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Dual Role for the *zeste-white3/shaggy*-Encoded Kinase in Mesoderm and Heart Development of *Drosophila*

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ABSTRACT A *Drosophila* homolog of the serine / threonine kinase GSK-3β, encoded by the *zest-white3 / shaggy* gene (*zw3*), has been implicated as a maternally provided antagonist of zygotic signaling by the secreted segmentation gene *wingless* (*wg*). The *wg* signal apparently causes a spatially localized inhibition of the ubiquitous repressor function of *zw3*. This double negative mechanism of signal transduction has been shown to mediate the patterning function of Wg in a number of developmental processes. Although *wg* is absolutely required for specifying the heart progenitors within the mesoderm of *Drosophila,* the role of *zw3* in this process has been unclear. Here, we present evidence that *zw3* has a dual role in mesoderm development: (1) *zw3* acts as an antagonist in cardiogenic *wg* signal transduction, and (2) *zw3* also seems to be required to promote positively the formation of a larger mesodermal region, the *tinman*- and *dpp*-dependent ''dorsal mesoderm,'' which is a prerequisite not only for cardiogenesis, but also for visceral mesoderm formation. We also demonstrate that a recently identified proximal component of the *wg* cascade, which is a transcription factor encoded by *pangolin / dTCF* (*dTCF*), also seems to mediate *wg*-dependent cardiogenesis. Further, we present evidence that *Notch* (*N*), which opposes *wg* signaling in other situations, is unlikely to be directly involved in the cardiogenic *wg* pathway, but seems to have multiple other myogenic functions, one of which is to inhibit mesoderm differentiation altogether, when overexpressed as a constitutively active form. Dev. Genet. 22:201–211, 1998. © 1998 Wiley-Liss, Inc.

Key words: cardiogenesis; *wingless;* GSK-3β-kinase; Notch; pangolin / TCF / LEF-1; pattern formation

INTRODUCTION

The mechanisms involved in mesoderm patterning and subsequent heart formation are beginning to be understood in *Drosophila* [for review see Bodmer *et al.*, 1997]. As a result of maternal mechanisms of dorsal-ventral axis formation [for review see Govind and Steward, 1991; St. Johnston and Nusslein-Volhard, 1992], one of the first zygotic genes, *twist* (*twi*), a bHLH-type transcription factor, initiates its expression in the mesoderm anlagen in the ventral third portion of the blastoderm embryo [Thisse *et al.*, 1987, 1988]. The absolute requirement of *twist* for initial mesoderm formation comes from the observation that no mesoderm is formed in *twist* mutant embryos [Simpson, 1983]. Shortly after the mesodermal anlagen have invaginated during gastrulation along the ventral midline, they migrate dorsally in close apposition to the ectoderm. A gene, which is activated in a *twi*-dependent fashion before gastrulation and the beginning of dorsal migration, is *heartless* (*htl*), which encodes a fibroblast growth factor receptor (FGFR/DFR1) [Shishido *et al.*, 1993]. *htl* mutants have severe defects in dorsal mesodermal derivatives, in particular the heart and visceral muscles, but also lack many somatic muscles. This seems mainly to be due to a failure of dorsal mesodermal cell migration [Beiman *et al.*, 1996; Gisselbrecht *et al.*, 1996; Shishido *et al.*, 1997; see also Michelson *et al.*, 1998]. As with *htl,* the homeobox-containing gene *tinman* (*tin*) is expressed in the mesoderm anlagen at blastoderm in a *twi*-dependent fashion [Bodmer *et al.*, 1990]. *tin* function is absolutely required, not only for the specification of the heart primordium, but also for the formation of most visceral and dorsal somatic muscles [Azpiazu and Frasch, 1993; Bodmer 1993; Yin and Frasch, 1998]. In contrast to *htl, tin* is apparently not required for dorsal mesodermal migration, but

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rather for cell fate specification within the entire dorsal portion of mesoderm. Interestingly, *tin* expression is restricted to the dorsal mesoderm before cardiac and visceral mesoderm is specified and later becomes exclusively restricted to the differentiating heart [Bodmer *et al.*, 1990].

In addition to the control of mesoderm development at the transcriptional level, inductive mechanisms also play an important role in the specification of mesodermal cell fates. A factor secreted from the ectoderm in close apposition with the mesoderm is encoded by *decapentaplegic* (*dpp*), whose product belongs to the transforming growth factor-β (TGF-β) superfamily. *dpp* is normally expressed in the dorsal ectoderm exactly overlying the mesoderm to which *tin* expression becomes restricted. *dpp* apparently maintains *tin* expression in the dorsal mesoderm by acting as a signal from the dorsal ectoderm to the underlying mesoderm [Staehling-Hampton *et al.*, 1994; Frasch, 1995]. The results from ectopic expression of *dpp* and of loss of *dpp* function studies suggest that *dpp* is an essential signal for the formation of cardiac and visceral muscle progenitors [Staehling-Hampton *et al.*, 1994; Frasch, 1995]. Although these studies clearly demonstrate a role of *dpp* in conjunction with *tin* in determining dorsal mesoderm cell fates, they do not explain how heart, as opposed to visceral mesoderm and somatic muscle identities, is specified.

An additional signal that seems to be absolutely required for heart formation is encoded by the segment polarity gene, *wingless* (*wg*) [Wu *et al.*, 1995]. *wg* is expressed in 15 stripes in the trunk region of the embryo [Baker, 1987; van den Heuvel *et al.*, 1989], orthogonally in orientation to the expression of *dpp*. With the aid of a temperature-sensitive allele, Wu *et al.* [1995] have shown that *wg* is required for heart precursor formation at the stage when *tin* is restricted to dorsal mesoderm. Interestingly, the cardiogenic *wg* function is temporally distinct from other known *wg* functions, such as segmentation and neurogenesis. Moreover, the initial visceral mesoderm formation does not seem to be significantly affected in *wg* mutants. These studies and other evidence suggest that the intersect of *tin*, *dpp*, and *wg* is critical for cardiac specification [W. Lockwood and R. Bodmer, unpubl.].

Epistasis and biochemistry of the *wg* signaling pathway have been elucidated in considerable detail. A serpentine-like protein with seven membrane spanning domains, encoded by the *Drosophila frizzled* 2 gene (*Dfz2*), was proposed to be a *wg* receptor [Bhanot *et al.*, 1996]. The *wg* signal is then thought to be transduced to a cytoplasmic factor encoded by *disheveled* (*dsh*) [Woods and Bryant, 1991; Klingensmith *et al.*, 1994], which seems to become activated by phosphorylation [Yanagawa *et al.*, 1995]. Active *dsh* inhibits *zw3* function, a maternally supplied repressor related to the mammalian serine-threonine kinase GSK-3β [Bourouis *et al.*, 1990; Siegfried *et al.*, 1990, 1992, 1994]. Without a *wg* signal, *zw3* apparently represses *armadillo* (*arm*), a β-catenin homolog [Riggleman *et al.*, 1990]. This repression of *arm* is relieved upon activated *dsh*-mediated repression of *zw3*. Activated *arm* is then likely to function as a transactivation domain of the transcription factor *dTCF*, a *Drosophila* homolog of vertebrates transcription factors of the TCF/LEF family (T-cell factor/lymphocyte-enhancer-binding factor) [Behrens *et al.*, 1996; Huber *et al.*, 1996; Molenaar *et al.*, 1996; Brunner *et al.*, 1997; Riese *et al.*, 1997; van de Wetering *et al.*, 1997].

Some of these components of the *wg* signaling pathway have been shown to be involved in transducing the cardiogenic *wg* signal as well [Park *et al.*, 1996]. As in other developmental situations, *dsh* and *arm* seem to act as positive components of the *wg* pathway for heart development. However, upon removal of *zw3* function, instead of the expected increase of in heart progenitors, a reduction was observed [Park *et al.*, 1996]. From these data, it was postulated that *zw3* might not act as the usual negative transducer of the *wg* signal during cardiogensis. Since heart and other muscle development was clearly abnormal in *zw3* mutants, its exact role(s) in mesoderm development needed further examination.

Genetic studies have provided evidence that interactions between *wg* and *Notch* (*N*) are involved in the same developmental patterning events [Hing *et al.*, 1994; Couso and Martinez-Arias, 1994; Gonzalez-Gaitan and Jäckle, 1995; Axelrod *et al.*, 1996]. *N* encodes a transmembrane receptor protein containing 36 extracellular cysteine-rich, EGF-like tandem repeats as well as intracellular ankyrin repeats (also termed "cdc10/Sw16" repeats) [Wharton *et al.*, 1985; Kidd *et al.*, 1986]. Upon ligand-mediated activation, it is thought that the intracellular domain of *N* transduces a signal to the nucleus [Lieber *et al.*, 1993; Rebay *et al.*, 1993; Struhl *et al.*, 1993). By employing genetic and molecular techniques, it has been shown that *N* and *wg* signaling can be mutually inhibitory and that this inhibition may be due to an interaction between *dsh* and *N* [Axelerod *et al.*, 1996]. In that model, *N* activates *zw3* in the absence of *wg* signaling; but in the presence of *wg* signaling, *dsh* can repress *N*. Thus, *zw3* function may be inhibited in two ways: by repression from *dsh* and by a lack of activation from *N*.

In this report, we re-evaluate the role of *zw3* in mesoderm development, as well as the role of *N* in cardiogenic *wg* signaling. We present evidence that *zw3* plays a dual role in mesoderm development: (1) as a negative component of the cardiogenic *wg* pathway, and (2) as a positive regulator of dorsal mesoderm formation. In addition, we determined that *dTCF*, a newly identified component of the *wg* pathway, is also involved in mediating cardiogenesis. Additionally, we present evidence that *N* is unlikely to be involved in cardigenic *wg* signaling downstream of *dsh*, but can act,

when constitutively actived, as a general inhibitor of mesoderm differentiation.

MATERIALS AND METHODS

Drosophila Stocks

wg^{IL114} [Nusslein-Volhard *et al.*, 1984] is a strong temperature-sensitive allele (wg^{ts}) [Bejsovec and Martinez Arias, 1991; Chu-LaGraff and Doe, 1993]. *T8-Hsdsh* (insertion on the second chromosome) is described in Axelrod *et al.* [1996] and Park *et al.* [1996], and arm^{XM19} in Peifer and Wieschaus [1990]. Hs*zw3*, dsh^{75} FRT^{101}, $zw3^{M11}$ FRT^{101}, and $zw3^{M11}$ dsh^{75} FRT^{101} are described in Siegfried *et al.* [1992, 1994] and Klingensmith *et al.* [1994]. N^{1081} dsh^{v26} FRT^{101} and N^{1081} FRT^{101} are described in Axelrod *et al.* [1996]. HsN^{intra} and UAS-N^{intra} are described in Struhl *et al.* [1993] and Lieber *et al.* [1993]. A dominant negative form of *dTCF* (*dTCFΔN*) is described in van de Wetering *et al.* [1997]. $Notch^{264\text{-}40}$ FRT^{101} was used as a zygotic null mutant without generating germ-line clone. 24B-GAL4 is described in Brand and Perrimon [1993] and twist-GAL4 is described in Greig and Akam [1993].

Germ Line Clone Analysis

Germ line clones (GLC) were generated as described in Chou and Perrimon [1992]. dsh^{75} FRT^{101}, N^{1081} FRT^{101}, $zw3^{M11}$ dsh^{75} FRT^{101}, and N^{1081} dsh^{v26} FRT^{101} chromosomes (from the Perrimon lab) were used in conjunction with ovo^{D1} FRT^{101}; F38 to generate homozygous mutant germ line clones in females that were heat-shocked at first or second larval staged and later crossed to FM7 males. Of the eggs that these females laid, one-half were maternally and zygotically mutant and the other half were only maternally mutant for *dsh*, *zw3*, *arm*, *N*, *zw3 dsh*, or *N dsh*. Both types of progeny easily could be distinguished from one another because of the severe segmentation defects of maternally and zygotically mutant embryos evident after 7 hours of development (stage 11).

Immunocytochemistry

Procedures for antibody staining were carried out as described [Bodmer, 1993]. Antibodies and dilutions were as follows: anti-Eve 1:5,000 [Frasch *et al.*, 1987], anti-FasIII (2D5) 1:10 [Patel *et al.*, 1987], anti-Serpent (Srp) 1:2,000 (Sam *et al.*, 1996).

Tissue In Situ Hybridization

in situ hybridization was performed on wholemount embryos using digoxigenin-labeled RNA probes (Boehringer-Mannheim, Mannheim, Germany) according to Tautz and Pfeifle [1989] with the following modifications: (1) prior to hybridization, embryos were fixed with 4% formaldehyde in 100 mM sodium phosphate, pH 7.6, and (2) hybridization occurred at 53°C instead of 45°C. Embryos were hybridized with an antisense *tin* cDNA probe [Bodmer *et al.*, 1990]. RNA probes were synthesized according to Boehringer-Mannheim protocols.

Heat-shock Treatments

Heat-shock treatments of embryos containing the constructs Hs*zw3* and HsN^{intra} were carried out similarly as described previously [Park *et al.*, 1996]. Briefly, embryos were collected on plates with shallow grape agar at 1-hour intervals at 25°C and aged at 25°C. At or shortly after blastoderm formation (3–4 hours of development), embryos containing plates were covered and submerged in a water bath at 39°C (37°C for HsN^{intra}) for 40 minutes. The embryos were then aged at 25°C until fixation and antibody staining.

RESULTS

Role of Components of the Wg Pathway in Heart Development

For studying the cardiogenic role of potential effector gene functions of the *wg* pathway, the late expression of the pair-rule gene *even-skipped* (*eve*) in a subset of pericardial cells (EPCs) serves as a convenient marker for heart development (Fig. 1) [see also Park *et al.*, 1996]. In wild-type embryos, mesodermal *eve* expression is restricted to small, segmentally repeated cluster of cells at the dorsal margin (Fig. 1A). From these clusters, two tissue types differentiate and continue expressing *eve:* the EPC subset of heart cells and the dorsal somatic muscle number one (DA1; arrows in Fig. 1B). As is the case with other markers of heart development, mesodermal *eve* expression is drastically reduced when *wg* function was removed during the critical time of its requirement for heart development (Fig. 1C) [see also Wu *et al.*, 1995].

The segment polarity genes, *dsh*, *arm*, and *zw3* have been proposed to mediate the action of *wg* in the cells that have received the *wg* signal in cell autonomous fashion [Noordemeer *et al.*, 1990; Riggleman *et al.*, 1990; Siegfried *et al.*, 1990, 1992, 1994; Klingensmith *et al.*, 1994; Peifer *et al.*, 1994; also, for review see Perrimon, 1994; Ingham, 1996). In segmentation, *dsh* and *arm* are thought to act positively, whereas *zw3* acts antagonistically within the *wg* pathway. Since these genes are supplied maternally and are ubiquitously distributed in the early embryo, it is necessary to generate germline clones (GLC) to eliminate completely their gene function in early and midembryonic stages [Chou and Perrimon, 1992]. *dsh* or *arm* mutant embryos derived from GLC (see Materials and Methods) completely lack heart development, suggesting that *dsh* and *arm* are essential for heart development (Fig. 1D,F) [see also Park *et al.*, 1996]. As in *wg* mutant embryo, the initial specification of visceral and somatic mesoderm does not seem to be much affected in embryos derived from GLC of *dsh* and *arm* (data not

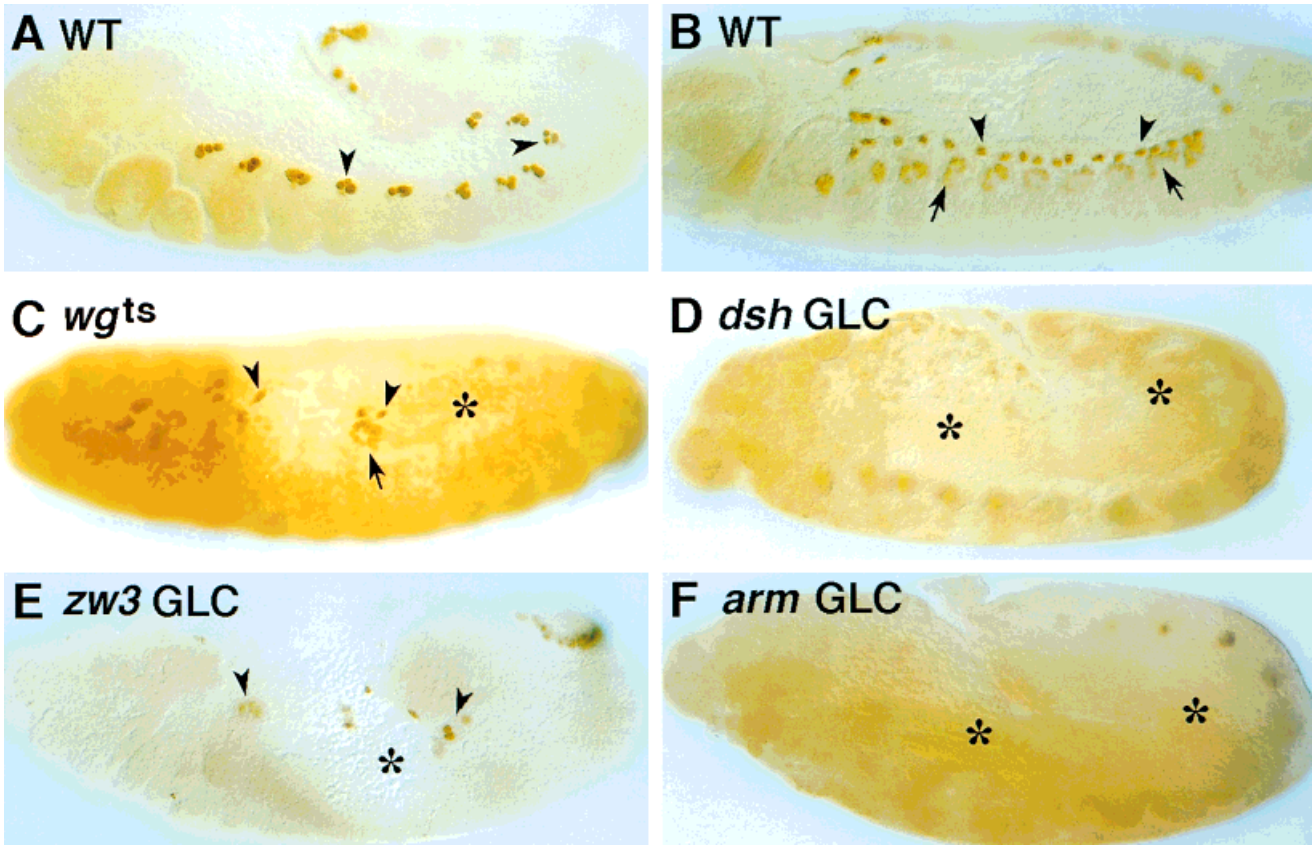

Fig. 1. Heart defects caused by mutations in the wg pathway. Lateral view of stage 11/12 (A, D, E, F) and stage 16 (B, C) embryos stained with anti-Eve antibodies. Anterior is to the left in all figures. **A.** Early, Eve is expressed in a small cluster of cells in each segment (arrowheads). **B.** Later, Eve is present in the subset of pericardial cells (EPCs, arrowheads) and in the syncytial nuclei of the dorsal DA1 muscle (arrows). **C.** Temperature-shifted wg^{IL114} embryo lacks most EPCs and DA1 muscles (asterisk). Arrowheads and arrow indicate remaining EPCs and DA1, respectively. **D.** Maternally and zygotically dsh^{75} mutant embryo (derived from GLC) display a complete absence of heart precursor formation (asterisks). **E.** Maternally and zygotically $zw3^{M11}$ mutant embryo showing the typical decrease in mesodermal Eve expression in these mutants. **F.** Maternally and zygotically arm^{XM19} mutant embryo exhibiting a complete lack of heart precursor formation (asterisks).

shown); [Wu *et al.,* 1995; Park *et al.,* 1996]. These data suggest these known components of the *wg* pathway in other situations also participate in specifying heart precursors.

The *zw3* function is thought to repress *arm* function in the absence of a Wg signal, and this repression is relieved by localized activation of *dsh* function [Siegfried *et al.,* 1992, 1994; Axelrod *et al.,* 1996; for review see Perrimon, 1994; Ingham, 1996). Therefore, in maternally and zygotically *zw3* mutant embryos, we expect to see an excess of heart formation. Instead, we observe that the number of cardiac progenitor cells is much reduced upon elimination of *zw3* function (Fig. 1E) [Park *et al.,* 1996]. These findings would be consistent with the interpretation that *zw3* is not involved in the cardiogenic Wg pathway and the cardiac abnormalities are secondary to segmentation defects, or alternatively, that *zw3* has multiple roles during mesoderm development.

Novel Role for *zw3* in Dorsal Mesoderm Formation

To study further the role of *zw3* in the development of cardiac and other mesodermal tissues, we used a variety of markers to analyze mesoderm differentiation in *zw3* mutants (Fig. 2A,C,E,G): *tin* RNA expression marks dorsal mesoderm (the primordium of heart and visceral muscles) at stage 10 and exclusively cardiac progenitors at stage 11/12 [Bodmer, 1990]; Fasciclin III (Fas III) protein visualizes visceral mesoderm at stage 11 [Patel *et al.,* 1987]; and Serpent (Srp) protein indicates the fatbody primordium at stage 11 [Sam *et al.,* 1996], which is a ventral mesodermal derivative [Azpiazu *et al.,* 1996]. Since mesodermal *eve* expression is reduced in *zw3* GLC embryos (Fig. 1E), we first examined cardiac-specific *tin* expression at stage 11/12 (Fig. 2A) to determine if all aspects of heart development are affected. As with mesodermal *eve*, cardiac *tin*

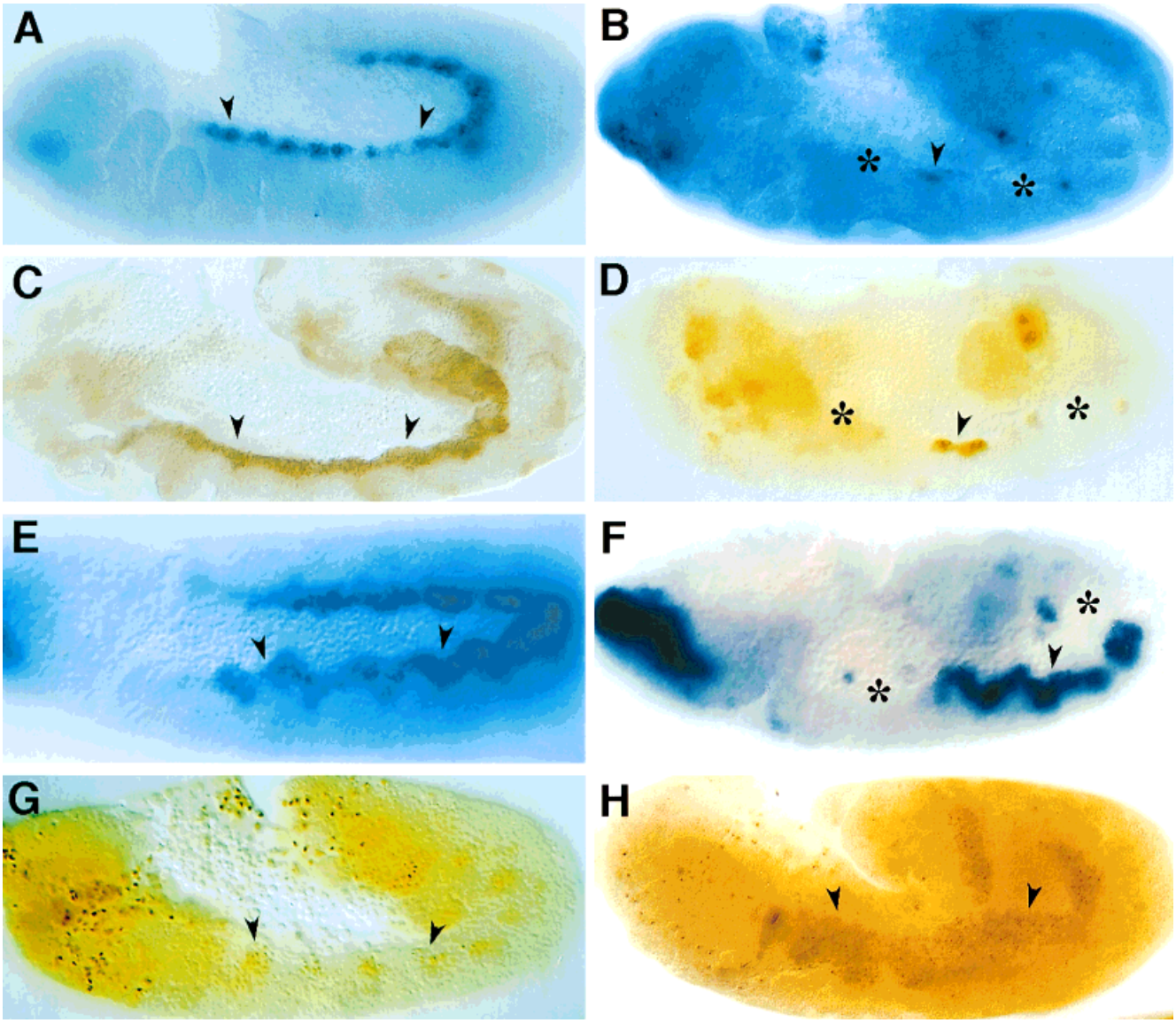

Fig. 2. *zw3* acts as a positive regulator for dorsal mesoderm formation. Lateral view of wild-type (A, C, E, G) and maternally and zygotically zw^{M11} mutant (B, D, F, H) embryos. All embryos are at stage 11/12, except in E and F (stage 10). **A.** *tin* RNA is restricted to the heart precursors at late stage 11 (arrowheads). **B.** *tin* expressing heart progenitors are reduced dramatically in $zw3^{M11}$ mutants (asterisks). Arrowhead points to residual cardiac *tin* expression. **C.** FasIII protein expression in visceral mesoderm (arrowheads). **D.** FasIII expressing visceral mesoderm (arrowhead) is substantially decreased (asterisks) in $zw3^{M11}$ mutant embryos. **E.** *tin* RNA in the dorsal mesoderm at stage 10. **F.** Typical reduction of *tin*-expressing dorsal mesoderm in a $zw3^{M11}$ mutant embryo (indicated by asterisks). **G.** Srp protein in fatbody progenitors (arrowheads). **H.** Expansion of Srp expressing mesoderm (arrowheads) in $zw3^{M11}$ mutant embryo.

expression is also severely reduced in stage 11/12 *zw3* GLC embryos (Fig. 2B), which suggests that the cardiac mesoderm fails to be specified.

Next, we examined visceral mesoderm formation in *zw3* mutant embryos. Although *dsh* or *arm* mutant embryos do not show an appreciable alteration in the initial FasIII expressing visceral mesoderm, *zw3* mutants exhibit a drastic reduction in FasIII (Fig. 2D). This suggests that in contrast to the other two *wg* effectors *dsh* and *arm*, *zw3* not only affects the heart, but also the visceral mesoderm primordium. Since both heart and visceral mesoderm are dorsal mesodermal derivatives, it may be that *zw3* function is required for initiating dorsal mesoderm specification. Indeed, *tin* RNA expression at stage 10, the time when it normally delineates the entire dorsal mesoderm (Fig. 2E), shows a severe reduction in *zw3* mutants (Fig. 2F). This suggests that dorsal mesoderm is not formed properly

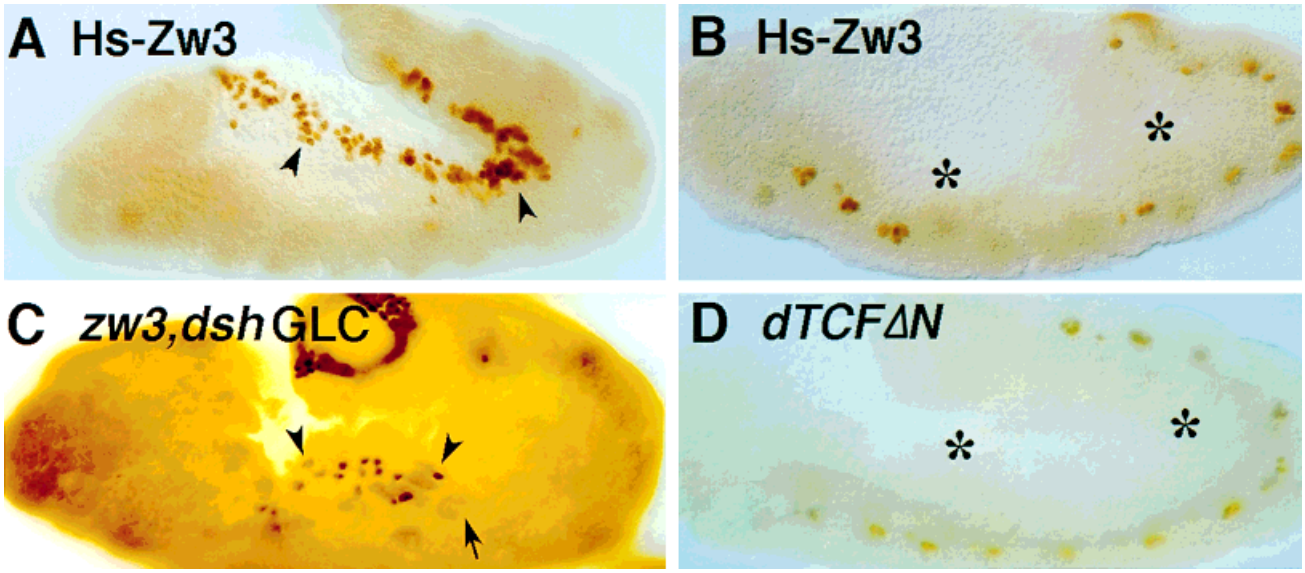

Fig. 3. *zw3* is a negative regulator of cardiogenic *wg* signaling downstream of *dsh*. **A,B.** Eve protein in Hs-*zw3* embryos heat-shocked during 3.5 and 4.5 hours of development for 40 min at 39°C: (A) embryo with an increase in heart formation (arrowheads), (B) embryo with a severe reduction in heart formation (asterisks). **C.** Reduction, but not complete lack of heart formation in maternally and zygotically dsh^{75}, $zw3^{M11}$ double mutant embryo. Arrowheads and arrows indicate the presence of EPCs (dark) and DA1 muscle (faint), respectively. **D.** *dTCFΔN* induction abolishes heart precursor formation (asterisks).

when *zw3* gene function is depleted from the embryo. These data are consistent with the interpretation that the observed cardiac hypotrophy in *zw3* mutants is primarily due a failure in dorsal mesoderm formation. It is possible that Zw3 acts as a transducer of Dpp, the ectodermal signal for dorsal mesoderm formation [Frasch, 1995]. However, Zw3 is probably not the only mediator of this process, because unlike in *dpp* mutants, some dorsal mesoderm is still formed in *zw3* mutants (Fig. 2F).

Next, we wanted to determine if ventral mesodermal derivatives are also affected in *zw3* mutants. In wild-type embryos, Srp is expressed in segmentally repeated clusters of fatbody progenitors (Fig. 2G). In *zw3* mutant embryos, the number of cells expressing Srp protein is dramatically increased (Fig. 2H). Taken together, these data suggest that in *zw3* mutants, more mesoderm with a ventral (fatbody-like) fate is formed, which seems to be at the expense of dorsal mesodermal fates. Thus, *zw3* apparently is required for mediating the correct specification of the dorsal mesoderm, or alternatively, for preventing the differentiation of ventral fates in the dorsal mesoderm. It is therefore possible that *zw3* is indeed involved in the cardiogenic *wg* pathway, but elucidation of such a role is complicated by its prior role in dorsal mesoderm specification.

Does *zw3* Function as a Negative Regulator of the Cardiogenic Wg Pathway?

To corroborate the requirement of *zw3* in dorsal mesoderm formation, we decided to attempt induction of an excess of *zw3* function by conditional *zw3* overexpression at various times of development under the control of a heat-shock promoter. Since *zw3* is required for dorsal mesoderm development, we speculate that a gain-of-*zw3*-function may produce more dorsal mesoderm and consequently more heart may be specified. When *zw3* is overexpressed immediately before the time when *wg* is required for heart formation (see Materials and Methods), three different classes of embryos were observed. The majority of the embryos (71%, n = 500) showed near normal Eve-expression (data not shown). It may be that in these cases insufficient levels of *zw3* function were achieved to have an appreciable effect. The other two classes of embryos exhibited phenotypes that were opposite to each other: either an excess (15% of embryos) or a drastic reduction/absence (14% of embryos) of mesodermal Eve cells was observed (Fig. 3A,B). A similar phenotype was observed with other heart markers as well (data not shown). Hardly any intermediate phenotypes were observed. Interestingly, it was not possible to achieve a temporal separation of the two phenotypes by altering the onset or duration of the heat-shocks. These data can be interpreted as follows: the cardiac hypertrophy (Fig. 3A) is consistent with the hypothesis that *zw3* acts as a positive regulator of dorsal mesodermal fates; in contrast, the lack of heart formation (Fig. 3B) is consistent with the idea that *zw3* acts as a repressor of cardiogenic *wg* signaling. If correct, this would suggest that dorsal mesoderm specification does not significantly precede the *wg*-dependent specification of the cardiac mesoderm, which is consistent with previous studies on the temporal requirement for *wg* itself [Wu *et al.*, 1995].

To test directly whether or not *zw3* also plays a conventional role during cardiogenic *wg* signaling (in addition to its postulated role in dorsal mesoderm formation), we examined heart formation in embryos that were doubly mutant for *zw3* and *dsh*. If *zw3* acts downstream of (and antagonistic to) *dsh*, we expect to see the same phenotype as *zw3* single mutant, which is a reduction (Fig. 1E), but not a complete absence of heart formation (Fig. 1D). In contrast, if *zw3* and *dsh* are not in the same pathway (or if *dsh* acts subsequent to *zw3*), we expect to see a *dsh* phenotype, i.e., a complete lack of heart formation. We find that in *zw3, dsh* double mutant embryos heart formation is reduced as in *zw3* mutants alone (compare Fig. 3C with Fig. 1E). The observed epistasis of *zw3* to *dsh* argues strongly for an involvement of *zw3* in cardiogenic Wg signaling. Taken together, these and the above studies provide strong evidence that *zw3* functions not only as a repressor of *wg*-dependent heart precursor formation, but also as a positive regulator in dorsal mesoderm development.

dTCF, a new component of the Wg pathway, plays a role in heart development

Recent studies have demonstrated that the vertebrate transcription factors TCF/LEF interact with β-catenin in mediating Wnt signaling [Behrens *et al.*, 1996; Huber *et al.*, 1996; Molenaar *et al.*, 1996; Riese *et al.*, 1997]. A *Drosophila* homolog, *dTCF*, has been cloned and shown to interact physically with Armadillo [van de Wetering *et al.*, 1997]. By generating a dominant negative form of *dTCF* (*dTCF dTCFΔN)*, which lacks the region required for β-catenin binding, these authors have shown that *dTCF* functions downstream of *arm* in the establishment of segment polarity by Wingless signaling. From the extracellular ligand, *wg*, to the most downstream-known component of *wg* signaling pathway, *dTCF*, the Wingless/Wnt cascade appears to be conserved between *Drosophila* and vertebrates in various developmental processes [Riese *et al.*, 1997; van de Wetering *et al.*, 1997].

To determine if *dTCF* function is also a mediator of cardiogenic *wg* signaling, we examined heart formation in embryos in which the dominant-negative *dTCFΔN* was mesodermally overexpressed. Since *dTCF* has been shown to be a positive effector of the Wg pathway, we expected that *dTCFΔN* causes a reduction in heart mesoderm formation. Indeed, overexpression of *dTCFΔN* in the mesoderm using the UAS GAL4 system (see Materials and Methods) [Brand and Perriman, 1993] causes a drastic reduction or complete absence of heart progenitors without affecting visceral mesoderm development (Fig. 3D, data not shown). This suggests that *dTCF* is an effector of *wg* signaling in heart development as it is in segmentation and midgut differentiation.

Is *Notch* Involved in the Cardiogenic Wg Pathway?

To determine whether *N* inhibits *wg* signaling by activating *zw3* function downstream of *dsh* not only in wing margin bristle formation [Axelrod *et al.*, 1996], but also in cardiogenesis, we examined heart development in embryos in which *N* function was either removed (alone or in conjunction with *dsh*) or constitutively activated. Since *N* appears to play several roles in mesoderm development [Corbin *et al.*, 1991; Hartenstein *et al.*, 1992; Bate *et al.*, 1993; Baker and Schubiger, 1996; M.P. and R.B. unpub.], we focused our analysis on the *wg*-dependent stage of heart formation. In zygotic *N* null mutants or embryos derived from *N* GLC (see Materials and Methods), as expected, larger Eve-expressing clusters are formed than in wild-type embryos (compare Fig. 4A with Fig. 1A). In contrast, when a constitutively activated form of *N* (N^{intra}) was mesodermally overexpressed, heart formation was completely absent (Fig. 4B). In these embryos, the specification and/or differentiation of many other tissues, including the visceral mesoderm (Fig. 4C) and somatic muscles (data not shown), was also abolished. Similar but less severe defects were observed when N^{intra} was induced conditionally with a heat-shock promoter (data not shown). These studies do not rule out a role for *N* as an antagonist of cardiogenic *wg* signaling.

To test directly whether or not *N* acts downstream of *dsh* (by activating *zw3*), we examined the phenotype of maternally and zygotically *dsh,N* double mutants. If the hypothesis that *N* is epistatic to *dsh* were correct, we would anticipated observing the *N* single mutant phenotype in these double mutants (as in Fig. 4A). However, we find a complete lack of heart formation (Fig. 4D) as in *dsh* single mutants (see Fig. 1D). This result argues against an involvement of *N* in the cardiogenic Wg pathway and rather suggests that *N* in this situation acts independently or at a level that is "upstream" of *dsh*. Nevertheless, an ubiquitous activation of *N* signaling seems to be able to completely inhibit mesoderm differentiation.

DISCUSSION

Cardiogenic Wg Signaling Pathway

In *Drosophila* and vertebrate development, the biochemical pathway of Wg/Wnt signaling appears to be well conserved from the extracellular ligand encoded by *wg* to a transcription factor encoded *dTCF*. Here, we demonstrated that this pathway is also conserved in *Drosophila* heart formation (Fig. 5). We showed that *wg* and its well-known downstream components, *dsh* and *arm*, are absolutely required for heart formation. We also presented evidence that *zw3* acts as the expected antagonist in the cardiogenic *wg* cascade. In addition, we presented evidence that *dTCF* plays a role in the cardiogenic *Wg* pathway, which is probably downstream of *arm*, given the evolutionary conservation of

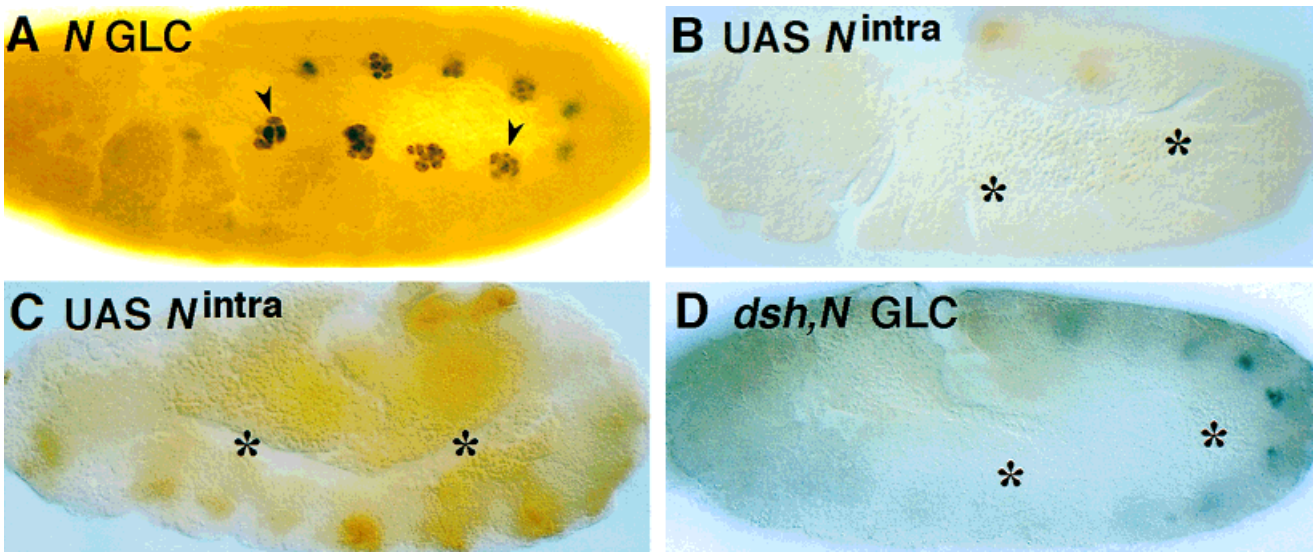

Fig. 4. *N* does not show an interaction with cardiogenic *wg* signaling. **A.** Maternally and zygotically N^{1018} mutant embryo showing an increased number of Eve-expressing cells in the dorsal mesodermal clusters (compare with Fig. 1A). Same results were obtained from zygotically $Notch^{264\text{-}40}$ null mutant embryo (data not shown). **B.** Constitutively active form of *N* (UAS-N^{intra}) has been expressed throughout the mesoderm by the use of *twi*-GAL4; 24B GAL4 (see Materials and Methods). Note the complete lack of mesodermal Eve-expression (asterisks). **C.** UAS-N^{intra} expression as in (B) showing the lack of FasIII-expressing visceral mesoderm (compare with Fig. 2C). **D.** Maternally and zygotically *dsh,N* double mutant showing the complete absence of Eve-expression (asterisks, compare with *dsh* phenotype in 1D).

the pathway (although the epistasis of *dTCF* within the pathway has not been tested).

zw3: Promoter of Dorsal Mesoderm and Repressor of Heart Development

zw3 has been well studied as a maternal repressor within the Wg cascade during pattern formation in *Drosophila* [Bourouis *et al.*, 1990; Siegfried *et al.*, 1990, 1992, 1994]. When we examined the potential function of *zw3* during cardiogenic *wg* signaling, we observed that a loss-of-zw3-function not only causes a reduction of heart formation, which was contrary to our expectation, but also results in a loss of visceral mesoderm. Thus, *zw3* seems to be prominently required for the formation of the dorsal mesoderm, in addition to its repressor function within the cardiogenic Wg pathway (Fig. 5). This interpretation is strongly supported by the fact that the stage 10 restriction of *tin* expression to the dorsal mesoderm is drastically reduced in *zw3* (Fig. 2F) and that *zw3* is epistatic to *dsh*, not only in segmentation (and other processes), but also with respect to heart formation. This dual role of *zw3* in dorsal mesoderm and heart development is further supported by the two phenotypes observed in embryos with *zw3* overexpression (Fig. 3A,B).

Taken together, it appears that *zw3* has two opposing functions with respect to heart development. Its requirement for dorsal mesoderm formation indicates that, indirectly, *zw3* is promoting heart formation. However, its antagonistic role within the Wg pathway suggests that it also represses, more directly, cardiogenesis. How can these two conflicting functions of *zw3* be reconciled during development so that normal heart formation occurs? In one model, it can be envisioned that the two roles of *zw3* are intimately linked to the patterned expression of *dpp* (a determinant of dorsal mesoderm) and *wg* (a determinant of cardiac mesoderm). The *dpp* signal transducers may depend on *zw3*-mediated phosphorylation for their activity, whereas the *wg* transducers (such as *arm*) may be inhibited by *zw3*-dependent phosphorylation. Although dorsal mesoderm formation depends on *zw3*, its function has to be subsequently eliminated to specifically allow heart formation within the dorsal mesoderm. This interpretation would require that the two *zw3* functions are temporally distinct. The *zw3* overexpression studies performed here do not allow us to address this question with the necessary temporal resolution.

Notch Does Not Appear to Interact With Cardiogenic Wg Signaling

Cell-cell communications through signaling molecules between neighboring cells are fundamental for cell fate determination during development. The products of *wg* and *N* are the two signaling molecules that have been proposed to interact genetically in some developmental contexts [Couso and Martinez-Arias, 1994; Hing *et al.*, 1994; Gonzalez-Gaitan and Jäckle, 1995; Axelrod *et al.*, 1996]. Since heart formation is abnormal in *N* mutants, we examined the possible participation of *N* in cardiogenic *wg* signaling. The expanded expression of cardiac markers in *N* loss-of-

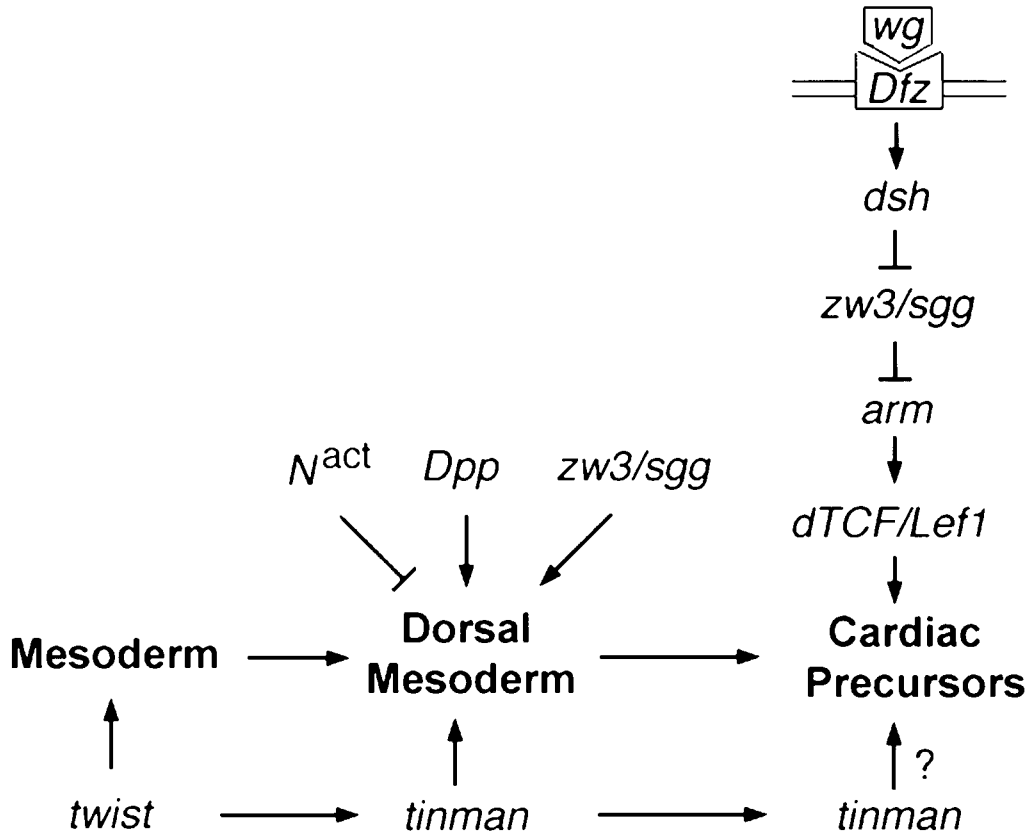

Fig. 5. Model for cardiac precursor specification. At blastoderm, the transcription factor encoded by *twist* defines the initial mesoderm and activates *tin* expression in all mesoderm. The specification of dorsal mesoderm is achieved by the combined function *dpp*, *tin*, and *zw3*. At this point, *dpp* and *tin* expression is confined to the dorsal ectoderm and mesoderm, respectively, whereas *zw3* is present ubiquitously. It is not known if *zw3* is participating in Dpp signal transduction, or if it functions in parallel to *dpp*. It is also not known if or in what way *N* normally affects dorsal mesoderm formation. Subsequent to dorsal mesoderm formation, the cardiac precursors are specified at the dorsal edge of the (dorsal) mesoderm. This process requires the wg signaling cascade, which again involves the function of *zw3*. We propose that *zw3* is first promoting dorsal mesoderm specification, but its function then needs to be eliminated in the prospective cardiogenic region of the dorsal mesoderm to allow heart formation. Although *tin* is restricted at this point to the cardiac mesoderm, it is not known if it is needed again for heart precursor specification.

function mutants together with the reduction (or absence) of heart and dorsal mesoderm in embryos with overexpression of activated *N* is consistent with the hypothesis that *N* might inhibit cardiogenic *wg* signaling. However, the complete absence of heart formation in *N*,*dsh* double mutants (similar to *dsh* single mutants), suggests that *Notch* is unlikely a (negative) component of cardiogenic Wg pathway downstream of *dsh*. The enlargement of the Eve-clusters observed in loss-of-*N*-function embryos is probably due a lack of lateral inhibition, thus causing an increased number of muscle founder cells to be recruited within a field of competence [Corbin *et al.*, 1991; Bate *et al.*, 1993), similar to the well-known function of *N* in neuroblast formation.

Drosophila Heart Development as a Model for Cardiogenesis

Despite the obvious differences of heart morphology between Drosophila and vertebrates, many fundamental similarities in its development and the molecular nature of its determinants have recently been described [for review see for Bodmer, 1995; Harvey, 1996; Bodmer *et al.*, 1997]. For example, *tin*-related genes have been cloned from various vertebrates including human, and many of these genes are expressed in pre-cardiac mesoderm similar to *tin* in *Drosophila*. Furthermore, TGF-β based signaling in vertebrates is also crucial for cardiac-specific expression of *tin*-related genes. In addition, in vitro studies have suggested that *Wnt* signaling also may play a role in vertebrate heart development [McMahon and McMahon, 1989; Augustine *et al.*, 1993]. Taken together, *Drosophila* has been proven to serve as an excellent model system and rich resource to study fundamental mechanisms of heart development.

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