

The multitype zinc-finger protein U-shaped functions in heart cell specification in the *Drosophila* embryo

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Multitype zinc-finger proteins of the Friend of GATA/U-shaped (Ush) class function as transcriptional regulators of gene expression through their modulation of GATA factor activity. To better understand intrinsic properties of these proteins, we investigated the expression and function of the *ush* gene during *Drosophila* embryogenesis. *ush* is dynamically expressed in the embryo, including several cell types present within the mesoderm. The gene is active in the cardiogenic mesoderm, and a loss of function results in an overproduction of both cardial and pericardial cells, indicating a requirement for the gene in the formation of these distinct cardiac cell types. Conversely, ectopic expression of *ush* results in a decrease in the number of cardioblasts in the heart and the inhibition of a cardial cell enhancer normally regulated by the synergistic activity of the Pannier and Tinman cardiogenic factors. These findings suggest that, similar to its known function in thoracic bristle patterning, Ush functions in the control of heart cell specification through its modulation of Pannier transcriptional activity. *ush* is also required for mesodermal cell migration early in embryogenesis, where it shows a genetic interaction with the Heartless fibroblast growth factor receptor gene. Taken together, these results demonstrate a critical role for the Ush transcriptional regulator in several diverse processes of mesoderm differentiation and heart formation.

GATA zinc-finger transcription factors are expressed in a variety of cell types, where they regulate gene expression during their control of cell commitment and differentiation (reviewed in refs. 1 and 2). Members of the GATA4, -5, and -6 subfamily are expressed in overlapping patterns during early heart development in the mouse, where they likely function in the establishment of the cardiac cell lineage. However, genetic studies thus far have failed to show a definitive role for any of the genes in cardiomyocyte specification. Loss of *GATA4* function does result in aberrant cardiac morphogenesis, because null mice form bilateral heart tubes that fail to fuse at the ventral midline (3, 4). Nonetheless, normal specification of the cardiac cell lineage occurs because these tubes contain differentiated cardiomyocytes that express numerous contractile protein genes. Likewise, insights have not been gained from the analysis of other gene mutants as *GATA5* null mice are viable (4) and *GATA6* homozygotes die shortly after implantation (5, 6). Because functional redundancies may exist between GATA4, -5, and -6, proof of a requirement for these proteins in cardiomyocyte specification may occur only through the analysis of mice mutant for at least two of the genes.

Recent studies in zebrafish and *Drosophila* have demonstrated an essential function for two GATA factors in the early events of heart formation. The zebrafish *faust* (*fau*) gene, which encodes the GATA5 transcription factor, is required for the formation of normal numbers of myocardial precursors and the proper expression of several cardiac-expressed genes, including *nkx2.5* (7). *fau* function is also needed for heart and endoderm morphogenesis, including the production of ventricular tissue. Forced expression of GATA5 results in the *de*

novo activation of several myocardial genes and the ectopic production of contracting heart-like tissue. Such results demonstrate an integral role for this GATA factor in cardiac cell production, differentiation, and morphogenesis during zebrafish development.

During *Drosophila* embryogenesis, the GATA gene *pannier* (*pnr*; refs. 8 and 9) is expressed in the cardiogenic mesoderm, where it is required for cardial cell formation while repressing the overproduction of a pericardial cell type (10). *pnr* can function as a cardiac identity gene because its forced expression results in supernumerary cardial cells at the expense of other dorsal mesodermal cell lineages. Pnr works synergistically with the Tinman (Tin) homeodomain protein (11–13) in the activation of a heart enhancer for the *D-mef2* differentiation gene and the specification of the cardioblast fate (10, 14, 15). Mouse GATA4 can also perform as a cardiogenic factor in the *Drosophila* system, suggesting an evolutionarily conserved function between Pnr and GATA4 in the early stages of heart development.

In addition to this functional synergism of Pnr and Tin, cardiac-expressed GATA proteins are known to interact with heterologous transcription factors, including NFAT3, Nkx-2.5, and Friend of GATA-2 (FOG-2; refs. 16–22). FOG-2 is a multitype zinc-finger protein that is coexpressed with the GATA factors in several cell types in the mouse, including the developing and mature heart. FOG-2 can bind to the amino-terminal zinc-finger of GATA4 and affect GATA4 transcriptional activity in its regulation of various cardiac promoters in cell culture studies (20–22). These results suggest the involvement of FOG-2 as a modulator of GATA factor function in the activation of cardiac-expressed genes during cardiomyocyte specification and subsequent heart development.

U-shaped (Ush) is a multitype zinc-finger protein of *Drosophila* that is structurally related to FOG-2 (23). Comparable to the interactions of GATA4 and FOG-2, Pnr and Ush heterodimerize through the amino-terminal zinc finger of Pnr (24), and genetic studies have shown that Ush antagonizes the function of Pnr in the establishment of the thoracic bristle pattern in the adult (24, 25). Given the molecular and functional similarities between the GATA and multitype zinc-finger proteins of vertebrates and flies and the recent demonstration of a cardiogenic function of *pnr*, we looked for a possible role for *ush* in *Drosophila* heart development. In this report, we show that *ush* is expressed in the embryonic mesoderm including the cardiogenic region. Genetic studies demonstrate a requirement for *ush* function in the normal migration of the mesoderm and in the proper formation

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Abbreviations: D-MEF2, *Drosophila* myocyte enhancer factor 2; Eve, Even-skipped; FOG-2, Friend of GATA-2; *htl*, *heartless*; Pnr, Pannier; Tin, Tinman; UAS, upstream activation sequence; Ush, U-shaped.

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of cardiac cells. Our results are consistent with a model in which Ush modulates the function of Pnr in heart cell specification, similar to its known function in neuronal precursor cell determination.

Materials and Methods

Fly Strains. *y w^{67c23}* served as our wild-type stock. The *ush* strains used in this study were obtained from the Umea Stock Center (Umea, Sweden) and included the presumed point mutations *ush¹ cn¹ bw¹ sp¹/CyO* and *ush² cn¹ bw¹ sp¹/CyO* and the *ush* deletion *Df(2L)al/CyO, ftz-lacZ*. These three mutations have been classified previously as embryonic lethals and germ band retraction amorphs (26). The *ush¹/SM6, Roi, eve-lacZ* and *ush²/SM6, Roi, eve-lacZ* stocks were further established to discriminate between the mutant and balancer chromosomes. The *D-mef2* enhancer-*lacZ* gene used was *IIA341* (10, 15). The Gal4 driver strain used to direct gene expression in the mesoderm was *twi-Gal4* (27). The *UAS-ush* stock was generated in this study by standard PCR amplification of a full-length *ush* cDNA cloned in pBluescript (Research Genetics, Huntsville, AL), by using oligonucleotides (Sigma-Genosys) to engineer 5' *XhoI* (pUASTush1: ATATACTCGAGGAATAATTGTATTACAGTGAGTAGTGAG) and 3' *XbaI* (pUASTush2: AAATATCTAGACTCCGGGAATACTGTCGTCGATTG) restriction sites at the ends of the 4.5-kb cDNA insert. This fragment was then cloned into the corresponding sites of the pUAST transformation vector (28), with insert orientation and integrity confirmed by DNA sequencing. The pUAST-*ush* DNA was injected into *y w^{67c23}* embryos, and multiple transgenic lines were established by using standard transformation procedures (14). Eight lines were tested and showed a moderate to strong dorsal vessel phenotype. The *heartless (htl)* allele used in this study was *htl^{AB42}/TM3, Sb, ftz-lacZ*, characterized as a null allele based on molecular and phenotypic analyses (29).

RNA in Situ Hybridization and Immunohistochemical Staining of Embryos. DNAs containing *ush* or *pnr* cDNAs cloned in pBluescript were linearized by restriction digestion and used as templates to generate antisense cRNA probes by using the DIG RNA labeling kit (Roche Molecular Biochemicals). RNA *in situ* hybridization and sectioning of embryos were performed as described (10). The same study also describes the methods used for immunostaining of embryos with mouse anti- β -galactosidase, rabbit anti-*Drosophila* myocyte enhancer factor 2 (D-MEF2), and mouse anti-Even-skipped (Eve) antibodies.

Gene Expression Analyses in Mutant and Gal4/UAS Embryos. Embryos obtained from the mating of *twi-Gal4* and *UAS-ush* flies were collected at 29°C, whereas all other embryos were collected at 23°C. To analyze the function of the *D-mef2* cardinal cell enhancer in *ush* mutant embryos, *ush¹/SM6, Roi, eve-lacZ* or *Df(2L)al/CyO, ftz-lacZ* males were crossed to *y w; IIA341-44* females. Non-Cy F₁ virgins of the genotype *ush¹/+; IIA341-44/+* or *Df(2L)al/+; IIA341-44/+* were then backcrossed to males of the respective parental strains. Expression of the *D-mef2-lacZ* fusion gene was assayed by staining for β -galactosidase activity. Homozygous embryos were identified by the failure of germ band retraction, and the number of cardinal cells present per hemisegment was averaged over 15 mutant embryos.

ush embryos were also stained for D-MEF2 and β -galactosidase protein to determine whether there was an increase in the number of cardinal cell nuclei. Homozygous embryos were identified by the germ band retraction phenotype and the absence of the β -galactosidase marker associated with the balancer chromosome. Nuclei counts were obtained from an average of 10 wild-type or *ush* mutant embryos. To determine the number of Eve-expressing cells, embryos were collected from the mating of *ush* stocks and stained for Eve and β -galactosidase with ho-

mozygous embryos identified by the absence of the marker protein. The average number of Eve-expressing pericardial cells was determined from the count of 20 mutant embryos. To analyze the activity of cardiac cell markers in embryos with forced *Ush* expression, *UAS-ush* males were crossed to *twi-Gal4* or *twi-Gal4; IIA341-44* females, with the resulting embryos stained with anti-D-MEF2 or anti- β -galactosidase antibodies, respectively.

Expression Vectors, Cell Transfections, and CAT Assays. *Drosophila* S2 cells were grown in M-3 medium (Quality Biologicals, Gaithersburg, MD) supplemented with 10% FBS. Cells (1×10^5 per transfection) were cotransfected with the indicated expression vectors (2 μ g per transfection) and the D-mef2-CAT reporter plasmid (1 μ g per transfection) by using the calcium phosphate precipitation method. Normalization of transfection efficiency with β -galactosidase activity and CAT assays with a CAT ELISA kit (Roche Molecular Biochemicals) were performed as described (30). For the construction of expression vectors, full-length cDNAs for *pnr*, *ush*, and *tin* were subcloned into the pIZ/V5-His plasmid (Invitrogen). For the Tin (N-Q) construct, the coding region for the homeodomain in the wild-type Tin expression vector was replaced by a DNA fragment from the A6-NK4HD(NQ) plasmid (31) containing the mutated sequence corresponding to an Asn to Gln substitution at amino acid position 351. The D-mef2-CAT reporter plasmid was constructed by inserting the DNA fragment from the *D-mef2-341* vector (15) into the *BamHI* site of the pBLCAT2 plasmid (Promega).

Analysis of a Mesodermal Cell Migration Phenotype. Embryos were collected from the independent matings of the *ush¹/SM6, Roi, eve-lacZ* and *htl^{AB42}/TM3, Sb, ftz-lacZ* stocks and from an intercross of these flies to produce *ush¹/+; htl^{AB42}/+* transheterozygotes. Embryo populations were stained for D-MEF2 and β -galactosidase proteins. Homozygous or transheterozygous mutant embryos were identified by the absence of β -galactosidase activity associated with the presence of the balancer chromosome. The migration of mesodermal cells marked by D-MEF2 expression was analyzed in embryo cross sections as described (10).

Results

ush Expression in the Embryonic Mesoderm. The *ush* gene exhibits a dynamic pattern of expression during embryogenesis. Gene transcripts are first detected at high levels in the primordium of the amnioserosa at stage 5 (Fig. 1A). Additional expression is observed in germ band extending embryos in cells of the developing anterior and posterior midgut and in hemocyte precursors present in the cephalic mesoderm (Fig. 1B). By stage 11, *ush* RNA is detected in the dorsal ectoderm and in precursor cells of the hemocytes and fat body (Fig. 1C and D). By late embryogenesis, *ush* expression is greatly diminished, but transcripts are still observed in the dorsal ectoderm during dorsal closure (Fig. 1E) and cells within, or associated with, the central nervous system (Fig. 1F). To investigate the possible expression of *ush* in mesodermal cells underlying the dorsal ectoderm, we examined cross sections of embryos at stage 11. *ush* RNA is detected in a changing pattern in this germ layer, initially throughout most of the mesoderm (Fig. 1G) and then in subpopulations of cells, including precursors of the fat body, visceral mesoderm, and cardiogenic mesoderm (Fig. 1H). As a comparison, the presence of *pnr* RNA in the dorsal ectoderm and cardiogenic mesoderm is also shown (Fig. 1I; ref. 10). Therefore, *ush* is expressed in the dorsal mesoderm, where it could function in the early stages of heart formation.

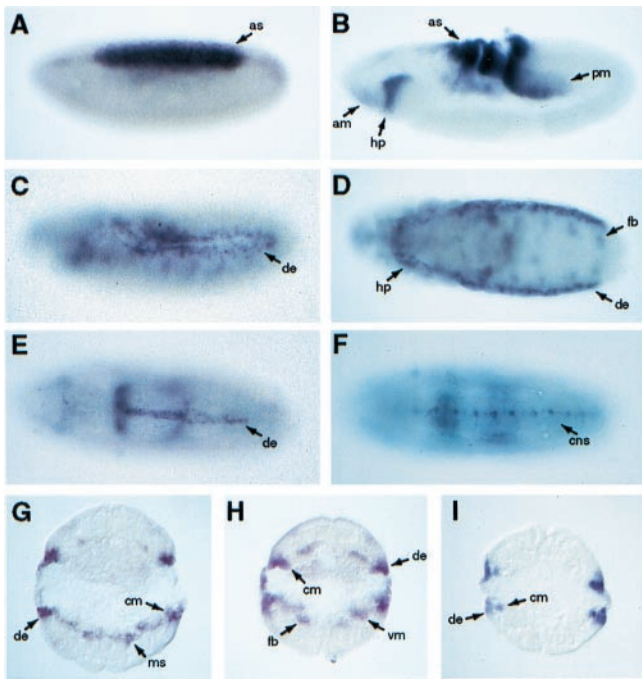


Fig. 1. *ush* gene expression during *Drosophila* embryogenesis. (A–C) Lateral views of *ush* RNA in stage 5, 8, and 11 whole-mount embryos. (D and F) Ventral views of *ush* RNA in stage 11 and 16 whole-mount embryos. (E) Dorsal view of *ush* RNA in a stage 16 whole-mount embryo. (G and H) Cross sections of stage 11 embryos stained for *ush* RNA. (I) Cross section of a stage 11 embryo stained for *pnr* RNA. Abbreviations: am, anterior midgut primordium; as, amnioserosa primordium; cm, cardiogenic mesoderm; cns, central nervous system; de, dorsal ectoderm; fb, fat body precursors; hp, hemocyte primordium; ms, mesoderm; pm, posterior midgut primordium; vm, visceral mesoderm precursors.

***ush* Function Is Required for Proper Cardiac Cell Specification.** *pnr* mutant embryos show a loss of contractile cardiac cells and an overproduction of certain nonmuscle pericardial cells in the heart-forming region (10). To identify a possible role for *ush* in

these cardiogenic processes, we looked for alterations in cardiac cell production in mutant embryos. The *ush* alleles used in this analysis were *ush¹* and *ush²*, believed to be hypomorphic mutations of the gene, and *Df(2L)al*, a chromosome deletion that represents a *ush* null mutation. The *D-mef2* heart enhancer-*lacZ* fusion gene serves as a cardiac cell marker as it is detected in progenitors of these cells around stage 11 and thereafter in two, then four cardioblasts per hemisegment of the forming dorsal vessel (14, 15, 32). Embryos homozygous for either a point mutation or deletion of the gene showed an increase in the number of cells expressing the reporter gene as compared with the wild-type embryo (Fig. 2A). In *ush¹* embryos, a few hemisegments contained up to nine positive cells with an average of six cardiac cells present in many clusters (Fig. 2B). In *ush*-deficiency embryos, a comparable increased density of cardiac cells was obtained (Fig. 2C).

D-MEF2 protein also marks cardioblasts as it is detected in the nuclei of all cardiac, but not pericardial cells of the forming dorsal vessel. In wild-type embryos at stage 13, the germ band has retracted with cardioblasts migrating dorsally, separating from the dorsal somatic muscles. A lateral view at this stage shows a single row of cells that contains six stained nuclei per hemisegment (Fig. 2D). In contrast, *ush* mutant embryos possess supernumerary cardioblast nuclei. *ush¹* (Fig. 2E) and *ush²* (Fig. 2F) embryos contain up to 12 nuclei per hemisegment with eight cells per cluster observed on average. Similar results were also obtained with *ush*-deficiency embryos (data not shown). Therefore, reducing or completely eliminating *ush* function leads to an increased production of cardiac cells. Intriguingly, the *ush* heart phenotype uncovered by the analysis of these two markers directly contrasts the absence of cardiac cells observed in *pnr* loss-of-function embryos.

We also assayed for the production of pericardial cells in *ush* mutant embryos, using *Eve* protein as a marker for a subset of these cells. In wild-type embryos at stage 12, there exist 11 *Eve*-positive clusters within the dorsal mesoderm, each containing about three cells (Fig. 2G). In contrast, the number of *Eve*-expressing pericardial cells increased in homozygous *ush¹* and *ush²* embryos to an average of 5–6 per cluster (Fig. 2H and I). A similar increase in pericardial cell number was also

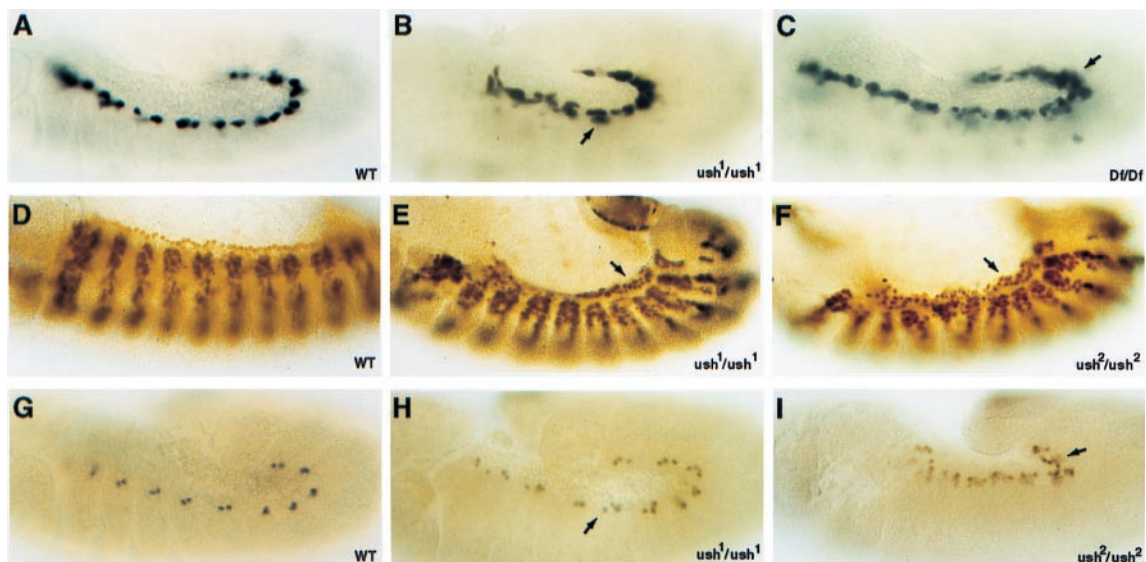


Fig. 2. *ush* gene functions during *Drosophila* cardiogenesis. (A, D, and G) WT, wild-type embryos. (B, C, E, F, H, and I) Homozygous *ush* embryos of the indicated genotype. (A–C) Lateral views of stage 12 embryos stained for *lacZ* reporter gene expression under the control of a *D-mef2* cardiac cell enhancer. (D–F) Lateral views of stage 13 embryos stained for D-MEF2 protein in cardiac cells. (G–I) Lateral views of stage 12 embryos stained for *Eve* protein in a subset of pericardial cells. Arrows point to the increased numbers of cardiac or pericardial cells in the *ush* mutant embryos.

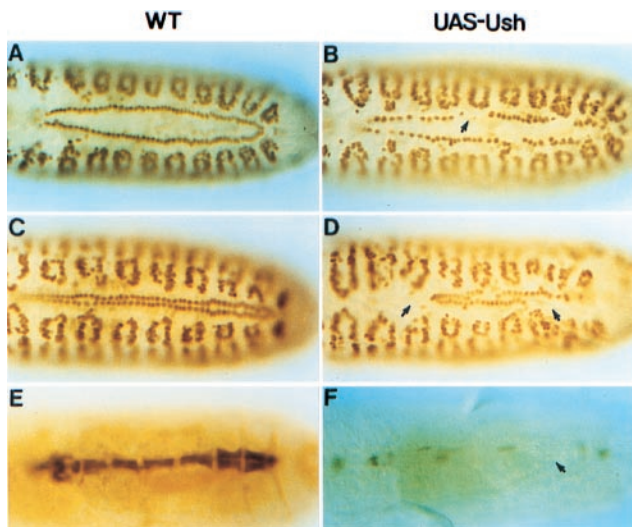


Fig. 3. Forced mesodermal expression of the *ush* gene affects heart development. (A, C, and E) WT, wild-type embryos. (B, D, and F) UAS-Ush, embryos expressing *ush* in the mesoderm under the control of a *twi-Gal4* driver. (A–D) Expression of *ush* results in a reduced number of cardiac cells in the forming dorsal vessel. Stage 14 (A and B) and 16 (C and D) embryos stained for D-MEF2 protein. (E and F) Expression of *ush* results in a decreased activity of the *D-mef2* cardiac cell enhancer. Stage 16 embryos were stained for β -galactosidase protein in cardiac cells of the dorsal vessel. Arrows point to missing cardiac cells or cardioblasts that fail to express the *lacZ* reporter gene.

observed in homozygous *Df(2L)al* embryos (data not shown). Thus, *ush* gene activity is required to prevent the overproduction of this pericardial cell type, a function that has also been ascribed to the *pnr* gene.

Forced Expression of *ush* Results in a Diminution of Cardiac Cells.

Because the loss of *ush* function resulted in a supernumerary cardiac cell phenotype, we wanted to monitor the effect of expressing the gene throughout the mesoderm using the Gal4/UAS binary system (28). D-MEF2 was used to assess the status of cardiac cells, with two contiguous rows of 52 cells present in the forming or mature dorsal vessel of wild-type embryos (Fig. 3 A and C). In comparably staged embryos expressing *ush* throughout the mesoderm, a significant reduction in cardiac cells was observed. In the two representative embryos shown, a 37% (Fig. 3B) and 50% (Fig. 3D) decrease occurred with small gaps or large deletions present within the heart tubes. The *D-mef2* heart enhancer-*lacZ* fusion gene was used as a second marker for the cardiac cells and also to assay the effect of *ush* expression on enhancer activity. In wild-type embryos at stage 16, the enhancer is active in eight cardiac cells in most segments of the dorsal vessel (Fig. 3E). In contrast, β -galactosidase activity is greatly diminished in the hearts of *ush*-expressing embryos, most likely a combination of the decrease in cardiac cell number and the reduced activity of the *D-mef2* cardiac enhancer (Fig. 3F). Thus, forced expression of *ush* has a potent negative effect on cardiac cell formation and enhancer function.

Ush Decreases the Synergistic Activation of the *D-mef2* Heart Enhancer by Pnr and Tin.

It was previously shown that Pnr could function combinatorially with Tin in the regulation of the *D-mef2* heart enhancer in *Drosophila* embryos (10). We further analyzed this synergistic activation using a transient transfection assay in cultured *Drosophila* cells. The activation of a CAT reporter gene linked to the *D-mef2* enhancer was monitored in cells transfected independently with Pnr, Tin, and Ush or with various combinations of the factors. The expression of Tin alone activated the

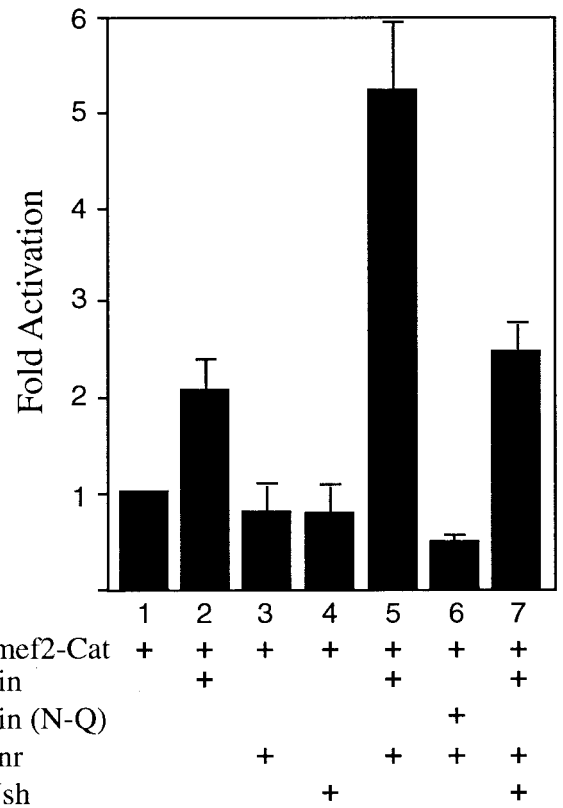


Fig. 4. Ush antagonizes Pnr transcriptional synergy with Tin in the regulation of the *D-mef2* cardiac enhancer. *Drosophila* S2 cells were cotransfected with a D-mef2-CAT reporter plasmid along with Tin, Pnr, and Ush expression plasmids, as indicated. The normalized CAT activity obtained from the transfections of a test effector(s) with the reporter plasmid was divided by the corresponding value obtained with an empty vector (pIZ/V5-His) and reporter, with the value shown as fold activation. The data show that Pnr collaborates with Tin to activate the *D-mef2* enhancer, and this effect is diminished by the inclusion of Ush.

enhancer about 2-fold above the basal level, whereas neither Pnr nor Ush affected enhancer activity (Fig. 4, lanes 1–4). Coexpression of Pnr and Tin resulted in a synergistic activation of the enhancer to a level 5–6 times that of the basal activity (Fig. 4, lane 5), and this strong induction required the binding of Tin to the *D-mef2* enhancer as a Tin DNA binding mutant, Tin (N-Q), failed to synergize with Pnr in the assay (Fig. 4, lane 6). In contrast, adding Ush as a third transfected factor significantly inhibited the synergistic activation of the enhancer by Pnr and Tin (Fig. 4, lane 7). This result demonstrates that Ush can antagonize the positive functional interaction of Pnr and Tin in the regulation of the cardiac cell enhancer, which is consistent with the *in vivo* data shown in Fig. 3F.

ush Embryos Exhibit a Mesodermal Cell Migration Phenotype.

As previously noted, *ush* mutants contain an increased number of cardiac cells. However, we also noticed in about half of the embryos a disparity in the cardiac cell populations, ranging from a high of 8–12 per hemisegment down to regions completely devoid of cells. This complex phenotype was observed with both *ush* hypomorphic (Fig. 5A) and null (Fig. 5B) alleles. This sporadic loss of cells from the dorsal-most part of the mesoderm was reminiscent of a *htl* mutant phenotype, where the absence of the encoded fibroblast growth factor receptor homologue results in an incomplete dorsal migration of mesodermal cells (29, 33, 34). In this event, cells fail to receive the ectodermal signal

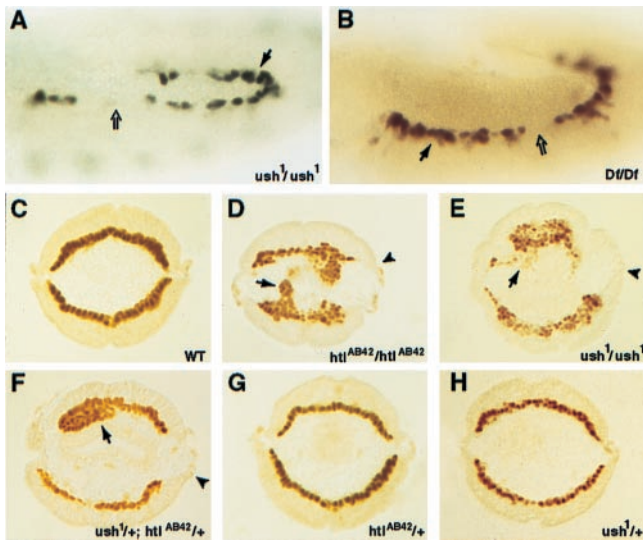


Fig. 5. *ush* function in mesodermal cell migration. (A and B) Lateral views of stage 11 and 12 embryos of the genotypes *ush¹/ush¹*; *IIA341-44/+* and *Df(2L)al/Df(2L)al*; *IIA341-44/+*, respectively, stained for β -galactosidase produced in cardiac cells under the control of a *D-mef2* heart enhancer. Solid arrows point to increased numbers of cardiac cells whereas open arrows indicate the absence of cells in some hemisegments. (C–H) Analysis of mesodermal cell migration defects. Stage 10 embryos of the indicated genotypes were stained for D-MEF2 protein and sectioned to visualize the normal or abnormal spreading of the mesodermal cell layer. Arrows point to clusters of mesodermal cells, whereas arrowheads indicate the dorsal-most ectodermal cells that correspond to the normal position of cell spreading in wild-type embryos. Strong migration defects are observed in homozygous *htl* and *ush* mutants and in embryos transheterozygous for the two genes.

needed for their further commitment, resulting in a loss of dorsal mesodermal derivatives, including cardioblasts.

To determine whether the variable absence of cardiac cells in *ush* embryos was because of a cell migration defect, we stained wild-type and mutant embryos for D-MEF2 protein present in the invaginated population of mesodermal cells. In cross sections of normal embryos at stage 10, a uniform layer is observed where the dorsal-most mesodermal cells have migrated to a position adjacent to the dorsal-most ectodermal cells (Fig. 5C). In contrast, both *htl* (Fig. 5D) and *ush* (Fig. 5E) homozygous embryos display an irregular layer where mesodermal cells remain clustered and fail to undergo their complete dorsal migration. This result suggested *ush* function is required for the directional migration of the mesoderm. To investigate a potential genetic interaction of *htl* and *ush* in this process, we next examined embryos that were heterozygous for mutations in each of the genes. These embryos also presented a strong mesodermal migration phenotype (Fig. 5F), suggesting the two genes function in a common genetic pathway that controls this aspect of mesoderm differentiation. As was observed with homozygous *ush* embryos, slightly less than half (47%; $n = 46/97$) of the transheterozygous embryos showed a loss of cells from the dorsal mesoderm. As a control, we likewise examined embryos individually heterozygous for *htl* or *ush* gene mutations, and such embryos exhibited a normal pattern of mesodermal cell migration (Fig. 5G and H).

Discussion

In this report, we show that the transcriptional regulator Ush is expressed and functions during *Drosophila* embryogenesis. Consistent with its known requirement for germ band retraction (26), *ush* is abundantly expressed in the forming amnioserosa. Current results further demonstrate a function for the gene in the dorsal



Fig. 6. Model of the functional interactions of Pnr, Tin, and Ush during cardiac and pericardial cell specification, based on loss- and gain-of-function phenotypes. The arrow indicates a positive requirement for Tin and Pnr in cardiac cell formation, whereas the solid blocked line indicates a negative role for the *pnr*, *ush*, and/or *tin* genetic combination in this process. The hatched blocked line indicates a negative function for these genes in the Eve-expressing pericardial cells. The model is a simplification of the total genetic complexity of cardiac cell specification in *Drosophila* and does not necessarily list all components required to control the production of these distinct cell types.

migration of mesodermal cells during the early development of this germ layer and the production of two cardiac cell types that contribute to the dorsal vessel. Because *ush* is expressed in additional cells in the embryo, the gene is likely to function in the commitment and/or differentiation of other lineages as well.

Ush is structurally similar to the vertebrate FOG proteins, and it has been shown to negatively regulate Pnr function within the larval wing disk during sensory bristle development (24, 25). When expressed alone, Pnr functions as a transcriptional activator of an enhancer for the proneural genes *achaete* (*ac*) and *scute* (*sc*), turning on these loci in the dorsocentral cluster. In cellular domains where Pnr and Ush overlap, heterodimers form between the two proteins, and these complexes serve as transcriptional repressors. Thus, Ush can antagonize the activator function of Pnr in cells where they are coexpressed, and this property suggests that Ush is functionally similar to the FOG proteins because they also modulate the activity of GATA factors in the control of gene expression (20–22, 35). Given this proven interaction of Pnr and Ush in regulating *ac* and *sc* during thoracic bristle patterning, and the recent demonstration of *pnr* function in cardiac cell production, we looked for *ush* expression in the dorsal mesoderm. Our results show that, like *pnr*, *ush* is expressed in the heart-forming region, where it could interact with *pnr* in regulating the production of heart precursor cells.

pnr has a dual requirement in the cardiogenic mesoderm because it is needed for the formation of cardiac cells although simultaneously limiting the production of Eve-expressing pericardial cells (10). Based on these dissimilar phenotypes, we postulated Pnr might work with different combinations of factors to promote or repress the formation of cells within the distinct lineages. Recent studies have shown that Pnr and Tin act synergistically to induce cardiac cells and activate gene expression, and the loss of function of either of these genes results in an absence of cardiac cells. Therefore, the two work together in cardiac cell specification (Fig. 6).

In contrast, Ush is a factor that normally suppresses cardiac cell production. *ush* homozygous and hemizygous embryos show an increase in cardiac cell number, and the latter finding suggests Ush control of this cell population is dose-dependent, as is the case for Ush regulation of Pnr during sensory bristle development (24). Furthermore, forced expression of Ush decreases cardiac cell production and *D-mef2* heart enhancer activity, whereas ectopic expression of Pnr produces extra cardiac cells and expands the domain of enhancer activity. Thus, Ush displays phenotypes that are in direct opposition to those of Pnr, suggesting that it can function as an antagonist of Pnr's cardiogenic activity (Fig. 6). This conclusion is supported by the ability of Ush to inhibit the synergy of Pnr and Tin in the activation of the *D-mef2* heart enhancer in cell transfection studies. As for the second cardiac phenotype, both *pnr* and *ush* are required to limit the number of Eve-expressing pericardial cells, consistent with a

model in which Ush and Pnr function as corepressors in the control of these cells (Fig. 6). To summarize, our genetic studies predict that, in the wild-type embryo, *pnr* is expressed and functions independently of *ush* in precursors of the cardioblast lineage. However, in neighboring cells that include the Eve lineage precursors, the expression and function of the two most likely overlap. Future expression analyses of the two regulatory proteins at the resolution of single mesodermal cells will be required to elaborate on this genetic model in molecular terms.

About half of the *ush* embryos show a complex phenotype wherein certain hemisegments contain supernumerary cardiac cells, whereas others are devoid of the heart precursors. Because this latter observation was reminiscent of a *htl* phenotype, we examined mesodermal cell migration in the mutants and also looked for a genetic interaction between *ush* and *htl*. These studies showed that *ush* activity is needed for the proper formation of the mesodermal monolayer, but the phenotype is only partially penetrant as the normal dorsal migration of many cells does occur. This conclusion is based on the position of the D-MEF2-expressing cells and the formation of cardiac precursors, which requires the mesodermal cells be present within the Dpp signaling domain of the dorsal ectoderm (36). Intriguingly, embryos transheterozygous for the *htl* and *ush* genes showed a strong mesodermal migration phenotype, suggesting they function in a common genetic pathway used in the movement of the cells. A possible explanation for this interaction would be that Ush functions in the activation of *htl* gene expression in the early

mesoderm. Alternatively, it could be involved in the production of the ligand for the Htl fibroblast growth factor receptor in cells of the dorsal ectoderm, providing an attractant source for the mesodermal cells (29).

In conclusion, our current and recent results contribute to the expanding evidence for the conservation of genes controlling cardiogenesis in *Drosophila* and higher eukaryotes. Two common themes appear to be emerging, and such hypotheses can be further investigated both molecularly and genetically. First, it is likely that positive combinatorial interactions occur between GATA and Tin class factors in the specification of cardiac cell lineages and the activation of cardiac gene expression. Second, it is possible that antagonistic functions exist between GATA and Ush/FOG-2 type proteins in the transcription of cardiac expressed genes and in cell fate specification during early heart development. Relevant to the latter view, it will be of clear interest to compare the phenotypes of *ush* and *fog-2* mutant embryos to identify their common or different functions in the genetic control of cardiogenesis.

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