofold in *ush* heterozygotes compared to wild-type controls (Fig. 2G to H′). The mean crystal cell number was 29.8 ± 9.3 in wild-type lymph glands, whereas in heterozygous lymph gland, the mean cell number was 78.2 ± 13.3 ($P < 0.01$). When the mean crystal cell number was adjusted to account for lymph gland size, heterozygous lymph glands averaged twice the size of wild-type lymph glands (59.9 ± 19.6 [$P < 0.05$]). These results showed that the increase in crystal cell numbers was not due solely to the size of the heterozygous lymph gland and indicated that Ush limits the number of prohemocytes that develop into crystal cells during lymph gland hematopoiesis. This again is consistent with the role of Ush during embryonic crystal cell production (18, 45). In contrast, crystal cell numbers decreased in *ush trans* heterozygotes (Fig. 2G to I′). Together, these results showed that both plasmatocytes and crystal cells respond similarly to changes in the level of Ush expression, increasing in *ush* heterozygotes and decreasing in *ush trans* heterozygotes. This demonstrates that the increase in numbers of mature hemocytes is independent of lymph gland size. Increased lymph gland size is most likely a result of overproliferation. However, the mitotic indices of the late-third-instar heterozygous and *trans*-heterozygous lymph glands are not significantly different than those of the wild type, suggesting that overproliferation occurs at an earlier stage of lymph gland development (56; data not shown). The dynamics of this process have not been fully characterized across the entire third larval instar; however, it is unlikely that the number of these cells increases solely as a result of overproliferation because numbers of mature hemocytes are independent of lymph gland size. Altogether, these data support our hypothesis that Ush limits the differentiation of the prohemocyte pool and regulates hemocyte development.

The lamellocyte, a third class of hemocyte, is not observed in large numbers except during specific immune challenges or in particular genetic backgrounds (2, 9, 11, 13, 22, 36, 39, 42, 47, 54, 55, 59). Like plasmatocytes and crystal cells, lymph gland-derived lamellocytes develop from medullary zone prohemocytes (27, 32, 36). Furthermore, extensive la-
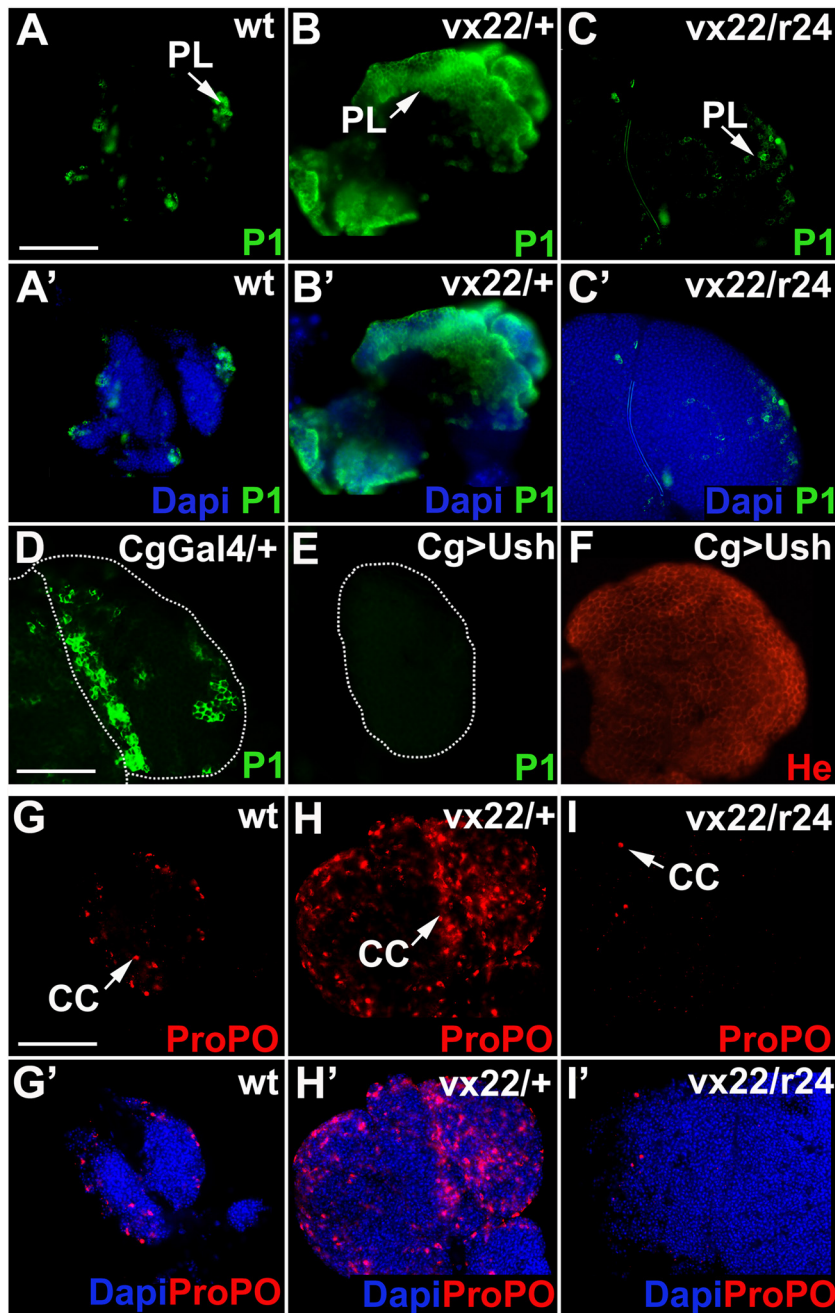
FIG. 2. Ush blocks plasmatocyte and crystal cell differentiation in the lymph gland. Shown is plasmatocyte and crystal cell differentiation in lymph glands with altered levels of *ush* expression (fluorescence microscopy) (magnification, ×40). (A to F) Plasmatocyte differentiation was monitored using P1 in animals with the following genotypes: wild type (wt) (A and A′), *ush* heterozygotes (*ush*^{vx22}/+; vx22/+) (B and B′), *ush trans* heterozygotes (*ush*^{vx22}/*ush*^{r24}; vx22/r24) (C and C′), *Cg-Gal4*/+ (D), and *Cg-Gal4/UAS-ush* (Cg>Ush) (E). Arrows mark plasmatocytes. (F) He expression in Cg>Ush lymph glands. (G to I′) Crystal cell differentiation was monitored using ProPO in animals with the following genotypes: wild-type (G and G′), *ush* heterozygotes (*ush*^{vx22}/+; vx22/+) (H and H′), and *ush trans*-heterozygotes (*ush*^{vx22}/*ush*^{r24}; vx22/r24) (I and I′). Arrows mark crystal cells. Lymph gland hyperplasia produced by the loss of one (heterozygote) or more (*trans*-heterozygote) copies of *ush* often prevented the visualization of the entire lymph gland. Abbreviations: CC, crystal cells; PL, plasmatocytes. Scale bars: 100 μm (A and G) and 170 μm (D).

mellocyte differentiation depletes the prohemocyte pool (32). These observations raised the possibility that Ush would also block lamellocyte differentiation as a means of maintaining prohemocyte potency.

To address the role of Ush in lamellocyte differentiation, we first analyzed Ush expression levels in 16 lamellocytes from

wild-type lymph glands. Lamellocytes were identified using the specific marker L1 (35). Fourteen of the 16 lamellocytes showed markedly reduced Ush expression levels compared to those of neighboring cells that lacked L1 expression (Fig. 3A and A′). However, two of the 16 lamellocytes appeared to have levels of Ush expression that were comparable to those of
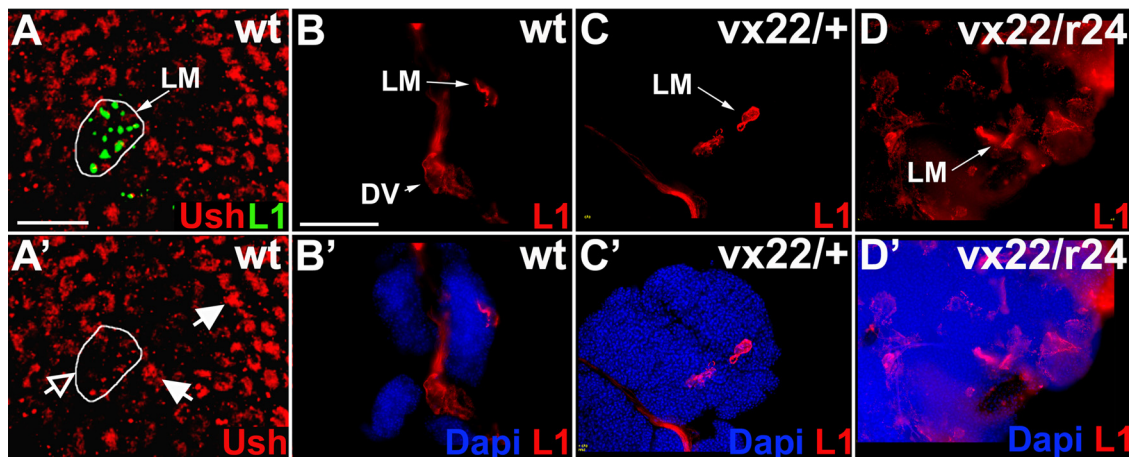
FIG. 3. Reduction in *ush* expression levels promotes lamellocyte differentiation. Lamellocytes were identified using the cell-specific marker L1. (A and A′) Ush and L1 expression in cortical zone hemocytes (confocal microscopy) (magnification, ×60). (A) Ush and L1 coexpression. (A′) Same field showing only Ush expression. Closed arrows mark Ush-expressing hemocytes; the open arrow marks a lamellocyte with reduced Ush expression levels. (B to D′) Lamellocyte differentiation in animals with altered levels of *ush* expression (fluorescence microscopy) (magnification, ×40). Data for L1 expression in animals with the following genotypes are shown: wild type (wt) (B and B′), *ush* heterozygotes (C and C′), and *ush* trans heterozygotes (D and D′). Arrows mark lamellocytes and the dorsal vessel, both identified using L1. Abbreviations: DV, dorsal vessel; LM, lamellocyte. Scale bars, 12 μm (A) and 100 μm (B).

neighboring hemocytes (data not shown). Overall, these results are consistent with the hypothesis that *ush* expression is down-regulated to permit lamellocyte differentiation and predict that lamellocyte numbers would increase in a *ush* mutant background. To test this hypothesis, we assayed lymph gland-derived lamellocyte differentiation in *ush* heterozygotes and *trans* heterozygotes.

As with plasmatocytes and crystal cells, we observed striking differences in the numbers of lamellocytes produced in *ush* heterozygotes compared to those produced in *trans* heterozygotes. However, in contrast to plasmatocytes and crystal cells, lamellocyte differentiation was greater in *trans* heterozygotes than in heterozygotes. Heterozygous lymph glands had only a marginal increase in lamellocyte differentiation. We observed that 19 of 26 *ush* heterozygous lymph glands tested had lamellocyte numbers that were comparable to those of wild-type controls, which averaged two lamellocytes per lymph gland (Fig. 3B to C′), whereas 5 of 26 lymph glands tested had a 10-fold increase above wild-type numbers (data not shown). In contrast, *trans*-heterozygous lymph glands showed a dramatic increase in lamellocyte differentiation. Of the 30 *trans*-heterozygous lymph glands tested, all had at least a 10-fold increase above wild-type numbers. Moreover, 20 of 30 lymph glands were covered with large lamellocyte aggregates that prevented the determination of the precise number of cells (Fig. 3D and D′ and data not shown). Collectively, our results showed that two functional copies of the *ush* gene are required to limit plasmatocyte and crystal cell production, whereas only one copy is required to limit lamellocyte production.

Ush prevents prohemocyte loss in the medullary zone. We showed that Ush was expressed in medullary zone prohemocytes and limited the production of all three hemocyte classes. These results suggested that Ush limits prohemocyte differentiation to preserve the multipotent state. To test this hypothesis, we assayed for the presence of prohemocytes in *ush* mutant lymph glands using the specific markers DE-cad and Ptc

(40). We observed a loss of DE-cad expression levels and a concomitant increase in levels of plasmatocyte production in *ush* heterozygous lymph glands compared to levels in wild-type controls (Fig. 4A to C). DE-cad and Ptc were also downregulated in *ush* trans-heterozygous lymph glands, which showed a corresponding increase in lamellocyte differentiation (Fig. 4D to H). These results show that in *ush* mutants, the prohemocyte pool is dramatically reduced, while the mature hemocyte population increases. This shift in the cell population from prohemocytes to mature hemocytes indicates that the loss of Ush function leads to increased prohemocyte differentiation and thereby supports the hypothesis that Ush preserves the multipotent prohemocyte pool by limiting differentiation.

Previous work by others showed that the PSC preserves prohemocyte potency by blocking differentiation (32, 40). Accordingly, we asked if the increase in prohemocyte differentiation resulted from disrupting PSC function in *ush* loss-of-function lymph glands. Antp is required for the specification of the PSC and serves as a marker for these cells (40). Ush was not detected in the PSC, as determined by its failure to colocalize with Antp (Fig. 5A). This is consistent with data from a previous report (27) and demonstrates that Ush is not an intrinsic regulator of the PSC. Furthermore, we did not detect any change in Antp expression or PSC size in *ush* trans heterozygotes (Fig. 5B, C). This indicates that a prohemocyte loss in *ush* hypomorphic backgrounds does not result from a disruption of the PSC. Instead, we concluded that Ush acts either independently of the PSC or as a downstream effector of the PSC to regulate prohemocyte differentiation. In the latter case, signaling from the PSC would upregulate Ush within the prohemocyte population to limit differentiation.

JAK/STAT activation maintains Ush expression in the lymph gland. The PSC regulates prohemocyte differentiation through the JAK/STAT and Hedgehog signaling pathways (32, 40). If Ush functions as a downstream effector of the PSC, then at least one of these signal transduction pathways could acti-
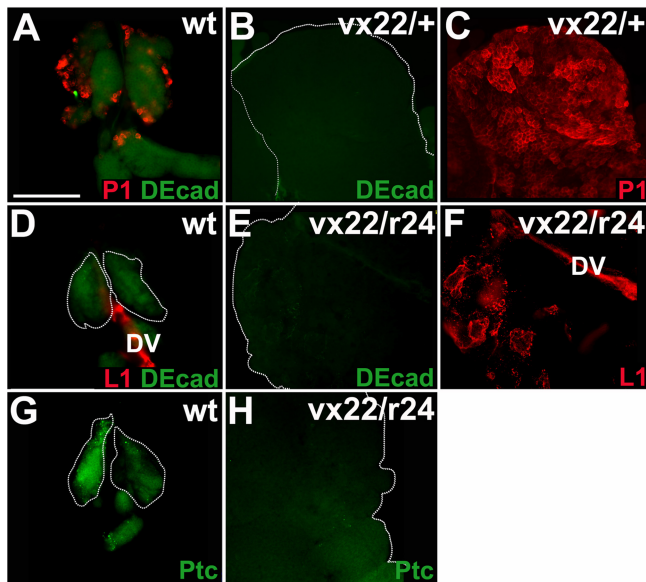
FIG. 4. Prohemocyte markers are downregulated in *ush* mutant lymph glands. Prohemocyte differentiation was monitored in lymph glands of animals with altered levels of *ush* expression (fluorescence microscopy) (magnification, ×40). (A to C) Medullary zone prohemocytes and plasmatocytes were identified using DE-cad and P1, respectively, in the following genotypes: wild type (wt), both prohemocytes and plasmatocytes (A); *ush* heterozygotes, prohemocytes only (B); and *ush* heterozygotes, plasmatocytes only (C). (D to F) Medullary zone prohemocytes and lamellocytes were identified using DE-cad and L1, respectively, in the following genotypes: wild type, prohemocytes and lamellocytes (D); *ush trans* heterozygotes, prohemocytes only (E); and *ush trans* heterozygotes, lamellocytes only (F). The dorsal vessel also expressed L1. (G and H) Medullary zone prohemocytes were identified using Ptc in the following genotypes: wild type (G) and *ush trans* heterozygotes (H). Scale bar, 100 μm.

vate *ush* expression in prohemocytes. The following observations suggested that *ush* might be upregulated by JAK/STAT signaling. First, *stat* and *ush* heterozygous lymph glands have similar phenotypes. This is supported by a comparison of our data with data reported previously by Krzemień and coworkers that showed both a loss of prohemocytes and an increase in crystal cell differentiation in *stat* mutants (32). We observed a similar phenotype for *ush* heterozygous lymph glands (Fig. 2H and 4B). Additional similarities between *stat* and *ush* mutants, including lymph gland hyperplasia and increased plasmatocyte
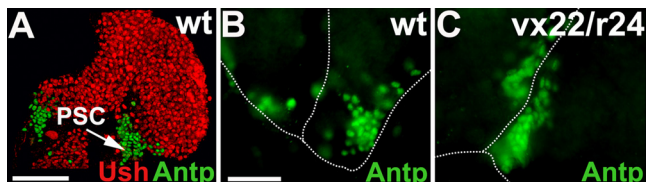

FIG. 5. The reduction in *ush* expression levels has no effect on PSC cell number or size. (A) Ush expression in the PSC was monitored by assessing the level of Ush coexpression with the PSC-specific marker Antp (confocal microscopy) (magnification, ×60). (B and C) PSC cell number was monitored using Antp in animals with altered levels of *ush* expression (fluorescent microscopy) (magnification, ×40). Shown are the wild type (wt) (B) and *ush trans* heterozygotes (C). Scale bars, 50 μm (A) and 25 μm (B).
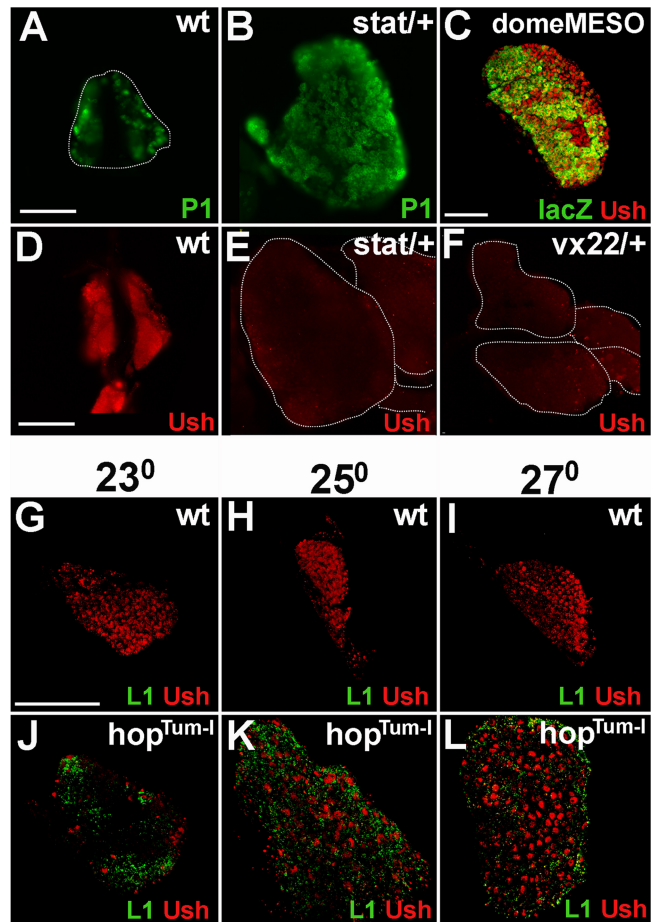

FIG. 6. Activated STAT is required for Ush expression in the lymph gland. (A and B) Plasmatocyte differentiation was monitored using P1 in animals with altered levels of *stat* expression (fluorescence microscopy) (magnification, ×40). Shown are wild-type (wt) (A) and *stat* (*stat*[397-6]/+) (B) heterozygotes. (C) pSTAT activity in Ush-expressing prohemocytes was assessed by monitoring the coexpression of DomeMESO *lacZ* and Ush (confocal microscopy) (magnification, ×60). (D to F) Ush expression was monitored in animals with altered levels of *stat* and *ush* expression (fluorescence microscopy) (magnification, ×40). Shown are data for the wild type (D), *stat* heterozygotes (E), and *ush* heterozygotes (F). (G to L) Ush expression and lamellocyte differentiation were monitored in wild-type and *hop*[Tum-l] second-instar larval lymph glands of animals cultured at three different temperatures (confocal microscopy) (magnification, ×60). (G to I) Wild-type lymph glands cultured at the following temperatures: 23°C (G), 25°C (H), and 27°C (I). (J to L) *hop*[Tum-l] lymph glands cultured at the following temperatures: 23°C (J), 25°C (K), and 27°C (L). Scale bars, 100 μm (A and D) and 50 μm (C and G).

differentiation, offered further support for a connection between Ush and JAK/STAT signaling (Fig. 6A and B). Moreover, neither *ush* nor *stat* heterozygous lymph glands showed a marked increase in lamellocyte differentiation (Fig. 3C and C′ and data not shown). Furthermore, prohemocytes with JAK/STAT activity also express Ush. This was demonstrated by showing that Ush was coexpressed in the medullary zone with domeMESO *lacZ* (Fig. 6C), which is a marker for JAK/STAT activity (24, 32). Collectively, these observations pointed to the JAK/STAT pathway as being a regulator of *ush* expression in the medullary zone, which prompted us to determine the level

of *ush* expression in *stat* heterozygous lymph glands. The level of Ush was markedly reduced in *stat* heterozygotes compared to wild-type controls (Fig. 6D and E). We determined the relative levels of Ush expression in wild-type and *stat* heterozygotes by measuring the densitometric mean of fluorescent lymph gland images. On average, the mean was reduced three-fold in *stat* heterozygotes (1,870 ± 865) compared to wild-type controls (5,902 ± 1,933 [$P < 0.01$]). The level of Ush was also markedly reduced in *ush* heterozygotes (Fig. 6D and F). These results indicated that STAT is required for *ush* expression and predicted that *ush* expression would increase in STAT gain-of-function mutants.

A temperature-sensitive mutation of *Drosophila* JAK (Hopscotch[Tum-l] [Hop[Tum-l]]) hyperphosphorylates STAT, producing increased STAT activity with increasing temperatures (39). We tested *ush* expression in wild-type and *hop*[Tum-l] lymph glands cultured at three different temperatures, 23°C, 25°C, and 27°C (Fig. 6G to L). Because *hop*[Tum-l] lymph glands disperse prior to the beginning of the third larval instar (56), we tested *ush* expression in second-instar *hop*[Tum-l] and wild-type lymph glands.

We noted a difference in both hemocyte composition and Ush expression between wild-type and *hop*[Tum-l] lymph glands. Wild-type second-instar lymph glands were previously reported to contain primarily prohemocytes and plasmatocyte precursors (27). In addition, we did not detect any lamellocytes in these lymph glands (Fig. 6G to I). In contrast, *hop*[Tum-l] lymph glands had considerable numbers of lamellocytes at all three culture temperatures (Fig. 6J to L), consistent with data from a previously reported study (56). Our analyses of Ush expression showed that Ush was detected throughout wild-type lymph glands and that the level of Ush was not affected by temperature (Fig. 6G to I). In contrast, *hop*[Tum-l] lymph glands cultured at 23°C contained a small number of Ush-expressing lamellocytes (Fig. 6J). Similarly, we observed that Ush was downregulated in the majority of wild-type lamellocytes from late-third-instar larval lymph glands (Fig. 3A and A′). However, when the culture temperature was increased to 25°C and 27°C, we observed a progressive increase in the numbers of Ush-expressing lamellocytes (Fig. 6K and L). Because the increase in culture temperature was coupled with the upregulation of both STAT activity and Ush expression (39) (Fig. 6J to L), we propose that activated STAT upregulates Ush expression. However, the increased level of Ush was not sufficient to block *hop*[Tum-l]-induced lamellocyte production, which is consistent with data from a previous report (56).

Ush is a downstream effector of the JAK/STAT pathway. The results of *stat* loss- and gain-of-function studies prompted us to ask if activated STAT directly regulates *ush* gene expression. To address this question, we surveyed the *ush* hematopoietic CRM for STAT binding sites. Ush expression in embryonic and larval lymph gland hemocytes is upregulated by a minimal CRM located between positions −174 and −25 (44) (Fig. 7A). The ability of this CRM to drive reporter gene (*lacZ*) expression in the majority of cells that express endogenous Ush strongly indicates that it contains *cis*-regulatory elements required to drive *ush* expression in the lymph gland (44) (Fig. 7B and B′). The activity of the *ush* −174/−25 CRM is dependent on three GATA sites located at positions −156/−153, −122/−119, and −111/−108 (44). Our survey of this CRM iden-
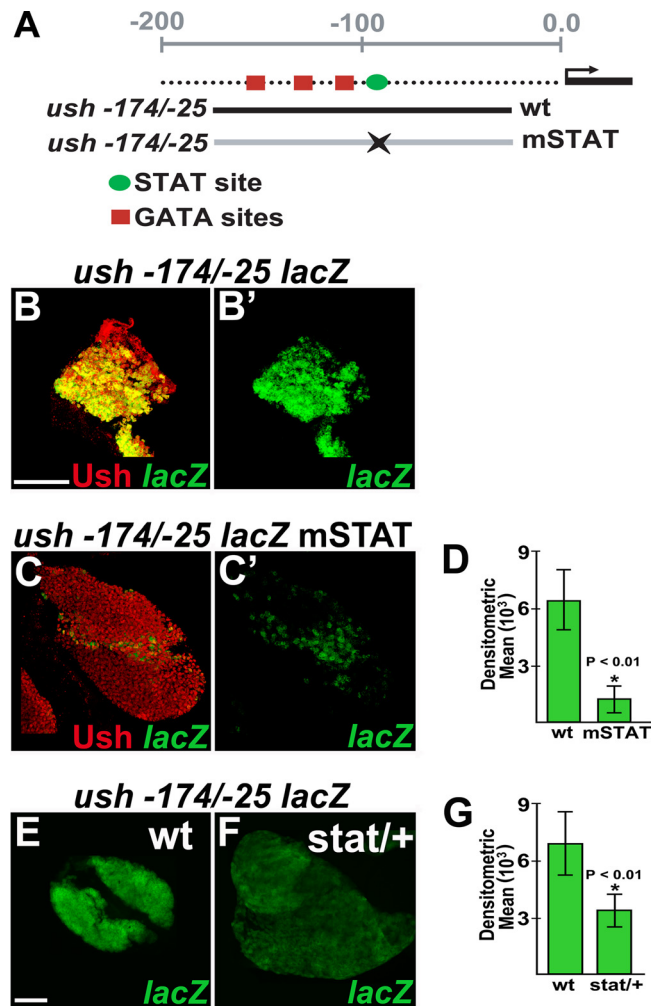

FIG. 7. The STAT binding site in the minimal *ush* hematopoietic CRM is required for activity. (A) Schematic showing the relative positions of the GATA and STAT sites within the *ush* −174/−25 CRM. The wild-type (wt) and STAT site mutant (mSTAT) CRMs differ by 2 bp within the STAT core sequence. (B to C′) *ush* −174/−25 *lacZ* activity and Ush expression in the primary lobe of the lymph gland (confocal microscopy) (magnification, ×60). (B and B′) *ush* −174/−25 *lacZ* wild-type sequence. (C and C′) *ush* −174/−25 *lacZ* STAT site mutant. (D) Densitometric means for lymph glands carrying the *ush* −174/−25 *lacZ* wild-type (wt) and *ush* −174/−25 *lacZ* STAT site mutant (mSTAT) constructs were 6,526 ± 1,579 and 1,285 ± 842, respectively ($P < 0.01$). (E and F) *ush* −174/−25 *lacZ* activity in wild-type (E) and *stat* heterozygous (F) lymph glands (fluorescence microscopy) (magnification, ×40). (G) Densitometric means of *ush* −174/−25 *lacZ* activity in wild-type and *stat* heterozygous (stat/+) lymph glands were 7,043 ± 1,700 and 3,463 ± 840, respectively ($P < 0.01$). Scale bars, 50 μm.

tified a putative STAT binding site 9 bp downstream of the 3′ GATA site at position −98/−90 (Fig. 7A). This site has a single mismatch in the 3′ half-palindromic sequence (G-to-T substitution), which is a high-affinity variant used by vertebrate STAT1 to regulate a variety of target genes (14).

To determine if the STAT site was required for CRM activity, we introduced two single-base-pair substitutions into this site. We then compared *lacZ* expression levels in the lymph glands of animals harboring the STAT site mutant version of

the ush $-174/-25$ CRM (mSTAT) with those in the lymph glands of animals carrying the wild-type version (Fig. 7A). Ten different strains were analyzed, four harboring wild-type and six harboring mSTAT CRMs. For each strain, a minimum of six lymph glands were assayed. In all cases, animals carrying the mSTAT CRM had markedly reduced $lacZ$ expression levels compared to those of animals carrying wild-type CRM (Fig. 7B to C′ and data not shown). The activity of the wild-type CRM was on average fivefold greater than that of the mSTAT CRM as determined by measuring the densitometric mean from the fluorescence images of lymph glands carrying wild-type and mSTAT CRMs (Fig. 7D). Thus, the STAT site at position $-98/-90$ is required for ush $-174/-25$ $lacZ$ activity. To confirm these results, we assayed the activity of wild-type CRM in $stat$ heterozygous mutant lymph glands and observed a twofold reduction in the densitometric mean compared to that of the wild-type controls (Fig. 7E to G). These results predicted that increased levels of STAT activity in hop^{Tum-l} mutants would upregulate the wild-type but not the mSTAT CRM. To test this hypothesis, we assayed wild-type and mSTAT CRM activities in hop^{Tum-l} lymph glands from animals cultured at three different temperatures (Fig. 8). In wild-type lymph glands, the wild-type CRM was significantly more active than the mSTAT CRM regardless of the culture temperature (Fig. 8A, A′, and E and data not shown). In contrast, we observed no difference between wild-type and mSTAT CRM activities in hop^{Tum-l} lymph glands from animals cultured at 23°C (Fig. 8B, B′, and E). However, when the culture temperature was increased to 25°C and 27°C, we observed a statistically significant increase in levels in wild-type CRM activity compared to that of the mSTAT CRM (Fig. 8C to E). Based on these results, we propose that activated STAT upregulates wild-type ush CRM activity through the consensus STAT site. Altogether, these results indicate that the JAK/STAT pathway upregulates ush expression to maintain prohemocyte potency.

DISCUSSION

In this study, we showed that a decrease in ush expression levels resulted in a loss of medullary zone prohemocytes and a concomitant increase in hemocyte differentiation. In addition, we provide evidence that ush expression is regulated directly by activated STAT. Collectively, these observations indicate that ush is a target of the JAK/STAT signaling pathway and that Ush functions to restrict the forward momentum of prohemocyte differentiation. Considering that the PSC maintains prohemocyte potency through the JAK/STAT pathway (32), our findings provide an important link between the stem cell niche and the intrinsic regulation of prohemocyte differentiation. Furthermore, these results may have implications for the regulation of mammalian HSCs because FOG-1 is expressed in mouse HSCs (8). The role of FOG-1 in these cells is unknown; however, like Ush, FOG-1 may block stem cell differentiation and maintain the multipotent state. In addition, FOG-1 expression may be regulated by the stem cell niche. Finally, the modulation of FOG expression by the JAK/STAT pathway may be part of a conserved strategy for the regulation of HSCs and other stem cell types. In this regard, STAT5 regulates mammalian HSC self-renewal (28). Perhaps, similar to their
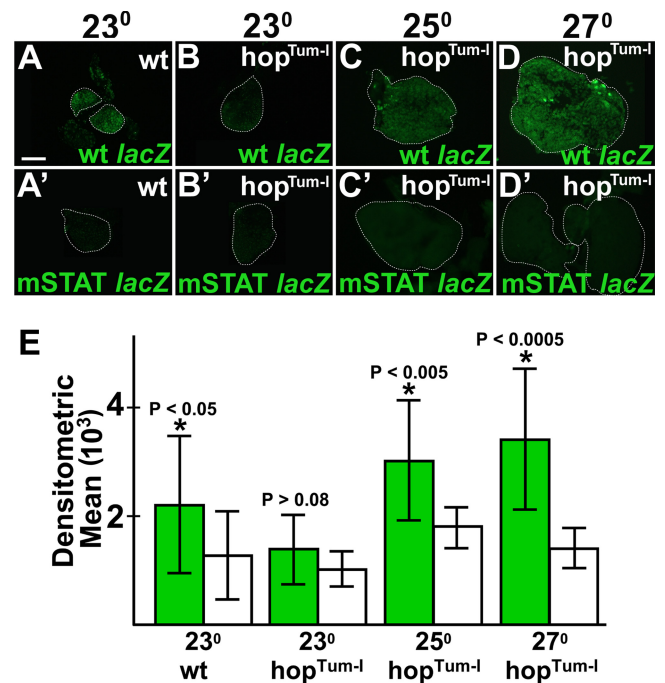

FIG. 8. ush hematopoietic CRM activity in hop^{Tum-l} mutant lymph glands. Shown are data for wild-type (wt)and mutant STAT binding site (mSTAT) ush hematopoietic CRM activity in wild-type or hop^{Tum-l} second-instar larval lymph glands (fluorescence microscopy) (magnification, ×40). (A and B′) Wild-type and mSTAT ush hematopoietic CRM activity in wild-type second-instar larval lymph glands of animals cultured at 23°C. (B, C, and D) Wild-type hematopoietic CRM activity in hop^{Tum-l} lymph glands cultured at the following temperatures: 23°C (B), 25°C (C), and 27°C (D). (B′, C′, and D′) mSTAT hematopoietic CRM activity in hop^{Tum-l} lymph glands cultured at the following temperatures: 23°C (B′), 25°C (C′), and 27°C (D′). (E) Densitometric means of wild-type (green bars) and mSTAT (white bars) ush hematopoietic CRM activity in wild-type and hop^{Tum-l} lymph glands. Densitometric means of wild-type lymph glands carrying the wild-type and mSTAT ush hematopoietic CRM were $2,216 \pm 1,258$ and $1,272 \pm 810$, respectively ($P < 0.05$). Densitometric means of hop^{Tum-l} lymph glands carrying the wild-type or mSTAT ush hematopoietic CRM were as follows: $1,392 \pm 636$ (wild type) and $1,017 \pm 323$ (mSTAT) for lymph glands cultured at 23°C ($P > 0.08$), $3,046 \pm 1,126$ (wild type) and $1,787 \pm 369$ (mSTAT) for lymph glands cultured at 25°C ($P < 0.005$), and $3,391 \pm 1,298$ (wild type) and $1,435 + 369$ (mSTAT) for lymph glands cultured at 27°C ($P < 0.0005$). Asterisks indicate a significant difference (Student t test) between wild-type and mSTAT CRMs. Scale bar, 50 μm.

counterparts in the fly, STAT5 activates FOG-1 expression to maintain mammalian HSC potency.

Ush not only regulates the choice between the maintenance of potency and differentiation in prohemocytes but also regulates the choice between lineage-specific developmental pathways. In this regard, we observed that a change in the level of ush expression dramatically altered the composition of the lymph gland hemocyte population. Plasmatocyte and crystal cell differentiation increased with the loss of one functional ush gene (heterozygote), whereas the loss of more than one functional ush gene ($trans$ heterozygote) produced fewer crystal cells and plasmatocytes and a corresponding increase in numbers of lamellocytes. These results indicate that a threshold level of Ush is required to promote the crystal cell and plas-

matocyte developmental programs and are consistent with the observation that Ush is expressed in a subpopulation of these cells. Maintaining Ush at this threshold level may also limit lamellocyte differentiation to prevent the depletion of the prohemocyte population. In this regard, we observed that an increase in numbers of lamellocytes was accompanied by a decrease in numbers of prohemocytes, which is consistent with data from a previous report (32). *ush trans*-heterozygous lymph glands contain large aggregates of lamellocytes and a substantial number of cells that are not terminally differentiated lamellocytes (L1-negative cells). In addition, we observed few plasmatocytes and crystal cells and no prohemocytes in *ush trans*-heterozygous lymph glands. From these observations, we conclude that most of the L1-negative cells are lamellocyte precursors.

The ability of Ush to control prohemocyte fate choice is achieved through a complex gene regulatory strategy. Srp is an intrinsic regulator of *ush* expression in the lymph gland and, like activated STAT, is part of a larger regulatory cascade. Srp directly activates *ush* expression through clustered GATA sites within the hematopoietic CRM (44). Recently, *srp* expression was shown to be negatively regulated by the transforming growth factor β homolog Decapentaplegic (Dpp). Dpp blocks zinc finger homeodomain protein 1 expression, which is required for the expression of *srp*. As a result, *dpp* mutant larvae have elevated Ush levels and are refractory to *Salmonella*-induced lamellocyte differentiation (19). Together, these observations suggest that the *ush* hematopoietic CRM acts as a nexus between the Dpp and JAK/STAT pathways, and antagonism between these pathways modulates *ush* expression (Fig. 9A). One key element of this gene regulatory network is the architecture of the *ush* hematopoietic CRM, in which the STAT binding site is located 9 bp from the clustered GATA sites. The proximity of the STAT and GATA binding sites may facilitate a cooperative interaction between Srp and STAT in much that same way as adjacent GATA and RUNX sites promote a cooperative interaction between Srp and Lz (45). Thus, this network may operate as a graded rheostat mechanism to regulate the level of *ush* expression through combinatorial interactions involving Srp and STAT and thereby control prohemocyte potency and lineage choice (Fig. 9).

FOG proteins bind the N-terminal zinc finger of their respective GATA partners to modify GATA-activated gene expression (16). During embryonic crystal cell development, Ush converts SrpNC from an activator to a repressor, which limits commitment and maturation by blocking *lz* expression (18, 45). Similarly, Ush may interact with SrpNC during lymph gland hematopoiesis to maintain prohemocyte potency and control lineage choice. In order to perform these diverse functions during the different stages of hematopoiesis, the SrpNC-Ush repressor complex may interact with additional protein binding partners to confer gene regulatory specificity, as is the case with the vertebrate GATA-FOG complex (4, 7, 12, 25, 26, 30, 38, 50, 51). Conversely, a reduction in the level of Ush may free SrpNC to act with lineage-specific factors to drive commitment and differentiation, much as the downregulation of Ush in embryonic crystal cell precursors allows SrpNC to act with Lz to drive crystal cell maturation (18, 45). In this regard, we observed that Ush was downregulated in crystal cells and plas-
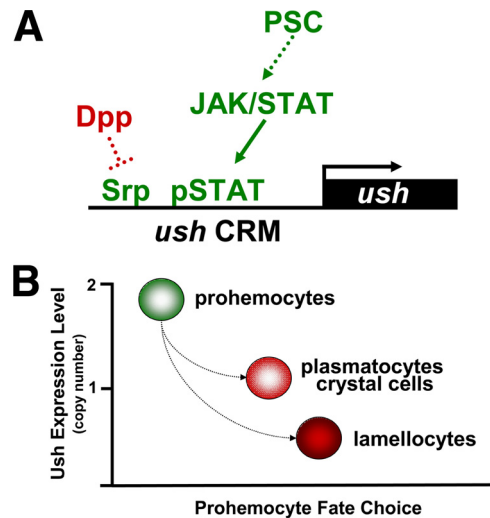

FIG. 9. Ush is a key component of a gene regulatory network that controls prohemocyte fate choice. (A) Model showing regulation of *ush* expression levels by the convergence of the Dpp and JAK/STAT pathways. The positive regulatory pathway (green) begins with a signal from the PSC, which maintains JAK/STAT activity in the lymph gland medullary zone. *ush* gene expression is coactivated by Srp and activated STAT. Ush expression is negatively regulated through the inhibition of *srp* expression by Dpp (red). The solid arrow indicates a direct link between pathway components. The dotted arrow and blocked line indicate additional unspecified components that comprise the pathway. (B) Model showing that prohemocyte fate choice is determined by the level of *ush* expression. Two copies of Ush maintain prohemocyte potency. The loss of one copy favors plasmatocyte and crystal cell differentiation. The loss of more than one copy favors lamellocyte differentiation. Together, these models describe key components of a larger integrated genetic network that adjusts hemocyte composition in response to the needs of the organism.

matocytes. Furthermore, this loss of *ush* expression is most likely due to decreased STAT activity in these cells (32).

In conclusion, Ush is a key component of the overall gene regulatory network that controls the blood organ's response to the changing needs of the organism. We show that *ush* expression is regulated directly by activated STAT and that changes in *ush* expression alter prohemocyte fate. Current models of *Drosophila* lymph gland hematopoiesis describe a stem cell niche that regulates prohemocyte maintenance through the Hedgehog and JAK/STAT signaling pathways (32, 40). Most importantly, our findings identify a downstream target of the niche and provide a mechanism for the processing of these signals to control prohemocyte potency and differentiation.

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