Development 130, 3039-3051

doi:10.1242/dev.00484

Myogenic cells fates are antagonized by Notch only in asymmetric lineages of the *Drosophila* heart, with or without cell division

Zhe Han* and Rolf Bodmer†

Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI 48109, USA
*Present address: Department of Molecular Biology, UT Southwestern Medical Center, 6000 Harry Hines Boulevard, NA8.510, Dallas, TX 75390-9148, USA
†Author for correspondence (e-mail: rolf@umich.edu)

Accepted 14 March 2003

SUMMARY

During the formation of the *Drosophila* heart, a combinatorial network that integrates signaling pathways and tissue-specific transcription factors specifies cardiac progenitors, which then undergo symmetric or asymmetric cell divisions to generate the final population of diversified cardiac cell types. Much has been learned concerning the combinatorial genetic network that initiates cardiogenesis, whereas little is known about how exactly these cardiac progenitors divide and generate the diverse population of cardiac cells. In this study, we examined the cell lineages and cell fate determination in the heart by using various cell cycle modifications. By arresting the cardiac progenitor cell divisions at different developing stages, we determined the exact cell lineages for most cardiac cell types. We found that once cardiac progenitors are specified, they can differentiate without further divisions. Interestingly, the progenitors of asymmetric cell lineages adopt a myocardial cell fate as opposed to a pericardial fate when they are unable to divide. These progenitors adopt a pericardial cell fate, however, when cell division is blocked in *numb* mutants or in embryos with constitutive *Notch* activity. These results suggest that a *numb/Notch*-dependent cell fate decision can take place even in undivided progenitors of asymmetric cell divisions. By contrast, in symmetric lineages, which give rise to a single type of myocardial-only or pericardial-only progeny, repression or constitutive activation of the *Notch* pathway has no apparent effect on progenitor or progeny fate. Thus, inhibition of *Notch* activity is crucial for specifying a myogenic cell fate only in asymmetric lineages. In addition, we provide evidence that whether or not Suppressor-of-Hairless can become a transcriptional activator is the key switch for the Numb/Notch activity in determining a myocardial versus pericardial cell fate.

Key words: *Drosophila*, Heart, Cell cycle regulators, Asymmetric cell division, *numb*, *Notch*, *CycA*, *Rca1*, *dacapo*, *fizzy-related*

INTRODUCTION

How cell-fate diversity is generated is a central issue in developmental biology. Recently, this question has been more specifically asked during the development of individual organs. During *Drosophila* heart development, a limited number of progenitors generate a defined set of cell types that are arranged in a stereotyped and segmentally repeated pattern (Ward and Skeath, 2000; Han et al., 2002), making it an excellent model with which to address this question.

The *Drosophila* heart originates from bilaterally symmetrical rows of precursor cells in the dorsal mesoderm, which express the homeobox gene *tinman* (Bodmer, 1993; Azpiazu and Frasch, 1993). As a consequence of inductive signals from the adjacent ectoderm, *tinman* expression is confined to the dorsalmost region of the mesoderm, defined as the cardiac mesoderm (Frasch, 1995; Wu et al., 1995; Lockwood and Bodmer, 2002). Within this competence domain, cardiac progenitors emerge as cell clusters that express a distinct combination of transcription factors, including *even-skipped* (*eve*), *ladybird* (*lb*), *seven-up* (*svp*), *odd-skipped* (*odd*) and *tinman* itself, which are thought to be involved in conferring the appropriate differentiation pathway to these clusters (Ward and Skeath, 2000; Jagla et al., 2002; Han et al., 2002). The initiation of these cell clusters of different developing fates is probably mediated by extrinsic, inductive mechanism, which is determined by their position in the cardiogenic domain. Subsequently, a defined pattern of lineages generates the final diversity of cell types (Ward and Skeath, 2000). Some cardiac progenitors divide symmetrically, whereas others undergo stereotyped asymmetric cell divisions.

Two types of asymmetric cell division in the cardiac mesoderm have been described: the progenitors of the Svp lineage divide asymmetrically to generate Svp myocardial cells (SMC) and Svp-Odd pericardial cells (SOPC) (Ward and Skeath, 2000); the progenitors of the mesodermal Eve lineage generate Eve pericardial cells (EPC) and muscle founders DA1 and DO2 (Park et al., 1998; Halfon et al., 2000; Carmena et al., 2002; Han et al., 2002). These two types of asymmetric cell division are similar in the sense that they both generate myogenic versus non-myogenic sibling cell fates. The myocardial and muscle founder cells have many characteristics

of muscle identity in common, which is excluded in pericardial cell differentiation. For example, they both express the muscle differentiation gene *Mef2* (Bour et al., 1995; Lilly et al., 1995). Previous studies suggest that the Notch pathway plays an important role in determining these alternative cell fate decisions (Park et al., 1998; Carmena et al., 1998; Carmena et al., 2002; Ward and Skeath, 2000). In mutants of the Notch antagonist encoded by *numb* (reviewed by Jan and Jan, 1998), the number of pericardial SOPC and EPC is increased, accompanied by a loss of myocardial SMC and of DA1 muscles. Conversely, when *numb* is overexpressed in the mesoderm, EPCs are not formed, only DA1 muscles. Moreover, when a constitutively active form of Notch, Notch intracellular domain or N(icd), is expressed in the mesoderm, the mesodermal Eve lineage is almost completely eliminated, suggesting that Notch activity inhibits the formation of progenitors at an early stage. However, when a temperature-sensitive allele of *Notch* is used to eliminate *Notch* function at the time when the Eve progenitors divide, EPCs but not DA1 muscle fail to form (Park et al., 1998). Thus, *Notch* seems to have a dual function, as it is required both for progenitor specification and for asymmetric cell fate determination of the same lineage. It also appears that the *Notch* pathway is required for specifying pericardial as opposed to myocardial or muscle founder cell fate in both the Svp and the Eve lineages. It is not known, however, if the Notch-dependent cell fate decision is made after cell division in the progeny cells or if it is already initiated in the progenitors before division.

Studying these *Notch*-mediated asymmetric cell divisions in the context of the cell cycle may provide insights into the coordination of cell fate and cell division. Thus, preventing cell division of asymmetric cell divisions allows us to investigate whether precursors blocked in cell cycle progression are predetermined or biased in their cell fate decision, or whether this is a necessary prerequisite for alternative cell fate determination. Moreover, blocking the mesodermal cell division at progressively later stages will enable us to get a better understanding of the cell lineages in the heart. Previous studies have followed marker gene expression and used the Flp-FRT-based lineage tracing method to address this question (Park et al., 1998; Ward and Skeath, 2000; Carmena et al., 1998; Carmena et al., 2002), but some of the details of these lineages are still unclear.

Several genes have been shown to arrest cell divisions at different stages and cell cycle number within the ectoderm. It is believed that most cells in *Drosophila* undergo three rounds of cell division after blastoderm that are partially synchronous; e.g. slightly earlier in the mesoderm than in the ectoderm (Foe, 1989; Campos-Ortega and Hartenstein, 1997). Later, specialized embryonic tissues, such as the nervous system, undergo further cell divisions (Bodmer et al., 1989; Foe, 1989; Lu et al., 2000). The last round of global cell division, mitosis 16, is blocked in *CyclinA* (*CycA*) or *Rca1* mutants (Knoblich and Lehner, 1993; Dong et al., 1997). By contrast, mitosis 16 is not obviously affected by *CyclinB* (*CycB*) mutants. However, double mutants of *CycA* and *CycB* act synergistically and arrest cell division of most ectodermal cells at the G2/M transition of cycle 15 (Knoblich and Lehner, 1993). In *string* (*stg*, *cdc25* in yeast) mutant embryos, mesodermal cells fail to enter metaphase of mitosis 14 (Foe, 1989; Edgar and O'Farrell, 1989; Edgar and O'Farrell, 1990). In addition to these genes that are required for cell cycle progression, some other cell cycle genes have been shown to be required for cells to exit the cell cycle. These include *dacapo* (*dap*) (Lane et al., 1996) and *fizzy related* (*fzr*; *rap* – FlyBase) (Sigrist and Lehner, 1997). *dap* encodes a CDK inhibitor that is necessary for exiting the cell cycle at the appropriate time, whereas *fzr* negatively regulates the levels of cyclins A, B and B3, and is required for cyclin removal during G1 (when cell proliferation stops). Loss of either gene causes cell division progression through an extra cycle. Conversely, premature overexpression of *dap* or *fzr* in transgenic embryos inhibits mitosis and results in cell division arrest (Lane et al., 1996; Sigrist and Lehner, 1997).

In this study, we examined the formation of cardiac cell types in cell cycle mutants and in embryos in which cell cycle inhibitors are overexpressed. We found that the cardiac progenitors continue to differentiate in the absence of cell division. Interestingly, the progenitors of the asymmetric Eve and Svp lineages always adopt a myogenic cell fate, presumably because Notch activation is prevented in the progenitor because of the presence of Numb. To test this, we arrested cell division in *numb* mutants or overexpressed N(icd) in these progenitors, and found that they adopt a non-myogenic pericardial cell fate. These data indicate that genes normally involved in the cell fate decisions during asymmetric cell division also determine the progenitor cell fate. By contrast, cell fate decisions with symmetric cardiac lineages are not influenced by the presence or absence of Notch activity. Our data suggest that cell cycle genes act in concert with the Notch pathway to generate the diversity of cell types in the *Drosophila* heart, and that the bHLH transcription factor Suppressor-of-Hairless [Su(H)] mediates this activity. We speculate that an increase in cell type diversity can be achieved by adopting the Numb/Notch system to generate asymmetry within any lineage of an organism.

MATERIALS AND METHODS

Drosophila strains

The following mutant stocks were used: $Rca1^{03300}$, stg^{7} and $numb^{1}$ are from the Bloomington Stock Center; $CycA^{C8}$ and $CycB^{2}$ (described by Knoblich and Lehner, 1993) are gifts from C. Lehner; $Notch^{ts}$ has been described previously (Park et al., 1998). Overexpression of transgenes was accomplished by using the Gal4-UAS system (Brand and Perrimon, 1993). The following fly lines were used: *twi*-Gal4 and 24B-Gal4 (conferring pan-mesodermal expression) (Greig and Akam, 1993; Brand and Perrimon, 1993), eme-Gal4 (which confers expression in the mesodermal Eve lineage) (Han et al., 2002), UAS-*dap* (Lane et al., 1996), UAS-*fzr* (Sigrist and Lehner, 1997), UAS-*Su(H)* and UAS-*Su(H)-vp16* (Furriols and Bray, 2000), and UAS-*N(icd)* and UAS-*numb* (Yaich et al., 1998; Park et al., 1998). Fly lines carrying emeA-*lacZ* (380 bp *eve* mesodermal enhancer directing *lacZ* expression) (Han et al., 2002) is used to label EPCs and the DA1 and DO2 founder cells. Oregon-R was used as the wild-type reference strain.

Immunohistochemistry and microscopy

Embryos from different lines were collected and stained with various antibodies as previously described (Han et al., 2002). The following primary antibodies were used: anti-β-galactosidase 1:300 (Promega); anti-Eve 1:10,000 (Frasch et al., 1987); anti-Tin 1:500 (Venkatesh et al., 2000); anti-Mef2 1:1000 (Lilly et al., 1995); and anti-Lbe 1:40

(Jagla et al., 1997). FITC- or Cy3-conjugated secondary antibodies (from Jackson Laboratories) were used to recognize the primary antibodies. Images were obtained with a Zeiss LSM510 confocal microscope.

RESULTS

Mesodermal Eve lineages and the fate of Eve progenitors in the absence of cell division

The *Drosophila* heart progenitors are specified at the dorsal mesodermal edge and are coincident with the cells that maintain expression of *tinman* at stage 11. These progenitor cells undergo symmetric or asymmetric cell divisions to generate a diverse set of cardiac cell types (Ward and Skeath, 2000). From stage 13 onwards, each cell type is positioned at a specific dorsoventral and anteroposterior location and is distinguished by the expression of a unique combination of transcription factors (see Han et al., 2002). Of these, the MADS-box transcription factor Mef2 (Bour et al., 1995; Lilly et al., 1995) marks all the myocardial cells, whereas the homeobox gene *even-skipped* (*eve*) labels the EPCs (a pericardial cell type), DA1 (a dorsal muscle) and transiently the founder of the DO2 muscle (Fig. 1A) (Frasch et al., 1987; Carmena et al., 2002; Han et al., 2002). The lineage giving rise to the mesodermal *eve*-expressing cells has been studied intensely (e.g. Carmena et al., 1998; Park et al., 1998; Su et al., 1999; Halfon et al., 2000). Nevertheless, the exact lineage of these cells has not been unambiguously resolved, which is reflected in two different models (Fig. 1E,F) (Park et al., 1998; Carmena et al., 1998).

Here, we study the Eve and other lineages of the heart by arresting the cell cycle at various stages. We first examined formation of cardiac cell types in *CycA* mutant, which causes cell cycle arrest at mitosis 16 in the ectoderm (Knoblich and Lehner, 1993). In *CycA* mutants, EPCs are absent as judged by the absence of *eve* expression, but DA1 muscles form as in wild type (Fig. 1A,B). A similar phenotype is observed in *Rca1* mutants (arrest also at ectodermal cycle 16 as *CycA*) (Dong et al., 1997; Wai et al., 1999), but with somewhat less penetrance (Fig. 1C). In *Rca1* mutants, 86% (n=144) of hemisegments show no EPCs and 14% show one EPC; all of them have normal DA1 muscles (Fig. 1C). The one-EPC phenotype suggests that a common progenitor exists for the two EPCs in each hemisegment. This conclusion is further supported by examining embryos in which the cell cycle inhibitor *dap* or *fzr* is overexpressed specifically in the mesoderm [using the twist-Gal4 driver line (Greig and Akam, 1993) in conjunction with the UAS-Gal4 system (Brand and Perrimon, 1993)]. In these embryos (progeny from the cross between twist-Gal4 crossed UAS-*dap*, twi>*dap*) most hemisegments show only one EPC (94%, n=150; Fig. 1D) suggesting that the last division generating two EPCs did not occur. This division apparently is symmetrical. A similar phenotype is observed in 70% twi>*fzr*

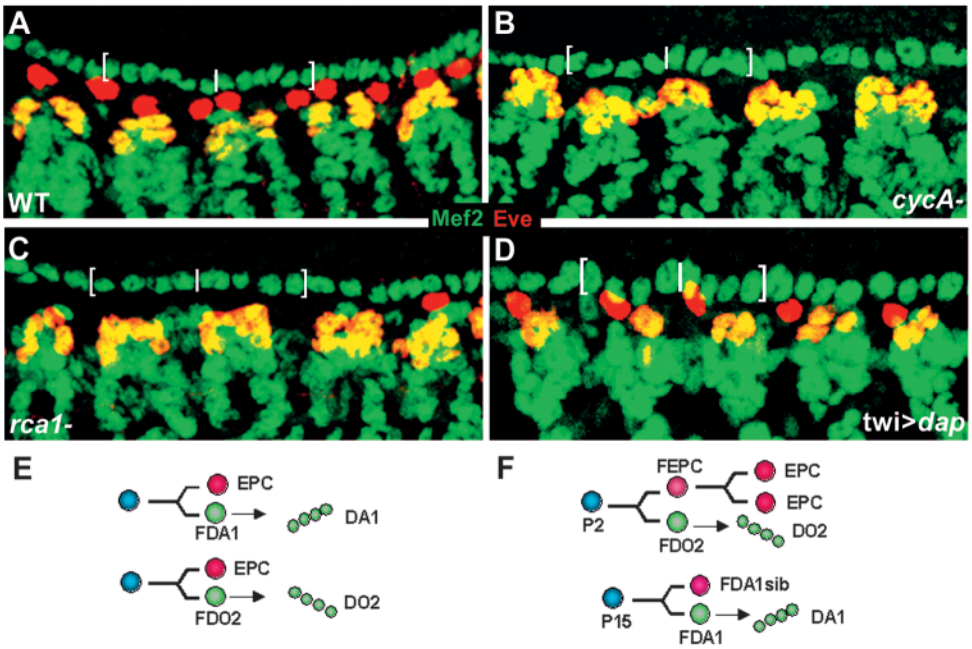

Fig. 1. Progenitors of the mesodermal Eve lineage adopt a muscle cell fate when their asymmetric divisions are blocked. (A-D) Antibody staining of Eve (red) and Mef2 (green) in stage 13 embryos. Five hemisegments of segment A2-A7 are shown in each panel. (A) Six *Mef2*-expressing myocardial cells, two EPCs, and one DA1 muscle are present in each hemisegment in wild-type stage 13 embryos. (B) In *CycA* mutants, the number of Mef2 myocardial cells is reduced to four per hemisegment; no EPCs are specified, but the DA1 muscles are present. (C) In *Rca1* mutants, four myocardial cells are present in each hemisegment; most EPCs are absent. (D) Pan-mesodermal expression of *dap* reduces the number of myocardial cells to four and the EPCs to one per hemisegment. The DA1 muscles are present. (E) In the mesodermal Eve lineage model of Park et al. (Park et al., 1998), two progenitors are formed per hemisegment, each giving rise to an EPC and a muscle founder. (F) In the model of Carmena et al. (Carmena et al., 1998), two progenitors are also formed in each hemisegment, but one (P2) gives rise to both EPCs and one muscle founder, whereas the other (P15) generates only the DA1 founder. This EPC progenitor is a daughter cell of an asymmetric cell division that also generates the DO2 muscle founder.

embryos (*n*=150, data not shown). Because it is thought that Dacapo causes arrest of the last division (Lane et al., 1996), and that CycA and Rca1 are required for the cell cycle 16, we propose that the EPCs are generated at cycle 17 by symmetric cell division and the progenitor of EPCs and a muscle founder are generated at cycle 16 by asymmetric cell division. Thus, these findings together with previous data support the model depicted in Fig. 1F (see also Fig. 9). More importantly, these data also suggest that in *CycA* or *Rca1* mutants, the undivided Eve progenitors of cycle 16 adopt a myogenic cell fate.

Undivided progenitors of the Svp lineage also adopt a myocardial fate

In all the four cell cycle arrest situations examined so far (*CycA* and *Rca1* mutants, overexpression of *dap* or *fzr* in the mesoderm), four instead of six myocardial cells form in each hemisegments of the abdominal segments, A2-A7 (Fig. 1B-D), indicating that the progenitors of myocardial cells differentiate as myocardial cells when the last cell division is blocked (Fig. 9). It also indicates that the last division of myocardial cells is mitosis 16, as twi>*dap* or twi>*fzr* embryos exhibit the same myocardial cell phenotype as *CycA* or *Rca1* mutants. Previous studies suggest that in each hemisegment the four Tinman myocardiac cells (TMC) are generated from symmetrical divisions, whereas two Svp progenitors divide asymmetrically to give rise to two Svp myocardial cells (SMC) and Svp-Odd pericardial cells (SOPC; Fig. 2A,E) (Ward and Skeath, 2000) (see also Fig. 9C). If this is the case, the number of TMC in *CycA* or *Rca1* and twi>*dap* embryos should be two, and the number of SMC should be either two or none, depending of the fate of the undivided Svp precursors. Indeed, in these cycle 16 arrest situations, only two TMC per hemisegment form (Fig. 2B-D). Because the total number of myocardial cells is four (Fig. 1B-D), the other two myocardial cells are probably SMC. In order to test this, we examined the number of *odd*-expressing pericardial cells, two out of a total of four co-express *svp* (SOPC) in wild-type embryos (Fig. 2E). In *CycA* mutants, a single *odd*-expressing pericardial cell (OPC) is present per hemisegment (Fig. 2F, Fig. 9B), consistent with the prediction that the two Svp-Odd progenitors assume a myocardial fate when cycle 16 is blocked. A third Odd-only progenitor is likely to divide symmetrically, as suggested previously (Ward and Skeath, 2000).

tinman-expressing myocardial progenitors are likely to divide symmetrically

In each abdominal hemisegment of A2-A7 there are four *ladybird*-expressing heart cells (Fig. 9A,C), two that are myocardial, co-expressing *tinman* and *Mef2* (TLMC), and the other two that are pericardial, co-expressing *tinman* only (LPC) (Jagla et al., 1997). These cells are adjacent to the Eve cells but never overlap with them (Fig. 2G) (Han et al., 2002). In *CycA* mutants, two of the myocardial cells express *tinman* (Fig. 2B), and of only one expresses *ladybird* (TLMC in Fig. 2H; Fig. 9B), suggesting that the two *tinman*-expressing myocardial progenitors divide symmetrically, but each assumes a distinct molecular identity distinguished at least in part by the presence or absence of *ladybird* expression (summarized in Fig. 9).

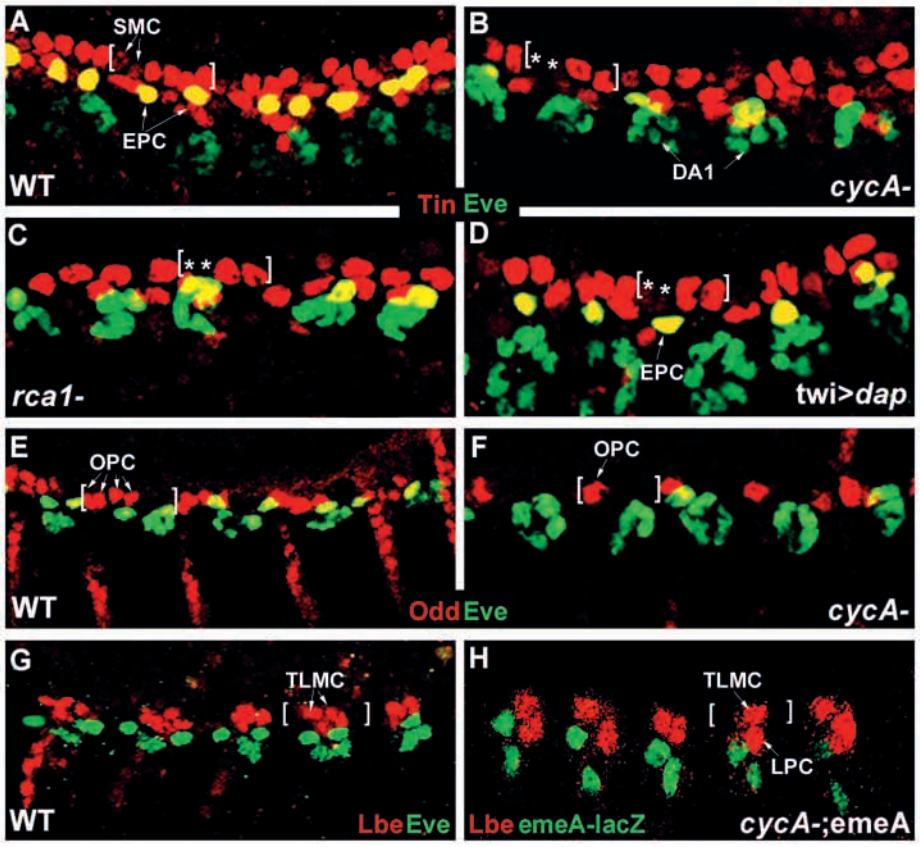

Fig. 2. Progenitors of the Svp lineage adopt a myocardial cell fate when their asymmetric divisions are blocked. (A-D) Antibody staining of Tinman (red) and Eve (green), (E,F) Odd (red) and Eve (green), (G) Lbe (red) and Eve (green), and (H) Lbe (red) and β-Gal (green). Five stage 13 A2-A7 hemisegments are shown in each panel. (A) In wild-type embryos, four out of six myocardial cells are labeled with Tinman. (B) In *CycA* mutants, the number of Tinman myocardial cells is reduced to two per hemisegment; no EPCs, only DA1 muscles, are specified. (C) In *Rca1* mutants, two Tinman myocardial cells are present per hemisegment; EPCs are usually absent but sometimes appear as a single cell. (D) Mesodermal overexpression of *dap*: two Tinman myocardial cells, one EPC and normal DA1 muscles forms in each hemisegment. (E) In wild type, four pericardial cells express *odd* (OPC) in each hemisegment. (F) In *CycA* mutants, the number of Odd pericardial cells is reduced to one per hemisegment. (G) In wild type, two *lbe*-expressing myocardial cells (TLMC) and two *lbe*-expressing pericardial cells (LPC) are present in each hemisegment. (H) In *CycA* mutants, only one TLMC and one LPC are present in each hemisegment. emeA-*lacZ* shows two Eve lineage derived muscle founders form in each hemisegment (see also Fig. 6C).

Distinct lineages of the *tinman*-expressing myocardial cells in the anterior two segments of the heart

By comparing the *Mef2* and *tinman* expression in both wild-type and cell cycle mutants, we noticed that the pattern of cardiac cell types is different in the anterior two segments (T3 and A1). The embryonic heart is formed approximately within segment T3 to A8, and is composed of six myocardial cells per hemisegment, except in A8, where only four are present (Fig. 3A). Therefore, the total number of the myocardial cells expressing *Mef2* is 104 [(6×8+4×1)×2] (number of cells per hemisegment × number of segments × 2 sides) (Fig. 3A). Two of the myocardial cells in each A2-A8 hemisegment express *svp* but not *tinman* (SMC). Therefore, the total number of SMC is 28 (2×7×2) (see Gajewski et al., 2000; Lo et al., 2002). In the anterior two segments, all myocardial cells express *tinman* (Fig. 9A), which adds up to a total number of 76 TMCs [(6×2+4×6+2×1)×2], (Fig. 3C; Fig. 9A) (Alvarez et al., 2003). In embryos in which *dap* is expressed throughout the mesoderm, thus blocking the last division, the number of myocardial cells is reduced in T3-A1 (average 3.2 per hemisegment, *n*=20), as it is in the A2-A7 abdominal segments (two per hemisegment, *n*=76) (Fig. 3B,D). In *CycA* or *Rca1* mutant embryos, however, which are normally blocked in cell cycle 16, the number of TMCs in T3-A1 remains unchanged (six per hemisegment, *n*=26), unlike in A2-A7 where the number of TMCs is reduced to half (two per hemisegment, *n*=82) (Fig. 3E,F; Fig. 9B). This suggests that in the anterior two segments, the TMC precursor divisions are already complete after cycle 15, thus not affected in *CycA* and *Rca1* mutants, or the anterior myocardial lineages are different, as suggested by a recent lineage study (Alvarez et al., 2003), or both. Whatever turns out to be the case, these conclusions are consistent with recent observation that the T3-A1 heart precursors are specified under homeotic control that is distinct from that of the other abdominal segments (Lovato et al., 2002; Lo et al., 2002). Not only is the myocardial cell number in T3-A1 unaffected in *CycA* or *Rca1* mutants, but as observed in a late stage embryos, these anterior myocardial cells assemble into a tube as in wild type (Fig. 3F). By contrast, the fewer than normal abdominal myocardial cells do not align properly, suggesting that a reduction in myocardial cell number adversely affects heart tube morphogenesis.

CycA;*CycB* double mutant arrests mesodermal division at mitosis 15

In order to study the cardiac lineages further, we examined *CycA*;*CycB* double mutants, which are thought arrest cell division before cycle 16. CycA and CycB function synergistically during the G2-M transition (Knoblich and Lehner, 1993). Even though *CycB* mutants do not seem to affect the three post-blastoderm divisions, *CycA*;*CycB* double

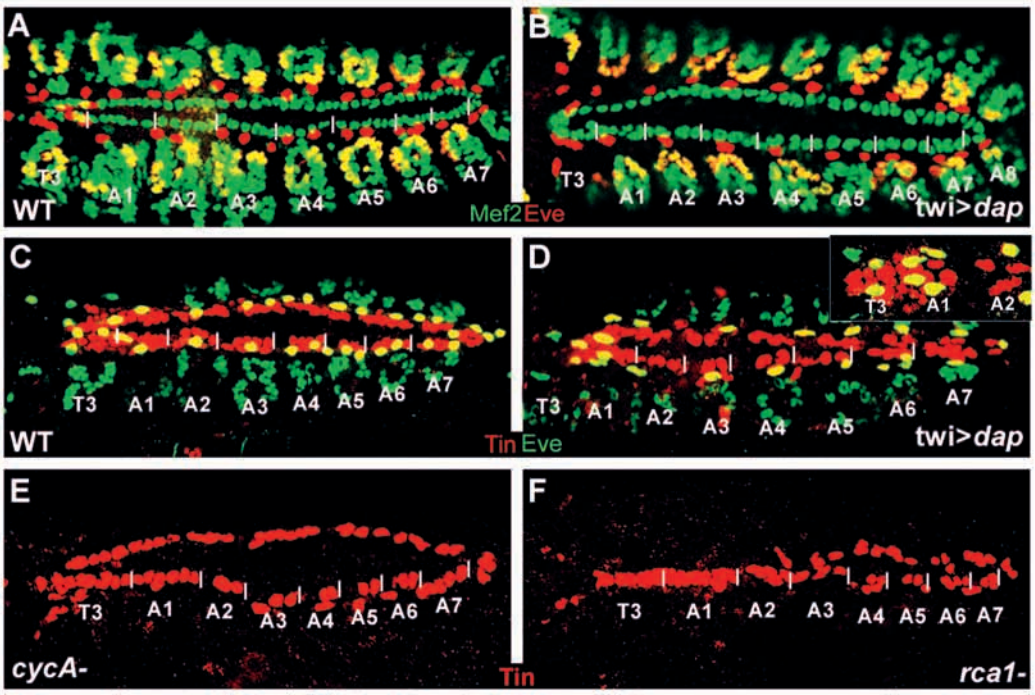

Fig. 3. Segmental differences of the *Drosophila* cardiac lineages. Stage 15 embryos stained for (A,B) Mef2 (green) and Eve (red), (C,D) Tinman (red) and Eve (green), or Tinman only (E,F). (A) In the wild-type embryos, six myocardial cells and two EPCs are present in each hemisegment from T3 to A7. (B) Overexpression of *dap* in the mesoderm reduces the myocardial cells from six to four per hemisegment in A2-A7 segments, and in T3-A1 to about three. (C) In wild-type segments A2-A7, four out of six myocardial cells express *tinman* in each hemisegment; in segment A8, two out of four express *tinman*; however, in segments T3-A1, all six myocardial cells express *tinman*. (D) Overexpression of *dap* in the mesoderm reduces the *tinman*-expressing myocardial cells in A2-A7 from four to two, in A8 from two to one, and in T3-A1 from six to about three (see insert). (E,F) In *CycA* or *Rca1* mutants, *tinman*-expressing myocardial cells are reduced from four to two in hemisegments A2-A7, from two to one in A8. However, there is no change in hemisegments T3-A1 in that all six myocardial cells express *tinman*. Note that the change in myocardial cell number in A2-7 affects heart tube assembly in the posterior abdominal segments, but not in T3-A1 (F).

mutants are more severe than *CycA* mutant alone, in that ectodermal cell division is arrested at cycle 15 (Knoblich and Lehner, 1993). In the heart, *CycA*;*CycB* double mutants exhibit a further reduction in the number of myocardial cells when compared with *CycA* alone (Fig. 4A-D). Instead of six myocardial cells in wild type and four myocardial cells in *CycA* mutants, only two myocardial cells are observed in 75% of 120 observed hemisegments in *CycA*;*CycB* double mutants (Fig. 4B). In addition, only one TMC forms in 64% of 120 observed hemisegments (Fig. 4D), indicating that the cell divisions of myocardial lineages are indeed blocked at cycle 15 in *CycA*;*CycB* double mutants. These data also suggest that the four TMC that are formed in each hemisegment originate from one super progenitor (TSP) and the two SMC plus two SOPC originate from another super progenitor (SSP, Fig. 9C). Both super progenitors appear to be specified before cycle 15. Although one of the two TMC per hemisegment in *CycA* mutants expresses *ladybird early*, this homeobox gene product is no longer detected in double mutants of *CycA* and *CycB* (Fig. 4E,F). These observations are consistent with the idea that initially one TMC super-progenitor is specified in each hemisegment, which divides asymmetrically during cycle 15, giving rise to a Ladybird-positive and Ladybird-negative TMC progenitor.

In segments T3-A1, the number of TMCs is not altered by *CycA* or *CycB* mutants. By contrast, *CycA;CycB* double mutants exhibit a reduction of TMC from six to three in 70% of observed hemisegments (n=46; Fig. 4G,H). These data are consistent with the hypothesis that the T3-A1 TMC progenitor divisions occur during cycle 15, which also fits with the idea that the identity of these two cardiac segments is specified differently (Lo et al., 2002; Alvarez et al., 2003). Interestingly, the DA1 muscles are specified similarly in *CycA* single or in *CycA*;*CycB* double mutants (Fig. 1B; Fig. 4B,D), suggesting that Eve progenitor specification is unaffected by arresting mitosis at cycle 15. This is reminiscent of the observation made previously in *stg* mutants, in which the cell division is arrested at cycle 14, the first post-blastoderm division: up to two Eve progenitors are specified per hemisegment (Carmena et al., 1998; Su et al., 1999), one of which will give rise to the DA1 muscle founder and the other to the immediate precursor of two EPCs per hemisegment (Fig. 9C). Re-examining the cardiac phenotype in *stg* mutants we found that the *eve*-expressing cells often appear in pairs, although in some segments *tinman*- or *eve*-expressing progenitors fail to form in this early arrest mutant. The pairs of *eve*-expressing cells seem to adopt a muscle founder cell fate, as most of them express *Mef2* but not *tinman* in stage 13/14 embryos (Fig. 4I,J). However, unlike *CycA*;*CycB* double mutants, *stg* mutants show severe segmentation and other patterning defects, presumably

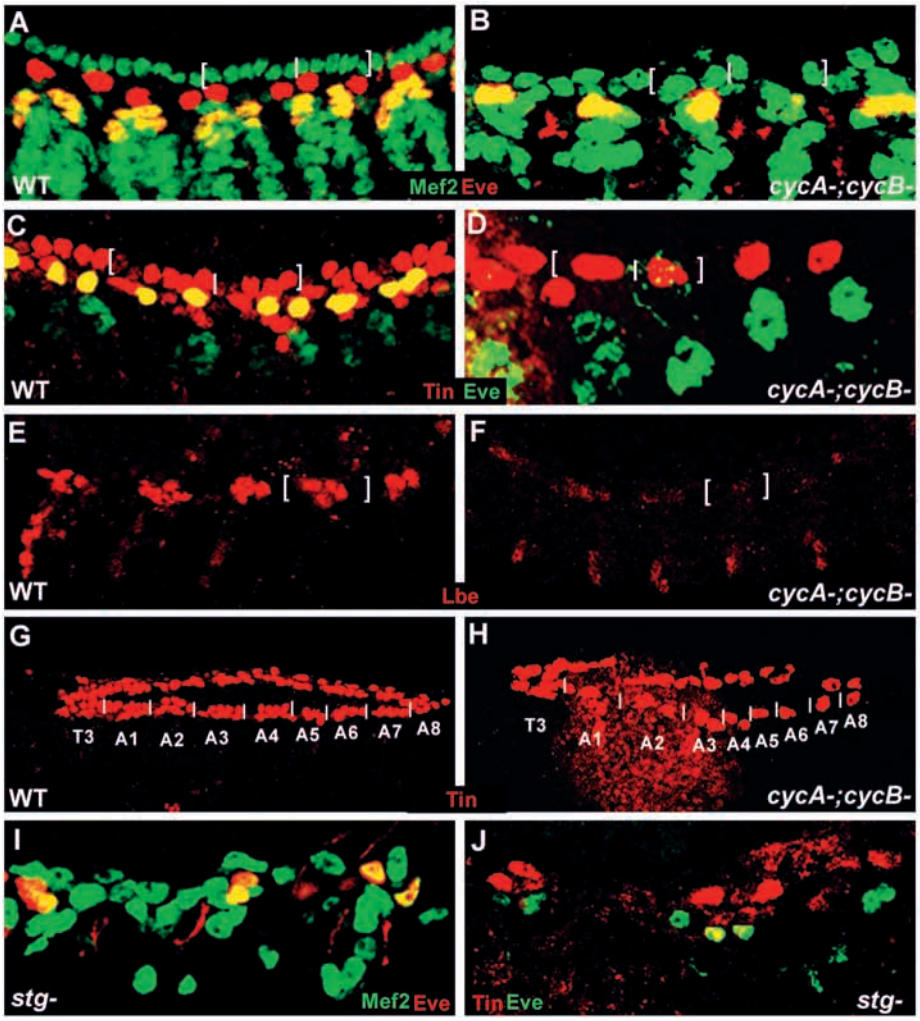

Fig. 4. Cardiac cell types in *CycA*-;*CycB*-double mutants and in *stg* mutants. (A-F,I-J) A2-A7 hemisegments of stage 13 embryos are labeled for (A,B,I) Mef2 (green) and Eve (red), (C,D,J) Tinman (red) and Eve (green), or (E,F) Lbe (red) alone. (G,H) Stage 15 embryos labeled for Tinman (red). (A,B) In *CycA*;*CycB* double mutants, two instead of six myocardial cells are present in each hemisegment, DA1 muscles but no EPCs are formed. (C,D) Double labeling for Tinman and Eve shows that only one of the two myocardial cells per hemisegment in the *CycA*;*CycB* double mutant expresses *tinman* (D). (E,F) Lbe staining shows that the single *tinman*-expressing myocardial cells in each hemisegment are not Lbe positive. (G,H) About three *tinman*-expressing myocardial cells remain in each T3-A1 hemisegment in the mutant (H), but only one in A2-A7. Note that some hemisegments show one TMC cell and one *tinman*-expressing pericardial cell. (I,J) In *stg*$^{-}$ mutants, the overall mesodermal segmentation is significantly affected, in addition to the arrest at cell cycle 14. The *Mef2*-, *tinman*- and *eve*-expressing cells are reduced dramatically. However, the cells that maintain *eve* expression often appear in pairs and co-express *Mef2*, but not *tinman*, indicating that they acquire a myogenic cell fate.

aggravated by the paucity of cells that are formed, which precludes further interpretation of the cardiac lineages.

Function of Numb and Notch in the asymmetric cardiac cell lineages

Previous studies have shown that the asymmetric cell divisions in both the Svp lineage and the Eve lineage are *numb* dependent and involve the Notch pathway (Park et al., 1998; Carmena et al., 1998; Carmena et al., 2002; Ward and Skeath, 2000). In *numb* mutants, the number of myocardial cells in segments A2-A7 is reduced from six to four per hemisegment (Fig. 5A,B; Fig. 9B), whereas the number of myocardial cells in T3-A1 is not affected (data not shown) (Alvarez et al., 2003). The reduction of myocardial cells is paralleled by an increase in EPC number from two to four in 80% of 200 hemisegments counted, accompanied by a loss of DA1 muscles (Fig. 5B; Fig. 9B) (Park et al., 1998). By using a Gal4 driver under the control of the mesodermal *eve* enhancer (eme-Gal4) (Han et al., 2002), Numb and constitutively active N(icd), were expressed exclusively in the mesodermal Eve lineage (Fig. 5C,D). In eme>*numb* embryos, no EPCs are formed, only the DA1 muscles (Fig. 5C). By contrast, no DA1 muscles are formed in eme>*N(icd)* embryos, and 60% of 160 hemisegments exhibit an increase in EPC number from two to three or four (Fig. 5D), supporting a cell autonomous action of Numb and Notch in this lineage. Interestingly, the remaining segments show no expression of *eve*, suggesting the corresponding *eve* progenitors have not formed, as observed previously with pan-mesodermal overexpression of N(icd) (Park et al., 1998). It is likely that the variation in phenotype is the result of slight regional differences in the onset of N(icd) expression: earlier expression eliminates the progenitor, while later expression results in a sibling fate transformation opposite to that observed in *numb* mutants.

Notch pathway functions in the cardiac mesoderm through Su(H)

As Su(H) is the only transcription factor that has been characterized so far in mediating Notch activation, we wanted to know if it has a similar function in the cardiac lineages. Unexpectedly, overexpression of Su(H) in the mesodermal Eve lineage or throughout the mesoderm does not alter the normal cardiac/muscle fate determination (Fig. 5E; data not shown). Recent data suggest that Su(H) is converted from a transcription repressor to an activator when the Notch receptor is activated (reviewed by Bray and Furriols, 2001). Therefore, the mere presence of more wild-type Su(H) protein may not cause activation of the Notch pathway in the mesoderm, as it does in parts of the nervous system (Wang et al., 1997; Nagel et al., 2000). To circumvent this limitation, we expressed an activated form of Su(H), Su(H)vp16, which has been generated by fusing wild-type Su(H) to vp16 (Furriols and Bray, 2000), a strong transcriptional activation domain. Interestingly, eme>Su(H)vp16 embryos exhibit a phenotype similar to that of eme>*N(icd)*: in some segments no Eve cells form, whereas in others more EPCs and no DA1 muscles form (Fig. 5F). These data suggest that the dual function of *Notch*, lateral inhibition and asymmetric cell fate determination, are both

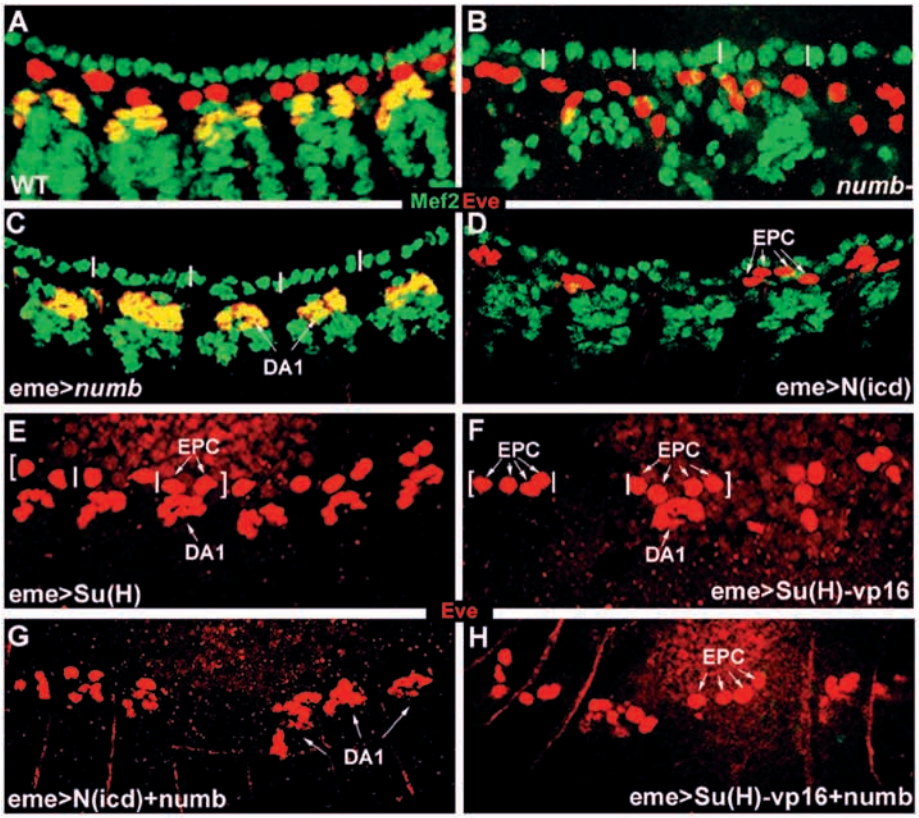

Fig. 5. The Notch pathway functions through activation of Su(H) to specify a pericardial cell fate in asymmetric lineages. A2-A7 hemisegments of stage 13 embryos were labeled with (A-D) Mef2 (green) and Eve (red), or (E-H) Eve only. (A,B) In *numb* mutants, the number of myocardial cells per hemisegment is reduced from six to four; the number of EPC is increased from two to an average of 3.6 per hemisegment; DA1 muscles are not formed. (C) Overexpression of *numb* in the mesodermal *eve* lineage (eme>*numb*) abolishes the formation of all EPCs and sometimes more than one DA1 muscle per hemisegments seems to be generated. (D) Overexpression of the eme>*N(icd)* either generate three or four EPCs per hemisegment, or abolish *eve* expression altogether. No DA1 muscle founders are formed, as judged by the absence of Eve and Mef2 double labeling. (E) Overexpression of eme-*Su(H)* does not seem to alter *eve* expression. (F) By contrast, eme>*Su(H)vp16* exhibits a similar phenotype as eme-*N(icd)*, in that no *eve*-expressing cells or only EPCs form in most segments. Occasionally, four EPCs as well as one forming DA1 muscle are observed, suggesting that these two cell types are specified independently (see text). (G) Overexpression of *numb* together with the *N(icd)* [eme>*N(icd)+numb*] in the mesodermal Eve lineage generates an intermediate phenotype. (H) By contrast, overexpression of *numb* together with Su(H)vp16 [eme>*Su(H)vp16+numb*] generates a phenotype that is indistinguishable from overexpression of *Su(H)vp16* alone [eme>*Su(H)vp16*].

mediated by a mechanism that involves the conversion of Su(H) into a transcriptional activator.

As the function of Numb is thought to interfere with Notch activation in the sibling cell it segregates into (Fig. 9C), we wanted to examine if this activity is at the level of Notch itself or downstream in the pathway at the level of Su(H). We reasoned that if Numb acts at the level of activated Notch, overexpression of *numb* attenuates the *N(icd)* overexpression phenotype, but not that of *Su(H)vp16*. Indeed, it seems that increasing the level of Numb protein by eme-Gal4-mediated expression counteracts the effect of *N(icd)*, in that more DA1 muscles and less EPCs form (Fig. 5G). By contrast, overexpression of *numb* together with *Su(H)vp16* generates a phenotype similar to that of *Su(H)vp16* overexpression alone (Fig. 5H), which suggests that Numb functions upstream of activated Su(H).

The phenotype of eme>Su(H)vp16 embryos is not as strong as with eme>N(icd), in that some DA1 muscles are still formed in some segments. This allowed us to address the question, whether or not DA1 muscle founders are siblings of EPCs, from a new angle: if they derived from the same precursor, DA1 muscle formation would never occur simultaneously with EPC duplication. By contrast, we did occasionally observe four EPCs and one DA1 muscle in the same hemisegment (Fig. 5F), consistent with an independent lineage of EPC and DA1 progenitors (Fig. 1F; Fig. 9C).

emeA-*lacZ* as a marker for sibling cell fates of the mesodermal Eve lineage

A recent study suggests that the sibling cell of the EPC progenitor is the DO2 muscle founder (Carmena et al., 2002). However, muscle founders fuse with surrounding myoblasts and *eve* expression disappears in the DO2 founder after the asymmetric cell division is completed, which makes it difficult to follow cell fate transformations between the proposed siblings. To circumvent this problem, we took advantage of a mesodermal *eve* enhancer, emeA (Han et al., 2002), which labels the EPCs and both the DA1 and DO2 founder nuclei, and founder expression persists even after myotubes begin to form (but, unlike with *eve*, the myoblast nuclei that have fused with the founders are unlabeled; Fig. 6A). In eme>*dap* embryos, both DA1 and DO2 founders form, but the number of EPCs is reduced to half (Fig. 6B), similar to the phenotype observed with *dap* overexpression throughout the mesoderm (Fig. 1D). In the *CycA* mutant, only the two muscle founders are present in each hemisegment (Fig. 6C), suggesting that when the asymmetric divisions of cycle 16 are blocked both progenitors adopt muscle founder cell fate. In *numb* mutants, neither DA1 and nor DO2 founders form; instead, four EPCs are present (Fig. 6D). We note that the occasional formation of a DO2 founder (identified by position and absence of Eve protein) is always accompanied by the presence of two instead of four EPCs (data not shown), consistent with a lineage relationship between DO2 and EPC (Fig. 1F; Fig. 9C). Conversely, in eme>*numb* embryos, two pairs of DA1 and DO2 muscle founders, but no EPCs, are observed in 28% of 120 hemisegments counted (Fig. 6E). In the remaining segments, only one DO2 is observed accompanied always by two EPC with or without a duplicated DA1 founder (data not shown). This suggests again that DO2 rather than DA1 is related by lineage to the EPCs. Finally, N(icd) expression in the mesodermal Eve lineage results in a loss of DO2 and DA1 founders accompanied by the concomitant formation of four EPC in 60% of 120 hemisegments counted (Fig. 6F), or no *eve* or *lacZ* expression in the remaining segments (see also Fig. 5D).

numb directs a myogenic cell fate of undivided progenitors only in asymmetric cardiac lineages

The loss-of-function phenotype of *numb* is opposite to that of *CycA* within the EPC lineage, in that *numb* mutants produce only pericardial EPCs, whereas in *CycA* mutants only myogenic fates are specified (Fig. 6A,C,D; Fig. 7A-C). We wished to determine the epistatic relationship between these genes in the mesodermal Eve lineage by examining *numb*;*CycA* double mutants. In such double mutant embryos, we observe formation of one non-myogenic, *eve*-expressing cell per hemisegment, apparently at the expense of DA1 muscle formation (Fig. 7D, 100% penetrance, n=110). This phenotype is different from a EPC-only *numb* phenotype (Fig. 7B, 100% penetrance, n=100) or a muscle founder-only *CycA* phenotype (Fig. 7C, 100% penetrance, n=96), indicating that *numb* function is required in the asymmetric Eve lineages to specify a myogenic fate regardless whether the progenitor divides or not. Similarly, when *N(icd)* is expressed after the initial specification of mesodermal cardiac progenitors using a heatshock promoter [Hs-*N(icd)*], it can generate a similar cell fate transformation as that of *numb* or a *numb;CycA* mutant (Fig. 7E, 84% penetrance, n=64; Fig. 7F, 90% penetrance, n=42) (see Park et al., 1998). As other evidence suggests that the EPC progenitor is the sibling of the DO2 founder, which undergoes an asymmetric cell division at cycle 16, it is likely that the observed non-myogenic *eve*-expressing cells of

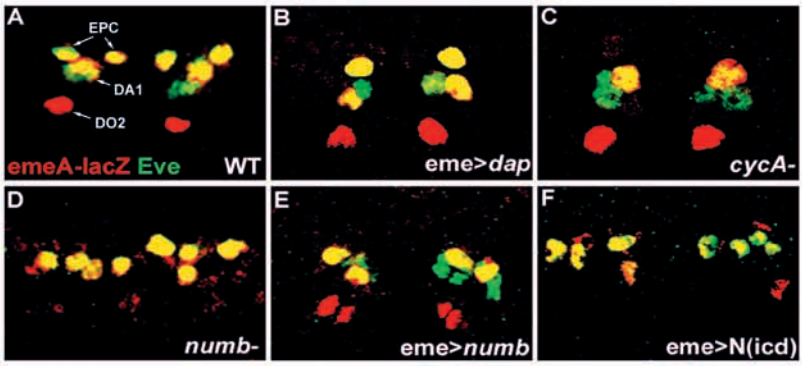

Fig. 6. emeA-*lacZ* marks the mesodermal Eve lineage. (A-F) Double labeling for Eve (green) and β-Gal (marking emeA-*lacZ* expression in red) of two hemisegments in stage 13 embryos. (A) In wild type, emeA-*lacZ* labels the two EPCs, as well as both the DA1 and DO2 muscle founders. (B) In embryos with ectopic *dap* in the mesoderm, the number of EPCs is reduced to one per hemisegment, but the DA1 and DO2 founders are unaffected. (C) In *CycA* mutants, no EPCs are specified but both muscle founder cells are unaffected. (D) In *numb* mutants, the number of EPCs is increased from 2 to average 3.6 per hemisegment with loss of both muscle founders. (E) eme-specific overexpression of *numb* causes a twofold increase in the number of DA1 and DO2 founders concomitantly with a loss of EPCs. (F) eme-specific overexpression of N(icd), as in *numb* mutants, causes a loss of the eve muscle founder and a doubling of EPCs.

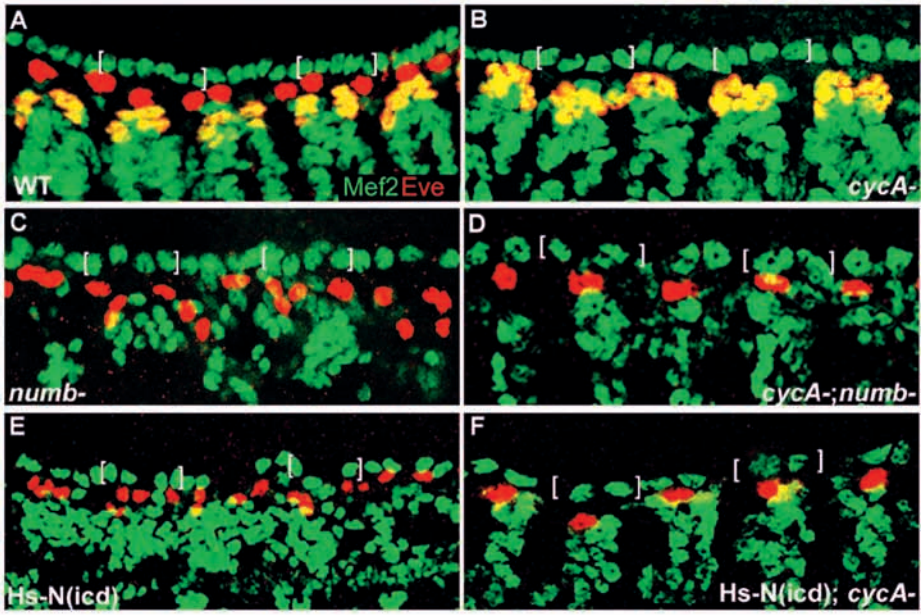

Fig. 7. Progenitors of asymmetric Eve lineage adopt a pericardial EPC cell fate in *numb,CycA* double mutants. (A-F) A2-A7 hemisegments of stage 13 embryos labeled for Mef2 (green) and Eve (red). (A-C) In *CycA* mutants, no EPCs only DA1 muscles, whereas in *numb* mutants, up to four EPCs but no DA1 muscles are present in each hemisegment. Both *CycA* and *numb* single mutants show four instead of six myocardial cells per hemisegment. (D) Double mutants of *numb* and *CycA* show two myocardial cells and a EPC in each hemisegment. (E) Embryos overexpressing *N(icd)* with heat shock promoter (see Park et al., 1998) exhibit twice the normal number of EPCs as do *numb* mutants. (F) Overexpression of *N(icd)* in *CycA* mutant embryos, as in *numb,CycA* double mutants, also produces one EPC and two myocardial cells per hemisegment.

numb;*CycA* or of Hs-*N(icd);CycA* mutants are the undivided EPC/DO2 progenitors, rather than the siblings of the DA1 founders, which apparently no longer express *eve* (see Carmena et al., 2002). This DA1 sibling is likely to die, because eme-*lacZ* expression, unlike in DO2, does not perdure in this DA1 sibling cell (Figs 6, 9).

When myocardial cell lineages were examined, we found that the number of myocardial cells in either *numb* or *CycA* mutants is four per hemisegment in segment A2-A7 (Fig. 7B,C). However, the identities of these four myocardial cells in *numb* mutants are different from those in *CycA* mutants, as indicated by Tinman expression (Fig. 8B,C; see also Fig. 2F and Fig. 9) (Ward and Skeath, 2000): in *CycA* mutants, two out of the four myocardial cells are SMC and the other two are TMC (Fig. 7A; Fig. 8B); whereas in *numb* mutants, all four myocardial cells are TMCs (Fig. 8C). In *numb*;*CycA* double mutant embryos, only two *Mef2*-expressing myocardial cells are specified in each A2-7 hemisegment (Fig. 7D, 94% penetrance, *n*=66), and both of them express *tinman* (Fig. 8D), suggesting the symmetrically dividing TMC progenitors

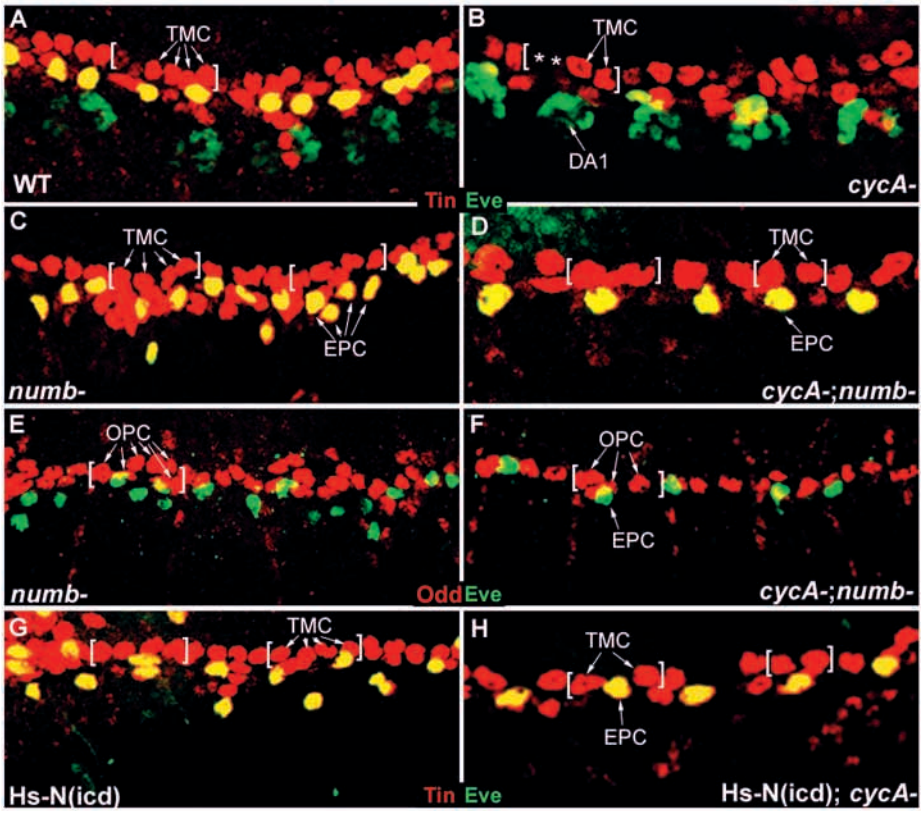

Fig. 8. Progenitors of the Svp lineage adopt a pericardial cell fate in *numb,CycA* double mutants. A2-A7 hemisegments of stage 13 embryos labeled for (A-D,G,H) Tinman (red) and Eve (green), or (E-F) Odd (red) and Eve (green). (A-D) Although both *numb* and *CycA* mutants exhibit four myocardial cells per hemisegment, only two express *tinman* (TMC) in *CycA* mutants (B) (asterisks indicate position of SMC), but in *numb* mutants (C) all four express *tinman* (no SMC form). In *numb,CycA* double mutants (D), two myocardial cells are formed, both expressing *tinman*; thus, no SMCs form. (E) The number of *odd*-expressing pericardial cells (OPC) is increased from four (Fig. 2E) to six in *numb* mutants. (F) In *CycA* mutants, only one OPC is formed (Fig. 2F), in *CycA;numb* double mutants, three OPCs are usually observed. (G) Overexpression of *N(icd)* not only increases the number of EPCs, but also reduces the number of the myocardial cells from six (Fig. 7E) to four, all of which exhibit TMC characteristics. (H) Overexpression of *N(icd)* in *CycA* mutants generates one EPC and the two myocardial cells (seen in Fig. 7F), both of which express *tinman* (TMC).

maintain their myogenic fates in the absence of *numb* function, whereas the asymmetrically dividing SMC progenitors may assume a pericardial, non-myogenic fate. In agreement with this conclusion, the number of *odd*-expressing pericardial cells increases from four to six per A2-7 hemisegment of *numb* mutants (Fig. 8E, average 5.8, *n*=48), but only one is specified in the *CycA* mutant (Fig. 2F, average 1.1, *n*=55; see also Fig. 9B). In *numb*;*CycA* double mutants, the average number of *odd*-expressing cells per hemisegment is three (Fig. 8F, average 3.2, *n*=42), consistent with the two SMC progenitors acquiring a non-myogenic SOPC-type fate in these double mutants (see also Fig. 9B). Similarly, expression of *N(icd)* in *CycA* mutants also generates two TMC per hemisegment (Fig. 7H, 94% penetrance, *n*=86; Fig. 8H, 92% penetrance, *n*=62), instead of four in a otherwise wild-type background (Fig. 8G, 91% penetrance, *n*=46). Taken together, these data suggest that *numb* is only required to inhibit Notch activity in asymmetric lineages that produce both myogenic and non-myogenic cell fates to promote the myogenic pathway of differentiation in the sibling that inherits Numb. By contrast, the presence or absence of Notch activation has no influence on cell fates of the symmetric myogenic-only lineages (summarized in Fig. 9).

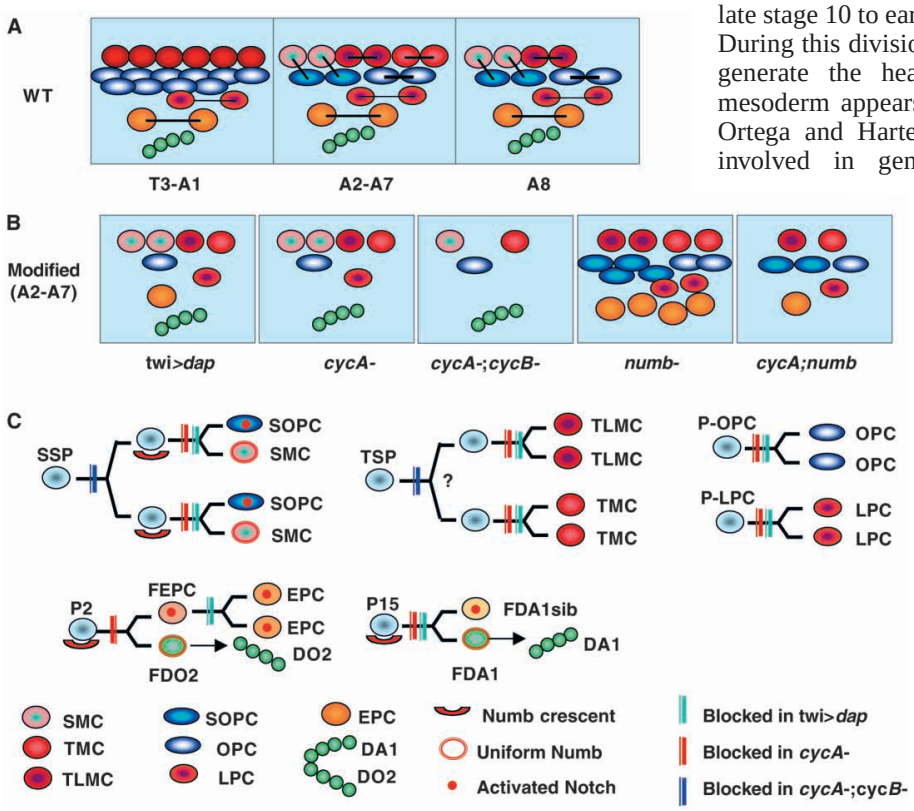

Fig. 9. Model of cardiac lineages, the effect of cell cycle arrest and the function of Numb-Notch in determining cardiac cell fates. (A) The *Drosophila* heart is from T3-A8 segments. Black lines indicate lineage relationships based on this study, Ward and Skeath (Ward and Skeath, 2000), and Alvarez et al. (Alvarez et al., 2003). (B) The effects of cell cycle arrest, *numb* mutants and double mutants of *numb* and *CycA*. (C) Block of cell divisions in A2-A7 abdominal segments are as indicated. Asymmetric segregation of Numb into one daughter cell, or blocking precursor division, promotes myocardial cell fate by inhibition of Notch signaling. By contrast, activation of Notch signaling or the absence of Numb causes the daughter cell, or undivided precursor, to adopt a non-myogenic pericardial cell fate. SMC, Svp myocardial cell; TMC, Tinman myocardial cell; SOPC, Svp-Odd pericardial cell; EPC Eve pericardial cell; TMLC, Tinman-Lbe myocardial cell; LPC, Lbe pericardial cell; OPC, Odd pericardial cell; DA1, dorsal acute muscle; DO2, dorsal oblique muscle 2; DA1sib; SSP, Svp-positive super progenitor; TSP, Tin-positive super progenitor. FEPC, FDO2, FDA1 and FDA1sib are founders of EPCs, DO2, DA1 and DA1sib, respectively. P2 and P15 are progenitors of the above founder cells.

DISCUSSION

Postblastoderm cell divisions in the cardiac mesoderm

All mesodermal cells go through three postblastoderm cell cycles (cycle 14-16) (Campos-Ortega and Hartenstein, 1997). The mesodermal cells enter the first postblastoderm division (mitosis 14) at 210 minutes AED as domain 10 (Foe, 1989). They are the first embryonic cells to go through the second postblastoderm division at about 250 minutes AED. The mesodermal cells are thought to divide in an approximately synchronous fashion in the first two postblastoderm cell divisions. The third division (mitosis 16) takes place during late stage 10 to early stage 11 between 280-300 minutes AED. During this division, a continuous longitudinal zone that may generate the heart precursors and part of the visceral mesoderm appears as a subdomain of mitosis 16 (Campos-Ortega and Hartenstein, 1997). It is likely that *tinman* is involved in generating this subdomain because it is specifically expressed in such a continuous longitudinal zone at stage 10 (Bodmer et al., 1990).

Mesodermal Eve lineages

We show that the asymmetric cell divisions of the Eve lineage that generates the EPC progenitors and DO2 founders are arrested in *CycA* or *Rca1* mutants, in which cell cycle 16 is blocked (Knoblich and Lehner, 1993; Dong et al., 1997), but not in the twi>*dap* embryos, in which the subsequent division of the EPC progenitor is inhibited (cycle 17). Based on the effects of these genes in the ectoderm, it is likely that *CycA* mutant blocks the progression of cell cycle also in the mesoderm, but ectopic *dap* induces early exit from the cell cycle. Therefore, in *CycA* mutants mitosis may be blocked at a certain time point during development (such as at G2/M transition of mitosis 16), but the ectopic *dap* may induce skipping of the last division. It has been shown that the Numb crescent in the precursor P2, which generates the DO2 founder and the EPC progenitor, appears at late stage 10 and the division happens between late stage 10 and early stage 11 (Carmena et al., 1998), consistent with this being mitosis 16 of the mesodermal cells. Therefore, it seems that many cells of the cardiac mesoderm go through three postblastoderm cell divisions (mitosis

14-16), but some of them (such as the EPC lineage) undergo an additional division (mitosis 17).

Two different models have been proposed for the mesodermal Eve lineages. One model suggested that each EPC share a progenitor with a muscle founder (Park et al., 1998). The other model suggested that the two EPCs per hemisegment share a progenitor, which in turn share a progenitor with one of the muscle founder cells (DO2), whereas the other DA1 muscle founder derives from the second progenitor (Carmena et al., 1998). The data presented in this paper strongly support the latter model (depicted in Fig. 9). The most direct evidence derives from pan-mesodermal *dap* overexpression, which results in the formation of a single EPC, probably because of a block the last division. By contrast, the first model predicts formation of either no EPC or two EPCs, clearly not what is observed. These conclusions are also supported by recent lineage tracing experiments (A. Alvarez and J. B. Skeath, unpublished).

Myocardial lineages along the anterior-posterior axis

Recent studies have suggested that cardiac specification along the anterior-posterior axis is under the control of homeotic genes (Lovato et al., 2002; Ponzielli et al., 2002; Lo et al., 2002) (A. Alvarez and J. B. Skeath, unpublished). For example, Svp myocardial cells are only present in the abdominal segments of the heart, but not in the thoracic segments (Gajewski et al., 2000; Lo et al., 2002); probably under the control of *Antennapedia* which is active in these segments (A. Alvarez and J.B. Skeath, unpublished). In this study we found that in addition to cell identity differences between the anterior two and the posterior heart segments, the lineage of the T3-A1 myocardial cells are also distinct. In cycle 16 blocking *CycA* or *Rca1* mutants, the last cycle of myocardial divisions in T3-A1 is not arrested unlike it is the case for the posterior myocardial cells. The anterior myocardial progenitors may either undergo the last division during cycle 15 or they may be less susceptible to a loss-of-*CycA*-function. The first possibility is consistent with the observation that embryos with overexpression of last division inhibitor *dap*, but not cycle 16 arrested *CycA* mutants, exhibit a reduction of TMCs in T3-A1. Alternatively, it is possible that all T3-A1 myocardial lineages are asymmetric, as the Svp lineages more posterior, except they all express *tinman*, due to the lack of *svp* in these two segments (Lo et al., 2002). Thus, blocking division in *CycA* mutants would not alter the number of TMC in these two segments. Recent lineage tracing data are consistent with this view (A. Alvarez and J. Skeath, unpublished), but further experiments are needed to elucidate these lineages.

Extension of cardiac lineages

The second postblastoderm cell division of the mesodermal cells (mitosis 15) seems to be arrested if both *CycA* and *CycB* functions are lost. In *CycA*;*CycB* double mutants, only two of the normally six myocardial cells are formed in A2-A7 segments, one exhibiting TMC and the other SMC characteristics. Therefore, we propose that in each hemisegment two myocardial superprogenitors are specified (Fig. 9): the TMC superprogenitor (TSP) divides twice symmetrically, whereas the SMC superprogenitor (SSP) first divides symmetrically and then asymmetrically. Present and previous studies suggest there are probably five progenitors in each hemisegment that give rise to 14 heart-associated cells (six myocardial and eight pericardial): the TSP gives rise to four myocardial cells (two of which are TLMC), the SSP generates two myocardial and two SOPC, the EPC progenitor to two EPCs; the remaining four pericardial cells, two OPC and two LPC, probably derive from two symmetrically dividing precursors, although their lineage is not as well understood.

Asymmetric cell division and cell cycle progression

Asymmetric divisions have previously been studied in the context of cell cycle progression in the *Drosophila* PNS and CNS (Vervoort et al., 1997; Wai et al., 1999; Tio et al., 2001). In the PNS, Notch activity is required for specification of a type I versus type II neuronal fate. When sensory organ progenitor cell division is blocked in *stg*$^-$ mutants, the undivided precursor adopts a type II neuronal fate, whereas in *numb*;*stg* double mutants, a type I fate is chosen (Vervoort et al., 1997). In the CNS, Notch is required for specification of the sib cell fate versus the RP2 cell fate of the GMC1 asymmetric cell division. In *Rca1* mutants, the undivided GMC1 adopts a RP2 fate, whereas in *numb*;*Rca1* double mutants, the undivided GMC1 often adopts the sib cell fate (Buescher et al., 1998; Wai et al., 1999; Lear et al., 1999). Both experimental outcomes are analogous to what we observe in *CycA* mutants: the undivided P2 progenitor adopts a pericardial fate in the absence of *numb* function instead of a myogenic fate in a wild-type background. Taken together, these observations suggest that arrest of an asymmetric cell division leads the undivided progenitor to adopt the fate of the daughter cell that inherits Numb, and in the absence of Numb the alternative fate is chosen.

Notch activity promotes autonomously a non-myogenic fate in asymmetric lineages of the cardiac mesoderm irrespective of cell division

Previous studies suggested that Notch activity controls two distinct processes during the specification of cardiac cell fates (Park et al., 1998). First, it is required to single initial progenitors out of a field of competence by supporting the selection and inhibiting surrounding cells from adopting the same fate (Culi and Modolell, 1998). Subsequent to the progenitor specification, Notch is required again for the specification of alternative cell fates of sibling cells produced during asymmetric cell divisions (reviewed by Jan and Jan, 1998). In this study, we have examined the cell autonomy of Notch, by using eme-Gal4 to drive activated forms of Notch and Su(H) exclusively in the mesodermal Eve lineages. We also used conditional ubiquitous expression of activated Notch to examine its lineage-specific function in other cardiac lineages. We find that Notch is required for specification of a non-myogenic fate in both the Eve and the Svp lineages of the cardiac mesoderm. By contrast, activation or inhibition of the Notch pathway did not affect cell fate decisions within the symmetric lineages. This suggests a mechanism by which cell type diversity may be increased during evolution by co-opting the Notch pathway during cell division to distinguish between alternative fates of the daughter cells. The inability of activated Su(H) to autonomously influence cell fates in symmetric cardiac lineages further suggests that other factors or activities, not present in symmetric lineages, are crucial for the asymmetric lineage-specific functions of Notch and Su(H).

Interestingly, this influence of the Notch pathway on cell fate decision in asymmetric cardiac Eve and Svp lineages is not altered when cell division is arrested. Thus, cell division is not essential to distinguish between alternative cell fates. The data also suggest that the default cell fate of a asymmetrically dividing cardiac precursor in *Drosophila* is determined to assume a myogenic fate, owing to Numb-mediated inhibition of Notch, unless that fate is switched by the activation of target genes downstream of Su(H). Moreover, in a double mutant of *Notch* and *numb* we would expect to observe the same lineage phenotype as of *Notch* alone, i.e. a myogenic cell fate, as the primary role of Numb is to inhibit Notch signaling (see also Spana and Doe, 1996). Unfortunately, analysis of such double mutants is complicated by the earlier role of Notch in lateral inhibition.

Another unresolved issue is the source of the Notch ligand that activates signal transduction within asymmetric cardiac lineages. If the myogenic cell were to produce the ligand for Notch activation in its pericardial sibling, then the undivided progenitor would have to secrete its own Notch ligand. This is unlikely, as production of the ligand is usually inhibited within the cell that experiences Notch signaling (see Culi and Modellel, 1998). In the asymmetric MP2 lineage of the *Drosophila* CNS, for example, ligand production appears to be required in cells outside the MP2 lineage (Spana and Doe, 1996). A similar scenario may be operating in the asymmetric cardiac lineages.

Numb acts at the level of Notch in preventing Su(H) activation

Within the Eve lineages, Notch activation is mimicked by Su(H) fused to the VP16, a potent transcriptional activation domain. Recent studies suggest that in the absence of Notch activity, DNA-bound Su(H) prevents activators from promoting transcription. When Notch ligands, such as Delta, bind to its receptor, Notch is cleaved to produce an intracellular domain fragment, N(icd), which is thought to enter the nucleus and interact directly with Su(H) to recruit transcriptional co-activators and alleviate Hairless-mediated repression, thus promoting transcription (for a review, see Bray and Furriols, 2001; Barolo and Posakony, 2002). In support of this model, we find that Su(H) overexpression can mimic Notch activation only when linked directly to a transcriptional activator, but not in its wild-type form when it presumably associates with co-repressors, such as Hairless and Groucho (Barolo et al., 2002), that prevent Su(H)-dependent transcriptional activation in the absence of Notch signaling.

The role of the PTB-containing, membrane-associated protein Numb in preventing Notch activation in the nervous system is well established (for a review, see Kopan and Turner, 1996; Jan and Jan, 1998; Jafar-Nejad et al., 2002). To explore at which level Notch signaling is inhibited by Numb in the cardiac lineages, we overexpressed *numb* simultaneously with *N(icd)* or *Su(H)vp16* within the mesodermal Eve lineages. Excess Numb was able to counteract activated Notch but not activated Su(H) function, suggesting that Numb can inhibit Notch activity after it has been cleaved, possibly by preventing its nuclear translocation, but is unlikely to prevent the transcriptional activator function of Su(H) directly. Recent data suggest that Numb is involved in stimulating endocytosis of Notch, thus removing it from the cell surface and inhibiting its function (Berdnik et al., 2002). It is not clear, however, if this inhibition by endocytosis is at the level of the entire Notch receptor, or (also) at the level of N(icd) after it is cleaved off. Our experiments provide strong evidence that Numb can indeed interfere with N(icd) function, but it remains to be determined if endocytosis is an obligatory intermediate in this inhibition of activated Notch.

Notch activity may specify a pericardial cell fate in both *Drosophila* and vertebrates by similar mechanisms

A recent study in *Xenopus* suggests that the Notch pathway may also have a role in vertebrates in specifying pericardial and other non-myogenic cell fates within the dorsolateral cardiogenic region of the anterolateral plate mesoderm (Rones et al., 2000). As in the Eve and Svp lineage of the *Drosophila* heart, activation of the Notch pathway decreased myocardial gene expression and increased expression of a pericardial marker, whereas inhibition of Notch signaling resulted in an increase of cardiac myogenesis. Similar results were obtained with an activated form of RBP-J [a vertebrate homolog of *Drosophila* Su(H) fused to vp16, as in our study] (Rones et al., 2000). These data indicate that the Notch pathway may play a role in the specification of myocardial versus pericardial cell fates in both *Drosophila* and vertebrates. This raises the question of whether the mechanism of Notch mediated cell identity determination is also conserved between vertebrates and flies. Because it is not yet known if (Numb-controlled) asymmetric cell divisions are also involved in vertebrate heart development, the answer awaits future studies. However, recent studies on the role of Numb during cortical development suggest that it is likely to have a similar control function in cell fate specification in vertebrates as it does in flies (Shen et al., 2002).

We thank C. Lehner for helpful suggestions and discussions; the Bloomington stock center and C. Lehner for sending *Drosophila* stocks; J. Skeath for sharing data prior to publication; and C. Lehner, M. Mercola and J. Skeath for comments on the manuscript in preparation. This work was supported by an American Heart Association Predoctoral Fellowship to Z.H. and by grants from the NHLBI of NIH to R.B.

REFERENCES

Alvares, A. D., Shi, W. S., Wilson, B. A. and Skeath, J. B. (2003). Pannier and pointedP2 act sequentially to regulate Drosophila dorsal vessel development. *Development* **130**, IN THE SAME ISSUE

Azpiazu, N. and Frasch, M. (1993). *tinman* and *bagpipe*: two homeo box genes that determine cell fates in the dorsal mesoderm of *Drosophila*. *Genes Dev*. **7**, 1325-1340.

Barolo, S. and Posakony, J. W. (2002). Three habits of highly effective signaling pathways: principles of transcriptional control by developmental cell signaling. *Genes Dev.* **16**, 1167-1181.

Barolo, S., Stone, T., Bang, A. G. and Posakony, J. W. (2002). Default repression and Notch signaling: Hairless acts as an adaptor to recruit the corepressors Groucho and dCtBP to Suppressor of Hairless. *Genes Dev*. **16**, 1964-1976.

Berdnik, D., Torok, T., Gonzalez-Gaitan, M. and Knoblich, J. A. (2002). The endocytic protein alpha-Adaptin is required for numb-mediated asymmetric cell division in Drosophila. *Dev. Cell* **3**, 221-231.

Bodmer, R., Carretto, R. and Jan, Y. N. (1989). Neurogenesis of the peripheral nervous system in Drosophila embryos: DNA replication patterns and cell lineages. *Neuron* **3**, 21-32.

Bodmer, R., Jan, L. Y. and Jan, Y. N. (1990). A new homeobox-containing gene, *msh-2* (*tinman*), is transiently expressed early during mesoderm formation in *Drosophila*. *Development* **110**, 661-669.

Bodmer, R. (1993). The gene *tinman* is required for specification of the heart and visceral muscles in *Drosophila*. *Development* **118**, 719-729.

Bour, B. A., O'Brien, M. A., Lockwood, W. L., Goldstein, E. S., Bodmer, R., Taghert, P. H., Abmayr, S. M. and Nguyen, H. T. (1995). *Drosophila mef2*, a transcription factor that is essential for myogenesis. *Genes Dev*. **9**, 730-741.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Bray, S. and Furriols, M. (2001). Notch pathway: making sense of suppressor of hairless. *Curr. Biol.* **11**, 217-221.

Buescher, M., Yeo, S. L., Udolph, G., Zavortink, M., Yang, X., Tear, G. and Chia, W. (1998). Binary sibling neuronal cell fate decisions in the Drosophila embryonic central nervous system are nonstochastic and require inscuteable-mediated asymmetry of ganglion mother cells. *Genes Dev.* **12**, 1858-1870.

Campos-Ortega, J. A. and Hartenstein, V. (1997). *The Embryonic Development of* Drosophila melanogaster. 2nd edn, pp. 287-308. Berlin: Spinger-Verlag.

Carmena, A., Murugasu-Oei, B., Menon, D., Jimenez, F. and Chia, W. (1998). Inscuteable and numb mediate asymmetric muscle progenitor cell divisions during Drosophila myogenesis. *Genes Dev.* **12**, 304-315.

Carmena, A., Buff, E., Halfon, M. S., Gisselbrecht, S., Jimenez, F., Baylies, M. K. and Michelson, A. M. (2002). Reciprocal regulatory interactions between the Notch and Ras signaling pathways in the *Drosophila* embryonic mesoderm. *Dev. Biol.* **244**, 226-242.

Culi, J. and Modolell, J. (1998). Proneural gene self-stimulation in neural precursors: an essential mechanism for sense organ development that is regulated by Notch signaling. *Genes Dev.* **12**, 2036-2047.

Dong, X., Zavitz, K. H., Thomas, B. J., Lin, M., Campbell, S. and Zipursky, S. L. (1997). Control of G1in the developing *Drosophila* eye: rca1 regulates Cyclin A. *Genes Dev*. **11**, 94-105.

Edgar, B. A. and O'Farrell, P. H. (1989). Genetic control of cell division patterns in the Drosophila embryo. *Cell* **57**, 177-187.

Edgar, B. A. and O'Farrell, P. H. (1990). The three post blastoderm cell cycles of Drosophila embryogenesis are regulated in G2 by string. *Cell* **62**, 469-480.

Foe, V. E. (1989). Mitotic domains reveal early commitment of cells in *Drosophila* embryos. *Development* **107**, 1-22.

Furriols, M. and Bray, S. (2000). Dissecting the mechanisms of suppressor of hairless function. *Dev. Biol*. **227**, 520-532.

Frasch, M. (1995). Induction of visceral and cardiac mesoderm by ectodermal Dpp in the early *Drosophila* embryo. *Nature* **374**, 464-467.

Frasch, M., Hoey, T., Rushlow, C., Doyle, H. and Levine, M. (1987). Characterization and localization of the even-skipped protein of *Drosophila*. *EMBO J*. **6**, 749-759.

Gajewski, K., Choi, C. Y., Kim, Y. and Schulz, R. A. (2000). Genetically distinct cardial cells within the *Drosophila* heart. *Genesis* **28**, 36-43.

Greig, S. and Akam, M. (1993). Homeotic genes autonomously specify one aspect of pattern in the *Drosophila* mesoderm. *Nature* **362**, 630-632.

Halfon, M. S., Carmena, A., Gisselbrecht, S., Sackerson, C. M., Jimenez, F., Baylies, M. K. and Michelson, A. M. (2000). Ras pathway specificity is determined by the integration of multiple signal-activated and tissue-restricted transcription factors. *Cell* **103**, 63-74.

Han, Z., Fujioka, M., Su, M., Liu, M., Jaynes, J. B. and Bodmer, R. (2002). Transcriptional integration of competence modulated by mutual repression generates cell-type specificity within the cardiogenic mesoderm. *Dev Biol*. **252**, 225-240.

Jafar-Nejad, H., Norga, K. and Bellen, H. (2002). Numb: 'adapting' notch for endocytosis. *Dev. Cell* **3**, 155-156.

Jagla, K., Frasch, M., Jagla, T., Dretzen, G., Bellard, F. and Bellard, M. (1997). *ladybird*, a new component of the cardiogenic pathway in *Drosophila* required for diversification of heart precursors. *Development* **124**, 3471-3479.

Jagla, T., Bidet, Y., da Ponte, J. P., Dastugue, B. and Jagla, J. (2002). Cross-repression interactions of identity genes are essential for proper specification of cardiac and muscular fates in *Drosophila*. *Development* **129**, 1037-1047.

Jan, Y. N. and Jan, L. Y. (1998). Asymmetric cell division. *Nature* **392**, 775-778.

Knoblich, J. A. and Lehner, C. F. (1993). Synergistic action of Drosophila cyclins A and B during the G2-M transition. *EMBO J*. **12**, 65-74.

Kopan, R. and Turner, D. L. (1996). The Notch pathway: democracy and aristocracy in the selection of cell fate. *Curr. Opin. Neurobiol*. **6**, 594-601.

Lane, M. E., Sauer, K., Wallace, K., Jan, Y. N., Lehner, C. F. and Vaessin, H. (1996). Dacapo, a cyclin-dependent kinase inhibitor, stops cell proliferation during Drosophila development. *Cell* **87**, 1225-1235.

Lear, B. C., Skeath, J. B. and Patel, N. H. (1999). Neural cell fate in rca1 and cycA mutants: the roles of intrinsic and extrinsic factors in asymmetric division in the Drosophila central nervous system. *Mech. Dev*. **88**, 207-219.

Lilly, B., Zhao, B., Ranganayakulu, G., Paterson, B. M., Schulz, R. A. and Olson, E. N. (1995). Requirement of MADS domain transcription factor D-MEF2 for muscle formation in Drosophila. *Science* **267**, 688-693.

Lo, P. C., Skeath, J. B., Gajewski, K., Schulz, R. A. and Frasch, M. (2002). Homeotic genes autonomously specify the anteroposterior subdivision of the Drosophila dorsal vessel into aorta and heart. *Dev. Biol*. **251**, 307-319.

Lockwood, W. K. and Bodmer, R. (2002). The patterns of *wingless*, *decapentaplegic*, and *tinman* position the Drosophila heart. *Mech. Dev.* **114**, 13-26.

Lovato, T. L., Nguyen, T. P., Molina, M. R. and Cripps, R. M. (2002). The Hox gene abdominal-A specifies heart cell fate in the Drosophila dorsal vessel. *Development* **129**, 5019-5027.

Lu, B., Jan, L. and Jan, Y. N. (2000). Control of cell divisions in the nervous system: symmetry and asymmetry. *Annu. Rev. Neurosci.* **23**, 531-556.

Nagel, A. C., Maier, D. and Preiss, A. (2000). Su(H)-independent activity of hairless during mechano-sensory organ formation in Drosophila. *Mech. Dev.* **94**, 3-12.

Park, M., Yaich, L. E. and Bodmer, R. (1998). Mesodermal cell fate decisions in *Drosophila* are under the control of the lineage genes *numb*, *Notch*, and *sanpodo*. *Mech. Dev*. **75**, 117-126.

Ponzielli, R., Astier, M., Chartier, A., Gallet, A., Therond, P. and Semeriva, M. (2002). Heart tube patterning in Drosophila requires integration of axial and segmental information provided by the Bithorax Complex genes and hedgehog signaling. *Development* **129**, 4509-4521.

Rones, M. S., McLaughlin, K. A., Raffin, M. and Mercola, M. (2000). Serrate and Notch specify cell fates in the heart field by suppressing cardiomyogenesis. *Development* **127**, 3865-3876.

Shen, Q., Zhong, W., Jan, Y. N. and Temple, S. (2002). Asymmetric Numb distribution is critical for asymmetric cell division of mouse cerebral cortical stem cells and neuroblasts. *Development* **129**, 4843-4853.

Sigrist, S. J. and Lehner, C. F. (1997). Drosophila fizzy-related down-regulates mitotic cyclins and is required for cell proliferation arrest and entry into endocycles. *Cell* **90**, 671-681.

Spana, E. and Doe, C. Q. (1996). Numb antagonises Notch signaling to specify sibling neuron cell fate. *Neuron* **17**, 21-26.

Su, M. T., Fujioka, M., Goto, T. and Bodmer, R. (1999). The *Drosophila* homeobox genes *zfh-1* and *even-skipped* are required for cardiac-specific differentiation of a numb-dependent lineage decision. *Development* **126**, 3241-3251.

Tio, M., Udolph, G., Yang, X. and Chia, W. (2001). cdc2 links the Drosophila cell cycle and asymmetric division machineries. *Nature* **409**, 1063-1067.

Venkatesh, T. V., Park, M., Ocorr, K., Nemaceck, J., Golden, K., Wemple, M. and Bodmer, R. (2000). Cardiac enhancer activity of the homeobox gene tinman depends on CREB consensus binding sites in Drosophila. *Genesis* **26**, 55-66.

Vervoot, M., Merritt, D., Ghysen, A. and Dambly-Chaudiere, C. (1997) Genetic basis of the formation and identity of type I and type II neurons in *Drosophila* embryos. *Development*. **124**, 2819-2828.

Wai, P., Truong, B. and Bhat, K. M. (1999). Cell division genes promote asymmetric interaction between Numb and Notch in the Drosophila CNS. *Development* **126**, 2759-2770.

Wang, S., Younger-Shepherd, S., Jan, L. Y. and Jan, Y. N. (1997). Only a subset of the binary cell fate decisions mediated by Numb/Notch signaling in *Drosophila* sensory organ lineage requires Suppressor of Hairless. *Development* **124**, 4435-4446.

Ward, E. J. and Skeath, J. B. (2000). Characterization of a novel subset of cardiac cells and their progenitors in the *Drosophila* embryo. *Development* **127**, 4959-4969.

Wu, X., Golden, K. and Bodmer, R. (1995). Heart development in *Drosophila* requires the segment polarity gene wingless. *Dev. Biol*. **169**, 619–628.

Yaich, L. E., Ooj, J., Park, M., Landry, C., Bodmer, R. and Margolis, B. (1998). Functional analysis of the Numb phosphotyrosine-binding domain using site-directed mutagenesis. *J. Biol. Chem*. ***273***, 10381-10388.