Gene Deletion Screen for Cardiomyopathy in Adult *Drosophila* Identifies a New Notch Ligand

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Abstract

Rationale—*Drosophila* has been recognized as a model to study human cardiac diseases.

Objective—Despite these findings, and the wealth of tools that are available to the fly community, forward genetic screens for adult heart phenotypes have been rarely performed due to the difficulty in accurately measuring cardiac function in adult *Drosophila*.

Methods and Results—Using optical coherence tomography to obtain real-time analysis of cardiac function in awake *Drosophila*, we performed a genomic deficiency screen in adult flies. Based on multiple complementary approaches, we identified *CG31665* as a novel gene causing dilated cardiomyopathy. *CG31665*, which we name *weary (wry)*, has structural similarities to members of the Notch family. Using cell aggregation assays and γ-secretase inhibitors we show that Wry is a novel Notch ligand that can mediate cellular adhesion with Notch expressing cells and transactivates Notch to promote signaling and nuclear transcription. Importantly, Wry lacks a DSL (Delta-Serrate-Lag) domain that is common feature to the other *Drosophila* Notch ligands. We further show that Notch signaling is critically important for the maintenance of normal heart function of the adult fly.

Conclusions—In conclusion, we identify a previously unknown Notch ligand in *Drosophila* that when deleted causes cardiomyopathy. Our study suggests that Notch signaling components may be a therapeutic target for dilated cardiomyopathy.

Keywords

Heart failure; Cardiomyopathy gene; *Drosophila*; Notch signaling; Optical coherence tomography

Introduction

Mammalian animal models are useful to investigate the molecular pathways and pathophysiology of cardiomyopathy. However, genetic screens in mammalian models are complicated by time and effort required to identify the gene responsible for disease-related

phenotypes [1-3]. As a model system of human diseases, *Drosophila* has several advantages including efficient genetic screening and mapping, a well-annotated genome, transposon insertion and molecularly-defined genomic deficiency stocks, and conserved signaling pathways [4-7]. Additionally, the fruit fly has been recognized as a model to study human cardiac diseases [6, 8]. The adult *Drosophila* heart develops from the embryonic dorsal vessel and shares similarities with vertebrate myocardium [9-11]. For example, alterations in structural proteins such as troponin I, tropomyosin, δ-sarcoglycan, and dystrophin result in impaired *Drosophila* cardiac function [8, 12-14]. Moreover, mutations in ion channels including the potassium channel genes *KCNQ* and *HERG* result in increased arrhythmia index and shortened lifespan [15-17]. Additionally, transcription factors including *Tin/Nkx2.5, Twist, Mef*, and *Hand* have been identified in *Drosophila* and have orthologs that are critical to vertebrate heart development [6, 18-20]. Despite these findings, and the wealth of tools that are available to the fly community, forward genetic screens for adult heart phenotypes have been rarely performed due to the difficulty in accurately measuring cardiac function in adult *Drosophila*. Recently, we developed a strategy to obtain rapid and accurate real-time measurements of cardiac function in the awake adult fly by optical coherence tomography (OCT) [8]. Using OCT, we performed a screen of adult flies that have genomic deficiencies and identified a novel gene, *CG31665* that has structural similarities to members of the Notch family.

In *Drosophila*, Notch signaling is essential for cell differentiation and tissue development [21, 22]. The membrane-spanning Notch (N) receptor is stimulated by the ligands Delta (Dl) or Serrate (Ser), which are present on the surface of neighboring cells. After ligand binding, presenilin proteins mediate proteolytic cleavage of the Notch Intracellular domain (NICD) [21, 23]. Subsequently, the cleaved NICD undergoes nuclear translocation, where it functions as a transcriptional coactivator for the Suppressor of Hairless [Su(H)] transcription factor, resulting in the expression of specific target genes such as *Enhancer of split* [*E(spl)*]. This activation is strictly controlled, and deregulation causes extreme developmental defects [24-27]. The stringency of the control system is provided by the general Notch-antagonist Hairless (H) that assembles in a repressor complex on Notch target genes [21, 22]. Although Notch signaling is crucial in the development of many different *Drosophila* tissues including the heart [22, 28-32], the role of Notch signaling in adult *Drosophila* cardiac function is unknown. In mammals, Notch signaling regulates many aspects of embryonic development, tissue homeostasis, and disease [33-36]. In both humans and mice, mutations in components of the Notch signaling pathway play a role in the development of a number of congenital cardiovascular diseases [33, 37].

Here we identified a new gene that when deleted causes dilated cardiomyopathy. The phenotype observed in deletion mutants of *CG31665* is consistent with a tired or fatigued heart, and therefore we name the gene *weary* (*wry*). We show that Wry functions as a Notch ligand, but is structurally distinct from Delta and Serrate. We further show that Notch signaling is critically important for the maintenance of normal heart function of the adult fly.

Methods

Molecular-defined genomic deficiency strains and transposon insertion stocks were obtained from the FlyBase repository at Bloomington, IL and PiggyBac insertion mutants were obtained from the Exelixis repository at Harvard Medical School. Transgenic RNAi strains were obtained from the Vienna *Drosophila* RNAi Center.

Detailed descriptions of fly stocks, cardiac measurements in adult *Drosophila*, QRT-PCR, imaging wings, *Drosophila* cell culture and cell aggregation assays, ligand-dependent luciferase assay, and statistical analysis are provided in the supplemental materials.

Results

Identification of a haploinsufficient region on chromosome 2L that causes dilated cardiomyopathy in adult *Drosophila*

We used OCT to measure heart function in a screen of adult *Drosophila* with molecularly-defined genomic deficiencies along chromosome 2L. We identified a deletion mutant, Df(2L) Exel7007, with the phenotype of dilated cardiomyopathy defined as enlarged end-diastolic and end-systolic dimensions, and impaired fractional shortening (Figure 1A and 1B). This mutant is haploinsufficient for a 193kb genomic region corresponding to 22B1-22B5 on chromosome 2 and covers 23 annotated genes.

To narrow down the candidate gene interval responsible for the cardiomyopathic phenotype of Df(2L)Exel7007, we examined three overlapping deletion stocks. Df(2L)Exel8005 had a dilated phenotype that was similar to Df(2L)Exel7007 and allowed us to narrow the candidate interval to 16 genes (Figure 1C through 1E).

Because the majority of homozygous deletions are lethal, deficiency stocks are maintained as heterozygotes in the presence of a balancer chromosome. Since the cardiomyopathic phenotype in Df(2L)Exel7007 and Df(2L)Exel8005 could be theoretically attributed to the balancer chromosome, we tested both deficiency strains in the context of w^{1118} to remove the possible contribution of the balancer to cardiac dysfunction. Both stocks retained the abnormal cardiac phenotype in the absence of balancer chromosomes (Online Figure I, A through C), supporting our interpretation that a gene deletion in Df(2L)Exel7007 is causative for a cardiomyopathic phenotype in the fly.

Transposon insertions f00122 and 22697 identify *CG31665* as a cardiomyopathy candidate gene

To narrow the 130Kb genomic region that included the 16 candidate genes, we examined heart function in seven transposon insertion lines within the genomic region. Of the seven insertion lines screened, we identified two stocks (f00122 and 22697) that phenocopied the original deletion Df(2L)Exel7007 (Figure 1F and 1G). The remaining five insertion lines inserted outside the *CG31665* gene had a normal cardiac phenotype (Figure 1F, 1G and data not shown).

Since both f00122 and 22697 are inserted in the gene *CG31665*, we examined *CG31665* gene expression by QRT-PCR in Df(2L)Exel7007, Df(2L)Exel8005, f00122, and 22697. All four stocks displayed a reduction in *CG31665* expression (Figure 1H). Additionally, f00122 had normal expression levels of two neighboring genes (*c-cup* and *CG31663*) while the two deficiency stocks showed reduced expression of *c-cup* and *CG31663* as expected (data not shown).

To validate that the piggyBack transposon insertion altered cardiac function through its effect on *CG31665*, we excised the piggyBac element from f00122 using precise excision [38]. Precise excision of the piggyBac element resulted in full restoration of normal heart function (Online Figure I, E). These data suggest that f00122 and 22697 interrupt the gene *CG31665* and that *CG31665* is the gene responsible for the dilated cardiac phenotype in Df(2L)Exel7007 and Df (2L)Exel8005.

Transgenic *Drosophila* harboring RNAi directed against *CG31665* have dilated cardiomyopathy

We next obtained transgenic *Drosophila* strains that contain UAS-RNAi targeted to genes within the candidate interval. We crossed each stock with Gal4 driver lines using *tinC*, *actin* or *hsp70* promoters and evaluated heart function in the F1 generation. To test whether knocking

down CG31665 would result in a cardiomyopathic phenotype, we crossed UAS-CG31665RNAi flies with the various Gal4 driver lines. *Actin* or *tinC* promoter-induced *CG31665* RNAi expression resulted in abnormal cardiac function (Figure 2A and 2B). Using quantitative real-time RT-PCR, we confirmed that UAS-CG31665RNAi flies with *actin*-Gal4 had decreased *CG31665* expression compared to w^{1118} controls (Figure 2C).

Since the expression of the *CG31665* RNAi transgene was under the control of Gal4 drivers that are expressed early during *Drosophila* development, we wanted to eliminate the possibility that the abnormal heart function observed resulted from altered gene expression during embryogenesis. To test whether knocking down *CG31665* gene expression post-developmentally causes dilated cardiomyopathy, we engineered *w*;p{*tub*-Gal80^ts};p{*tinCΔ4*-Gal4}/p{UAS-CG31665RNAi} flies based on the temperature-sensitive Gal80 protein in the context of the bipartite Gal4/UAS transgenic expression system in *Drosophila* employing the cardiac-specific driver, *tinCΔ4*-Gal4 [39-43]. The Gal80^ts protein is functional at 18°C and binds to Gal4 thereby preventing Gal4 binding to a UAS-CG31665RNAi transgene. However, at 26°C, the Gal80^ts/Gal4 complex dissociates and Gal4 then binds to the UAS-CG31665RNAi to activate its transcription [39, 41]. A temperature shift from 18°C to 26°C resulted in the deterioration of cardiac function that was corrected by a second temperature shift back to 18°C (Figure 2 D and 2E). These data suggest that post-developmental loss of expression of *CG31665* in adult fly heart results in inducible and reversible dilated cardiomyopathy. Similar data was found using *hsp70* promoter to induce *CG31665* RNAi expression (Online Figure II, A through C). We also examined other RNAi lines in our candidate interval and show that cardiac induced RNAi expression for these other genes has no effect on cardiac function (Online Figure III). Lastly, we used the Gal80^ts/Gal4 temperature-sensitive system for a UAS-RNAi line targeting a neighboring gene (*c-cup*) and showed no change in cardiac function with temperature shift (data not shown). These data demonstrate that *CG31665* is the causative gene responsible for the dilated cardiomyopathic phenotype of Df(2L)Exel7007, and we name this gene *weary* (*wry*).

Expression of recombinant Wry restores cardiac function in the deletion mutant Df(2L)Exel7007

To demonstrate that *wry* is directly responsible for the cardiomyopathic phenotype, we examined the ability of cardiac-specific expression of Wry to rescue the cardiac abnormality in Df(2L)Exel7007. Expression of Wry in the heart completely rescues the abnormal cardiac function of Df(2L)Exel7007 supporting our conclusion that *wry* is the causative gene (Figure 2F and 2G).

Disrupting components of the Notch signaling pathway causes dilated cardiomyopathy

We performed bioinformatic analyses of Wry using the Stockholm Bioinformatics Centre InParanoid (http://inparanoid.sbc.su.se/cgi-bin/index.cgi), Ensembl (http://www.ensembl.org), and PANTHER (http://www.pantherdb.org) databases to examine for sequence homologies across multiple species. Wry contains EGF repeats and a transmembrane domain but does not possess a DSL (Delta-Serrate-Lag) domain (Online Figure IV), suggesting that it may be involved in Notch signaling. To determine the transcriptional pattern of *wry* expression, we performed QRT-PCR analysis and show that *wry* is expressed in all four life stages and parallels the expression pattern of the known Notch ligand Ser (Online Table I and Supplemental Results Section). We next examined the heart function in *w*;p{*tub*-Gal80^ts};p{*tinCΔ4*-Gal4} flies harboring p{UAS-Dl.DN(dominant-negative)} or UAS-Ser.DN to determine if Notch signaling is important for post developmental cardiac function. Temperature shift from 18°C to 26°C resulted in deterioration in cardiac function and a second temperature shift back to 18°C restored normal function (Figure 3). We examined deficiency stocks for Notch signaling components and observed that stocks with deficiencies of Notch

ligands or Su(H) had abnormal cardiac function (Online Figure V, A and B). Additionally, we observed that the cardiac-specific expression of RNAi directed against Notch receptor and ligands caused abnormal cardiac function (Online Figure V, C, D and data not shown). Since mutants in Notch signaling regulators often have wing phenotypes [44], [45], we next examined the wings of Df(2L)Exel7007. Interestingly, Wry deficiency stocks had normal appearing wings (Online Figure V, E). These data support our hypothesis that disrupting Notch signaling in the adult fly heart can result in an inducible and reversible dilated cardiomyopathy.

Cardiac-specific expression of Notch signaling components rescues the abnormal phenotype of Df(2L)Exel7007

Since *wry* appears to be the causative gene for the phenotype of Df(2L)Exel7007, and may be involved in Notch signaling, we tested whether specific components of the Notch pathway can rescue Df(2L)Exel7007. The introduction of a *Notch* (*N*) transgene driven by a cardiac-specific promoter into the context of Df(2L)Exel7007 deletion restored normal cardiac function (Figure 4A through 4B). Additionally, cardiac-specific expression of Notch ligands rescues the abnormal phenotype of Df(2L)Exel7007 (Figure 4C through 4F). Finally, cardiac-specific expression of Su(H), a positive regulator of Notch signaling, rescues the abnormal phenotype of Df(2L)Exel7007 whereas the Notch antagonist Hairless (H), a negative regulator of Notch signaling, could not restore normal cardiac function (Figure 5A through 5D).

To demonstrate the specificity of Wry in restoring abnormal cardiac function, we show that transgenic flies harboring either human delta-sarcoglycan (Dsg) or fly Medea, a positive regulator of BMP signaling [46], is unable to restore normal cardiac function in Df(2L)Exel7007 (Online Figure VI).

Wry functions as a Notch ligand

Since cardiac-specific expression of Notch ligands rescues the abnormal phenotype of Wry deficiency stocks, we hypothesized that Wry acts as a functional Notch ligand. To test this hypothesis, we conducted cell-aggregation assays with cells expressing Notch, Delta, Serrate or Wry and examined the ability of Notch to bind different ligands. Confocal images of immunofluorescent cells containing Delta (Dl), Serrate (Ser) or Wry indicate that these proteins are each able to promote cellular adhesion with Notch expressing cells as shown by the clustering of ligand and receptor at sites of cellular contact (Figure 6A). Dl, Ser and Wry induced approximately 20-35% cell aggregation compared to <5% in Notch only containing cells (Figure 6B). Next, we examined whether Wry activates downstream nuclear transcription by performing ligand-dependent Notch-activity reporter assays using S2 cells. We transfected S2N cells with the reporter construct, previously shown to be responsive to transactivation by Delta [47]. When S2N cells were mixed with S2 cells overexpressing Wry, promoter activity was transactivated by approximately 3-fold compared with those mixed with untransfected cells (Figure 6C). As a positive control, cells overexpressing Dl or Ser mixed with S2N cells showed a 4-5 fold increase in promoter activity. Importantly, Wry-mediated transactivation of Notch was blocked by the γ-secretase inhibitors, compound E and DAPT (Figure 6C). Interestingly, it does not appear that Wry can transactivate Notch signaling in mammalian C2C12 cells (Online Figure VII and Supplemental Results Section). Collectively, our results suggest that Wry functions as a Notch ligand in *Drosophila* to induce cleavage of Notch and effectively activate transcriptional events in response to Notch signaling.

Cardiac-specific expression of Wry rescues the abnormal phenotype of SerrateRNAi flies

Since *in vitro* cell adhesion assays (Figure 6) and *in vivo* rescue studies (Figure 4C through 4F) suggest that Wry acts as a Notch ligand, we tested whether Wry can rescue the abnormal cardiac function in Serrate loss-of-function mutants. The cardiac-specific expression of a *wry* transgene into the context of SerrateRNAi restored normal cardiac function. In contrast,

we show that the expression of Wry by a wing-specific *vgMQ* promoter did not rescue the wing morphologic abnormalities associated with SerrateRNAi flies whereas Serrate restored both the cardiac and the wing phenotype in the context of SerrateRNAi (Figure 7). These results suggest that Wry functions as a novel Notch ligand in a somewhat tissue-specific manner.

Discussion

In this study, we have identified *wry* as a gene associated with dilated cardiomyopathy in adult *Drosophila* through the examination of *Drosophila* mutants that have genomic haploinsufficiencies, transposon insertions, or cardiac-specific RNAi expression. The protein encoded by *wry* has motifs that include EGF repeats and a transmembrane domain consistent with the Notch family of proteins, but lacks a DSL domain. Using cell aggregation assays and γ-secretase inhibitors we show that Wry is a novel Notch ligand that can mediate cellular adhesion with Notch expressing cells and transactivate Notch to promote signaling and nuclear transcription.

Notch signaling is important in directing the specification of tissues including the heart in almost all developmental stages in *Drosophila* [22, 28, 29, 31, 32]. There are two types of Notch ligands, which correspondingly activate distinct signaling mechanisms [48-50]. Typical Notch ligands, which include Serrate and Delta in *Drosophila* and Jagged 1-2 and Delta-like 1,3,4 in mammals, contain a DSL domain and transduce canonical Notch signaling pathway to the CSL (CBF1/Suppressor of Hairless/Lag-1)-NICD-Mastermind complex for the maintenance of stem or progenitor cells through transcriptional activation of Notch target genes such as *E (spl)* in *Drosophila* and *HES1* in mammals [35, 51-53]. In contrast, atypical Notch ligands like DNER, F3/Contactin and NB-3 in mammals have no DSL domain, and transduce non-canonical Notch signaling to the CSL-NICD-Deltex complex for the differentiation of progenitor cells through MAG transcriptional activation [48-50]. Notch signals are transduced to the canonical pathway (CSL-NICD-Mastermind signaling cascade) or the non-canonical pathway (CSL-NICD-Deltex signaling cascades) based on the expression profile of Notch ligands, Notch receptors, and Notch signaling modifiers [36]. Interestingly, Wry does not have a DSL domain although Wry does share EGF domain homology with the two *Drosophila* Notch ligands.

Our results suggest that Wry regulates Notch signaling in a DSL domain-independent manner which has not been previously recognized to occur in *Drosophila*. Interestingly, the identification of Notch signaling in normal adult *Drosophila* cardiac function has not been previously described. Our results show that the cardiac-specific expression of Notch ligands can rescue the abnormal cardiac phenotype observed in Df(2L)Exel7007, supporting the concept that Wry functions as in the Notch pathway. Interestingly, Df(2L)Exel7007 does not have abnormal wing vein morphology, a phenotype that is associated with deficiency mutants for Notch ligands [44, 45, 54]. In addition, Df(2L)Exel7007 does not possess the characteristic abnormalities in external sensory organs including bristle morphology defects (data not shown) that have previously been identified in mutations of Notch regulators [55, 56]. These results are consistent with a recent study that employed genome-wide RNAi screens of Notch signaling and showed no apparent abnormalities in wing vein morphology or external sensory organs in flies that expressed UAS-CG31665RNAi under the control of a *pnr* (notum specific)-Gal4 or *MS1096* (wing specific)-Gal4 driver [30].

In general, an identification of abnormalities that cause dilated cardiomyopathies in adult *Drosophila* requires a distinction between mutations that alter heart formation from post-developmental effects on cardiac function. During larval stages, the *Drosophila* cardiovascular system is composed of a dorsal vessel containing 52 pairs of cardiomyocytes that display both endothelial and muscle cell characteristics. The cardiomyocytes form a tubular structure

flanked by pericardial cells [9, 10]. Abnormal cardiac function in adult *Drosophila* can result from defects in embryonic dorsal vessel development mimicking congenital abnormalities [20] and abnormalities arising in post-development. To address this issue we used a temperature sensitive Gal80[ts] system to drive expression of UAS-WryRNAi, Dl.DN, and Ser.DN in the fully developed adult fly heart. The transgene expression in *tinC*-expressing cells could have effects on cardiomyocytes in an autonomous manner, or could affect neighboring cells to influence heart function in a non-autonomous manner. Indeed, non-autonomous influence of the epi/pericardial cells on the function of the fly heart has been postulated [57, 58]. However, in both cases, our results demonstrate that the inhibition and subsequent restoration of Notch signaling results in the induction and reversal of abnormal cardiac function, respectively. Thus, the regulation of Notch signaling appears to be an important component in the maintenance of normal cardiac function in the adult fly. In mammals, Notch signaling is involved in many aspects of development and disease [33-36]. Although human mutations in Notch signaling and mutant mouse models of Notch signaling have already been implicated in congenital heart disease [33, 37], the involvement of Notch signaling in adult cardiac disease remains unclear. Our study suggests that gene orthologues involved in Notch signaling may be important in pathogenesis of mammalian dilated cardiomyopathy and that Notch signaling components may be a therapeutic target for dilated cardiomyopathy.

Novelty and Significance

What Is Known?

- The Notch signaling pathway is important in many different tissues at all developmental stages in *Drosophila*.

- Whether Notch signaling is important to maintain normal heart function in adult *Drosophila* is not known.

What New Information Does This Article Contribute?

- This study identifies a new gene that causes dilated cardiomyopathy in adult *Drosophila*.

- This gene, which we name *weary* (*wry*), functions as a novel Notch ligand to maintain normal heart function.

- Disrupting Notch signaling in the adult fly heart causes dilated cardiomyopathy.

A genomic-deficiency screen along chromosome 2L was performed to identify genes that cause dilated cardiomyopathy in awake adult *Drosophila*. This search identified *CG31665* as a cardiomyopathy candidate gene, which we name *weary* (*wry*). The Wry has EGF repeat motifs and a transmembrane domain similar to that found in Notch ligands, but it is structurally distinct from Delta and Serrate because Wry lacks a DSL (Delta-Serrate-Lag) domain common to other Notch ligands. Despite the absence of a DSL domain, Wry can mediate cellular adhesion with Notch expressing cells, and transactivate Notch signaling in *Drosophila* S2 cells like the two other known Notch ligands. Wry has not been previously described, nor has it been shown in the fly that a Notch ligand can regulate Notch signaling in a DSL domain-independent manner. Additionally, deficiencies in Wry do not possess the characteristic abnormalities in external sensory organs and wings that have been identified in mutations of Notch regulators. Based on the findings, this newly identified Notch ligand may play a fundamental role in maintaining normal cardiac function, and gene orthologues involved in Notch signaling may be important in the pathogenesis of human dilated cardiomyopathy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Ikeda Y, Ross J Jr. Models of dilated cardiomyopathy in the mouse and the hamster. Curr Opin Cardiol 2000;15:197–201. [PubMed: 10952428]

2. Ross J Jr. Dilated cardiomyopathy: concepts derived from gene deficient and transgenic animal models. Circ J 2002;66:219–224. [PubMed: 11922267]

3. Yarbrough WM, Spinale FG. Large animal models of congestive heart failure: a critical step in translating basic observations into clinical applications. J Nucl Cardiol 2003;10:77–86. [PubMed: 12569335]

4. Reiter LT, Potocki L, Chien S, Gribskov M, Bier E. A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster. Genome Res 2001;11:1114–1125. [PubMed: 11381037]

5. Simon MA, Bowtell DD, Dodson GS, Laverty TR, Rubin GM. Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase. Cell 1991;67:701–716. [PubMed: 1934068]

6. Bodmer R, Venkatesh TV. Heart development in Drosophila and vertebrates: conservation of molecular mechanisms. Dev Genet 1998;22:181–186. [PubMed: 9621426]

7. Davidson B, Levine M. Evolutionary origins of the vertebrate heart: Specification of the cardiac lineage in Ciona intestinalis. Proc Natl Acad Sci U S A 2003;100:11469–11473. [PubMed: 14500781]

8. Wolf MJ, Amrein H, Izatt JA, Choma MA, Reedy MC, Rockman HA. Drosophila as a model for the identification of genes causing adult human heart disease. Proc Natl Acad Sci U S A 2006;103:1394–1399. [PubMed: 16432241]

9. Ward EJ, Skeath JB. Characterization of a novel subset of cardiac cells and their progenitors in the Drosophila embryo. Development 2000;127:4959–4969. [PubMed: 11044409]

10. Molina MR, Cripps RM. Ostia, the inflow tracts of the Drosophila heart, develop from a genetically distinct subset of cardial cells. Mech Dev 2001;109:51–59. [PubMed: 11677052]

11. Lo PC, Frasch M. A role for the COUP-TF-related gene seven-up in the diversification of cardioblast identities in the dorsal vessel of Drosophila. Mech Dev 2001;104:49–60. [PubMed: 11404079]

12. Colombo MG, Botto N, Vittorini S, Paradossi U, Andreassi MG. Clinical utility of genetic tests for inherited hypertrophic and dilated cardiomyopathies. Cardiovasc Ultrasound 2008;6:62. [PubMed: 19099557]

13. Bier E, Bodmer R. Drosophila, an emerging model for cardiac disease. Gene 2004;342:1–11. [PubMed: 15527959]

14. Duan D. Challenges and opportunities in dystrophin-deficient cardiomyopathy gene therapy. Hum Mol Genet 2006;15(Spec No 2):R253–261. [PubMed: 16987891]

15. Sanguinetti MC, Tristani-Firouzi M. hERG potassium channels and cardiac arrhythmia. Nature 2006;440:463–469. [PubMed: 16554806]

16. Johnson E, Ringo J, Bray N, Dowse H. Genetic and pharmacological identification of ion channels central to the Drosophila cardiac pacemaker. J Neurogenet 1998;12:1–24. [PubMed: 9666898]

17. Ocorr K, Reeves NL, Wessells RJ, Fink M, Chen HS, Akasaka T, Yasuda S, Metzger JM, Giles W, Posakony JW, Bodmer R. KCNQ potassium channel mutations cause cardiac arrhythmias in Drosophila that mimic the effects of aging. Proc Natl Acad Sci U S A 2007;104:3943–3948. [PubMed: 17360457]

18. Lyons I, Parsons LM, Hartley L, Li R, Andrews JE, Robb L, Harvey RP. Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. Genes Dev 1995;9:1654–1666. [PubMed: 7628699]

19. Zaffran S, Reim I, Qian L, Lo PC, Bodmer R, Frasch M. Cardioblast-intrinsic Tinman activity controls proper diversification and differentiation of myocardial cells in Drosophila. Development 2006;133:4073–4083. [PubMed: 16987868]

20. Schott JJ, Benson DW, Basson CT, Pease W, Silberbach GM, Moak JP, Maron BJ, Seidman CE, Seidman JG. Congenital heart disease caused by mutations in the transcription factor NKX2-5. Science 1998;281:108–111. [PubMed: 9651244]

21. Maier D. Hairless: the ignored antagonist of the Notch signalling pathway. Hereditas 2006;143:212–221. [PubMed: 17362357]

22. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. Science 1999;284:770–776. [PubMed: 10221902]

23. Mumm JS, Kopan R. Notch signaling: from the outside in. Dev Biol 2000;228:151–165. [PubMed: 11112321]

24. Bailey AM, Posakony JW. Suppressor of hairless directly activates transcription of enhancer of split complex genes in response to Notch receptor activity. Genes Dev 1995;9:2609–2622. [PubMed: 7590239]

25. Jarriault S, Le Bail O, Hirsinger E, Pourquie O, Logeat F, Strong CF, Brou C, Seidah NG, Isra l A. Delta-1 activation of notch-1 signaling results in HES-1 transactivation. Mol Cell Biol 1998;18:7423–7431. [PubMed: 9819428]

26. Lecourtois M, Schweisguth F. Role of suppressor of hairless in the delta-activated Notch signaling pathway. Perspect Dev Neurobiol 1997;4:305–311. [PubMed: 9171444]

27. Wettstein DA, Turner DL, Kintner C. The Xenopus homolog of Drosophila Suppressor of Hairless mediates Notch signaling during primary neurogenesis. Development 1997;124:693–702. [PubMed: 9043084]

28. Bray S. A Notch affair. Cell 1998;93:499–503. [PubMed: 9604926]

29. Portin P. General outlines of the molecular genetics of the Notch signalling pathway in Drosophila melanogaster: a review. Hereditas 2002;136:89–96. [PubMed: 12369105]

30. Mummery-Widmer JL, Yamazaki M, Stoeger T, Novatchkova M, Bhalerao S, Chen D, Dietzl G, Dickson BJ, Knoblich JA. Genome-wide analysis of Notch signalling in Drosophila by transgenic RNAi. Nature 2009;458:987–992. [PubMed: 19363474]

31. Corbin V, Michelson AM, Abmayr SM, Neel V, Alcamo E, Maniatis T, Young MW. A role for the Drosophila neurogenic genes in mesoderm differentiation. Cell 1991;67:311–323. [PubMed: 1913825]

32. Kwon C, Han Z, Olson EN, Srivastava D. MicroRNA1 influences cardiac differentiation in Drosophila and regulates Notch signaling. Proc Natl Acad Sci U S A 2005;102:18986–18991. [PubMed: 16357195]

33. High FA, Zhang M, Proweller A, Tu L, Parmacek MS, Pear WS, Epstein JA. An essential role for Notch in neural crest during cardiovascular development and smooth muscle differentiation. J Clin Invest 2007;117:353–363. [PubMed: 17273555]

34. High FA, Lu MM, Pear WS, Loomes KM, Kaestner KH, Epstein JA. Endothelial expression of the Notch ligand Jagged1 is required for vascular smooth muscle development. Proc Natl Acad Sci U S A 2008;105:1955–1959. [PubMed: 18245384]

35. Kopan R, Ilagan MX. The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 2009;137:216–233. [PubMed: 19379690]

36. D'Souza B, Miyamoto A, Weinmaster G. The many facets of Notch ligands. Oncogene 2008;27:5148–5167. [PubMed: 18758484]

37. High FA, Epstein JA. The multifaceted role of Notch in cardiac development and disease. Nat Rev Genet 2008;9:49–61. [PubMed: 18071321]

38. Hacker U, Nystedt S, Barmchi MP, Horn C, Wimmer EA. piggyBac-based insertional mutagenesis in the presence of stably integrated P elements in Drosophila. Proc Natl Acad Sci U S A 2003;100:7720–7725. [PubMed: 12802016]

39. Kim IM, Wolf MJ. Serial Examination of an Inducible and Reversible Dilated Cardiomyopathy in Individual Adult Drosophila. PLoS ONE 2009;4:e7132. [PubMed: 19771157]

40. Duffy JB. GAL4 system in Drosophila: a fly geneticist's Swiss army knife. Genesis 2002;34:1–15. [PubMed: 12324939]

41. McGuire SE, Le PT, Osborn AJ, Matsumoto K, Davis RL. Spatiotemporal rescue of memory dysfunction in Drosophila. Science 2003;302:1765–1768. [PubMed: 14657498]

42. McGuire SE, Roman G, Davis RL. Gene expression systems in Drosophila: a synthesis of time and space. Trends Genet 2004;20:384–391. [PubMed: 15262411]

43. Yin Z, Frasch M. Regulation and function of tinman during dorsal mesoderm induction and heart specification in Drosophila. Dev Genet 1998;22:187–200. [PubMed: 9621427]

44. de Celis JF, Garcia-Bellido A, Bray SJ. Activation and function of Notch at the dorsal-ventral boundary of the wing imaginal disc. Development 1996;122:359–369. [PubMed: 8565848]

45. Huppert SS, Jacobsen TL, Muskavitch MA. Feedback regulation is central to Delta-Notch signalling required for Drosophila wing vein morphogenesis. Development 1997;124:3283–3291. [PubMed: 9310323]

46. Raftery LA, Wisotzkey RG. Characterization of Medea, a gene required for maximal function of the Drosophila BMP homolog Decapentaplegic. Ann N Y Acad Sci 1996;785:318–320. [PubMed: 8702167]

47. Mukherjee A, Veraksa A, Bauer A, Rosse C, Camonis J, Artavanis-Tsakonas S. Regulation of Notch signalling by non-visual beta-arrestin. Nat Cell Biol 2005;7:1191–1201. [PubMed: 16284625]

48. Eiraku M, Hirata Y, Takeshima H, Hirano T, Kengaku M. Delta/notch-like epidermal growth factor (EGF)-related receptor, a novel EGF-like repeat-containing protein targeted to dendrites of developing and adult central nervous system neurons. J Biol Chem 2002;277:25400–25407. [PubMed: 11950833]

49. Hu QD, Ang BT, Karsak M, Hu WP, Cui XY, Duka T, Takeda Y, Chia W, Sankar N, Ng YK, Ling EA, Maciag T, Small D, Trifonova R, Kopan R, Okano H, Nakafuku M, Chiba S, Hirai H, Aster JC, Schachner M, Pallen CJ, Watanabe K, Xiao ZC. F3/contactin acts as a functional ligand for Notch during oligodendrocyte maturation. Cell 2003;115:163–175. [PubMed: 14567914]

50. Cui XY, Hu QD, Tekaya M, Shimoda Y, Ang BT, Nie DY, Sun L, Hu WP, Karsak M, Duka T, Takeda Y, Ou LY, Dawe GS, Yu FG, Ahmed S, Jin LH, Schachner M, Watanabe K, Arsenijevic Y, Xiao ZC. NB-3/Notch1 pathway via Deltex1 promotes neural progenitor cell differentiation into oligodendrocytes. J Biol Chem 2004;279:25858–25865. [PubMed: 15082708]

51. Androutsellis-Theotokis A, Leker RR, Soldner F, Hoeppner DJ, Ravin R, Poser SW, Rueger MA, Bae SK, Kittappa R, McKay RD. Notch signalling regulates stem cell numbers in vitro and in vivo. Nature 2006;442:823–826. [PubMed: 16799564]

52. Iso T, Kedes L, Hamamori Y. HES and HERP families: multiple effectors of the Notch signaling pathway. J Cell Physiol 2003;194:237–255. [PubMed: 12548545]

53. Katoh M. Identification and characterization of human HESL, rat Hesl and rainbow trout hesl genes in silico. Int J Mol Med 2004;14:747–751. [PubMed: 15375612]

54. Jack J, DeLotto Y. Effect of wing scalloping mutations on cut expression and sense organ differentiation in the Drosophila wing margin. Genetics 1992;131:353–363. [PubMed: 1353736]

55. Bang AG, Hartenstein V, Posakony JW. Hairless is required for the development of adult sensory organ precursor cells in Drosophila. Development 1991;111:89–104. [PubMed: 2015800]

56. Schweisguth F, Posakony JW. Antagonistic activities of Suppressor of Hairless and Hairless control alternative cell fates in the Drosophila adult epidermis. Development 1994;120:1433–1441. [PubMed: 8050354]

57. Buechling T, Akasaka T, Vogler G, Ruiz-Lozano P, Ocorr K, Bodmer R. Non-autonomous modulation of heart rhythm, contractility and morphology in adult fruit flies. Dev Biol 2009;328:483–492. [PubMed: 19233157]

58. Fujioka M, Wessells RJ, Han Z, Liu J, Fitzgerald K, Yusibova GL, Zamora M, Ruiz-Lozano P, Bodmer R, Jaynes JB. Embryonic even skipped-dependent muscle and heart cell fates are required for normal adult activity, heart function, and lifespan. Circ Res 2005;97:1108–1114. [PubMed: 16239588]

Non-standard Abbreviations and Acronyms

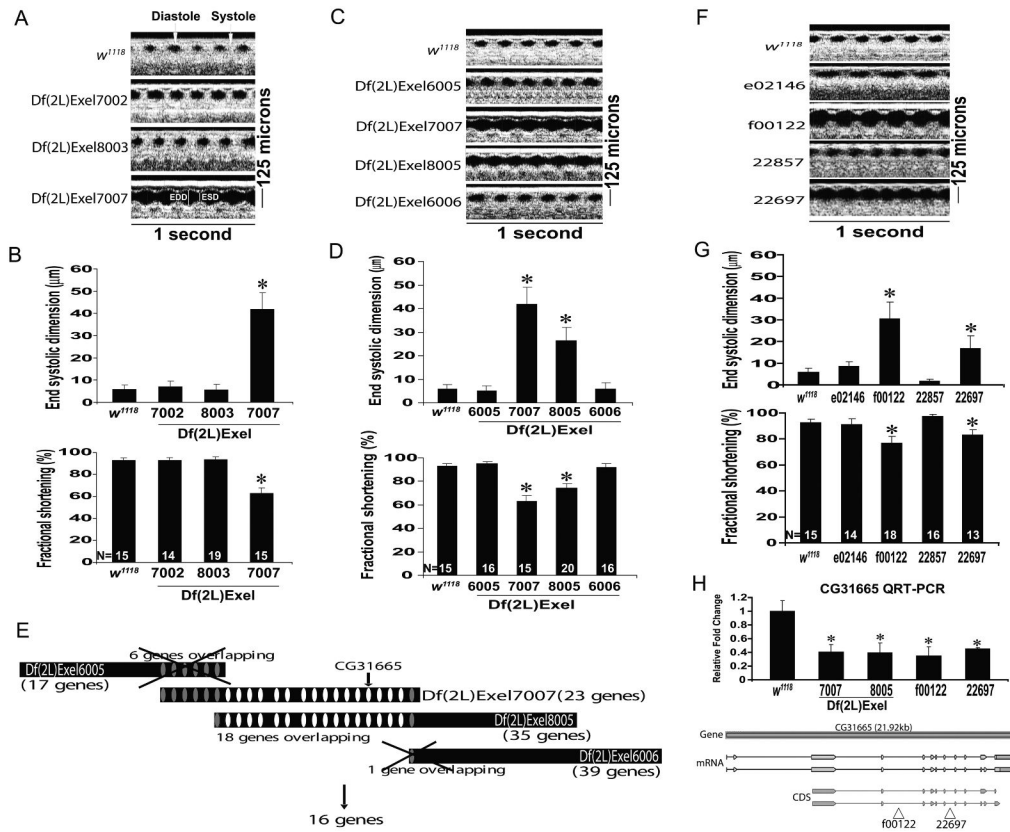|	
CSL	CBF1/Suppressor of Hairless/Lag-1
Dl	Delta
DN	Dominant-Negative
E(spl)	Enhancer of split
N	Notch receptor
NICD	Notch Intracellular Domain
H	Hairless
OCT	Optical Coherence Tomography
Ser	Serrate
Su(H)	Suppressor of Hairless

Figure 1. Screening of exelixis deletions and transposon insertions identified *CG31665* as a cardiomyopathy candidate gene
(A) Representative OCT M-mode images from indicated stocks. (B) Summary data for M-mode images showing that Df(2L)Exel7007 has an increased end systolic dimension and impaired fractional shortening. (C) Representative OCT M-mode images from indicated flies. (D) Summary data for M-mode images. Df(2L)Exel8005 has dilated cardiac dimensions similar to Df(2L)Exel7007. (E) Schematic diagram of overlapping deletion stocks. Because Df(2L)Exel8005 also shows an abnormal phenotype, candidate interval is narrowed down to 16 genes. (F) Representative OCT M-mode images from indicated stocks. (G) Summary data for M-mode images. f00122 and 22697 display abnormal cardiac function. (H) Representative quantitative real-time RT-PCR. f00122 and 22697 are inserted in the intron of *CG31665* gene as shown in cytological map of the *CG31665* gene. The map is modified from GBrowse at FlyBase. *CG31665* gene is disrupted in two deficiency stocks as shown in E. Summary data show a significant reduction in *CG31665* expression in all four stocks vs. w^{1118} controls. Data represent mean ± SE of OCT measurements (B, D, and G) and three independent experiments, each performed in triplicate (H). A 125 micron standard and 1 second scale bar is shown. *p<0.05 vs. w^{1118}.

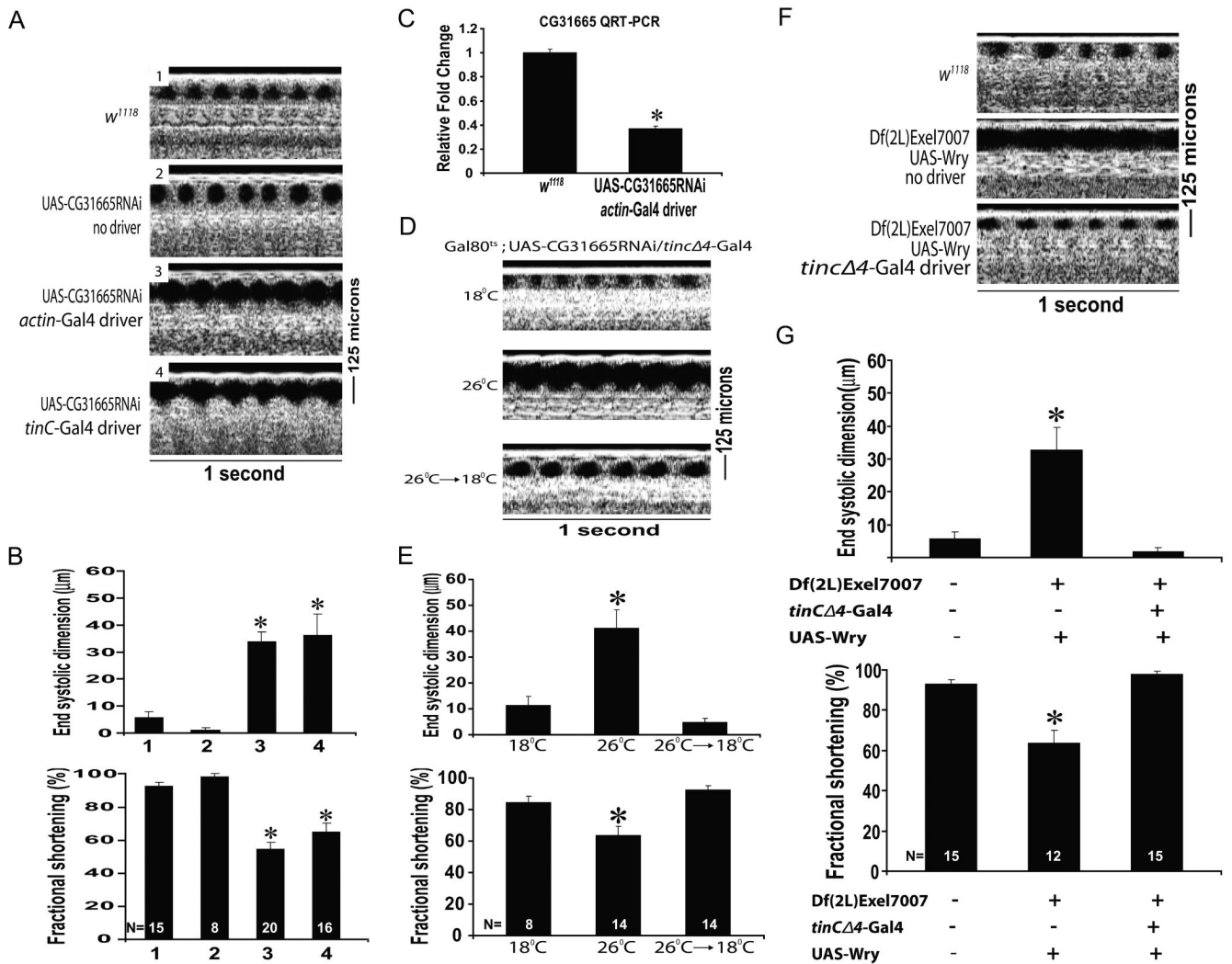**Figure 2.** *Actin* **promoter-induced or cardiac-specific** *tinC* **promoter-induced CG31665RNAi expression resulted in dilated cardiomyopathy and cardiac-specific promoter-induced Wry expression rescues the abnormal phenotype of Df(2L)Exel7007**
(A) Representative OCT M-mode images from indicated flies (B) Summary data for M-mode images. *Actin* or *tinC* promoter-induced CG31665RNAi expression caused abnormal cardiac function. (C) CG31665RNAi flies with *actin*-Gal4 have decreased *CG31665* expression. Representative quantitative real-time RT-PCR. Summary data show a significant reduction in *CG31665* expression in CG31665RNAi flies with *actin*-Gal4 vs. *w^{1118}* controls. (D) Representative OCT M-mode images from indicated flies. Flies were maintained at 18°C until eclosion. After flies were eclosed, the first set of flies was kept at 18°C and the second set of flies was kept at 26°C for 7 days. A third set of flies was kept at 26°C for 7 days and then switched back to 18°C for an additional 7 days. (E) Summary data for M-mode images. Temperature shift from 18°C to 26°C results in deterioration in cardiac function and a second temperature shift back to 18°C restores cardiac function. (F) Representative OCT M-mode images from indicated flies (G) Summary data for M-mode images. The introduction of *wry* transgene with cardiac-specific promoter into the context of Df(2L)Exel7007 restored normal cardiac function. Data represent mean ± SE of OCT measurements (B, E, and G) and three independent experiments, each performed in triplicate (C). *p<0.05 vs. controls.

A

Gal80^{ts} ; UAS-Dl.DN/$tinc\Delta4$-Gal4
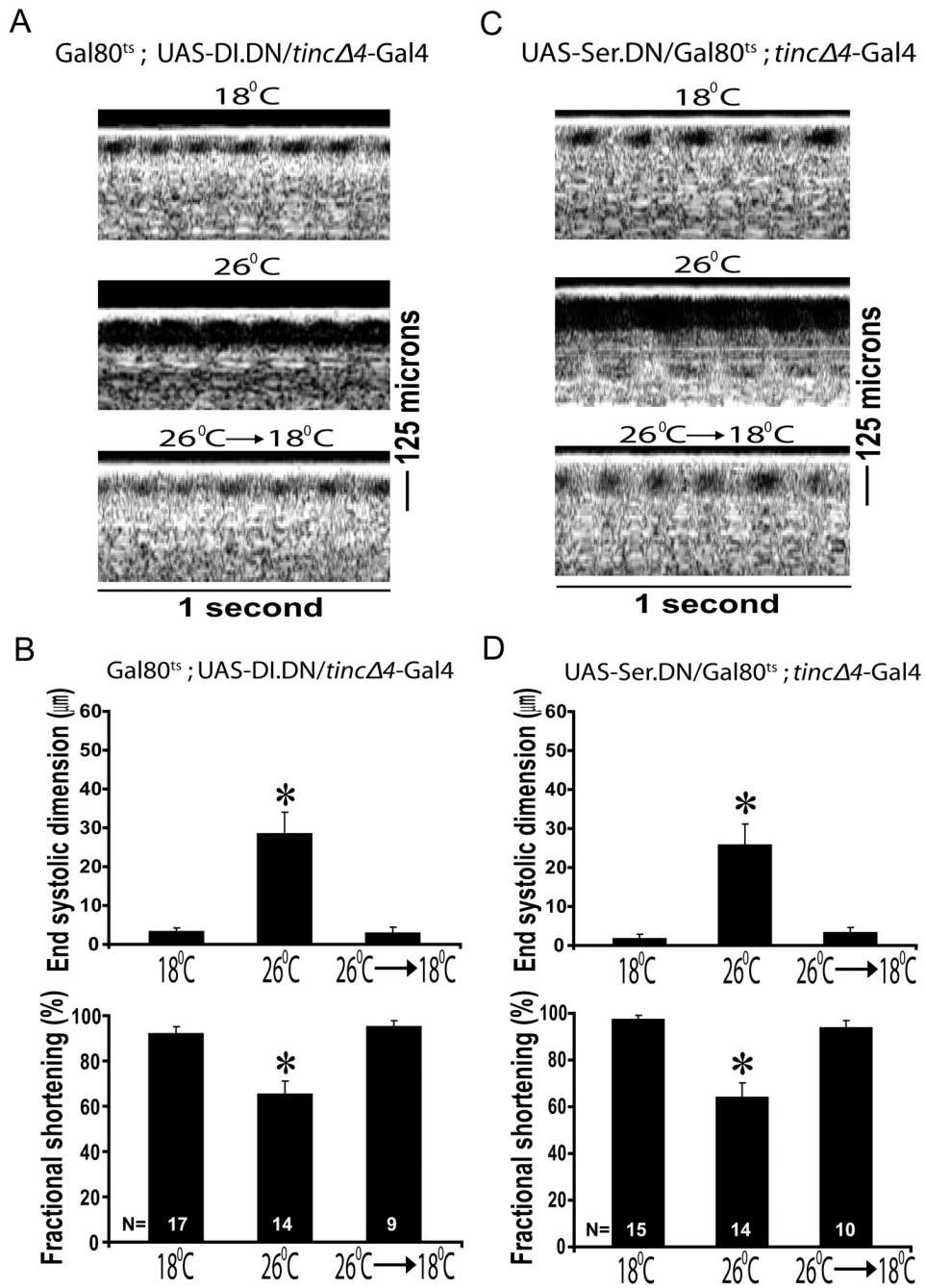
C

UAS-Ser.DN/Gal80^{ts} ; $tinc\Delta4$-Gal4

Figure 3. Temperature sensitive dominant-negative Delta or dominant-negative Serrate expression in adult fly heart resulted in inducible and reversible dilated cardiomyopathy
(A) Representative OCT M-mode images from indicated flies. (B) Summary data for M-mode images. (C) Representative OCT M-mode images from indicated flies. (D) Summary data for M-mode images. Temperature shift from 18°C to 26°C results in deterioration in cardiac function and a second temperature shift back to 18°C results in restoration of cardiac function. Data represent mean ± SE of OCT measurements. *$p<0.05$ vs. 18°C or 26°C to 18°C.
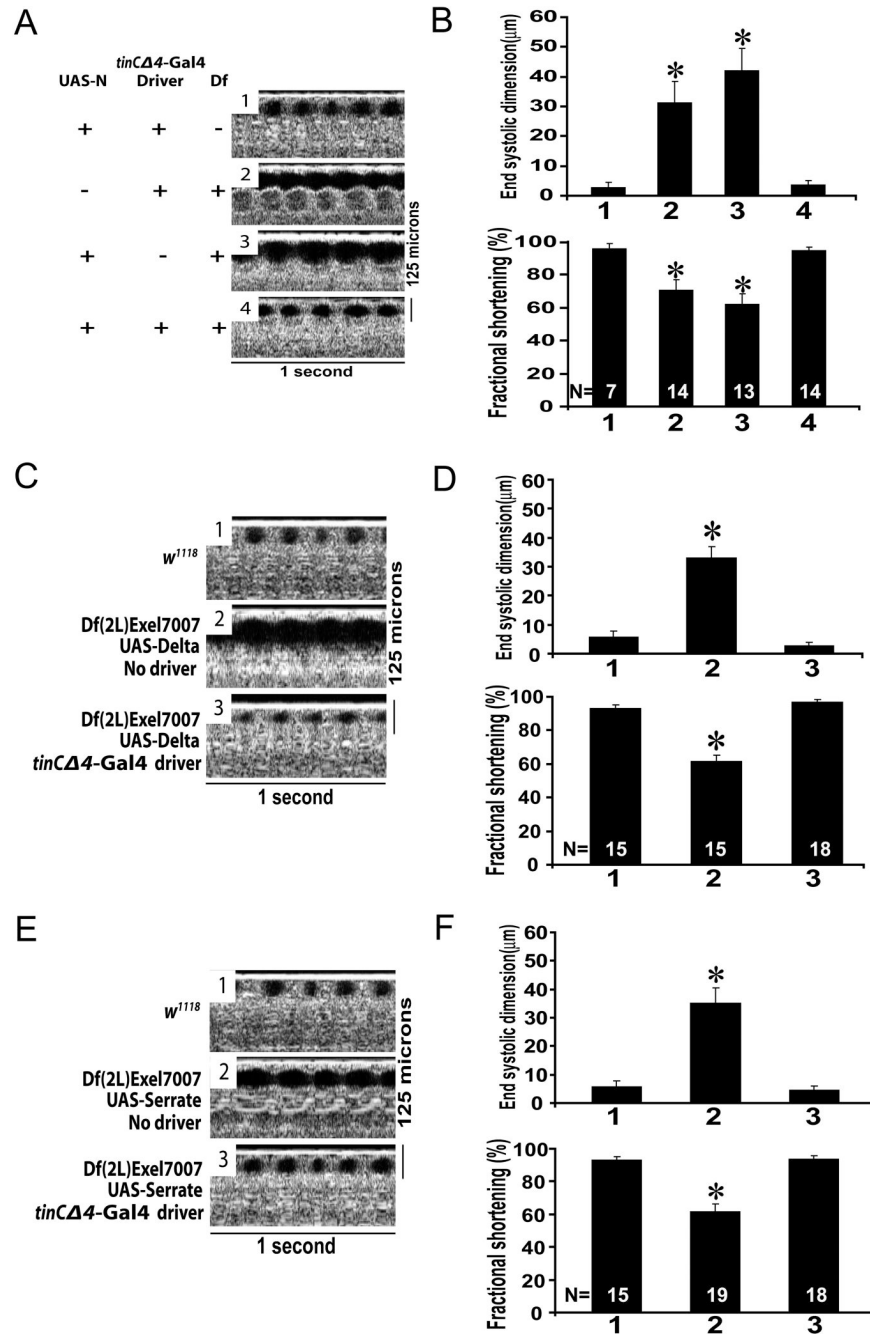
Figure 4. Cardiac-specific promoter-induced expression of Notch or Notch ligands can rescue the abnormal phenotype of Df(2L)Exel7007

(A) Representative OCT M-mode images from indicated flies. (B) Summary data for M-mode images. (C) Representative OCT M-mode images from indicated flies. (D) Summary data for M-mode images. (E) Representative OCT M-mode images from indicated flies. (F) Summary data for M-mode images. The introduction of *Notch (N) or Delta or Serrate* transgene with cardiac-specific promoter into the context of Df(2L)Exel7007 deletion restored normal cardiac function. Data represent mean ± SE of OCT measurements. *$p < 0.05$ vs. controls.
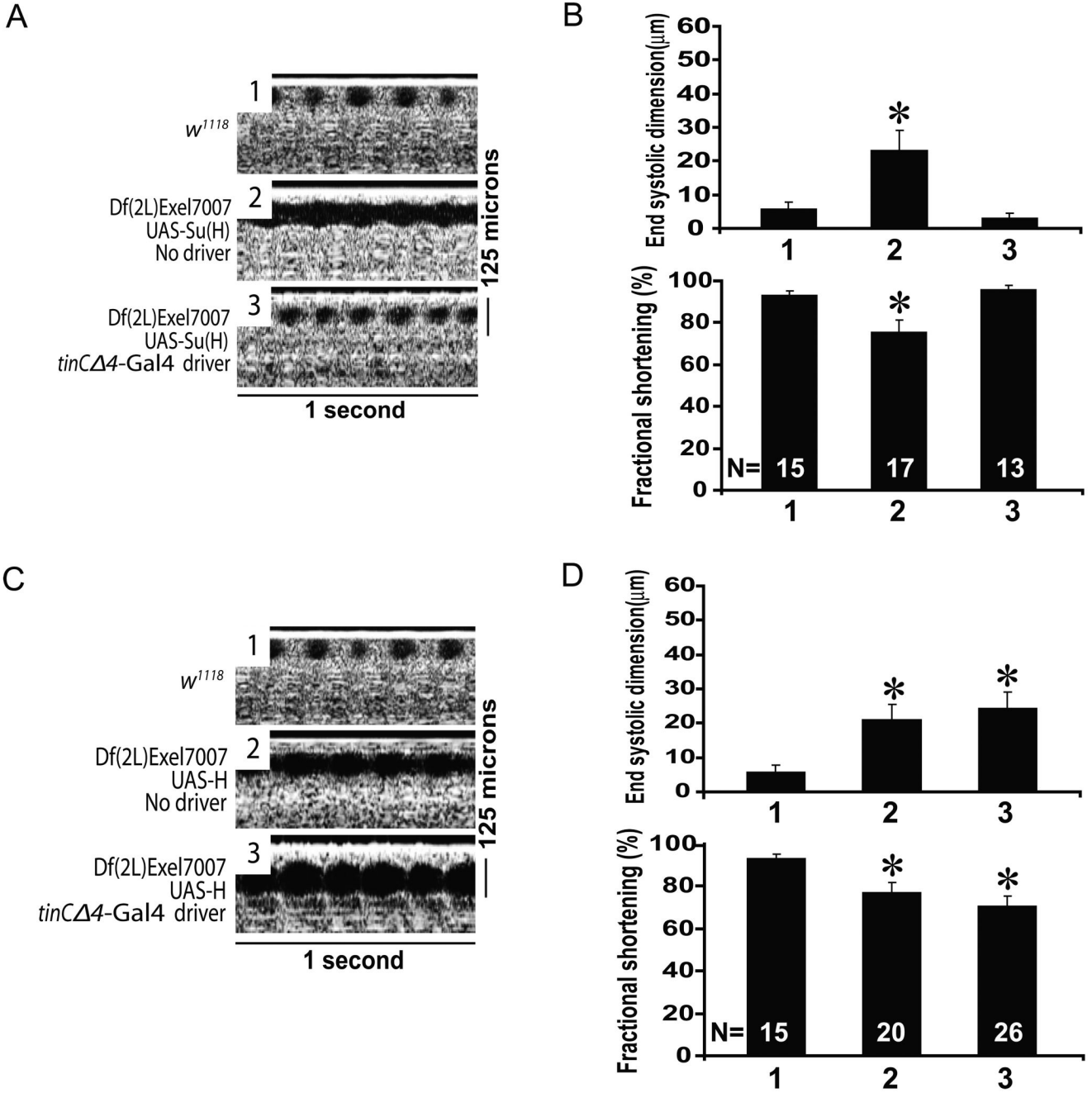
A

B

C

D

Figure 5. Cardiac-specific promoter-induced expression of Su(H) can rescue the abnormal phenotype of Df(2L)Exel7007
(A) Representative OCT M-mode images from indicated flies. (B) Summary data for M-mode images. (C) Representative OCT M-mode images from indicated flies. (D) Summary data for M-mode images. The introduction of *Su(H)* transgene [not *Hairless* (*H*) transgene] with cardiac-specific promoter into the context of Df(2L)Exel7007 deletion restored normal cardiac function. Data represent mean ± SE of OCT measurements. *p<0.05 vs. controls.
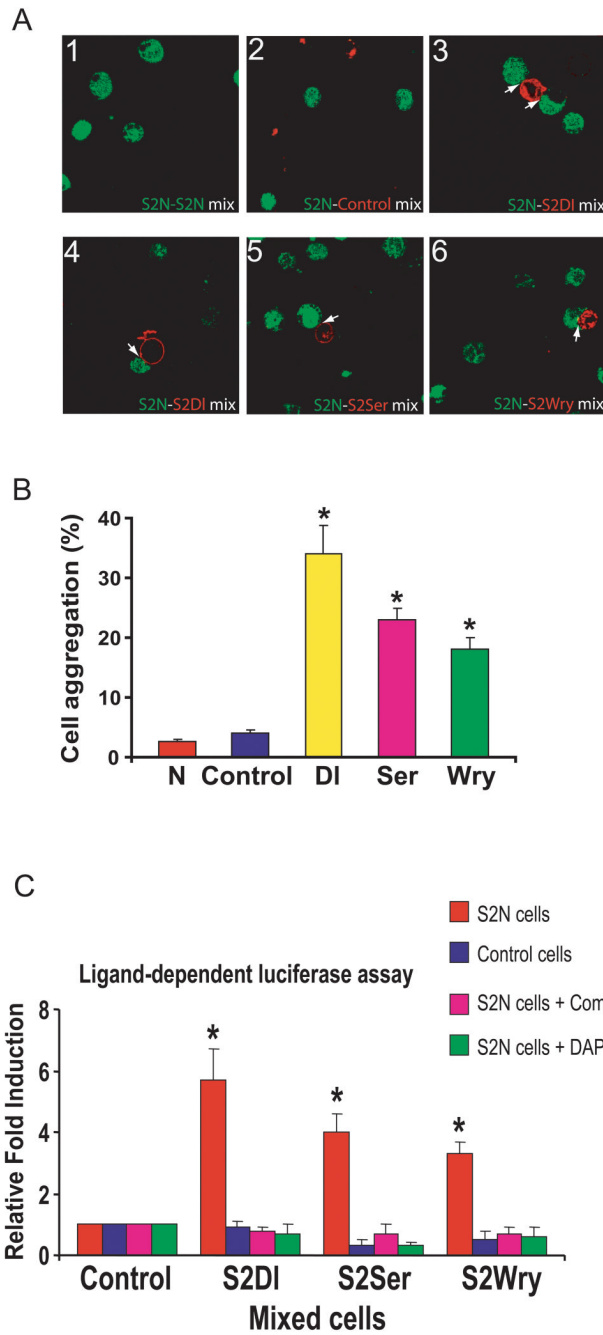
Figure 6. Wry can mediate cellular adhesion with Notch expressing cells and transactivate Notch signaling pathway in S2 cells

(A) Panels 1-3: representative cell-aggregation data from indicated cell mix. The cell mix used is labeled in each panel. Panels show merged confocal images of green cell tracker-labeled Notch cells and red cell tracker-labeled control (S2) or Delta cells. Panels 4-6: cell-aggregation assay of the Notch binding potential of different ligands. The cell mix used is labeled in each panel. Panels show merged cell tracker fluorescent images of Notch cells (green) and immunofluoresence images of ligand cells (red). Arrows represent the clustering of ligand and receptor at sites of cellular contact. (B) Graph shows the percentage of total Notch-expressing cells bound to cells that express different proteins or control cells. (C) Relative induction of

luciferase activity. Control or S2N cells were transfected with a reporter construct 2 × m3-Luc and treated with vehicle only (DMSO) or Compound E or DAPT for 24 hours and then mixed with S2 cells expressing Delta, Serrate or Wry. Activity was measured 4 h after mixing. Activation of Notch by S2Dl, S2Ser or S2Wry cells resulted in a significant increase in luciferase signal over controls, which were incubated with control or S2N cells treated with γ-secretase inhibitors. Data represent mean ± SE of at least four independent experiments, each performed in triplicate. *p<0.05 vs. controls. Original magnification × 150.
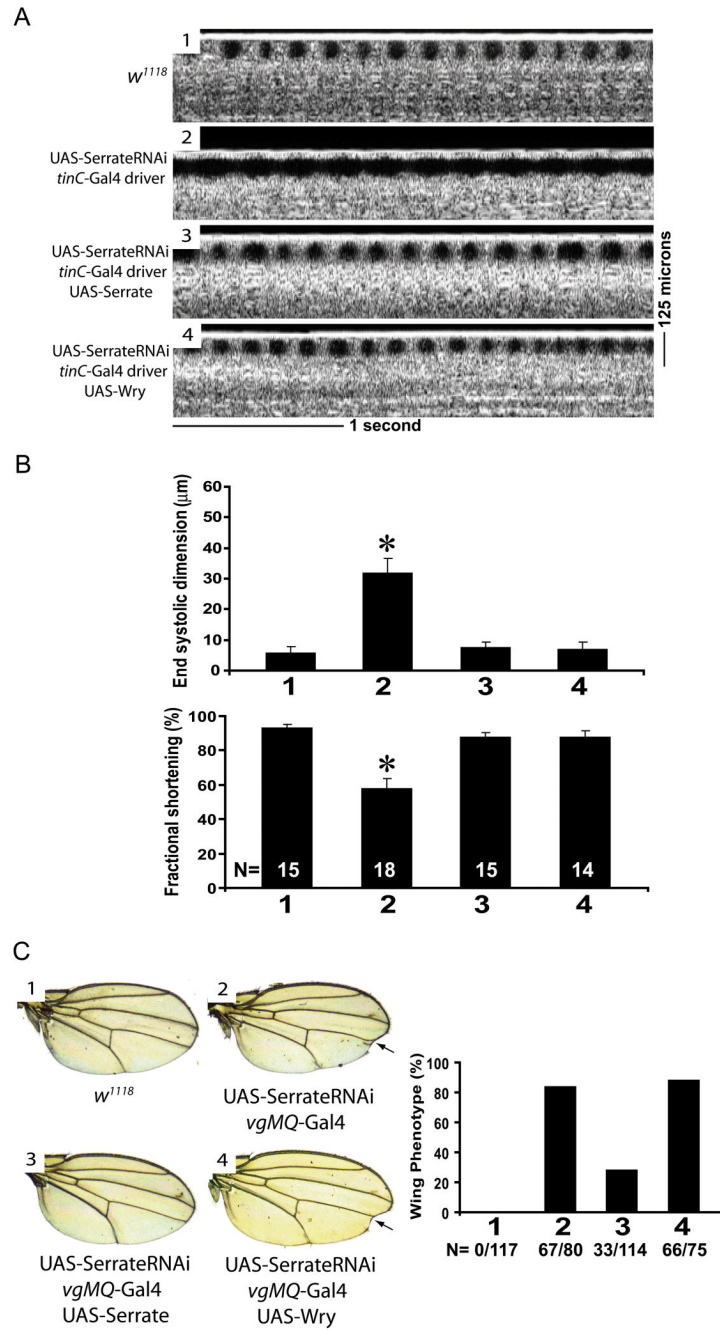
Figure 7. Cardiac-specific promoter-induced expression of Wry rescues the abnormal cardiac phenotype of SerrateRNAi flies

(A) Representative OCT M-mode images from indicated flies. (B) Summary data for M-mode images. Data represent mean ± SE of OCT measurements. *p<0.05 vs. controls. (C) Serrate loss-of-function mutants had abnormal wing vein morphology shown as arrows. Only Serrate is able to rescue the abnormal wing phenotype induced by Serrate knockdown. N are numbers of flies having a wing phenotype divided by numbers of total counted flies.