Excessive Dpp signaling induces cardial apoptosis through dTAK1 and dJNK during late embryogenesis of *Drosophila*

Sheng-An Yang and Ming-Tsan Su*

**Abstract**

**Background:** To identify genes involved in the heart development of *Drosophila*, we found that embryos lacking raw function exhibited cardial phenotypes. *raw* was initially identified as a dorsal open group gene. The dorsal open phenotype was demonstrated to be resulted from the aberrant expression of *decapentaplegic (dpp)*, a member of the tumor growth factor beta (TGF-β), signaling pathway. Despite the role of *dpp* in patterning cardioblasts during early embryogenesis of *Drosophila* have been demonstrated, how mutation in *raw* and/or excessive *dpp* signaling involves in the differentiating heart of *Drosophila* has not been fully elaborated at late stages.

**Results:** We show that *raw* mutation produced a mild overspecification of cardial cells at stage 14, but these overproduced cells were mostly eliminated in late mutant embryos due to apoptosis. Aberrant *dpp* signaling is likely to contribute to the cardial phenotype found in *raw* mutants, because expression of *dpp* or constitutively activated *thickven (tkv)*, the type I receptor of Dpp, induced a *raw*-like phenotype. Additionally, we show that *dpp* induced non-autonomous apoptosis through TGFβ activated kinase 1 (*TAK1*), because mis-expression of a dominant negative form of *Drosophila TAK1* (*dTAK1DN*) was able to suppress cell death in *raw* mutants or embryos overexpressing *dpp*. Importantly, we demonstrated that *dpp* induce its own expression through *dTAK1*, which also leads to the hyperactivation of *Drosophila JNK (DJNK)*. The hyperactivated DJNK was attributed to be the cause of Dpp/DTAK1-induced apoptosis because overexpression of a dominant negative DJNK, *basket (bsk)*, suppressed cell death induced by Dpp or DTAK1. Moreover, targeted overexpression of the anti-apoptotic P35 protein, or a dominant negative proapoptotic P33 (*P53DN*) protein blocked Dpp/DTAK1-induced apoptosis, and rescued heart cells under the *raw* mutation background.

**Conclusions:** We find that ectopic Dpp led to DJNK-dependent cardial apoptosis through the non-canonical TGF-β pathway during late embryogenesis of *Drosophila*. This certainly will increase our understanding of the pathogenesis of cardiomyopathy, because haemodynamic overload can up-regulate TGF-β and death of cardiomyocytes is observed in virtually every myocardial disease. Thus, our study may provide possible medical intervention for human cardiomyopathy.

**Background**

The *Drosophila* heart is a simple tubular organ located at the dorsal midline beneath the epidermis, and it is therefore alternatively termed the dorsal vessel. The fly heart consists of two major cell types, myocardial cells and pericardial cells, which arise from two bilateral rows of cardiac primordia at the leading edge of the migrating mesoderm. The contractile myocardial cells which form the lumen are arranged in a segmental repeat comprised of six cells per hemisegment in the mature embryonic heart. The pericardial cells, which are essential for normal cardiac function, are aligned alongside the myocardial cells. Despite its simple structure, fly heart has recently emerged as an excellent model system for dissecting the complex pathway that determines cardiogenic cell fate, and for investigating the physiologic function of the adult heart [1,2].

* Correspondence: mtsu@ntnu.edu.tw
Department of Life Science, National Taiwan Normal University, Taipei 11677, Taiwan

© 2011 Yang and Su; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
Extensive study has revealed that a combinatory action of extrinsic signaling and intrinsic transcription network is required for correct specification of cardial precursors and differentiation of mature heart (reviewed in [3]). Of all external signalings, Dpp, a member of the mammalian Transforming growth factor superfamily (TGF-β), has been shown to play a pivotal role during cardiogenesis of Drosophila [4]. The cardiogenic function of Dpp begins when it is expressed in the dorsal epidermis in a broad band along the anterior-posterior axis during germ band extension in Drosophila [5]. This spatiotemporal pattern of Dpp specifies the underling dorsal mesodermal cell fate by maintaining the expression of the transcription factor, tinman (tin) [4,6-8]. Dpp also regulates the expression of several other cardiogenic transcription factors, including pannier (pnr) and dorsocross (doc) [9,10]. For further specification of the cardiogenic mesoderm, Wg signaling together with the combinatorial action of several transcription factors, including tin, pnr, doc and tailup, are required [11-20].

Around stage 10, Dpp expression in the dorsal ectoderm vanishes briefly, but reappears in the leading edge (LE) cells of the dorsal ectoderm at stage 11. This second round of Dpp expression in LE cells persists through stage 17 [21]. Interestingly, pMad, the activated Dpp signal transducer, can be detected in a subset of cardial progenitors in stages 12 to 14 [22]. This indicates that a second round of Dpp activity is required for further differentiation of Drosophila heart. Indeed, dpp mutants with alleles that affect the expression of Dpp in LE cells have impaired embryonic heart development and larval cardiac function [23,24]. These findings indicate a biphasic requirement for Dpp during cardiogenesis of Drosophila, in which it is required early for dorsal mesoderm patterning and later for differentiating heart cells.

Dpp regulates many developmental processes, including cell fate determination, alteration of cell shape, proliferation, and apoptosis. Morphogenic function of Dpp in cell fate determination has been shown to be mediated through the canonical pathway, in which it interacts with a type I receptor, Tkv, and a type II receptor, Punt. Upon formation of ligand-receptor complex, activated Punt phosphorylates Mad, which subsequently interacts with Medea. The resultant complex containing pMad, and Medea is then translocated into the nucleus where it activates transcription of Dpp target genes [25]. Other than the canonical pathway, it has been found that mammalian Dpp homolog, TGF-β transduce its signaling that is independent of Smad, a homolog of Drosophila pMad. The Smad-independent pathway is designated as the non-canonical TGF-β pathway. In the non-canonical pathway, TGF-β activated kinase 1 (TAK1) forms a multiple protein complex protein complex with TRAF6, TAB2, and TAB3. Upon binding of TGF-β to its receptor, TRAF6 exerts its E3 ubiquitin ligase activity together with ubiquitin-conjugating enzymes to catalyze Lys63-linked polyubiquitination. Subsequently, the Lys63-linked polyubiquitin chain associates with TAB2 which leads to autophosphorylation and activation of TAK1 [26,27]. Despite it is less clear in Drosophila, many functionally-conserved non-canonical signaling transducers, including DTAK1 and TAB2, have been identified in Drosophila [28,29]. Moreover, Dpp signaling has also been shown to control the viability of cells. A lack of Dpp signaling activates c-Jun N-terminal kinase (JNK)-dependent apoptosis in wing discs [30]. Interestingly, Dpp is also likely to function as a pro-apoptotic signal because increased Dpp activity leads to both non-autonomous JNK activation and cell death [31]. However, the link between Dpp signaling and JNK-mediated apoptosis is currently unclear in Drosophila.

Dpp is a downstream target of the JNK pathway, a conserved and pleiotropic signaling system whose function governs many different biological activities, including morphogenesis, differentiation, proliferation and apoptosis. Components of the DJNK pathway, including Djun and Raw, have been shown to participate in Drosophila heart development by modulating the expression of Dpp [14,16]. In Djun mutant embryos, the expression of dpp is not maintained at dorsal edge, which leads to down-regulation of cardiac tin at later stages [16]. By contrast, pericardial cells are overspecified in raw loss-of-function mutant embryos [14]. The excessive differentiation of cardial cells has been attributed to the ectopic Dpp activity induced by dysregulated DJNK signaling.

In our effort to identify genes involved in the heart development of Drosophila, we observed that embryos lacking raw function exhibited cardial phenotypes, in which heart cells were overspecified in moderately degree during mid-embryogenesis, and that the overproduced heart cells had disappeared at late stages. We show here that the elimination of heart cells in late raw mutant embryos was a result of excessive apoptosis, and ectopic Dpp signaling was responsible for the cardial apoptosis phenotypes of raw mutant embryos. We also found that elevated Dpp can function as a pro-apoptotic signal to promote non-autonomous apoptosis in a dose-dependent manner. Importantly, ectopic Dpp auto-regulate its own expression through DTAK1. The autocrine Dpp further enhanced the expression of DJNK and consequently led to P53-dependent cell death. Our study defined a novel pathway which linked ectopic Dpp signaling and DJNK-dependent apoptosis during late cardiogenesis of Drosophila.

**Methods**

**Fly stocks and genetics**

Fly stocks were raised and crossed at 25°C. Gal4 drivers: Act5C-gal4 (constitutive), 24B-gal4 (mesodermal), and
mediated germ-line transformation procedure using
firmed by restriction enzyme digestion. Flies carrying
The orientation of the inverted DNA fragment was con-
[34].

CAGCA-3

transcriptase (Invitrogen). PCR was performed using
and
rps17
primers (5'-TACCATAAGCACC-GCAG-3' and 5'-ATGGCAACTGGCAGAGGATC-3') and
AGAAG-3' and 5'-CC TGCAACTTGATGGAGA-
TACC-3'). PCR condition: at 94°C for 2 min, 30 cycles
(at 94°C for 30 sec, at 46°C for 30 sec, at 72°C for 1
min), and at 72°C for 7 min.

Cell death detection
Apoptosis was detected by acridine orange (AO) stain-
ing or terminal deoxynucleotidyl transferase-mediated
dUTP nick end labeling (TUNEL). For AO staining, the
same protocol was followed as used elsewhere [40].
Briefly, dechorionated embryos were stained by placing
in an equal volume of 1-heptane and 1 × PBS contain-
ing 5 mg/ml of acridine orange (Sigma) for 5 minutes
on a shaking platform. For TUNEL analysis, an in situ
cell death kit was used according to the manufacturer's
instructions (Roche Applied Science). To detect cardiac
apoptosis in embryos, a TUNEL and immunofluores-
cence double-labeling protocol was followed as
described [41]. Stained embryos were mounted either
with mineral oil (Sigma) or series 700 Halocarbon oil
(Halocarbon Products, Hackensack, NJ). Samples were
viewed either with a fluorescence or TCS SP2 confocal
microscope (Leica Microsystems).

Plasmid construction and generation of transgenic flies
The UAS/Gal4 binary expression system was used to drive
the expression of transgenic constructs [37]. For genera-
tion of the UAS-raw transgenic construct, the full-length
raw cDNA was amplified using pNB3301 (obtained from A.
Letso) as template with a pair of primers (5'-CAC-
CATGAAAACATGAGAGCAGACT-3' and 5'-CGAGC
GGTCGCGGTTGTT-3') by PCR. The amplified DNA
fragment was first cloned into a pENTR/D-TOPO vector
and subsequently into a pTWF vector (The
Drosophila Genomics Resource Center). The plasmid con-
struct was confirmed by sequencing before germ-line
transformation. The raw-RNA interference construct, UAS-
raw-RNAi, was generated by amplification of a 693
bp DNA fragment using the following primers, 5'-
GCTCTAGACCTGGAGCGCCAGAGTCTC-3' and 5'-
GCTCTAGATGACGAAGAGCAACACTCG-3'. The
amplified DNA fragment was digested with XbaI and
cloned into the AvrII site of a pWIZ vector. The correct
clone was used to clone the same XbaI-digested DNA
fragment into the Nhel site, as described elsewhere [38].
The orientation of the inverted DNA fragment was con-
firmed by restriction enzyme digestion. Flies carrying
transgenic constructs were generated by P-element-
mediated germ-line transformation procedure using w^{1118}
as the parental line [39].

RT-PCR
Total RNA was purified from embryos carrying raw-
RNAi transgene driven by Act5C-gal4 driver using Tri-
Zol (Invitrogen). 10 μg of total RNA was reverse tran-
scribed using oligo(dT) primers and SuperScript reverse
transcriptase (Invitrogen). PCR was performed using
raw specific primers (5'-TACCATAAGCACC-
CAGCA-3' and 5'-ATGGCAACTGGCAGAGGATC-3') and
rps17 primers (5'-CGAACAAAGACCGGTGA

Immunohisocytochemistry, X-gal staining and cuticle
preparation
Immunohistochemical staining was performed as
described [42]. Antibodies and dilutions were as follows:
Anti-Eve, anti-Wg 4D4 and anti-EC11 (1:10) obtained
from the Developmental Studies Hybridoma Bank, anti-
Tin (1:2000, provided by M. Frasch), anti-LacZ, anti-
GFP (1:200, Molecular Probes), and anti-pMad (1:20,
Cell Signaling) [43]. Appropriate anti-mouse or anti-rab-
bit HRP-conjugated secondary antibodies were used at a
dilution of 1:200 (Jackson ImmunoResearch). The stain-
ing pattern was visualized using the Vectastain ABC kit
(Vector Laboratories). X-gal staining was performed as
described previously with slight modifications [44].
Briefly, collected embryos were dechorionated in 50%
bleach for 90 s and fixed in 4% formaldehyde buffered
with 1× PBS for 20 min. Fixed embryos were washed
briefly and incubated with X-gal staining solution (10
mM sodium phosphate, pH 7.2, 150 mM NaCl, 1 mM
MgCl2, 3 mM K3[Fe(CN) 6], 3 mM K4[Fe(CN) 6], 0.3%
Triton X-100, 0.2% X-gal) at 25°C. Immuno- and X-gal
stained embryos were mounted in 50% glycerol. For pre-
paration of cuticle, embryos were fixed in glycerol-acetic
acid (1:4) and cleared in Hoyer’s medium overnight at
60°C [45]. Stained embryos and cuticle were visualized
with a light microscope (Leica DMR A2).

Image acquisition and processing
Epifluorescence images were acquired using a digital
camera (CoolSnap 5.0, Photometrics) steered by the
Northern Eclipse 6.0 software (EMPIX Imaging, Mississauga, Ontario, Canada). When necessary, Z-series of optical or fluorescent images were acquired at 2 µm increments with a piezo-electric motor (LVDTr, Physik Instruments). The Helicon Focus program was applied to combine the focused images (Helicon Soft Ltd. Kharkov, Ukraine). All the figures were arranged in Adobe Illustrator CS3 (Adobe Co.).

Results
Loss of raw function impairs heart and muscle development
In our effort to identify genes involved in the heart development of Drosophila, we found that many cardiac cell types were missing in raw mutant embryos. For instance, using anti-Eve antibodies we found that Even-skipped positive pericardial cells (EPCs) were aligned normally along the dorsal vessel in wild-type embryos (Figure 1A). However, these EPCs almost completely disappeared in the raw mutant at stage 16 (Figure 1D). Similarly, absence of pericardial cells was observed in raw mutant using anti-EC11 and anti-Odd antibodies, which labelled extracellular matrix and Odd-skipped pericardial cells (OPCs), respectively, of pericardial cells (Figure 1B, E and Additional file 1, Fig. S1). Additionally, using a heart-specific enhancer trap line, E2-3-9, we found that sup-expressing myocardial cells were reduced and/or missing under the raw mutant background (Figure 1C vs. 1F, arrow). To further investigate whether raw is involved in the early cardiogenesis of Drosophila, we used Tin as a marker because it is expressed initially in all cardiac progenitors and later in four of six cardioblasts per hemisegment as well as a subset of pericardial cells in the mature embryonic heart (Figure 1H and 1I; see also [46-49]). We found that the expression of Tin in the dorsal mesoderm was normal in both wild-type and raw mutant embryos at stage 12 (Figure 1G and 1J). However, Tin-expressing heart cells were mildly over-produced in raw mutants at stage 14 (Figure 1K, braces). Nevertheless, these overproduced heart cells were reduced and/or absent in raw mutants during late embryogenesis (Figure 1L). Since the above markers label most of the cardiac and pericardial cell types, our results suggest that the raw mutation affects all the cardiac cell types in developing Drosophila heart. Based on the above observations, we thus concluded that heart cells were over-specified mindedly during mid-embryogenesis, and that these overproduced heart cells disappeared at late stages in raw mutant embryos.

To further confirm our findings, we used a him-GFP reporter in which a cardiac-specific enhancer of him was placed upstream of nuclear GFP [33]. The GFP reporter was expressed in the precursors of both muscle and heart cells at stage 12 and its cardiac expression persisted till late embryogenesis in the differentiated heart cells under the control of tin, while its muscle expression was greatly reduced at stage 14 (Figure 1M and 1N; and M-T. Su unpublished results). We found that the expression of him-GFP was increased in both heart and musculature at stage 14 under the raw mutation background (Figure 1P). Consistent with the above data, cardiac expression of him-GFP decreased significantly in mutant embryos at stage 16 (Figure 1O and 1Q). Taken together, these data show that heart precursors are overspecified during mid-embryogenesis, but are missing in late mutant embryos.

Down-regulation of raw causes cardiac apoptosis
How could the cardiac cells disappear in the raw mutant embryos at later stages? One possibility is that loss of raw function induces programmed cell death (PCD). To investigate whether cardiac apoptosis occurred in raw mutant embryos, we stained embryos with a vital dye, acridine orange (AO), which provides a rapid visual assessment of apoptosis in live Drosophila [40]. Epi-fluorescence micrography showed apoptotic cells, mainly in the cephalic ganglia and in head regions in wild-type embryos at stage 14 (Figure 2A). A similar AO staining pattern was detected in raw‘ mutant embryos at the same stage (Figure 2D). Excessive cell death in the dorsal mesoderm was observed in raw‘ mutant embryos at stage 16 (Figure 2B vs. 2E). To verify the above findings, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was applied. Indeed, TUNEL positive nuclei were identified in the dorsal mesoderm of raw‘ mutant embryos at stage 16 (Figure 2C vs. 2F). We then made use of RNA interference to specifically knock down the expression of raw (Figure 2G and 2H), and the results were consistent with the above findings, founding that silencing the expression of endogenous raw in ectoderm using 69B-gal4 successfully induced localized apoptosis in the dorsal mesoderm of embryos (Figure 2H).

Since the embryonic heart of Drosophila is located in the dorsal mesoderm, which was also the region where cell death was present in the raw mutants, we suspected that PCD is the cause of eliminating cardiac cell types in the raw mutant. To test this, we double-labeled raw mutant embryos with TUNEL and heart-specific him-GFP reporter (Figure 2I-L). By confocal microscopy analysis, the results showed that many him-GFP expressing cells were co-labeled with TUNEL, indicating that mesodermally-derived tissues did undergo apoptosis (Figure 2I, yellow nuclei surrounded with green cytoplasm). Since the expression of him-GFP was mainly in cardiac and muscle cells under raw mutation background at late stages (Figure 1Q), these results indicate that raw mutation does induce death of heart cells. Apart from the
Figure 1 Cardial phenotypes of raw mutant embryos. (A, B, C, G, I, M, N) Wild-type (WT) embryos; (D, E, F, J, K, L, P, Q) raw1 mutant embryos. (A) EPC was visualized with anti-Eve antibodies at stage 16. (D) EPC was completely absent in raw1 mutant embryos. (B) The EC11 antibody was used to label pericardial cells at stage 16. (E) Pericardial cells were abolished in raw1 mutant embryos. (C) E2-3-9 enhancer trap line shows the presence of 2 svp-specific cardioblasts per hemisegment in WT embryos (arrows). (F) Most svp-expressing cardioblasts were absent in raw mutants. (G) The expression of Tin in dorsal mesoderm was revealed using anti-Tin antibodies in WT embryos at stage 12. (J) The expression of Tin was not affected in raw1 mutant embryos at similar stages. (H) Tin was expressed in heart cells at stage 14 in wild type embryos. (K) Overproduction of heart precursors was observed in raw1 mutant embryos at stage 14 (braces). (L) Tin was confined in matured myocardial cells at stage 16 in the wild type (L). The number of myocardial cells was greatly reduced in raw1 mutants during late embryogenesis (brackets). (M) Cardial and muscle cells were monitored by the expression of him-GFP reporter at stage 14. (P) The number of him-GFP expressing cardial precursors increased dramatically (braces). Note that the enhancer activity of him was also enhanced in musculature of raw1 mutants at stage 14. (N) The expression of him-GFP was confined to all cardial and pericardial cells, while its expression in muscle cells was reduced greatly at stage 16. (Q) The him-GFP expressing heart cells were dramatically reduced (brackets). Note that the expression of him-GFP in muscle persisted in raw1 mutants during late embryogenesis. (O) Number of heart cells in wild-type (gray bar) and raw1 mutant (black bar) embryos at stage 14 and 16 was revealed by Tin immunoreactivity and expression of him-GFP reporter. Data were expressed as the mean ± SD and were analyzed by Student’s t-test (n = 15). * indicates p < 0.01; ** indicates p < 0.001. st, stage.
him-GFP-expressing cardial and muscle cells, we noticed few unidentified TUNEL-positive cells (Figure 2I, red, arrowheads). Since heart is the major defective tissue in the mesoderm of raw mutant, we have focused our attention on how loss of raw function leads to cardial apoptosis in Drosophila during late embryogenesis.

Function of raw is required in ectoderm
Previous study showed that mutation of raw resulted in dorsal open phenotype [35]. Ventral denticle belts are also missing in embryos homozygous for the null raw allele (Figure 3B, see also [50]). As demonstrated above, lack of raw function leads to apoptosis of dorsal mesodermal tissues, including heart. These findings suggest that raw is a pleiotropic gene which is required for the normal development of multiple tissues in Drosophila. To determine the spatial requirement of raw, we performed rescue experiments by expressing raw using 69B- or 24B-gal4 driver, which direct the expression of UAS-raw in ectoderm or mesoderm respectively (69B>raw or 24B>raw). Consistent with a previous study, we found that the cuticular phenotypes of raw mutant could be rescued by targeted expression of Raw protein under the control of 69B-gal4 (Figure 3A, C, E, see also [50]). In fact, 55% of the raw mutant flies survived to adulthood after rescue of the cuticular phenotype (data not shown). By contrast, expression of the UAS-raw transgene driven by 24B-gal4 failed to restore the dorsal open or the loss of denticle belt phenotypes (Figure 3D and 3E). Conversely, we were able to replicate the cuticular phenotype by knocking down endogenous raw using a 69B-gal4 driver (Figure 3F). However, no cuticle defect was observed when the raw-RNAi was driven with the pan-mesoderm driver 24B-gal4 (Figure 3G).

Rescue experiments were also conducted to determine whether raw functions cell-autonomously for the survival of wild-type wild-type; st, stage.
Ectodermal raw is sufficient for the normal development of Drosophila (A-D, F, G). Cuticular phenotype. (A) Wild type (WT). (B) raw1. (C) UAS-raw; raw1/69B-gal4; raw1. (D) UAS-raw; raw1/24B-gal4; raw1. (E) Bar chart showing that ectodermally, but not mesodermally, expressed raw suppressed dorsal open phenotype in raw mutants. Statistical analysis of the percentage of embryos with dorsal open phenotype in the indicated genetic background. Number of scored embryos for each genotype: raw1 (n = 544); 69B>raw; raw1 (n = 414); 24B>raw; raw1 (n = 324). Chi-square test, ** indicates p < 0.001. (F) UAS-raw/69B-gal4. (G) UAS-raw-RNAi/24B-gal4. (H-K) Apoptosis phenotype revealed by AO staining. Ectodermally (H), but not mesodermally (I), expressed raw blocked cardial apoptosis (brackets) in raw mutants. raw-RNAi transgene driven by 69B-gal4 (J), but not 24B-gal4 (K), caused cardial apoptosis (brackets).
of mesodermally-derived tissues. We found that epidermal expression of raw suppressed cardial apoptosis under raw mutant background (Figure 3H). However, mesodermally-overexpressed raw was unable to completely inhibit cardial apoptosis in the raw mutant (Figure 3I, brackets). Additionally, transgenic raw-RNAi construct driven by 69B-gal4, but not 24B-gal4, produced a cardial apoptosis phenotype (Figures 2H, 3J vs. Figure 3K, brackets). These results strongly suggest that ectodermal Raw is sufficient for the proper development of Drosophila.

Raw affects Dpp and Wg signalings

Having established that ectodermal Raw functions in a cell non-autonomous manner to affect the viability of dorsal mesodermal cells, we speculated that ectodermally-secreted factors might be responsible for the cardial apoptosis observed in the raw mutant. In this regard, Wg and Dpp are good candidates because they are essential for patterning dorsal mesodermally-derived tissues, including cardial progenitor cells. Previous study has shown that Dpp is ectopically expressed at the dorsal epidermis of raw mutant embryos [35]. Using a dpp-LacZ reporter, we confirm that Dpp signaling is ectopically activated in raw mutant embryos at stage 14 (Figure 4A, B vs. Figure 4C, D). To further determine if Dpp signaling is altered in raw mutants, we conducted immunocytochemistry experiments using a monoclonal antibody against pMad [43], a Dpp-activated Smad protein. pMad immunoreactivity was detected as a broad band in the dorsal ectoderm of both wild-type and raw mutant embryos at stage 13 (Figure 4E and 4G). The broad band expression pattern disappeared in wild-type embryos but not in the dorsal ectoderm of raw mutants at stage 14 (Figure 4F vs. 4H, brackets). Moreover, optical sectioning through the stained embryo showed the same broad band expression pattern of pMad in mutant but not in wild-type embryos (Figure 4I and 4K, brackets). At late stages, pMad was not detected in the dorsal ectoderm of either mutant or wild-type embryos (Figure 4J and 4L).

Immunostaining was also used to investigate if Wg signaling is affected by the raw mutation. We found that Wg was expressed in transverse striped domains of the ectoderm along the anteroposterior axis in wild type embryos at stages 13 (Additional File 2, Fig. S1). The expression pattern of Wg was not altered in raw mutants by stage 13. However, the expression level of Wg had significantly decreased in raw mutants at stage 14 (Additional File 2, Fig. S2). At stage 16, residual Wg was detected in the ectoderm of wild-type embryos, but was completely absent in raw mutant embryos (Additional File 2, Fig. S2).

Ectopic Dpp signaling promotes cell death

To investigate if down-regulation of Wg signaling causes cardial apoptosis, we conducted a temperature shift experiment using a temperature-sensitive wg^{L114} allele. Inactivation of Wg at 9-15 hr, the time window during which Wg expression is missing in raw mutant embryos, did not cause the cardial apoptosis seen in raw mutant embryos, and overexpression of wg did not suppress the apoptosis phenotype under raw mutation background (Additional File 2, Fig. S2). We thus concluded that defective Wg signaling is not the cause of cardial apoptosis.

We then turned our attention to the question of whether ectopic Dpp signaling can induce cardial apoptosis, and found that overexpression of dpp, using 69B-gal4 (69B>dpp), phenocopied the raw mutant phenotypes (Figure 5A-F). For instance, him-GFP expressing heart cells were overproduced at stage 14, but they had disappeared in 69B>dpp embryos at stage 16 (Figure 5A-B and Figure 5D-E). Naked cuticle and dorsal open phenotypes were also observed in 69B>dpp embryos (Figure 5C). However, unlike raw mutant embryos in which only him-GFP expressing cardioblasts were lost, both heart and muscle cells were completely abolished in 69B>dpp embryos at stage 16 (Figure 1Q and 5E). This loss of him-GFP-expressing myoblasts and cardioblasts in 69B>dpp embryos is very likely to have resulted from apoptosis, because extensive cell death was detected throughout the entire 69B>dpp embryos, whereas dead cells were located mainly in the dorsal mesoderm in raw mutants (Figure 2E and 5F). The difference in the apoptosis phenotype between raw mutant and 69B>dpp embryos might reflect the fact that the ectopic dpp was mainly found in the dorsal ectoderm of raw mutant whereas dpp was expressed in the entire ectoderm under the control of 69B-gal4. These findings reinforce our hypothesis that Dpp activity is correlated with cardial apoptosis.

Dpp is a secretory protein which can be transduced to the underlying mesoderm. If the apoptotic phenotype is associated with the ectopic Dpp activity, it would expect that mesodermal overexpression of Dpp should replicate the apoptosis phenotypes seen in 69B>dpp embryos. Indeed, mis-expression of dpp in mesoderm using a pan mesodermal driver, 24B-gal4, induced excessive cell death accompanied with elimination of him-GFP-expressing cells during late developmental stages (Additional File 3, Fig. S3). These results suggest that excessive dpp in ectoderm or mesoderm can cause apoptosis and removal of cardial cells.

Dpp transduces its signaling by binding to a heteromeric type I/type II transmembrane serine/threonine kinase receptor complex, encoded by tkv and punt. To further demonstrate that ectopic Dpp signaling is the cause of apoptosis in raw mutants, we targeted overexpression of a constitutively-active form of tkv, tkv^{CA}, using a 24B-gal4 driver (24B>tkv^{CA}). We found that
Figure 4 Dpp signalings was affected in raw mutants. (A, C) The expression of dpp-LacZ was normal in both wild-type and raw mutants at stage 13. (B, D) At stage 14, dpp-LacZ was expressed in the leading edge of wild-type embryos whereas dpp-LacZ was ectopically expressed in dorsal ectoderm of raw mutant embryos. (E, G) At stage 13, pMad was detected at the dorsal ectoderm in both wild-type (WT) and raw mutants. (F, H) Expression of pMad in epidermis was reduced in the wild-type embryos but was greatly increased in raw mutant embryos at stage 14 (brackets). (I, K) Optical section through the stained embryos revealed that the broad-band expression pattern of pMad was persisted in the dorsal mesoderm of raw mutant embryos but not in wild-type embryos. (J, L) At stage 16 pMad was detected in developing midgut and hindgut in wild-type embryos. Its expression in raw mutant embryos was mostly abolished. WT, wild-type; st, stage.
Figure 5 Ectopic Dpp signaling induces apoptosis. (A) him-GFP reporter is expressed in muscle and heart precursors in control 69B-gal4 drivers at stage 14. (B) The expression of him-GFP reporter was confined to cardiac and pericardial cells at stage 16. (C-F) Overexpression of dpp in ectoderm using 69B-gal4 driver mimicked the raw mutant phenotype. (C) Ectodermal expression of dpp resulted in naked cuticle with dorsal open phenotype. (D) Cardiac him-GFP was overexpressed in embryos overexpressing dpp at stage 14 (brackets). (E) Cardial and muscular him-GFP was lost in embryos overexpressing dpp at stage 16. (F) Ectopic dpp induced excessive cell death. (G) Overexpression of tkvCA using 24B-gal4 increased the expression of him-GFP reporter at stage 14. (H) The expression of him-GFP was abolished in embryos overexpressing tkvCA at stage 16. (I) Normal developmental PCD revealed by AO staining in control 24B-gal4 driver. (J) AO-positive cells were moderately increased in tkv7 mutant embryos. (K) RNAi silenced tkv under the control of 24B-gal4 driver induced apoptosis. (L) Mesodermal overexpression of tkvCA caused excessive cell death. (M) Scattered AO-positive cells were present in tkv7, raw1 double mutant embryos. Note that the mutant embryos did not exhibit cardiac apoptosis, but showed a dorsal open phenotype. (N) Mesodermal knockdown of the expression of tkv suppressed cardiac apoptosis in raw mutant. (O-U) Ectopic dpp-induced apoptosis was dose-dependent. (P-R) Control Act5C-gal4 driver showed normal AO staining pattern at different temperatures: (P, S) 18°C; (Q, T) 25°C; (R, U) 30°C. (S-U) Constitutive overexpression of dpp induced apoptosis. (O) Dead cells were quantified and expressed as the mean ± SD values. Comparison of dead cells among three groups was assessed by one-way analysis of variance (ANOVA) followed by Dunnett’s post-hoc test (n = 15). * indicates p < 0.05; ** indicates p < 0.001. WT, wild-type; st, stage.
24B>tkvCA embryos exhibited the same phenotype as 69B>dpp or 24B> dpp embryos, in which him-GFP-expressing cardial cells were increased in 24B> tkvCA embryos at stage 14 (Figure 5G), while him-GFP-expressing heart and muscle cells were eliminated at stage 16 (Figure 5H). Extensive cell death was also detected throughout 24B> tkvCA embryos (Figure 5L).

In a complementary experiment, we depleted the expression of tkv by RNAi using a transgenic fly carrying inverted repeats corresponding to tkv, under the control of a UAS sequence inducible by 24B-gal4 (24B>tkv-RNAi, Additional file 4, Fig. S4). Down-regulation of mesodermal tkv induced a moderate degree of apoptosis in which the AO-positive cells were randomly distributed in the entire 24B-tkv-RNAi embryos (Figure 5I vs. 5K). A similar pattern of scattered cell death was also detected in loss-of-function of tkv mutant embryos (Figure 5L). These data suggest that Dpp is required for cell survival during embryogenesis in Drosophila. To test whether inhibiting Tkv activity can block excessive Dpp-induced apoptosis, we compared the AO staining pattern in raw, tkv and raw;tkv double mutant embryos. Both tkv and raw;tkv double mutant embryos exhibited the same scattered AO staining pattern, unlike the cardiac apoptosis phenotype observed in raw mutant (Figure 5E vs. 5J and 5M). Moreover, mesodermal knockdown of the expression of tkv was able to suppress cardiac apoptosis under the raw mutant background (Figure 5N). These results indicate that ectopic Dpp induced apoptosis is transduced through Tkv.

As above-mentioned, ectopic tkv expression promoted apoptosis in 24B>tkvCA embryos, indicating that Dpp can function as a pro-apoptotic signaling. To directly test this hypothesis, Dpp was constitutively overexpressed under the control of the ubiquitous Act5C-gal4 driver (Act5C>dpp). In control Act5C-gal4 embryos, AO staining revealed that apoptotic cells were mainly present in the ventral nerve cord and head regions at stage 16 (Figure 5P-R). Raising the temperature slightly increased the amount of cell death in the central nervous system of late Act5C-gal4 embryos (Figure 5O-R). Constitutive overexpression of Dpp caused a remarkable degree of apoptosis in Act5C>dpp embryos (Figure 5S-U). Notably, increasing the dpp expression level by raising the temperature significantly increased the number of dying cells (Figure 5O-U). This result suggests that Dpp can function as a pro-apoptotic signaling in a dose-dependent manner.

Dpp induced apoptosis is mediated through DTAK1

As shown above, reducing Tkv activity successfully blocked dpp-mediated cardial apoptosis in raw mutants (Figure 5M and 5N). This data encouraged us to further examine whether overexpression of brinker (brk), a transcriptional repressor of Dpp, could inhibit the cardial apoptosis phenotype in raw mutants. Overexpression of brk using 24B-gal4 driver (24B>brk) caused a moderate degree of apoptosis (Figure 6A). The AO staining pattern in 24B>brk embryos was similar to the pattern in tkv and 24B-tkv-RNAi embryos (Figures 6A, 5, and 5K), suggesting that ectopic brk-induced apoptosis was likely to be a result of the inhibition of Dpp signaling. Unexpectedly, mis-expression of brk in mesoderm failed to suppress the cardial apoptosis phenotype of the raw mutant (Figure 6B, brackets), suggesting that the suppressor activity of Brk may not be strong enough to block Dpp-mediated cardial apoptosis. Nevertheless, it is equally possible that Dpp-induced apoptosis is mediated through a distinct pathway which can not be repressed by Brk.

A previous study showed that TGF-β activated kinase 1 (TAK1), a member of the JNKK kinase superfamily that activates the JNK cascade, can transduce TGF-β signaling and induce apoptosis in vertebrates [51]. To determine if TAK1 induces apoptosis in response to Dpp signaling, a Drosophila TAK1 homolog, DTAK1, was mis-expressed using either Act5C- or 24B-gal4. Ectopic DTAK1 activity was sufficient to induce apoptosis in embryos (Figure 6C and 6D). In contrast, ectodermal or mesodermal overexpression of a dominant negative form of dTAK1 (dTAK1DN) was capable of suppressing developmental programmed cell death (Figure 6E and 6F). Constitutive overexpression of dTAK1DN also effectively inhibited ectopic Dpp-induced apoptosis (Figure 6G). Moreover, mesodermally overexpressed dTAK1DN suppressed cardial apoptosis under the raw mutation background (Figure 6H). Taken together, our results clearly demonstrate that DTAK1 is a downstream effector of Dpp-mediated cardial apoptosis.

DTAK1 induces JNK dependent apoptosis

Although our data suggest that dTAK1 acts downstream of dpp to promote apoptosis, it is interesting to note that dTAK1 stimulates dpp expression, suggesting that dpp is a downstream target of dTAK1 [29]. Despite the discrepancy in these epistatic relationships, these observations strongly suggest that dTAK1 and dpp act in the same genetic pathway. Using a dpp-LacZ reporter, we found that dTAK1 activated the transcription of dpp to the same degree as basket (bsk), the Drosophila Jun amino-terminal kinase (DJNK) homolog encoded gene which activates the expression of dpp in the dorsal-most epidermal cells (Figure 7A-C). These results indicate that dTAK1 acts upstream of dpp. Since dpp is also activated by bsk, these results suggest that dTAK1 activates bsk and thereby dpp. Moreover, we found that overexpression of dTAK1 using 24B-gal4 driver stimulated the expression of pMad in the entire mesoderm.
(Figure 7D vs. Figure 7E), indicating that the ectopic
Dpp induced by DTAK1 can transduce its own signal-
ing through Tkv and result in the expression of ectopic
pMad. As Dpp can transduce its signaling through
DTAK1 to promote apoptosis (Figure 6G), the ectopic
Dpp induced by DTAK1 is likely to autoregulate itself
through DJNK. In other words, a positive autoregulatory
loop for Dpp expression is formed when DTAK1 is
activated.

The JNK cascade is a well-known pro-apoptotic sig-
naling that participates in stress-related apoptosis in
Drosophila. Together with the fact that TAK1 can acti-
vate the JNK cascade, the autocrine Dpp is expected to
enhance the activity of DTAK1 and thereby hyperactivi-
tates the DJNK pathway. In other words, the ectopic
dpp-induced cell death could be a consequence of
hyperactivated JNK signaling. To test this hypothesis,
bsk was overexpressed in the entire embryo or
specifically in mesoderm using either Act5C- or 24B-gal4 driver (Act5C>bsk; 24B>bsk). Ectopic bsk activity dramatically increased levels of apoptosis in the embryos (Figure 7F and 7G). Conversely, overexpression of a dominant negative bsk, bskDN, again using either Act5C- or 24B-gal4 driver, was able to suppress ectopic Dpp/DTAK1-induced apoptosis (Figure 7H and 7I). These results demonstrate that Dpp exerts its pro-apoptotic functions through DTAK1 as well as through DJNK.

Overexpression of anti-apoptotic P35 or dominant negative P53 blocks Dpp induced apoptosis
As demonstrated above the missing of cardial cells in raw mutants is likely to be a consequence of excessive dpp induced apoptosis. If this is the case, we expect that blocking cell death would reverse the cardial apoptosis phenotype in raw mutant embryos. The baculovirus P35 protein has been shown to suppress normal and induced apoptosis by inhibiting caspases in animals [36]. We found that overexpression of the anti-apoptotic P35 could prevent apoptotic cell death induced by either ectopic Dpp or DTAK1 (Figure 8A and 8C). P35 also significantly reduced the death of heart cells under the raw mutation background (Figure 8E). Moreover, it has been shown that aberrant JNK-induced apoptosis is mediated through P53 (reviewed in [52]). To further demonstrate if Dpp/DTAK1-induced apoptosis is P53-dependent, a dominant negative form of p53 (p53DN) was ectopically expressed using either Act5C- or 24B-gal4 driver (Act5C>p53DN or 24B> p53DN). We found that overexpression of p53DN suppressed Dpp- or DTAK1-induced apoptosis successfully (Figure 8B and

Figure 7 DTAK1 activates DJNK pathway. (A) dpp-LacZ showed a typical expression pattern in dorsal and lateral spots along the anterior-posterior axis in control 24B-gal4 driver embryos. (B) Mesodermal bsk induced the expression of dpp-LacZ. (C) dpp-LacZ reporter was ectopically expressed in 24B>dTAK1 embryos. (D) pMad was not ectopically expressed in control 24B-gal4 driver. (E) Targeted overexpression of DTAK1 in mesoderm induced ectopic pMad. (F, G) Excessive apoptosis was induced by the expression of bsk using Act5C- or 24B-gal4 drivers. (H) Expression of bskDN inhibited ectopic dpp-induced apoptosis. (I) Mesoderml expression of bskDN inhibited DTAK1-induced cell death.
suggesting that Dpp/DTAK1-induced apoptosis is mediated through P53. We also showed that mesodermal overexpression of P53DN significantly reduced the death of heart cells in raw mutant embryos (Figure 8F). Consistent with the above observation, immunostaining experiments revealed that mesodermally-overexpressed P53DN was able to rescue Tin-positive cardiac cells in raw mutant embryos (Figure 8G and 8H). In fact, superfluous Tin-positive cells were usually observed when cardiac apoptosis was blocked by ectopic P53DN expression (Figure 1I vs. Figure 8H, arrow), suggesting that the overspecified cardiac cells during mid-embryogenesis in raw mutants survived when apoptosis was blocked.

These results also indicate that the Dpp-DTAK1-DJNK-mediated apoptosis pathway is likely to be P53-dependent.

**Discussion**

**Function of Raw in DJNK signaling pathway**

Analysis of the amino acid sequence of Raw revealed that it does not comprise any specific functional domain or motifs. Despite the fact that the C-terminus of Raw protein is rich in glutamine residues, a characteristic feature of some transcription factors, Raw protein was mainly detected in cytoplasm, suggesting that it may not play a role in transcriptional regulation. The most
prominent feature of Raw protein is that it contains two duplicated domains with a 32 amino acid core repeat (designated as Raw repeats) that is highly conserved in invertebrates [35]. The structure of Raw is currently unavailable, so no further information regarding the function of Raw can be deduced through sequence analysis.

Epistasis analysis has demonstrated that raw negatively regulates jra activity in parallel to DJNK signaling in the epidermis of Drosophila [35-50]. As demonstrated above, Dpp-induced apoptosis is mediated through activation of DJNK signaling (Figure 7H). If raw can negatively regulate DJNK, it would be expected that forced expression of raw would inhibit the DJNK cascade and reverse the apoptotic phenotype in the raw mutant. Ectodermal overexpression of raw did rescue the cardial apoptosis in raw mutants (Figure 3H). Nevertheless, targeted expression of raw in the mesoderm was unable to prevent cell death under the raw mutation background (Figure 3I). Why is the effect of mesodermal Raw so different from that of ectodermal Raw? As demonstrated above, Dpp can trigger its own expression in the mesoderm through DTAK1 and DJNK (Figure 7A-E). This auto-regulated Dpp is expected to further enhance DJNK activity, which may out-compete the suppressive activity of Raw. This might explain why mesodermal expression of raw was unable to suppress raw mutation-induced apoptosis. However, if the initial Dpp signaling in ectoderm is suppressed then it will not initiate the autocrinal Dpp signaling in mesoderm and result in cell death. Consistent with our notion, we found that ectodermally-overexpressed raw suppressed the expression of pMad in raw mutants. In contrast, targeted expression of raw in mesoderm did not inhibit the expression of pMad under the raw mutation background (Additional File 5, Fig. S5).

**Dpp functions as survival and pro-apoptotic signals**

The morphogenetic function of Dpp patterns cell fates across the developing field by forming a gradient which provides position identity for the receiving cells. Similarly, it seems that Dpp controls the viability of cells in the same concentration-dependent manner. Mutant cells deprived of Dpp signaling are lost from wing disc epithelium due to DJNK activation and apoptosis [30,53]. These observations have suggested that Dpp functions as a survival factor by preventing activation of the DJNK-dependent apoptotic pathway. Additionally, down-regulation of mad activated JNK and caspase-3, indicating that Dpp functions as a survival factor mediated through Mad [54]. Consistent with these findings, we found that down-regulation of tkv or overexpression of brk induced moderate cell death in embryos (Figure 5I, K and 6A). Nevertheless, Dpp seems to act as a double-edged sword because increased Dpp signaling induces DJNK-mediated apoptosis in the proximal wing [30]. Similarly, in this study, we show that ectopic dpp or tkvCA expression promotes DJNK-mediated apoptosis in embryos (Figure 5F and 5L). Moreover, the apoptotic propensity of Dpp is proportional to its own expression level (Figure 5O and 5S-U). Taken together, these results suggest that Dpp can act as both survival and death signals and thus, an appropriate expression level of Dpp is indispensable for the survival of cells during development.

**TAK1 is a key transducer that mediates ectopic Dpp induced apoptosis**

TAK1 is also a member of the MAPKKK family, which was originally identified as a mediator of TGF-β signaling pathway [55]. It has also shown to regulate a great variety of cellular processes through activating many downstream kinase cascades, including I-kappa B kinase complex (IKK), p38 MAPK, JNK, and AMP-activated protein kinase (AMPK) (reviewed in [56]). Unlike the canonical pathway in which members of TGF-β family elicit phosphorylation of Smad proteins, activation of TAK1 was found to function in a receptor kinase-independent manner [26]. The existence of the non-canonical pathway may explain why mesodermal overexpression of brk was unable to block cardial apoptosis in raw mutants (Figure 6B), because it seems that brk only suppresses Dpp target genes containing Mad consensus binding sites [57]. Interestingly, mesodermal pMad was increased in raw mutant or in embryos overexpressing dTAK1 (Figure 4K, and 7E), indicating that both canonical and non-canonical pathways were activated simultaneously in response to ectopic Dpp signaling. The fact that apoptotic cell numbers increased dramatically in raw mutants or in embryos overexpressing dTAK1 (Figure 2E, F, 6C and 6D) suggests that the non-canonical pathway suppresses the canonical pathway when cells are exposed to excessive Dpp levels. Together with the fact that the apoptotic propensity of Dpp is dose-dependent (Figure 5O), these data imply that higher levels of Dpp elicit stronger DTAK1 activity and result in apoptosis. In support of this argument, homozygous raw mutant animals had significant apoptosis in the dorsal-most tissues, such as the heart, where Dpp activity is at its peak (Figure 2E and 2F), and global overexpression of Dpp signaling led to ubiquitous cell death (Figure 5F, L and 5S-U).

**Late Dpp signaling in heart development**

Compared to the early function of Dpp in patterning cardiogenic mesoderm, the function of Dpp during late cardiogenesis is less explored. Previously, studies had showed that numbers of various pericardial cell types,
but not cardiac cells, were increased in fly embryos with the dpp\textsuperscript{66} mutant allele, whose expression was not maintained in the dorsal ectoderm during germ-band retraction [23,24]. The expression of the mitosis marker phospho-histone 3 was concomitantly increased in the dpp\textsuperscript{66} mutant, suggesting that Dpp restricts the proliferation of pericardial cells specifically during late cardiogenesis [24]. As shown above, dpp signaling is ectopically-activated at stages 14-15 in raw mutants (Figure 4D, E and 4K, see also [58]); this also gives us a good opportunity to decipher late Dpp function in the developing heart of Drosophila. As demonstrated above, ectopic Dpp signaling leads to both overproliferation of cardiac cells during mid-embryogenesis and cardiac apoptosis during late embryogenesis because overexpression of dpp or tkv\textsuperscript{CA} replicated the raw mutant phenotypes (Figure 5A-H). Interestingly, both cardiac and pericardial cells were eliminated in either raw mutant, 69B>dpp or 24B>tkv\textsuperscript{CA} embryos (Figure 1D-F, I, O, Q, 5E and Figure 5H). Our data contradict the observation that targeted overexpression of tkv\textsuperscript{CA} under the control of cardioblast-specific tinC\Delta4-gal4 did not reduce the number of cardiac cells significantly [24]. The disparate results may be due to differences in the temporospatial expression of the transgene driven by different gal4 drivers. Alternately, if the pro-apoptotic propensity of Dpp is concentration-dependent (Figure 5O and 5S-U), as discussed above, it is also possible that the expression of tkv\textsuperscript{CA} driven by tinC\Delta4-gal4 is not strong enough to trigger the apoptosis response in cardioblasts.

**Model for raw mutation mediated apoptosis**

Based on our data, we propose a model to depict the genetic pathways involved in raw mutation-mediated apoptosis (Figure 9). At stage 14, Dpp activity in leading edge cells activates cardiogenic factors in the underlying mesoderm which are essential for the differentiation of dorsal mesodermally derived tissues, including the heart. Deficits in Raw function cause overexpression of dpp which increases the activities of cardiogenic factors and results in overgrowth of cardiac cells at stage 14. At stage 15, the expression of Dpp in the LE cells is maintained by the DJNK cascade, and heart cells are continuously differentiated by the function of cardiogenic factors in wild-type embryos. In raw mutant embryos, ectopic Dpp activates DTAK1 which triggers the expression of Bsk as well as Dpp. The induced Dpp functions in an autocrine manner to further enhance activity of Bsk and eventually lead to P53-mediated apoptosis.

In support of our model, it has been found that TGF-β/SMAD signaling exerts its apoptotic function in an autocrine loop manner in rat cardiomyocytes [59]. Induction of cardiomyocyte apoptosis by caspase overexpression has been shown to cause lethality and dilated cardiomyopathy in mice [60]. In contrast, inhibition of cardiomyocyte apoptosis by treating with a caspase

---

**Figure 9** The genetic network that leads to cardiac apoptosis in raw mutation. At stage 14, Raw restricts bsk which limits the expression of dpp at ectodermal leading edge cells. Minimal Dpp activity maintains the activation of cardiogenic factors which are essential for the differentiation of cardiac cells. In raw mutation, Bsk is de-regulated which causes overexpression of dpp in dorsal ectoderm at stage 14. Ectopic dpp signaling increases the expression of cardiogenic factors which results in overgrowth of cardiac cell types at stage 14. At stage 15, while the expression of Dpp in the LE cells gradually decreases, heart cells are continuously differentiated with the function of cardiogenic factors. At stage 15, DTAK1 is activated by the ectopic Dpp signaling in raw mutant. DTAK1 activates DJNK and thereby Dpp. Autocrine Dpp further enhances DJNK and eventually leads to P53-dependent apoptosis.
inhibitor reduced apoptosis, improved cardiac function, and delayed progression of heart failure in a cardiomyopathy animal model [61]. Prolong haemodynamic overload can up-regulate TGF-β [62,63], and death of cardiomyocytes is observed in virtually every myocardial disease (reviewed in [64]). The pathway unraveled in this study is the first report that links ectopic Dpp and DJNK-dependent cardiac apoptosis through the non-canonical pathway and dTAK1 activation. Our findings may thus suggest possible medical interventions for human cardiomyopathy.

Conclusion

By analyzing the heart defect phenotype of raw mutant embryos, we demonstrate that overexpression of dpp lead to cardiac apoptosis during late embryogenesis of Drosophila. We also demonstrate that Dpp induces its own expression through dTAK1. The activation of dTAK1 causes the hyperactivation of Drosophila JNK (DJNK) thereby cardiac apoptosis. This is the first report that links ectopic Dpp and DJNK-dependent cardiac apoptosis through the non-canonical Dpp signaling pathway and dTAK1 activation. Since haemodynamic overload usually up-regulates TGF-β, a mammalian homolog of Dpp, and death of cardiomyocytes, the pathway delineated in this study may suggest possible medical interventions for human cardiomyopathy.

Additional material

Additional file 1: Fig. S1. Odd-skipped pericardial cells (OPCs) are missing in raw mutant embryos at late stages. (A) wild-type embryos show the presence of OPCs. (B) OPCs are completely absent in raw mutants.

Additional file 2: Fig. S2. Loss-of Wg function does not lead to localized apoptosis. (A, D) Wg was expressed in a series of ectodermal cells at dorsal and ventral sites of embryos at stage 13. The expression pattern was not altered in raw mutant. (B, E) Lateral expression of Wg became a transverse stripe in the dorsal ectoderm of wild-type embryos. However, its expression decreased significantly in raw mutant embryos at stage 14. (C, F) At stage 16, residual Wg staining was detected in the dorsal epidermis of wild-type embryos, but its expression was completely lost in raw mutants at stage 16. (G) raw mutation shows cardiac apoptosis phenotype (brackets). (H) wg1214 is a temperature-sensitive allele that mimics the null wg allele at non-permissive temperatures. Removal of wg function does not lead to cardiac apoptosis phenotype in temperature shift experiment using wg1214 allele. Incubation times (9-15 hr) were normalized to development at 25°C. (I) Ectopic wg expression driven by 698-gal4, did not suppress cardiac apoptosis in raw mutants (brackets).

Additional file 3: Fig. S3. Mesodermally overexpression of Dpp induces raw-like phenotypes. (A) him-GFP reporter was expressed in muscle and heart precursors in 248-gal4 control flies at stage 14. (B) Expression of him-GFP was limited in heart cells in control 248-gal4 driver at stage 16. (C) Mesodermal overexpression of dpp induced ectopic heart cells at stage 14. (D) him-GFP expressing heart cells were lost in embryos expressing dpp using 248-gal4 at stage 16. (E, F) Normal AO staining pattern was observed in 248-gal4 control driver at stage 14 and 16. (G) Mesodermal overexpression of dpp does not induce apoptosis at stage 14. (H) Excessive cell death was detected in embryos overexpressing dpp using 248-gal4 at stage 16.
cardiac and muscular tissues in Drosophila. Development 2002, 129:1037-1047.

14. Klinedinst SL, Bodmer R: Gata factor Pannier is required to establish competence for heart progenitor formation. Development 2003, 130:3027-3038.

15. Lee HH, Frasch M: Wingless effects mesoderm patterning and ectoderm segmentation events via induction of its downstream target sloppy paired. Development 2000, 127:5497-5508.

16. Lockwood WR, Bodmer R: The patterns of wingless, decapentaplegic, and tinman position the Drosophila heart. Mech Dev 2002, 114:13-26.

17. Mann T, Bodmer R, Pandur P: The Drosophila homolog of vertebrate Isl1 is a key component in early cardiogenesis. Development 2009, 136:317-326.

18. Park M, Wu X, Golden K, Axelrod JD, Bodmer R: The wingless signaling pathway is directly involved in Drosophila heart development. Dev Biol 1996, 177:104-116.

19. Reichenmann V, Ison U, Wilson R, Grosskosthenhaus R, Leptin M: Control of cell fates and segmentation in the Drosophila mesoderm. Development 1997, 124:2915-2922.

20. Wu X, Golden K, Bodmer R: Heart development in Drosophila requires the segment polarity gene wingless. Dev Biol 1995, 169:619-628.

21. Newfeld SJ, Takasu NT: An analysis using the hobo genetic system reveals that combinatorial signaling by the Dpp and Wg pathways regulates dpp expression in leading edge cells of the dorsal ectoderm in Drosophila melanogaster. Genetics 2002, 161:685-692.

22. Knir S, Frasch M: Molecular integration of inductive and mesoderm-intrinsic inputs governs even-skipped enhancer activity in a subset of pericardial and dorsal muscle progenitors. Dev Biol 2001, 238:13-26.

23. Johnson AN, Bergman CM, Kreitman M, Newfeld SJ: Embryonic enhancers in the dpp disk region regulate a second round of Dpp signaling from the dorsal ectoderm to the mesoderm that represses Zfh-1 expression in a subset of pericardial cells. Dev Biol 2003, 262:137-151.

24. Johnson AN, Burnett LA, Sellin J, Paululat A, Newfeld SJ: Defective decapentaplegic signaling results in heart overgrowth and reduced cardiac output in Drosophila. Genetics 2007, 176:1609-1624.

25. Affolter M, Basler K: The Decapentaplegic morphogen gradient: from pattern formation to growth regulation. Nat Rev Genet 2007, 8:663-674.

26. Sorrentino A, Thakur N, Grimsby S, Marcusson A, von Bulow V, Schuster N, Hay BA, Wolff T, Rubin GM: Expression of baculovirus P35 prevents cell death in Drosophila. Development 1994, 120:2121-2129.

27. Brand AH, Perrimon N: Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 1993, 140:411-415.

28. Lee YS, Cartew RW: Making a better RNAi vector for Drosophila: use of intron spacers. Methods (San Diego, Calif) 2005, 30:322-329.

29. Spalding KC, Rubin GM: Transposition of cloned P elements into Drosophila germ line chromosomes. Science 1982, 218:341-347.

30. Abrams JM, White K, Fessler LJ, Steller H: Programmed cell death during Drosophila embryogenesis. Development 1993, 117:29-43.

31. Booth GE, Kinrade EF, Hidalgo A: Glia maintain follower neuron survival during Drosophila CNS development. Development 2000, 127:237-244.

32. Su MT, Fujikura M, Goto T, Bodmer R: The Drosophila homeobox genes zfh-1 and even-skipped are required for cardiac-specific differentiation of a nub-dependent lineage decision. Development 1999, 126:3241-3251.

33. Cao J, Pellock BJ, White K, Rafferty LA: A commercial phospho-Smad antibody detects endogenous BMP signaling in Drosophila tissues. Drosophila Information Services. 2006, 89:131-155.

34. Su MT, Golden K, Bodmer R: Drosophila embryos viable for 12 days at 25 °C are phenotypically normal and exhibit a high rate of pericardial cell death. Development 2002, 129:4913-4923.

35. Byars CL, Bates KL, Letsou A: The dorsal-open group gene raw is required for restricted DJunK signaling during closure. Development 1999, 126:4913-4923.

36. Hay BA, Wolff T, Rubin GM: Expression of baculovirus P35 prevents cell death in Drosophila. Development 1994, 120:2121-2129.

37. Mann T, Bodmer R, Pandur P: The Drosophila homolog of vertebrate Isl1 is a key component in early cardiogenesis. Development 2009, 136:317-326.

38. Park M, Wu X, Golden K, Axelrod JD, Bodmer R: The wingless signaling pathway is directly involved in Drosophila heart development. Dev Biol 1996, 177:104-116.
60. Wencker D, Chandra M, Nguyen K, Miao W, Garantziotis S, Factor SM, Shirani J, Armstrong RC, Kitsis RN: A mechanistic role for cardiac myocyte apoptosis in heart failure. J Clin Invest 2003, 111:1497-1504.

61. Hayakawa Y, Chandra M, Miao W, Shirani J, Brown JH, Dorn GW, Armstrong RC, Kitsis RN: Inhibition of cardiac myocyte apoptosis improves cardiac function and abolishes mortality in the peripartum cardiomyopathy of Galpha(q) transgenic mice. Circulation 2003, 108:3036-3041.

62. Dai RP, Dheen ST, He BP, Tay SS: Differential expression of cytokines in the rat heart in response to sustained volume overload. Eur J Heart Fail 2004, 6:693-703.

63. Takahashi N, Calderone A, Izzo NJ, Maki TM, Marsh JD, Colucci WS: Hypertrophic stimuli induce transforming growth factor-beta 1 expression in rat ventricular myocytes. J Clin Invest 1994, 94:1470-1476.

64. Dorn GW: Apoptotic and non-apoptotic programmed cardiomyocyte death in ventricular remodelling. Cardiovascular Research 2009, 81:465-473.

doi:10.1186/1423-0127-18-85

Cite this article as: Yang and Su: Excessive Dpp signaling induces cardiac apoptosis through dTAK1 and dJNK during late embryogenesis of Drosophila. Journal of Biomedical Science 2011 18:85.