Hand is a direct target of Tinman and GATA factors during Drosophila cardiogenesis and hematopoiesis

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Summary

The existence of hemangioblasts, which serve as common progenitors for hematopoietic cells and cardioblasts, has suggested a molecular link between cardiogenesis and hematopoiesis in Drosophila. However, the molecular mediators that might link hematopoiesis and cardiogenesis remain unknown. Here, we show that the highly conserved basic helix-loop-helix (bHLH) transcription factor Hand is expressed in cardioblasts, pericardial nephrocytes and hematopoietic progenitors. The homeodomain protein Tinman and the GATA factors Pannier and Serpent directly activate Hand in these cell types through a minimal enhancer, which is necessary and sufficient to drive Hand expression in these different cell types. Hand is activated by Tinman and Pannier in cardioblasts and pericardial nephrocytes, and by Serpent in hematopoietic progenitors in the lymph gland. These findings place Hand at a nexus of the transcriptional networks that govern cardiogenesis and hematopoiesis, and indicate that the transcriptional pathways involved in development of the cardiovascular, excretory and hematopoietic systems may be more closely related than previously appreciated.

Key words: Hand, tinman, pannier, serpent, Drosophila, Heart development, Hematopoiesis, Lymph gland, Transcription regulation

Introduction

The fruit fly, Drosophila melanogaster, has a simple open circulatory system composed of circulating blood cells (hemocytes) and a dorsal vessel surrounded by pericardial cells. The dorsal vessel is a contractile tube lined by a layer of myoepithelial vascular cells called cardioblasts. The anterior part, called the aorta, functions as a major blood vessel; the posterior part, called the heart, pumps hemocytes through the aorta into the body cavity. The pericardial cells flanking the aorta and heart are excretory cells, so-called pericardial nephrocytes. Anterior to the pericardial nephrocytes, there are two pairs of cell clusters flanking the aorta, which comprise the lymph and ring gland. The lymph gland is made up of hematopoietic progenitor cells that generate all three blood cell types in the adult. The cardioblasts, pericardial nephrocytes and the lymph gland hematopoietic progenitors all arise from the same cardiac mesoderm that is specified by signaling pathways involving bone morphogenetic protein (Bmp), Decapentaplegic (Dpp), Wingless (Wg) and fibroblast growth factor (Fgf) (Cripps and Olson, 2002; Evans et al., 2003), hinting at a possible link between cardiogenesis and hematopoiesis.

Several transcription factors have been shown to play key roles in cardiogenesis and hematopoiesis in flies and vertebrates. The Drosophila NK-type homeobox gene tinman (tin), the earliest marker of the cardiac lineage, is initially expressed in the entire mesoderm before becoming restricted to the dorsal mesoderm and later to the cardiac mesoderm, in response to ectodermal Dpp and Wg signals. After all the cardiac cell types are specified, tin expression is extinguished in many cardiac cell types and maintained in only a subset of cardiac and pericardial cells (Han et al., 2002; Han and Bodmer, 2003). In tin mutant embryos, the entire cardiogenic region and the lymph gland fail to form (Bodmer, 1993, Mandal et al., 2004), indicating the essential role of Tinman in early specification of the cardiac and hematopoietic lineages.

There are several NK-type homeobox genes in vertebrates, which are named Nkx2.3-Nkx2.10 (Evans, 1999). Nkx2.5 is expressed in the early cardiac crescent and continues to be expressed throughout heart development. Mouse embryos lacking Nkx2.5 show early cardiac defects and arrested cardiogenesis before looping morphogenesis (Lyons et al., 1995). Furthermore, overexpression of a dominant-negative form of Nkx2.5 in Xenopus blocks cardiogenesis (Grow and Krieg, 1998) and mutations in Nkx2.5 cause congenital heart disease in humans (Schott et al., 1998). As tin is no longer expressed in hematopoietic progenitors after stage 13, its function in hematopoiesis is limited to the early specification of the cardiogenic mesoderm containing the progenitor cells for the lymph gland (Mandal et al., 2004).

Members of the GATA family of zinc-finger transcription factors play crucial roles in both cardiogenesis and hematopoiesis in Drosophila and vertebrates. The Drosophila GATA factor Pannier is expressed in the cardiac mesoderm as well as the overlaying ectoderm and functions primarily in cardiogenesis. Embryos lacking pannier (pn) show a dramatic reduction of cardiac progenitor cells (Gajewski et al., 1999; Alvarez et al., 2003; Klinedinst and Bodmer, 2003). In vertebrates, GATA4, GATA5 and GATA6 are expressed in the
cardiogenic region. Loss-of-function assays in mouse, *Xenopus* and zebrafish have shown that these GATA factors are required for myocardial differentiation and normal heart development (Molkentin et al., 1997; Gove et al., 1997; Reiter et al., 1999). Another *Drosophila* GATA factor Serpent (Srp) functions mainly in hematopoiesis. It is expressed in all hematopoietic progenitors formed in the head mesoderm and the lymph gland. In serotonin (srp) mutant embryos, hematopoiesis from both the head mesoderm and the lymph gland is inhibited (Lebestky et al., 2000; Mandal et al., 2004), indicating that Serpent plays an essential role in hematopoietic progenitor cell specification. In vertebrates, GATA1, GATA2 and GATA3 play fundamental roles in various aspects of hematopoietic development (Tsai et al., 1994; Ting et al., 1996; Ferreira et al., 2005). It is likely that the functions of Pannier and Serpent in cardiogenesis and hematopoiesis, respectively, reflect the highly conserved but simplified developmental processes in *Drosophila* compared with vertebrates.

Several transcription factors that are directly regulated by Tinman and Pannier have been identified, including *Mef2* and even-skipped, through enhancer mutagenesis studies (Gajewski et al., 1997; Gajewski et al., 1998; Nguyen and Xu, 1998; Knirr and Frasch, 2001; Han et al., 2002). These studies have begun to establish a transcriptional network that governs *Drosophila* cardiogenesis. In this network, Tinman and Pannier function in parallel as key cardiogenic factors at the top of the hierarchy. Although several transcription factors, such as Lozenge (Lz) and Glial-cells-missing (Gcm), appear to act ‘downstream’ of Serpent, there is as yet no evidence for direct activation of these genes by Serpent.

The *Drosophila* Hand gene encodes a highly conserved basic helix-loop-helix (bHLH) transcription factor. Interestingly, Hand is the only gene identified so far that is expressed in a specific pattern in all the cardioblasts, pericardial nephrocytes and hematopoietic progenitors in the lymph gland (Kolsh and Paululat, 2002). The vertebrate Hand genes have been shown to play essential roles during heart development (Srivastava et al., 1995; Srivastava et al., 1997; Hamburger et al., 1998; Yelon et al., 2000; Davidson and Levine, 2003). The conserved cardiac expression patterns of Hand genes across vertebrates, GATA1, GATA2 and GATA3 play fundamental roles during heart development (Srivastava et al., 1995; Srivastava et al., 1997; Hamburger et al., 1998; Yelon et al., 2000; Davidson and Levine, 2003). The conserved cardiac expression patterns of Hand genes across vertebrates suggest that these genes play conserved roles during cardiogenesis and may be regulated by conserved genetic pathways.

In an effort to understand the position of Hand in the genetic networks that govern cardiogenesis and hematopoiesis, we searched for and identified the cis-regulatory region of the *Drosophila* Hand gene. We describe a minimal Hand enhancer that completely recapitulates endogenous Hand expression in cardioblasts, pericardial nephrocytes and lymph gland prehemocytes. This enhancer contains consensus binding sites for the NK factor Tinman and the GATA factors Pannier and Serpent, which are conserved across evolutionarily divergent *Drosophila* species. Mutagenesis of these consensus binding sites shows that Hand is directly activated by Tinman and Pannier in the heart, and by Serpent in the lymph gland. Overexpression of Tinman, Pannier or Serpent induces ectopic Hand in muscle progenitors, dorsal vessel and hematopoietic progenitors, respectively, indicating that Hand is activated separately by Tinman, Pannier and Serpent in distinct cell types. These findings place Hand at a central position to link the transcriptional networks that govern cardiogenesis and hematopoiesis.

**Materials and methods**

**Drosophila strains**

The following mutant stocks were used: tin<sup>Ec40</sup> (Bodmer, 1993), pnr<sup>VX6</sup> (Ramain et al., 1993), srp<sup>neo5</sup> (the Bloomington stock center). Different *Drosophila* species were provided by the Tucson species center. Overexpression of transgenes was accomplished by using the Gal4-UAS system (Brand and Perrimon, 1993). The following fly lines were used: twi-Gal4; 24B-Gal4 (Greig and Akam, 1993), UAS-tin (Ranganayakulu et al., 1998), UAS-pnr (Gajewski et al., 1999), UAS-Srp (Wältli et al., 2002), UAS-TinEnR (Han et al., 2002), UAS-PnrEnR (Klinedinst and Bodmer, 2003). Oregon-R was used as the wild-type reference strain.

**Generation of transgenic fly lines**

The various Hand enhancer fragments (Fig. 2A) were PCR amplified and subcloned into pC4LZ (containing the lacZ reporter gene) or pPelicant (containing the GFP reporter gene) (Barolo et al., 2000), using SphI/XhoI or KpnI/NolI sites, respectively. The constructs were injected according to standard procedures. Germline transformed, transgenic flies were selected by red eye color (w<sup>+/+</sup>) and maintained as homozygotes. At least four independent transgenic lines were analyzed for each construct.

**Immunohistochemistry and microscopy**

Embryos from different lines were collected and stained with various antibodies as previously described (Han et al., 2002). The following primary antibodies were used: mouse anti-F-b-galactosidase 1:300 (Promega); rat anti-Even-1:200 (from D. Kosman); rabbit anti-Tinman 1:500 (from R. Bodmer); rabbit anti-Dmel2 1:1000 (from B. Peterson); rabbit anti-GFP 1:2000 (Abcam); and rabbit anti-Srp 1:500 (from R. Reuter). Cy2, Cy3, Cy5 or Biotin-conjugated secondary antibodies (from Jackson Lab) were used to recognize the primary antibodies. Images were obtained with a Zeiss LSM510-meta confocal microscope or a Leica DMRXE compound microscope.

**Electrophoretic mobility shift assays**

GST-Tin and GST-Pnr fusion proteins were prepared according to standard procedures. Complimentary oligonucleotides containing Tin or GATA consensus site were radio labeled using Klenow fill-in reaction as probes. Complimentary oligonucleotides containing wild-type consensus binding sites or binding-site mutations were used as non-labeled competitors to compete for the binding of GST fusion proteins in the presence of the radio-labeled probe. After 30 minutes incubation of the protein, probe and competitor oligonucleotides at 4°C, the products were electrophoresed in 7.5% non-denaturing polyacrylamide gels at 4°C. The sense strand DNA sequences of the oligonucleotides used are shown as follows with consensus binding sites in parentheses and mutated nucleotides underlined: Tin1, TTT CCA AAA AGG (CACTTAA) TTA ATC AAA CCC; Tin2: TTT CTG AAG CAC (CACTTAG) ACA CIT TGT TCT; Tin3, TTT CCT ATG AAG (TCAAGTG) CIT TTT TTT CCT; Tin4/G5: ATA ATA AAC AAA (CACATTGA) (GATA) TCT ACG GCC CAG; G1, CTT CTG GC CAG (CACATTGA) ACA CIT TGT TCT; Tin5, CCT TTT ATG AAG (CCTCGATTG) CTT TTT CCT; Tin6, ATA ATA AAC AAA (CATTCCA) (GATA) TCT ACG CCC CAG; G1m, CTT CTG TGT TCA (TCCC) TAA AAC CAG
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ATT; G2m, GCG TCT GCG GTT (TCCC) ACT TCC GAA ATT; G3m, CCA TTA GGA ATA (TCCC) TAC AAT CAA TCG; G4m, CAA TCG AGT TTT (TCCC) TGC GGA TTA CAA; G5m, ATA ATA AAC AAA (CAATTGA) (GGGA) TCT ACG CCC CAG.

Transfection assays

Cell transfection and luciferase assays were performed as described (Han et al., 2004). Reporter plasmid (100 ng) and 100 ng of each activator plasmid were used. The Hand-luciferase was generated by cloning the minimal Hand enhancer identified in this study into the pGL3 vector (Promega). Tin-pAc5.1, Pnr-pAc5.1 or SrpNC-pAc5.1 were generated by cloning the full length tin, pnr or srp cDNAs into the pAc5.1-HisA vector (Invitrogen), respectively. Luciferase activities are expressed as mean±s.d. from three experiments.

Results

Expression of Hand in cardioblasts, pericardial nephrocytes and lymph gland hematopoietic progenitors

We and others (Moore et al., 2000; Kolsh and Paululat, 2002) have identified the Drosophila Hand gene by homology to its vertebrate orthologs. Similar to its vertebrate orthologs, Drosophila Hand is expressed in a specific pattern in the cardiogenic mesoderm. Hand expression is initiated in the cardiogenic region at late stage 12, immediately following the differentiation of Even-skipped (Eve)-positive mesodermal progenitors into segmentally repeated Eve pericardial cells (EPCs) and DA1 muscles, which marks the completion of progenitor cell divisions that give rise to the cardioblasts and pericardial nephrocytes (Han and Bodmer, 2003). Cardiac expression of Hand is initially weak and segmental, but soon becomes strong in most cardioblasts and pericardial cells from stage 13 (Fig. 1A-C). At the end of embryogenesis, when the heart is completely formed, Hand is expressed in all the cardioblasts that also express Dmef2 (Fig. 1D-F) and in all the pericardial nephrocytes that express even-skipped (eve) (Fig. 1D-F).

At stage 15, tin is expressed in four of the six cardioblasts in each hemisegment from segment A1 to A5, and all the Eve-positive pericardial cells, as well as all cardioblasts in from segment T2 to T3, but not in the lymph gland (Fig. 1G). Hand expression is detected in all the Tinman-positive cardiac cells (Fig. 1H). Hand is likely to be expressed in all the pericardial nephrocytes as all Zfh-1-positive pericardial cells express Hand (Fig. 1I). odd-skipped (odd) is expressed in both the lymph gland hematopoietic progenitor cells and a subset of pericardial nephrocytes (Fig. 1J). Hand expression is also detected in all the Odd-skipped-positive hematopoietic progenitors and pericardial nephrocytes (Fig. 1K). In addition, Hand is co-expressed with Serpent in all the lymph gland progenitors (Fig. 1L). The secreted extracellular protein Pericardin (Prc) labels the ring gland and the extracellular matrix surrounding the pericardial nephrocytes (Fig. 1M). Hand expression is not detected in the ring gland, but Hand-
expressing cells are surrounded by Prc from segment T2-A6 (Fig. 1N-O). Hand expression also appears in the visceral mesoderm (Fig. 1A-C, data not shown), the garland cells (data not shown) and in a subset of central nervous system cells (data not shown).

**Cardiac and hematopoietic expression of Hand is controlled by a 513bp enhancer**

To search for cis-regulatory elements capable of conferring the specific expression pattern of Hand in cardioblasts, pericardial nephrocytes and lymph gland hematopoietic progenitors, we generated a series of reporter genes containing lacZ and the hsp70 basal promoter linked to genomic fragments within a 13 kb genomic region encompassing the gene and examined reporter gene expression in transgenic embryos. As shown in Fig. 2A, we identified a 513 bp minimal enhancer, referred to as Hand cardiac and hematopoietic (HCH) enhancer, between exons 3 and 4 of the Hand gene (see Fig. 2B for the sequence), which was both necessary and sufficient to direct lacZ expression in the entire embryonic heart and lymph gland in a pattern identical to that of the endogenous Hand gene (Fig. 2C, parts a-c). Further deletions of this enhancer caused either a partial or complete loss of activity (data not shown). The 513 bp HCH enhancer showed the same expression pattern in the heart and lymph gland as larger genomic fragments that were positive for enhancer activity (data not shown). We conclude that this enhancer fully recapitulates the temporal and spatial expression pattern of Hand transcription in the distinct cell types derived from the cardiogenic region.

Replacing the lacZ reporter gene with a GFP reporter made it possible to examine HCH enhancer activity after embryogenesis. The HCH-GFP is expressed in embryos in the...
same pattern as Hand transcripts (Fig. 2C, part d). After embryogenesis, the enhancer activity remains strong in the lymph gland, cardioblasts and pericardial nephrocytes in larvae (Fig. 2C, part e), and GFP expression persists in the heart throughout the Drosophila life cycle (data not shown).

**Conservation of the Hand enhancer among different Drosophila species**

As Hand expression in the heart is conserved from Drosophila to humans, we reasoned that possible evolutionary conservation of cis-regulatory sequences in the minimal HCH enhancer could guide us towards the identification of upstream transcriptional activators of Hand transcription. We therefore searched for the Hand enhancer sequence in other Drosophila species, taking advantage of the fact that the enhancer is located between two conserved exons, which allowed us to perform PCR of genomic DNA using a series of nested primers. Sequence alignment of genomic DNA obtained by PCR from five other Drosophila species – D. sechellia, D. yakuba, D. erecta and D. virilis (in order of increasing evolutionary distance from D. melanogaster) showed that the sequence of the HCH enhancer was conserved in these different species, whereas the surrounding sequence was divergent. Alignment of the sequences with homology to the HCH enhancer revealed four consensus sequences for binding of Tinman (CAC/ATTNA/G) and five potential GATA (GATAA/T) binding sites (Fig. 3A, see Fig. S1 for sequence and alignments). The spacing between these consensus sequences is similar from D. melanogaster to D. erecta, except that one of the five GATA sites is not conserved in D. erecta, indicating possible redundancy of the consensus binding sites. In D. virilis, although the spacing and order of the consensus sites are different from that of D. melanogaster because of variable intervening sequences, the number of consensus sites is the same as that of D. melanogaster, suggesting the importance of these consensus-binding sites.

To examine if the HCH enhancer-like sequence of other Drosophila species could confer cardiac and lymph gland expression of Hand, we tested the D. virilis HCH enhancer sequence (vir-HCH) because it is the most evolutionarily divergent Hand enhancer sequence we identified. The vir-HCH enhancer sequence directed GFP expression in an identical pattern to that of the D. melanogaster Hand enhancer (Fig. 3B, parts a-d). These findings suggest that the regulation of Hand expression in the heart and lymph gland is evolutionarily conserved among Drosophila species that diversified ~60 million years ago.

**Tinman, Pannier and Serpent bind directly to the consensus sites in the HCH enhancer**

To test for binding of Tinman protein to the Tinman consensus-binding sites in the HCH enhancer, we performed gel mobility shift assays with GST-Tinman fusion protein and a radiolabeled probe corresponding to first Tinman consensus site (Tin1). GST-Tinman bound avidly to this site, and binding could be competed by unlabeled oligonucleotides corresponding to any of the Tinman consensus-binding sites in the HCH enhancer (Fig. 4A). We then tested whether the GATA factor Pannier could bind to the GATA consensus sites. By using a radiolabeled probe containing the second GATA consensus site (G2), we found that GST-Pannier fusion protein could bind this probe, and binding could be competed by unlabeled oligonucleotides corresponding to any of the GATA consensus-sites in the HCH enhancer (Fig. 4B). Next, we tested whether the same GATA consensus sites could be bound by the hematopoietic GATA factor Serpent. As expected, both forms of the Serpent protein (SrpnC and SrpC) could bind to the radiolabeled probe containing the second GATA consensus site (G2), and the binding could be competed by any of the unlabeled GATA consensus-sites in the HCH enhancer (Fig. 4C). Mutation of the Tinman and GATA consensus-sites severely diminished their ability to compete for binding of the corresponding proteins to the labeled probes (Fig. 4A-C). As not all of these consensus-binding sites are conserved in all the Drosophila species, but they were all bound by the corresponding proteins, it is likely that some of the NK and GATA consensus-binding sites are functionally redundant.

**Activation of the HCH enhancer by Tinman, Pannier and Serpent in Drosophila S2 cells**

To examine if the HCH enhancer could be activated by Tinman and GATA factors in vitro, we generated a luciferase reporter construct using the HCH enhancer and tested it in Drosophila S2 cells. Remarkably, Tinman was able to activate this enhancer over 100-fold, whereas Pannier and Serpent activated the enhancer approximately sixfold (Fig. 4D). Although previous studies suggested that Tinman and Pannier function synergistically to activate cardiac gene expression (Gajewski et al., 1998), we did not detect significant synergy between these factors on the HCH enhancer when transfected simultaneously (Fig. 4D).
In order to show that the activation occurred specifically through binding of the three transcription factors to their consensus-binding sites, we mutated the Tinman and GATA-binding sites in the HCH enhancer. Tinman could still activate the HCH enhancer with all the GATA-binding sites mutated, but could not activate the enhancer with all the Tinman-binding sites mutated, whereas Pannier or Serpent could activate the enhancer with the Tinman-binding sites mutated, but not with the GATA-binding sites mutated (Fig. 4D). An enhancer with both Tinman- and GATA-binding sites mutated could not be activated by either Tinman, Pannier or Serpent (Fig. 4D). These results further support the conclusion that the HCH enhancer is a direct transcriptional target of Tinman, Pannier and Serpent.

**Ectopic expression of Tinman, Pannier and Serpent induces distinct expansion of Hand expression**

To further investigate the potential of Tinman, Pannier and Serpent to activate the HCH enhancer, we overexpressed these three transcription factors in the mesoderm of Drosophila embryos and examined the expression of the HCH-GFP reporter. Surprisingly, ectopic expression of Tinman in the mesoderm using the twi-Gal4; 24B-Gal4 driver strongly induced GFP expression in all the somatic muscles, in a pattern nearly identical to that of Mef2 (Fig. 5D-F). Interestingly, ectopic Tinman did not cause any significant over-proliferation of cardioblasts or pericardial nephrocytes (Fig. 5D-F), indicating that Tinman alone is not sufficient to induce cardiogenesis. We did not detect an inhibitory role of Tinman on lymph gland progenitor development as was shown in a previous study (Mandel et al., 2004), probably owing to different experimental conditions.

In contrast to Tinman, Pannier overexpression in the mesoderm using the same Gal4 driver induced the formation of ectopic Mef2-positive cardioblasts (indicated by arrows in Fig. 5H,I), as shown in a previous study (Klinedinst and Bodmer, 2003). Ectopic expression of HCH-GFP was also detected in all the extra cardioblasts (Fig. 5G-I). The expanded HCH-GFP pattern also showed that more pericardial nephrocytes were induced by ectopic Pannier (indicated by the arrowhead in Fig. 5G-I). We did not detect supernumerary Eve-positive pericardial cells (Fig. 5I), but Odd-positive pericardial cells were significantly increased (data not shown). Although ectopic expression of HCH-GFP was detected randomly in a few muscle cells, this effect was insignificant compared with the ectopic HCH-GFP expression induced by Tinman. We did not detect an expansion of the lymph gland when Pannier was overexpressed in the mesoderm.

Unlike Tinman or Pannier, ectopic Serpent driven by twi-Gal4; 24B-Gal4 did not induce any cardioblasts or pericardial
nephrocytes, but instead repressed their formation (indicated by arrows in Fig. 5J-L). By contrast, cell clusters forming the lymph gland (identified by their position, shape and Hand-GFP expression) were significantly expanded by ectopic Serpent (indicated by arrowheads in Fig. 5J-L). Furthermore, in embryos with ectopic mesodermal Serpent, pericardial nephrocytes around the aorta and heart often failed to align along the dorsal vessel, but formed cell clusters like hematopoietic progenitors in the lymph gland (Fig. 5J-L), suggesting a cell fate transformation from pericardial nephrocytes to hematopoietic progenitors. A gain-of-function study of Srp using a mef2-Gal4 driver showed similar results with Odd as a marker (Mandal et al., 2004).

Tinman, Pannier and Serpent are required for HCH enhancer activity in different tissues

As ectopic expression of Tinman, Pannier and Serpent caused distinct changes of Hand expression, we sought to determine whether Tinman, Pannier or Serpent were required in different cell types for Hand expression. A previous study has suggested the important role of Tinman and Pannier in specification of cardiogenic and hematopoietic progenitors (Mandal et al., 2004). Here, we examined HCH-GFP expression in mutants of tin, pnr and srp (Fig. 6B-D), by using a balancer chromosome containing actin-GFP and aging collected embryos for 24 hours before observation for easy identification of homozygous embryos or larvae. In tin-null mutants (tinEC40), the lymph gland and heart fail to form and no HCH-GFP expression was detected (Fig. 6B), indicating that Tinman is required for the specification of the progenitor cells for both cardiogenesis and hematopoiesis. In the pnr-null mutant (pnrVX56), HCH-GFP expression was only detectable in a few cells that could be the remnants of heart and lymph gland that are not formed normally (Fig. 6C), indicating that Pannier is required for the majority of cardiac and hematopoietic cells to form. By contrast, in an allele of serpent (srp neo45) that specifically abolishes Srp expression in hemocyte precursors, most of the cardioblasts and pericardial nephrocytes formed, but HCH-GFP expression was no longer detected in the lymph gland (Fig. 6D), indicating that the HCH enhancer activity is dependent on Srp in this cell type.

As the dependence of the HCH enhancer on Tinman and Pannier could result secondarily from defects in tin or panner mutant embryos, we established a system to test the requirement of Tinman and Pannier for activation of Hand expression specifically in the cells that express Hand. Using the HCH enhancer, we generated HCH-Gal4 transgenic flies, which could drive a UAS-GFP reporter in a pattern identical to that of the endogenous Hand gene (data not shown). We then overexpressed dominant-negative forms of Tinman or Pannier specifically in the Hand-expressing cardiac and hematopoietic cells using the HCH-Gal4 driver. The dominant-negative forms of Tinman (Tin-EnR) or Pannier (Pnr-EnR) were made by fusing the Engrailed repression domain (EnR) to the Tinman or Pannier DNA-binding domain (Han et al., 2002; Klinedinst and Bodmer, 2003). Overexpression of Tin-EnR in the Hand-expressing cells nearly abolished HCH-GFP expression in cardioblasts and pericardial nephrocytes, and also reduced HCH-GFP in the lymph gland but less dramatically (Fig. 6F), indicating that dominant-negative Tinman can suppress HCH activity more efficiently in cardiac cells than in hematopoietic cells. Overexpression of Pnr-EnR in the Hand-expressing cells using HCH-Gal4 abolished most of the HCH activity in the heart and lymph gland (Fig. 6G), indicating that dominant-negative Pannier is able to suppress HCH activity efficiently in both heart and lymph gland, probably by competing with both endogenous Pannier and Serpent for binding to the HCH enhancer. Ectopic expression of Tin-EnR or Pnr-EnR in the
Fig. 6. Tinman, Pannier and Serpent are required for HCH enhancer activity during development. (A) The HCH-GFP is expressed in the lymph gland (arrow) and the heart (arrowhead) in the first-instar larva. (B) In homozygous tin mutant larvae, the lymph gland and heart fail to form and no HCH-GFP is detected. (C) Only residual activity of the HCH enhancer is detected in homozygous pannier mutant larvae, in which no lymph gland is formed (indicated by arrow) and the few surviving cardiac cells fail to fuse at the dorsal midline (indicated by arrowheads). (D) In homozygous serpent mutant larvae, the lymph gland does not form (indicated by the arrow), but most cardiac cells form and express HCH-GFP (indicated by arrowhead). (E) In first-instar larvae that express a dominant-negative form of Tinman in the Hand-expressing cells using HCH-Gal4 driver, HCH-GFP expression is dramatically suppressed in the heart (arrowhead), and less dramatically suppressed in the lymph gland (arrow). (F) Overexpression of a dominant-negative form of Pannier in the Hand-expressing cells using the HCH-Gal4 driver dramatically suppressed the HCH-GFP expression in both the heart (arrowhead) and the lymph gland (arrow). All panels are dorsal view of embryos/ larvae carrying the HCH-GFP with anterior towards the left.

*Hand*-expressing cells did not ablate these cells in the embryos but rather appeared to induce some kind of cell fate changes that we are currently investigating (data not shown).

**Functional analysis of Tinman and GATA consensus-binding sites in the HCH enhancer**

To further assess the potential importance of Tinman and GATA factors for activation of the Hand enhancer in vivo, we generated transgenic flies carrying the HCH enhancer with various combinations of binding site mutations. Mutation of any single Tinman or GATA consensus-binding site, or combination of single Tinman site mutations and single GATA site mutations, did not alter enhancer activity (data not shown), suggesting that the enhancer is robustly activated through redundant Tinman and GATA sites. Therefore, we mutated all four Tinman consensus-binding sites simultaneously and examined enhancer activity in transgenic flies. This mutant enhancer (HCH-4T) retained the ability to direct expression of GFP in a majority of cardiac cells and all the lymph gland cells (Fig. 7D-F). However, the overall GFP expression level was reduced compared with the wild-type HCH enhancer (comparing Fig. 7E with Fig. 7B). HCH-4T-GFP expression was also frequently missing or dramatically reduced in the Tinman-positive cardioblasts (indicated by parallel arrows in Fig. 7D-F) and pericardial nephrocytes (indicated by joined arrows in Fig. 7D-F). These data indicate that the direct binding of Tinman to this enhancer is required for its full activity and the Tinman consensus-binding sites are more crucial for mediating enhancer activity in the cells with higher expression levels of Tinman. These data also suggest that activation of this enhancer by other factors can support its activity at a reduced level in most of the heart and at normal level in the lymph gland.

To examine the in vivo function of the GATA consensus-binding sites, we generated transgenic flies carrying the HCH enhancer with all five GATA sites mutated (HCH-5G). Interestingly, this mutant enhancer activated GFP expression only in Tinman-positive cardiac cells (Fig. 7G-I). The expression pattern of HCH-5G-GFP was almost identical to that of Tinman (Fig. 7G). The level of GFP expression in these Tinman-positive cardioblasts and pericardial nephrocytes (Fig. 7H-J) was the same as that of the wild-type HCH enhancer (compare Fig. 7H with Fig. 7B). The absence of HCH-5G-GFP activity in the Tinman-negative cardioblasts and pericardial nephrocytes indicates that the binding of Pannier to the consensus GATA sites is necessary to activate Hand expression in Tinman-negative cardioblasts and pericardial cells. However, the absence of the HCH-5G-GFP in the lymph gland hematopoietic progenitors (Fig. 7G) suggests that the binding of Serpent to the consensus GATA-binding sites is required for Hand expression in the lymph gland hematopoietic progenitors.

In order to test whether the Tinman and GATA sites are necessary for all the expression of Hand in the cardioblasts, pericardial nephrocytes and lymph gland hematopoietic progenitors, we created a mutant HCH enhancer with all the four Tinman-binding sites and five GATA-binding sites mutated. This enhancer, HCE-4T5G, was completely devoid of activity in cardioblasts, pericardial nephrocytes and the lymph gland (Fig. 7J-L), demonstrating that the activation of Hand in these three closely linked cell types is absolutely dependent on the binding of Tinman, Pannier and Serpent to the Hand cardiac and hematopoietic (HCH) enhancer.

**Discussion**

**Regulation of Hand expression in the heart and lymph gland**

In this study, we have identified a 513 bp minimal Hand cardiac and hematopoietic (HCH) enhancer that is necessary and sufficient to drive reporter expression in cardiac cells and lymph gland hematopoietic progenitors. This enhancer contains conserved consensus binding sites for the NK factor Tinman and the GATA factors Pannier and Serpent, which bind and directly activate this enhancer.
Fig. 7. Requirement of Tinman and GATA sites for activity of the HCH enhancer during development. (A–C) The wild-type HCH enhancer drives GFP expression strongly in the lymph gland hematopoietic progenitors (indicated by arrowhead in A), cardioblasts (indicated by arrowheads in B and C, labeled in C by Mef2 antibody in blue) and the pericardial nephrocytes (arrows in B and C indicate a subset of pericardial nephrocytes labeled in C by Eve antibody in red). (D–F) The HCH enhancer with all four Tinman binding sites mutated (HCH-4T) drives GFP expression in a similar pattern to the wild-type HCH, but at a lower level. The activity of this enhancer is not affected in the lymph gland (indicated by the arrowhead in D), but is frequently missing from the Tinman-positive cardioblasts (parallel arrows in D–F; the four Tinman-positive cardioblasts in each hemisegment come from a common progenitor cell), as well as Tin/Eve-positive pericardial nephrocytes (joined arrows in D–F; two Tin/Eve-positive pericardial cells are formed in each hemisegment). (G–I) The HCH enhancer with all five GATA-binding sites mutated (HCH-5G) fails to drive GFP expression in the lymph gland (indicated by arrowhead in G). In contrast to HCH-4T-GFP, HCH-5G-GFP is expressed in an identical pattern to that of Tinman (shown by Tinman antibody in red, which totally overlaps with the HCH-5G-GFP pattern in green). (H,I) Higher magnified panels co-labeled with Mef2 in blue and Eve in red show that HCH-5G-GFP is only expressed in four out of six cardioblasts (parallel arrows) and two Eve pericardial cells (joined arrows) in each hemisegment. (J–L) Mutation of both Tinman and GATA-binding sites totally abolishes HCH enhancer activity in the lymph gland, cardioblasts and pericardial nephrocytes. Each row of panels shows a different enhancer activity in green as indicated. The left column (A,D,G,J) shows dorsal views of stage 15 embryos carrying the enhancer-GFP (green) and labeled by Tinman antibody in red. The right two columns are dorsal/lateral views of three hemisegments of stage 14 embryos carrying different enhancer GFP (green) and co-labeled with DMe2 antibody (blue) and Eve antibody (red). Anterior is towards the left in all panels.

The homeobox-containing protein Tinman is essential for the formation of the cardiac mesoderm, from which the heart and blood progenitors arise (Bodmer, 1993). However, its potential late functions remain unknown. It is believed that Tinman is not required for the entirety of heart development in flies, because it is not maintained in all the cardiac cells at late stages. Our data reveal at least one function for the late-embryonic Tinman expression, which is to maintain Hand expression. The fact that ectopic Tinman can turn on Hand expression dramatically in the somatic muscles is striking and suggests the existence of a Tinman-co-factor in muscle cells that can cooperate with Tinman to activate Hand expression; this co-factor would not be expected to be expressed in pericardial cells or the lymph gland. This co-factor should also be expressed in Drosophila S2 cells, as transfected Tinman can increase activity of the HCH S2 enhancer in S2 cells by more than 100-fold. The generally reduced activity of the HCH enhancer that results from mutation of the Tinman-binding sites also suggests that Tinman activity is required to fully activate the Hand enhancer.

Although Pannier and Serpent bind to the same consensus sites, these GATA factors produce distinct phenotypes when overexpressed in the mesoderm. Ectopic Pannier induces cardiogenesis, shown by the extra number of cardioblasts and pericardial nephrocytes, but does not affect the lymph gland hematopoietic progenitors. Ectopic Serpent, however, induces ectopic lymph gland hematopoietic progenitors, but reduces the number of cardioblasts and pericardial cells. Interestingly, pericardial cells with ectopic Serpent expression have a tendency to form cell clusters such as the lymph gland progenitors, suggesting a partial cell fate transformation. These results suggest that Pannier functions as a cardiogenic factor, whereas Serpent functions as a hematopoietic factor. Although both can activate Hand expression, Pannier and Serpent activate the HCH enhancer in different cell types. This assumption is also supported by the specific expression pattern of Serpent and Pannier in late embryos. We and others (Mandel et al., 2004) have detected Serpent specifically in the lymph gland hematopoietic progenitors but not in any cardiac cells. Pannier expression in the cardiogenic region of late embryos is not clear because of the interference by the high level Pannier expression from the overlying ectoderm. However, we examined the lymph gland in late stage embryos and did not detect any Pannier expression in these cells (data not shown). Together with the evidence from loss-of-function and gain-of-function experiments with Serpent, we conclude that the HCH-5G-GFP transgene is not expressed in the lymph gland because Serpent could not bind to the mutant enhancer in the lymph gland cells; whereas the lack of HCH-5G-GFP expression in cardiac cells is due to the inability of Pannier to bind the mutant enhancer in these cardiac cells.

As tin and pnr are not expressed in all the cardiac cells of late stage embryos but the Hand-GFP transgene is expressed in these cells, it is likely that additional factors control Hand.
expression in the heart. One group of candidates is the T-box family. As Doc1, Doc2 and Doc3 genes (Drosophila orthologs to vertebrate Tbx5) are expressed in the Svp-positive cardioblasts where tin is not expressed (Lo and Frasch, 2001), but H15 and midline (Drosophila orthologs to vertebrate Tbx11) are expressed in most of the cardiac cells in late embryos (Miskolczi-McCallum et al., 2005; Qian et al., 2005), it is likely that the T-box genes activate Hand expression in cells that do not express tin and pannier. However, the enhancer lacking GATA and Tinman sites has no activity, indicating that the additional factors that may activate Hand expression in the heart and lymph gland also requires these crucial Tinman and GATA sites, probably through protein interaction between Tinman and the GATA factors.

**Evolution of the HCH enhancer**

We have identified putative Hand enhancers from divergent Drosophila species. In most of these species, the entire 513 bp Hand enhancer region is highly conserved. However, the D. virilis HCH enhancer does not exhibit highly conserved sequence between the consensus binding sites, even though it has a similar number of consensus binding sites for both Tinman and Pannier. The fact that this D. virilis enhancer can also drive reporter gene expression in the heart indicates that these Tinman and GATA-binding sites are the crucial elements for enhancer activity. Besides the enhancers with all Tinman or all GATA binding sites mutated, we also generated transgenic flies carrying one or two mutations of the Tinman or GATA-binding sites. None of these transgenic lines shows significant changes in enhancer activity (data not shown), indicating that this enhancer is robustly activated by Tinman, Pannier and Serpent through functionally redundant binding sites. These data also explain why the Hand enhancers from different Drosophila species have different numbers of Tinman or GATA-binding sites.

Interestingly, Hand expression is also dependent on GATA factors in vertebrates. We have previously described an enhancer necessary and sufficient to direct cardiac expression of the mouse Hand2 gene, which contains two essential GATA-binding sites (McFadden et al., 2000). Thus, we propose that the Hand genes are directly regulated by GATA factors in an evolutionarily conserved developmental pathway in both Drosophila and mice. Although no functional NK binding sites were identified in the mouse Hand2 enhancer, there are perfectly matched NK consensus sites in the Hand2 locus that may function in a redundant or refined way to regulate Hand2 expression (Z.H. and E.N.O., unpublished).

**Identification of Hand as a common target of transcriptional cascades that govern cardiogenesis and hematopoiesis**

In mammals, the adult hematopoietic system originates from the yolk sac and the intra-embryonic aorta-gonad-mesonephros (AGM) region (Medvinsky and Dzierzak, 1996). The AGM region is derived from the mesodermal germ layer of the embryo in close association with the vasculature. Indeed, the idea of the hemangioblast, a common mesodermal precursor cell for the hematopoietic and endothelial lineages, was proposed nearly 100 years ago without clear in vivo evidence. Recently, this idea was substantiated by the identification of a single progenitor cell that can divide into a hematopoietic progenitor cell in the lymph gland and a cardioblast cell in the dorsal vessel in Drosophila (Mandal et al., 2004). In addition to providing the first evidence for the existence of the hemangioblast, this finding also suggested a close relationship between the Drosophila cardiac mesoderm, which gives rise to cardioblasts, pericardial nephrocytes and pre-hemocytes, and the mammalian cardiogenic and AGM region, which gives rise to the vasculature (including cardiomyocytes), the excretory systems (including nephrocytes) as well as adult hematopoietic stem cells (Evans et al., 2003). In fact, in both Drosophila and mammals, the specification of the cardiogenic and AGM region requires the input of Bmp, Wnt and Fgf signaling (Cripps and Olson, 2002; Evans et al., 2003). In addition to the conserved role of the NK and GATA factors, GATA co-factors (U-shaped in Drosophila and Fog in mice) also play important roles in...
cardiogenesis and hematopoiesis in both Drosophila and mammals (Fossett et al., 2001; Sorrentino et al., 2005). Recent studies have shown that the Notch pathway is required for both cardiogenic and hematopoietic progenitor specification in Drosophila (Han and Bodmer, 2003; Mandel et al., 2004), as well as for mammalian embryonic vascular development (Fischer et al., 2004). It is likely that Notch also plays an important role in mammalian hematopoiesis.

In this study, we found that Drosophila Hand is expressed in cardioblasts, pericardial nephrocytes and pre-hemocytes, and is directly regulated by conserved transcription factors (NK and GATA factors) that control both cardiogenesis and hematopoiesis. The bHLH transcription factor Hand is highly conserved in both protein sequence and expression pattern in almost all organisms that have a cardiovascular system. In mammals, Hand1 is expressed at high levels in the lateral plate mesoderm, from which the cardiogenic region and the AGM region arise, in E9.5 mouse embryos (Firulli et al., 1998). Functional studies of Hand1 and Hand2 using knockout mice have demonstrated the essential role of Hand genes during cardiogenesis (Srivastava et al., 1995; Srivastava et al., 1997; Yamagishi et al., 2001; McFadden et al., 2005), whereas the functional analysis of Hand genes during vertebrate hematopoiesis has not yet been explored. It will be interesting to determine whether mammalian Hand genes are also regulated in the AGM region by GATA1, GATA2 and GATA3 (vertebrate orthologs to Drosophila Serpent), and whether they play a role in mammalian hematopoiesis.

In summary, this study places Hand at a pivotal point to link the transcriptional networks that govern cardiogenesis and hematopoiesis, as shown in Fig. 8. As the Hand gene family encodes highly conserved bHLH transcription factors expressed in the cardiogenic region of widely divergent vertebrates and probably in the AGM region in mouse, these findings open an avenue for further exploration of the conserved transcriptional networks that govern both cardiogenesis and hematopoiesis, by studying the regulation and functions of Hand genes in vertebrate model systems.

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Supplementary material
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