The *Drosophila* homeobox genes *zfh-1* and *even-skipped* are required for cardiac-specific differentiation of a *numb*-dependent lineage decision

Ming-Tsan Su¹, Miki Fujioka², Tadaatsu Goto²,⁎ and Rolf Bodmer¹,‡

¹Department of Biology, University of Michigan, Ann Arbor, MI 48109-1048, USA
²Kimmel Cancer Institute, Thomas Jefferson University, 1020 Locust Street, Philadelphia, PA 19107, USA
*Dr Goto passed away January 31, 1997
†Author for correspondence (e-mail: rolf@umich.edu)

Accepted 30 April; published on WWW 21 June 1999

SUMMARY

A series of inductive signals are necessary to subdivide the mesoderm in order to allow the formation of the progenitor cells of the heart. Mesoderm-endogenous transcription factors, such as those encoded by *twist* and *tinman*, seem to cooperate with these signals to confer correct context and competence for a cardiac cell fate. Additional factors are likely to be required for the appropriate specification of individual cell types within the forming heart. Similar to *tinman*, the zinc finger- and homeobox-containing gene, *zfh-1*, is expressed in the early mesoderm and later in the forming heart, suggesting a possible role in heart development. Here, we show that *zfh-1* is specifically required for formation of the *even-skipped* (eve)-expressing subset of pericardial cells (EPCs), without affecting the formation of their siblings, the founders of a dorsal body wall muscle (DA1). In addition to *zfh-1*, mesodermal *eve* itself appears to be needed for correct EPC differentiation, possibly as a direct target of *zfh-1*. Epistasis experiments show that *zfh-1* specifies EPC development independently of *numb*, the lineage gene that controls DA1 founder versus EPC cell fate. We discuss the combinatorial control mechanisms that specify the EPC cell fate in a spatially precise pattern within the embryo.

Key words: *zfh-1*, *even-skipped*, *numb*, *Notch*, *spitz*, EGF receptor, Lineage, Cell fate, Zinc finger, Homeodomain, Heart, Pericardial cell, Myocardium, *Drosophila*, Mesoderm, Cardiogenesis, Myogenesis

INTRODUCTION

The *Drosophila* heart is linear and thus very different in appearance from the looped and chambered heart of vertebrates. By contrast, remarkable similarities are revealed when comparing embryonic origins and genes involved in cardiac specification (Bodmer, 1995; Harvey, 1996; Olson and Srivastava, 1996; Bodmer and Venkatesh, 1998). For example, both the homeobox gene *tinman* in *Drosophila*, as well as its counterparts Nkx2.5 and Nkx2.3 in vertebrates, have essential roles in heart development (Bodmer, 1993; Azizazu and Frasch, 1993; Lyons et al., 1995; Fu et al., 1998; Grow and Krieg, 1998; Tanaka et al., 1999). Moreover, vertebrate *tinman*-related genes can substitute, at least in part, for *tinman* function when expressed as transgenes in *Drosophila* (Park et al., 1998a; Ranganayakulu et al., 1998). Similarly, equivalent members of the TGF- family are involved in cardiac induction in both *Drosophila* and vertebrates (Staehling-Hampton et al., 1994; Frasch, 1995; Schultheiss et al., 1997). Thus, basic mechanisms of cardiogenesis may be considerably conserved between vertebrates and invertebrates.

The *Drosophila* heart consists only of a small number of cell types, thus well suited for studying the genetic basis of cell-type diversification during organogenesis. Two major cardiac cell types have been described (Rizki, 1978): the inner contractile (myo)cardiac cells form a central cavity, the lumen of the heart, whereas the outer, non-myogenic pericardial cells align alongside the cardiac cells. A subset of the pericardial cells contain enlarged nuclei and express the homeobox gene *even-skipped* (*eve*, Frasch et al., 1987). Recent advancements have provided a model of how heart development is initiated in *Drosophila* (for review see Bodmer et al., 1997; Bodmer and Frasch, 1999): The bHLH protein Twist and the Zinc finger protein Snail are essential for the mesoderm to form (Simpson, 1983; Thisse et al., 1988; Kosman et al., 1991; Leptin, 1991). Several potential downstream targets of *twist*, which also code for putative transcription factors, are expressed ubiquitously, as *twist*, in the early mesoderm and participate in mesoderm differentiation. These genes include *Dmef2* (Bour et al., 1995; Lilly et al., 1995), *tinman* (Bodmer et al., 1990), *heartless* (Shishido et al., 1993) and *zfh-1* (Lai et al., 1991). The early mesoderm flattens and spreads dorsally into a monolayer closely apposed to the ectoderm under the control of *heartless*, which codes for a FGF receptor (Shishido et al., 1993; Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997). Subsequently, the mesoderm subdivides into four major groups of cells with restricted cell fates: the somatic mesoderm, which forms the skeletal body wall muscles, the visceral mesoderm, which forms the gut muscles, the fatbody/gonadal mesoderm and the cardiac mesoderm, which forms the heart.
Studies of the spatial domains of gene expression indicate that the mesoderm is organized in parasegmental units similar to the ectoderm (Azpiazu et al., 1996; Riechmann et al., 1997). The early expression in transverse stripes of the pair-rule genes *even-skipped* (eve) and *sloppy-paired* (slp), as well as of the segment polarity genes *wingless* (wg) and *hedgehog* (hh), have been shown to be crucial for anteroposterior patterning of the developing mesoderm (Wu et al., 1995; Azpiazu et al., 1996; Riechmann et al., 1997; for review see Bodmer and Frasch, 1999). By contrast, the TGF- factor encoded by *dpp* and the homeobox gene *tinman* (at stage 9/10) are expressed perpendicularly to these four genes in a broad dorsal domain along the anteroposterior axis. *Dpp* is secreted from the dorsal ectoderm and necessary to maintain *tinman* expression in the mesoderm. Both are required for subdividing the mesoderm and for specifying dorsal mesodermal cell fates, including the heart (Azpiazu and Frasch, 1993; Bodmer, 1993; Frasch, 1999). Unlike *dpp* and *tinman*, which are essential not only for heart but also for visceral mesoderm formation, *wg* is needed for the cardiac (and some skeletal) but not the visceral mesodermal precursors (Wu et al., 1995; see also Baylles et al., 1995; Ranganayakulu et al., 1996). Thus, it has been postulated that the orthogonal overlap of *wg* and *dpp* signaling in the context of dorsal mesodermal *tinman* expression is sufficient for cardiac cell type specification (W. K. Lockwood and R. B., unpublished).

Because *wg*, *dpp* and *tinman* functions are necessary for all aspects of heart formation, they are unlikely to be sufficient for specifying the distinction between different cardiac cell types. Other spatially restricted cues and mesodermal context information, perhaps in conjunction with these three gene functions, must be involved in further cellular diversification of the heart. It has recently been shown that the lineage gene *numb*, which distinguishes between alternative cell fates during asymmetric cell divisions of the nervous system (Uemura et al., 1989; Rhyu et al., 1994), also acts within the mesoderm (Ruiz Gomez and Bate, 1997; Carmena et al., 1998a), including the heart (Park et al., 1998b).

A possible candidate for providing additional mesodermal context is the zinc finger- and homeobox-containing gene *zfh-1*, since it is as *tinman* widely expressed in the early mesoderm and later prominently in (the pericardial cells of) the forming heart tube (Lai et al., 1991; this study). Previous genetic studies showed that *zfh-1* is required for body wall muscle patterning and gonadal mesoderm (Lai et al., 1993; Brohieter et al., 1998; Moore et al., 1998). A vertebrate homolog of *zfh-1*, *DEFI*, is also involved in certain aspects of mesoderm differentiation (Takagi et al., 1998). In contrast to *zfh-1*, the homeobox gene *eve* (aside from its early metameric pattern at blastoderm) is expressed in a small subset of dorsal mesodermal cells within the cardiac primordium (Frasch et al., 1987). Two *eve*-expressing mesodermal progenitor cells per hemisegment undergo asymmetric divisions under the control of *numb* to give rise to a subset of pericardial cells (the EPCs) and the founder cells of the dorsal muscles (DA1) (Fig. 1A,C; Park et al., 1998b; see also Carmena et al., 1998a,b).

Here, we have studied the function of *zfh-1* and *eve* in cell type specification during heart development. In *zfh-1* mutants, the EPC subset of pericardial cells are selectively missing, apparently without affecting formation of the EPC progenitor cells or the *eve*-expressing DA1 muscles. The cardiac and the other pericardial cells of the heart do form, but the heart tube suffers from minor to moderate morphological abnormalities. Using a temperature-sensitive allele of *eve*, we show that *eve* function itself is required for EPC differentiation. In addition, *eve* overexpression in *zfh-1* mutants partially restores EPC formation, suggesting that *eve* acts downstream of *zfh-1*. Moreover, the *zfh-1* homeodomain binds in vitro to a *zfh-1* consensus binding site in the *eve* enhancer element, which drives expression specifically in the EPC progenitors and their progeny. Since the initial number of EPC progenitors is unchanged in *zfh-1* mutant embryos, we suggest that *zfh-1* is necessary to maintain *eve* expression and to promote differentiation of the EPC cell type. The phenotype of double mutant combinations of *zfh-1* and *numb* supports the idea that *zfh-1* does not take part in the alternative lineage decision controlled by *numb*, but rather contributes to the appropriate mesodermal context, along with *tinman*, for correct EPC differentiation. Therefore, a precise temporal and spatial combination of multiple positional and context cues are necessary for correct cell type specification.

MATERIALS AND METHODS

**Drosophila** stocks

The *Drosophila* mutants eveD1 (or *eve*1, a temperature-sensitive allele of *eve*, Nüsslein-Volhard et al., 1984), spitzDΔ (Mayer and Nüsslein-Volhard, 1988), zfh-12 (a protein null allele of *zfh-1*, Lai et al., 1993), DmedE2 (Bour et al., 1995), numbD (Rhyu et al., 1994; Park et al., 1998b), spdo227 (Skeath and Doe, 1998) and string785 (Edgar and O’Farrell, 1989) have been described. Overexpression of transgenes in the mesoderm was achieved by using the UAS/Gal4 described by Brand and Perrimon (1993). UAS-*eve* was obtained from N. Perrimon; UAS-*numb* (Yaiich et al., 1998), twist-Gal4 (Greig and Akam, 1993), and 24B-Gal4 (Brand and Perrimon, 1993) have been described. twist-Gal4 (transgene inserted on second chromosome) was also used in combination with 24B-Gal4 (transgene inserted on third chromosome) to achieve higher, more uniform and persistent mesodermal expression (data not shown). For identification of homozygous mutant embryos, we used balancer chromosomes that contain *wg-lacZ, abdA-lacZ* or *ftz-lacZ* transgenes.

**Generation of transgenic flies**

The fragment from +5.8 kb to +6.6 kb downstream of the *eve* coding region was inserted downstream of the tubulin poly(A) signal of a modified CaSpeR vector, C3D (Fujioika et al., 1996). Therefore, the relative position of the fragment to the promoter is similar to the in vivo situation (for more information, see Fujioika et al., 1999a). The *eve* enhancer-lacZ reporter construct (100 µg/ml) was injected along with a transposase-containing helper plasmid (50 µg/ml) into *yw;+;+;+* flies according to standard procedures (Spradling 1986) with some modifications (Fujioika et al., 1999b). The successful transgenic flies were selected by their red eye color (*w*+) and maintained as homozygotes.

**Immunocytochemistry and whole-mount embryo in situ hybridization**

Immunohistochemical staining and in situ hybridization were performed as described (Bodmer, 1993). Antibodies and dilutions were as follows: anti-Zfh-1 1:1000 (Lai et al., 1991), anti-*Eve* 1:5000 (Frasch et al., 1987), anti-Dmed-2 1:1000 (Lilly et al., 1995), pericardial cell surface specific anti-PC (mAb No. 3, Yamnitzky and Volk, 1995; see also Wu et al., 1995) 1:10, anti-β-galactosidase 1:4000 (Cappel Labs). Embryos were then double labeled with 5-bromo, 4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) and tissue-specific antibodies as
described in Su et al. (1998). Homozygous mutant embryos (ie lacking the balancer chromosome) were negative for lacZ expression. For double antibody staining, the same protocol by Su et al. (1998) was followed, except that during the color reaction the substrate for horse-radish peroxidase, dianaminobenzidine (Sigma), was used first (brown staining), followed by the Chromogen (Vector SG substrate kit) color reaction (blue-gray staining). For some antibody stainings, the ABC Elite kit (Vector labs) was used to enhance the signal according to the manufacturer’s instructions. Tissue sections of stained embryos were performed as described in Wu et al. (1995). Briefly, embryos were stained with antibodies, dehydrated in ethanol, embedded in OCT containing 20% sucrose and cut into 5-10 μm frozen sections. For in situ and antibody double stainings, a digoxigenin-labeled antisense lacZ mRNA probe was used in conjunction with anti-Eve antibodies according the protocol described in Mullen and DiNardo (1995). Embryos and sections were mounted in Fluoromount (Southern Biotechnology Associates) and examined with Nomarski optics.

temperature-shift experiments

Temperature-shift treatments were carried out as follows: eve^D embryos were collected on shallow grape agar plates at 1-2 hour intervals at 18°C and aged at 18°C. At the desired developmental stage (see text), embryo-containing plates were covered and submerged in a water bath at 29°C or 32°C for a period of 4 or 2 hours, respectively, with essentially the same result. The embryos were then aged further at 18°C at least until heart tube formation before they were fixed and stained with antibodies. Ages indicated in the text were adjusted for a standard at 25°C (two-fold slower development at 18°C and 1.4 times faster development at 29°C).

gel mobility shift assay

Sequence analysis of the 800 bp mesodermal eve enhancer (+5.8 kb to +6.6 kb) revealed that it contained a single RCS1 consensus site (CTAATYRRNNT, Y= C or T; R= G or A; Fortini et al., 1991; mismatch is doubly underlined). To test if the Zfh-1 homeodomain can specifically bind (in vitro) to the RCS1 site within the 800 bp enhancer, the following oligonucleotides (plus their inverse complement) were used in gel mobility shift assays: 5'-GCCCTGCTAATGGATCCCGG-3' contains the native RCS1 sequence (underlined; mismatch is doubly underlined); 5'-GCCTGCTAATGTCGGG-3' contains a perfect RCS1 sequence (underlined), but a mismatch in position 7 (phenylalanine to valine) is created. These probes were band-shifted by the Zfh-1 homeodomain in vitro and in vivo, as shown by electrophoretic mobility shift. The 5'-GCCCTGCTAATGGATCCCGG-3' contains a mutated RCS1 sequence (underlined) flanked by native sequence. Gel shifts with either the native or the perfect consensus RCS1-containing oligos gave similar results. Oligos were labeled with polynucleotide kinase and [γ-32P]ATP. The Zfh-1-homeodomain-GST fusion proteins were purified as described in Lai et al. (1991). Protein-DNA binding was carried out in 20 mM Hepes (pH 7.5), 50 mM KCl, 1 mM MgCl2, 1 mM DTT, 0.5 mg/ml of BSA, 5% NP-40, 100 mg/ml of poly[d(I-C)] for 20 minutes at 4°C. In the competition experiments, the wild- and mutant oligos were added to the reaction mixture 20 minutes before adding labeled oligos (a mutant oligo with only 4/11 consensus base pairs changed the same results, Z. Han and R. B., unpublished). The mixture was loaded onto a 7.5% non-denaturing acrylamide gel (29:1, polyacrylamide: bis-acrylamide) buffered in 0.5x TBE and electrophoresed at 180 V for 2 hours at 4°C.

Results

Cardiac phenotype of zfh-1 mutant embryos

Previous phenotypic analysis suggested that zfh-1 mutant embryos not only suffer from moderate abnormalities in skeletal muscle patterns but also from occasional kinks in the linear heart tube (Lai et al., 1993; see also Fig. 1E-H). In contrast, early steps in mesoderm differentiation, such as the somatic, visceral and cardiac mesoderm formation, appear to be normal in zfh-1 mutant embryos (Fig. 1A,B; data not shown), which suggests that zfh-1 is probably not required for the initial mesodermal subdivision and subtype specification, but perhaps for later aspects of differentiation, including the heart. Using cardiac-specific markers, we re-evaluated heart formation in zfh-1 mutants. We found that the EPC-specific eve expression is selectively missing without affecting the earlier pattern of mesodermal eve expression in Eve progenitors (Fig. 1A-D). In contrast, the number of other cardiac cell type markers is not significantly affected (Fig. 1E-H), and the assembly of the heart tube at the dorsal midline and the formation of somatic muscle fibers does occur, albeit with morphological defects (Fig. 1F,H; Lai et al., 1993). We do not know if the heart morphology defects are due to the missing EPCs or because of a direct effect of zfh-1 on the other cardiac cell types.

Mesodermal eve and Zfh-1 overlap in the early mesoderm but not in pericardial cells

The EPCs are localized within the bilateral rows of pericardial cells, where they are morphologically distinguishable because of their large nuclei (Fig. 2). Since at the heart tube forming stage (stage 14-15) zfh-1 is primarily expressed in pericardial cells, we wanted to know if cardiac Zfh-1 coincides with Eve in EPCs. Double-labeling for Eve protein and a pericardial cell-specific epitope (using anti-PC antibodies; see materials and methods) revealed that the EPCs are a subset of pericardial cells (Fig. 2C). Co-labeling for Eve and Dmef2, a marker of all contractile muscle cells including the cardiac cells, showed that the EPCs are aligned laterally to the cardiac cells but dorsally to the skeletal muscles (Fig. 2D). Finally, co-labeling for Eve and Zfh-1 showed that EPCs and the zfh-1-expressing cells are two distinct, non-overlapping subsets of pericardial cells (Fig. 2E). Therefore, it is unlikely that zfh-1 directs eve expression in the EPCs during these late stages of heart development.

In order to determine if zfh-1 expression at earlier stages coincides with eve expression in the cardiogenic region, we observed doubly labeled embryos at the stage when Eve is first expressed and zfh-1 is present in the entire trunk mesoderm (early stage 11). At that stage, eve and zfh-1 expression clearly overlap (Fig. 2F-H), which means that zfh-1 is probably required for EPC formation or for EPC-specific eve expression at the stage when the EPC progenitors first form.

EPC progenitor cells form normally in zfh-1 mutants

Since zfh-1 expression overlaps with that of eve in the EPC progenitor cells but not after the EPCs and DA1 muscles are distinguishable, we wanted to know if the number of EPC progenitor cells in the absence of cell division and muscle fusion is affected in zfh-1 mutants. For this purpose, we used string (stg) mutants, in which cell division but not the overall embryonic development is arrested at blastoderm (Edgar and O’Farrell, 1989). In stg mutants, as well as in stg; zfh-1 double mutants, the number of mesodermal Eve cells is similar, which is up to two cells per hemisegment (Fig. 3A,B). This suggests that zfh-1 is not required to specify the number of EPC progenitors but rather a later process that leads to EPC differentiation.

It is possible that, in zfh-1 mutants, the EPCs are transformed into muscle founder cells. In order to address this question, we used a Dmef2 mutant background in which muscle founders do
not fuse with surrounding myocytes (Fig. 3E; see also Bour et al., 1995). Examining embryos doubly mutant for \(zfh-1\) and \(Dmef2\) revealed that the number of EPC progenitors is unchanged compared to that of wild-type or single \(Dmef2\) mutants (Fig. 3C,D). At later stages, however, when EPCs and DA1 founders are distinct, we observe only one \(eve\)-expressing cell per hemisegment in the double mutant instead of three in the \(Dmef2\) single mutant (Fig. 3E,F). Therefore, it is unlikely that the EPCs are transformed into DA1 founders in \(zfh-1\) mutants. These data support the hypothesis that without a functional \(zfh-1\) gene, \(eve\) fails to be maintained in the differentiating EPCs.

**\(zfh-1\) is required for EPC differentiation independently of the numb-controlled EPC progenitor lineage**

It has previously been shown that \(numb\) controls alternative cell fate decisions during asymmetric cell divisions of the EPC progenitors, in which Numb protein is asymmetrically localized (Park et al., 1998b). In analogy to the situation in the nervous system (Uemura et al., 1989; Ryhu et al., 1994), the daughter cell that inherits Numb assumes the DA1 muscle founder fate whereas its sibling is destined for an EPC fate. Thus, in \(numb\) mutants, the number of EPCs is doubled and no DA1 muscles form (Fig. 4A,C; Park et al., 1998b). Conversely, no EPCs form in embryos where \(numb\) is overexpressed, or that are mutant for \(sanpodo\) (\(spdo\)), a tropomodulin-encoding gene that functions downstream of and opposite to \(numb\) (Dye et al., 1998; Skeath and Doe, 1998; Park et al., 1998b). This latter phenotype is similar to that of \(zfh-1\) mutants (Fig. 4B).

Although the \(Dmef2;\!zfh-1\) double mutant phenotype is consistent with the idea that \(zfh-1\) acts independently of the \(numb\)-controlled lineage decision, since EPCs are not transformed to a DA1 fate in \(zfh-1\) mutants (Fig. 3F), we wanted to determine directly whether or not \(zfh-1\) is associated with the genetic pathway controlled by \(numb\). If \(zfh-1\) acted downstream of \(numb\) in a linear pathway, we would expect a \(zfh-1\) phenotype in \(numb;\!zfh-1\) double mutants (as in Fig. 4B). Conversely, if \(numb\) were downstream, we would expect to see twice the number of EPCs as in \(numb\) single mutants (Fig. 4C).

Interestingly, the phenotype that we observe in \(numb;\!zfh-1\) double mutants was neither of the two (Fig. 4D): \(eve\) expression both in EPCs as well as in DA1 muscles is absent in these double mutant embryos. Only rarely are there one or two EPCs present (as in Fig. 4D). These findings strongly suggest that \(zfh-1\) is not a component of the \(numb\) pathway, at least not in the same way as \(spdo\) is (\(numb;\!spdo\) double mutants have a \(spdo\) phenotype; Park et al., 1998b; see also Dye et al., 1998; Skeath and Doe, 1998). Rather, \(zfh-1\) appears to be required for promoting EPC differentiation, once the \(numb\)-controlled lineage decision has been made. Thus, in the absence of \(numb\), the EPC progenitor division produces only daughter cells with an EPC fate. These prospective EPCs, however, seem to be unable to differentiate correctly in the absence of \(zfh-1\) function. Consistent with this interpretation is the finding that the number of EPC progenitors earlier on in development is normal in \(numb;\!zfh-1\) double mutants (data not shown). These findings suggest that \(zfh-1\) is essential for providing the appropriate mesodermal context for EPC differentiation.

**The EGF pathway is required for DA1 muscle differentiation independently of \(zfh-1\) and numb**

\(zfh-1\) and the components of the \(numb\) pathway are not the only factors required for specifying EPC or DA1 founder fates (or for...
A transcription factor encoded by the lethal-of-scute gene is expressed in a cluster of mesodermal cells out of which the EPC and other muscle progenitors emerge aided by a laterally inhibitory mechanism (Corbin et al., 1991; Carmena et al., 1995, 1998b; Park et al., 1998b). lethal-of-scute, however, as well as another transcription factor encoded by the Krüppel gene, which is expressed in the DA1 (and other muscle) founder cells, are only weakly required for the corresponding muscles to form (Carmena et al., 1995; Ruiz Gomez et al., 1997). In contrast, the Drosophila EGF signal transduction pathway plays an essential role in DA1 specification (Buff et al., 1998). For example, in the absence of the secreted EGF-receptor (DER) ligand spitz, the number of EPCs is normal but nearly all the DA1 muscles fail to form (Fig. 4E; see Buff et al., 1998 for a comprehensive analysis of the EGF pathway in DA1 formation).

Since DA1 founders and EPCs are likely to derive from common precursors and the phenotype of spi mutants is opposite to that of zfh-1 (compare Fig. 4B and E), we wanted to determine whether or not zfh-1 and spitz were part of a

Fig. 2. Localization of eve and zfh-1 expression during heart development. (B-E) Stage 15/16 embryos. (F-H) Stage 11 embryos. (A) Diagram of a late stage embryonic heart in Drosophila. EPC, eve-expressing gericardial cells; PC, pericardial cells; CC, myocardial cells; RG, ring gland; LG, lymph glands. (B,C) Localization of EPC-characteristic Eve nuclei (brown, indicated by arrowheads in C-E) within pericardial cells, marked with anti-PC (green-blue). (D) Localization of EPC (brown) adjacent to Dmef2-expressing myocardial cells (grey-blue). (D,E) Eve-containing nuclei of a DA1 muscle are indicated by a forked arrow. (E) Co-labeling of late stage embryos with anti-Eve (brown) and anti-Zfh-1 (grey-blue) shows no overlap within the same cell. The apparent coincidence of some (large) brown and (small) grey-blue nuclei is due to two the overlay of two focal planes. (F-H). At earlier stages, EPC progenitors (indicated by the arrows) co-express eve (brown) and zfh-1 (grey-blue) at the dorsal edge of the mesoderm. (H) Cross section of the double-stained embryos.

Fig. 3. EPC progenitor formation in the absence of post-blastoderm cell division or myoblast fusion. All embryos are stained for Eve protein. In both stg7869 single (A) and stg7869; zfh-12 double mutant embryo (B) a maximum of two EPC progenitors per hemisegment (arrows) are formed. Dmef-22520 single (C,E) and Dmef-22520; zfh-12 double mutant embryo (D,F). At stage 11, both single and double mutants (C,D) show a wild-type number of eve-expressing nuclei (arrows; see Fig. 1A). At later stages, two EPCs (arrowheads) and one DA1 founder cell (arrows) forms per hemisegment in Dmef-2 single mutants (E). In late Dmef-2; zfh-1 double mutants, the DA1 founder differentiates but not the EPCs (arrows).
common genetic pathway. In a similar epistasis experiment to that described above, we examined the phenotype of spitz;zfh-1 double mutants. In these double mutants, neither EPC- nor DA1-specific eve expression is present (Fig. 4F), suggesting that the DER pathway is required for DA1 differentiation independently of zfh-1. This raises the question whether or not DER pathway activation is required for providing the correct DA1 differentiation context in a way that is reminiscent of zfh-1 function in providing context for EPC differentiation. If yes, we would expect that spitz, like zfh-1, functions independently of the numb pathway. Indeed, when numb is mesodermally overexpressed in spitz mutant embryos (spitz;UAS-numb;twist-Gal4;24B-Gal4), we observe a phenotype similar to that of spitz;zfh-1 double mutants, in that neither EPC- and nor DA1-specific eve expression is observed (Fig. 4G). As is the case for either zfh-1, numb or spitz single mutants, the early eve expression in numb;zfh-1, spitz;zfh-1 and spitz;UAS-numb double mutant embryos appears unchanged (Fig. 4H; data not shown). Taken together, these results suggest that correct cell type-specific differentiation depends on both asymmetric segregation of cell fate determinants during cell division as well as on the appropriate regional context. In this case, the context information (zfh-1 or DER activity) does not need to be originating from a spatially localized source, but may act in concert with other mesodermal context determinants (e.g., tinman).

**eve function is required for EPC development**

eve is well known for its function in ectodermal segmentation. It has recently been shown that eve also participates in patterning of the early mesoderm (for review see Bodmer and Frasch, 1999). eve null mutants lack visceral and cardiac mesoderm altogether (Azpiazu et al., 1996). As shown above, zfh-1 is required for the formation and/or expression of eve in EPCs. Thus, mesodermal eve expression itself may be needed for correct EPC differentiation. To address this question, we used a temperature-sensitive allele of eve (eveD), which produces a non-functional but nevertheless antigenic protein at the non-permissive temperature (see Materials and methods). When eveD mutant embryos were shifted to the non-permissive temperature for 2 hours at early stage 11, the number of EPCs (expressing eveD) was drastically reduced (Fig. 5A,D,E): on average only 24% of EPCs were present as compared to wild type. DA1 muscle formation seems to be also affected, but to a lesser degree (data not shown). The EPC deficiency was less severe when the temperature shifts occurred earlier or later in development (Fig. 5B,C). Interestingly, some of the remaining EPCs in early stage 11 shifted embryos are located at some distance from the heart tube (Fig. 5A,D,E), suggesting that eve function during this critical time period is required for correct differentiation of the EPCs. Thus, in the absence of eve, the forming EPCs lose their association with the heart and disappear.

Temperature shifts during the temperature-sensitive period for EPC formation also affected the overall pericardial cell population (Fig. 5D,E), as seen in zfh-1 mutant embryos (Fig. 1F,H), perhaps due in part to the lack of EPCs. In contrast to the zfh-1 phenotype, however, the number of cardiac cells, heart tube formation and overall body muscle formation are not significantly affected in early stage 11 shifted eveD embryos (Fig. 5E), which is not surprising since eve is not expressed in these tissues during the critical period for EPC formation. Although eve inactivation at earlier stages also perturbs neural development (eg. RP2 neurons), in addition to visceral and somatic muscle formation (data not shown), stage 11 shifts show no morphologically detectable CNS defects (data not shown). We conclude that eve, in addition to zfh-1, is required for the proper differentiation of the EPCs at the time when the eve progenitors normally appear.

**Overexpression of eve restores EPC formation in zfh-1 mutant embryos**

Since zfh-1 is required for eve expression in the forming EPCs and eve function is required for EPC differentiation, we wondered if eve function were sufficient to promote EPC development in the absence of zfh-1. Mesodermal expression of eve may be autoregulated, as is the case for the eve late stripe expression element (Goto et al., 1989, Harding et al., 1989; Fujioka et al., 1995). Thus, eve expression may need to be activated at least until autoregulation is initiated. Again, we used the Gal4 system (Brand and Perrimon 1993) to ectopically express eve in the all mesoderm of zfh-1 mutant embryos until but not beyond stage 11. At stage 14/15, we then assayed for EPC-specific endogenous Eve protein expression. In order to achieve this, we used the twist promoter to drive Gal4 expression (Greig and Akam, 1993), which in turn drives the eve cDNA under the control of UAS Gal4-binding sites. This protocol to overexpress eve in the mesoderm of zfh-1 mutant embryos restores partially the formation of EPCs (Fig. 5F). These results support the hypothesis that eve acts downstream of zfh-1 and that it is required itself for the proper formation of EPCs. Since the rescue is partial, we can not rule out that normally the combination of both zfh-1 and eve functions are necessary for EPC development.

**eve is likely a direct target of Zfh-1**

We have presented genetic evidence that eve function is essential for the appropriate development of the EPCs. Embryos lacking eve at the critical time exhibit a loss of EPCs. Since zfh-1 seems to be required for maintaining eve expression in the forming EPCs, and zfh-1 and eve expression coincide during Eve progenitor formation, it is possible that the Zfh-1 transcription factor directly regulates eve gene expression in the mesoderm. An 800 bp enhancer element sufficient to drive expression in the mesoderm has been identified 3' of the eve coding region (see Materials and methods; Fujioka et al., 1999a). This mesodermal eve enhancer causes reporter gene expression in an identical mesodermal pathway (as the endogenous eve gene (Fig. 6A,B). zfh-1 encodes a protein with two distinct DNA-binding motifs, zinc fingers and a homeodomain. A putative consensus Zfh-1 homeodomain-binding sequence (P3/RCS1; Fortini et al., 1991) is present within the eve mesodermal enhancer. To test whether the homeodomain of zfh-1 can bind to the putative P3/RCSI consensus site in this enhancer, a gel mobility shift assay was conducted. The purified zfh-1 homeodomain-GST fusion protein (Lai et al., 1991) binds specifically to this sequence of the enhancer as determined by competition experiments (Fig. 6C; see Materials and methods). This finding is consistent with the hypothesis that EPC-specific eve expression is under the direct control of Zfh-1.
DISCUSSION

Much is known about the molecular components of a number of signaling pathways that participate in many processes including the development of the Drosophila mesoderm (reviewed in Bodmer and Frasch, 1999). In addition, the asymmetric distribution of the numb gene product directs alternative cell fates during many lineage decisions (Rhyu et al., 1994; Guo et al., 1996; Spana and Doe, 1996; Ruiz Gomez and Bate, 1997; Carmena et al., 1998a; Park et al., 1998b). Thus, these general mechanisms of cell fate diversification must be accompanied by additional, tissue-specific cues for the correct specification of individual cellular identities. We have provided here evidence that positional cues and lineage mechanisms cooperate with regional- or tissue-specific factors to define the correct differentiation pathway of a heart-associated cell type: zfh-1, in addition to tinman, seems to provide mesoderm-specific context for differentiation of the EPCs, which are generated by a numb-dependent lineage (see model in Fig. 7).

The role of zfh-1 and eve in heart development

We have shown that zfh-1 is required for the differentiation of the EPCs but not for the formation of their precursors or their sibling DA1 muscle founder cells (Fig. 1). eve appears to also play a role in EPC differentiation, since lowering eve function when the EPC progenitors divide disrupts the association of the EPCs with the developing heart (Fig. 5). Although the subsequent fate of these cells is not known, we speculate that they are likely to die, since Eve antibodies localize them at various distances from the heart before they disappear (see Fig. 5A,D,E). Another possibility is that the Eve protein that is generated from the eve<sup>ts</sup> allele, when the embryos are shifted to the non-permissive temperature, may be unstable and degraded rapidly. This is less likely, however, since a shift of eve<sup>ts</sup> embryos to the non-permissive temperature at a later time of development does not affect either the antigenicity (i.e. presence) of Eve protein within the EPCs or their correct positioning (Fig. 5B). Thus, eve and zfh-1 are required for EPC-specific differentiation. Although the other pericardial cells and the (myo)cardial cells of the heart are formed in zfh-1 and eve<sup>ts</sup> mutants, their correct morphology is disrupted (Figs 1,F,H, 5D,E). It is possible that other heart cells are directly affected by the lack of zfh-1 function. Alternatively, since eve is normally not expressed in these other heart-associated cells, the abnormalities in heart morphology may be an indirect effect due to a destabilization of the heart structure, consistent with the idea that EPCs anchor the heart. The structural relationship between EPCs and the other heart cells is unknown.

zfh-1 is not only needed for EPC formation but also for the development of a number of other mesodermal derivatives, including some of the body wall muscles (Lai et al., 1993; Broihier et al., 1998; Moore et al., 1998; see also Fig. 1H). Although in zfh-1 mutants, DA1 muscle-specific eve expression appears to be present, we can not rule out that normal differentiation of this muscle is affected. Unlike zfh-1, eve itself seems to be needed for promoting not only EPC but also DA1 muscle differentiation, since eve expression is lost from some DA1 muscle cells following removal of eve function (Fig. 5A).

Both zfh-1 and eve are required for EPC differentiation, and maintaining eve expression in the forming EPCs appears to depend on zfh-1 function. Therefore, the role of zfh-1 in EPC development may be via maintaining eve expression. Indeed, mesodermal overexpression of eve apparently restores the formation of some of the EPCs in zfh-1 mutant embryos (Fig. 5F), suggesting that eve is genetically epistatic to zfh-1 in this situation (and maintains its own expression in the EPCs). The finding that the Zfh-1 homeodomain binds in vitro to a consensus site (Fortini et al., 1991) within the mesodermal eve enhancer (Fig. 6) is consistent with the hypothesis that eve is a direct downstream target of zfh-1. However, maintaining eve expression may not be the only role of zfh-1 during EPC development, since the rescue of EPC formation is only partial. The incomplete rescue may be due to a direct requirement for zfh-1, in parallel to that of eve. Alternatively, incorrect timing and levels of ectopic eve expression may also account for the lack of complete rescue.

zfh-1 acts independently of numb and the EGF-receptor pathway

Although the EPC phenotype of zfh-1 mutants resembles the spdo loss-of-function and the numb gain-of-function phenotype, zfh-1 function does not seem to participate in the numb-dependent alternative cell fate decision: embryos doubly mutant for zfh-1 and numb do not exhibit the phenotype of either mutant alone (i.e. neither EPCs nor DA1 muscles are formed, see Fig. 4D). Thus, no epistatic relationship appears to exist between these two genes. What is the difference between the genetic functions of numb and zfh-1? Clearly, the numb pathway is not tissue-specific, as it plays a role in many asymmetric lineages. Therefore, it is necessary to invoke other factors that confer specificity as to the exact differentiation pathway to be chosen following alternative cell fate decisions. zfh-1 is likely to provide the necessary tissue-specific context for those eve-expressing mesodermal cells that do not experience a numb function, i.e. those destined to differentiate as EPCs (Park et al., 1998b; see also Fig. 7). Thus, if numb function is absent, both daughter cells of the EPC progenitors are destined become EPCs but, if zfh-1 activity is also absent, the EPC-specific differentiation is not executed.

In contrast to the EPCs, correct differentiation of their sibling DA1 founders require numb function in addition to DER pathway activity (Fig. 4E). Most of the DER signaling components are ubiquitously expressed, including the relevant (TGFα-like) ligand encoded by spitz. Although in our model, DER activation does not need to be locally restricted, a necessary component for spitz activation, which is encoded by rhomboid (rho; Bier et al., 1990), appears to be expressed in the vicinity of the DA1 founders (Buff et al., 1998). Since zfh-1;spitz double mutants lack differentiation of both daughter cell fates (Fig. 4F), it seems that zfh-1 does not take part in DER signaling. Therefore, we propose the model that the mesodermal context for EPC and DA1 differentiation is independently interpreted by each daughter cell of the EPC progenitors, depending on the presence or absence of numb: DER signaling provides contextual information for DA1 differentiation as zfh-1 does for EPC differentiation (Fig. 7).

Combinatorial control of specification and differentiation of a mesodermal cell lineage

Previous studies have shown that the specification of the entire
Fig. 4. Genetic epistasis of zfh-1 in EPC and DA1 development. All embryos are stained for Eve protein at stage 15/16, except (H) which is stage 11. The heart is indicated by open arrows, EPCs by arrowheads and DA1 muscles by brackets. (A) Wild-type embryo. (B) zfh-1^I mutant showing no EPCs, only DA1 muscles. (C) numb^I mutant showing no DA1 muscles but twice the number of EPCs (Park et al., 1998b). (D) numb^I;zfh-1^I double mutant showing no EPCs and no DA1 muscles, except for rare occurrences (arrowhead). (E) spitz^II^A mutant showing the normal number of EPCs, but no DA1 muscles. (F) spitz^II^A;zfh-1^I mutant showing no EPCs and no DA1 muscles (with few exceptions, arrowheads). (G) spitz^II^A mutant embryo expressing numb throughout the mesoderm (twi24B-Gal4·UAS-numb). Note the absence of EPCs and DA1 muscles. (H) Embryo with same genotype as G but at an earlier stage (11). Note that the initial eve expression appears to be normal. Normal eve expression at stage 11 was also observed in numb^I;zfh-1^I, spitz^II^A;spdo ZZ27 or spitz^II^A;zfh-1^I double mutants (data not shown).

Fig. 5. Temporal requirement of eve during heart development. (A-E) Temperature-sensitive eve^II^ embryos were collected and aged at 18°C, then shifted to the restrictive temperature (32°C) for 2 hours at the indicated times of embryonic development (see materials and methods). Embryos were then aged further until stage 15/16 (14-16 hours of development normalized to 25°C) and stained with anti-Eve antibodies alone (A-C,F), or in conjunction with anti-PC (D), or anti-Dmef2 (E) antibodies. (A,B,D,E) eve^II^ mutant embryos shifted to the restricted temperature at 5-6 hours (early stage 11) or 7-8 hours (late stage 11) of development, respectively. Note that in the early but not the late shifts most EPCs (and DA1 muscles) are missing or have lost their association with the heart (arrowheads in A,D; location of the heart between open arrows). (C) The wild-type heart contains an average of 42 Eve-positive pericardial cells, which are associated with the heart. 40-50 embryos were scored at each time point for the presence of EPCs. (F) Ectopic expression of eve (twi-Gal4·UAS-eve) in zfh-1 mutant background. Note that some EPCs have been restored in the zfh-1 mutant embryos (indicated by arrowheads).
cardiac mesoderm, including the EPC progenitors, depends on mesoderm endogenous transcription factors (twist and tinman) as well as on inductive signals encoded by wg and dpp that are expressed in specific patterns (reviewed in Bodmer and Frasch, 1999). Moreover, embryonic tissue at the intersection of both wg and dpp signaling and in the presence of tinman has the potential to differentiate with a cardiac-specific cell fate, including in non-mesodermal regions (W. K. Lockwood and R. B., unpublished).

How do the two mechanisms, i.e., global specification of (cardiac) competence and cell type-specific differentiation, interface with each other? In the model that we are proposing (Fig. 7), specification of a particular cell type (that of EPCs in this case) is a step-wise process and the sequential elements of specification are integrated on a defined enhancer element (Fig. 6). First, the spatially coincident activity of the transcription factor, Tinman, together with the mesoderm-specific response induced by the patterning signals, Wg and Dpp, are necessary to specify and position the most dorsal portion of the mesoderm, which includes the EPC progenitors and other cardiac precursors. The EPC progenitors then divide and produce two types of progeny cells under the control of the lineage gene numb. The daughter cell that inherits Numb protein will differentiate as the DA1 muscle founder, because Notch and spdo encoded functions are inhibited, allowing DER signaling (spitz) to be effective (perhaps in conjunction with Eve). In the daughter cell without Numb, Notch signaling is operational and the transcription factors Zfh-1 together with and/or mediated by Eve can effectively contribute the correct differentiation of the EPC fate. Thus, three levels of information appear to cooperate in the specification of a particular cell fate: prepatterning or positional information, asymmetric lineages and tissue context information.

As a consequence of the convergent functions of dpp, wg and tinman at the dorsal edge of the mesoderm, eve expression is initiated in the EPC progenitors. Moreover, the mesodermal eve enhancer contains consensus binding sites for Tinman as well as wg and dpp consensus response elements (Z. Han, M.-T. S. and R. B., unpublished). This is consistent with the hypothesis that these gene functions directly affect mesodermal
eve expression. eve expression is not initiated in all heart progenitors, however, which may be due in part to the inhibitory function of the ladybird homeobox genes, which are expressed in heart precursors adjacent to the EPC progenitors (Jagla et al., 1997). The presence of Ladybird consensus sites in the mesodermal eve enhancer suggests that regulation of eve expression by ladybird may be direct (Z. Han, M.-T. S. and R. B., unpublished).

With the initiation of the EPC progenitor lineage, Numb is localized asymmetrically in the progenitors and segregates to only one of the progeny. In the daughter cell that receives Numb, DA1 founder differentiation is initiated, in cooperation with DER activity. In the Numb-deficient daughter, EPC differentiation ensues, aided by Zfh-1. Taken together, the data presented suggest that a two-tiered context information system (first tinman then zfh-1) operates in conjunction with global mechanisms of specifying position (dpp and wg) and of generating asymmetric lineages (numb) to specify tissue- and position-specific cell fates within the developing embryo.

We thank M. Akam, A. Brand, B. Edgar, M. Frasch, Y. N. Jan, Z. Lai, B. Patterson, N. Perrimon, T. Volk and the Bloomington stock center for sending fly stocks, DNA constructs and antibodies. We also thank K. Golden for excellent assistance in making the figures, and Z. Han, K. Hardiman, J. B. Jaynes, M. Liu, W. K. Lockwood for comments on the manuscript. M.-T. S. was supported by a fellowship from the Organogenesis Center of the University of Michigan, T. G. by a grant from by NSF, and R. B. by grants from NIH and the American Heart Association.

REFERENCES


Goto, T., MacDonald, P. and Maniatis, T. (1989). Early and late patterns of even-skipped expression are controlled by distinct regulatory elements that respond to different spatial cues. Cell 57, 413-422.


