Cardioblast-intrinsic Tinman activity controls proper diversification and differentiation of myocardial cells in Drosophila

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The NK homeobox gene tinman (tin) is required for the specification of the cardiac, visceral muscle and somatic muscle progenitors in the early dorsal mesoderm of Drosophila. Like its vertebrate counterpart Nkx2.5, the expression of tin is maintained in cardiac cells during cardiac maturation and differentiation; however, owing to the complete lack of a dorsal vessel in tin mutant embryos, the function of tin in these cells has not been defined. Here we show that myocardial cells and dorsal vessels can form even though they lack Tin, and that viable adults can develop, as long as Tin is provided in the embryonic precardiac mesoderm. However, embryos in which tin expression is specifically missing from cardial cells show severe disruptions in the normal diversification of the myocardial cells, and adults exhibit severe defects in cardiac remodeling and function. Our study reveals that the normal expression and activity of Tin in four of the six bilateral cardioblasts within each hemisegment of the heart allows these cells to adopt a cell fate as ‘working’ myocardium, as opposed to a fate as inflow tract (ostial) cells. This function of tin involves the repression of Dorsocross (Doc) T-box genes and, hence, the restriction of Doc to the Tin-negative cells that will form ostia. We conclude that tin has a crucial role within myocardial cells that is required for the proper diversification, differentiation, and post-embryonic maturation of cardiomyocytes, and we present a pathway involving regulatory interactions among seven-up, midline, tinman and Dorsocross that establishes these developmental events upon myocardial cell specification.

KEY WORDS: Dorsal vessel, Drosophila, tinman, Dorsocross, Nkx2.5, seven-up, Heart, Repressor

INTRODUCTION

In a wide variety of organisms, both vertebrate and invertebrate, cardiogenesis requires the action of regulators that belong to the NK2, GATA and T-box families of transcription factors (Cripps and Olson, 2002; Buckingham et al., 2005; Reim and Frasch, 2005). In most cases, the expression of each of these cardiogenic factors spans a large developmental window of cardiogenesis and continues in the mature heart. Consequently, the specific function of a cardiogenic factor can change during the progression of cardiogenesis because, for example, it depends on the presence of additional factors and signals that modulate its activity. Although several examples of differential and stage-specific functions of cardiogenic factors have already been described (Pashmforoush et al., 2004; Zeisberg et al., 2004; Oka et al., 2006), our overall knowledge of these dynamic changes in regulatory activities is still very limited.

The apparent conservation of cardiogenic factors during evolution suggests strongly that some of their specific molecular functions within the regulatory network of cardiogenesis and heart differentiation may also be shared among different vertebrate and invertebrate species. Prime examples of conserved cardiogenic factors include the Drosophila NK homeodomain factor Tinman and the homologous Nkx2.5 proteins in vertebrates, which have been shown to play key roles during early stages of heart formation in the respective organisms (Harvey, 1996). In Drosophila, the tinman (tin) gene is essential for the specification of all cardiac progenitors in the early dorsal mesoderm, and some of its target genes during this event, and the functional Tin-binding sites in their regulatory sequences have been defined by genetic and molecular analyses (Azpiazu and Frasch, 1993; Bodmer, 1993; Xu et al., 1998; Halfon et al., 2000; Gajewski et al., 2001; Knirr and Frasch, 2001; Han et al., 2002). The requirement for tin during the earliest steps of cardiogenesis is reflected in its early expression in the mesoderm, which is controlled by two distinct enhancer elements (Yin et al., 1997). Initially, during the invagination and spreading of the mesoderm, tin expression is activated by the bHLH protein Twist through an intronic enhancer in the entire trunk mesoderm (Bodmer et al., 1990; Yin et al., 1997). Thereafter, tin expression becomes dependent on a Dpp-responsive enhancer located downstream of tin, which leads to the restriction of tin expression to dorsal mesodermal cells that receive Dpp signals from the ectoderm (Xu et al., 1998). This corresponds to the stage when tin is required for the specification of myocardial and pericardial progenitors within the dorsally located cardiogenic mesoderm. In addition, tin is essential for the formation of other dorsal mesodermal derivatives during this stage, which include trunk visceral mesoderm precursors and dorsal somatic muscle progenitors. Even later, tin expression is further restricted to cardiac progenitors and this expression persists in myocardial and pericardial cells of the mature dorsal vessel. tin expression in cardioblasts depends on the Tbx20-related genes midline (mid) and H15, and is driven by another downstream enhancer (Yin et al., 1997; Reim et al., 2005). It is thought that tin plays a role in the differentiation of the heart progenitors during this late phase of expression, which is likely to include the direct activation of MeF2 and Hand, which regulate normal differentiation, as well as of cardiac differentiation genes such as β3-tubulin (Bour et al., 1995; Lilly et al., 1995; Gajewski et al., 1997; Kremsner et al., 1997).
The Drosophila heart is a relatively simple linear tube that, in spite of its overt simplicity, is highly structured and consists of a variety of myocardial and pericardial cell types (Rizki, 1978; Ward and Skeath, 2000). This organization is illustrated by the presence of different chamber-like regions with distinct functions (the heart in the posterior and the aorta in the anterior), and by the controlled posterior-to-anterior flow of the larval hemolymph, which enters through valvular openings (ostia) into the heart region. In addition to its broad anteroposterior (AP) organization, the dorsal vessel maintains a segmental organization, with units that, in most of the tube (i.e. from segments A2 to A7), consist of six pairs of cardioblasts in each segment. The segmental organization and AP polarity within each segment is revealed by the restricted expression of several transcription factor-encoding genes (reviewed by Lo and Frasch, 2003). In fact, the myocardial expression of tin within the dorsal vessel is restricted to the four posterior pairs of cardioblasts within each of these segments. Conversely, the anterior two pairs of cardioblasts in each segment are marked by the expression of seven-up (svp), which encodes an orphan nuclear receptor, and of the Dorsocross T-box genes (Doc1, Doc2 and Doc3, henceforth referred to as Doc) that are downstream ofsvp in these cells (Gajewski et al., 2000; Lo and Frasch, 2001; Reim et al., 2003; Reim and Frasch, 2005). In the heart region, this differential organization correlates with the formation of distinct subtypes of myocardial cells within each segment. Specifically, the two Svp/Doc-positive cardioblasts in each segment differentiate into ostial cells to form inflow valves, whereas the four Tin-positive cells form ‘working’ cardiomyocytes of the heart (Molina and Cripps, 2001). Currently, it is not known whether the Tin- versus Svp/Doc-positive cells in the major part of the aorta are also distinct with regard to their function or physiology. Likewise, it is still unclear whether the two central pairs of cardioblasts, which express the ladybird (lb) homeobox genes in addition to tin (Jagla et al., 1997), are functionally distinct from the two posterior pairs of Lb/Tin+ cells within each segment.

In the present study, we test genetically whether tin possesses a later cardiogenic function specifically within the four Tin+ pairs of cardioblasts in each segment of the dorsal vessel. Based upon the known arrangement of the different enhancer elements, we generated genomic tin constructs that support normal patterns of tin expression in the early, or early plus dorsal, mesoderm, without supplying tin expression in any cardioblasts of the dorsal vessel at later stages. By analyzing the phenotype of embryos that carry within each of these segments. Conversely, the anterior two pairs of cardioblasts in each segment differentiate into myocardial cells within each segment. Specifically, the two later cardiogenic function specifically within the four Tin+ pairs of cardioblasts, which are likely to contribute to the differential properties of the ‘working’ myocardium. We present a model in whichsvp in cardiac progenitors and their descendents repress tin in two cardioblast pairs within each segment from A2 to A7; this allows the expression of Doc by default, which in turn contributes to the continued repression of tin in these cells. Together, these interactions lead to the establishment and maintenance of two mutually exclusive differentiation states of cardioblasts, which are modulated further by differential homeotic gene activities in the aorta versus heart regions of the dorsal vessel. In addition, we show that the function of tin is required for normal remodeling and growth of the dorsal vessel during metamorphosis in pupal stages and, thus, is needed for generating the enlarged myocardium observed in adult flies.

MATERIALS AND METHODS
Drosophila strains and genetics
tin
(Azpiazu and Frasch, 1993), tin
(Bodmer, 1993) and mid
(Buescher et al., 2004), and the deficiency lines Df(3R)GCC14 (Azpiazu and Frasch, 1993; Bodmer, 1993), Df(3L)DocAc and Df(3L)29A6 (Reim et al., 2003) and svp
(svp-lacZ; from Y. Hiromi, National Institute of Genetics, Mishima, Japan) were balanced with CyO, wg-lacZ or TM3, eve-lacZ.

Ectopic expression experiments with UAS-tin (Yin and Frasch, 1998), UAS-Doc1, UAS-Doc2 (Reim et al., 2003), UAS-svpI (from M. Hoch, Bonn University, Germany) or UAS-Svp2.5 (lines 1, 2b, and 3a) were carried out at 28°C. UAS-Svp2.5 was generated from a full-length cDNA of Flag-Svp2.5 (Kasahara et al., 2001), initially subcloned as a HindIII/Iol blunt fragment into EcoRV of pbS-SK and then into EcoRI of pUAST vector (Brand and Perrimon, 1993). S59-Mef2-Ht3AD-Gal4 (line 10–2a) contains a minimal cardioblast enhancer of Mef2 (Hanh Nguyen, AECOM, Bronx, NY, unpublished) and a minimal skeletal muscle enhancer of S59/slouch (M.F., unpublished) in pGAL4-221 (from Christian Klämbt, Münster University, Germany), and is active in cardioblasts, in S59-positive somatic muscles and, weakly, in some pericardial cells.

Embryo staining
We used rabbit anti-β-Tubulin (1:1500, TSA; from Renate Renkawitz-Pohl, Philips University Marburg, Germany), rat anti-Bin (1:500) (Zaffran et al., 2001), anti-α-actinin (Saide et al., 1989), rabbit anti-Homothorax (1:500) (from Richard Mann, Columbia University, New York, NY), monoclonal rat anti-Tropomyosin (1:500), mouse anti-β-galactosidase 40-1a (1:60, TSA), rabbit anti-Toll (1:500, TSA; from Steve Wasserman, UC San Diego, CA) and mouse anti-α-Spectrin 3A9 (1:10, TSA) (from the Developmental Studies Hybridoma Bank, University of Iowa, developed under the auspices of NICHD). Other antibodies and the in situ probes for bhh, mid, H15, svpI and Sur are described by Reim et al. (Reim et al., 2005) and by Lo and Frasch (Lo and Frasch, 2001). A Zeiss Axiohot and the confocal Leica TCS-SP and Zeiss LSM 510 META systems were used for analysis.

Generation of tin rescue constructs
Restriction fragments from the genomic region of tin in pCaSpeR-Re28 (Azpiazu and Frasch, 1993) were subcloned into pBluescript KS (Stratagene). For, tin-AB, the large genomic EcoRI fragment was cloned into pCaSpeR-3. The tin-D enhancer element (Yin et al., 1997) was cloned downstream of the tin-AB fragment to generate tin-ABD. For tin-D, the EcoRI/EcoRI fragment from the tin cDNA was used to replace the corresponding genomic fragment in tin-ABD, leaving intact the minimal promoter. All pCaSpeR constructs were sequenced and several transformant lines from each were analyzed. For rescue with tin-ABD, the insertions T003-1B2 (2nd chromosome) and T003-1C1 (3rd chromosome) were used.

Development 133 (20)
Cardiac pacing and survival assays
Females and males were separated and aged to 2-3 days post-eclosion. Flies were aligned between two electrodes on a glass microscope slide and then paced to 6 Hz for 30 seconds using a square wave stimulator (Wessells and Bodmer, 2004; Wessells et al., 2004). Heart failure rate is defined as the percentage of flies that either arrest or fibrillate during or immediately after pacing. Flies that have undergone heart failure were observed for 2 minutes to calculate the percentage of flies that recover to a normal resting heartbeat [recovery rate; see Wessells et al. (Wessells et al., 2004) for the survival assay].

RESULTS
The early expression of Tinman is necessary and sufficient to specify all dorsal mesoderm derivatives
Embryonic tin expression can be separated into the three major phases (Azpiazu and Frasch, 1993; Bodmer, 1993), each regulated by a separable enhancer module that is subject to distinct regulatory inputs (Yin et al., 1997; Venkatesh et al., 2000). The earliest tin expression, which is observed in the entire trunk mesoderm during gastrulation, is recapitulated by constructs containing the enhancer element tinB (Fig. 1A,D). The Dpp-dependent expression in the dorsal mesoderm, which includes the primordia of the dorsal vessel, visceral muscles and somatic dorsal muscles, during stage 10-11 is driven by tinD (Fig. 1B,D) (Xu et al., 1998). At later stages, the enhancer tinC mediates expression in a segmental subset of cardioblasts of the developing and mature dorsal vessel (Fig. 1C,D) (Lo and Frasch, 2001).

Altogether, these and other data suggest that the early phase of tin expression is necessary and sufficient to specify the progenitors of all cell types generated from the dorsal mesoderm, but specification occurs with reduced efficiency. This activity of tin in tin-AB; tin mutant embryos in the absence of the second, dorsal-specific phase of tin expression is probably due to Tin protein perduring in the dorsal and cardiac mesoderm from its earlier phase of twist-driven mRNA expression. However, the combination of the first two phases of tin expression, in both early in the entire early mesoderm and subsequently in the dorsal mesoderm, leads to an improved rescue of cardiac specification and allows the formation of lymph glands (shown below). This demonstrates that Dpp-induced tin does contribute to the full biological activity of tin. We conclude that the absolute requirement for dpp during the specification of dorsal
mesodermal derivatives is mainly due to the requirement of synergistic Tin and activated Smads during the induction of Tin/Dpp targets, and to a lesser degree to the induction of tin expression itself by Dpp.

Expression of *tinman* in cardioblasts is dispensable for many aspects of cardiomyocyte differentiation

Next, we used a transgenic construct expressing tin throughout its two early phases (twist and Dpp dependent, respectively) with the aim of obtaining robust expression close to wild-type levels of Tin in the cardiogenic mesoderm in otherwise tin mutant animals. Staining of such embryos for several markers that normally show expression in all cardiomyocytes shows that the absence of cardiac tin expression has little effect on dorsal vessel formation as such. Notably, an almost normal number of cells expressing the pan-myogenic transcription factor Mef2 (Bour et al., 1995; Lilly et al., 1995) is detectable along the dorsal midline of tin-ABD; tin^{346} mutant embryos (compare Fig. 3B with 3A). The slight and variable synergistic Tin and activated Smads during the induction of Tin/Dpp mesodermal derivatives is mainly due to the requirement of Tin. The slight and variable expression in all cardiomyocytes shows that the absence of cardiac tin expression has little effect on dorsal vessel formation as such.

*tinman* expression in developing cardioblasts is required for the diversification of myocardial cell types

Based on the expression patterns of various genes, cardioblasts are thought to acquire at least three different identities within each segment of the dorsal vessel, the Tin-positive cells, the Tin/Lb-positive cells and the Svp/Doc-positive cells (Lo and Frasch, 2003). The Svp/Doc-positive cells in the posterior three segments of the dorsal vessel become different from the remaining cardioblasts morphologically and functionally, as these are the cells that form the ostia (inflow valves) of the larval heart (Molina and Cripps, 2001). In addition, based on the restricted expression of certain genes specifically in the Tin-positive cells such as Sulfonylurea receptor (*Sur*), which encodes a K+ channel subunit (Nasonkin et al., 1999), and the structural protein β3-tubulin (Kremser et al., 1999). Tin-positive and Tin-negative cells are thought to possess different physiological properties.
To test for possible alterations in the identities of myocardial cells in mutants lacking tin expression in the developing dorsal vessel, we examined the expression of the available identity markers in tin-ABD; tin346 mutant embryos (Fig. 4). Strikingly, Doc, which is normally expressed in the two Tin+ pairs of cells within each segment, is expressed ectopically and detected in all cardioblasts in these embryos (compare Fig. 4B with 4A). Conversely, the expression of Lbe, which is normally co-expressed with Tin in subsets of cardioblasts, is lost (Fig. 4C,D) (Jagla et al., 1997). The expanded expression of Doc is highly reminiscent of what has recently been described for mid mutants (Reim et al., 2005). These mutants also lose Tin from developing cardioblasts, which explains the similarities of the phenotypes with regard to Doc expression and of other phenotypes such as disrupted cardioblast diversification in the ‘heart’ region (Fig. 3H) (Reim et al., 2005). Furthermore, in both cases high-level expression of the Sir gene, a likely target of Tin in the Tin-positive cells, is lost (Fig. 4E,F) (Reim et al., 2005; Akasaka et al., 2006). Surprisingly, β-Tubulin at 60D (βTub60D, also known as β3-tubulin), which has also been reported to be a target gene activated by Tin in the dorsal vessel (Kremser et al., 1999), is still expressed in the tin-ABD; tin346 mutant background and maintains its intrasegmental modulation, albeit with a slightly disturbed pattern (Fig. 4G,H). This observation suggests that the β3-tubulin gene is driven by both tin-dependent and tin-independent enhancers in the Tin+ cardioblasts. In addition, the segmental pattern of β3-tubulin in tin-ABD; tin346 mutants shows that there must be factors other than Tin and Doc that can still generate a patterned expression of certain differentiation genes in the dorsal vessel. sVP, which is activated during early stage 12 possibly in response to Hh signaling (Ponzio et al., 2002), also maintains its segmental expression in this genetic background (Fig. 4I,J), and hence may act as a repressor that antagonizes the activation of β3-tubulin by a uniformly expressed activator in myocardial cells. We predict that other markers like β3-tubulin exist that are regulated differentially by sVP but do not depend on tin.

The anterior aorta, which lacks the Svp+ cardioblasts (Fig. 4I) (Lo and Frasch, 2003), also shows ectopic Doc expression in the absence of myocardial Tin (Fig. 4B,D), whereas the expression of the anterior-specific marker Homothorax (Hth; Perrin et al., 2004) is unaffected (see Fig. S1 in the supplementary material). In addition, the near absence of Tin in pericardial cells causes a loss of pericardial ladybird and a reduction of pericardial zfh-1 expression, but the only known marker specific for Tin+ pericardial cells, Odd, does not exhibit any concomitant ectopic expression (see Fig. S2 in the supplementary material).

**Mutual repression of Tin and Doc maintains differential cardiac cell identities**

The observed expansion of Doc expression, as well the morphological abnormalities in the heart region, suggest a switch in cardiac cell identities in dorsal vessels lacking Tin. In order to test this notion further and to establish the underlying regulatory mechanisms, we examined the expression of regulatory factors associated with cell diversifications into different cardioblast subtypes under various genetic conditions. Fig. 5A visualizes the expression of the three known regulatory genes, tin, Doc and sVP (as detected by sVP-lacZ expression from a heterozygous lacZ enhancer trap insertion in sVP), which are expressed in the described ‘4+2’ pattern in an otherwise wild-type embryo. Whereas in the absence of Tin in developing cardioblasts Doc expression is expanded into all cardioblasts, sVP-lacZ maintains its restriction to two pairs of cardioblasts per segment (Fig. 5B, yellow arrows). Although we have shown previously that sVP is normally required for Doc expression in the two Svp+ cells in each hemisegment (Lo and Frasch, 2001), our present observation shows that Doc expression can occur in Svp-negative cardioblasts, provided that Tin is not present. Hence, we propose that Doc is under the repressive control of Tin rather than being activated directly by Svp. To confirm this hypothesis, we analyzed Doc expression in double mutants lacking Tin and Svp in cardioblasts. Although the dorsal vessels of such
embryos (tin-ABD; svp\textsuperscript{AE127} tin\textsuperscript{346}) display some morphological defects, we still observe an expansion of Doc expression into all cardioblasts present (Fig. 5C). Thus, in the normal situation, the presence of Tin in four cardioblasts per hemisegment is essential for the repression of Doc in these cells. Accordingly, analysis of embryos in which \textit{tin} is ectopically expressed in most (although not all) cardioblasts using S59-Mef2-H\textit{Id}-\textit{Gal4} confirms the repressive activity of Tin towards Doc (Fig. 5D,E; the S59 enhancer-driven component serves as an internal expression control). The presence of \textit{svp-lacZ}-positive Tin\textsuperscript{+}/Doc\textsuperscript{−} cells suggests that Tin did not repress \textit{svp} in these experiments. In conclusion, these data demonstrate that Tin acts as a repressor of Doc but not \textit{svp} expression in cardioblasts.

Previous loss- and gain-of-function studies have shown that Svp acts as a repressor of \textit{tin} (Gajewski et al., 2000; Lo and Frasch, 2001; Molina and Cripps, 2001) (see also Fig. 6B,C). This repressive effect of Svp towards \textit{tin} is confirmed upon ectopic expression of Svp in the cardioblasts of the dorsal vessel (Fig. 5F). In addition, ectopic Svp leads to an expansion of \textit{svp-lacZ} expression into some, but not all, of the Doc-positive cells, arguing for the presence of a positive feedback loop in those cells.

We further considered the possibility that Doc can also act as a repressor of \textit{tin}. When UAS-\textit{Doc2} is ectopically expressed throughout the dorsal vessel (Fig. 5G), we observe a reduction or loss of \textit{tin} expression in most of the Doc-expressing cells, particularly in the posterior aorta and heart regions. Repression of \textit{tin} by Svp in the misexpression assay was consistently more robust throughout the dorsal vessel when compared with repression of \textit{tin} by Doc. However, the repression of \textit{tin} by Doc is independent of \textit{svp}, as \textit{svp} is not activated downstream of \textit{Doc} in Tin-negative cardioblasts (Fig. 5G), and \textit{tin} repression is also seen when \textit{Doc2} is misexpressed in a homozygous \textit{svp}\textsuperscript{AE127} mutant background (see Fig. S3 in the supplementary material). In addition, analyses of embryos with genotypes in which \textit{Doc1} is deleted and the gene dose of \textit{Doc2} and \textit{Doc3} is reduced by half (Reim et al., 2003; Reim and Frasch, 2005) also support the notion that Doc acts as a repressor during cardioblast diversification. In these embryos, we detect ectopic expression of \textit{tin} in a number of Svp-positive cells, presumably because the lowered dose of Doc provided insufficient repressive activity towards \textit{tin} (Fig. 5H). Taken together these findings show that Doc and Tin have mutually repressive functions in cardioblasts of the dorsal vessel.

The expression of Wingless (Wg) in the late stage embryonic heart in three segmentally repeated double pairs of cardioblasts (Fig. 6A) marks the differentiation of the Svp/Doc-positive cardioblasts within the heart region into ostia (inflow valves) (Lo et al., 2002). Previously, it has been shown that \textit{wg} expression depends on \textit{svp} and \textit{abd-A} activities; it is missing in \textit{svp} mutants, but expanded if \textit{svp} is expressed ectopically (Lo et al., 2002; Perrin et al., 2004) (see also Fig. 6B,C). However, based on these data, it is not possible to discriminate between a direct activation of \textit{wg} by Svp, an indirect activation by Svp via Doc or an absence of repression of \textit{wg} by Tin in the \textit{svp}\textsuperscript{+} cardioblasts. In order to distinguish between these possibilities, \textit{Doc2} was activated ectopically in the dorsal vessel (Fig. 6D), which produced a moderate expansion of \textit{wg}, with some but not all \textit{Doc}\textsuperscript{+} cardioblasts in the ‘heart’ region being positive for \textit{Wg}. \textit{tin-ABD}; \textit{tin}\textsuperscript{346} mutant embryos, in which \textit{svp} is not affected, display ectopic activation of \textit{wg} in all cardioblasts of the ‘heart’ region (Fig. 6E). Furthermore, \textit{svp}\textsuperscript{AE127} \textit{tin}\textsuperscript{346} double mutant embryos (Fig. 6F) display the same pattern of \textit{wg} expression as \textit{svp}\textsuperscript{AE127} \textit{tin}\textsuperscript{346} double mutant embryos, in which \textit{svp} is not affected, display ectopic activation of \textit{wg} in all cardioblasts of the ‘heart’ region (Fig. 6E). Furthermore, \textit{svp}\textsuperscript{AE127} \textit{tin}\textsuperscript{346} double mutant embryos (Fig. 6F) display the same pattern of \textit{wg} expression as \textit{svp}\textsuperscript{AE127} \textit{tin}\textsuperscript{346} double mutant embryos, in which \textit{svp} is not affected, display ectopic activation of \textit{wg} in all cardioblasts of the ‘heart’ region (Fig. 6E).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4}
\caption{Markers for distinct cardioblast sub-types reveal a requirement of \textit{tin} for cardiac cell diversifications. Dorsal views of stage 15-16 wild-type (left column) and \textit{tin-ABD}; \textit{tin}\textsuperscript{346} mutant embryos (right column). (A,B) Staining with antibodies against Mef2 (green) and Doc2\textsuperscript{+3} proteins (red/red arrowheads). Doc2\textsuperscript{+3} expands into all cardioblasts in mutant embryos that lack \textit{tin} expression in the dorsal vessel. (C) Ladybird (Lbe) is detected in two cardioblasts per hemisegment (green arrowheads) just posterior to the Doc-positive pair in the wild type. (D) Cardiac Lbe expression is absent in \textit{tin-ABD}, \textit{tin} mutants except for the outflow region (*). (E) In normal stage 16 embryos, \textit{Sur} mRNA is detected at high levels in Tin cardioblasts and at very low levels in Doc\textsuperscript{+} cells (red arrowheads). (F) \textit{Sur} mRNA is barely detectable in mutants lacking cardiac \textit{tin} expression. (G) In the wild type, \textit{B3-Tubulin} (\textit{B3-Tub}) is detected in \textit{Tin}\textsuperscript{+}/Doc\textsuperscript{−} cells of the dorsal vessel (bracket) and in somatic muscles. (H) \textit{B3-Tubulin} is maintained with a slightly irregular segmental pattern (bracket) in cardioblasts of \textit{tin-ABD}, \textit{tin}\textsuperscript{346} mutants. (I,J) Detection of \textit{svp} mRNA and Tin protein. \textit{svp} is normally expressed in the Tin-negative cardioblasts (I, green arrowheads). In \textit{tin-ABD}, \textit{tin} mutant embryos (J) \textit{svp} expression retains its normal pattern (red arrow indicates sporadic pericardial cell expression of Tin in \textit{tin-ABD}, \textit{tin} mutants; rg, ring gland).}
\end{figure}
embryos carrying the tin-ABD transgene also show expanded wg expression (Fig. 6F). Hence, wg may either be activated by Doc, or it may be repressed by Tin while being activated by another factor that could be active in all cardioblasts. In summary, these data prove that syp is not directly required for Doc and wg gene activation, but rather as a repressor of tin, which in turn represses Doc and perhaps wg. These findings agree with a role of Svp as a transcriptional repressor, a function also fulfilled by its mammalian homolog COUP-TFI1 (Nrl2) (Pereira et al., 1999).

Conserved activity of mammalian Nkx class homeobox genes in altering the diversity of Drosophila cardiac cells

To test directly whether the tin-related Nkx2.5 gene from the mouse possesses a repressive function equivalent to tin, and to assess whether such a function is active in the heterologous system, we expressed Nkx2.5 throughout all cardioblasts in late stage embryos. Although Nkx2.5 could not promote cardiogenesis in Drosophila (Park et al., 1998; Ranganayakulu et al., 1998), we observed that Doc and Wg expression are strongly repressed when Nkx2.5 is expressed in cardioblasts, similar to the effects seen with ectopic tin (compare Fig. 6H with 6I). These data indicate that Nkx2.5, like Tin, can have a repressive function towards some cardiac regulators.

Cardiac tinman function is required for proper ultrastructure, remodeling and functionality of the larval and adult heart

In both larval and adult dorsal vessels of wild-type animals, the myofibrils are arranged spirally with essentially transverse orientations around the heart and aortic tubes (Fig. 7A, A') (Molina and Cripps, 2001; Monier et al., 2005). By contrast, larval dorsal vessels from animals in which tin was never expressed in myocardial cells (tin-ABD, tin346) show a very different ultrastructure. In these dorsal vessels, the myofibrils are arranged almost exclusively in an AP orientation, which leads to the appearance of striations (Fig. 7B, B'). The only exceptions are seen in the heart, where several abnormal cross-shaped or ‘knotted’ patterns of myofibrils are present, particularly near the posterior end. In addition, the aortae in these larvae appear thinner when compared with the wild type, whereas the heart frequently has a wider diameter (Fig. 7B, B'), see Fig. S4 in the supplementary material; data not shown). Hence, myocardial activity of tin is required for establishing the normal ultrastructure of the contractile fibers and is, perhaps, related to this function, for generating a morphologically normal dorsal vessel. An almost identical alteration of the myofibril orientation has been reported for adult animals with ectopic expression of abd-A in the aorta (Monier et al., 2005); however, it is presently not known whether there is any mechanistic connection with our observation.

In adult animals lacking cardiac tin expression, we observe a much thinner heart tube (Fig. 7D, F, compare with 7C, E). As in larvae, the myofibrils are arranged longitudinally and transverse spirally arranged myofibrils are almost completely absent in these mutants (Fig. 7F, compare with 7E) (see also Molina and Cripps, 2001; Monier et al., 2005). The adult heart is generated by remodeling of the posterior larval aorta, which is accompanied by a significant widening of the tube and myocardial cell growth and the histolysis of the larval heart (Molina and Cripps, 2001; Monier et al., 2005; Sellin et al., 2006). Obviously, the absence of tin activity in myocardial cells impedes this process of heart remodeling. As a consequence, the small adult dorsal vessel in tin-ABD, tin346 flies probably represents the largely unchanged larval aorta after the larval heart has been histolysed.

The altered ultrastructure of myocardial cells, as well as the observed changes in the expression of cardiac differentiation genes and in cardiac cell identities, would be expected to affect the functionality of the dorsal vessel. In order to examine the role of tin in the function of the adult heart, we measured cardiac responses to acute stress in tin-depleted adult flies and in controls. External electrical stimuli were applied to pace the heart of the fly to an elevated rate compared with wild type (Wessells et al., 2004). In response to this stress, flies either recover to a regular heart beat or...
else fail (defined as cardiac arrest or fibrillation). tin-deficient hearts showed a dramatic increase in heart failure rate, while those that are heterozygous for tin in the heart failed at the same rate as wild-type controls (20-30%; Fig. 7G; see Movies 1 and 2 in the supplementary material). In parallel, we monitored the recovery of flies that underwent either fibrillation or arrest. Two minutes after the pacing protocol, almost all wild-type and heterozygous flies recovered to a normal resting heartbeat, whereas in the absence of cardiac tin, only 40% of flies were able to recover (Fig. 7H). This suggests that tin function is required for a properly functioning adult heart. Moreover, flies without cardiac tin have a reduced lifespan (Fig. 7I), consistent with a possible link between cardiac function and aging. Taken together, these data suggest that tin is required for the formation of the adult heart, in addition to its early requirement for the embryonic dorsal vessel. As a consequence, the lack of cardiac tin causes severe disruptions in cardiac contractility and rhythmicity (see Movies 1 and 2 in the supplementary material), and leads to a much elevated risk of heart failure in response to stress.

**DISCUSSION**

Our current data demonstrate that a major function of tin during later stages of embryonic cardiogenesis is the establishment of non-ostial, i.e. ‘working’ myocardial cells. Doc and Svp restrict this function by repressing tin and, in the heart region, enable Tin-negative cells to form ostia (summarized in Fig. 6G). These data fill an important gap in the clarification of the full pathway of the regulation and function of tin during cardiogenesis. In this pathway, the early functions of tin (together with the GATA gene pnr and the Doc T-box genes) are required for activating the Tbx20 genes mid and H15 within early cardioblasts (Miskolczi-McCallum et al., 2005; Qian et al., 2005; Reim and Frasch, 2005; Reim et al., 2005), and mid then feeds back to activate tin expression specifically in myocardial cells (Reim et al., 2005). However, the expression of the COUP-TFiI-related gene svp, which is activated during stage 12 in two cardioblast progenitors in each hemisegment between A2 and A7, prevents mid from activating tin in these cells (Gajewski et al., 2000; Lo and Frasch, 2001). tin then represses Doc, which appears to be activated by default (via yet unknown activators) in the ‘Tin’ cardioblasts and in turn contributes to the continued repression of tin in them. These interactions lead to the stabilization of two mutually exclusive differentiation states of myocardial cells that are defined presumably to a large extent by their differential expression of tin versus Doc and of their respective target genes. The particular features of these two segmental types of myocardial cells are further modulated by the activities of Hox genes such as abd-A, which leads to the differential formation of ‘working’ (Tin+) versus ostial myocardial cells (Doc+, Wg+) that form the inflow valves in the heart region (Fig. 6G).

![Fig. 6. Control of Wg expression in ostial cardioblasts and summary of regulatory interactions in the heart region. Detection of Wg (green), Doc2+3 (red) and Tin (blue) in the dorsal vessel of stage 16 embryos. (A) In the wild type, Wg marks the three posterior pairs of Doc+/Tin– cardioblasts as ostia (green arrows). (B) Wg and Doc proteins are not detectable in the dorsal vessel of homozygous svpAE127 mutants, whereas Tin is expanded. (C) Embryo expressing svp1 ectopically throughout the dorsal vessel. All cardioblasts of the ‘heart’ region express Doc and Wg (bracket). (D) Misexpression of Doc2 causes less efficient ectopic activation of Wg when compared with svp (arrow). (E) tin346 mutant and (F) tin346, svpAE127 double mutant embryos carrying tin-ABD. Wg is expressed in all Doc-labeled cardioblasts of the posterior dorsal vessel (brackets). (G) Top: schematic representation of the dorsal vessel with corresponding epidermal segment numbers and expression domains of homeotic selector proteins. Middle: in the wild type, tin is activated in cardioblasts downstream of the Tbx20-homolog mid. This activation is blocked by the COUP-TFI homolog Svp (which itself depends on Hh inputs during stages 11-12) in presumptive ostial cells. Doc is expressed by default in these cells and contributes to the repression of tin. Myocardial Tin represses Doc and prevents wgp expression. Wg is expressed only in Doc-positive ostial cells that also express Abd-A. These cells, which feature an elongated shape, differentiate into inflow valves. Bottom: in embryos that lack cardiac tin expression (either owing to the absence of the required cis-regulatory element tinC or because of the missing tin trans-activator Mid), all cardioblasts express Doc independently of Svp and Wg is activated in all cardioblasts of the Abd-A domain. (H) Misexpression of tin, which causes repression of Doc in Svp+ cardioblasts, leads to loss of Wg in those cells (red arrows; green arrows are as in A). (I) Forced expression of mouse Nkx2.5 in the dorsal vessel using S59-Mef2-HtaD-Gal4 and UAS-Nkx2.5 leads to repression of Doc and Wg in the heart, similar to UAS-tin.
Whereas previous data have documented that Tin functions as a direct activator of specific target genes, our present data implicate Tin strongly in the repression of certain myocardial genes, including Doc and potentially wg. Indeed, interactions of Tin with the corepressor Groucho have been demonstrated in biochemical and cell culture experiments (Choi et al., 1999), and the N-terminal TN domain of Tin is proposed to function as an EH1 repressor domain (Copley, 2005). We propose that the activity of Tin either as an activator or a repressor is context-specific and is ultimately determined by the enhancer architecture of a particular target gene. For example, during early stages of cardiac induction by Dpp, combinatorial binding of Tin and Smads promotes activating functions of Tin, whereas in the Tin'+ cardioblasts during later stages, Tin can either activate (e.g. Mef2, Sur) or repress (e.g. Doc). These opposite activities in the same cells are probably determined by the presence of additional binding sites for tissue-specific or ubiquitous co-factors that can switch Tin activity.

Our findings with the ‘cardiac-specific knockout’ of tin are reminiscent of some of the phenotypes reported for mouse mutant phenotypes of Nkx2.5. For example, in both situations, cardiomyocytes and a linear embryonic heart tube with a largely normal morphology are formed (Lyons et al., 1995; Tanaka et al., 1999) (our present data). Although at first it was thought that, in the mouse, closely related Nkx genes could partially compensate for the functional loss of Nkx2.5, it is now widely assumed that cardiogenic genes encoding other classes of transcription factors, particularly those of the GATA and T-box families, exert most of the compensatory effects (Tanaka et al., 1998; Olson and Schneider, 2003). The latter type of compensation appears also to operate upon cardioblast-specific ablation of tin function, where the derepressed Doc T-box genes are presumably able to regulate myocardial development in the cells that would normally be under the control of tin. It will be instructive to determine the phenotype of animals in which the cardioblast expression of both tin and Doc has been ablated simultaneously. Likewise, the analysis of cardiogenesis with analogous double mutants in the mouse will be highly informative.

Additional phenotypic similarities between our tin mutant animals and mouse Nkx2.5 mutants include the failure to remodel the linear heart tube, which in the mouse leads to defects in looping morphogenesis and chamber formation and in Drosophila to a failure of converting the larval aorta into an adult heart. As in adult flies, a conditional knockout of mouse Nkx2.5 also leads to defects in ventricular cell lineage specification and maturation, which is accompanied by the aberrant down- or upregulation of cardiac differentiation genes (Pashmforoush et al., 2004). Notably, a major difference between the vertebrate and Drosophila systems is the early and broad mesodermal expression of Tin, which is essential for Drosophila cardiac induction and is not compensated for by other factors.

We thank Seigo Izumo and Patrick Jay for the Flag-Nkx2.5 plasmid. We are grateful to Margaret Buckingham for allowing Stéphane Zaffran to carry out part of this work in her laboratory. The research was supported by grants from NIH-NICHD (HD30832) to M.F and from NIH-NIA (2P01AG015434) to R.B. Confocal laser-scanning microscopy at the MSSM-Microscopy Shared Resource Facility was supported by NIH-NIC (R24 CA095823) and NSF (DBI-9724504).


