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

Il-Man Kim, Matthew J. Wolf, Howard A. Rockman

**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 because of 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. (Circ Res. 2010;106:1233-1243.)

**Key Words:** heart failure ■ cardiomyopathy gene ■ Drosophila, Notch signaling ■ optical coherence tomography

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 because of 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 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 Su(H) (suppressor of Hairless) transcription factor, resulting in the expression of specific target genes such as E(spl) (enhancer of split). 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 that assembles in a repressor complex on Notch target
Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSL</td>
<td>CBF1/suppressor of Hairless/Lag-1</td>
</tr>
<tr>
<td>DI</td>
<td>Delta</td>
</tr>
<tr>
<td>DN</td>
<td>dominant negative</td>
</tr>
<tr>
<td>E(spl)</td>
<td>enhancer of split</td>
</tr>
<tr>
<td>H</td>
<td>Hairless</td>
</tr>
<tr>
<td>N</td>
<td>Notch</td>
</tr>
<tr>
<td>NICD</td>
<td>Notch intracellular domain</td>
</tr>
<tr>
<td>OCT</td>
<td>optical coherence tomography</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>Ser</td>
<td>Serrate</td>
</tr>
<tr>
<td>Su(H)</td>
<td>suppressor of Hairless</td>
</tr>
</tbody>
</table>

genesis.\textsuperscript{21,22} Although Notch signaling is crucial in the development of many different Drosophila tissues including the heart,\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 DI and Ser. 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 RNA interference (RNAi) strains were obtained from the Vienna Drosophila RNAi Center.

Detailed descriptions of fly stocks, cardiac measurements in adult Drosophila, quantitative RT-PCR, imaging wings, Drosophila cell culture and cell aggregation assays, ligand-dependent luciferase assay, and statistical analysis are provided in the expanded Methods section, available in the Online Data Supplement at http://circres.ahajournals.org.

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 3 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. Because 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$^{118}$ 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 130-Kb 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 2 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 and 1G and data not shown).

Because both f00122 and 22697 are inserted in the gene CG31665, we examined CG31665 gene expression by quantitative RT-PCR in Df(2L)Exel7007, Df(2L)Exel8005, f00122, and 22697. All 4 stocks displayed a reduction in CG31665 expression (Figure 1H). Additionally, f00122 had normal expression levels of 2 neighboring genes (c-cup and CG31663), whereas the 2 deficiency stocks showed reduced expression of c-cup and CG31663 as expected (data not shown).

To validate that the piggyBac transposon insertion altered cardiac function through its effect on CG31665, we excised the piggyBac element from f00122 using precise excision.\textsuperscript{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 w1118 controls (Figure 2C).

Because 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 postdevelopmentally causes dilated cardiomyopathy, we engineered w; p[tub-Gal80ts]; p[tinC Δ4-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 using the cardiac-specific driver, tinC Δ4-Gal4. The Gal80 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/Gal4 complex dissociates and Gal4 then binds to the UAS-CG31665RNAi to activate its transcription. 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 postdevelopmental loss of expression of CG31665 in adult fly heart results in inducible and reversible dilated cardiomyopathy. Similar data were 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 Gal80ts/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 named 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
Wry contains EGF repeats and a transmembrane domain but does not possess a DSL (Dl-Ser-Lag) domain (Online Figure IV), suggesting that it may be involved in Notch signaling. To determine the transcriptional pattern of wry expression, we performed quantitative RT-PCR analysis and show that wry is expressed in all 4 life stages and parallels the expression pattern of the known Notch ligand Ser (Online Table I and Online Results). We next examined the heart function in w;ptub-Gal80ts;p{tinC/H9004}4-Gal4 flies harboring p{UAS-Dl.DN(dominant-negative)} or UAS-Ser.DN to determine whether 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 and D and data not shown). Because 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

Because 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 transgene driven by a cardiac-specific promoter
into the context of Df(2L)Exel7007 deletion restored normal cardiac function (Figure 4A and 4B). Additionally, cardiac-specific expression of Notch ligands rescued the abnormal phenotype of Df(2L)Exel7007 (Figure 4C through 4F). Finally, cardiac-specific expression of Su(H), a positive regulator of Notch signaling, rescued the abnormal phenotype of Df(2L)Exel7007, whereas the Notch antagonist Hairless, a negative regulator of Notch signaling, did 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 δ-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**

Because cardiac-specific expression of Notch ligands rescued 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, Dl, Ser, or Wry and examined the ability of Notch to bind different ligands. Confocal images of immunofluorescent cells containing Dl, 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% to 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 Dl.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 to 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 Online Results). 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 SerRNAi Flies

Because 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 Ser loss-of-function mutants. The cardiac-specific expression of a wry transgene into the context of SerRNAi restored normal cardiac function. In contrast, we show that the expression of Wry by a wing-specific vgMQ promoter did not rescue the wing morphological abnormalities associated with SerRNAi flies, whereas Ser restored both the cardiac and the wing phenotype in the context of SerRNAi (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 2 types of Notch ligands, which correspondingly activate distinct signaling mechanisms.48–50 Typical Notch ligands, which include Ser and Dl in Drosophila and Jagged 1 to 2 and Dl-like 1, 3, and 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
S2N cells treated with luciferase signal over controls, which were incubated with control S2Dl, S2Ser, or S2Wry cells resulted in a significant increase in Activity was measured 4 hours after mixing. Activation of Notch by 24 hours and then mixed with S2 cells expressing Dl, Ser, or Wry. S2N cells were transfected with a reporter construct 2 X m3-Luc and treated with vehicle only (DMSO) or Compound E or DAPT for

Figure 6. Wry can mediate cellular adhesion with Notch-expressing cells and transactivate Notch signaling pathway in S2 cells. A, Panels 1 to 3 are representative of cell-aggregation data from indicated cell mix. The cell mix used is labeled in each panel and shows merged confocal images of green cell tracker–labeled Notch cells and red cell tracker–labeled control (S2) or Dl cells. Panels 4 to 6 show cell-aggregation assay of the Notch binding potential of different ligands. The cell mix used is labeled in each and shows merged cell tracker fluorescent images of Notch cells (green) and immunofluorescence 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 X m3-Luc and treated with vehicle only (DMSO) or Compound E or DAPT for 24 hours and then mixed with S2 cells expressing Dl, Ser, or Wry. Activity was measured 4 hours 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 means±SE of at least 4 independent experiments, each performed in triplicate. *P<0.05 vs controls. Original magnification, ×150.

activation of Notch target genes such as E(spl) in Drosophila and HES1 in mammals. In contrast, atypical Notch ligands like DNER, F3/Contactin and NB-3 in mammals have no DSL domain, and transduce noncanonical Notch signaling to the CSL-NICD-Deltex complex for the differentiation of progenitor cells through MAG transcriptional activation. Notch signals are transduced to the canonical pathway (CSL-NICD-Mastermind signaling cascade) or the noncanonical pathway (CSL-NICD-Deltex signaling cascades) based on the expression profile of Notch ligands, Notch receptors, and Notch signaling modifiers. Interestingly, Wry does not have a DSL domain although Wry does share EGF domain homology with the 2 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. 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. These results are consistent with a recent study that used 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.

In general, an identification of abnormalities that cause dilated cardiomyopathies in adult Drosophila requires a distinction between mutations that alter heart formation from postdevelopmental 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. Abnormal cardiac function in adult Drosophila can result from defects in embryonic dorsal vessel development mimicking congenital abnormalities and abnormalities arising in postdevelopmental. To address this issue we used a temperature sensitive Gal80t 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 nonautonomous manner. Indeed, nonautonomous influence of the epi-/pericardial cells on the function of the fly heart has been postulated. 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. 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 orthologs 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.

Acknowledgments

We thank Weili Zou for technical assistance, Michelle Casad for generating Drosophila reagents, and Teresa Lee for cloning.

Sources of Funding

This work was supported by NIH grants HL083065 (to H.A.R.) and K08 HL085072 (to M.J.W.), American Heart Association National Innovative Research Grant 0970391N (to M.J.W.), American Heart...
Disclosures

References

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-Ser-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 2 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 orthologs involved in Notch signaling may be important in the pathogenesis of human dilated cardiomyopathy.
Gene Deletion Screen for Cardiomyopathy in Adult Drosophila Identifies a New Notch Ligand
Il-Man Kim, Matthew J. Wolf and Howard A. Rockman

Circ Res. 2010;106:1233-1243; originally published online March 4, 2010;
doi: 10.1161/CIRCRESAHA.109.213785

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/106/7/1233

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2010/03/04/CIRCRESAHA.109.213785.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Supplemental Materials:

Detailed Methods

Fly stocks. 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. w*; P{tubP-GAL80[ts]}10; TM2/TM6B, Tb[1] was obtained from the Bloomington *Drosophila* Stock Center. Transgenic p{UAS-N}, p{UAS-Serrate}, p{UAS-Delta}, p{UAS-Su(H)}, and p{UAS-H} fly lines were kindly provided by Dr. Spyros Artavanis-Tsakonas. For UAS transgene expression, we used several Gal4 driver lines. The flies express Gal4 by the cardiomyocyte-specific constitutive tinC promoter or the ubiquitous actin 5c promoter or the heat-shock inducible hsp70 promoter or the wing-specific vgMQ promoter. Transgenic flies harboring p{UAS-Wry} were engineered as previously described. Briefly, the cDNA encoding wry was obtained by RT-PCR of total RNA, sequenced, and subcloned into pUAST. All fly stocks were maintained on yeast paste nutrient media (yeast, cornmeal, molasses, and agar with tegosept) and kept at either 18°C or 26°C according to standard procedures.

Cardiac measurements in adult *Drosophila*. Cardiac function in adult *Drosophila* was measured using a custom built OCT microscopy system (Bioptigen, Inc) as previously described. Briefly, adult *Drosophila* at 7 days post eclosion were briefly subjected to CO₂, gently placed on a soft gel support, and allowed to fully awaken based on body movement. Multiple OCT m-modes of heart function from awake *Drosophila* were recorded along with a 125 micron standard. All images were processed using ImageJ software using Bioptigen software. M-mode images were used to measure cardiac parameters determined from 3 consecutive heart beats. Cardiac chamber dimensions were measured from the edge of the superior and inferior walls during mid-diastole for EDD (end-diastolic dimension) and mid-systole for ESD (end-systolic dimension). Fractional shortening (FS) was calculated as [(EDD - ESD)/EDD] × 100 and represents a surrogate measurement of contractility. All OCT imaging was conducted at room temperature.

QRT-PCR. Thirty flies of 7 days age were individually collected from each group and embryos, larvae, pupae, and dissected adult tissues were collected from *w*¹¹¹⁸ to define the transcriptional expression pattern of wry and ser. Total RNA was prepared using RNA-Bee (Tel-Test "B," Inc.) and treated with RNase-free DNaseI to remove genomic DNA. cDNA was synthesized using invitrogen SuperScript II reverse transcriptase. Applied Biosystems Taqman Gene expression assays were used to perform quantitative (real time) RT-PCR (fly wry, Dm01808177_m1, fly ser, Dm02151542_m1, and fly ribosomal protein L32, Dm02151827_g1 for endogenous control). The following reaction components were used for each probe: 2 uL cDNA, 12.5 uL 2X TaqMan Universal PCRMaster Mix (Applied Biosystems), 1.25 uL probe, and 9.25 uL water in a 25 µL total volume. Reactions were amplified and analyzed in triplicate using an ABI PRISM® 7000 Sequence Detection System. PCR reaction conditions were as follows: Step 1: 50°C for 2 minutes, Step 2: 95°C for 10 minutes, Step 3: 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. Expression relative to ribosomal protein L32 was calculated using 2⁻ΔΔCt and levels were normalized to baseline.

Imaging wings. Adult wings were removed and placed on a cover slide. Images were taken on a Zeiss Microscope with a x5 objective using PXCam ARC software and assembled using Adobe Photoshop.
**Drosophila cell culture and cell aggregation assays.** *Drosophila* S2 cells were maintained at 25°C in standard Schneider M3 medium (Invitrogen) supplemented with 10% fetal bovine serum. S2N cells and S2DI cells were described previously\(^{11,12}\) and were kindly provided by Dr. Spyros Artavanis-Tsakonas. For Ser and Wry expression, S2 cells were transiently transfected with pMT/Myc-Ser (S2Ser) or pUAST/HA-Wry and ubiquitin-Gal4 (S2Wry). All transfections were performed using Cellfectin reagent (Invitrogen). Cell aggregation assays were performed as described previously\(^{11,13}\). Briefly, S2N cells were induced with 1 mM CuSO\(_4\). In parallel, separate cultures of S2, S2DI, S2Ser and S2Wry cells were induced. After induction, S2N cells were incubated with green cell tracker (Invitrogen) and S2 or S2DI cells were incubated with red cell tracker (Invitrogen) as described previously\(^{14}\). And then, cells were mixed and aggregated with gentle rotation before transferring the cell suspension to confocal dishes coated in collagen (Roche) for confocal micrographs. For immunostaining, S2N cells were incubated with green cell tracker and mixed with S2DI, S2Ser, or S2Wry cells. And then, cells were incubated in Rabbit anti-DI (1:50, Santa Cruz) or anti-Myc (1:1000, Sigma) or anti-HA (1:1000, Sigma) polyclonal antibody, followed by incubation with Texas-red-conjugated anti-rabbit (1:500, Invitrogen) secondary antibody. Confocal images were obtained as described previously\(^{15}\).

**Ligand-dependent luciferase assay.** Ligand-dependent Notch luciferase assays in S2 cells were performed as described previously\(^{12}\). Briefly, S2N cells were transfected with 2 X m3-Luc construct and induced with 1 mM CuSO\(_4\). In parallel, separate cultures of S2, S2DI, S2Ser and S2Wry cells were induced and 0.5 ml of the cultures were mixed with 0.5 ml of transfected S2N cells and rocked at room temperature. Samples of 100 \(\mu\)l were taken at 0h and 4 h, and luciferase signals were developed using a Luc-Screen assay (Applied Biosystems) and measured using a NOVOSTar plate reader (BMG) as described previously\(^{16}\). To quantify the fold induction after ligand activation, values at 0 h were subtracted from values at 4 h to correct for basal expression, then the corrected values at 4 h for S2DI, S2Ser or S2Wry cells were divided by the corrected values at 4 h for S2 cells. To examine the effect of \(\gamma\)-secretase inhibitors, S2N cells were pretreated with 100nM of Compound E (Alexis Biochemicals) or DAPT (Sigma) or vehicle DMSO alone before mixing with ligand-expressing cells as described previously\(^{17,18}\). Luciferase assays in C2C12 cells were performed as described previously\(^{19}\). Briefly, C2C12 cells were transfected with a reporter construct pGa981-6 using FuGENE6 reagents (Roche Molecular Biochemicals). Fourty-eight hours after transfection, cells were co-cultured with ligand-expressing HEK293 cells. Luciferase signals were measured as above.

**Statistical Analysis.** Data are expressed as mean±SE. Statistical significance was determined using one-way ANOVA with Bonferroni correction for multiple comparisons or Student’s unpaired t-tests. A P value of < 0.05 was considered statistically significant.
Supplemental Results

**Wry is expressed from embryonic stages through adulthood**

To determine the transcriptional pattern of wry expression, we performed QRT-PCR analysis in four life stages of w¹¹¹; embryo, larva, pupa, and adult. Wry was expressed in all four stages and highly expressed in 16-24 hour embryo, pupa, and adult head. The expression of wry in dissected adult heart was detectable but low compared to other tissues such as muscles (Online Table I). We also examined the transcriptional pattern of ser expression using QRT-PCR. The Notch ligand Ser was expressed in all four life stages, highly enriched in pupa, and at a low level in the adult heart which paralleled the expression of wry (Online Table I). These Notch ligands appear to play an important role in maintaining the normal cardiac function in the adult fly despite the relatively low expression in the heart.

**Wry can not transactivate Notch signaling in mammalian cells**

The fact that Wry acts as a functional Notch ligand in flies prompted us to examine whether Wry activates transcription downstream of Notch signaling in mammalian cells. To assess the ligand activity of Wry, we performed luciferase reporter assays using C2C12 mouse myoblast cell lines that endogenously express Notch and its downstream signaling molecule RBP-J. We transfected C2C12 cells with a reporter construct pGa981-6 carrying RBP-J binding sites that has been shown to be transactivated by NICD and suppressed by a dominant-negative form of RBP-J. When C2C12 cells were co-cultured with HEK 293 cells overexpressing Wry, promoter activity was not transactivated compared with those mixed with control cells. As a positive control, cells overexpressing DNER (a mammalian Notch ligand) mixed with C2C12 cells showed a 7-fold increase in promoter activity compared to control cells (Online Figure VII). These results suggest that Wry may not function as a Notch ligand in mammalian cells.
Supplemental References


Supplemental Figure Legends

Online Figure I. Both the Df(2L)Exel7007 and Df(2L)Exel8005 display the abnormal cardiac phenotype with absence of balancer chromosomes and precise excision of piggyBac element from f00122 restores normal cardiac function. (A) Breeding strategy to separate effects of balancer vs. deletion chromosome. (B) Representative OCT M-mode images of F1 flies from the cross in A. A 125 micron standard and 1 second scale bar is shown. (C) Summary data for M-mode images. Both the Df(2L)Exel7007/+ and Df(2L)Exel8005/+ have dilated cardiomyopathy phenotypes. Each graph represents the summary data for OCT measurements and is expressed as the mean +/- SE (n=15, 20, and 11 as shown in the order). (D) Breeding strategy to excise the piggyBac element from f00122. The excision of the piggyBac element from f00122 is based on the introduction of the piggyBac transposase into the germ line. Viable offsprings that have the piggyBac element excised are identified by eye color change. (E) Summary data for M-mode images from w^{1118}, f00122, and precise excision line Ex(f00122). Ex(f00122) restores normal cardiac function. Each graph represents the summary data for OCT measurements and is expressed as the mean +/- SE (n=15, 18, and 15 as shown in the order). * : p<0.05 vs CyO/+ or w^{1118} or EX(f00122).
Online Figure II. Heat shock-induced CG31665 RNAi expression in adult fly resulted in dilated cardiomyopathy. (A) Representative OCT M-mode images from Hsp70-Gal4;UAS-CG31665 RNAi with either no heat shock or heat shock and UAS-CG31665 RNAi flies with heat shock. We subjected 3 day old flies to daily 1h heat shocks at 37°C for 3 days and examined cardiac function at 7 days post eclosion. A 125 micron standard and 1 second scale bar is shown. (B) Summary data for M-mode images. Heat shock-induced CG31665 RNAi expression caused abnormal cardiac function. Each graph represents the summary data for OCT measurements and is expressed as the mean +/- SE (n=8, 18, and 13 as shown in the order). (C) Hsp70-Gal4;UAS-CG31665 RNAi flies with heat shock have decreased CG31665 expression. Representative quantitative real-time RT-PCR of either no heat shock or heat shock in UAS-CG31665 RNAi flies with hsp70-Gal4. Summary data show a significant reduction in CG31665 expression in heat shock vs. no heat shock controls. Data represent mean ± SE of at least three independent experiments, each performed in triplicates. *: p<0.05 vs. controls.
Online Figure III. Cardiac-specific tinC promoter-induced RNAi expression for other candidate genes displayed normal cardiac function. (A) Representative OCT M-mode images of F1 flies from the cross in UAS-Dicer2; tinC-Gal4 and UAS-RNAi for candidate genes. The numbers indicate Vienna RNAi stock numbers for each gene. A 125 micron standard and 1 second scale bar is shown. (B) Summary data for M-mode images. Cardiac induced RNAi expression for other candidate genes caused normal cardiac phenotypes. Each graph represents the summary data for OCT measurements and is expressed as the mean +/- SE (n=15, 11, 12, 10, 13, 13, 13, 10, 12, 15, 9, 10, 11 and 10 as shown in the order).
Online Figure IV

A

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delta</td>
<td>KGFTNPIQFPFSWPGTFLIVEAWHDTNNNSGNARTNKLLIGQRLVQDGSVEVSSEMKTN 167</td>
</tr>
<tr>
<td>Serrate</td>
<td>---VGAIVLPTFRKTSSFHILQALDMYNTS---YPDAERLIETSYSVILPSFBKGL 223</td>
</tr>
<tr>
<td>Wry</td>
<td>DVMLAQQQTASLNLIVDPLKIDQRIPIIROKINDLQETQQTQHTQHENAEYKQNLMPN 240</td>
</tr>
<tr>
<td>Delta</td>
<td>KSESQYTSLELYIFRTCDNLYYGSGCAKPCPRDD---------SFQHSTCSETTEFILCLT 219</td>
</tr>
<tr>
<td>Serrate</td>
<td>DHIGNARNAYVRQVAVYNTTCTTFPRPRDD---------QFGHACGSESQKLCLN 275</td>
</tr>
<tr>
<td>Wry</td>
<td>EVEMMVDKEVIEMKIQNQIEKQSGSTTVKPTTSPHCQGTTADFTQKLLADQKDLITEI 300</td>
</tr>
<tr>
<td>Delta</td>
<td>GWQGDYCHIPKCAKGEHGHCDKPNQVCQQLGWGKALGCEVLNPCHTNKPT 276</td>
</tr>
<tr>
<td>Serrate</td>
<td>GWQGVNGEAICKAGCPVHGKDCRPGECEFCRGFWGKPLCNCMDYFPGCKHSNCNGSAW 335</td>
</tr>
<tr>
<td>Wry</td>
<td>ESRFQHLHETTTLRPRTTVVQHPLGNTFIDRVRKSSFEFPQMRASSVMAMPHIDFANTKFQ 360</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Protein</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delta</td>
<td>823aa</td>
</tr>
<tr>
<td>Serrate</td>
<td>1407aa</td>
</tr>
<tr>
<td>Wry</td>
<td>1157aa</td>
</tr>
</tbody>
</table>

Legend:
- DSL domain
- EGF repeat
- WVC domain
- Transmembrane domain
Online Figure IV. Wry does not have a DSL domain. (A) Amino acid alignments in Delta, Serrate, and Wry. * represents amino acid identity in three proteins. The DSL (Delta-Serrate-Lag) domain is underlined and characters in red colors represent the consensus sequence. There are some homologies in EGF repeats shown as yellow boxes. (B) Schematic representation of domain architecture in Delta, Serrate, and Wry. While two Notch ligands have a DSL domain, Wry does not have a DSL domain. Additionally, Serrate has a VWC (von willebrand factor type C) domain.
Online Figure V

A

B

C

D

E

Online Figure V
Online Figure V. Disrupting Notch signaling components caused dilated cardiomyopathy and Wry deficiency stocks have no wing phenotypes. (A) Representative OCT M-mode images from w1118, Serrate Df, Delta Df, and Su(H)Df. A 125 micron standard and 1 second scale bar is shown. (B) Summary data for M-mode images. Df(3R)Exel6208, Df(3R)BSC475, and Df(2L)BSC299 displayed abnormal cardiac function. Each graph represents the summary data for OCT measurements and is expressed as the mean +/- SE (n=15, 14, 19, and 23 as shown in the order). (C) Representative OCT M-mode images from w1118 and UAS-N RNAi/tincΔ4-Gal4. A 125 micron standard and 1 second scale bar is shown. (D) Summary data for M-mode images. Cardiac-specific promoter-induced N RNAi expression caused abnormal cardiac function. Each graph represents the summary data for OCT measurements and is expressed as the mean +/- SE (n=15 and 18 as shown in the order). (E) While Delta loss-of-function mutants have thickening of wing vein shown as arrows, Wry deficiency stocks have normal wing morphology. *: p<0.05 vs. w1118.
Online Figure VI. Cardiac-specific promoter-induced human Dsg or Medea expression can not rescue the abnormal phenotype of Df(2L)Exel7007. (A) Representative OCT M-mode images from flies harboring UAS-Dsg and tinc\textsuperscript{Δ4}-Gal4 driver, flies harboring Df(2L)Exel7007 and UAS-Dsg, and flies harboring Df(2L)Exel7007, UAS-Dsg and tinc\textsuperscript{Δ4}-Gal4 driver. A 125 micron standard and 1 second scale bar is shown. (B) Summary data for M-mode images. The introduction of Dsg transgene with cardiac-specific promoter into the context of Df(2L)Exel7007 deletion did not restore normal cardiac function. Each graph represents the summary data for OCT measurements and is expressed as the mean +/- SE (n=8, 7, and 9 as shown in the order). (C) Representative OCT M-mode images from flies harboring UAS-Medea and tinc\textsuperscript{Δ4}-Gal4 driver, flies harboring Df(2L)Exel7007 and UAS-Medea, and flies harboring Df(2L)Exel7007, UAS-Medea and tinc\textsuperscript{Δ4}-Gal4 driver. A 125 micron standard and 1 second scale bar is shown. (B) Summary data for M-mode images. The introduction of Medea transgene with cardiac-specific promoter into the context of Df(2L)Exel7007 deletion did not restore normal cardiac function. Each graph represents the summary data for OCT measurements and is expressed as the mean +/- SE (n=11, 7, and 9 as shown in the order). *: p<0.05 vs. controls.
Online Figure VII

Ligand-dependent luciferase assay

Relative Fold Induction

Control  DNER  Wry

*
Online Figure VII. Wry can not transactivate Notch signaling pathway in mammalian cells. Relative induction of luciferase activity. C2C12 cells were transfected with a reporter construct pGa981-6 carrying RBP-J binding sites and then co-cultured with HEK 293 cells expressing DNER (a mammalian Notch ligand), Wry, or a control vector (CMV-empty). Activity was measured 48 h after start of co-culture. Data represent mean ± SE of at least three independent experiments, each performed in triplicate. *p<0.05 vs. controls.
Online Table I. Transcriptional expression patterns of wry and ser in wild-type flies at four life stages.

<table>
<thead>
<tr>
<th></th>
<th>wry</th>
<th>ser</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-8h</td>
<td>1 ± 0.03</td>
<td>17.9 ± 4.7</td>
</tr>
<tr>
<td>8-16h</td>
<td>176.3 ± 18.6</td>
<td>86.4 ± 10.3</td>
</tr>
<tr>
<td>16-24h</td>
<td>347 ± 66.7</td>
<td>17.8 ± 2.3</td>
</tr>
<tr>
<td>Larva</td>
<td>25.1 ± 6.8</td>
<td>5.6 ± 2.2</td>
</tr>
<tr>
<td>Pupa</td>
<td>352 ± 90.3</td>
<td>626.9 ± 30.4</td>
</tr>
<tr>
<td>Adult</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head part</td>
<td>470 ± 102.6</td>
<td>5.1 ± 1.2</td>
</tr>
<tr>
<td>Thorax part</td>
<td>155.3 ± 41.3</td>
<td>1.3 ± 0.9</td>
</tr>
<tr>
<td>Abdomen part</td>
<td>2.9 ± 0.7</td>
<td>7.2 ± 1</td>
</tr>
<tr>
<td>Heart</td>
<td>0.3 ± 0.06</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>Leg Muscle</td>
<td>61.7 ± 12.5</td>
<td>30.5 ± 4.4</td>
</tr>
<tr>
<td>Wing</td>
<td>5.8 ± 1.7</td>
<td>2.7 ± 0.3</td>
</tr>
</tbody>
</table>

Representative quantitative real-time RT-PCR. Total RNA and cDNA were prepared from embryos in indicated time points after birth, larvae, pupae, and dissected adult tissues of w1118. Summary data show relative gene expression of wry and ser at all four stages (expressed as fold-change compared to the wry expression level at 0-8h embryo). Data represent mean ± SE of at least three independent experiments, each performed in triplicate.