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Control of Cardiac Development by an Evolutionarily Conserved Transcriptional Network

Richard M. Cripps*,[1] and Eric N. Olson†

Department of Biology, University of New Mexico, Albuquerque, New Mexico 87131-1091; and †Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9148

Formation of the heart is dependent on an intricate cascade of developmental decisions. Analysis of the molecules and mechanisms involved in the specification of cardiac cell fates, differentiation and diversification of cardiac muscle cells, and morphogenesis and patterning of different cardiac cell types has revealed an evolutionarily conserved network of signaling pathways and transcription factors that underlies these processes. The regulatory network that controls the formation of the primitive heart in fruit flies has been elaborated upon to form the complex multichambered heart of mammals. We compare and contrast the mechanisms involved in heart formation in fruit flies and mammals in the context of a network of transcriptional interactions and point to unresolved questions for the future.

Key Words: **heart; cardiogenesis; dorsal vessel; Drosophila; review; gene network; transcription factor; transcription network.**

Introduction

Understanding the mechanisms that control the formation of the heart, with its spontaneous contractility that supports the life of the organism, represents a fundamental challenge in developmental biology. Over the past ten years, there has been rapid progress in our understanding of the molecular details of this process. These advances can be attributed in part to the remarkable conservation between distinct phyla in many of the embryological events involved in cardiogenesis, which is paralleled by conservation in the sequence and function of the cardiogenic transcription factors that orchestrate the early steps in heart formation. In this review, we describe the genetic networks that control the formation of the primitive heart of the fruit fly, *Drosophila melanogaster,* and consider how this framework has been elaborated upon to generate the hearts of vertebrates. While the vertebrate heart is more complex than that of the fruit fly, many of the basic elements of the transcriptional programs for cardiac specification and differentiation are conserved. In contrast, developmental pathways that underlie cardiac morphogenesis are specific to vertebrates and appear to involve transcriptional circuits not found in fruit flies or other invertebrates.

At its core, the process of heart formation, as for all pathways of organogenesis, relies on a transcriptional circuit "hard-wired" into the DNA and dependent on combinatorial associations of cell type-specific and widely expressed transcription factors that interpret cell identity, extracellular signals, and positional information within the embryo (Davidson, 2001). While we are many years away from fully understanding the detailed molecular events required to generate the heart, or any other organ, certain fundamental principles are apparent and provide a blueprint for thinking about the logic for tissue-specific transcription as a basis for organ formation. One realization from recent studies is that cardiac gene regulation is highly modular, such that multiple independent and interdependent *cis*-regulatory modules are required to direct the complete pattern of expression of an individual cardiac gene. This modularity is so specific that many cardiac enhancers have been found to direct transcription within subsets of cardiogenic cells that were previously unknown to be molecularly distinct from their neighbors. It is also not uncommon for a cardiac gene to be regulated by multiple enhancers that

[1] To whom correspondence should be addressed. Fax: (505) 277-0304. E-mail: rcripps@unm.edu.

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direct overlapping or identical expression patterns. This type of modularity provides opportunities for fine-tuning of transcriptional responses and also serves as a foundation for diversification of gene expression programs during evolution.

Embryology of the Drosophila Dorsal Vessel

The circulatory system in *Drosophila*, as with all insects, is limited to a large dorsomedial muscular tube termed the dorsal vessel or heart, which pumps blood anteriorly toward the brain (Wigglesworth, 1984). Blood deposited at the brain then percolates posteriorly through the body cavity until it reenters the heart through inflow tracts termed ostia. In contrast to vertebrate embryos, which require a functional heart early in embryogenesis, the *Drosophila* embryo is capable of developing in the absence of a functional dorsal vessel, permitting the detailed analysis of the effects of mutant phenotypes upon the mature organ. However, a functional dorsal vessel is required for viability after embryogenesis.

The dorsal vessel arises from the mesoderm. This germ layer is specified at the beginning of embryogenesis in response to cues laid down in the oocyte, which result in nuclear localization of the maternally derived Rel-domain transcription factor Dorsal (Rushlow *et al.*, 1989; Steward, 1989; Roth *et al.*, 1989). Dorsal then directly activates expression of the zygotic regulatory genes *twist* and *snail* (Thisse *et al.*, 1991; Jiang *et al.*, 1991; Ip *et al.*, 1992; Pan *et al.*, 1991), which encode basic helix–loop–helix (bHLH) and zinc-finger transcription factors, respectively, that are required for specification and morphogenesis of the mesoderm (Leptin, 1991). Mesodermal cells subsequently invaginate and spread laterally to form a monolayer closely apposed to the overlying ectoderm.

Cues from the ectoderm then instruct a subset of dorsal mesodermal cells in each hemisegment to acquire dorsal vessel fate through a series of interactions of signaling effector molecules with the *cis*-regulatory elements of a number of genes. Programmed cells from each side of the embryo subsequently migrate to the dorsal midline where they converge to form the dorsal vessel (Fig. 1A). During this dorsal migration, activation of specific gene networks results in the specification of numerous cell types that ultimately give rise to the mature dorsal vessel, which consists of two central rows of cardial cells that form the muscular tube surrounded by pericardial cells (Fig. 1B).

The simplicity implicit in a single circulatory vessel belies the complexity of the *Drosophila* cardiac organ, which is comprised of genetically distinct cell types that repeat along the anterior–posterior axis coincident with the segmental units of the animal. Whereas all six cardial cells in each hemisegment express the MADS (MCM1, Agamous, Deficiens, serum response factor) box transcription factor gene *Myocyte enhancer factor-2* (*Mef2*; Lilly *et al.*, 1995; Bour *et al.*, 1995), only four of the six cells per hemisegment express the homeobox gene *tinman* (*tin*; Bodmer, 1993; Azpiazu and Frasch, 1993) and the remaining two cells express the orphan nuclear receptor gene *seven-up* (*svp*; Bodmer and Frasch, 1999). Within the Tin-expressing cardial cell population, two of the four cells also express the two *ladybird* (*lb*) homeobox genes, *ladybird-early* and *ladybird-late* (Jagla *et al.*, 1997).

Superimposed upon this segmentally repeating motif in the cardial cell population is an anterior–posterior pattern, which results in morphological diversity along the length of the dorsal vessel. In the posterior two segments, the dorsal vessel is termed the heart proper, and is structurally and functionally distinct from the anterior region, termed the aorta. The heart and aorta are separated by a pair of cells that form a cardiovascular valve. In addition, the ostia through which hemolymph enters the circulation are located exclusively in the heart during embryonic and larval stages (Fig. 1B; Rizki, 1978; Molina and Cripps, 2001).

There is also significant molecular complexity in the remaining group of cells that form the dorsal vessel: the pericardial cells. The function of these cells is not fully understood, although a subset may function as stationary macrophages, and possibly in blood filtration (Miller, 1950). Genetic marker analyses have demonstrated that this cell type consists of at least five subtypes. Patterns of gene expression in the cardial and pericardial cells are indicated in Fig. 1C.

In anterior segments, there are fewer pericardial cells and the dorsal vessel instead is surrounded by accessory structures termed the lymph glands and the ring gland (Fig. 1B). The lymph glands are major blood-forming organs (Rizki, 1978; Lanot *et al.*, 2001), and are recognizable as a paired cluster of cells expressing several pericardial cell markers, including *odd* (Ward and Coulter, 2000). Manipulating expression of the *Hox* gene *Ultrabithorax* indicates that the lymph glands and pericardial cells form from equivalent precursors, since mutation of *Ubx* results in an increase in the number of lymph glands at the expense of pericardial cells (Rodriguez *et al.*, 1996). The other glandular structure, the ring gland, is an endocrine organ (Rugendorff *et al.*, 1994).

Specification of the Heart Field in Drosophila

How are mesodermal cells committed to becoming precursors of the dorsal vessel? Much of our understanding of this process has come from the analysis of *tin*, which is required for specification of all heart cells. The *tin* gene encodes a homeodomain transcription factor whose broad initial expression in the mesoderm is sequentially restricted to dorsal mesoderm, then heart precursors, and subsequently to a subset of cardial and pericardial cells. In the absence of *tin* function, no dorsal vessel precursors are generated, and mutant embryos die at the end of embryogenesis, likely from a lack of circulation (Bodmer, 1993; Azpiazu and Frasch, 1993).

Understanding the transcriptional regulatory events converging on the *tin* gene has provided important insight into

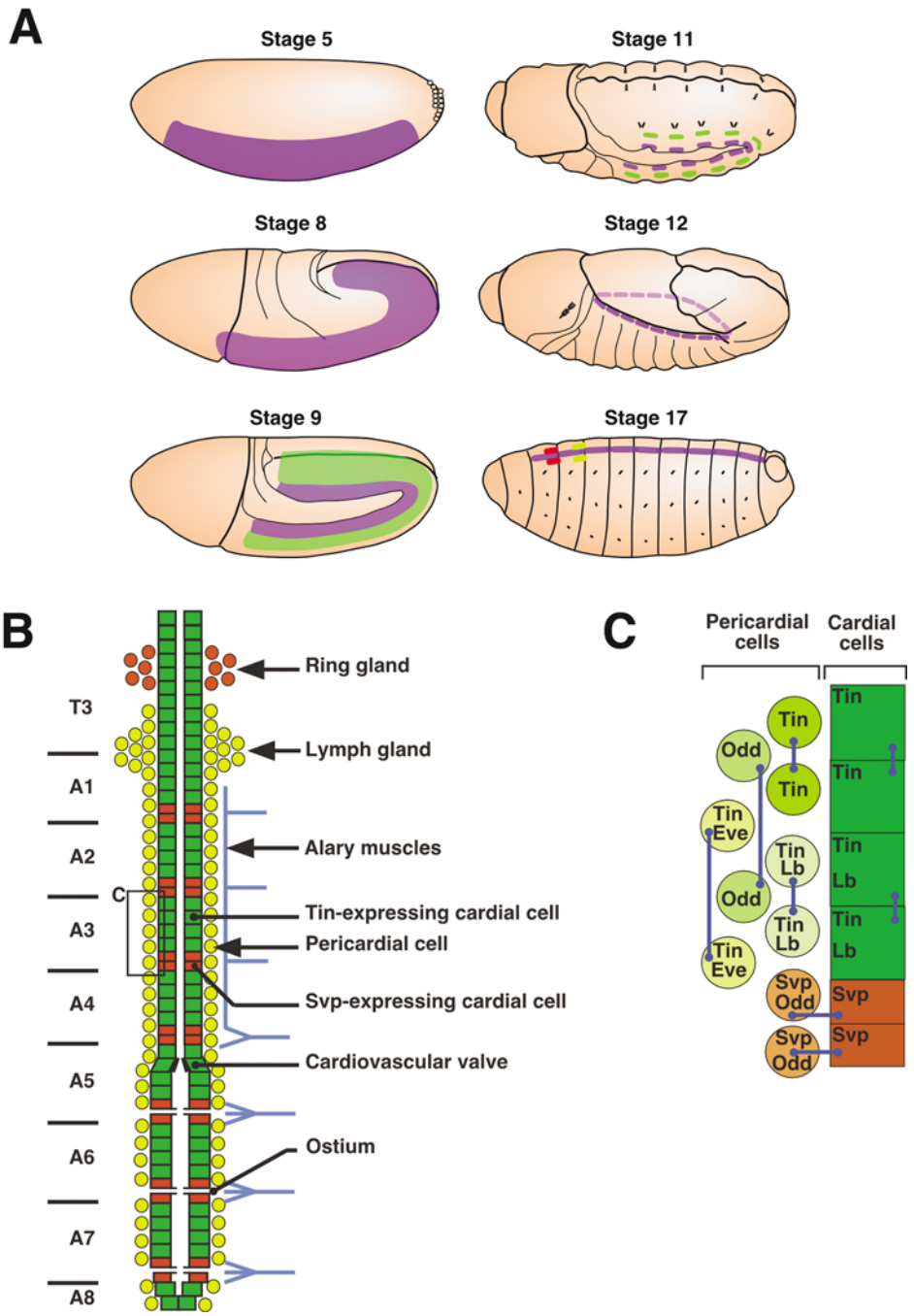

FIG. 1. Formation and structure of the dorsal vessel in Drosophila. (A) Summary of *tin* expression (purple) during dorsal vessel formation. Early during embryogenesis (stage 5), the mesoderm is specified on the ventral side of the embryo, and *tin* expression is uniform throughout the mesoderm. By stage 8, the mesoderm has invaginated and spread dorsally along the inside of the ectoderm. Shortly thereafter (stage 9), *tin* expression is restricted to the dorsal mesoderm by Dpp signaling; the rest of the mesoderm is shown in green. At stage 11, the visceral mesoderm precursors (green) move to the interior of the embryo, leaving just the Tin-expressing heart precursors close to the epidermis. During dorsal closure (stage 12), the distinct heart precursors begin to coalesce to form a continuous row of dorsal vessel cells on each side of the embryo. At stage 17, the differentiated heart tube is located at the dorsal midline, surrounded at the anterior end by the lymph glands (yellow) and ring gland (red). All images are dorsolateral views with anterior to the left. (B) Ultrastructure of the dorsal vessel. The dorsal vessel consists of an inner row of cardial cells which express either *tin* (green) or *svp* (red). The posterior end of the dorsal vessel (the heart proper) has a larger lumen than the aorta and is more muscular. Ostia, inflow tracts for hemolymph, are formed in the heart by the Svp-expressing cells, and a cardiovascular valve separates the heart from the aorta. The cardial cells are surrounded by pericardial cells shown schematically here (yellow) and in more detail in (C) (boxed region). The heart is associated along its length with fine alary muscles (blue). Anterior is to the top. (C) Cell-type diversity within the dorsal vessel. Shown are all of the known dorsal vessel cells within a hemisegment (cardial and pericardial), summarizing marker gene expression in the cells. Note also that all pericardial cells identified to date express *Zfh-1* (Lai *et al.*, 1991). Cells which derive from a common precursor cell ("siblings") are joined by a blue line. Anterior is to the top, and the dorsal midline is to the right.

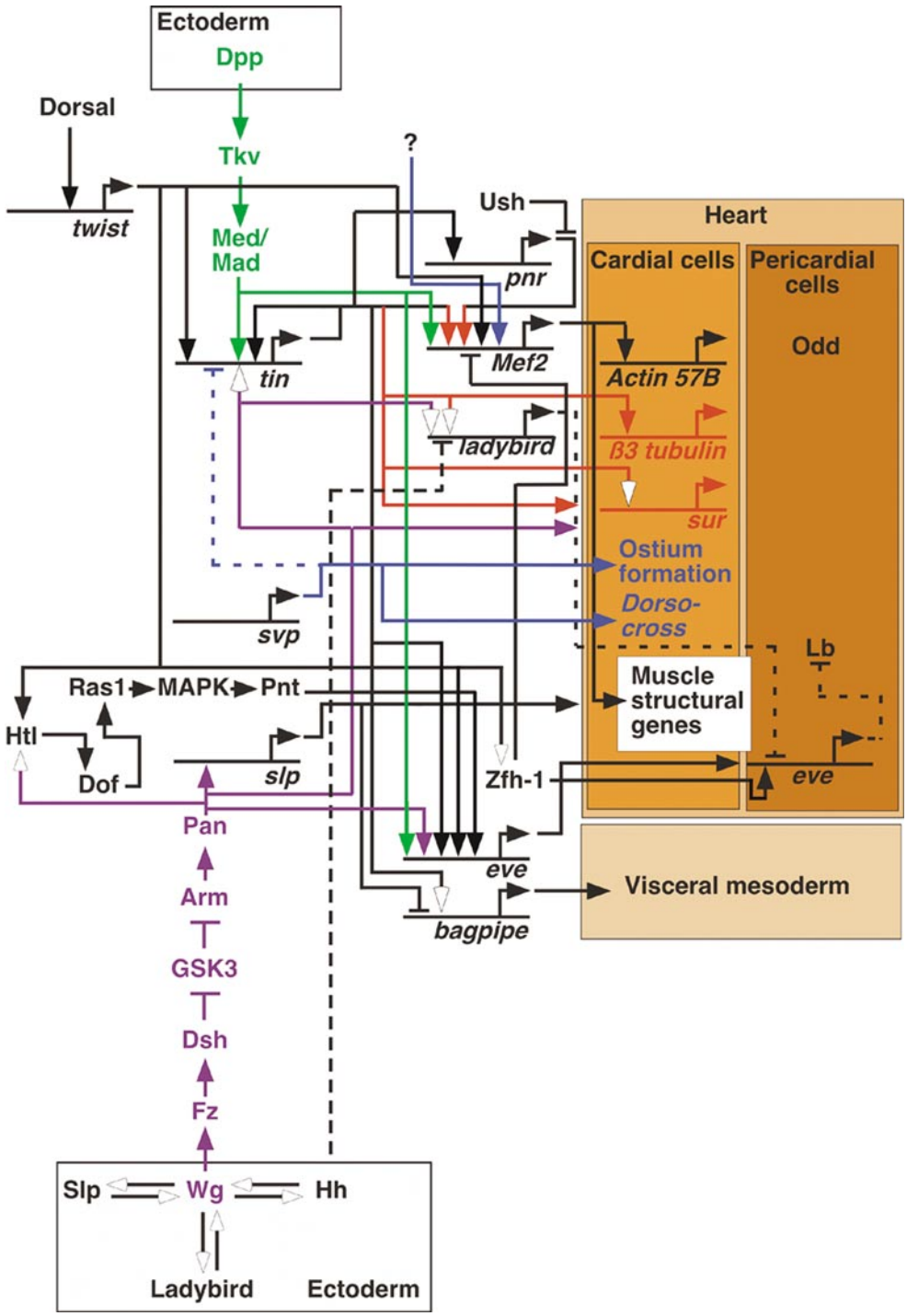

FIG. 2. A transcriptional network for cardiogenesis in *Drosophila*. Shown in the diagram are the cardiac gene regulatory interactions that have been characterized to date. Direct interactions of transcriptional activators with target genes are signified by solid arrowheads, and positive regulatory effects for which there is no evidence of a direct interaction are indicated with open arrowheads. Repressive influences that are direct are shown with solid lines and those that have not been shown to be direct are shown with broken lines. Target genes are shown as lines with rightward pointing arrows signifying initiation of transcription. Activation of proteins along a signal transduction pathway is also indicated by a closed arrow. Colors: Green indicates the pathway and effects of Dpp signaling; Purple indicates the pathway and effects of Wg signaling; Blue indicates interactions that occur specifically in the two Svp-expressing cardial cells per hemisegment (for which the activator of *Mef2* is unknown, indicated by ?); Red indicates the interactions that occur in the four Tin-expressing cardial cells per hemisegment. See text for references, but also see Su *et al.* (1999a) and Postigo *et al.* (1999).

how heart field specification is hard-wired in the genome (Fig. 2). At least six separable regulatory elements control *tin* expression during embryogenesis (Lee *et al.,* 1997; Yin *et al.,* 1997; Venkatesh *et al.,* 2000), and each controls a different phase of the changing *tin* expression pattern. The earliest detectable expression of *tin* is controlled by an enhancer that directs expression throughout the mesoderm from the onset of gastrulation. Activation of this enhancer occurs through the direct binding of Twist to three conserved E-boxes (Yin *et al.,* 1997).

Subsequently, *tin* transcription is restricted to cells of the dorsal mesoderm that are fated to form the cardiac and visceral muscle lineages. This dorsal restriction occurs in response to Decapentaplegic (Dpp), a member of the bone morphogenetic protein (BMP) family (Frasch, 1995). Dpp is expressed in the dorsal ectoderm and activates the type I TGF-β receptor Thickveins (Tkv). Activation of Tkv causes phosphorylation of the Dpp effectors Mad and Medea, which then translocate to the nucleus and directly activate *tin* expression via a second *cis*-regulatory element (Yin and Frasch, 1998; Xu *et al.,* 1998; Raftery and Sutherland, 1999). Full activation of *tin* through this enhancer also requires the autoregulatory function of Tin, suggesting that earlier expression of *tin* throughout the mesoderm acts to render mesodermal cells competent to respond to the Dpp signal (Xu *et al.,* 1998; Frasch, 1999).

The importance of this dorsal signal in determining heart and visceral muscle phenotypes is underscored by the effects upon mesodermal fate of mutation of the *heartless* (*htl*) FGF receptor gene. In *htl* mutants, dorsal migration of the mesoderm fails to occur, and cells do not receive the instructive Dpp signal. As a result, dorsal mesodermal cell fate is not specified, resulting in a lack of cardiac and visceral muscle cells (Gisselbrecht *et al.,* 1996; Beiman *et al.,* 1996; Shishido *et al.,* 1997). Mutation of the *downstream of FGF* gene (*dof*) results in a similar phenotype (Vincent *et al.,* 1998). Furthermore, mutations in the *sugarless* and *sulfateless* genes, which are thought to function in the synthesis of heparin sulfate proteoglycan cofactors for FGF signaling, also cause defects in mesodermal migration and a loss of dorsal mesoderm derivatives (Lin *et al.,* 1999).

Among the dorsal mesodermal cells that respond to the Dpp signal by maintaining *tin* expression, some move to the interior of the embryo and form the splanchnic visceral mesoderm. In these cells, *tin* activates the expression of a related homeobox gene *bagpipe* (*bap*), which functions to direct visceral mesoderm fate (Azpiazu and Frasch, 1993). The remaining dorsal mesodermal cells constitute the heart field, and many of these cells maintain *tin* expression through the end of embryogenesis (Azpiazu and Frasch, 1993; Bodmer, 1993).

The signal that controls the decision to form cardiac or visceral muscle has been well characterized. The secreted Wingless (Wg) protein, a homolog of the vertebrate Wnts, is released to the mesoderm from the overlying ectoderm in a series of segmentally repeating stripes, which overlie the presumptive dorsal vessel cells (Lawrence *et al.,* 1995). Abrogation of *wg* function results in the absence of the dorsal vessel, indicating that this signal is essential for heart formation (Wu *et al.,* 1995). Although *wg* expression in the mesoderm can function in dorsal vessel specification, it is most likely that ectodermal *wg* expression constitutes the major cardiac determinant (Lawrence *et al.,* 1995).

Many of the factors that transduce the Wg signal in other tissues, including the Wg-dependent transcriptional regulator Pangolin (Pan/dTCF/LEF-1), also function in the mesoderm to specify cardiac fate among dorsal mesodermal cells (Park *et al.,* 1996, 1998a). The two genes of the *sloppy-paired* locus, *slp-1* and *slp-2* (Lee and Frasch, 2000), which have overlapping functions in segmentation and mesoderm development (Grossniklaus *et al.,* 1992; Cadigan *et al.,* 1994; Riechmann *et al.,* 1997), are direct targets of Pan in the mesoderm.

Activation of the *slp* genes in mesodermal cells underlying the ectodermal Wg stripe has numerous developmental consequences. In addition to maintaining high levels of *twist* expression in the forming skeletal muscle lineage (Riechmann *et al.,* 1997), *slp* functions in the formation of dorsal vessel fate by directly down-regulating *bap*, thereby suppressing visceral mesoderm fate in the presumptive heart cells (Lee and Frasch, 2000). *slp* also functions in the ectoderm to maintain expression of *wg*. This is important, since Wg signals are required for heart development at multiple times during embryogenesis (Wu *et al.,* 1995; Jagla *et al.,* 1997). However, Slp alone cannot substitute for Wg signaling, since mesodermal expression of *slp* in *wg* mutants does not result in the activation of dorsal vessel marker genes such as *even-skipped* (*eve*). More likely, striped mesodermal *slp* expression renders the cells capable of interpreting further Wg signals during the generation of the dorsal vessel (Lee and Frasch, 2000). The mechanisms by which *slp* initiates heart competence and how this competence is realized in response to the Wg signal have yet to be determined.

The Function of tin

Clearly, activation of *tin* expression is a central event in the control of heart development in *Drosophila,* and the central position of *tin* in the cardiogenic network is illustrated by the many functions of *tin* in the mesoderm. In the absence of *tin* function, there is a complete loss of dorsal mesodermal structures, including cardiac and visceral muscle cells, as well as a subset of dorsal skeletal muscles (Azpiazu and Frasch, 1993; Bodmer, 1993). The failure of *tin* mutants to generate cardiac and visceral muscles is not due to defects in mesoderm morphogenesis (as is the problem in *heartless* mutant embryos), since dorsal spreading of the mesoderm occurs normally in *tin* mutants. However, shortly after the separation of splanchnic mesodermal cells from the heart precursors, there is a failure of the visceral cells to express the visceral marker *fasciclin III*. Defects in heart development are also apparent shortly afterwards as a

failure to express a number of markers of the cardial and pericardial cell populations of the mature dorsal vessel (Bodmer, 1993; Azpiazu and Frasch, 1993). Taken together, these findings indicate that the earliest function of *tin* is to specify the identity of dorsal mesodermal cells. Genetic experiments have not been able to address roles of *tin* later in heart development.

Tin binds to the consensus sequence 5′-TYAAGTG-3′ (Chen and Schwartz, 1995), and has been shown to be both an activator and a repressor of transcription (Ranganayakulu *et al.*, 1998; Choi *et al.*, 1999; Fossett *et al.*, 2000). To date, four Tin target genes have been identified: *tin* itself (via the dorsal mesoderm enhancer discussed above), the GATA factor gene *pannier* (*pnr*), *Mef2*, and the structural gene *β3-tubulin* (Xu *et al.*, 1998; Gajewski *et al.*, 1997, 1998, 2001; Cripps *et al.*, 1999; Kremser *et al.*, 1999). In all cases, there are two consensus Tin binding sites, both of which are required for full activity of the enhancer. Given the complete genomic sequence of *D. melanogaster* (Adams *et al.*, 2000), this finding suggests that it may be possible to identify additional Tin target genes by looking for the close (~300 bp) apposition of two consensus Tin binding sites.

The nature of these target genes illustrates the critical roles of *tin* in multiple phases of heart development. Tin has an early autoregulatory role in specifying dorsal mesodermal fate; Tin activates *pnr*, and the Pnr protein subsequently collaborates with Tin to activate *Mef2*, which is required for heart differentiation (see below). Tin also activates *β3-tubulin*, a structural gene expressed in the mature heart. That *tin* expression in the dorsal vessel persists to the adult stage suggests additional important roles in heart growth and metamorphosis (Molina and Cripps, 2001).

Despite structural and functional similarities between Tin and a vertebrate homolog Nkx2.5, the latter is incapable of compensating for Tin function in heart formation in *Drosophila*, although visceral mesoderm development can be rescued by expressing Nkx2.5 in *tin* mutants (Ranganayakulu *et al.*, 1998; Park *et al.*, 1998c). On the contrary, Nkx2.5 inhibits heart formation in wild-type embryos, which may reflect the fact that it binds the same target sites as Tin, but cannot activate transcription through those sites. The failure of Nkx2.5 to rescue heart development is due to the presence in Tin of a unique N-terminal domain which, when transferred to Nkx2.5, renders the chimeric protein capable of rescuing heart and visceral muscle fate (Ranganayakulu *et al.*, 1998). It has been suggested that the N terminus of Tin interacts with factors required for heart development in *Drosophila*, but that Nkx2.5 lacks this cofactor interaction during mammalian heart development (Ranganayakulu *et al.*, 1998). This suggests that, despite their similar structures and roles in cardiogenesis, there has been significant divergence during evolution in the proteins with which Tin and Nkx2.5 interact to promote heart formation.

An understanding of at least one factor that collaborates with Tin in heart development has come from an analysis of the Tin-dependent *Mef2* enhancer (Gajewski *et al.*, 1998, 1999). The *pnr* gene encodes a GATA transcription factor which is expressed in the dorsal ectoderm and dorsal mesoderm and which is required for heart development (Gajewski *et al.*, 1999). In the heart, Pnr interacts with Tin to directly activate *Mef2* expression in cardial cells (Gajewski *et al.*, 1999). It is not yet known whether Pnr also collaborates with Tin to control other Tin-responsive genes.

Fossett *et al.* (2000) have also shown that the function of Pnr in promoting cardial cell differentiation is antagonized by a multitype zinc finger transcription factor, U-shaped (Ush), expressed in the dorsal ectoderm and mesoderm. In the absence of *ush* function, there is an increase in cardial cell number, and upon overexpression of *ush*, there is a loss of cardial cells (Fossett *et al.*, 2000). These effects result from a direct interaction of Ush with Pnr to render Pnr transcriptionally inactive. Clearly, modulating Pnr function is essential in the delineation of cardial cells from pericardial cells, the latter of which do not express *Mef2* (Fossett and Schulz, 2001).

The alteration in numbers of cardial cells relative to pericardial cells in *ush* mutants is reminiscent of the role of the immunoglobulin family gene *faint sausage* (*fas*), which is expressed in heart precursors. In *fas* mutants, there are excess cardioblasts at the expense of pericardial cells; however, the mechanism for this defect is not known (Haag *et al.*, 1999).

Cell Lineage Specification within the Heart Field

Upon specification of a cluster of heart-forming cells in each hemisegment in the dorsal mesoderm, individual cells are subsequently assigned to particular fates. Within a short period of time, 16 cells within each cluster give rise to 6 cardial and 10 pericardial cells. Although the specific mechanisms for cell fate specification have been described for some of these cells, a complete picture of heart field patterning has yet to emerge.

Much is known concerning the specification of the two Eve-positive pericardial cells (EPCs) present in each hemisegment. The progenitor for these cells arises from a precluster of cells, named pre-C2, which expresses the proneural bHLH factor *lethal of scute* (*l'sc*; Carmena *et al.*, 1998b). Dorsal cells within pre-C2 then express *eve* as well as *l'sc*, and this pattern of coexpression is subsequently limited to a single cell in the cluster through lateral inhibition via the Notch signaling pathway. This single cell is termed the P2 progenitor. P2 then divides, but only a single daughter cell (termed F2) maintains *eve* expression; the other daughter cell loses *eve* expression and assumes an unknown fate (Carmena *et al.*, 1998b; Halfon *et al.*, 2000). F2 divides again to form the two Eve pericardial cells (Carmena *et al.*, 1998b).

A complex set of intrinsic and extrinsic signals converges at the Eve-expressing P2 cells to program this specific cell fate. The P2 cells arise in the region of the dorsal mesoderm

that receives both Dpp and Wg signals, which renders them competent to express *eve* and to activate the ras/MAPK cascade. Localized ras/MAPK activation, as well as activities of the mesodermal gene products Twist, Tin, and Pan, result in maintenance of *eve* expression in only a single cell of pre-C2 (Halfon *et al.*, 2000). *eve* activation and maintenance in an adjacent cluster, C15, likely occurs by an analogous mechanism.

Upon division of the P2 cell, maintenance of *eve* expression within the F2 cell and not within its sibling results from the action of the *numb* (*nb*) gene product. In *nb* mutants, supernumerary EPCs are detected, whereas upon *nb* overexpression, the EPC population is reduced, suggesting that *nb* controls cell identity following asymmetric cell division (Park *et al.*, 1998b; Carmena *et al.*, 1998a). Indeed, Nb protein is asymmetrically localized in P2 such that, upon division, it is inherited predominantly by the sibling cell and not by F2. Within the sibling cell, the functions of the signaling molecule Notch (N) and of Sanpodo (Spdo), a tropomodulin homolog, perhaps important for transducing the N signal, are both inhibited. The daughter lacking Numb becomes F2 presumably through action of the N pathway (Park *et al.*, 1998b; Carmena *et al.*, 1998a,b).

The role of *nb* in asymmetric cell divisions in the mesoderm is not limited to the EPCs. The antagonistic functions of *nb* and *spdo* have been exploited to demonstrate that Nb promotes Svp-expressing cardial cell identity at the expense of Svp/Odd-expressing pericardial cells (Gajewski *et al.*, 2000; Ward and Skeath, 2000). The opposite effect is seen in *spdo* mutant embryos. In contrast, the two Tin-expressing cardial cells and the two Tin/Lb-expressing cardial cells in each hemisegment are identical daughter cells, since their numbers are not affected by mutation of *nb* nor *spdo* (Gajewski *et al.*, 2000; Ward and Skeath, 2000; Jagla *et al.*, 2002). These findings were also confirmed by lineage-tracing analyses (Ward and Skeath, 2000).

Clearly, asymmetric cell division is a highly effective mechanism for generating cell diversity with the group of cells fated to form the dorsal vessel. Superimposed on this mechanism are the inhibitory effects of *svp*, *lb*, and *eve* on specific cell fates. Lo and Frasch (2001) demonstrated that Svp-expressing cells initially appear within the Tin-expressing cluster, whereupon *tin* expression is downregulated in the Svp cells. This potent repression results from *svp* function since expression of *svp* in the Tin-positive cardial cells results in a loss of *tin* expression in those cells. Conversely, mutation of *svp* results in *tin* expression in all six cardial cells per hemisegment (Gajewski *et al.*, 2000; Lo and Frasch, 2001).

Jagla *et al.* (2002) have also demonstrated that cross-repressive interactions occur between *lb*, *eve*, and a dorsal muscle transcriptional regulator *msh-1*. Loss-of-function in any one of these genes results in supernumerary cells expressing the other markers. Furthermore, overexpression of any one of these three genes results in a loss of identify of cells expressing each of the other two markers. These findings are consistent with the mutually exclusive expression patterns of *lb*, *eve*, and *msh-1* within the dorsal mesoderm.

Taken together, these findings indicate that single cell identity is likely specified from among a group of cells, such as P2 identity for the EPCs. Once cell identity has been determined, cross-repressive interactions among different transcription factors function to maintain that cell identity. A challenge in the future will be to determine precisely how single cell identities are established within this group of cells, and how it is ensured that each of eight unique cell types arises in each hemisegment.

Differentiation of the Dorsal Vessel

As presumptive dorsal vessel cells arrive at the dorsal midline from either side of the embryo, those expressing *Mef2* align to form a linear tube two cells wide, which is surrounded by the MEF2-negative pericardial cells. Subsequently, muscle structural genes are expressed in the cardial cells and, by the end of embryogenesis, a beating heart can be observed.

Mutations affecting the differentiation of the heart can be classified into two categories: those affecting the formation of the heart tube, and those affecting the differentiation of cardiomyocytes. Genes encoding cell adhesion molecules are prominent in the first category: for example, *laminin-A* chain (Yarnitzky and Volk, 1995) and the *shotgun/DE cadherin* gene are required for the formation of a heart tube (Haag *et al.*, 1999). Two other genes, *apontic* (Su *et al.*, 1999b) and *brokenhearted* (Fremion *et al.*, 1999), are also required for the formation of an uninterrupted tube.

The predominant transcription factor involved in heart muscle differentiation is MEF2. MEF2 plays a central role in the differentiation of all muscle types within the embryo; however, it is not required for the formation of the heart tube (Lilly *et al.*, 1995; Bour *et al.*, 1995; Ranganayakulu *et al.*, 1995). Expression of *Mef2*, like *tin*, initiates in the early mesoderm as a direct target of Twist (Taylor *et al.*, 1995; Cripps *et al.*, 1998; Nguyen and Xu, 1998). Shortly afterwards, *Mef2* expression is also enriched in the dorsal mesoderm through Dpp signaling (Nguyen and Xu, 1998), before being activated directly by Tin (Gajewski *et al.*, 1997, 1998; Cripps *et al.*, 1999). Activation of *Mef2* by Tin (in collaboration with Pnr) continues through the end of embryogenesis in the four Tin-expressing cardial cells per hemisegment, while *Mef2* expression in the remaining two cardial cells per hemisegment is regulated through a more distal enhancer. However, the direct transcriptional regulator in this cell type is not known (Gajewski *et al.*, 2000).

In the absence of *Mef2* function, several structural genes are not expressed, including *Myosin heavy chain*, *Myosin light-chain alkali*, *Myosin light-chain-2*, and *Actin57B* (Lilly *et al.*, 1995; Bour *et al.*, 1995; Ranganayakulu *et al.*, 1995; Kelly *et al.*, 2002), suggesting that MEF2 controls differentiation through activation of these structural genes. However, a direct role for MEF2 in the transcription of structural genes in the heart has only been demonstrated for

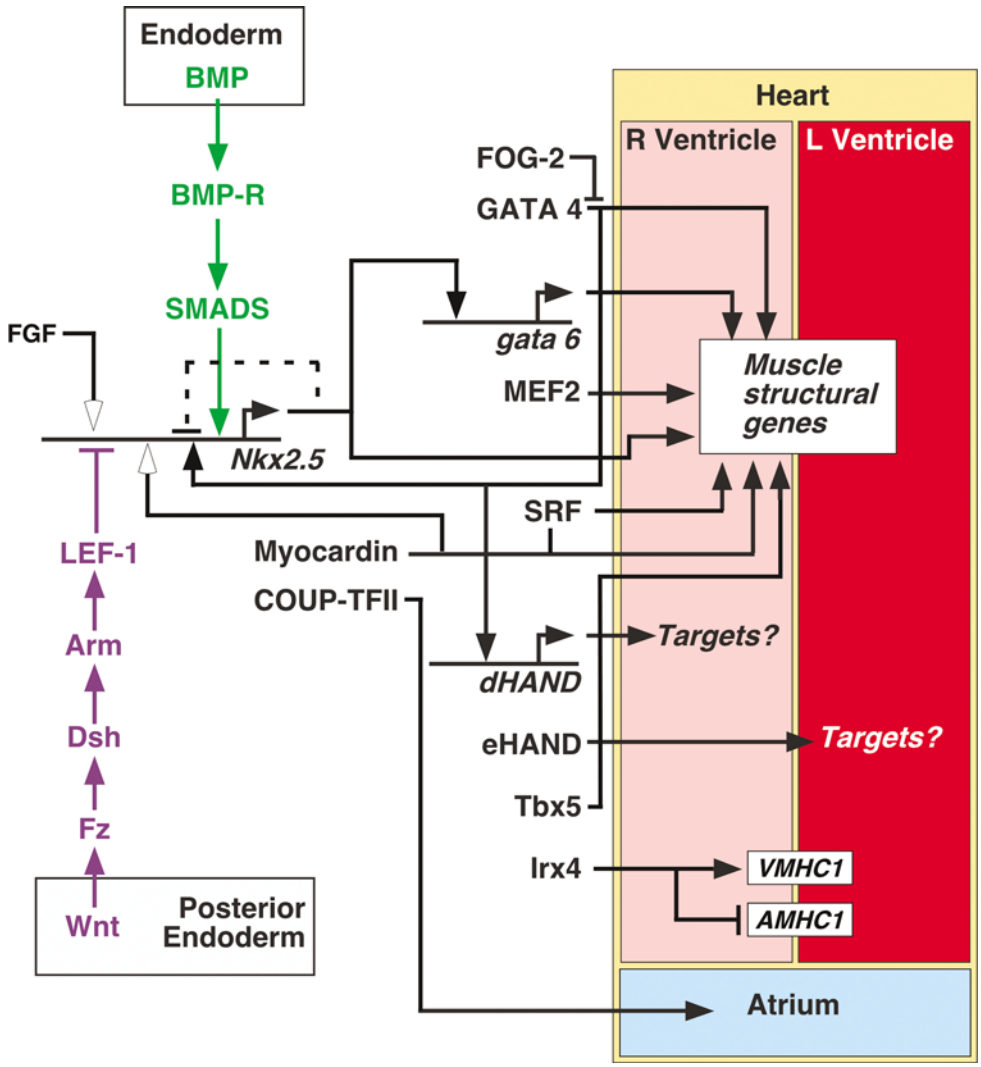

FIG. 3. A transcriptional network for early cardiogenesis in vertebrates. Transcriptional interactions involved in the early steps of vertebrate heart development are shown. See legend to Fig. 2 for details.

Act57B, which contains a conserved MEF2 binding site in its promoter (Kelly *et al.*, 2002). It should also be pointed out that some muscle structural genes appear to be independent of MEF2, such as *Tropomyosin I*, which is broadly expressed in the heart and is unaffected in *Mef2* mutant embryos (Lin *et al.*, 1996).

In addition to *Mef2*, *tin* also is required for the activation of structural genes in the dorsal vessel. The *β3-tubulin* gene is expressed in the dorsal vessel only in the Tin-expressing cardial cells; this expression is mediated by direct binding of Tin to a cardiac enhancer element (Kremser *et al.*, 1999). The *sur* gene, which encodes a sulfonylurea receptor (Nasonkin *et al.*, 1999), is also expressed in the Tin-positive cardial cells and is likely to be a direct target for Tin activation.

Importantly, there are numerous genes encoding transcription factors expressed in the mature dorsal vessel for which no functional information is yet available. These include *Hand* (Moore *et al.*, 2000), the T-box gene *H15* (Griffin *et al.*, 2000), and *dorsocross* (Lo and Frasch, 2001). Whether these genes control heart specification, tube formation, or cardial cell differentiation is not known, nor are the potential epistatic relationships between these genes and others described in this review.

Cross-Species Comparisons of the Transcriptional Network for Cardiogenesis

Based on our current understanding of cardiogenesis in *Drosophila*, it is possible to begin to outline the transcrip-

tional regulatory circuits involved in this developmental process (Fig. 2). These circuits are obviously oversimplified and do not take into account thresholds of transcriptional activity required for different genetic responses or regulated interactions between those DNA-binding factors and accessory factors. Nevertheless, the circuitry is instructive in illustrating regulatory relationships between genes, in pointing to gaps in our understanding, and in allowing comparisons between the genetic underpinnings of cardiac development in *Drosophila* and vertebrates.

Although the events involved in forming the vertebrate multichambered heart are much more involved than for the formation of the *Drosophila* dorsal vessel, many of the same signals, families of transcription factors, and regulatory circuits that mediate dorsal vessel development also appear to operate during the early steps in vertebrate heart formation. Below, we describe the similarities and differences in the regulatory strategies for specification of cardiac cell fate, differentiation of cardiac myocytes, and morphogenesis of the linear heart tube in fruit flies and vertebrates, and we consider how this network of gene interactions has been modified over the evolutionary distance between *Drosophila* and vertebrates. A simplified transcriptional network for the genetic interactions involved in the initial steps of vertebrate heart development is shown in Fig. 3. After the linear heart tube forms in the vertebrate embryo, it undergoes complex events of looping morphogenesis, chamber specification, septation, and diversification, as well as integration with the vascular system. These events, which are specific to vertebrates, have been reviewed elsewhere and are beyond the scope of this review (Fishman and Olson, 1997; Srivastava and Olson, 2000; Yelon and Stainier, 1999).

Signals That Mediate Cardiogenic Induction

The heart is the first organ to form during mammalian development. Like the dorsal vessel in *Drosophila*, the heart in vertebrates is derived from a subpopulation of mesodermal precursor cells that become committed to a cardiogenic fate in response to inductive cues from adjacent cell types. However, in contrast to *Drosophila*, the vertebrate heart is derived from ventral mesodermal cells, reflecting the dorsoventral inversion of the vertebrate body plan compared with that of insects (Bodmer, 1995).

BMPs expressed in the endoderm adjacent to the heart-forming region play an instructive role in cardiogenic induction (Andree *et al.*, 1998; Schultheiss *et al.*, 1995, 1997) and appear to cooperate with additional growth factors, including FGF (Reifers *et al.*, 2000) and undoubtedly others that are not yet identified. The boundaries of the heart-forming region, known as the cardiac crescent, are also delineated by repressive signals mediated by members of the Wnt family. The expression of secreted Wnt antagonists, such as Crescent and Dkk1, in the cardiac-forming region eliminates this repressive influence, leading to commitment of cells to a cardiac fate (Tzahor and Lassar, 2001; Schneider and Mercola, 2001; Marvin *et al.*, 2001). In this respect, Wnt signaling in vertebrates and flies appears distinct—Wnts inhibit cardiogenesis in vertebrates, but enhance cardiogenesis in flies. The molecular basis for this apparent divergence remains to be determined.

Nkx2.5 and tinman

Identifying the cardiogenic target genes that respond to inductive cues for cardiogenesis remains a fundamental challenge in the field. *Nkx2.5*, a vertebrate ortholog of *tin*, is expressed in the cardiogenic mesoderm concomitant with specification of the lineage and its expression is maintained in the heart until adulthood (Lints *et al.*, 1993; Komuro and Izumo, 1993). *Nkx2.5* is also expressed in a subset of other tissues, including the pharynx, spleen, stomach, and thymus.

Like *tin*, *Nkx2.5* is regulated by an extraordinarily complex series of positive and negative regulatory modules (reviewed in Schwartz and Olson, 1999). Two independent enhancers have been shown to direct transcription in subtly different patterns within the cardiac crescent (Searcy *et al.*, 1998; Lien *et al.*, 1998). Later, as the heart tube undergoes looping and chamber specification, these enhancers and others come to demarcate distinct transcriptional territories (Reecy *et al.*, 1999; Tanaka *et al.*, 1999). Both early cardiac enhancers contain tandem GATA binding sites that are required for cardiac expression.

One of the early *Nkx2.5* cardiac enhancers is activated in response to BMP signaling (Liberatore *et al.*, 2002). Mutational analysis of this enhancer has revealed two sets of Smad binding sites that appear to direct expression at distinct stages of cardiac development (Liberatore *et al.*, 2002; Lien *et al.*, 2002). Smad sites near the 5′ end of the enhancer, as well as in the center of the enhancer, are required for initial activation of *Nkx2.5* transcription in the cardiac crescent. However, the more 5′ sites are dispensable for expression later in development and actually appear to play a negative role, since mutations in these sites enhance expression at later developmental stages. In contrast, the central Smad sites are required for *Nkx2.5* transcription at all stages of cardiac development.

Transcriptional Control of Myocyte Differentiation

As in *Drosophila*, members of the GATA and MEF2 families of transcription factors play key roles in cardiomyocyte differentiation in vertebrates. The ability of these factors to directly interact also provides mechanisms for cooperative activation of target genes that contain binding sites for only one of these families of transcription factors. In addition to their roles in activation of cardiac structural genes, GATA and MEF2 factors have been implicated in the early steps of morphogenesis of the heart tube.

Three members of the GATA family, GATA4, -5, and -6, are expressed in the cardiac lineage at various stages of development (Laverriere *et al.*, 1994; Jiang and Evans, 1996).

In addition to regulating *Nkx2.5* expression, as described above, GATA factors control numerous downstream cardiac muscle structural genes. GATA factors do not possess the ability to activate the cardiac gene program alone; rather they form complexes with other transcription factors, including Nkx2.5 and MEF2, to activate specific sets of target genes (Durocher *et al.,* 1997; Lee *et al.,* 1998; Sepulveda *et al.,* 1998).

Among the cardiac GATA genes, the regulation of *GATA6* has been analyzed in greatest detail. The *GATA6* gene contains multiple cardiac enhancers that depend on the direct binding of Nkx2.5 for activity (He and Burch, 1997; Molkentin *et al.,* 2000). This ability of Nkx2.5 and GATA factors to mutually activate one another's expression and to physically interact to activate cardiac transcription provides a potential mechanism for amplifying and stabilizing the transcriptional program for cardiac gene regulation.

The activity of cardiac GATA factors is influenced by association with the zinc-finger protein FOG-2, an ortholog of Drosphila Ush, expressed in the developing myocardium (Lu *et al.,* 1999; Svensson *et al.,* 1999; Tevosian *et al.,* 1999). FOG-2 has been shown to be required for late steps in cardiac morphogenesis and for signaling from the myocardium to the epicardium, which is essential for proper development of coronary arteries (Svensson *et al.,* 2000; Tevosian *et al.,* 2000). Like USH, FOG-2 can repress the activity of GATA factors; such repression is gene context-dependent since some genes are stimulated by GATA together with FOG.

There are four MEF2 genes in vertebrates, *mef2a*, *-b*, *-c*, and *-d*. Mice homozygous for a null mutation in *mef2c* die at the looping heart tube stage due to cardiovascular abnormalities, with failure to express a subset of muscle structural genes (Lin *et al.,* 1997, 1998). Thus, the functions of MEF2 as a direct activator of myocyte differentiation genes have been conserved from fruit flies to mammals. However, the existence of multiple MEF2 factors in vertebrates, which are expressed in overlapping patterns in muscle cell lineages and which exhibit the same DNA binding specificities and transcriptional activities has made it difficult to fully assess the functions of these factors *in vivo* (Black and Olson, 1998). Nothing is yet known about the upstream activators of *MEF2* gene expression in the vertebrate cardiac lineage.

Transcription Factors That Control Heart Morphogenesis and Patterning

Several transcription factors have been shown to be required for morphogenesis and patterning of the vertebrate heart, based on the phenotypes of mutant mice or zebrafish. The bHLH factors dHAND and eHAND are expressed throughout the linear heart tube before becoming restricted to the right and left ventricular chambers, respectively (Srivastava *et al.,* 1995). Loss-of-function mutations of mouse dHAND result in ablation of the right ventricular chamber (Srivastava *et al.,* 1997). In the zebrafish, which has a single ventricular chamber, there is only a single HAND gene, *dHAND*, which is essential for the formation of this segment of the heart (Yelon *et al.,* 2000). The direct target genes for HAND factors remain to be identified. A single *HAND*-like gene is expressed throughout the dorsal vessel of *Drosophila*, but its function has not yet been determined (Moore *et al.,* 2000).

The *HAND* genes are particularly interesting because of their chamber-restricted expression patterns, which indicate that the *cis*-regulatory elements for these genes must encode not only cardiac-specificity but also be capable of interpreting right vs left ventricular chamber identity. Right ventricular expression of *dHAND* is dependent on a cardiac-specific enhancer that contains two essential GATA binding sites (McFadden *et al.,* 2000). Since GATA factors show no chamber-specificity, additional factors must cooperate with GATA factors to govern the activity of this enhancer. Whether the right ventricular specificity of this enhancer reflects the existence of right ventricular activators or left ventricular suppressors of *dHAND* transcription remains to be determined.

The T-box transcription factor Tbx5 is also expressed in the developing heart and acts synergistically with Nkx2.5 to activate the *atrial natriuretic factor* (*ANF*) and *connexin 40* genes (Durocher *et al.,* 1996; Bruneau *et al.,* 2001). Mutations in *Tbx5* in mice and humans result in severe abnormalities in posterior segments of the heart that include atrial and ventricular septal defects and conduction abnormalities. Based on the expression of the related T-box gene, *H-15*, in the *Drosophila* dorsal vessel (Griffin *et al.,* 2000), it is tempting to speculate that the functions of T-box proteins have been evolutionarily conserved in the heart, but this has not yet been demonstrated.

The Irx4 transcription factor is expressed specifically in the ventricular chambers of the heart (Bao *et al.,* 1999). Ectopic expression of Irx4 in the atrial chambers results in activation of the ventricular-specific myosin heavy chain gene (*VMHC1*), whereas elimination of Irx4 expression in the ventricular chambers results in activation of atrial myosin heavy chain (*AMHC1*) gene expression. Whether Irx4 activates the entire program for ventricular gene expression and represses the entire atrial program, or whether its activity is limited to activation and repression of the *VMHC1* and *AMHC1* genes, respectively, remains to be determined. An enhancer associated with the slow myosin heavy chain gene, which is expressed specifically in the atrium, is repressed in ventricular myocytes by Irx4 in combination with the vitamin D and retinoid X receptor (Wang *et al.,* 2001a). The orphan nuclear receptor COUP-TF II, an ortholog of *Drosophila* Seven-up, has also been implicated in atrial development (Pereira *et al.,* 1999).

Myocardin

Recently, a novel transcription factor, called myocardin, was identified and shown to be coexpressed with Nkx2.5 in

the early heart field (Wang *et al.*, 2001b). Myocardin, which belongs to the SAP (SAF-A/B, Acinus, PIAS) family of chromatin-remodeling proteins, forms a ternary complex with serum response factor (SRF). Recruitment of myocardin to SRF binding sites associated with cardiac-specific genes results in potent transcriptional activation. Dominant negative mutants of myocardin that can associate with SRF but lack the transcription activation domain prevent expression of cardiac genes, including *Nkx2.5*, in injected *Xenopus* embryos, suggesting that myocardin is an essential early regulator of cardiac lineage specification. Two myocardin-related genes have been identified in *Drosophila*, but to date there is no evidence for their involvement in development of the dorsal vessel. On the contrary, SRF is required for the formation of the tracheal system in flies (Guillemin *et al.*, 1996), which mediates oxygen transfer to internal tissues. Defects in dorsal vessel formation have not been described in *Drosophila* mutants lacking SRF.

A Transcriptional Network for Vertebrate Cardiogenesis

Comparison of the genetic networks for cardiac development in *Drosophila* and vertebrates (Figs. 2 and 3) makes it clear that there are numerous parallels in the molecules and mechanisms that control the identity of cardiac cells and the differentiation of cardiomyocytes. That some of these molecules have divergent functions in the formation of the heart can be taken as evidence that although the heart "tool kit" is conserved, how it works has evolved independently. While there are still many regulatory interactions to be traced, a striking observation is that very few of the genes functioning in this process act solely within the cardiogenic mesoderm. Signaling by Dpp, Wg, and FGF, for example, occurs in numerous cell types other than mesodermal precursors of muscle, and even Tin and MEF2, the key regulators of cardiac specification and differentiation, are expressed in additional cell types. Many of the signaling molecules and transcription factors that control cardiac development also act at multiple developmental times within the mesoderm. Transcriptional specificity at a given time and in a specific cell type must be achieved by interpretation of inter and intracellular signals combined with cell identity. Understanding how gradients and thresholds of signals are coupled with combinatorial interactions among transcriptional regulators on individual transcriptional modules remains a major challenge for the future.

Finally, we have focused in this review on the fundamental role of *cis*-regulatory interactions as the basis for cardiac gene regulation. However, it should not be forgotten that this represents only the platform upon which the program for cardiac development (and development of any other specialized tissue) is built. Equally important are the additional layers of regulation that are imposed on the *cis*-regulatory information and its associated transcription factors. For example, alterations in chromatin conformation, as well as epigenetic mechanisms, such as DNA methylation, play key roles in the interpretation of DNA sequence information. In addition, protein–protein interactions among transcriptional activators and repressors that do not bind DNA directly are involved in modulating the activities of transcription factors bound to DNA. The convergence of these different regulatory strategies provides for the rich diversity of gene expression patterns that serve as the basis for development.

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