The Dorsocross T-box genes are key components of the regulatory network controlling early cardiogenesis in *Drosophila*

Ingolf Reim and Manfred Frasch*

Brookdale Department of Molecular, Cell and Developmental Biology, Box 1020, Mount Sinai School of Medicine, New York, NY 10029, USA

*Author for correspondence (e-mail: manfred.frasch@mssm.edu)

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Summary

Cardiac induction in *Drosophila* relies on combinatorial Dpp and Wg signaling activities that are derived from the ectoderm. Although some of the actions of Dpp during this process have been clarified, the exact roles of Wg, particularly with respect to myocardial cell specification, have not been well defined. Our present study identifies the Dorsocross T-box genes as key mediators of combined Dpp and Wg signals during this process. The Dorsocross genes are induced within the segmental areas of the dorsal mesoderm that receive intersecting Dpp and Wg inputs. Dorsocross activity is required for the formation of all myocardial and pericardial cell types, with the exception of the Eve-positive pericardial cells. In an early step, the Dorsocross genes act in parallel with *tinman* to activate the expression of *pannier*, a cardiogenic gene encoding a Gata factor. Our loss- and gain-of-function studies, as well as the observed genetic interactions among Dorsocross, *tinman* and *pannier*, suggest that co-expression of these three genes in the cardiac mesoderm, which also involves cross-regulation, plays a major role in the specification of cardiac progenitors. After cardioblast specification, the Dorsocross genes are re-expressed in a segmental subset of cardioblasts, which in the heart region develop into inflow valves (ostia). The integration of this new information with previous findings has allowed us to draw a more complete pathway of regulatory events during cardiac induction and differentiation in *Drosophila*.

Key words: Mesoderm patterning, Heart development, T-box genes, *Drosophila*

Introduction

Studies in several vertebrate models and *Drosophila* have uncovered a surprising degree of evolutionary conservation of regulatory mechanisms and genes that control early cardiogenesis. In all these organisms, cardiac induction in the lateral mesoderm involves the concerted action of signals mediated by Bmp, Wnt and Fgf family members, which act upon cardiogenic genes encoding NK homeodomain, Gata and T-box transcription factors (reviewed by Zaffran and Frasch, 2002). Together, these signaling and transcriptional pathways set up complex and only partially defined regulatory networks that result in the progressive delineation and specification of heart progenitors.

In *Drosophila*, the primordial cells generating myocardial cells (cardioblasts) and pericardial cells are induced within mesodermal areas receiving a combination of Dpp (i.e. BMP) and Wg (i.e. Wnt) signals (Zaffran and Frasch, 2002). Dpp is secreted from a dorsal domain of the ectoderm on either side of the embryo, whereas Wg derives from transversely striped segmental domains; hence, the cardiac progenitors are generated in bilateral, segmentally repeated areas of the dorsal mesoderm of the early embryo. The NK homeobox gene *tinman* (*tin*), which is crucial for the generation of all types of cardiac progenitors and all other dorsal mesodermal derivatives, is a direct downstream target of the Dpp signaling cascade and, as such, is induced in the entire dorsal mesoderm (Azpiazu and Frasch, 1993; Bodmer, 1993; Frasch, 1995; Xu et al., 1998). In addition, Tinman protein is required to provide mesodermal cells with the competence to respond to Dpp and other signals during additional events in cardiac induction (Halfon et al., 2000; Knirr and Frasch, 2001; Han et al., 2002). *tin*, potentially in combination with Dpp, is required for the transcriptional activation of the Gata factor-encoding gene *pannier* (*pnr*) in the cardiogenic mesoderm (Gajewski et al., 2001). *pnr* is crucial for the specification of both cardioblasts and pericardial cells (Gajewski et al., 1999; Alvarez et al., 2003; Klinedinst and Bodmer, 2003). One of the earliest regulatory genes turned on specifically in all cardioblasts downstream of *pnr* is the *Tbx20* ortholog *midline* (*mid*) (Miskolczi-McCallum et al., 2005; Qian et al., 2005; Reim et al., 2005). Together with its paralog *H15*, *mid* serves to activate late-stage expression of *tin* specifically within cardioblasts, which in turn is required for the normal patterning of the heart tube and the differentiation of myocardial cells (Reim et al., 2005).

When compared with Dpp, the exact role of Wg during cardiac induction is less well understood. For example, the lack of spatial correlation between *pnr* and *wg* expression suggests that *pnr* is not a direct target of Wg signals. Whereas ectodermal *wg* is striped, *pnr* expression initiates almost uniformly along the anteroposterior axis near the dorsal margin of the mesoderm (Gajewski et al., 1999; Klinedinst and

Bodmer, 2003), at a time when the segmentally distributed cardiac progenitors coalesce into a continuous band of cells. However, one important and direct consequence of Wg signals is the induction of *sloppy paired* (*slp1* and *slp2*) in striped domains in the early mesoderm (Riechmann et al., 1997; Lee and Frasch, 2000). The *slp* genes encode forkhead domain repressor factors, which prevent the induction of visceral mesoderm regulators by Dpp that would otherwise interfere with cardiac induction in the presumptive cardiogenic areas (Zaffran et al., 2001).

In addition to excluding these inappropriate regulators from the cardiogenic areas, *wg* is thought to promote the formation of both pericardial and myocardial progenitors also in a direct fashion. Indeed, such a mechanism operates during the specification of one specific subset of pericardial cells, termed Eve-PCs, and the induction of the *even-skipped* (*eve*) gene in progenitors of these cells requires binding sites for the Wg effector Pangolin (Pan, also known as dTCF) in combination with binding sites for Tinman and the Dpp-effectors Mad and Medea (Halfon et al., 2000; Knirr and Frasch, 2001; Han et al., 2002). Hence, the combinatorial inputs from Wg, Dpp and the cardiogenic competence factor Tinman are directly integrated at the level of the enhancer of a pericardial regulatory gene. Based upon the available genetic data on cardioblast specification, it is likely that an analogous integration of Wg and Dpp signals also occurs during the induction of certain early-acting myocardial regulatory genes. However, clear candidates for common targets of Wg and Dpp in myocardial development have not been described.

Our current study identifies the Dorsocross T-box genes as crucial new components that mediate the combinatorial activities of Wg and Dpp during early steps of myocardial induction. The Dorsocross (Doc) locus encodes a cluster of three genes, *Doc1*, *Doc2* and *Doc3*, that are closely related in terms of their T-box sequences and embryonic expression patterns (Reim et al., 2003). Our previous work has identified important roles of these genes during amnioserosa development and epidermal patterning of the embryo (Reim et al., 2003). We now show that, within the mesoderm, the Doc genes have an essential role for the formation of cardioblasts and a subset of pericardial cells. The induction of Doc gene expression occurs in dorsal mesodermal quadrants of cells at the intersections of the Dpp and Wg domains, and requires the activities of both Dpp and Wg, but not *tin*. We demonstrate that, in the absence of all three Doc genes, only very few cardioblasts become specified and no dorsal vessel is formed. In addition, the subpopulations of *odd-skipped* (*odd*)-expressing pericardial cells (Odd-PCs), *odd*-positive lymph gland cells and *tin*-only-expressing pericardial cells (Tin-PCs) require Doc gene activity. However, *eve* + *tin*-positive pericardial cells (Eve-PCs) and dorsal somatic muscle founders develop independently of Doc. We observe genetic interactions among Doc, *tin* and *pnr*, which suggests that these three cardiogenic regulators synergize during the specification of myocardial and certain pericardial cells. Accordingly, simultaneous ectopic expression of *Doc2* with *tin*, *pnr* or both generates large numbers of ectopic cardioblasts in the mesoderm. We demonstrate that one of the key functions of the Doc genes during cardiogenesis, which they exert in combination with *tin*, is the activation of *pnr* expression in the cardiogenic mesoderm. Altogether, the incorporation of the Doc genes into the cardiogenic network has allowed us to close important gaps in our understanding of the regulatory circuits operating during the induction of myocardial and pericardial cells.

Materials and methods

Drosophila strains and crosses

Df(3L)DocA, which contains *EP(3)3556* at its breakpoint, and the *Doc1*-deleting deficiency *Df(3L)29A6* have been described by Reim et al. (Reim et al., 2003). *Df(3L)29A6* and the null allele pnr^{VX6} were obtained from the Bloomington Stock Center. The null allele tin^{346} was described in Azpiazu and Frasch (Azpiazu and Frasch, 1993). Stocks with the following genotypes in a w^- background were generated via recombination: *Df(3L)DocA* pnr^{VX6}, *Df(3L)DocA* tin^{346}, pnr^{VX6} tin^{346} and *Df(3L)DocA* pnr^{VX6} tin^{346}. Mutant stocks were balanced with *TM3, eve-lacZ* for the identification of homozygous mutant embryos. Alternatively, genotypes causing protein nulls were confirmed by appropriate antibody staining. Oregon R was used as wild-type control unless indicated otherwise. The following enhancer-*lacZ* reporter lines were used: *Mef2-HtΔD-lacZ* #2 and #6 (2nd and 3rd chromosome insertions, respectively, unpublished; a gift from H. Nguyen, Albert Einstein College, New York), *tinCΔ7-lacZ* #23b (3rd chromosome) (Lo and Frasch, 2001), *tinD-lacZ* #25 (X chromosome) (Yin et al., 1997), *1010 B2-lacZ* (Yin and Frasch, 1998), AE127 (*svp-lacZ* enhancer trap; gift from Yasushi Hiromi, National Institute of Genetics, Mishima, Japan). The following *GAL4* and *UAS* lines were used: *c381-GAL4* (2nd chromosome) (Manseau et al., 1997), *tinD-GAL4* (*TMEN6-GAL4* #52D1; 2nd chromosome, unpublished; a gift from J. Weiss, OHSU, Portland, OR), *2xPE-twi-GAL4* (2nd chromosome) (Baker and Schubiger, 1996), *UAS-dpp* #4 and *UAS-wg* (X and 3rd chromosomes, respectively) (Frasch, 1995), *UAS-Doc1 #F2*, *UAS-Doc2* #I2, #K3 and #M2, and *UAS-Doc3* #C2 (Reim et al., 2003), *UAS-pnr* (2nd and 3rd chromosome insertions; obtained through R. A. Schulz, University of Texas, M. D. Anderson Cancer Center, Houston), UAS-*tin* #1 (Yin and Frasch, 1998) and *UAS-*tkv^{Q253D} (Nellen et al., 1994). For expression of multiple *UAS* constructs, *UAS-Doc2* #I2 (2nd chromosome) was combined with *UAS-tin* or *UAS-pnr* on chromosome 3 or with a recombinant *UAS-tin, UAS-pnr* 3rd chromosome. Except for embryos carrying *c381-GAL4*, which were kept at 25°C, embryos for UAS/GAL4 experiments were left to develop at 28°C.

Staining of embryos

Immunostaining of embryos using DAB, fluorescent immunostaining and in situ hybridization were carried out essentially as described by Knirr et al. (Knirr et al., 1999). Primary antibodies were detected with FITC-, Cy3- or Cy5-conjugated AffiniPure goat anti-rabbit IgG (H+L) (1:200; Jackson ImmunoResearch Laboratories). If necessary, Tyramide Signal Amplification (TSA) was performed using biotinylated secondary antibodies (1:500) in combination with the Vectastain ABC Kit (Vector Laboratories) and fluorescent Tyramide Reagent (PerkinElmer). Primary antibodies included rabbit anti-Bap (1:500 with TSA) (Zaffran et al., 2001), rabbit anti-Doc2 (1:2000), guinea pig anti-Doc2+3 (1:400), guinea pig anti-Doc3+2 (1:600) (Reim et al., 2003), rabbit anti-Eve (Frasch et al., 1987), guinea pig anti-Eve (1:400), rat anti-Odd (1:500), guinea pig anti-Runt (1:300) (Kosman et al., 1998), rabbit anti-β-Galactosidase (1:1500; Promega), mouse polyclonal anti-β-Galactosidase (1:200; Sigma), rabbit anti-Mef2 (1:750; a gift from Hanh Nguyen, Albert Einstein College, Bronx, NY) (Bour et al., 1995), rabbit anti-Phospho-Smad1/PMad (1:2000 with TSA; gift from C.-H. Heldin, Ludwig Inst., Uppsala), rabbit anti-Pnr (1:3000 with TSA) (Herranz and Morata, 2001), rabbit anti-Srp (1:800) (gift from D. Hoshizaki), rabbit anti-Tin (1:750) (Yin et al., 1997) and rabbit anti-Zfh-1 (1:2000) (Broihier et al., 1998). Monoclonal anti-Wg 4D4 (1:40 with TSA) and anti-β-Galactosidase

40-1a (1:60 with TSA) were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. Digoxigenin-labeled RNA in situ probes for Doc (Reim et al., 2003), *bkh*, *hand* (S. Zaffran and M.F., unpublished) and *H15* were prepared from cloned genomic fragments, and the *mid* in situ probe from the BDGP EST RE27439 (Reim et al., 2005). Images of DAB-stained embryos were taken using Nomarski optics and images from fluorescent staining using confocal laser scanning microscopy with Leica TCS-SP and Zeiss LSM 510 META microscope systems.

Results

Dynamic expression and regulation of *Dorsocross* in the early dorsal and cardiogenic mesoderm

The three Dorsocross (Doc) genes *Doc1*, *Doc2* and *Doc3* are co-expressed in a number of embryonic tissues, including the amnioserosa, the dorsolateral ectoderm and the dorsal mesoderm (Reim et al., 2003). Our present study focuses on the role of the Doc genes in the development of the dorsal vessel, which derives from the Doc-expressing areas of the early dorsal mesoderm.

In the trunk mesoderm, Doc expression emerges first at stage 10 within segmentally repeated patches of cells located in dorsal areas (Reim et al., 2003). As shown in Fig. 1A for *Doc3* mRNA, the ventral borders of Doc expression coincide with the ventral border of nuclear phosphorylated Mad (PMad) signals in the dorsal mesoderm. This indicates that *Doc* expression in the dorsal mesoderm is induced by Dpp signals

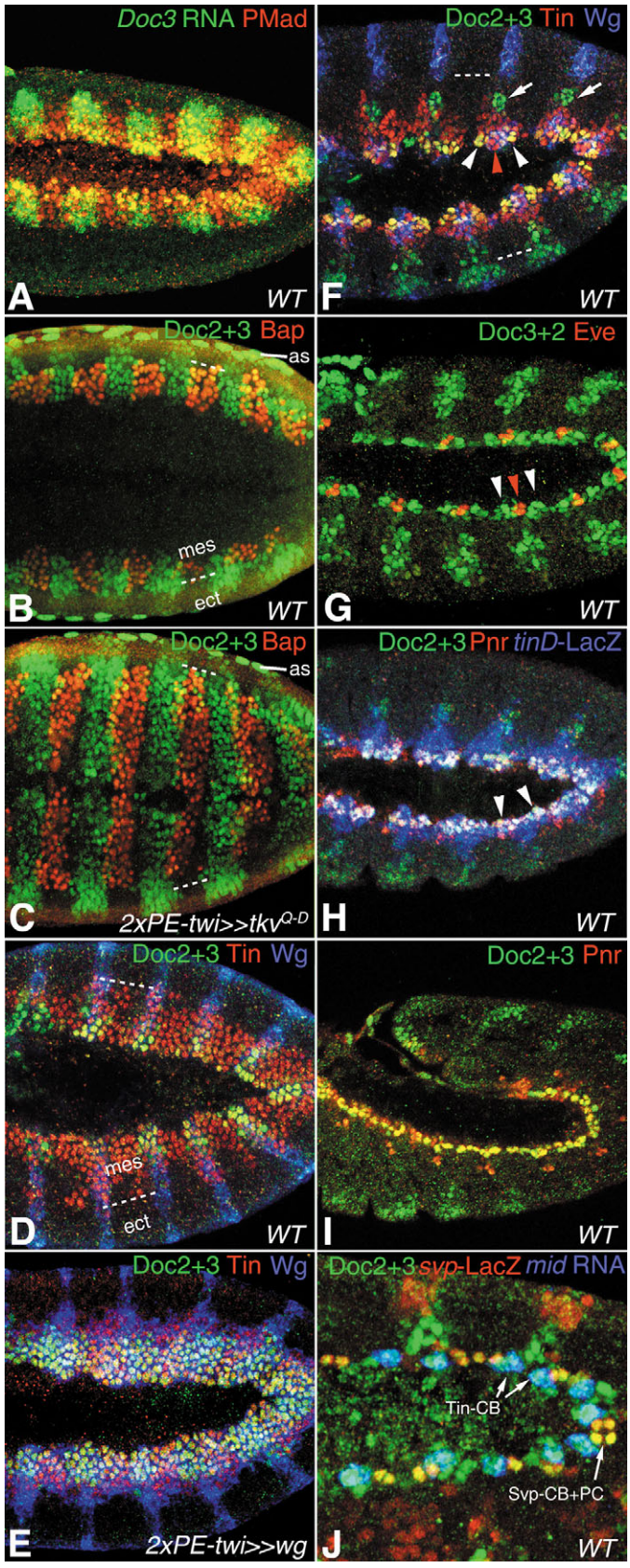

Fig. 1. Mesodermal expression pattern of Dorsocross. Dorsocross protein (shown in green) was detected by immunofluorescent staining using antisera recognizing Doc2 and Doc3 (anti-Doc2+3 or anti-Doc3+2 as indicated), except in A, which shows *Doc3* RNA detection by in situ hybridization. Images are merged optical sections of embryonic trunk mesoderm (lateral views, anterior towards the left and dorsal upwards, unless noted otherwise). Broken lines indicate borders between mesoderm (mes) and ectoderm (ect). (A) Stage 10 wild-type embryo showing overlapping expression of *Doc3* RNA and phospho-Mad in dorsal mesoderm. (B) Ventral view of late stage 10 wild-type embryo with alternating expression of Doc2+3 and the early visceral mesoderm marker Bap (red). as, amnioserosa. (C) Mesodermal expression of Doc and Bap extend towards the ventral midline (center) upon *2xPE-twi-GAL4*-driven ectopic expression of Tkv^{Q253D}. (D) Early stage 10 wild-type embryo stained for Doc2+3, Tin (red) and Wg (blue) proteins. Co-localized Doc and Tin in dorsal mesoderm appears yellow. Doc is strictly aligned with the Wg stripes. (E) Expanded Doc expression upon ectopically expressed *wg* (via *2xPE-twi-GAL4*) in the dorsal mesoderm at stage 10. (F) Wild-type stage 11 embryo stained as in D. Clusters of Doc^{+} cells in the dorsal mesoderm are marked by white arrowheads, a Doc^{-} Eve cluster by a red arrowhead and lateral (somatic) mesodermal clusters by arrows. (G) Mutually exclusive expression of Doc (white arrowheads) and Eve (red arrowheads) proteins along the dorsal mesodermal margin in wild type at stage 11. (H) Stage 11 *tinD-lacZ* embryo, labeled with anti-Doc2+3 (green), anti-Pnr (red) and anti-β-Galactosidase antibodies (blue). Overlap (white; arrowheads) occurs near the dorsal margin of the mesoderm. Additional Pnr^{+} nuclei (red) are from ectodermal invaginations. (I) Stage 12 wild-type embryo stained for Doc and Pnr proteins showing co-expression along the dorsal edge of the mesoderm (overlap appears yellow). (J) High magnification view of early stage 12 *svp-lacZ* embryo stained for Doc2+3 proteins, *mid* RNA (blue) and β-Galactosidase (red). All cardioblasts, which are either positive for *mid* (Tin-CB) or *svp*-LacZ (Svp-CB), express Doc, as do *svp-lacZ*$^{+}$ pericardial cells (Svp-PC). Differences in Doc levels within *svp-lacZ*-clusters (arrow) probably reflect continued expression of Doc in Svp-CBs versus its termination in Svp-PCs.

from the dorsal ectoderm. The fully developed clusters of Doc expression at mid stage 10 are reciprocal to the clusters of cells expressing the homeobox gene *bagpipe* (*bap*), which mark the precursors of the trunk visceral mesoderm (Fig. 1B). Ectopic activation of the Dpp signaling pathway throughout the mesoderm via a constitutively active Dpp receptor, Thickveins, Tkv^{Q253D} (Nellen et al., 1994), leads to ventral expansion of *bap* expression (Yin and Frasch, 1998), and Doc expression expands in a similar fashion upon pan-mesodermal Tkv^{Q253D} or Dpp expression (Fig. 1C, compare with 1B, and data not shown). Tkv^{Q253D}- or Dpp-induced ectopic Doc and *bap* occur only within their appropriate segmental domains that are defined by positive and negative Wg inputs, respectively (Azpiazu et al., 1996; Lee and Frasch, 2000; Reim et al., 2003). In the case of Doc, *wg* is essential for the segmental induction in both mesoderm and ectoderm (Reim et al., 2003). Accordingly, triple immunostaining with antibodies recognizing the Doc proteins Doc2/Doc3, the dorsal mesoderm marker Tin and Wingless (Wg) protein demonstrates that Doc co-localizes with Tin in the dorsal mesoderm, but unlike Tin is restricted to the areas that are close to the Wg-secreting cells of the ectoderm (Fig. 1D). In addition, forced pan-mesodermal wg expression causes the induction of Doc in a largely continuous fashion along the anteroposterior axis of the dorsal mesoderm, in a pattern that closely resembles that of *tin* (Fig. 1E). Altogether, these data confirm that early mesodermal Doc is induced by intersecting Dpp and Wg signaling pathways. As a consequence, Doc becomes expressed in the entire anterior dorsal quadrant of each mesodermal parasegment (i.e. the dorsal region of each mesodermal A domain) (Azpiazu et al., 1996). These areas will eventually generate the dorsal vessel as well as dorsal somatic muscles, as opposed to the *bap*-positive areas of the P domains that contain most of the primordial cells of the visceral musculature.

During stage 11, the trunk visceral mesoderm primordia separate from the A domains by moving inwards, while the Doc-positive cells of the dorsal A domains maintain contact with the ectoderm and spread out along the anteroposterior axis. Doc expression undergoes dynamic changes during this period. The highest levels of expression are retained along the dorsal margin of the mesoderm, except in the areas that express the homeobox gene *even-skipped* (*eve*), which remain directly underneath each Wg stripe (Fig. 1F and data not shown). Eventually, *Doc* expression becomes completely excluded from the *eve*- and *runt*-expressing dorsal muscle progenitors that derive from this cluster (Fig. 1G; data not shown). At the same time, new clusters of high-level Doc expression appear at more lateral positions, which are not considered part of the dorsal mesoderm as they lack *tin* expression and later contribute to lateral somatic muscles (Fig. 1F, arrows; I.R. and M.F., unpublished).

For additional clarification of the developmental events in the early cardiogenic mesoderm, we examined Doc expression relative to other cardiogenic regulators, including the Gata factor-encoding gene *pannier* (*pnr*). In addition to its expression in the cardiogenic mesoderm, *pnr* is expressed in a broad band of cells along the adjacent dorsal ectoderm (Winick et al., 1993; Herranz and Morata, 2001). We first detect Pnr protein in the mesoderm at early stage 11, which is notably later than the earliest Doc expression but coincides with the time when Doc expression starts to refine along the dorsal margin. Doc and Pnr protein colocalize within the most dorsal areas of the *tin*-positive portion of the mesoderm (Fig. 1H; *tin* expression is monitored with *tinD-lacZ*, a reporter driven by the Dpp-responsive enhancer of *tin*) (Yin et al., 1997). Like Doc, Pnr does not overlap with Eve (data not shown). Hence, the cardiac mesoderm in the *Drosophila* embryo (in a more stringent definition excluding the Eve-positive cells, which also produce dorsal muscles) is characterized by the co-expression of *tin, pnr* and Doc.

During stage 12, Doc maintains its co-expression with *pnr* and *tin*, although the expression of all three genes becomes further restricted to the dorsal margin (Fig. 1I) (Bodmer and Frasch, 1999). For a brief period, Doc is expressed in all cardioblasts that form during this time and are marked by expression of the *Tbx20* ortholog *midline* (*mid*) or the *COUP-TFII*-related gene *seven-up* (*svp*) (Fig. 1J) (Reim et al., 2005). Doc and Pnr gradually disappear during stage 12-13, except for two out of six cardioblasts per hemisegment that maintain Doc expression until the end of embryogenesis (Lo and Frasch, 2001; Reim et al., 2003). *tin* expression is maintained in a complementary set of cardioblasts, as well as in certain pericardial cells (Yin and Frasch, 1998; Ward and Skeath, 2000; Lo and Frasch, 2001).

Next we asked whether *tin* and *pnr* regulate Doc expression in the dorsal and cardiogenic mesoderm. Despite the importance of *tin* for the specification of all dorsal mesodermal derivatives, Doc expression initiates normally in the dorsal mesoderm of *tin* null mutants (Fig. 2B, compare with 2A). Likewise, early Doc expression is also unaffected in *pnr*-null mutants (Fig. 2F; compare with 2E), which is consistent with the observed onset of *pnr* expression after that of Doc. Therefore, Doc induction at stage 10 seems to be a direct result of Dpp and Wg signaling and, unlike *bap* (Lee and Frasch, 2005), does not a require Tin as a mesodermal competence factor. We presume that Dpp and Wg induce Doc expression in a similar fashion in both the dorsal mesoderm and the dorsal ectoderm.

In contrast to stage 10, *Doc* expression is affected in both *tin* and *pnr* mutants at later stages, and fades prematurely from the dorsal mesoderm. In stage 11 *tin* mutants, we see only few irregularly arranged cells that still express *Doc* in the dorsal mesoderm (Fig. 2D; compare with 2C), and by mid stage 12 Doc expression is absent in this area. We also find that *pnr* is never activated in the mesoderm of *tin* mutants (Fig. 2D′; compare with 2C′), which is consistent with previous findings that a *pnr-lacZ* reporter construct requires *tin* and essential Tin-binding sites in order to be activated in the mesoderm (Gajewski et al., 2001). Indeed, loss of *Doc* expression in *tin* mutants might be caused indirectly by the absence of *pnr* products, as high-level Doc expression also fails to emerge in the dorsal mesoderm of stage 11 *pnr* mutants and is virtually absent from stage 12 onwards (Fig. 2H; compare with 2G). The loss of Doc expression in stage 11-12 *pnr* mutants is accompanied by decreased *tin* expression (Fig. 2H) (Alvarez et al., 2003; Klinedinst and Bodmer, 2003), with the strongest reduction in the dorsal-most cells that would normally show high levels of Pnr and Doc.

As *pnr* is also required for *dpp* expression in the dorsal ectoderm at this stage (Herranz and Morata, 2001), it is conceivable that the loss of *Doc* expression is caused indirectly by the lack of the ectodermal Dpp inputs. To address this

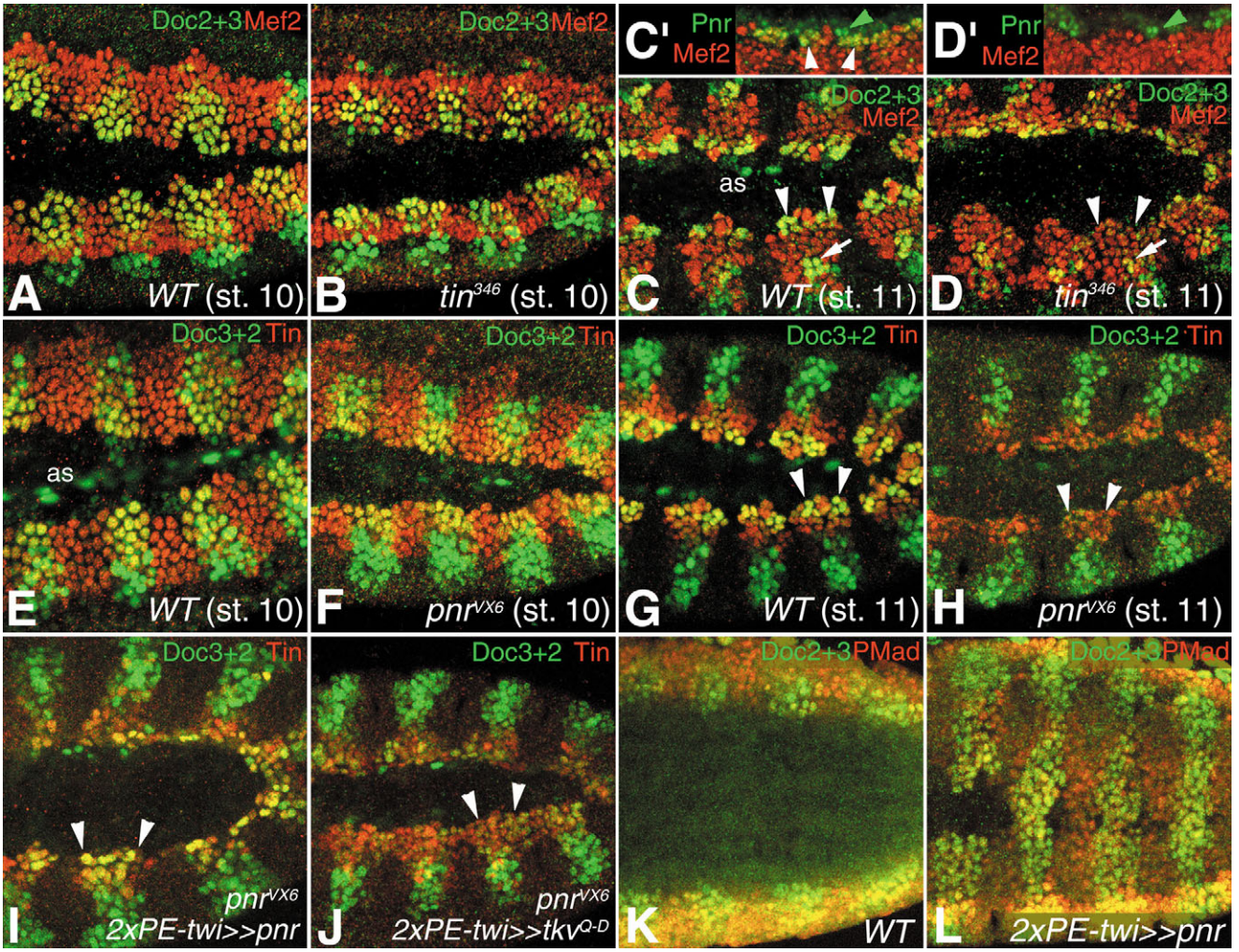

Fig. 2. *pnr*/*tin*-dependent and independent phases of *Doc* expression. (A-D) Expression of Doc proteins (green) in the Mef2-labeled mesoderm (red) in wild-type and tin^{346} mutant embryos. Images are merged optical sections of embryonic trunk mesoderm (lateral views), which occasionally include ventrolateral ectoderm and amnioserosa (as). (A,B) Doc expression initiates normally in the early dorsal mesoderm in *tin* mutants at stage 10. (C) At stage 11 strong Doc expression is seen in the dorsal-most areas of the mesoderm in wild-type embryos (arrowheads; arrow indicates somatic mesodermal Doc). (D) In *tin* mutant embryos, mesodermal Doc fades away during stage 11, except for longitudinal gut muscle founders (in dorsoposterior region of germ band) and the lateral somatic Doc clusters (arrow). (C′,D′) Detection of Pnr and Mef2 protein in wild-type and tin^{346} mutants at stage 11. *tin* mutants (D′) do not express *pnr* in the mesoderm, as is seen in wild-type embryos (white arrowheads, C′). Ectodermal Pnr expression (partially present in these projections; green arrowheads) is not affected. (E-J) Detection of Doc (green) and Tin protein (red) in wild-type and pnr^{VX6} mutant embryos. (E,F) At stage 10, normal Doc expression is seen in *pnr* mutants. (G,H) Doc protein fades away during stage 11 in *pnr* mutant embryos (arrowheads) and Tin levels also begin to decrease. (I) Expression of UAS-*pnr* only in the mesoderm via *2xPE-twi-GAL4* can restore high levels of Doc expression in the cardiogenic mesoderm of *pnr* mutants (arrowheads). (J) No significant rescue of cardiogenic Doc expression is observed if the Dpp-pathway is activated by mesodermal $UAS\text{-}tkv^{Q253D}$ expression (arrowheads). (K,L) Ventral view of stage 10-11 wild-type embryo and (L) of *2xPE-twi-GAL4*; *UAS-pnr* embryo stained with anti-Doc2+3 (green) and anti-phospho-Smad1/PMad antibodies (red). Ectopic expression of *pnr* in the mesoderm causes striped ectopic *Doc* expression in the mesoderm along with ectopic Mad phosphorylation.

possibility, we restored either *pnr* expression or Dpp signaling exclusively in the mesoderm of *pnr* mutant embryos. We found that forced mesodermal *pnr* expression can significantly rescue high-level Doc expression in stage 11 pnr^{-} embryos (Fig. 2I, arrowheads; compare with 2H). There is also a significant rescue of cardioblast specification, as indicated by the activation of cardioblast-specific *midline* in this background (Reim et al., 2005) and by late stage Doc and Tin expression in a complementary pattern (as in wild-type cardioblasts, albeit in fewer cells; data not shown). By contrast, activating Dpp signaling in the mesoderm of *pnr* mutants with $UAS\text{-}tkv^{Q253D}$ or *UAS-dpp* does not result in a significant rescue of Doc expression in the dorsal mesoderm (Fig. 2J and data not shown; compare with 2H,I). These data suggest that *pnr* is required, perhaps directly, to regulate *Doc* expression in the dorsal mesoderm during stages 11-12, although it is not involved in the initial activation of Doc during stage 10.

Unexpectedly, forced mesodermal *pnr* expression can activate *Doc* ectopically at stage 10, when endogenous *pnr* is not yet expressed in the mesoderm (Fig. 2L; compare 2K). The

observed restriction of ectopic *Doc* expression to the mesodermal A domains indicates that the activation of *Doc* by Pnr in this situation additionally requires Wg signals, as it does normally. We also considered the possibility that *pnr* can activate *dpp* in the mesoderm, which in turn would induce Doc. Indeed we observe ectopic activation of phospho-Mad in embryos with pan-mesodermal Pnr, which correlates with expanded Doc expression (Fig. 2L; compare 2K). This effect may reflect the normal activity of *pnr* in activating *dpp* expression in the dorsal ectoderm, although it is possible that *pnr* normally also activates low levels of *dpp* expression in the dorsal mesoderm, which may have escaped detection so far.

Dorsocross is required for the specification of cardioblasts

We used a recently generated small deficiency, *Df(3L)DocA*, that deletes all three Doc genes, *Doc1*, *Doc2* and *Doc3* (Reim et al., 2003), to investigate the role of the Doc genes in dorsal vessel development. As described previously, *DocA* mutants (i.e. embryos homozygous for *Df(3L)DocA*) do not undergo germ band retraction owing to the requirement of Doc in the amnioserosa. To avoid this complication and rule out indirect influences of morphogenetic defects on cardiogenesis, we performed our analysis predominantly with *DocA* mutants in which germ band retraction has been rescued by amnioserosa-specific expression of *Doc2* via *c381-GAL4* (Reim et al., 2003). Dorsal vessel development in these embryos was analyzed through expression of genes that normally mark all or subsets of cardioblasts.

Fig. 3A shows a cardioblast-specific β-Galactosidase staining produced by *Mef2-HtΔD-lacZ* in a wild-type background, which reflects the expression of the MADS-box

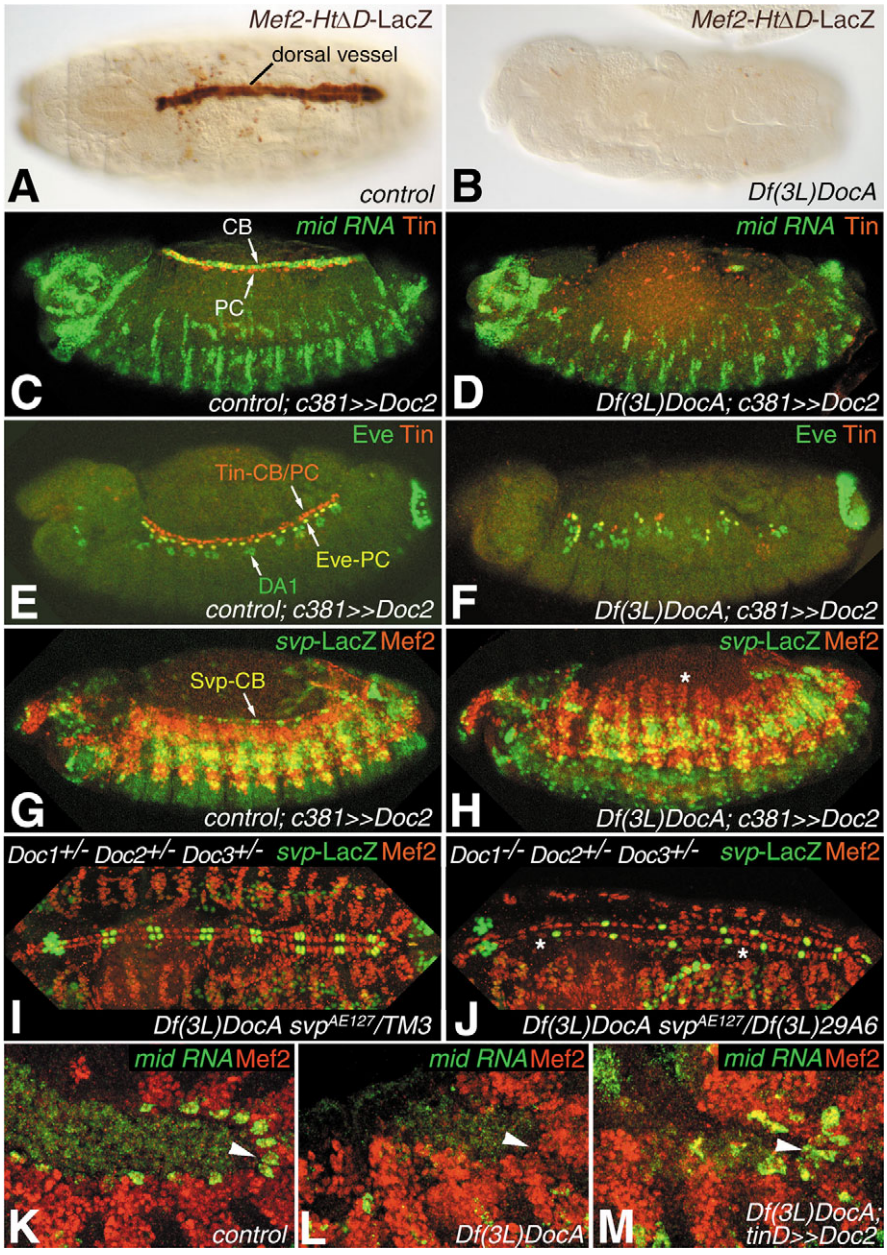

Fig. 3. Cardiac phenotype of *Dorsocross* loss-of-function mutants. (A,B) Stage 16 embryos carrying *Mef2-HtΔD-lacZ*, stained with anti-β-Galactosidase (dorsal view). β-Galactosidase signals mark all cardioblasts of the dorsal vessel of wild-type embryos (A), but are absent in homozygous *Df(3L)DocA* embryos (B). (C-H) Lateral views of stage 14 embryos fluorescently labeled for markers, as indicated by the color code. Control embryos (left column) and homozygous *Df(3L)DocA* mutant embryos (right column) carry *c381-GAL4* and *UAS-Doc2* used for amnioserosa rescue. (C,D) Detection of *mid* RNA and Tin protein as markers for cardioblasts (CB; *mid*-positive and Tin^+) and Tin^+ pericardial cells (PC; *mid*-negative). The number of Tin^+ cells is severely reduced and *mid*-expressing cardioblasts are almost absent in mutants. *mid* RNA is still detectable in ectodermally derived tissues. (E,F) Detection of Tin and Eve proteins. Tin^+ cardioblasts (Tin-CB, red) and pericardial cells (Tin-PC, red) are largely lost in the *DocA* mutant. Most of the remaining Tin^+ cells are Eve-pericardial cells that express both markers (Eve-PC, yellow). Eve^+ dorsal muscles (DA1, green) are also present in *DocA* mutants. (G,H) Embryos carrying one copy of AE127 (*svp-lacZ*) stained for β-Galactosidase and Mef2 to identify Svp-cardioblasts (Svp-CB), which are absent in the *DocA* mutant (asterisk). (I) Dorsal vessel of *Df(3L)DocA svp-lacZ* heterozygous embryo at stage 16 stained as in G showing normal number of cardioblasts. (J) Defective dorsal vessel of *Df(3L)DocA svp-lacZ/Df(3L)29A6* transheterozygous embryo stained as in G and I. The reduced Doc gene copy number has caused the loss of numerous cardioblasts, especially of the Svp-CB type. Asterisks indicate positions of Tin-CB loss. (K-M) Dorsal trunk mesoderm of early to mid stage 12 embryos stained for Mef2 protein (red) and *mid* RNA (green). (K) *mid* expression initiates in small clusters along the dorsal edge of the mesoderm (arrowhead) in the wild-type (K) but not in the *DocA* mutant embryo (L). (M) Mesodermal expression of *Doc2* via *tinD-GAL4* rescues cardiac *mid* expression in a homozygous *Df(3L)DocA* mutant background.

transcription factor Mef2 in cardioblasts (Nguyen, 1998) (H. Duan and H. T. Nguyen, unpublished). In *DocA* mutants, almost no β-Galactosidase is detected from this reporter (Fig. 3B; except for the distortions, the results were essentially the same in both amnioserosa-rescued and non-rescued embryos for this and all other markers tested). Likewise, examination of *tin* expression at stage 14 or later reveals that *DocA* mutants have a greatly reduced number of Tin-positive cells (Fig. 3D, compare with 3C). Consistent with our findings with the *Mef2-HtΔD-lacZ* marker, no or very few Tin-positive cells express the cardioblast marker *mid* (Fig. 3D, compare with 3C) or *H15* (data not shown) in *DocA* mutants. Double staining for the pericardial cell marker *even-skipped* (*eve*) shows that most of the residual Tin-positive cells co-express *tin* and *eve*, and, hence, correspond to Eve-pericardial cells (Eve-PCs) (Fig. 3F). Like the Eve-PCs, the *eve*-expressing somatic muscles #1 (also known as DA1) are also not affected by the loss of Doc activity (Fig. 3F).

Two distinct types of cardioblasts can be discriminated by their mutually exclusive expression of either *tin* or *svp*. We performed anti-β-Galactosidase/anti-Mef2 double staining of embryos carrying one copy of the *svp-lacZ* enhancer trap insertion AE127 to confirm that not only the Tin-cardioblasts (Tin-CBs) but also the Svp-cardioblasts (Svp-CBs) are affected by the loss of Doc genes. As shown in Fig. 3H, Svp-CBs are absent in *DocA* mutants, as is the Mef2-stained row of cardioblasts above the somatic mesoderm (compare with Fig. 3G). Heterozygous *DocA* embryos usually have normal numbers of cardioblasts (Fig. 3I), although a small fraction of embryos do have reduced numbers, which indicates less robust development of cardioblasts with decreased Doc activity. Indeed if Doc dose is further reduced by combining *Df(3L)DocA* with *Df(3L)29A6*, a deletion that removes *Doc1*, there is a significant reduction from normally 104 cardioblasts in wild-type embryos to 80±6 cells (n=10) (Fig. 3J). Although all types of cardioblasts are affected by the reduction of Doc levels, Svp-CBs appear to be more sensitive than Tin-CBs. This suggests that Doc has an early function required for all cardioblasts as well as a more specific function for Svp-CBs, in which it is expressed at later stages.

To distinguish whether Doc genes are required for the specification versus survival of cardioblasts, we examined the expression of a number of markers for newly specified cardioblasts at early stages of cardiogenesis. Cardioblast expression of *mid* (Fig. 3L; compare with 3K), *svp-lacZ* (Lo and Frasch, 2001), the G_0 α-subunit gene *brokenhearted* (*bkh*) (Fremion et al., 1999) and *hand* (Kölsch and Paululat, 2002) (data not shown) never initiates during early stage 12 in *DocA* mutants, although *bkh* and *hand* are still expressed in Eve-PC progenitors as in wild-type embryos. Cardiac expression of several markers, including *mid*, *H15*, *Mef2-HtΔD-lacZ*, *svp-lacZ*, *hand* and *bkh*, is partially rescued by forced expression of either *Doc1*, *Doc2* or *Doc3* via *tinD-Gal4* in the dorsal mesoderm in the background of *Df(3L)DocA* (Fig. 3M; see also Fig. 7; other data not shown; *Doc1* and *Doc3* rescuing activity was only tested with *mid* and *Mef2-HtΔD-lacZ*). As observed previously for the amnioserosa (Reim et al., 2003), the best rescue was consistently obtained with *Doc2*. These data demonstrate that mesodermal expression of Doc is sufficient to rescue cardioblast specification and that it is the Doc genes and not any of the other genes uncovered by *Df(3L)DocA* that are responsible for the observed cardiac phenotypes. We conclude that Doc genes have an essential role and act in a dose-dependent manner in specifying cardioblasts.

Doc is required for Odd-positive pericardial and lymph gland cells but not for Eve-positive pericardial cells and dorsal somatic muscles

The dorsal quadrants of Doc-expressing mesodermal cells from stage 10 embryos not only give rise to cardioblasts, but also to pericardial cells and dorsal somatic muscle founders. The observed reduction of Tin-positive cells already indicates that the pericardial cells expressing *tin* but not *eve*, called Tin-PCs, are also drastically reduced in number in Doc loss-of-function mutants (Fig. 3D,F). To complete the analysis of dorsal mesodermal cell types, we analyzed *DocA* mutants for the presence of Tin^- pericardial cells that express *odd-skipped* (*odd*). Two out of the four Odd-positive pericardial cells (Odd-PCs) in each hemisegment are Svp-CB siblings, while the other cells derive from a different lineage (Ward and Skeath, 2000). All Odd-PCs are missing in the mutants, as are Odd-positive cells that originate from the thoracic dorsal mesoderm and normally form the lymph glands (Fig. 4B; compare with 4A). This result is consistent with an early defect in the specification of a common pool of precursors of pericardial/lymph gland cells and cardioblasts (see Mandal et al., 2004). By contrast, and as described above, progenitors that produce Eve-pericardial cells and Eve-expressing founders of muscle 1 (DA1) from a distinct pool of cells are specified normally in the absence of *Doc* (Fig. 4B; compare with 4A). Runt marks another somatic muscle founder and the corresponding muscle #10 (DO2) that are derived from the early Doc-expressing domains. As for Eve, the Runt pattern in *DocA* mutants with rescued germ band retraction is very similar to wild type, even at later stages (Fig. 4D, compare with 4C). Likewise, staining for *1010 B2-lacZ*, a reporter gene fortuitously expressed in all dorsal muscles (Yin and Frasch, 1998), shows that the dorsal muscles are present in *DocA* mutants, although they are distorted because of the defects in embryo morphology without the amnioserosa (Fig. 4F, compare with 4E). Therefore Doc genes, although present in the entire dorsal quadrants of the early mesodermal A domains, are specifically required for pure cardiac cell lineages, but not for those that generate in addition or solely somatic muscles. This function correlates with the restricted Doc expression at stage 11-12, where Doc expression is excluded from the Eve-positive clusters.

Genetic interactions between Doc, *pnr* and *tin*

The observed co-expression of Doc, *tin* and *pnr* during stages 11 and 12, and defective cardioblast specification upon loss of function of these genes suggest that these three genes act in a common pathway during early cardiogenesis. The occurrence of genetic interactions among these genes would be further indicative of this possibility. Because we observe mild defects in cardioblast specification in embryos with a reduced Doc dose (*Df(3L)29A6/Df(3L)DocA*, Fig. 3J), we asked whether reduction of *tin* and *pnr* gene dose would have any further impact on cardiogenesis. In order to test this possibility, the null alleles pnr^{VX6} and tin^{346} were individually recombined onto the *Df(3L)DocA* chromosome. In addition, triple mutants carrying *Df(3L)DocA*, pnr^{VX6} and tin^{346}, as well as pnr^{VX6} tin^{346} double mutants, were generated. Heterozygous individuals of

all combinations are viable, although heterozygous triple mutants have a slightly reduced viability (63%) when compared with *tin*^{346} heterozygotes. If *Df(3L)DocA/TM3* heterozygous flies are crossed to *Df(3L)29A6/TM3* flies, all non-balanced transheterozygotes die at pupal stages (Reim et al., 2003). We were also able to obtain pupae of the genotypes *Df(3L)29A6/Df(3L)DocA pnr*^{VX6} and *Df(3L)29A6/Df(3L)DocA tin*^{346}, but not with *Df(3L)29A6/Df(3L)DocA pnr*^{VX6} *tin*^{346} among about 200 progeny, indicating more severe defects with the latter combination.

Fig. 5 shows representative phenotypes of transheterozygous embryos. As described above, *Df(3L)29A6/Df(3L)DocA* embryos, with one copy each of *Doc2* and *Doc3* and no *Doc1*, have a reduced number of cardioblasts (Fig. 5B; compare with 5A). Although all types of cardioblasts are affected, the number of Doc-positive cardioblasts is most strongly reduced, and not all of the residual *svp-lacZ*-positive cells retain *Doc* expression (Fig. 5B and data not shown, compare also with Fig. 3J). If one copy of *tin* or *pnr* is removed in the *Df(3L)29A6/Df(3L)DocA* background, even fewer cardioblasts are specified, which frequently causes gaps in the dorsal vessel (Fig. 5C,D). This synergistic effect becomes very strong in *Df(3L)29A6/Df(3L)DocA pnr*^{VX6} *tin*^{346} embryos, in which less than half the normal number of cardioblasts are formed (Fig. 5E). However, if only one Doc gene copy (*Doc1*) is removed, the reduction of one copy of *pnr* and *tin* has only mild effects (*Df(3L)29A6/pnr*^{VX6} *tin*^{346}; Fig. 5F). Odd-pericardial cells and the lymph glands are also strongly reduced in *Df(3L)29A6/Df(3L)DocA pnr*^{VX6} *tin*^{346} embryos, suggesting that Doc interacts with *pnr* and *tin* already early during the specification of the cardiac mesoderm (Fig. 5H; compare with 5G). The impact on Eve-pericardial cells is much weaker (data not shown), which is consistent with the observation that these cells are not affected upon complete loss of the Doc genes in *DocA* mutants. These observations indicate that Doc genes, *tin* and *pnr* cooperate or depend on each other during the specification of cardiac progenitors.

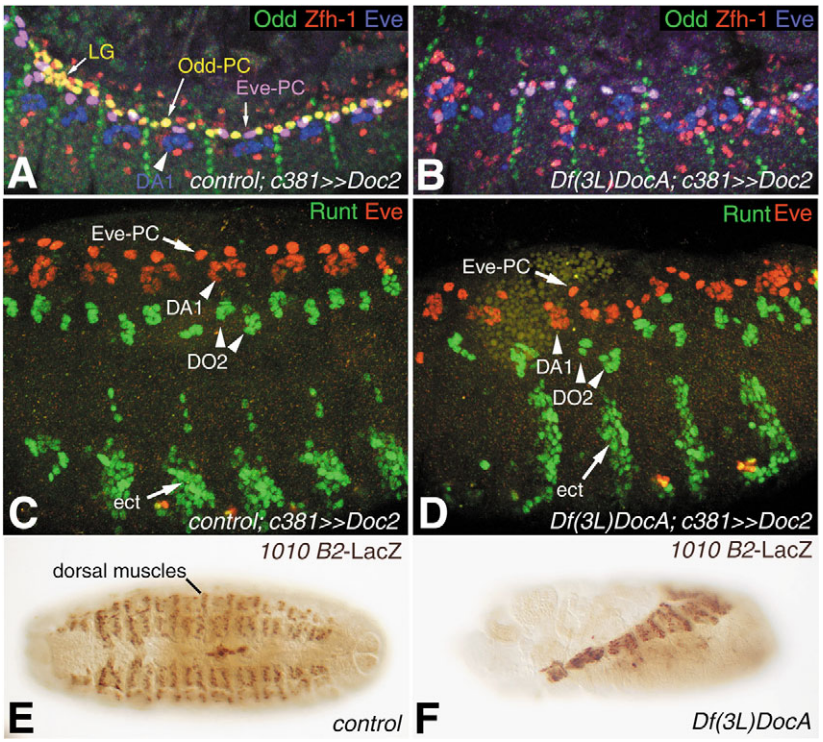

Fig. 4. Effects of *Dorsocross* mutation on pericardial cell and dorsal somatic muscle formation. (A) Lateral view of stage 14 control embryo that carries the amnioserosa rescue constructs *c381-GAL4/UAS-Doc2*, but is otherwise wild type. Staining for Zfh-1, Odd and Eve allows discrimination of lymph gland (LG) and Odd-pericardial cells (Odd-PC) (yellow, Odd$^+$ + Zfh-1$^+$) from Eve-pericardial cells (Eve-PC, purple, Eve$^+$ + Zfh-1$^+$) and from pericardial cells that express only Zfh-1 (Tin-PC and other tissues, red). Odd is also expressed in ectodermal stripes (green), and Eve in DA1 (#1) muscles (blue). (B) *DocA* mutant embryo with amnioserosa rescue constructs stained as in A. Odd$^+$ pericardial and lymph gland cells as well as the great majority of Tin-PCs are absent, while Eve-PCs and DA1 muscles are present. (C,D) Expression of the dorsal muscle marker proteins Eve (red) and Runt (green) in stage 14 embryos carrying amnioserosa rescue constructs. In control embryos (C), Eve is present in the nuclei of DA1 muscles and pericardial cells (Eve-PC, red) and Runt in DO2 (#10) muscles. In amnioserosa-rescued homozygous *DocA* mutants (D) muscles DA1 and DO2 are largely unaffected. Ectodermal *runt* (ect) is dorsally expanded owing to the function of Doc in the dorsolateral ectoderm. (E,F) Anti-β-Galactosidase staining of stage 16 embryos carrying the *1010 B2-lacZ*-reporter gene to visualize nuclei of dorsal muscles. (E) Wild-type embryo (dorsal view) with multi-nucleated dorsal muscles. (F) Homozygous *DocA* mutant embryo showing the presence of dorsal muscles, albeit distorted owing to the morphogenetic defects of these embryos.

Activation of *pnr* and maintenance of *tin* expression are major functions of *Dorsocross* during early cardiogenesis

The above-described expression patterns, phenotypes and genetic interactions among Doc, *pnr* and *tin* raise the possibility that *Doc* functions by activating or maintaining the expression of *pnr* and *tin*. *tin* and *pnr* expression in the cardiac mesoderm are also interdependent (Gajewski et al., 2001; Alvarez et al., 2003; Klinedinst and Bodmer, 2003), hence the reduced expression of either *tin* or *pnr* would reduce the products of both genes. In *DocA* mutants, Tin protein distribution appears normal until mid-stage 11, during which *tin* expression is activated from the *dpp*- and *tin*-dependent *tinD* enhancer (Fig. 6B; compare with 6A) (Xu et al., 1998). Accordingly, reporter gene expression driven by the *tinD* enhancer also appears to be normal (Fig. 6F), and *tinD-GAL4* can be used to rescue cardioblast specification via *UAS-Doc* transgenes (see Fig. 3M). By contrast, cardioblast-specific expression of *tin* driven by the *tinC* enhancer version *tinCΔ7* (active in all cardioblasts) (Lo and Frasch, 2001) is never detected (data not shown). This is consistent with the observation that progressively fewer Tin-positive cells are present in Doc mutants after mid-stage 11, in which *tin* expression is maintained at high levels only in cells that express *eve* (Fig. 6D; compare with 6C). Of note, the number of the Eve-positive cells is increased from 3-4 to 6-10 cells per cluster at mid-stage 11 (Fig. 6B; compare

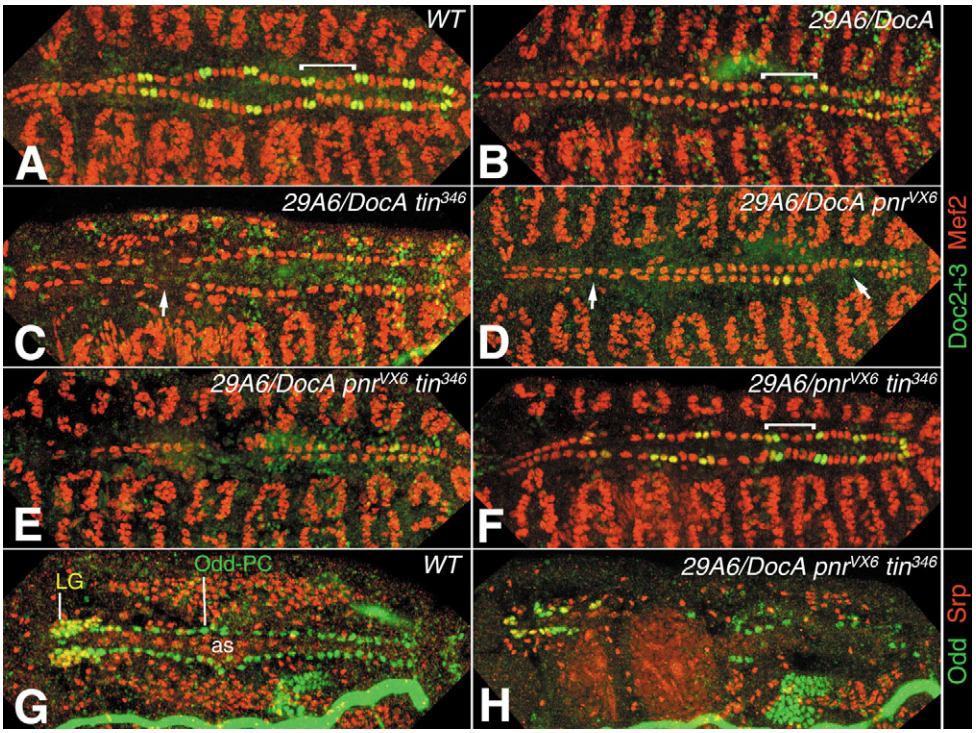

Fig. 5. *Dorsocross* interacts genetically with *pnr* and *tin*. (A-F) Presence of cardioblasts in the dorsal vessel of embryos with reduced copies of *Doc*, *pnr* and *tin* genes was examined in stage 16 embryos by anti-Mef2 (red) and anti-Doc2+3 (green) staining. For clarity, clearly identifiable regions of the Mef2-stained midgut lining were removed from the deepest sections prior to merging. (A) Wild type with two cardioblast rows along the dorsal midline. Doc is strongly expressed in the Svp-CBs. (B) Fewer cardioblasts, particularly of the Doc^{+} type, are seen in *Df(3L)29A6/Df(3L)DocA* transheterozygous embryos in which *Doc1* is deleted and only one copy each of *Doc2* and *Doc3* is present. Bracket indicates cardioblasts within one abdominal segment. Even fewer cardioblasts are specified in *Df(3L)29A6/Df(3L)DocA tin^{364}* (C) and *Df(3L)29A6/Df(3L)DocA pnr^{VX6}* (D) transheterozygous embryos, frequently leading to gaps in the cardioblast rows (arrows). (E) Cardioblast number is further reduced in *Df(3L)29A6/Df(3L)DocA pnr^{VX6} tin^{364}* transheterozygous embryos and only short regions of the dorsal vessel are formed. (F) Embryos with one copy of *tin*, *pnr* and *Doc1*, and two copies of *Doc2* and *Doc3* [*Df(3L)29A6/pnr^{VX6} tin^{364}*] also have a reduced number of cardioblasts, but there is no bias towards loss of Doc^{+} cardioblasts as seen in in B-D. (G,H) Dorsal view of stage 16 embryo stained for Odd (green) and Srp (red) proteins. In the wild type (G), Odd labels Odd-pericardial cells (Odd-PC) and the lymph gland (LG). Srp co-localizes with Odd in the lymph gland, but is also expressed in other tissues, including the amnioserosa (as). In *Df(3L)29A6/Df(3L)DocA pnr^{VX6} tin^{364}* transheterozygous embryos (H), only few Odd-pericardial and lymph gland cells are present.

with 6A). The broadened *eve* expression is only transient; it persists until stage 12 (Fig. 6D; compare with 6C) but at later stages nearly normal numbers of Eve-pericardial cells and lineage-related muscles (muscles #1/DA1 and #10/DO2) are present (see Fig. 4B,D).

In the wild type, mesodermal *pnr* expression starts later and in a more restricted domain when compared with *tinD*-driven *tin* or *lacZ* expression (Fig. 6E), but slightly earlier than *tinC*-driven *tin* expression. In *DocA* mutants we found that *pnr* is

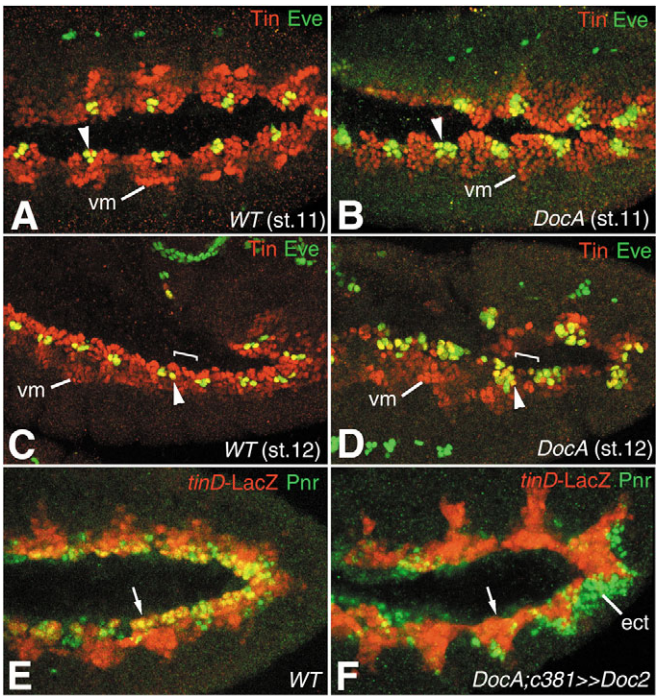

Fig. 6. Early defects in cardiogenesis caused by *Dorsocross* mutation. (A-D) Expression of Tin and Eve in dorsal mesoderm. In wild-type mid-stage 11 embryos (A) each segment contains a cluster of three or four Eve^{+} cells (arrowhead), and during mid-stage 12 (C), two or three Eve^{+} cells (progenitors of Eve-PCs and muscles #1 and 10) are present. The number of Eve^{+} cells is increased in *DocA* mutant embryos at stage 11 (B) and 12 (D). At stage 12, fewer *tin*-expressing cells are present in *DocA* mutants, and almost none of them within the area normally occupied by the cardiogenic mesoderm (D, bracket). Almost all dorsal cells that maintain *tin* expression are Eve^{+}. vm, visceral mesoderm. (E,F) Expression of *pnr* in the cardiogenic mesoderm visualized by anti-Pnr/anti-β-Galactosidase staining of wild-type (E) and *DocA* mutant (F) stage 11 embryos carrying *tinD-lacZ*. Arrow indicates overlapping expression of Pnr and *tinD-lacZ* along the dorsal margin of the mesoderm in wild-type embryos and missing Pnr expression in *DocA* mutants. Pnr is still present in the dorsal ectoderm (ect) of *DocA* mutants (partially present in projection). The embryo shown in F has been rescued for amnioserosa expression of *Doc2* via *c381-GAL4*, but results are identical without *c381*-driven *Doc2*.

not activated at all in the dorsal mesoderm, whereas its expression in the dorsal ectoderm is normal (Fig. 6F). We conclude that the Doc genes act upstream of *pnr* during a very early step in the specification of the cardiac mesoderm. As *pnr* is crucial for downstream events of cardiac specification, including the cardioblast-specific expression of *mid*, *H15*, *tin* and Doc, it is conceivable that many of the functions of Doc in cardioblast specification are mediated through *pnr*. We addressed this possibility by expressing *pnr* in the *DocA* mutant background. Restoring Pnr in the dorsal mesoderm of *Doc*-null mutants via *tinD-GAL4* and *UAS-pnr* is sufficient to rescue cardioblasts, which are identified as *mid* mRNA/Tin double-positive cells (Fig. 7C; compare with Fig. 7A,B) and with *Mef2-HtΔD-lacZ* (data not shown). The degree of rescue is not as high as with *UAS-Doc2* (Fig. 7E), but at least as efficient as that with *UAS-Doc3* (Fig. 7F). In an analogous experiment with *UAS-tin*, only very few *mid*-positive cells are detected (Fig. 7D), although their number appears still slightly higher as compared with *DocA* mutant controls (Fig. 7B). The observation of rescued cardioblasts and Odd-PCs (data not shown) in *DocA* mutants with ectopic Pnr in the mesoderm supports the notion that one of the major functions of *Doc* is the activation of *pnr* in the presumptive cardiac mesoderm. However, the failure of *pnr* to rescue Doc phenotypes fully indicates that Doc also functions in parallel with Pnr, for example, by synergizing with Pnr at common target enhancers, or that Doc activates additional targets that contribute to normal cardioblast development.

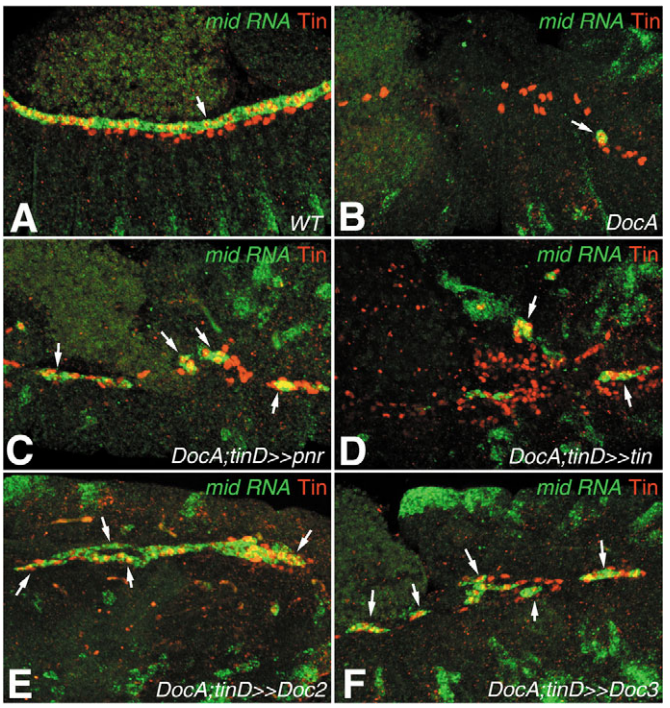

Fig. 7. Rescue of cardioblast specification by forced expression of cardiogenic regulators. Shown are stage 14-15 embryos double labeled for *mid* mRNA and Tin protein to monitor cardioblast (arrows) specification. (A) Wild type showing normal *mid* and Tin expression in cardioblasts, as well as Tin in pericardial cells. (B) In homozygous *DocA* mutants, very few cardioblasts are detectable, although Tin^+ Eve-PCs are still present. (C-F) *DocA* mutants expressing various *UAS*-rescue constructs under control of the dorsal mesoderm driver *tinD-GAL4*. (C) Cardioblast formation is moderately rescued by *UAS-pnr*. (D) In *DocA* mutants with *tinD*-driven *tin*, only few additional cardioblasts are formed compared with *DocA* mutants without any rescue construct. (E) *UAS-Doc2* causes efficient rescue of cardioblast specification in a *DocA* mutant background. (F) *UAS-Doc3* shows a moderate rescuing activity.

Ectopic *Doc2* promotes cardioblast cell fates in conjunction with *tin* and *pnr*

Given the observed requirement of Doc genes for cardioblast specification, we also asked whether misexpression of Doc can produce ectopic cardioblasts. Ectopic expression of *Doc2* with *tinD-GAL4*, a driver that is active in the dorsal mesoderm at stage 11-12 when Doc also functions in normal embryos, leads to formation of some additional cardioblasts (as monitored by co-expressed Mef2 protein and *mid* RNA; Fig. 8B, compare with 8A). Conversely, the number of Odd/Zfh1-positive pericardial cells is reduced, whereas the Eve-positive pericardial cells and dorsal muscles are less affected (Fig. 8J, compare with 8I). The number of extra cardioblasts is lower than the number of missing pericardial cells. In addition, the nuclei of the somatic mesoderm in dorsolateral areas are arranged irregularly. Together, these observations indicate that ectopic Doc2 can transform some pericardial and/or dorsal muscle progenitors into cardioblasts, although more frequently it disrupts the development of these progenitors without transforming them.

As the observed genetic interactions suggest a synergistic action of *Doc* with *tin* and *pnr*, we asked whether combined expression of *Doc2* with *tin* and *pnr* would increase the efficiency of generating ectopic cardioblasts. Ectopic expression of *pnr* alone in the entire mesoderm has been reported to increase cardioblast numbers (Gajewski et al., 1999), but with the more restricted and slightly weaker driver *tinD-GAL4*, we find only few extra cardioblasts (data not shown). No increase in cardioblast number was seen in embryos in which only *tin* is overexpressed (data not shown), but co-expression of *tin* and *pnr* produces more cardioblasts than *pnr* alone (Fig. 8C). Although this increase in cardioblasts is similar to that of *Doc2* expression, the numbers of pericardial cells are only slightly reduced (Fig. 8K). If *Doc2* is expressed together with *tin* or *pnr*, or with both, large numbers of extra cardioblasts are formed (Fig. 8D,E,F). Combined expression of *Doc2* and *pnr* consistently produces more cardioblasts than expression of *Doc2* and *tin*, and nearly as many as the triple co-expression of *Doc2*, *pnr* and *tin*, under which conditions cardioblast numbers are roughly doubled.

Broader ectopic expression of *Doc2*, via the early pan-mesodermal driver *2xPE-twi-GAL4*, causes even stronger expansion of early cardioblast markers such as *mid*, *H15, Toll305-clacZ* and *tinCΔ7-lacZ* in the mesoderm of stage 12-13 embryos (data not shown) (Wang et al., 2005). However, even under these conditions, the effects tend to be stronger in dorsal areas of the mesoderm, suggesting that Doc cooperates with other dorsally localized activities to promote cardioblast fates. Likely candidates include *tin* and *pnr*, because the combined ectopic expression of Doc2, *tin* and *pnr* causes widespread ectopic cardioblast formation (Fig. 8H; compare with 8G) to a much greater extent than compared with *Doc2* alone or the combination of *tin* plus *pnr* (data not shown)

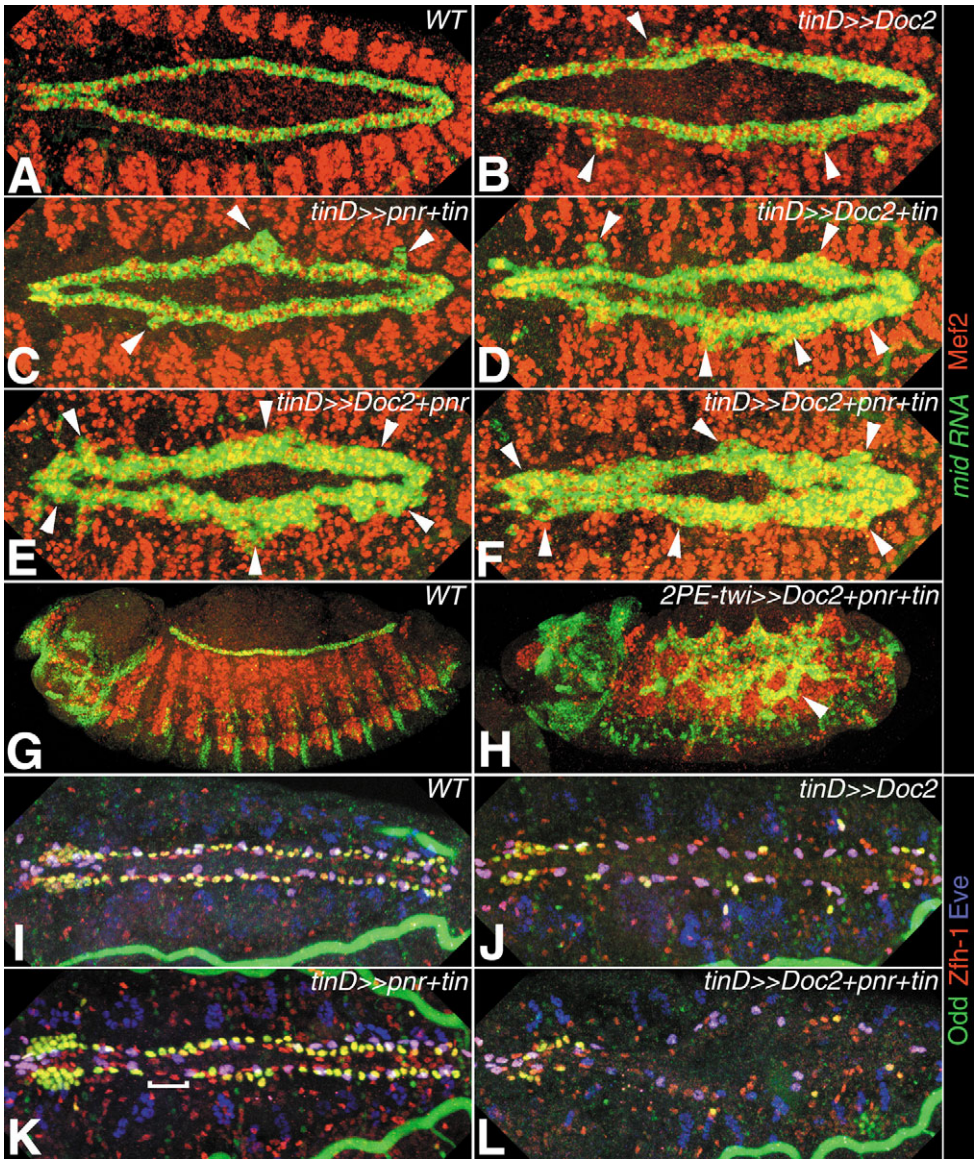

Fig. 8. Ectopic expression of *Doc* together with *pnr* and *tin* promotes cardioblast specification. (A-F) Cardioblasts of representative stage 15-16 embryos labeled for *mid* mRNA and Mef2 protein. All images are projections of confocal scans merged as in Fig. 5. (A) Wild-type embryo showing single-cell bilateral rows of cardioblasts. (B) Ectopic expression of *Doc2* throughout the dorsal mesoderm using *tinD-GAL4*/*UAS-Doc2* produces extra cardioblasts (arrowheads). (C) Combined expression of *pnr+tin* leads to a similar number of supernumerary cardioblasts as with *UAS-Doc2*. (D) Combined expression of *Doc2+tin* produces even more cardioblasts than with *UAS-Doc2* alone. (E) *UAS-Doc2+UAS-pnr* and (F) *UAS-Doc2+UAS-pnr+UAS-tin* driven by *tinD-GAL4* produce the strongest increase in cardioblast number, with almost twice as many cardioblasts as in wild type. (G) Lateral view of stage 14 wild-type embryo stained for *mid* mRNA and Mef2, showing a single row of cardioblasts. (H) Embryo as in G, but with *twi-GAL4*-driven expression of *Doc2*, *pnr* and *tin*, which shows a dramatic expansion of myocardial *mid* expression (arrowhead). (I) Stage 16 wild-type embryo stained for the pericardial cell markers Zfh-1, Odd and Eve as in Fig. 4A. (J) *tinD-GAL4*/*UAS-Doc2* embryo stained as in I, showing fewer pericardial cells, especially of the Odd-PC type (yellow). Eve-PCs (pink) and DA1 muscles (blue) are only mildly affected. A reduction of Odd^{+} cells is also seen in the lymph gland. (K) Combined expression of *pnr* and *tin* in the dorsal mesoderm leaves the majority of the pericardial cells intact, although some Eve-PCs and Odd-PCs are missing (bracket). (L) Combined overexpression with *UAS-Doc2+UAS-pnr+UAS-tin* leads to a severe reduction of all types of pericardial cells, although Eve-PCs (pink) are retained more frequently.

(Klinedinst and Bodmer, 2003). However, *twi*-driven *Doc2* by itself is sufficient to repress the visceral mesoderm markers *bagpipe* (*bap*) and *biniou* (*bin*) (data not shown).

Altogether, the ectopic expression data suggest that Doc genes synergize with *pnr*, and to some degree *tin*, in promoting cardioblast formation. Although the loss-of-function data show that Doc activity is required for the formation of both cardioblasts and Eve-negative pericardial cells, ectopic expression of Doc, especially in combination with *pnr* and *tin*, appears to transform these types of pericardial cells into cardioblasts. This may indicate that, after their initial role in generating progenitors of both cardioblasts and Eve-negative pericardial cells, Doc genes exert a second role in promoting cardioblast versus pericardial cell identities.

Discussion

In vertebrate species, genetic studies with loss-of-function alleles have implicated *Tbx1*, *Tbx2*, *Tbx5* and *Tbx20* in the control of heart morphogenesis and the regulation of cardiac differentiation markers (Basson et al., 1997; Li et al., 1997; Bruneau et al., 2001; Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001; Garrity et al., 2002; Harrelson et al., 2004; Brown et al., 2005; Cai et al., 2005; Singh et al., 2005; Stennard et al., 2005). In the case of *Tbx5*, a small number of cardiac differentiation genes have been identified as direct downstream targets (reviewed by Plageman and Yutzey, 2005). However, owing to the complexity of the system, the respective positions of these genes within a regulatory network during early cardiogenesis are still poorly understood.

Drosophila offers a simpler system to study regulatory networks in cardiogenesis. Recently, the *Tbx20*-related T-box genes *mid* and *H15* were shown to play a role in cardiac development downstream of the early function of the NK homeobox gene *tin* and the Gata gene *pannier* (*pnr*) (Miskolczi-McCallum et al., 2005; Qian et al., 2005; Reim et al., 2005). Whereas the role of these genes in the morphogenesis of the cardiac tube is minor, they are involved in processes of cardiac patterning and differentiation during the second half of cardiogenesis, which includes the activation of *tin* expression in myocardial cells (Reim et al., 2005). In our present report, we have characterized the roles of the *Tbx6*-related Dorsocross T-box genes (which may actually have arisen from a common ancestor of the vertebrate *Tbx4*, *Tbx5* and *Tbx6* genes) (Reim et al., 2003), in *Drosophila* cardiogenesis. We have shown that the Doc genes have a fundamental early role that is required for the specification of all cardiac progenitors that generate pure myocardial and pericardial lineages. They are not required for generating dorsal somatic muscle progenitors and lineages with mixed pericardial/somatic muscle, even though their early expression domains also include cells giving rise to these lineages.

The new information on the regulation and function of Doc fills a major gap in our understanding of early *Drosophila* cardiogenesis. Previous data have shown that the combinatorial activities of Wg and Dpp are required for the formation of both myocardial and pericardial cells (Frasch, 1995; Wu et al., 1995; Carmena et al., 1998). In addition, the homeobox gene *even-skipped* (*eve*) is a direct target of the combined Wg and Dpp signaling inputs in specific pericardial cell/dorsal somatic muscle progenitors (Halfon et al., 2000; Knirr and Frasch, 2001; Han et al., 2002). Our current data identify the Doc genes as downstream mediators and potential direct targets of combined Wg and Dpp signals during the induction of myocardial and Eve-negative pericardial cell progenitors. The induction of Doc expression by Wg and Dpp occurs concurrently with the induction of *tin* by Dpp alone, at a time when the mesoderm still consists of a single layer of cells (Fig. 9). As a result, *tin* and Doc are co-expressed in a segmental subset of dorsal mesodermal cells that include the presumptive cardiogenic mesoderm. Conversely, in the intervening subset of dorsal mesodermal cells (the presumptive visceral mesoderm precursors) *tin* is co-expressed with *bagpipe* (*bap*) and *biniou* (*bin*), which are negatively regulated by Wg via the Wg target *sloppy paired* (*slp*) (Lee and Frasch, 2000; Zaffran et al., 2001). Ultimately, these shared responses to Dpp, differential responses to Wg and the specific genetic activities of Doc versus *bap* and *bin* lead to the reciprocal arrangement of cardiac versus visceral mesoderm precursors in the dorsal mesoderm (Zaffran et al., 2002; Lee et al., 2004).

Although the Dpp signaling pathway (and likewise, the Wg pathway) is activated in both ectodermal and mesodermal germ layers, *tin* and *bap* respond to it only in the mesoderm. We have recently shown that the germ layer-specific response of these genes to Dpp relies on two probably interconnected mechanisms. The first of these involves the additional requirement for Tin protein as a mesodermal competence factor for Dpp signals, which is initially produced in the mesoderm downstream of *twist*. The second involves the specific repression of the responses of *tin* and *bap* to Dpp in the ectoderm by yet unidentified factors that bind to the Dpp-responsive enhancers of these two genes (Xu et al., 1998; Lee and Frasch, 2005). By contrast, the Doc genes are induced by Dpp and Wg with the same spatial and temporal expression patterns in both germ layers. This implies that the (yet unknown) Dpp and Wg-responsive enhancer(s) of the Doc genes are not subject to the ectodermal repressor activities acting on the *tin* and *bap* enhancers, and fits with the

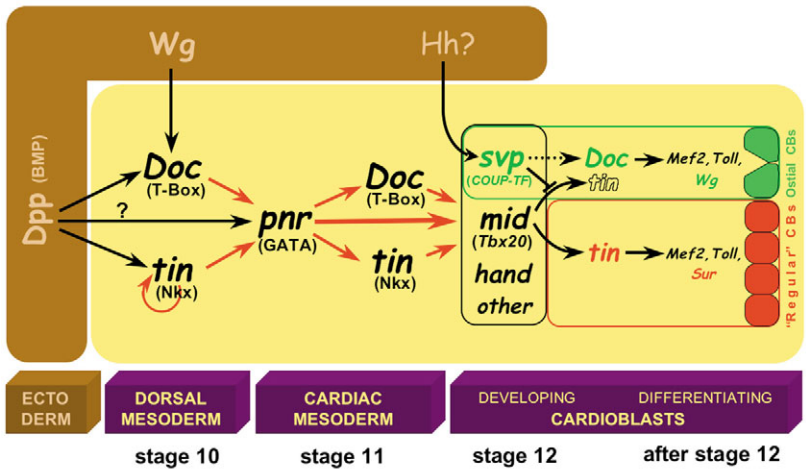

Fig. 9. Current model of cardioblast development and the roles of Dorsocross, *tinman* and *pannier*. Dorsocross is involved in multiple steps of early cardiogenesis. First (stage 10), positional information transmitted by Dpp and Wg signals is integrated within dorsal cells of the mesodermal A domain, in which the Doc genes are activated. In parallel, *tin* is activated by Dpp in the entire dorsal mesoderm. Second (stage 11), the cardiogenic mesoderm becomes delineated with the Doc- and *tin*-dependent activation of *pnr*, which itself is required for maintained expression of *tin* and Doc. During this stage, cells start to align at the dorsal mesodermal margin, while ectodermal *pnr* maintains *dpp* expression along the dorsal leading edge. The combined action of Doc, *tin* and *pnr* selects a pool of progenitors for all cells of the dorsal vessel, including those of the lymph gland, with the exception of the Eve-pericardial progenitors, which are Doc independent. Third (early stage 12), refined co-expression of Doc, *tin* and *pnr* leads to the activation of specific target genes in presumptive cardioblasts or their progenitor cells, thereby promoting cardioblast specification. Owing to additional localized inputs (particularly on *svp*, which additionally requires extrinsic Hh signals) (Ponzielli et al., 2002), cardioblast fate diversifications are also initiated during this process. Other regulators, including *mid* and *hand*, are turned on in all cardioblasts. During the second half of stage 12, *tin* and Doc are re-activated in complementary subsets of cardioblasts through the actions of *mid* and *svp*, respectively, as indicated (broken arrow indicates permissive role of *svp* on Doc; S. Zaffran, I.R. and M.F., unpublished). Tin and Doc, either alone or in combination with uniformly expressed cardioblast factors, then activate various regulatory and differentiation genes in the respective subtypes of cardioblasts. In the heart portion, which is defined by *abd-A* expression within cardioblasts, Doc-positive cardioblasts form ostial cells marked by *wg* expression, whereas the Tin-positive cells form 'regular' myocardial cells marked by *Sulfonylurea receptor* (*Sur*) expression.

observation that induction of Doc in the mesoderm does not require Tin as a mesodermal competence factor. However, because of the distinct roles of Doc in the ectoderm and mesoderm (Reim et al., 2003) (present study), this situation also implies that Doc must act in combination with germ layer-specific co-factors to exert its respective functions. Our data suggest that, in the early mesoderm, Doc acts in combination with *tin*.

A key gene requiring combinatorial Doc and Tin activities for its activation in the cardiac mesoderm is the Gata factor-encoding gene *pannier* (*pnr*) (Fig. 9). *pnr* expression is activated in the cardiac mesoderm shortly after the induction of Doc and *tin*, at a time when Doc expression has narrowed to the mesodermal precursors giving rise to pure cardiac lineages. The mechanisms restricting Doc expression to the cardiac mesoderm are currently not known, but as a consequence, *pnr* expression is also limited to the cardiac mesoderm. It is conceivable that Doc receives continued inputs during this period from the ectoderm through Dpp, whose expression domain narrows towards the dorsal leading edge by then (as was proposed for *pnr*) (Klinedinst and Bodmer, 2003). Together with the observed feedback regulation of *pnr* on *tin* and Doc, this situation leads to a prolonged co-expression of Tin, Doc and Pnr in the cardiac mesoderm of stage 11 to stage 12 embryos. Based upon the onset of the expression of early markers such as *mid* and *svp*, this is precisely the period when cardiac progenitors become specified.

We anticipate that the activation of some downstream targets in presumptive cardiac progenitors requires the combination of two, or perhaps all three, of these cardiogenic factors. Potential target genes include *mid*, *svp* and *hand*. However, none of these candidates is essential for generating cardiac progenitors, although *mid* and *svp* are known to be required for the normal diversification of cardioblasts within each segment.

Our observation that forced expression of Pnr in the absence of any Doc partially rescues cardiogenesis could indicate that the early, combinatorial functions of *tin* and Doc are primarily mediated by *pnr*. Alternatively, or in addition, this observation and the fact that a few cardioblasts can be generated without Doc could point to the existence of some degree of functional redundancy among these three factors. In the context of the latter possibility, it is tempting to speculate that the functional redundancy among T-box, Nkx and Gata factors during early cardiogenesis has further increased during the evolution of the vertebrate lineages. This would explain the less dramatic effects of the functional ablation of *Tbx5*, *Nkx2-5* and *Gata4/5/6* on vertebrate heart development (reviewed by Harvey, 1996; Peterkin et al., 2005; Plageman and Yutzey, 2005) as compared to the severe effects of Doc, *tin* or *pnr* mutations on dorsal vessel formation in *Drosophila*. Like the related *Drosophila* genes, these vertebrate genes are co-expressed in the cardiogenic region and developing heart of vertebrate embryos, which at least for Nkx2.5 and Gata6 also involves cross-regulatory interactions that reinforce their mutual expression (Molkentin et al., 2000) (reviewed by Bruneau, 2002).

The observed co-expression of Doc, Tin and Pnr allows for the possibility that, in addition to combinatorial binding to target enhancers, protein interactions among these factors play a role in providing synergistic activities during cardiac specification. Physical interactions of Tbx5 with Gata4 and Nkx2-5, as well between Nkx2-5 and Gata4 in vitro as well as synergistic activities cell culture assays have been demonstrated in mammalian systems and may be relevant to human heart disease (Durocher et al., 1997; Bruneau et al., 2001; Hiroi et al., 2001; Garg et al., 2003) (reviewed by Bruneau, 2002). In *Drosophila*, the genetic interactions between Doc, *tin* and *pnr* observed both in loss- and gain-of-function experiments reveal similar synergistic activities of the encoded factors during early cardiogenesis. Altogether, our observations make it likely that these *Drosophila* factors also act through combinatorial DNA binding and mutual protein interactions to turn on target genes required for the specification of cardiac progenitors.

Whereas *pnr* is expressed only transiently during early cardiogenesis, *tin* and Doc continue to be expressed in developing myocardial cells, suggesting that they act both in specification and differentiation events. We recently showed that the T-box gene *mid* is required for re-activating *tin* in cardioblasts (Fig. 9) (Reim et al., 2005). Of note, owing to the action of *svp*, Doc and *tin* are expressed in complementary subsets of cardioblasts within each segment (Lo and Frasch, 2001). This mutually exclusive expression of *tin* and Doc implies that they are not acting combinatorially but, instead, act differentially during later stages of myocardial development. Hence, their activities could result in the differential activation of some differentiation genes such as *Sulfonylurea receptor* (*Sur*), which is specifically expressed in the four Tin-positive cardioblasts in each hemisegment (Nasonkin et al., 1999; Lo and Frasch, 2001), and *wingless* (*wg*), which is only turned on in the two Doc-positive cells in each hemisegment of the heart that generate the ostia (Lo et al., 2002). Surprisingly, even the activation of some genes that are expressed uniformly in all cardioblasts has turned out to result from differential regulation within the Tin-positive versus Doc-positive cardioblasts. For example, regulatory sequences from the *Mef2* gene for the two types of cardioblasts are separable and those active within the four Tin-positive cells are directly targeted by Tin (Gajewski et al., 1997; Cripps et al., 1998; Nguyen, 1998; Gajewski et al., 2000). Likewise, regulatory sequences from a cardioblast-specific enhancer of *Toll* have been shown to receive differential inputs from Doc and Tin, respectively, in the two types of cardioblasts (Wang et al., 2005). In parallel with this differential regulation, we anticipate that yet unknown differentiation genes are activated uniformly in all cardioblasts downstream of *mid/H15* and *hand*. The integration of the new information on the roles of Doc in cardiogenesis has now provided a basic framework of signaling and gene interactions through all stages of embryonic heart development, which in the future can be further refined upon the identification of new components and additional molecular interactions.

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Note added in proof

Recent data showed that *twi-GAL4/UAS-Doc2* expression in *wg* mutant embryos partially rescues cardioblast specification by producing *mid/hand*/Mef2-positive cardioblasts in about one to three hemisegments per embryo on each side, further supporting the notion of *Doc* as a key downstream mediator of Wg signals during cardiogenesis.

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