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The *Drosophila* homolog of vertebrate *Islet1* is a key component in early cardiogenesis

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In mouse, the LIM-homeodomain transcription factor *Islet1* (*Isl1*) has been shown to demarcate a separate cardiac cell population that is essential for the formation of the right ventricle and the outflow tract of the heart. Whether *Isl1* plays a crucial role in the early regulatory network of transcription factors that establishes a cardiac fate in mesodermal cells has not been fully resolved. We have analyzed the role of the *Drosophila* homolog of *Isl1*, *tailup* (*tup*), in cardiac specification and formation of the dorsal vessel. The early expression of Tup in the cardiac mesoderm suggests that Tup functions in cardiac specification. Indeed, *tup* mutants are characterized by a reduction of the essential early cardiac transcription factors Tin, Pnr and Dorsocross1-3 (Doc). Conversely, Tup expression depends on each of these cardiac factors, as well as on the early inductive signals Dpp and Wg. Genetic interactions show that *tup* cooperates with *tin*, *pnr* and *Doc* in heart cell specification. Germ layer-specific loss-of-function and rescue experiments reveal that Tup also functions in the ectoderm to regulate cardiogenesis and implicate the involvement of different LIM-domain-interacting proteins in the mesoderm and ectoderm. Gain-of-function analyses for *tup* and *pnr* suggest that a proper balance of these factors is also required for the specification of Eve-expressing pericardial cells. Since *tup* is required for proper cardiogenesis in an invertebrate organism, we believe it is appropriate to include *tup/Isl1* in the core set of ancestral cardiac transcription factors that govern a cardiac fate.

KEY WORDS: *Drosophila*, Cardiogenesis, Islet1, Tailup, Second heart field

INTRODUCTION

In our effort to decipher the molecular network that determines a cardiac fate, we attempt to identify all the key players in this process. Some time ago, the LIM-homeodomain transcription factor *Islet1* (*Isl1*), known for its role in neural development, was introduced as a novel gene with a function in mouse heart development (Korzh et al., 1993; Pfaff et al., 1996; Thor and Thomas, 1997; Cai et al., 2003). Initial analyses of the murine *Isl1* expression pattern, combined with the missing right ventricle and outflow tract of the heart in *Isl1* knockout mouse embryos, suggested that *Isl1* demarcates a separate cardiac lineage, also called the second heart field (Cai et al., 2003) (reviewed by Buckingham et al., 2005; Abu-Issa and Kirby, 2007). However, in vitro studies in cell culture systems and analysis of *Xenopus Isl1* implicate that *Isl1* is part of the early transcriptional network that establishes a cardiac fate in mesodermal cells (reviewed by Anton et al., 2007; Brade et al., 2007). Here, we took advantage of the *Drosophila* model to genetically determine whether *tailup* (*tup*), the fly homolog of *Isl1*, is required for the specification of heart precursor cells.

The *Drosophila* heart, although a simple tube, has become a paradigm of studying complex genetic interactions that determine cell fate. Two major cell types comprise the fly heart, which forms at the dorsal midline of the embryo. The contractile myocardial cells form the lumen of the dorsal vessel. The six myocardial cells per hemisegment are flanked by a group of pericardial cells that are needed for normal heart function. During embryogenesis, two of the six myocardial cells further differentiate into specialized myocardial cells, the ostia, which serve as inflow tracts in the posterior heart portion of the fly. Heart development in *Drosophila* is initiated in the dorsal mesoderm when a particular group of cells in each hemisegment receives input from the ectodermal growth factors Wingless (Wg) and Decapentaplegic (Dpp) (Wu et al., 1995; Frasch, 1995; Park et al., 1996). These signaling pathways induce in Tinman (Tin)-positive mesodermal cells a complex network of transcription factors that distinguishes the cardiac mesoderm from the adjacent visceral mesoderm and dorsal somatic muscles (Frasch, 1995; Riechmann et al., 1997; Lee and Frasch, 2000; Lockwood and Bodmer, 2002; Jagla et al., 2002). In addition to the homeobox transcription factor *tin* (*Nkx2.5*), early specification requires the function of the T-box factors *Dorsocross1-3* (herein referred to as *Doc*) and of the GATA factor *pannier* (*pnr*) (Gajewski et al., 1999; Alvarez et al., 2003; Klinedinst and Bodmer, 2003; Reim and Frasch, 2005). Once cardiac specification has taken place, *tin*, *Doc* and *pnr* cross-regulate each other to maintain their expression and to initiate the differentiation of the cardiac cells (reviewed by Zaffran and Frasch, 2002; Qian et al., 2008). The latter requires additional transcription factors, including the *Tbx20*-related gene *neuromancer* (*nmr1* and *nmr2*; also known as *H15* and *mid*, respectively), the COUP-TFII-related gene *seven up* (*svp*), and the homeobox gene *ladybird* (*lb*), which are involved in regulating the diversity of myocardial and pericardial cell fates (Miskolczi-McCallum et al., 2005; Qian et al., 2005; Reim et al., 2005; Lo and Frasch, 2001; Jagla et al., 1997; Jagla et al., 2002). Most of these transcription factors have a fairly dynamic expression pattern during heart development, which suggests that their specific function in cardiogenesis can vary depending on the cellular context. For example, *tin* and *Doc* initially cooperate to properly specify cardiac progenitors (Reim and Frasch, 2005). However, during the differentiation of myocardial cells, *tin* represses *Doc* genes in four out of the six myocardial cells in each hemisegment, thereby restricting *Doc* expression to the two Tin-negative cells that form the ostia in segments A2 to A7 (Zaffran et al., 2006).

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A phenotypic characterization of *tup* mutants has shown that *tup* plays an important role in *Drosophila* heart and hematopoietic organ formation (Tao et al., 2007). However, these analyses, which included a description of the cardiac expression pattern of Tup, were restricted to stages well past the time when cardiac specification occurs. Hence, the question has remained whether *tup* is required for the proper specification of heart cells.

Here, we present a detailed study of Tup expression and function during cardiogenesis and show that *tup* is indeed required for the specification of a cardiac fate. Analyses of genetic interactions establish *tup* as a crucial factor that cooperates with *tin*, *pnr* and *Doc* during cardiogenesis. Germ layer-specific inhibition of Tup function shows that ectodermal Tup is also required for normal Tin expression at early stages. Rescue experiments suggest that there might be a different set of mesodermal and ectodermal factors with which Tup can interact through its LIM domains. Cell-specific inhibition of Tup function shows that Tup is required to maintain expression of Odd in pericardial cells. Overexpression experiments show that a balance of Tup and Pnr is required for the correct specification of Eve-expressing cell clusters. Taken together, these findings place *tup* as a crucial factor in the early cardiac transcriptional network.

MATERIALS AND METHODS

Drosophila stocks and crosses

The following mutant fly stocks were used: $tup^{isl\text{-}1}$ [isl^{37Aa} (Thor and Thomas, 1997], pnr^{VX6}, wg^{CX4}, dpp^{d6}, *Df(2L)OD15* (all from The Bloomington Stock Center), tin^{346} (Azpiazu and Frasch, 1993) and *Df(3L)DocA* (Reim et al., 2003). The $tup^{isl\text{-}1}$, wg^{CX4} and the *Df(2L)OD15* stocks were rebalanced with CyO, *wg-lacZ*, and the pnr^{VX6} stock was rebalanced with TM3, *ftz-lacZ* to identify homozygous mutant embryos. CantonS served as a wild-type stock. Analysis of cuticles of $tup^{isl\text{-}1}$/CyO embryos (n=415) showed that 19% of the homozygous *tup* mutants (n=104) had an obvious germ band retraction phenotype. These embryos were not included in our analyses. For the genetic interactions, embryos that were single or double heterozygous for the investigated allele(s) were selected based on the lack of staining for β-galactosidase activity present on the corresponding balancer chromosomes. Statistical computing was performed using R (www.r-project.org). The following Gal4 and UAS lines were used: twi-Gal4 (Greig and Akam, 1993), 69B-Gal4 (Brand and Perrimon, 1993), Dot-Gal4 (Kimbrell et al., 2002), tinCΔ4-Gal4 (Lo and Frasch, 2001), UAS-*tup*, UAS-*tupΔHD* (Thor and Thomas, 1997; O'Keefe et al., 1998), UAS-*tupΔLIM* (Biryukova and Heitzler, 2005), UAS-*pnrD4* (Haenlin et al., 1997) and UAS-*tin* (Ranganayakulu et al., 1998). For co-overexpression of UAS-*tup* and UAS-*pnrD4*, the individual UAS constructs were recombined on the third chromosome. The UAS-*tin*;UAS-*tup* stock was generated by standard genetic crossings.

Immunohistochemistry and in situ hybridization

Antibody staining (single and double labeling) was performed essentially as described (Qian et al., 2005; Liu et al., 2006). Primary antibodies were detected with a Cy3-conjugated AffiniPure donkey anti-rabbit IgG (H+L) (1:200) (Dianova, Hamburg, Germany). If amplification of the signal was necessary, biotinylated secondary antibodies were used (1:200) in combination with the Tyramide Signal Amplification System (Perkin Elmer) and dichlorotriazinylamino fluorescein (1:200) (Dianova). Embryos were mounted in Vectashield (Vector Laboratories). Embryos from single immunostainings were analyzed using Olympus BX60 (Olympus, Hamburg, Germany) or Keyence BZ-8000K epifluorescence microscopes, with the image-analyzing software BZ-Analyzer (Keyence, Neu-Isenburg, Germany). Embryos from double immunostainings were analyzed using a Leica TCS SP confocal microscope. Primary antibodies were used at the following dilutions: mouse anti-chicken Isl1 (Tup), 1:50 with TSA [Developmental Studies Hybridoma Bank (DSHB)]; rabbit anti-Dmef2, 1:2000 (Lilly et al., 1995); rabbit anti-Tin, 1:50 (Venkatesh et al., 2000); mouse anti-Pericardin (EC11), 1:10 with TSA (DSHB); rabbit anti-Eve, 1:3000 (Frasch et al., 1987); rabbit anti-Odd, 1:100 (Ward and Skeath, 2000); and mouse anti-Pnr, 1:400 with TSA (Herranz and Morata, 2001). Fluorescent in situ hybridization for *dpp* was performed essentially as described (Klinedinst and Bodmer, 2003). Double fluorescent in situ hybridization and immunostaining was adapted from Knirr et al. (Knirr et al., 1999). The digoxigenin-labeled *dpp* and *pnr* in situ probes were generated using the DIG RNA Labeling Mix from Roche (Mannheim, Germany).

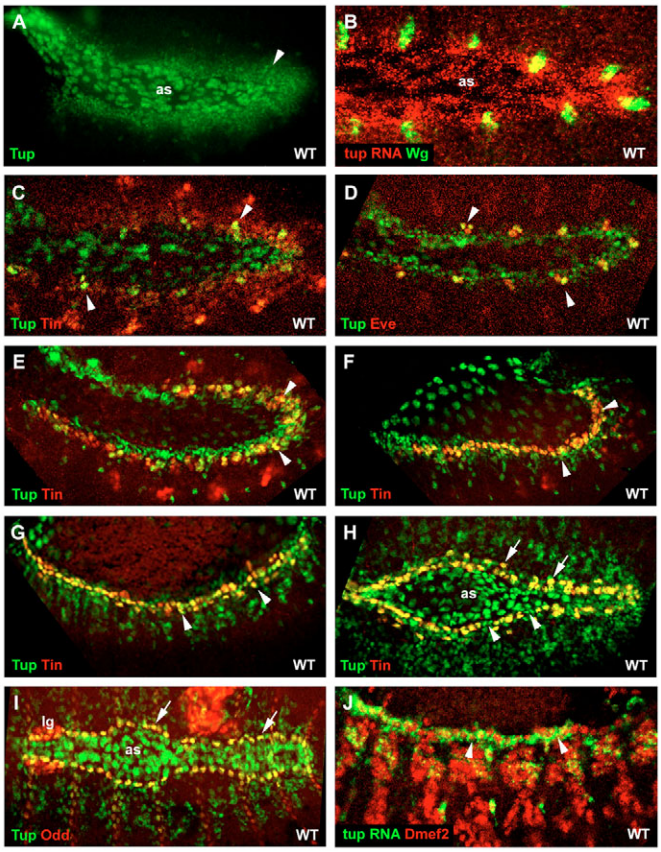

Fig. 1. Tup expression during cardiogenesis in wild-type *Drosophila* embryos. (**A**) At stage 10, Tup is expressed in a broad domain in the dorsal ectoderm (arrowhead). The expression in the amnioserosa (as) persists throughout embryogenesis. (**B**) Double labeling for Wg protein and *tup* RNA confirms the ectodermal expression of *tup*. (**C**) At mid-stage 11, Tup starts to be expressed in the cardiac mesoderm in ~10 small clusters of cells (arrowheads). (**D**) These clusters are also positive for Eve (arrowheads). (**E**) By late stage 11, Tup is co-expressed with Tin throughout the cardiac mesoderm (arrowheads). (**F-H**) Tup is expressed in all six myocardial cells (arrowheads) and in the Tin-positive pericardial cells (arrows in H). Arrowheads in H point to the two Tin-negative, Tup-positive myocardial cells. (**I**) Tup is expressed in all Odd-positive pericardial cells (arrows) and in a subset of Odd-expressing cells of the lymph glands (lg). (**J**) *tup* RNA expression in myocardial Dmef2-expressing cells matches Tup protein localization (arrowheads), as seen in G. Except for H and I, which are dorsal views of stage 15 embryos, all images are lateral views. Anterior is to the left. WT, wild type.

RESULTS

Expression pattern of Tup during dorsal vessel development

Tup protein, as detected by a monoclonal mouse antibody against chicken Isl1, was observed in a broad domain along the dorsal side of the *Drosophila* embryo at stage 10, within the ectodermal layer (Fig. 1A). This expression pattern is reminiscent of the expression domain of ectodermal Dpp, as well as of those of Tin (mesoderm)

and Pnr (initially only in the ectoderm), two transcription factors that are crucial for proper cardiac specification. Double labeling for *tup* transcripts and Wg protein demonstrated more clearly the ectodermal expression of *tup* (Fig. 1B). Double immunostainings for Tup and Tin were performed to identify Tup-positive cardiac cells within this broad domain. This analysis revealed that Tup expression in the cardiac mesoderm initiates at mid-stage 11, when Tin becomes restricted to the dorsal-most mesoderm, in ~10 clusters, each consisting of ~2 cells (Fig. 1C). Cells of the Tup clusters co-expressed Eve and therefore belong to the pericardial cell lineage (Fig. 1D). By late stage 11, Tup expression expanded within the Tin-expressing cardiac mesoderm (Fig. 1E) and continued in all myocardial cells during embryogenesis (Fig. 1F-I) (Tao et al., 2007). We also detected Tup in at least two of the Tin-positive pericardial cells and in all four Odd-expressing pericardial cells in each hemisegment (Fig. 1G-I). In the lymph glands, Tup was only expressed in some of the Odd-positive cells (Fig. 1I). *tup* transcripts were also present in the cytoplasm of the Dmef2 (Mef2)-positive myocardial cells, demonstrating that the expression patterns of *tup* RNA and Tup protein were identical (Fig. 1J). Consistent with its function in amnioserosa development, Tup was also detected in this tissue. Here, we focus on the role of *tup* in heart development and our analysis demonstrates that the observed heart phenotype is not primarily an effect of a defect in germ band retraction.

tup is required for the formation of the dorsal vessel

All analyses were performed using embryos harboring the $tup^{isl\text{-}1}$ allele. Molecular analysis of the $tup^{isl\text{-}1}$ allele suggests that it has a mutation in the transcriptional regulatory region (Tao et al., 2007). The extremely low expression level of Tup protein or *tup* RNA in mutant embryos indicates that the $tup^{isl\text{-}1}$ allele is a strong hypomorph (see Fig. S1A-F in the supplementary material). Consistent with its cardiac expression pattern, formation of the dorsal vessel is severely affected in $tup^{isl\text{-}1}$ embryos, as can be seen by the loss of Dmef2-expressing myocardial cells, as well as by the disrupted Pericardin (Pc) expression (Fig. 2A,B,D,E). Pericardin normally demarcates pericardial cells and accumulates at the basal membrane of the myocardial cells (Chartier et al., 2002). The loss of pericardial cells in $tup^{isl\text{-}1}$ embryos is shown by gaps in Odd and Eve expression (Fig. 2F-I). The late cardiac phenotype at stage 15/16 has essentially been described before (Tao et al., 2007). Our study aimed to determine the position of *tup* in the early cardiac transcriptional network, and whether the cause of the cardiac phenotype was distinct from secondary effects of problems in germ band retraction.

The loss of some Eve-expressing cell clusters is already seen by late stage 11 and indicates that the defects in heart development are not restricted to later stages when the two rows of myocardial cells come together at the dorsal midline. Reduced Tin and Dmef2 expression was also observed in $tup^{isl\text{-}1}/Df(2L)OD15$ transheterozygotes (Fig. 2C,J,K). Since *tup* is expressed in the dorsal mesoderm around the time when cardiac progenitor cells become specified, it is likely that *tup* plays a role in this process. Proper cardiac specification requires interactions between Tin, Pnr and Doc. Therefore, we examined whether the expression of these factors is affected in $tup^{isl\text{-}1}$ embryos. Indeed, *tup* mutants were characterized by a strong reduction in Tin-, Pnr- and Doc2-expressing cells at stage 11 (Fig. 3A-D,I,J). Since Tin and Doc are already expressed in the cardiac mesoderm at stage 10, before the onset of mesodermal Tup expression, these findings indicate that *tup* is required for their maintenance rather than their induction. The onset of cardiac

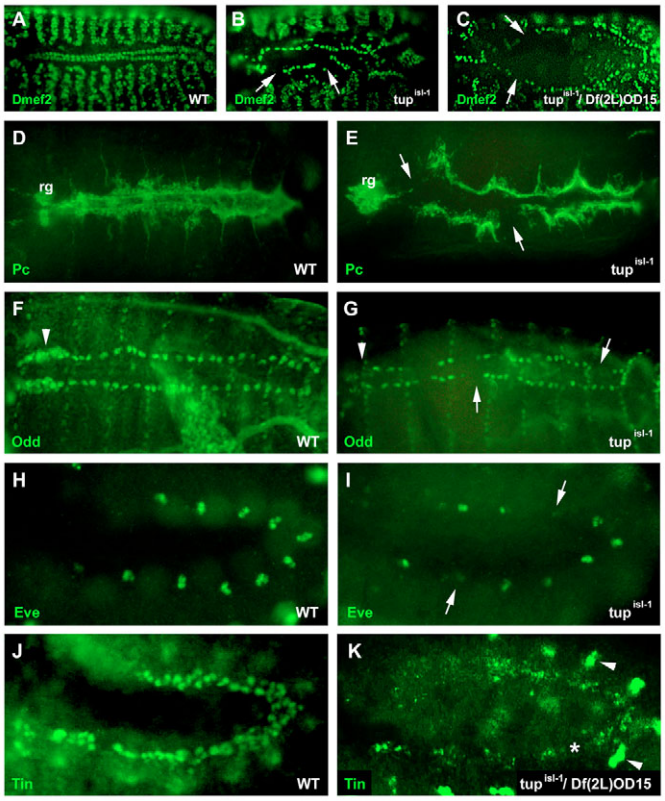

Fig. 2. Heart phenotypes in $tup^{isl\text{-}1}$ mutants. (**A**,**B**,**D-J**) Compared with wild-type *Drosophila* embryos, $tup^{isl\text{-}1}$ mutants are characterized by gaps in expression of all examined myocardial (Dmef2 and Tin) and all pericardial (Pc, Odd and Eve) cell markers. (**C**,**K**) Embryos that are transheterozygotic for $tup^{isl\text{-}1}$ and a deficiency that includes the *tup* locus, *Df(2L)OD15*, also show gaps in Dmef2 expression at stage 14 (arrows in C) and show a strong reduction of Tin-expressing cardiac cells at late stage 11 (asterisk in K). Arrowheads in K point to Tin-positive visceral mesodermal cells. as, amnioserosa; rg, ring glands.

expression of Pnr and Tup seems to coincide at stage 11 (Klinedinst and Bodmer, 2003; Reim and Frasch, 2005). Moreover, like Tup, Pnr is also expressed in the ectodermal layer and double staining for *pnr* RNA and Dmef2 or Wg protein demonstrated that *pnr* expression is reduced in the mesoderm and ectoderm in *tup* mutants (Fig. 3E-H). This suggests that Tup function is also required in the ectoderm to maintain Pnr expression.

To further evaluate the functional relationship between *tup*, *tin*, *pnr* and *Doc*, we analyzed the expression of Tup in tin^{346}, pnr^{VX6} and *Df(3L)DocA* embryos. Staining for Tup protein in tin^{346} embryos showed that the early Tup clusters are present, suggesting that they are initially independent of *tin* (Fig. 4A,B). However, Tup expression was not maintained (Fig. 4C,D). *Df(3L)DocA* and pnr^{VX6} mutants also showed a strong reduction in, or lack of, Tup-expressing cells (Fig. 4E,F). Together with the data above, these results point to an interdependency of all four factors: *tup*, *tin*, *pnr* and *Doc*.

Since Wg and Dpp are crucial growth factors in heart development, we also analyzed Tup expression in wg^{CX4} and dpp^{d6} embryos. Both mutants were characterized by a strong reduction in, or loss of, Tup expression (Fig. 4G,H). Similarly, as observed in tin^{346} mutants, Tup was initially present in the early cell clusters in wg^{CX4} embryos at stage 11 (data not shown). Early steps in visceral

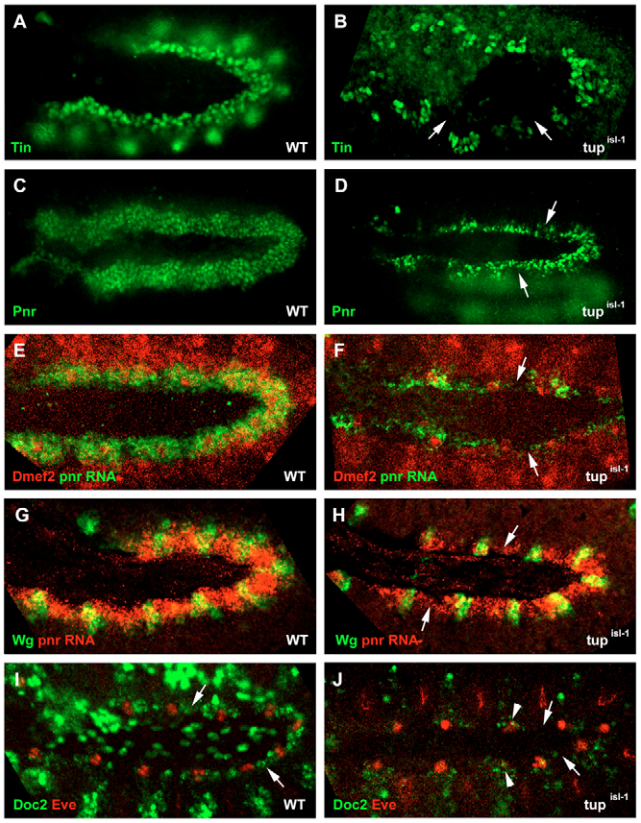

Fig. 3. *tup* is required for the normal expression of early cardiac transcription factors. (**A**,**B**) *Drosophila* stage 11 tup^{isl-1} mutants are characterized by a reduction in Tin-expressing cells (arrows). (**C**,**D**) The Pnr expression domain is strongly reduced in tup^{isl-1} mutants (arrows). (**E**,**F**) Double fluorescence labeling for Dmef2 protein and *pnr* RNA shows the mesodermal reduction of *pnr* expression in tup^{isl-1} mutants (arrows). (**G**,**H**) Reduced *pnr* expression (arrows) in the ectoderm is demonstrated by co-staining for Wg protein. (**I**,**J**) Stage 11 tup^{isl-1} mutants lack cardiac Doc2-positive cells (arrows). Arrowheads indicate missing Eve-expressing cells.

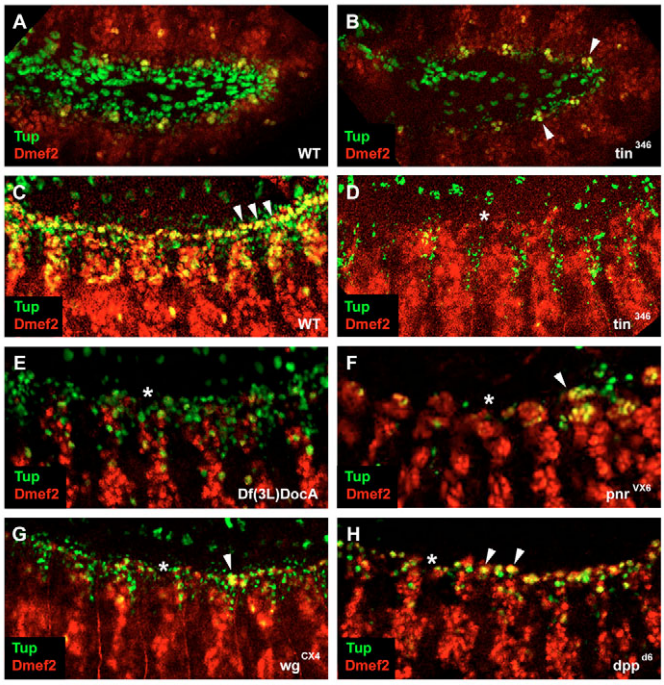

Fig. 4. Tup expression requires the presence of early cardiac transcription factors and depends on *wg* and *dpp* signaling. (**A-D**) Tup expression is initiated in the cell clusters in *Drosophila* tin^{346} mutants (arrowheads in B) but is not maintained at later stages (compare C with D). (**E**) Myocardial Tup and Dmef2 expression is absent in *Df(3L)DocA* mutants. Since *Doc* mutants have been shown to also lack pericardial cells, the remaining Tup-expressing cells (green) are unlikely to be cardiac-related cells. (**F**) pnr^{VX6} mutants also show a dramatic reduction in myocardial Tup- and Dmef2-expressing cells. (**G**,**H**) Tup expression at stage 13/14 depends on Wg (G) and Dpp (H) signaling. Arrowheads in all images point to Dmef2/Tup co-expressing cells, which appear yellow in the merged optical sections. Asterisks are placed in the region of the myocardial cell row, which has defects to various degrees in all mutants shown.

mesoderm formation seemed to be unaffected in tup^{isl-1} mutants, whereas *tup* might play a role in the specification of the Kr-expressing dorsal somatic muscle cells (see Fig. S2A-D in the supplementary material). In summary, these data demonstrate that *tup* is required for the proper specification of cardiac progenitor cells and for the formation of the dorsal vessel.

tup cooperates with *tin*, *pnr* and *Doc* during cardiogenesis

Next we tested whether *tup* interacts genetically with *tin*, *pnr* and *Doc*. For this purpose, we analyzed embryos that are transheterozygous for tup^{isl-1} and *Df(3L)DocA*, tup^{isl-1} and tin^{346}, or tup^{isl-1} and pnr^{VX6}. The phenotypes of these embryos were compared with the phenotypes of single heterozygotes for each of the investigated alleles. Each double transheterozygous combination resulted in obvious gaps within the Dmef2-expressing myocardial cell rows in ~30% of the embryos analyzed (Fig. 5A-D and Tables 1 and 2). Also, *tup* and *tin* cooperated to maintain normal Pnr expression, as tup^{isl-1}/+;tin^{346}/+ transheterozygotes showed reduced staining for Pnr (72%, *n*=102) (Fig. 5E,F). Tin expression was reduced in tup^{isl-1}/+;*Df(3L)DocA*/+ (51%, *n*=98) and tup^{isl-1}/+;pnr^{VX6}/+ (45%, *n*=111) embryos, demonstrating that the combined action of these factors is required for proper cardiac specification (Fig. 5G-I). Only ~8-13% of the single heterozygous embryos (~100 embryos for each combination were counted) had phenotypes comparable to the double transheterozygotes. Taken together, these results further indicate that *tup* is required in combination with *tin*, *pnr* and *Doc* to properly specify and maintain a cardiac fate.

Tissue- and cell-specific requirement for *tup* during cardiogenesis

As shown above, Tup is expressed in the dorsal ectoderm as well as in the cardiac mesoderm, and in *tup* mutants expression of Tup protein is almost absent in both germ layers. Therefore, we wanted to distinguish between the mesodermal and a possible ectodermal contribution of *tup* function in cardiogenesis. We interfered with endogenous Tup function by expressing a deletion construct of *tup*, which lacks the homeodomain but contains both LIM domains and is likely to act as a dominant-negative (UAS-*tupΔHD*) (O'Keefe et al., 1998). When expressing UAS-*tupΔHD* in the mesoderm, we observed a reduction in Tin-expressing cells (Fig. 6A,B). A similar phenotype was induced after expressing UAS-*tupΔHD* in the ectoderm (Fig. 6D). To further investigate the mesodermal and

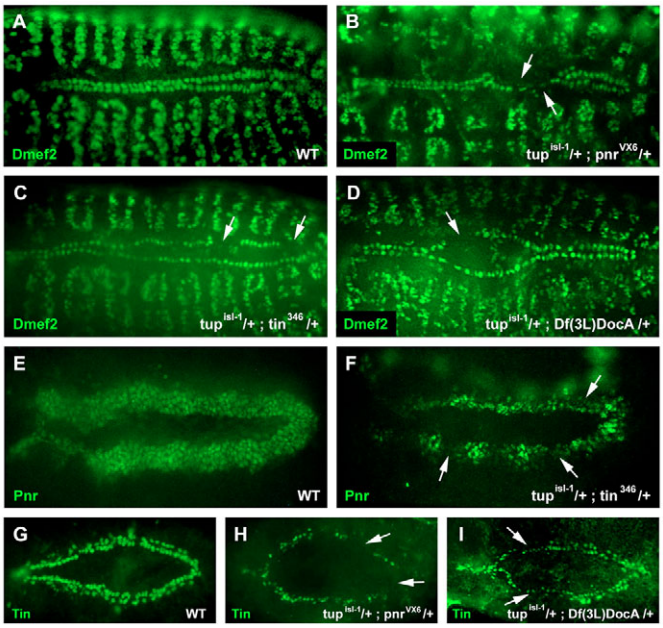

Fig. 5. Genetic interactions between *tup*, *tin*, *pnr* and *Doc*. The cardiac phenotypes in transheterozygotic *Drosophila* embryos demonstrate that *tup* interacts genetically with all three factors. The phenotypes were compared with those of the cardiac markers in single heterozygotes, and were evaluated statistically for Dmef2 (see Tables 1 and 2). (**A-D**) Dmef2 expression in the wild type (A) and in embryos transheterozygotic for tup^{isl-1} and pnr^{VX6} (B), tup^{isl-1} and tin^{346} (C), tup^{isl-1} and *Df(3L)DocA* (D). Dorsal views of embryos at stage 15/16 are shown. Arrows point to gaps in the myocardial rows of the dorsal vessel. (**E**,**F**) Pnr is reduced in *tup/tin* transheterozygotic embryos (arrows in F). A lateral view of a stage 11 embryo is shown. (**G-I**) Tin expression in the wild type (G), and in embryos transheterozygotic for *tup* and *pnr* (H), and *tup* and *DocA* (I). Reduced Tin expression is seen in both cases (arrows in H,I). Dorsal views of stage 14 embryos are shown.

ectodermal contribution of Tup for cardiogenesis, we aimed to rescue the Tin phenotype by co-expressing the full-length *tup* cDNA. When both constructs were expressed in the ectoderm, we observed a partial rescue in ~53% of the embryos (n=74) (Fig. 6E). However, ~82% of the embryos (n=48) in which UAS-*tupΔHD* and UAS-*tup* were co-expressed in the mesoderm, still exhibited a strong phenotype (Fig. 6C). Since the LIM domains are known to act as protein-interaction domains (Schmeichel and Beckerle, 1994; Kadrmas and Beckerle, 2004), this result implicates that the Tup LIM domains interact with, and thereby inhibit, other mesodermal factors, the functions of which appear to be required for normal cardiogenesis but cannot be rescued by simultaneous expression of *tup*. To test whether the LIM domains are required for Tup function in cardiogenesis, we expressed a UAS-*tupΔLIM* deletion construct in the mesoderm. This construct is expected to still bind to the DNA, but LIM domain-mediated interactions with other proteins are disrupted. Mesodermal expression of UAS-*tupΔLIM* also resulted in a reduction of Tin-expressing cells (Fig. 6F), demonstrating the requirement of the LIM domains to mediate proper interactions between Tup and other proteins in cardiogenesis.

The reduction of mesodermal Tin-expressing cells after inhibiting Tup in the ectoderm can only be explained if the function of a secreted cardiogenic factor is impaired. Owing to their highly similar expression patterns, Dpp is a likely candidate. Although *dpp* expression was reduced in embryos expressing UAS-*tupΔHD* in the ectoderm (Fig. 6G,H), we observed a stronger phenotype in tup^{isl-1} mutants (Fig. 6I). Hence, ectodermal Tup can regulate *dpp* expression, either directly or indirectly through Pnr. In contrast to the relatively strong downregulation of early Tin expression, there was still a considerable number of Dmef2-expressing myocardial cells, with only some segments that had fewer than the normal six Dmef2-positive cells (Fig. 6J,K,K′). Therefore, we analyzed Tin expression throughout embryogenesis and observed that the initial, strong reduction of Tin in embryos expressing UAS-*tupΔHD* in the mesoderm appears to recover over time, and by stage 16 Tin expression was comparable to that of wild-type embryos (see Fig. S3B,E,H in the

Table 1. The number of double transheterozygous embryos that have gaps in the myocardial cell rows is significantly higher than in embryos with only one mutant allele

Genotype (group 1)	# Embryos w/o gap	# Embryos with gap	Genotype (group 2)	# Embryos w/o gap	# Embryos with gap	Genotype (group 3)	# Embryos w/o gap	# Embryos with gap
tup^{isl-1}/+;*Df(3L)DocA*/+	50	20	pnr^{VX6}/tup^{isl-1}	38	18	tup^{isl-1}/+;tin^{346}/+	58	25
Df(3L)DocA/+	38	4	pnr^{VX6}/+	48	6	tin^{346}/+	63	7
tup^{isl-1}/+	60	6	tup^{isl-1}/+	60	6	tup^{isl-1}/+	60	6
Chi-square test (χ^2), P	χ^2=11.99, P_1=0.0005			χ^2=11.72, P_2=0.0006			χ^2=13.80, P_3=0.0002	

The χ^2 test revealed in all three groups that the proportion of embryos with and without gaps is statistically different for single heterozygous and double transheterozygous embryos. Adjustment for multiple comparisons was performed with the Bonferroni correction ($\tilde{P}_1$=0.0016, $\tilde{P}_2$=0.0019, $\tilde{P}_3$=0.00061).

Table 2. There is a significant difference between the number of Dmef2-positive cells in embryos with and without gaps in the myocardial cell rows

Genotype	# Dmef2$^+$ cells in embryos w/o gap*		# Dmef2$^+$ cells in embryos with gap*	
Wild type	105		–	
tup^{isl-1}/+;*Df(3L)DocA*/+	96	(91; 101)	90	(85.5; 95)
tup^{isl-1}/+;pnr^{VX6}/+	93	(89; 95)	84.5	(73; 92)
tup^{isl-1}/+;tin^{346}/+	100	(98; 104)	96	(86; 100.25)
Df(3L)DocA/+	100	(97; 102)	94.5	(93; 96)
pnr^{VX6}/+	100.5	(98; 105)	92.5	(87; 97)
tin^{346}/+	101	(95; 107)	93	(78; 98.75)
tup^{isl-1}/+	99	(96; 102)	94	(92; 98)

The data were statistically tested for significant differences using the Wilcoxon rank sum test (P=2.58^{-13}).
*Showing the median, with the interquartile range in parentheses.

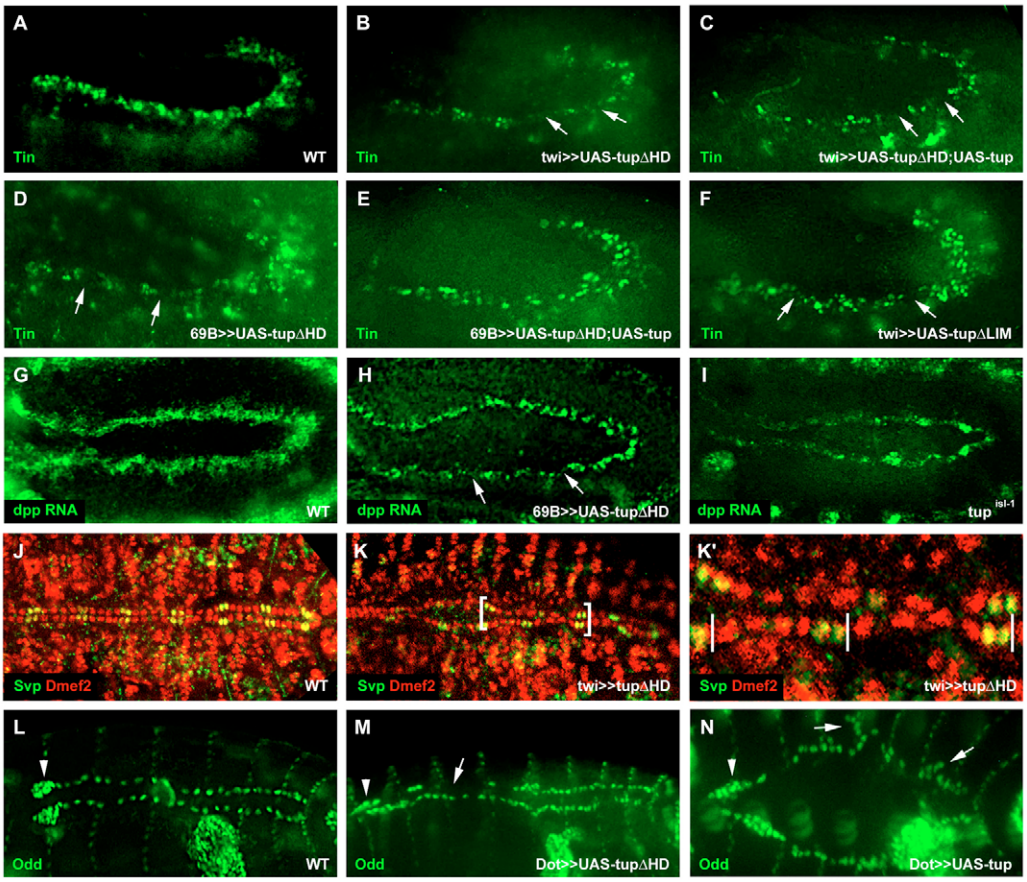

Fig. 6. Germ layer- and cell-specific requirements of Tup at various stages of cardiogenesis. *Drosophila* embryos at stage 12 (A-F) or between stages 10 and 11 (G-I), shown from the lateral side with anterior to the left; or at stage 15/16 (J-M) or 14 (N) shown from the dorsal side. (**A**,**B**,**D**) Mesodermal and ectodermal inhibition of Tup function by expressing UAS-*tupΔHD* results in a reduction of Tin-positive cells (arrows in B,D). (**C**,**E**) Full-length *tup* (UAS-*tup*) can partially restore Tin when co-expressed in the ectoderm but not in the mesoderm. (**F**) Mesodermal expression of a Tup construct lacking the LIM domains (UAS-*tupΔLIM*) also affects Tin expression (arrows). (**G-I**) Tup is required for normal *dpp* expression as shown by in situ hybridization. *dpp* is reduced after ectodermal inhibition of Tup function (arrows in H). *dpp* is strongly reduced in $tup^{isl\text{-}1}$ mutants (I). (**J-K′**) Mesodermal expression of UAS-*tupΔHD* results in a reduced number of Dmef2-positive myocardial cells. (K′) An enlargement of the two segments (as delineated by the vertical lines) indicated by the brackets in K. (**L-N**) Inhibition of Tup function in the pericardial cell lineage results in loss of Odd-positive cells (arrow in M), including a subset of Odd-expressing lymph gland cells (arrowhead in M). Overexpression of Tup in this lineage induces additional Odd-positive pericardial cells (arrows in N). Odd expression in the lymph glands appears unaffected (arrowhead in N).

supplementary material). This pattern of expression could either point to a temporal requirement of *tup* for Tin expression, or be due to the *twi-Gal4* driver, the activity of which becomes weaker during embryogenesis. Because we have observed a similar phenomenon for Tin expression in $tup^{isl\text{-}1}$ mutants (see Fig. S3C,F,I in the supplementary material), we favor the first possibility. Since Tup is expressed in pericardial cells throughout embryogenesis, we tested whether Tup function is required at later stages to maintain this pericardial fate. We expressed UAS-*tupΔHD* in the pericardial cell lineage using the *Dot-Gal4* driver (Kimbrell et al., 2002). Inhibition of Tup function in pericardial cells resulted predominantly in the loss of Odd-positive cells in one or more hemisegments in 63% of the embryos (n=122), as well as in lymph glands of reduced size (Fig. 6L,M). Conversely, overexpression of Tup in the pericardial cell lineage yielded additional Odd-expressing cells in several hemisegments in 42% of the embryos (n=119) (Fig. 6N).

Our data show that Tup functions in the mesoderm, as well as in the ectoderm regulating *dpp* expression to guarantee normal heart development. The experimental approach of inhibiting and rescuing Tup function implicates that Tup interacts with different proteins in the ectoderm and in the mesoderm to ensure normal cardiogenesis.

Mesodermal overexpression of Tup

The requirement of *tup* in cardiogenesis prompted us to investigate whether Tup might be sufficient to induce additional cardiac and/or pericardial cells. Early pan-mesodermal overexpression of Tup induced only a slight increase in Tin-positive cells (Fig. 7A,B). Expression of UAS-pnr^{D4} results in a strong induction of ectopic Tin expression, as reported by Klinedinst and Bodmer (Klinedinst and Bodmer, 2003) (Fig. 7C). Co-expression of UAS-pnr^{D4} and UAS-*tup* resulted in a similar phenotype to that seen upon mesodermal overexpression of UAS-pnr^{D4} alone (Fig. 7D). Double immunostaining for Tin and Tup in embryos overexpressing UAS-pnr^{D4} revealed that the ectopic Tin cells co-express Tup, but the clusters are heterogeneous because they also contain cells that only express Tup (Fig. 7C1-C3). Mesodermal overexpression of Tup induced an enlargement of the Eve-positive cell clusters in 46% of the embryos (n=112) (Fig. 7E,F,I), whereas mesodermal

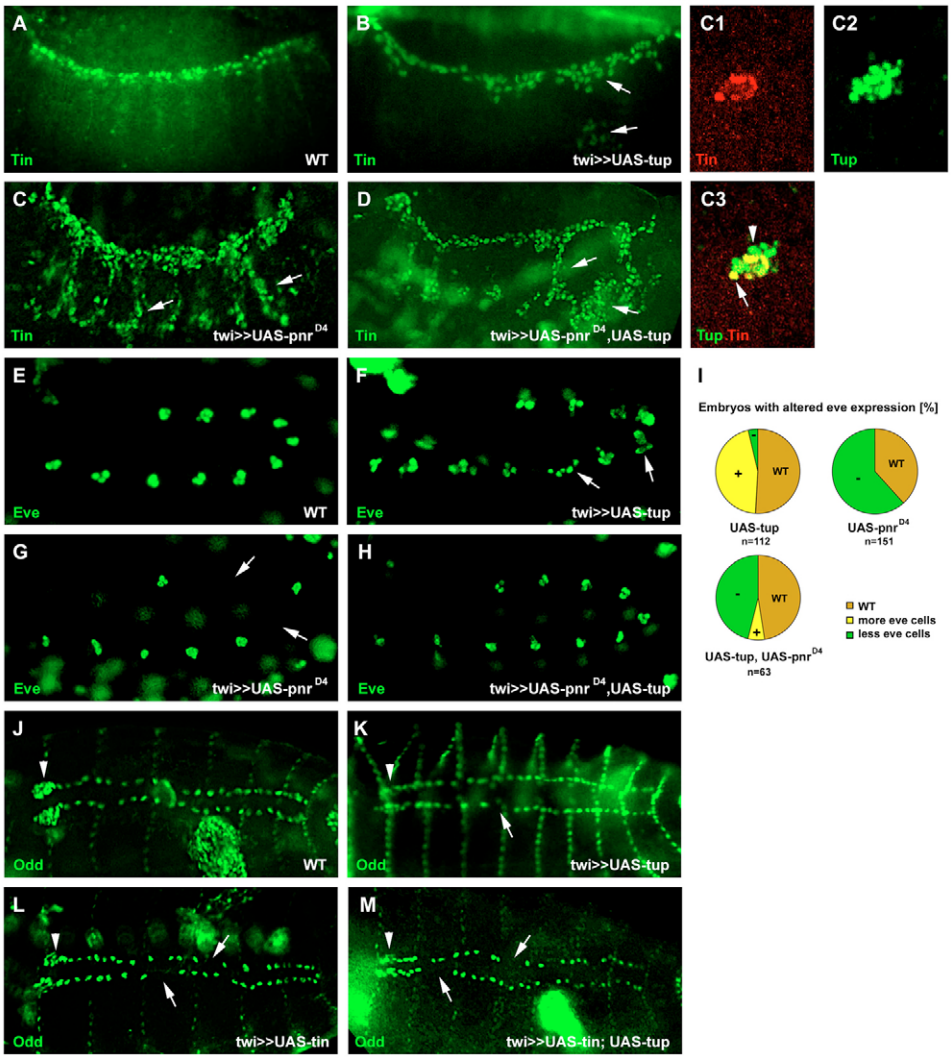

Fig. 7. Mesodermal overexpression of Tup reveals different functional relationships with other cardiac transcription factors. (**A-D**) Overexpression of Tup leads to a moderate expansion of Tin and some ectopic Tin-expressing cells on the lateral side of the embryo (arrows in B). Overexpression the Pnr allele *pnr^{D4}* results in a strong ectopic induction of Tin across the whole lateral side of the embryo (arrows in C). Co-overexpression of Tup and Pnr^{D4} mimics the phenotype of Pnr^{D4} overexpression alone (arrows in D point to ectopic Tin-expressing cells). (C1-C3) The ectopic Tin-positive cell clusters induced by overexpression of Pnr^{D4} alone are heterogenous. Some cells co-express Tin and Tup (arrow in C3), whereas others are only positive for Tup (arrowhead in C3). (**E-I**) Tup and Pnr counteract each other in Eve-positive pericardial cell specification. Overexpression of Tup results in additional Eve-positive cells within the clusters (arrows in F), whereas overexpression of Pnr^{D4} leads to the complete loss of Eve-positive cell clusters (arrows in G). (H) Co-overexpression of Tup and Pnr^{D4} can reduce the effects induced by each factor singly. (I) Pie charts showing the percentage of embryos with wild-type (WT, brown), expanded (+, yellow) or reduced (–, green) Eve-positive cell clusters. (**J-M**) Overexpression of Tup results in a moderate loss of Odd-positive pericardial cells (arrow in K) and to a strong reduction of Odd-positive lymph gland cells (arrowheads in J,K). Overexpression of Tin has a slightly stronger negative effect on the Odd-positive pericardial cells (arrows in L); however, the reduction of Odd-positive cells in the lymph glands appears to be less strong (arrow in L) than that caused by Tup overexpression (arrowhead in K). (M) Co-overexpression of Tup and Tin results in a similar phenotype to that seen for overexpression of Tin alone. Arrows point to the absence of Odd-positive pericardial cells; the arrowhead points to Odd-positive lymph gland cells.

overexpression of UAS-*pnr^{D4}* resulted in the opposite phenotype in 62% of the embryos (*n*=151) at stage 11 (Fig. 7G,I). Co-expression of UAS-*tup* and UAS-*pnr^{D4}* was able to rescue the effect induced by overexpression of each factor singly (Fig. 7H,I), but to different extents. It is important to note here that *pnr^{D4}* is a very active allele and therefore cannot be fully counteracted by *tup*. As a result, when both constructs are co-overexpressed, the phenotype of enlarged Eve-expressing clusters induced by UAS-*tup* was more efficiently 'rescued' (from 46% to 6%) than the phenotype induced by UAS-*pnr^{D4}* (from 62% to 46%). Mesodermal overexpression of UAS-*tup* resulted in a moderate loss of Odd-positive pericardial cells and in the complete loss of Odd-positive cells in the lymph glands (Fig. 7J,K). The same phenotype was observed when UAS-*tin* was expressed early throughout the mesoderm (Fig. 7L). When both factors were overexpressed, the effect on Odd-expressing pericardial cells did not appear to be synergistic (Fig. 7M).

In summary, an early pan-mesodermal overexpression of UAS-*tup* does not result in a dramatic overspecification of cardiac cells. Nonetheless, Tup can promote Tin expression, whereas it has a negative effect on Odd-positive cells. These results provide initial

clues that Tup regulates heart and hematopoietic organ development on a transcriptional level by acting as both an activator and a repressor, depending on the context.

DISCUSSION

The specification of a subset of mesodermal cells towards a cardiac fate requires well-orchestrated interactions of a plethora of factors. *Drosophila* is the model system of choice to decipher the complex transcriptional network that initiates and sustains a cardiac lineage. Our data place the LIM-homeodomain transcription factor *tup* as an essential component in the early transcriptional network that specifies cardiac mesoderm.

After the initially broad expression domain of Tin has become restricted to the dorsal mesodermal margin, we first see Tup expression in the cardiac mesoderm in ~10 small clusters, which co-express Eve. Slightly later, Tup is present throughout the Tin-positive cardiac mesoderm and gene expression analyses in tup^{isl-1}, tin^{346}, pnr^{VX6} and *Df(3L)DocA* embryos demonstrate that all four factors are required to maintain each other's expression (Fig. 8). Additionally, analyses of cardiac gene expression in embryos that are transheterozygotic for *tup* and *tin*, *pnr* or *Doc*, showed that these factors interact genetically to specify heart cells.

Although it might be expected that Tup expression is lost in *tin* mutants as these embryos are devoid of heart cells, it is interesting that Tup expression in the early cell clusters is still initiated. This finding is somewhat reminiscent of the observation that the initiation of Doc expression is also independent of *tin* (Reim and Frasch, 2005). According to the temporal appearance of Tup in the cardiac mesoderm with respect to Tin and Doc, *tup* is required for their maintenance rather than their initiation. By contrast, the onset of mesodermal Pnr and Tup expression appears to coincide (Klinedinst and Bodmer, 2003; Reim and Frasch, 2005). We did not resolve whether Tup is induced by Pnr or directly by Dpp. A direct regulation by Dpp was implicated by the reduced expression of Tup after mesodermal overexpression of UAS-*brinker* (data not shown), which is known to bind to *dpp*-response elements of *dpp* target genes (Kirkpatrick et al., 2001). Conversely, we show that *dpp* expression depends on *tup* and our present data suggest that this regulation requires *pnr*.

Germ layer-specific inhibition of Tup using a construct that lacks the homeodomain, but contains the two LIM domains, revealed that Tup can regulate cardiogenesis in the mesoderm as well as from the ectoderm. Since the *69B-Gal4* driver has been reported not to be strictly ectodermal (Klinedinst and Bodmer, 2003), it is possible that we also interfered with mesodermal Tup function. However, the mesodermal expression of 69B-Gal4 seems to be negligible (Baylies et al., 1995). The effect of ectodermal Tup inhibition on cardiogenesis in the mesoderm can only be explained if the function of a secreted growth factor is impaired. We have analyzed *dpp* expression and observed a slight downregulation of its transcripts in embryos expressing UAS-*tupΔHD* in the ectoderm. Since this effect might not be sufficient to account for the strong Tin phenotype, further experiments will be required to determine whether additional growth factors are affected.

To better determine the germ layer-specific contribution of Tup in cardiogenesis, we attempted to rescue the Tin phenotype by co-expressing the full-length *tup* cDNA. Somewhat unexpectedly, we obtained a better rescue when both constructs were expressed in the ectoderm rather than in the mesoderm. Since the LIM domains present in *tupΔHD* can sequester LIM-domain-binding proteins (O'Keefe et al., 1998), a simple explanation for this finding is that Tup interacts with proteins that are present in the mesoderm but not in the ectoderm. Based on the data published by O'Keefe et al. (O'Keefe et al., 1998), it is reasonable to hypothesize that in the mesoderm the LIM domains of *tupΔHD* not only act as a dominant-negative for Tup, but additionally for another, perhaps as yet unidentified, LIM-domain containing protein. Since it has been shown that Pnr can bind Tup through the LIM domains (Biryukova and Heitzler, 2005), we are likely to have interfered with Pnr function by overexpressing UAS-*tupΔHD*. The requirement of the LIM domains for proper cardiac specification is shown by the reduction of Tin-expressing cells after mesodermal expression of the UAS-*tupΔLIM* construct. Further experiments are under way to better resolve the molecular function of Tup in the different tissues.

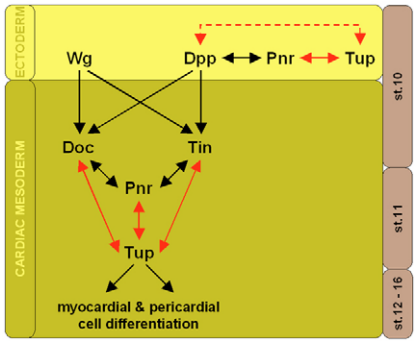

Fig. 8. Tup as a new component of the *Drosophila* early cardiac transcriptional network. At stage 10, Tup is expressed in the ectoderm and is required for normal Pnr and *dpp* expression. Regulation of *dpp* expression through Tup may be direct or indirect (dashed line). Likewise, ectodermal Tup expression may be regulated by Dpp directly or indirectly through Pnr. After Wg and Dpp have induced a cardiac fate in the dorsal mesoderm by initiating and maintaining Doc and Tin expression, respectively, Pnr and Tup start to be expressed in the cardiac mesoderm by stage 11. All four factors are required to ensure proper cardiac specification of mesodermal cells. Black arrows indicate previously characterized interactions; red arrows indicate novel interactions with Tup as proposed in this study.

Since the mesodermal expression of UAS-*tupΔHD* resulted in a strong reduction of Tin-expressing cells at early stages of cardiac mesoderm formation, it was surprising to observe a rather low reduction of Dmef2-positive myocardial cells at later stages (15/16). To exclude the possibility that the *twi-Gal4* driver does not sufficiently express UAS-*tupΔHD* throughout embryogenesis, we repeated this experiment using the combined mesodermal driver *twi-Gal4*; *24B-Gal4*. However, the phenotypes were not enhanced (data not shown). A time course for Tin expression in these crosses revealed that Tin appears to recover over time. A similar phenomenon can be seen in tup^{isl-1} mutants, although it might not be as obvious because the mutants also lack ectodermal *tup* expression. In any case, the data is suggestive of a different temporal requirement for *tup* with respect to *tin* expression. It is known that *tin* expression depends on different transcriptional activation events (Yin et al., 1997). Consistent with the onset of Tup expression in the cardiac mesoderm at mid-stage 11, the earlier phases of Tin expression are unlikely to depend on Tup. Hence, the initial Tin expression at stages 8-10 is sufficient to generate a considerable number of Dmef2-positive myocardial cells at later stages (Zaffran et al., 2006).

Our analyses further implicate that Tup might act as a transcriptional activator or repressor depending on the cellular context and on the factors with which it is co-expressed. This is most

strikingly observed with respect to the Odd-expressing pericardial and lymph gland cells. In *tup* mutants, Odd-positive cells are missing in both organs (Tao et al., 2007) (this study). A similar phenotype is seen when Tup is overexpressed in the mesoderm using the *twi-Gal4* driver. The loss of Odd-expressing cells in lymph glands is reminiscent of the phenotype observed in *tup* mutants, although it is less severe. This differential occurrence of the phenotype indicates that *tup* can differentially regulate factors involved in cardiogenesis versus lymph gland development. This is substantiated by the finding of Tao et al. (Tao et al., 2007), who showed that mesodermal overexpression of *tup* results in an increase in Hand expression in the lymph glands, while Hand expression throughout the dorsal vessel is only mildly affected. Despite the loss of Odd-positive cells after early mesodermal *tup* overexpression, Tup is required in the pericardial and lymph gland cells at later stages to maintain Odd expression. Moreover, overexpressing *tup* in the pericardial cell lineage yields additional Odd-expressing pericardial cells and rescues Odd expression in the lymph glands.

To obtain more insight into possible functional interactions with other cardiac transcription factors, we overexpressed *tup* in combination with pnr^{D4}. The latter is a highly active variant of wild-type *pnr* that contains an amino acid substitution in the N-terminal zinc finger, which abolishes binding of Ush to Pnr (Haenlin et al., 1997). Mesodermal overexpression of pnr^{D4} results in robust ectopic activation of Tin (Klinedinst and Bodmer, 2003) and embryos co-overexpressing *tup* and pnr^{D4} exhibit the same phenotype. Most likely, a possible influence of Tup on Pnr activity, regardless of whether it is positive or negative, is concealed by the strong gain-of-function *pnr* allele. However, analysis of Eve expression does provide insight into possible regulatory interactions between Tup and Pnr. Mesodermal overexpression of each factor alone yields opposing phenotypes, and when both factors are co-overexpressed Pnr^{D4} can efficiently counteract Tup activity and prevent the overspecification of Eve cells. Vice versa, Tup can, although only moderately, counteract the effect of Pnr^{D4}. It has been shown that during patterning of the thorax, Tup can antagonize the proneural activity of Pnr by forming a heterodimer, and that the physical interaction between Pnr and Tup is mediated by the two zinc fingers of Pnr (Biryukova and Heitzler, 2005). Hence, the somewhat weak, but possibly antagonistic, function of Tup towards Pnr^{D4} in Eve-positive cell specification could be due to the amino acid substitution encoded in the pnr^{D4} allele, which might weaken the interaction between the two factors, as compared with wild-type Pnr. Overexpression of a Tup construct that lacks both LIM domains did not result in expanded Eve-positive clusters (data not shown), which strongly suggests that the effect of Pnr on Tup activity, as seen when both factors are co-expressed, requires the presence of the LIM domains.

In summary, our data demonstrate the crucial role of *tup* in the proper specification of cardiac mesoderm in an invertebrate organism. Therefore, *tup*/*Isl1* should be added to the core set of ancestral cardiac transcription factors. Consequently, this implicates that the evolution of the vertebrate four-chambered heart does not necessarily require the acquisition of a novel network of cardiac transcription factors. At least, it is unlikely that *tup*/*Isl1* is part of a regulatory network separate from that of *tin*/*Nkx2.5*, *pnr*/*Gata4* and *Doc*/*Tbx5/6* because it is an essential factor for the formation of the simple linear heart tube in the fly.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/2/317/DC1

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