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Cardiac gene regulatory networks in Drosophila

Anton L. Bryantsev and **Richard M. Cripps**[*]
Department of Biology, University of New Mexico, Albuquerque, NM 87131-0001, USA

Abstract

The Drosophila system has proven a powerful tool to help unlock the regulatory processes that occur during specification and differentiation of the embryonic heart. In this review, we focus upon a temporal analysis of the molecular events that result in heart formation in Drosophila, with a particular emphasis upon how genomic and other cuttingedge approaches are being brought to bear upon the subject. We anticipate that systemslevel approaches will contribute greatly to our comprehension of heart development and disease in the animal kingdom.

Introduction

A central goal of developmental biology is to identify and characterize the genes which control the formation of specific cells, tissues, or organs within the body. Much recent progress has been driven by the increasing realization that genes controlling organ formation are implicated in congenital birth defects, as well as diseases in later life. Thus, genes whose normal function is to create cell types in the embryo, can also destroy those structures when mutated. Of equal importance, it is now apparent that genes showing evolutionary conservation in sequence frequently show evolutionary conservation in function. Thus, the analysis of development in model organisms has provided important insight into developmental mechanisms (reviewed in [1]).

It is also apparent that the complex tissues and organs of higher animals arise from the concerted actions of very large numbers of genes influencing cell behavior and function. These genes, many of them regulatory in nature, are known to function as components of regulatory networks; and there is increasing evidence that the genetic networks have also been conserved through evolution. Defining the parameters of such regulatory networks, and how the networks have evolved, has become a central challenge in the field (reviewed in [2]).

One of the earliest organs to form in the mammalian body is the heart. Significant progress has been made in defining the genes which contribute to heart development, in a diverse array of animal models (reviewed in [3-5]). Many of these critical genes play regulatory roles, and the roles of individual genes in contributing to the overall genomic regulatory network for cardiogenesis is becoming clear.

We recently presented a developmental regulatory network for cardiogenesis in Drosophila and mammals, drawing attention to the striking conservation in function for several named factors [3]. While this network provides insight into the cadre of genes controlling cardiac development, it does not provide a sense for how regulatory interactions change over time. In addition, it does not take into account the plethora of new information and new technologies available to the developmental cardiologist. As more data become available, it is incumbent

*Corresponding author. Email: E-mail: rcripps@unm.edu Tel.: +1 505 277 2822 Fax: +1 505 277 0304.

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upon us to find ways to analyze the data and to use it to generate predictive models regarding both normal cardiac development and cardiac disease mechanisms.

In this review, we have updated the gene regulatory interactions that are known to take place during *Drosophila* cardiac development. In doing this, we have parsed the embryonic period to a series of critical stages, and we have focused upon the known regulatory events taking place at each stage. This allows us to visualize cardiac development as a series of interdependent processes, starting prior to gastrulation, and culminating in the formation of a functioning heart tube only 20 hours later.

The embryology of Drosophila cardiac development

Cardiac tissue in Drosophila, as in other animals, arises from the mesoderm. The cellular details of cardiac cell specification and morphogenesis are described in Figure 1. Mesodermal cells are specified early in fly development as a ventral group of cells, which invaginates to form a layer within the embryo (Figure 1A-A”). Mesodermal cells then spread laterally and dorsally, and those that migrate the furthest become restricted to dorsal mesoderm fate via the action of the dorsally-derived TGFß signal, Decapentaplegic (Dpp). This process results in the formation of two bilateral lines of precardiac cells (Figure 1B-B”).

Cells within the dorsal mesoderm become directed to either cardiac fate or trunk visceral mesoderm fate. This diversification occurs in each segment of the embryo and is controlled by the actions of the ectodermally-derived signaling molecules Wingless (Wg), which promotes cardiac fate [6]; and Hedgehog (Hh), which promotes visceral mesoderm fate ([7]; Figure 1C-C”). Whereas visceral mesoderm precursors subsequently migrate internally, the cardiac cells remain close to the ectoderm [8]. Here, a series of intrinsic and extrinsic signals diversify the cardiac cell population to generate distinct subsets of cardiac cell types (Figure 1D). As this process elaborates, the bilateral cardiac precursors migrate dorsally and medially during the embryonic process of dorsal closure. Ultimately, the precursors meet at the dorsal midline to generate an antero-posteriorly oriented tube (Figure 1E, E').

The mature cardiac tube thus comprises a series of distinct cell types: cardial cells comprise the muscular portion of the vessel, and express either the homeodomain protein Tinman (Tin) or the orphan nuclear receptor Seven-up (Svp). In addition cardial cells are patterned along the antero-posterior (AP) axis, such that inflow tracts in the embryo form from the three most posterior sets of Svp cells [9-12]). Surrounding the cardial cells is a group of pericardial cells, which are characterized by diverse patterns of gene expression [13,14]. Pericardial cells are thought to function in detoxification of the hemolymph [15], although given their great diversity as defined by distinct patterns of gene expression, they probably have a number of distinct functions. Indeed, ablation of *even-skipped* (*eve*) -expressing pericardial cells affects cardiac physiology [16]. More recently, a subset of anterior *eve*-expressing pericardial cells was shown to contribute to the adult wing hearts [17]. As will be discussed, the embryonic origin of the Eve pericardial cells is a paradigm to understand how ectodermal and mesodermal cells interact in order to program the formation of a specific cell type within the cardiac mesoderm.

Viewed in this manner, the process of cardiogenesis can be considered as a series of distinct developmental decisions: dorso-ventral patterning of the embryo and mesoderm specification; specification of the dorsal mesoderm and of cardiac fate within the dorsal mesoderm; diversification of the cardioblast cell pool in each segment; AP diversification of cardial cells; and initiation of cardiac differentiation. In the following sections, we shall describe what is known concerning the regulatory circuits for each of these developmental events.

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The dorsoventral axis: several domains contribute to cardiac specification

The first critical decision made during the formation of cardiac tissue is to pattern the embryo along the dorsoventral axis. The dorsoventral patterning mechanism promotes the formation of a gradient of nuclear Dorsal protein from low in the dorsal region, through moderate in lateral regions, to high in ventral regions (Figure 2). On a superficial level, this decision is important since it results in the specification of the mesoderm as the ventral-most group of cells in response to the highest levels of nuclear Dorsal. However, as will be discussed, each dorsoventral domain is critical for cardiac development.

In the most ventral region, the mesoderm is specified. This occurs via the action of Dorsal upon the *twist* gene promoter, for which Dorsal is a direct transcriptional activator [18-20]. *twist* transcription is subsequently maintained by positive autoregulation [21]. Dorsal and Twist, in turn, activate expression of the zinc finger transcription factor *snail* [22] and the FGF receptor *heartless* (*htl*; [23]), among other targets. Snail is a transcriptional repressor of genes whose expression is not required in the ventral region, including *short gastulation* (*sog*) and the FGF ligand *thisbe* (*ths*; [23]). Direct transcriptional targets of Twist in the ventral mesoderm include the cardiogenic gene *tinman* and the muscle regulatory factor *Myocyte enhancer factor-2* [24-26]. Thus, the highest levels of nuclear Dorsal create a mesodermal cell fate, which is robustly maintained by positive autoregulation, and repression of non-mesodermal genes.

In lateral regions of the embryo, the neuroectoderm is specified. Here, low to moderate nuclear concentrations of Dorsal activate expression of at least two sets of genes defining dorsal and ventral subcomponents of the neuroectoderm. These genes include *sog* and *ths* [23,27], each of which encode secreted proteins critical to embryonic development. In this lateral region Twist and Snail are not activated, since their respective Dorsal binding sites are of lower affinity than those of the enhancers for markers of the neuroectoderm. By contrast, *sog* and *ths* are activated by Dorsal in lateral regions since the Dorsal binding sites in their enhancers are of higher affinity and they can thus be activated at lower nuclear concentrations of the Dorsal transcriptional activator.

The most dorsal regions of the embryo are specified by the relative lack of Dorsal protein in their nuclei. Cells in this region are fated to become dorsal ectoderm, and will express such genes as *dpp*.

What is the relationship between specification of these three broad embryonic regions, and cardiac development? Following invagination of the mesoderm, Twist-expressing cells spread laterally over the inside surface of the embryo, and many cells migrate dorsally. Cells that migrate the furthest become restricted to dorsal mesoderm fate, since they come into the vicinity of the dorsally-derived Dpp signal, which is essential for the next step in cardiac cell specification ([28]; see next section).

The importance of the dorsal migration of the mesoderm has been elegantly demonstrated by genetic analysis: mutant embryos which fail to exhibit mesoderm migration have been isolated and characterized, and most of these mutants show defects in the formation of cardiac tissue, since mesodermal cells in such embryos do not receive the Dpp signal. Analysis of the genes exhibiting such phenotypes has revealed that most are members of the fibroblast growth factor receptor (FGF-R) signaling pathway, implicating this signaling pathway in mesoderm migration and (indirectly) in cardiac specification [29-31].

While the FGF-R pathway is activated within the mesoderm, the ligand for the receptor is encoded by a pair of FGF-like genes named *pyramus* and *ths*. As indicated above, *pyr* and *ths* are expressed in the neuroectoderm, and their combined mutation also results in a failure of dorsal mesoderm specification [32]. These findings suggest that the release of ligand from

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the lateral ectoderm provides a source towards which the mesodermal cells might move. As development proceeds, expression of *pyr* and *ths* refines to more dorsal regions, potentially drawing mesodermal cells with them ultimately to contact the Dpp signal emanating from the dorsal ectoderm. The cellular details of this migration have yet to be fully established.

Figure 2 summarizes the regulatory network of events leading to the specification of the dorsoventral domains in the early embryo. A more complete treatment of this process can be found in [33]; here, we have concentrated upon events of direct relevance to cardiac specification.

What is the cadre of genes that is activated during dorsoventral axis specification? This question has been addressed by recent genome-scale studies, which have identified numerous new genes contributing to the realization of dorsoventral fate. The first study [34] used a microarray approach to identify genes expressed in response to either high, moderate, or low levels of nuclear Dorsal protein. This was achieved by comparing transcript levels for mutants which showed either fully ventralized phenotypes (high levels of nuclear Dorsal throughout the embryo in $Toll^{10B}$ mutants), fully dorsalized phenotypes (low levels of nuclear dorsal in *pipe* mutants), or mutants in which there was a uniform moderate level of nuclear Dorsal ($Toll^{rm9}$/$Toll^{rm10}$ mutants).

This study identified roughly 100 genes enriched in each region, several of which had been shown previously to be markers of the respective domains. Moreover, several new genes were identified, which were shown by *in situ* hybridization analysis to be expressed in the location predicted by their calculated levels of expression in the different mutants. Some of the newly-identified genes also have predicted regulatory regions with binding sites consistent with their patterns of activation.

A complementary approach carried out by Zeitlinger et al. [35] utilized ChIP-on-chip to identify targets of Dorsal, Twist and Snail. Here, antibodies against the regulatory factors were used to immunoprecipitate genomic fragments from young embryos. Immunoprecipitated DNAs were then labeled and used in a microarray analysis to identify at the genomic level the many targets of these factors. In addition to identifying enhancer regions predicted based upon existing information or bioinformatics, these studies indicated that there were significantly more target enhancers for each of the tested factors than were previously expected.

Specification of the cardiac field: integration of intrinsic and extrinsic events

The dorsal mesoderm

Specification of the dorsal mesoderm begins when the underlying mesodermal layer comes into the vicinity of the dorsal ectoderm (Figure 1B-B”). In response to the ectodermally produced Dpp signal, the dorsal ridge of the mesodermal layer is specified, as expression of a number of molecular markers becomes restricted to this region. Specifically, expression of the cardiogenic factor *tinman* (*tin*) narrows down from the broad expression in trunk mesoderm to a slimmer band corresponding to the dorsal mesoderm, as ventral mesoderm rapidly loses both *tin* RNA and protein [36-38]. Genetic evidence in support of this model is very strong. Dpp is both required and sufficient for the maintenance of the dorsal mesoderm in stage 9 embryos [28]. Moreover, the function of Dpp receptors is also required for dorsal mesoderm specification [39].

The change in *tin* expression from pan-mesodermal to dorsal mesoderm is a result of switching in its enhancer activities, and has been studied in some detail. Originally, expression of *tin* at the early stages of development is under control of *twist* (Figure 3A). Twist protein directly binds to the enhancer region termed TinB and located in the first intron of *tin*, and activates its

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broad expression in trunk mesoderm, similar to the Twist distribution pattern itself [26]. During formation of the dorsal mesoderm, however, transcription of *tin* comes under the control of a separate enhancer, TinD.

The dorsal mesoderm enhancer of *tin* is directly responsive to Dpp (Figure 3B). Dpp affects the transcriptional program of cells via binding to transmembrane receptors and activating intracellular messenger Smads (for general references see [40] and [41]). In *Drosophila* Dpp binds to the receptors encoded by *Thickvein* (*tkv*) and *punt* (*put*) (Dpp receptors type I and II, respectively [42, 43]), which leads to phosphorylation within the dorsal mesodermal cells of a Smad protein termed MAD (Mothers Against *Dpp*), heterooligomerization of the latter with yet another Smad protein, Medea, and translocation of this complex into the nucleus. In the nucleus of dorsal mesodermal cells the Smad complex binds to the TinD enhancer, located in the 3' region of the *tin* gene [44]. Since Smads are active only in the region where the mesoderm receives Dpp signals from the dorsal ectoderm, this makes the *tin* expression pattern narrower, confining it to the dorsal part of the mesoderm.

An interesting observation from this work was that in order for the Smads to activate the TinD enhancer, Tin protein must also be present [44]. Such autoregulation of *tin* is an important requirement for confining the cardiac field to the mesodermal cells, and not other germ layers which also receive the Dpp signal. Thus, pre-existing Tin protein, accumulated as a result of *tin* transcription and activated initially by Twist, provides a mesodermal context for reception of the Dpp signal (Figure 3B).

Several other genes with critical cardiogenic roles also become expressed at this stage of cardiac specification. These genes encode the GATA factor Pannier (Pnr; [45,46]); the three T-box genes Dorsocross 1-3 (Doc1-3, [47]); and the LIM homeodomain transcription factor Tailup/ Islet 1 (Tup; [48]). Other than the observation that Tin is a direct activation of the *pnr* gene [49], the direct regulators of these genes have yet to be defined in detail. Nevertheless, it is highly likely that both Tin and the Dpp pathways play major roles in their activation in the dorsal mesoderm.

The cardiogenic mesoderm

As development proceeds, the regions of the dorsal mesoderm that give rise to cardiac tissue are further refined. This confining of cardiac mesoderm is due to an interplay of activating and inhibitory stimuli received on top of the Dpp/Smad pathway, mediated by the segment polarity gene *wingless* (*wg*), encoding a member of the Wnt family [50,51]. Wg is produced in the ectoderm in transverse stripes that intersect perpendicularly with the Dpp secreting regions to generate ten clusters of cardiogenic cells (Figure 1C'). The Wg signaling is essential for cardiogenesis because in *wg* mutants, and in mutants for members of the Wg signaling pathway, no cardiac mesoderm is formed [6,52]. Meanwhile, dorsal mesoderm patches that do not receive the Wg signal but that still express Tin, differentiate into visceral mesoderm (Figure 3C; [7,28,53]).

Genetical analyses have identified several important intracellular transducers of the Wg signal in cardiac specification, including the scaffold protein Dishevelled (Dsh) and the genetically more distal transducer Armadillo [52,54,55]. Detailed mechanistic studies in several cell types have revealed that Dsh works through aggregating Wg-bound receptors (Arrow [56] and Frizzled-2 [57]) into aggresomes on the plasma membrane, followed by phosphorylation of intracellular receptor domains and thereby transduction of the Wg signal intracellularly [58]. Based upon the data of Park et al. [52] this pathway also functions in cardiac specification.

One of the terminal acceptors of the Wg signal is the beta-catenin transcriptional regulator Armadillo (Arm). In the absence of signaling, Arm is phosphorylated and rapidly degraded.

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However, upon stimulation (probably by recruiting the effector kinase, GSK3/zw3/shaggy, to the Wg receptor aggresomes), hypophosphorylated Arm translocates into the nucleus, where it performs multiple regulatory functions. One of the well-know partners of Arm is Pangolin (Pan), the only *Drosophila* member of the TCF family of transcriptional regulators [50]. Arm binds to Pan at its N-terminus and the resulting complex gains transcriptional activity. Interestingly without Arm, Pan mediates a quite opposite function of inhibition of the same set of target genes, because in its default state it is bound to the transcriptional repressor Groucho [59]. Hence, Armadillo is a classical example of a transactivational switch. There have been proposed additional partners of Armadillo (of both a positive and negative nature) including Pygopus, [60], CtBP [61], and others [50,51].

To date, direct transcriptional impact of the Wg signaling pathway in cardiogenesis has been demonstrated in only a few cases. The most striking, as well as the most studied, example is activation of the *even-skipped* (*eve*) gene. *eve* expression is commonly used as a genetical marker for progenitors that give rise to subpopulations of pericardial cells and dorsal muscles [62-64]. These progenitors appear first at places within the early cardiac mesoderm, where the Dpp and Wg signals intersect [38], and will be discussed in greater detail in the next section.

The role of the Wg signaling pathways in the activation of other cardiogenic genes has yet to be fully established. Indeed, given the critical role for Wg signaling in cardiac specification, surprisingly few direct targets of Wg in the cardiac cells have been identified. A possible explanation for this fact is that Wg acts, at least partially, through intermediary factors. One such pair of factors are the forkhead domain proteins encoded by the *sloppy paired* genes (*slp-1, slp-2*). Slp1 and Slp2 are critical to cardiac specification [65], and their genes are induced by the Wg signal in transverse mesodermal stripes corresponding to the overlying patterns of ectodermal Wg expression [53]. In the case of *slp-1*, this results from direct transcriptional activation by Pan [66].

A quite opposite situation is found in the Wg-dependent regulation of another dorsal mesodermal gene, *bagpipe* (*bap*; Figure 3C). *bap* codes for a NK-homeobox transcription factor participating in visceral mesoderm development [37]. The *bap* product is inhibitory to cardiogenesis and thereby must be suppressed in the cardiac mesoderm. Still, the early enhancer of *bap* bears a remarkable similarity to that of *eve*: there are Tin and Smad binding sites functioning in a similar manner to that of the TinD enhancer. However, unlike TinD, the *bap* enhancer additionally contains a binding site for Slp proteins. This high affinity Slp-binding site overlaps with a weaker binding site for the transcription factor Biniou and seems to have inhibitory function, repressing *bap* expression in cardiac mesoderm, where Slp proteins are present [53]. Thus, in this example, the Wg signal acts strictly as a suppressor, extending its action remotely via induction of secondary effectors, *Slp*.

Specification of distinct progenitors within the cardiac mesoderm: the Eve pericardial cell

By the end of stage 10 of *Drosophila* embryogenesis, the newly formed cardiac mesoderm comprises spaced clusters of cells situated at the dorsal ridge of the mesoderm, under the intersection points between Dpp and Wg ectodermal signals (Lockwood and Bodmer, 2002). From this presumptive cardiac field, three elements emerge: a subset of dorsal body wall muscles; cardial cells (cardiomyocytes); and pericardial cells. There is still much to learn about the mechanisms that specify cardial cell progenitors at this stage. By contrast, we have some appreciation of how combinatorial inputs of signaling networks specify the segregation and formation of a specific pair of pericardial cells: those which express the homeobox gene *even-skipped* (*eve*). This knowledge serves as a paradigm in our understanding of the complexity of

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cross-talk between different regulatory signals in the dorsal mesoderm, and several of the principles here probably also apply to cardiogenesis.

At late stage 10, mesodermal patches receiving Dpp and Wg signals start expressing the *lethal of scute* (*l'sc*) gene. Although originally *l'sc* has been reported to act in neurogenesis [67], its expression also can be found in the dorsal, lateral and ventral somatic mesoderm: in the mesoderm, expression of *l'sc* appears first in larger clusters of cells, which then condense into smaller groups, each comprising a few expressing cells - these groups of cells will ultimately be further restricted to single cells showing maximum expression of *l'sc* [64]. This temporo-spatial pattern of *l'sc* expression in the mesoderm is thought to mark and outline the processes of selection and specification of muscle and cardiac progenitors [64,68].

Cardiac expression of *eve* appears at early stage 11 in each hemisegment, in a small cell cluster shortly after initiation of *l'sc* expression [62]. *eve* expression is subsequently restricted, similarly to *l'sc*, to a single progenitor cell, named P2 [64]. The P2 cell then divides to produce two founder cells (F2) that give rise to the dorsal muscle DO2 and pericardial cells [69]. The spatial nature of *eve* expression that rapidly subsides to a single cell suggests a sequential programming of cell fate, that firstly establishes the *eve*-expressing cell clusters, and secondly restricts *eve* expression to a single progenitor.

It was found that in addition to the regulatory mechanisms that specify the cardiac field (Tin, Dpp signaling, and Wg signaling), expression of *eve* also depends on a locally activated receptor tyrosine kinase (RTK) transduction pathway, including the FGF receptor Htl [64, 69], the G-protein Ras, and MAP kinase signaling [63]. Thereby, the ectoderm continues to be actively involved in specification of the mesoderm. Moreover, it is clear that proper transduction and transcriptional interpretation of the signal from activated Htl can only be achieved in cells that have been made competent by Wg and Dpp signals [63]. The terminal effector of the RTK/Ras signal, Pointed, has been proposed to participate in full activation of the *eve* enhancer. Nevertheless, forced misexpression of activated Ras did not induce ectopic *eve* expression outside of Dpp-Wg intercross regions [64].

Consistent with the genetic evidence suggesting a plethora of regulatory factors that converge upon the *eve* gene, the enhancer responsible for cardiac expression of *eve* is particularly complex. A series of papers describing the identification and analysis of this enhancer have been published [63,70-72]. These several regulatory interactions are summarized in Figure 4.

The refining of *eve* expression from a cluster (potency group) to a single cell (progenitor) arises from the lineage specifying signaling of Notch (N), and its inhibitory effects upon the RTK/Ras signaling. Genetical studies have revealed that activated Ras induces expression of the membrane-bound Notch ligand Delta [69]. Delta binds to Notch receptors on the surface of neighboring cells and activates N signal (for review see [73]). Activated Notch receptors start suppressing the activating stimuli of the RTK/Ras pathway, while self-stimulating their own activity. According to the current view of the lateral inhibition model, the progenitor cell that becomes singled out of a potency group produces a fraction more Delta than its neighboring cells, thereby shifting the balance between activating (RTK/Ras) and inhibitory (Notch) signals in its neighbors in favor of the latter [74]. These interactions are detailed in Figure 4C.

Recently some progress has been achieved in our understanding of how different signaling pathways which participate in progeny cell specification are integrated. Besides the Notch ligand Delta that communicates between the Ras and N signal pathways [69], another factor Argos [75] inhibits RTK activities responsible for Ras activation [69]. In addition, Canoe has been shown recently to mediate cross-talks between Ras, N, and Wg pathways. Canoe is an intracellular protein that, via its multiple domains, is capable of physical binding to and

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modulating activities of important signal transducers: Notch, Ras, and Dsh (the most proximal component of the Wg signal) [76].

Another important contribution of the N signal in lineage establishment occurs at the first (asymmetrical) division, when individual progenitors (P) produce two founder cells (F) with unequal fates. In the case of the *eve*-positive P2 cells discussed above, one of the resulting F2 cells becomes a muscle founder and gives rise to the DO2 dorsal muscle; while the other daughter cells establishes a subpopulation of pericardial cells [69]. Besides Notch itself [77], genetical studies revealed two genes, Sanpodo and Numb, participating in regulation of asymmetrical division of pericardial progenitors [13,78].

In summary, although a comprehensive picture of how different types of cardiac progenitors emerge is far from being complete, we may assume that the process of progenitor specification follows this general scenario. Wg and Dpp (and probably Twist) signals create potency groups in the dorsal mesoderm, that further undergoes clustering through local activation of RTK signaling and *l'sc* expression. Within each cluster, or equivalence group, a progenitor is singled out as a result of lateral inhibition via the N signal. This concept, originally applicable to formation of a subset of pericardial and dorsal muscles progenitors, may be extended to cardioblasts. The lack of a reliable molecular marker for early cardioblast progenitors complicates studying mechanisms of cardial lineage specification. Nevertheless, recent studies of the origins of Ladybirdexpressing cardioblasts [79], and the identification of the NK homeobox gene *C15* as being expressed in the cardiac mesoderm [72] substantially improve these prospects.

Recent bioinformatic studies have also begun to shed light upon the identification of enhancers which integrate multiple intrinsic and extrinsic signals in the Drosophila embryo. These approaches depend upon the identification of *cis*-regulatory regions which show enrichment of binding sites for candidate transcriptional regulators (see for example [80]). This work was highly effective in predicting the locations of tissue-specific enhancers, in this instance for genes known to be expressed in skeletal muscle founder cells. A similar methodology should be effective for defining enhancers which function within the cardiac mesoderm.

Diversifying the cardiac tube: the anteroposterior axis

As the linear cardiac tube is being formed via the convergence of two rows of cardial cells, cells along the anteroposterior (AP) axis are programmed to assume distinct cell fates. This programming is most apparent in the patterning of Svp cells in the developing dorsal vessel: whereas ten trunk segments contribute cells to the cardiac tube, only in the most posterior seven segments do Svp cells form. There are additionally a number of genes, both regulatory and structural, whose patterns of expression show distinct AP diversification (Figure 5).

The specification of Svp cell fate is important, since Svp cells form the inflow tracts (also called ostia) of the cardiac tube: in the embryo and larva, the most posterior three sets of Svp cells express the Wg signaling molecule and develop into ostia [9,10,12]. Svp cells in the remaining four segments do not form ostia until pupal development, whereupon the Svp cells in these four segments are remodeled into ostia for the adult heart [9,81,82]. There are now a number of genes whose patterns of expression reflect AP fate in the cardiac tube [82,83], and a current challenge is to define how this pattern of gene expression is encoded at the genomic regulatory level.

Important insight into the identity of the factors which must control the AP-restricted expression of cardiac genes has come from analyses of homeotic selector genes of the Bithorax- and Antennapedia Complexes (BX-C and ANT-C, respectively). Studies from several groups have demonstrated that Hox genes are responsible for the AP patterning of the cardiac tube

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(Figure 5). Firstly, *abdominal-A (abd-A)* expression in the posterior heart region is required and sufficient for heart fate. This was demonstrated by both loss- and gain- of functions experiments using *abd-A*, and the analysis of such treatments upon patterns of gene expression and cell function [10-12]. Secondly, *Ultrabithorax (Ubx)* expressed in four segments of the aorta is required and sufficient for Svp cell fate in those segments. This was proven by removal of *Ubx* expression, which resulted in a loss of most of the Svp cells in the aorta; and by ectopic expression of *Ubx*, which resulted in supernumerary Svp cells in the anterior-most three segments [84,85].

A reasonable conclusion from these studies is that Hox gene products directly impact the enhancers of AP-restricted cardiac genes. However, the direct influence of a Hox protein upon a cardiac enhancer has yet to be demonstrated, perhaps because several of the target genes are large and complex, thus complicating traditional approaches for enhancer analysis.

Some progress on this front has come from the recent identification of the *svp* cardiac enhancer (SCE). Ryan et al. [86] used a simple bioinformatics approach to identify a candidate regulatory region for *svp*: locate a 1-kb sequence in the vicinity of *svp* that contains at least two consensus binding sites for the cardiac regulator Tinman, and determine if this sequence is conserved in other Drosophila species. The analysis identified a single location which might correspond to the *svp* enhancer, from a genomic region in excess of 50kb. Testing of the candidate enhancer region confirmed that it was active in the developing Svp cells of the dorsal vessel. Ryan et al. [86] also demonstrated that the SCE was a direct transcriptional target of Tinman during Svp cell specification, however they did not yet determine if this enhancer is a direct target of Hox gene products [86].

Based upon the observation by Ponzielli et al. [10] that *svp* expression is activated within each segment under the positive influence of Hedgehog signaling from the ectoderm, we can hypothesize that the *svp* cardiac enhancer must integrate at least three distinct types of signals: a cardiac-restricted signal, presumably Tinman; an axial signal such as Ubx and abd-A; and a segment-polarity signal from the Hedgehog pathway.

Once activated in the cardiac tissue, *svp* expression must very rapidly become independent of Tin, since studies have indicated that over-expression of *svp* in the cardiac tube functions to inhibit *tin* transcription [87,88]. How *svp* expression is maintained in the cardiac tissue remains to be determined.

Gene expression studies have also identified several additional cardiac structural genes which show AP polarity in their expression patterns ([83]; Figure 5). Based upon the in situ hybridization data, and upon the known expression patters of regulatory genes expressed in the cardiac mesoderm, it is possible to predict the gene regulatory interactions that are acting. Further, given the recent publication of the genome sequences of several Drosophila species, the identification and characterization of enhancers for Hox targets in the dorsal vessel should proceed rapidly.

Cardiac structural genes: a common code for a common pattern of expression?

Expression of a set of genes that directly determine cardiac function is the ultimate goal of the differentiation process, yet as described below, significant diversity is apparent both in patterns of structural gene expression and mechanisms of structural gene regulation. Cardiac structural genes can be tentatively grouped into sub-categories of (i) general muscle-specific genes and (ii) cardiac-specific genes, although there are also several genes, such as *svp* and *brokenhearted*, which show expression in rather disparate tissues including the cardiac

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mesoderm and the central nervous system, among others. In the first category are genes that encode proteins forming and regulating the contractile apparatus: muscle-specific actin (*Act57B*), myosin heavy-chain (*Mhc*), and several others. The second category contains genes comprising diverse functions, including those whose products code for ion transporters (*Sur, Ih*; [83,89,90]), structural proteins (*Pericardine*; [91]), receptors (*Toll*; [92]), and enzymes (*Transglutaminase*; [93]).

The number of genes that are expressed in the dorsal vessel is under continuous growth, as more in situ hybridization data become available via public databases, mainly the Berkeley Drosophila Genome Project [94]. A recent query via the FlyExpress search engine (url: http://www.flyexpress.net) for genes associated with the ontology term `embryonic dorsal vessel' returned over 60 hits. More candidate cardiac genes may be obtained using a computational prediction approach: recently a prediction for the identification of novel genes expressed in muscle was made, based on microarray expression data and employing machine learning algorithms [95]. A similar methodology might be used for the identification of genes expressed in cardiac tissue.

For the transcriptional activation of pan-muscular structural genes, the crucial input most likely comes from the activity of Myocyte enhancer factor-2 (MEF2). In *Mef2* mutants all types of muscles (including cardioblasts) are specified normally, but the mutants entirely lack expression of muscle structural genes [96-98]. In several cases MEF2-binding sites within an enhancer region of the target structural gene contribute much of the transcriptional activity [99-101] [102]. However, despite a strong requirement for MEF2 function in terminal muscle differentiation, ectopic expression of *Mef2* was unable to induce the myogenic program [103]. A possible explanation for such a failure may be a requirement for other co-factors that could synergize with MEF2 upon its target genes. Thus, for early stages of development a role for Twist as a co-activator acting along with MEF2 was deduced, based upon ChIP-on-chip assay results [104]. For the late stages of muscle development, another MEF2 co-regulator - zinc-finger domain factor Chorion Factor-2 - was recently reported [105].

Consistent with the single-gene identification of MEF2 targets, ChIP-on-chip analysis revealed active MEF2 binding sites in the intronic and/or flanking genomic sequences of many known and putative structural muscle genes [98], and it is anticipated that a large number of these target genes are expressed in cardiac tissue. In addition, over 600 total genomic regions were shown to interact with MEF2 during embryogenesis, confirming that MEF2 plays a major role in gene activation.

In contrast to our understanding of the regulation of structural muscle genes, there is much less known about the regulation of cardiac-specific or cardiac-enriched structural genes. The expression of these genes is MEF2-independent in many cases, and more likely depends upon cardiac factors such as Tinman. Indeed, it has been shown that Tinman directly regulates expression of a number of such genes. These include: *Sulfonylurea receptor* (*Sur*; [89,90]); the trans-membrane receptor gene *Toll*, which predominantly requires the function of a single Tinman-binding site to drive expression in cardioblasts, [92]; the ß3tubulin gene [106]; and the basic, helix-loop-helix factor Hand [71]. These examples suggest a significant role of Tinman in the late stages of heart development.

To test this notion, Zaffran et al [107] generated a genomic rescue fragment of *tin*, comprising the entire transcribed region, and all of the *tin* enhancers with the exception of TinC, which is active in the mature cardiobasts. *tin* null mutants rescued with this construct showed normal cardiac specification and tube formation, however significant aspects of cardiac differentiation were abrogated. This study confirmed a critical and sustained role for *tin* in cardiac development.

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Since *tin* is only expressed in a subset of muscular cells in the dorsal vessel, one must also consider how genes expressed in the Svp cells are regulated. To date, relatively few genes whose expression is restricted to these cells have been characterized as to their regulators. One of these is *svp* itself, for which the SCE is activated at stage 12 by Tin [86]. Activity of this enhancer does not persist at stage 16, however, when *tin* and *svp* expression are mutually exclusive [87]. A Svp cell marker active in the mature dorsal vessel is the crosslinking factor encoded by *Transglutaminase* (*Tg*), which is expressed predominantly in Svp cells from stage 14 onwards. *Tg* is activated in the cardiac tube via a MEF2-dependent enhancer, yet how the Svp cell specificity of this enhancer is determined has yet to be clarified [93]. A summary of cardiac structural gene regulatory mechanisms is presented in Figure 6.

Concluding Comments

Significant progress has been made over the last few years in identifying how cardiac cells are specified in the embryo and how their differentiation is regulated. It is now apparent that the process of cardiogenesis, in organisms from flies to man, arises from the action of a conserved network of regulatory interactions. New technological approaches are further identifying genes which are expressed in the cardiac tissue, defining their functions in that tissue, and demonstrating how their expression might be regulated during development.

We can anticipate a time when the entire set of genes that are expressed in the cardiac tissue during specification and differentiation has been identified. The Berkeley Drosophila Genome Project is in the middle of a sustained effort to define the expression patterns of all predicted genes in the genome [94]. This information will be critical in identifying what genes contribute to the cardiac phenotype.

Coupled with this, the identification of transcriptional targets for all of the critical cardiogenic factors (Tinman, Pannier, T-box factors (H15, Midline, Doc1-3), Tailup/Islet-1 and Hand) should be uncovered. Data in this area is likely to arise from analysis of single-gene enhancers according to traditional methodologies. It is also likely to be overtaken by array-based studies of changes in transcription factor levels in mutant backgrounds, and ChIP-on-chip studies to define the cadre of genomic regions bound by candidate transcription factors. This has already been achieved for critical mesodermal factors such as Twist and MEF2; parallel studies for the cardiogenic factors discussed above cannot be far behind. Also, computational approaches are rapidly developing to a stage where the enhancers for cardiac-expressed genes can be identified by searching for clusters of bindings sites for cardiac regulatory factors.

These innovations will significantly move us towards solving the problems of cardiac development and disease discussed at the start of this communication. Nevertheless, while each new discovery brings us closer to a more systemic understanding of cardiac biology, we anticipate that there is still much more to be learned. The transcriptional techniques and studies that are currently predominant in the cardiac development field do not take into account the modifications that both primary transcripts and polypeptides undergoes within the cell, nor do they integrate epigenetic effects upon gene expression and regulation. How this problem will be solved at the systems level remains to be determined.

Furthermore, only recently have we become aware of the importance of non-coding RNAs in biological systems. While micro-RNAs are already being associated with gene regulation in the mesoderm in *Drosophila* [108], and in cardiac development in mammals [109,110], a concerted effort to identify and characterize micro-RNAs expressed in the cardiac tissue has yet to be carried out, and such studies could have a profound effect upon our appreciation of the cardiac developmental regulatory network. These considerations remind us that, the more we discover in our search for understanding a biological system, the more complex and elegant that system becomes.

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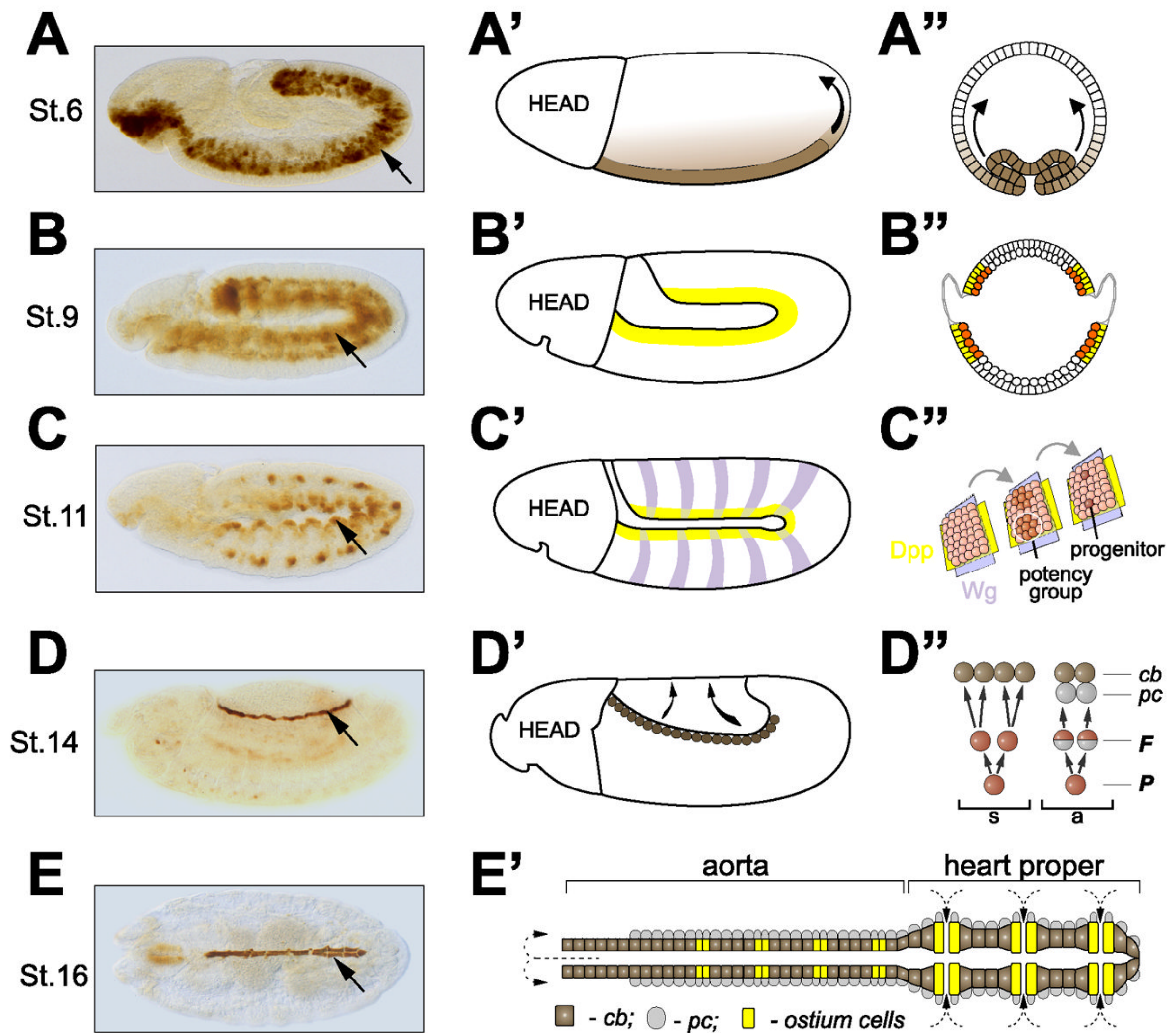

Figure 1. Morphological aspects of cardiogenesis in the *Drosophila* embryo

A. The lateral side of a stage 6 embryo immunohistochemically stained for expression of a *Mef2-lacZ* transgene, to outline the newly forming mesoderm (arrow). A'. Schematic representation of the lateral view of an early embryo (stages 4-6) outlining the position of the mesodermal layer (dark brown) and the gradient of nuclear Dorsal (brown). The arrow shows the direction of movement of the extending germ band. A". The same as in A' but transverse section is shown. Arrows indicate directions of spreading for invaginating mesodermal cells. B. The lateral view of a stage 9 embryo immunostained for *Mef2-lacZ* expression to reveal the dorsal mesoderm (arrow). B'. Representation of the embryo shown in B, highlighting in yellow the dorsal ectodermal region that produces Dpp. B". Transverse section of the same embryo, depicting zones of Dpp-producing dorsal ectoderm (yellow) and underlying Dpp-receiving dorsal mesoderm (orange). C. The lateral view of a middle-stage embryo immunostained for *Mef2-lacZ* expression to reveal clusters of cardiac mesoderm (arrow). C'. The cartoon of the embryo shown in C demarcating the ectodermal zones secreting Dpp (yellow) and Wg (blue), at the intersection of which the cardiac mesoderm is formed. C". Simplified flow chart of events leading to cardiac progenitor specification: cardiac mesoderm (pink) is specified under the intersection of Dpp (yellow stripe) and Wg (blue stripe) ectodermal signals; within cardiac mesoderm local clusters of potency groups (red) appear due to ectopic local activation of the

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RTK pathway; within each potency group a progenitor (dark red) is singled out as a result of the Notch signal activity. D. The lateral view of a late-stage transgenic embryo expressing *lacZ* under control of the cardioblast-specific *Sur* enhancer; immunostaining for beta-galactosidase reveals a row of cardioblasts. D'. The cartoon of a similar-stage embryo as shown in D depicting a row of cardioblasts migrating dorsally (arrows) to merge with cardioblasts from the opposite side to form the dorsal vessel. D". Predicted models of cell division events leading to formation of cardioblasts. The symmetrical division model (bracket s; which gives rise to Tin cardial cells) is where progenitors (P) give rise to identical founders (F) and, subsequently, cardioblasts (cb). In the asymmetrical model (bracket a; which specifies Svp cells) founder cells give rise to two cardiac cells subtypes: cardioblasts (cb) and adjacent pericardial cells (pc, grey color) due to Notch-mediated asymmetrical divisions. E. The dorsal view of a stage 16 embryo showing the mature embryonic dorsal vessel (arrow) visualized by immunostaining for beta-galactosidase expressed under the control of the cardioblast-specific *Sur* enhancer. E'. Organization of the embryonic dorsal vessel. The morphologically distinctive thinner aorta lies anteriorly; the thicker heart proper, containing inflow tracts (termed ostia; yellow), are indicated. Hemolymph flow is shown by dashed arrows. Note, that the number of grey pericardial cells (pc) is more than shown and additional cells are located both above and below of the cardioblasts (cb). Anterior is to the left and dorsal is uppermost, in all panels unless indicated.

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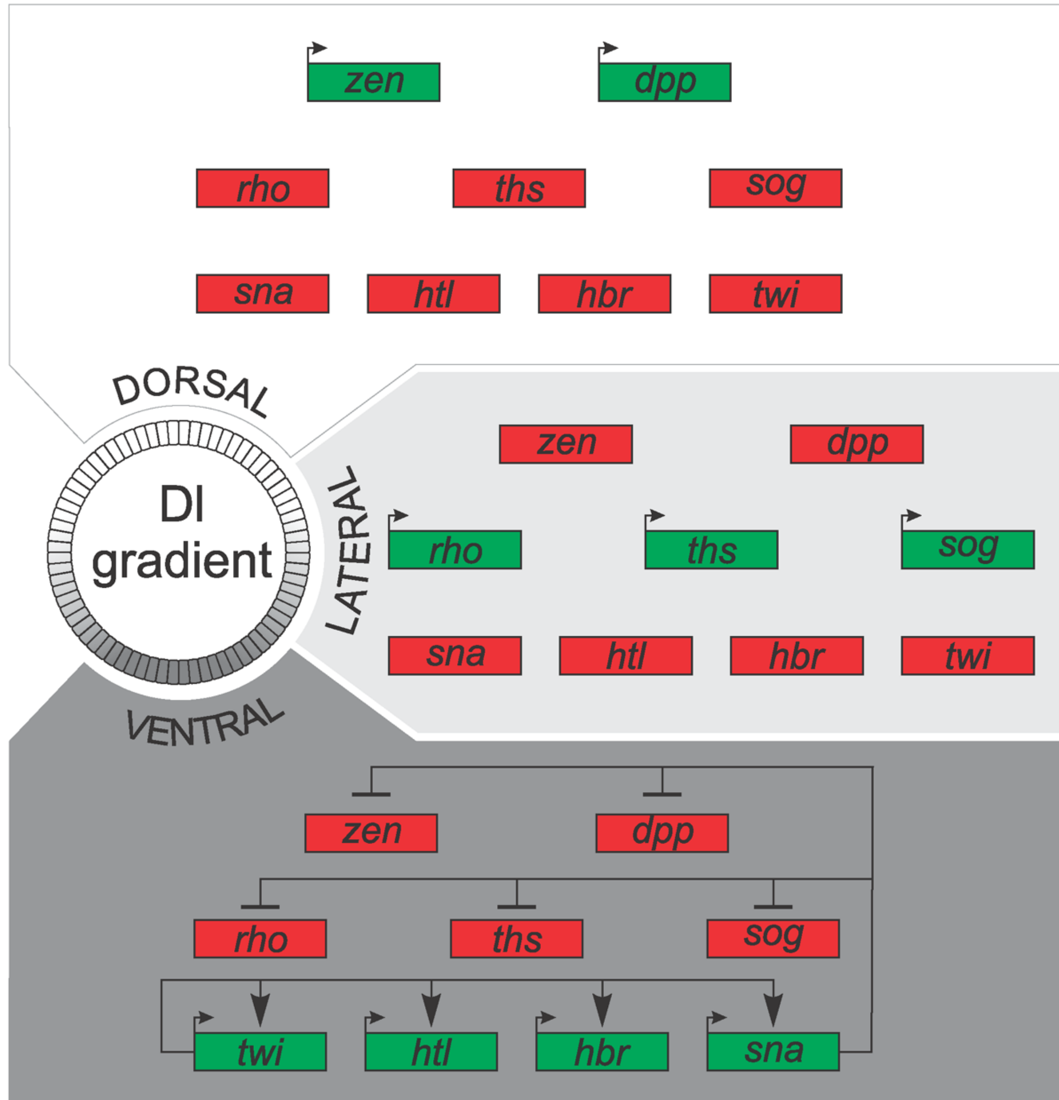

Figure 2. Gene regulatory networks controlling specification of the dorsoventral axis

The fertilized embryo can be functionally subdivided into three main regions. **Ventrally**, the mesoderm is specified by the highest levels of nuclear Dorsal protein, which results in the activation of genes characteristic of the mesoderm, and by active repression of neuroectodermal genes by the *sna* gene product. **Laterally**, the neuroectoderm is specified by moderate levels of nuclear Dorsal, which are sufficient to activate genes such as *rho* and *ths*, but which are at insufficient levels to activate the *sna* repressor gene. **Dorsally**, the relative lack of nuclear Dorsal protein permits expression of genes such as *zen* and *Dpp* which specify dorsal ectoderm and amnioserosa fate.

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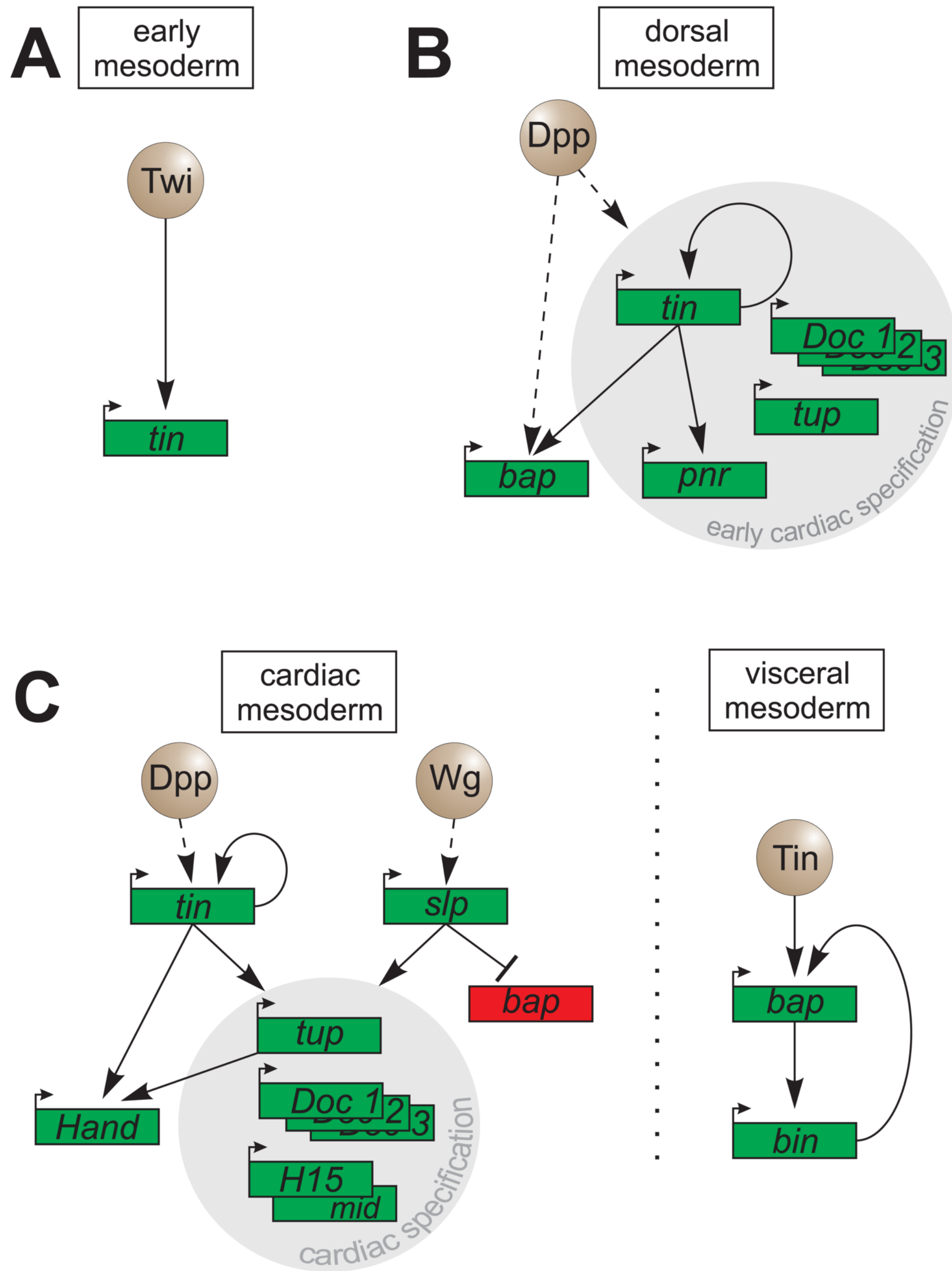

Figure 3. Gene regulatory interactions in the formation of the dorsal mesoderm and cardiogenic mesoderm

Direct and indirect interactions are shown in solid and dashed lines, respectively. Protein factors are shown as circles; genes are represented as boxes with their transcription status indicated in color. A. In early mesoderm Twi initiates expression of *tin*. B. In the dorsal mesoderm the *tin* expression is maintained through the Dpp signaling and self-activation. Dpp also probably stimulates a number of genes important for early cardiac specification (shaded in grey circle). Additionally, Dpp along with Tin activate expression of the pro-visceral mesodermal gene *bap*. C. At the moment when the dorsal mesoderm receives Wg signal it becomes cardiac mesoderm (left panel) in which cardiac specification genes (shaded in grey

circle) are activated by products of *tin* and *slp* genes, controlled by Dpp and Wg, respectively. At the same time Slp effectors suppress expression of *bap*. Genes expressed in cardial and pericardial cells, like *Hand*, also depend on *tin* activation and additionally require a product of one of the cardiac specification genes (e.g. *tup*). Meanwhile, in the absence of inhibitory Wg signal in the visceral mesoderm (right panel), the transcriptional activity of *bap* goes on, fed by stimulatory inputs from Tin and the genetically downstream gene *bin*. Bap suppresses the expression of pro-cardiac genes.

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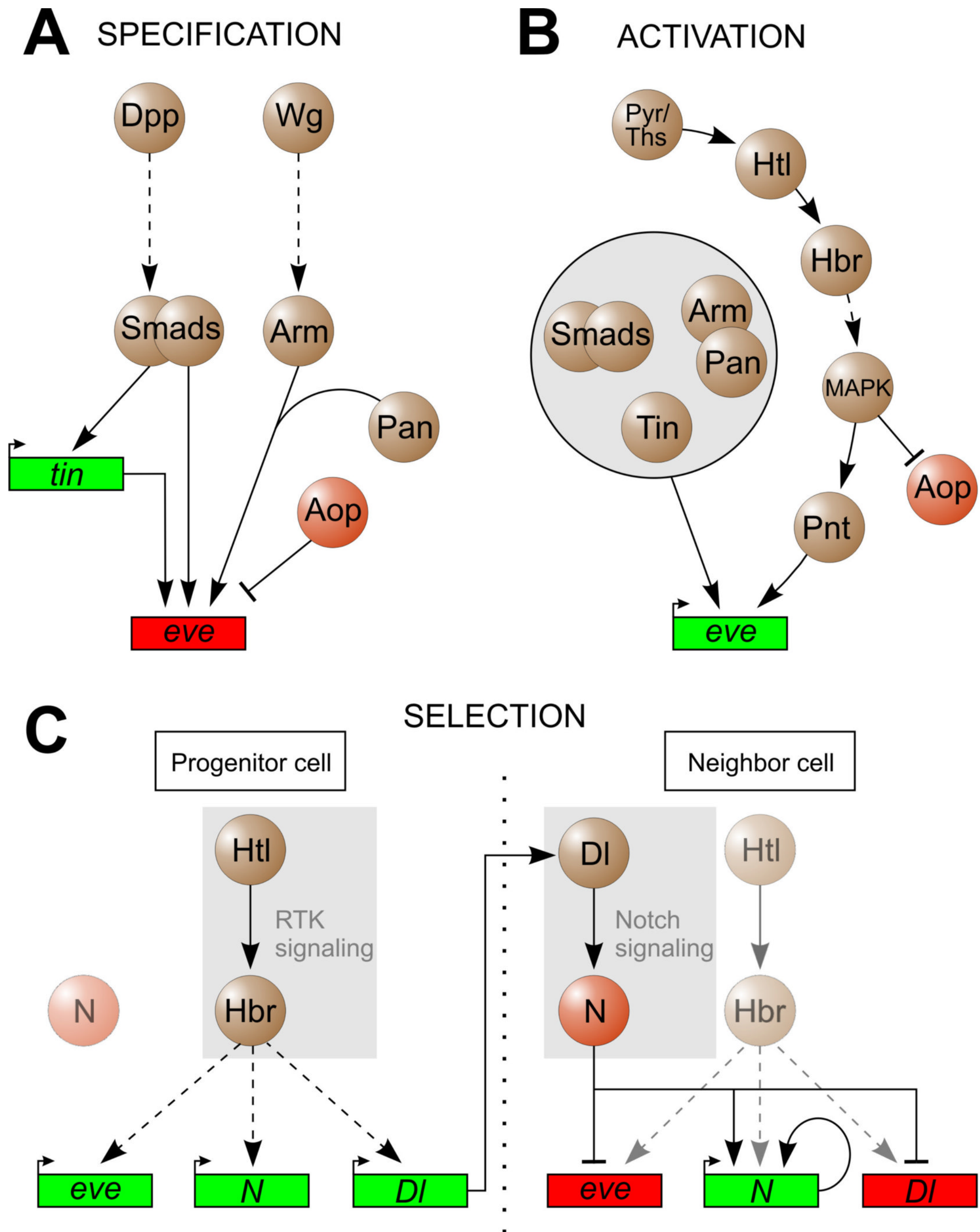

Figure 4. Interaction events governing expression of the *eve* gene

Direct and indirect interactions are shown in solid and dashed lines, respectively. Protein factors are shown as circles; genes are represented as boxes. A. The specification of future *eve* expression is mediated by the Dpp signaling that activates Smad effectors (Mad and Medea) that oligomerize and positively control *tin* expression. In parallel, Wg signaling stabilizes the transcription activator Arm and promotes its formation of a complex with the DNAbinding factor Pan and releasing it from a transcription repressor (not shown). Dpp and Wg specify the location of future *eve* expression as their contribution is equally important and thereby *eve* activation can be achieved only at Dpp and Wg signals intersection zones (see Fig.1 C'); the *tin* gene is active only in the mesoderm and thereby provides the mesodermal context to *eve*

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activation. However, the activity of the transcriptional repressor Anterior open (Aop) puts *eve* expression on hold (yellow shading). B. Within the cardiac mesoderm, a local activation of the receptor Htl by Pyr or Ths ligands subsequently leads to activation of the MAPK pathway, attendant activation of the transcription factor Pnt, and inactivation of the repressor Aop. The cooperative stimulatory inputs activate *eve* transcription (green) in small clusters of cardiac mesoderm. C. The final process of singling out a progenitor cell (left side) from adjacent neighboring cells (right side) within a cluster of *eve*-expressing cells. In the progenitor, the active RTK signaling pathway activates expression of the *eve* marker gene along with components of the Notch signal: the receptor Notch (N) and its ligand Delta (Dl). Here, the components of the RTK signaling, Htl and Hbr, are also self-maintained, while the Notch receptors are not active (shown in subdued colors) in the absence of Delta received from the neighboring cells. In contrast, neighboring cells receive the Delta signal produced by the progenitor cell, which leads to activation of N and suppression of the RTK signaling (subdued) and most of its targets, including the *eve* and *Delta* genes. Meanwhile, the Notch receptor stimulates its own expression to maintain and amplify the N signal. The neighboring cell cannot activate N signaling in the progenitor cell, as its own Delta production is suppressed by active Notch.

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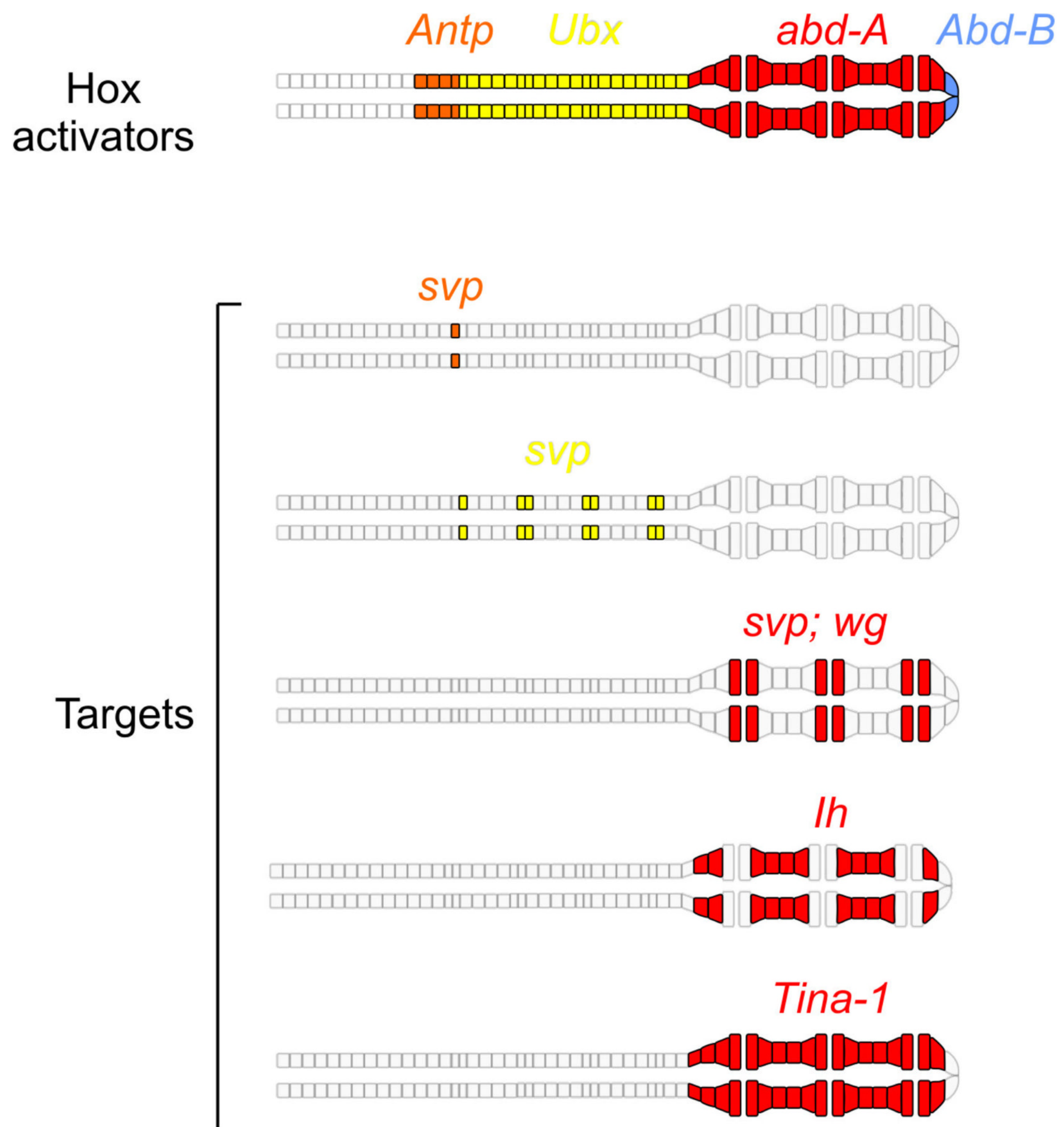

Figure 5. Schematic representation of the spatial expression of various Hox regulators and their putative targets in the Drosophila dorsal vessel

Top panel: a cartoon of dorsal vessel showing the color-coded expression patterns of indicated Hox genes: *Antennapedia* (*Antp*, orange), *Ultrabithorax* (*Ubx*, yellow), *abdominal-A* (*abd-A*, red), and *Abdominal-B* (*Abd-B*, blue). Note the sequential expression of *Antp* and *Ubx* in the aorta and mostly exclusive expression of *abd-A* in the heart proper. No Hox genes are expressed in the most anterior region of the aorta. Anterior is to the left. Lower panel: expression of putative Hox target genes, color-coded to match their potential Hox activators (from top to bottom): *Antp*-dependent *seven-up* (*svp*) expression in the anterior-most pair of Seven-up expressing cells (future adult ostia cells); *Ubx*-dependent *svp* expression in the rest of the aorta Seven-up cells; *svp* and *wg* expression in ostia cells in the heart proper depend on *abd-A*; expression of *Ih* and *Tina-1* are also *abd-A*-dependent although these genes are expressed in different patterns. There is no *Abd-B*dependent cardiac target found up to date.

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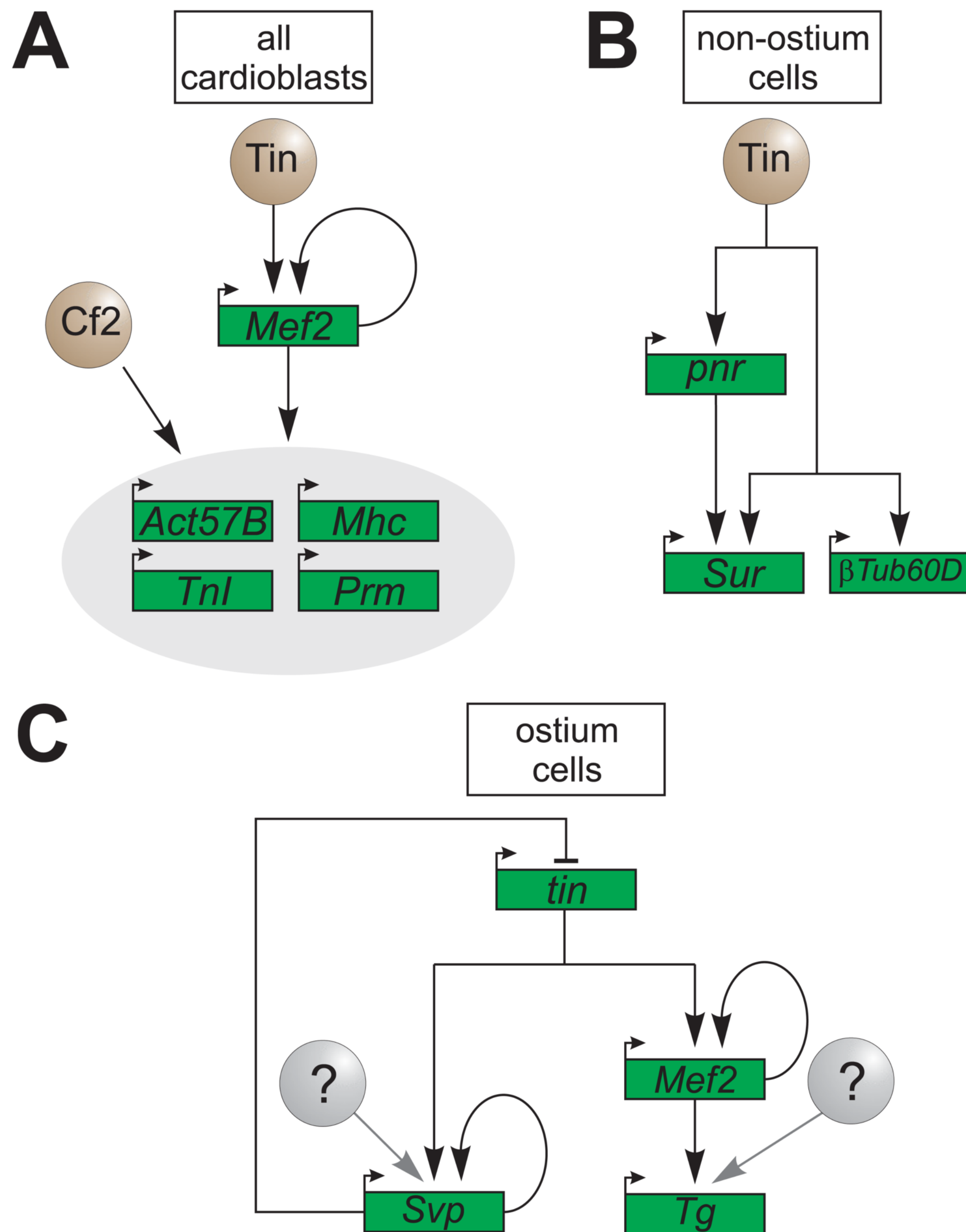

Figure 6. Transcriptional regulatory pathways in cardiac differentiation

The functioning of the cardiac tube is characterized by the expression of some genes throughout the cardiac tube and other genes detected only is specific subsets of the cardiac musculature, such as the Tin and Svp cells. Muscle structural genes, which in flies are expressed in all muscles types, depend centrally upon: MEF2, which is essential for muscle structural gene expression; and CF2, which collaborates with MEF2 in activation of structural genes. Of the genes whose expression is restricted to the Tin cells, all of those currently characterized are direct transcriptional targets of Tin. Tin can collaborate with the GATA factor Pnr (which is also activated by Tin) in activation of target genes. For the relatively few genes whose expression is predominant in the Svp cells *Tg* is a MEF2 target, whereas *svp* expression is initially activated by Tin but quickly becomes independent of it.