Pygopus Maintains Heart Function in Aging Drosophila Independently of Canonical Wnt Signaling

Min Tang, PhD; Wuzhou Yuan, PhD; Xiongwei Fan, PhD; Ming Liu, MD; Rolf Bodmer, PhD; Karen Ocorr, PhD; Xiushan Wu, PhD

Background—Heart function declines with age, but the genetic factors underlying such deterioration are largely unknown. Wnt signaling is known to play a role in heart development, but it has not been shown to be important in adult heart function. We have investigated the nuclear adapter protein encoded by pygopus (pygo), which mediates canonical Wnt signaling, for roles in aging-related cardiac dysfunction.

Methods and Results—Using the Drosophila heart model, we show that cardiac-specific pygo knockdown in adult flies causes a significant (4- to 5-fold) increase in cardiac arrhythmias (P<0.001) that worsened with age and caused a significant decrease in contractility (−54%; P<0.001) with systolic dysfunction. Immunohistochemistry revealed structural abnormalities that worsened with age, and both functional and morphological alterations were ameliorated by pygo overexpression. Unexpectedly, knockdown of 2 other Wnt signaling components, β-cat/armadillo or TCF/pangolin, had relatively milder effects on cardiac function. Double-heterozygous combinations of mutants for pygo and canonical Wnt signaling components had no additional effect on heart function over pygo heterozygotes alone. However, double knockdown of pygo and Ca2+/calmodulin-dependent protein kinase II caused additional arrhythmia compared with pygo knockdown alone, suggesting that some of the effects of pygo are mediated by Ca2+ signaling. In the isoproterenol-induced hypertrophic mouse model, we show that Pygo1 protein levels are increased.

Conclusions—Our data indicate that Pygo plays a critical role in adult heart function that is Wnt signaling independent and is likely conserved in mammals. (Circ Cardiovasc Genet. 2013;6:472-480.)

Key Words: arrhythmias, cardiac ■ atrial fibrillation ■ cardiac defects ■ cardiomyopathies ■ hypertrophy ■ physiopathology ■ systolic time interval

Cardiac disease and heart failure is the leading cause of death in industrialized countries. Aging is associated with many changes in the cardiovascular system, and it is a major risk factor for the development of cardiovascular disease and heart failure. Substantial improvements in life expectancy have led to a proportional increase in the population mostly at risk for heart disease, highlighting the need to understand the molecular mechanisms that contribute to age-related deterioration of cardiac function. In humans, the main age-related changes associated with cardiac insufficiency are reduced cardiac contractility and arrhythmias.1–4

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The wingless/Wnt (Wnt) signaling pathway plays critical roles in an array of biological processes, including maintenance of homeostasis in multiple tissues and regulation of stem cell proliferation.5,6 Interestingly, Wnt signaling seems to promote aging in mouse skeletal muscle stem cells but inhibits senescence in human fibroblasts.7 Thus, it seems clear that a link exists between Wnt signaling and aging, but the specific molecular effectors have not been identified.

Canonical Wnt signaling is initiated when Wnt ligands bind frizzled/lipoprotein receptor protein receptors, which prevents degradation and promotes stabilization of cytosolic β-catenin/armadillo (β-cat), a key mediator of Wnt signaling. β-cat subsequently translocates to the nucleus, where it forms a complex with the DNA-binding protein T-cell specific, HMG-box (TCF)/pangolin (Pan) and other cofactors, enabling transcription of Wnt/TCF-responsive genes, such as Ubx in Drosophila.8–10 The pygopus (pygo) gene encodes a transcriptional cofactor for canonical Wnt signaling in Drosophila, and pygo null mutations phenocopy the loss of Wnt signaling.11–14 In mammals, 2 pygo homologs, Pygo1 and Pygo2, have been identified: Pygo2 expression is ubiquitous, but Pygo1 is enriched in heart tissue, suggesting a possible cardiac-specific function.15 Double Pygo1 and Pygo2 mutant mice do not exhibit obvious developmental heart defects,16 but it is not known whether these molecules are required for

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From the Development and Aging Program, Sanford-Burnham Medical Research Institute, La Jolla, CA (M.T., R.B., K.O.); and the Center for Heart Development, Key Laboratory of MOE for Developmental Biology and Protein Chemistry, College of Life Sciences, Hunan Normal University, Changsha, Hunan, People’s Republic of China (M.T., W.Y., M.L., R.B., K.O., X.W.).

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Correspondence to Karen Ocorr, PhD, 10901 N Torrey Pines Rd, San Diego, CA 92037. E-mail kocorr@sbmri.org or Xiushan Wu, PhD, the Center for Heart Development, Hunan Normal University, Changsha 410081, Hunan, People’s Republic of China. E-mail xiushanwu@yahoo.com

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adult heart function under stress conditions or during aging. Interestingly, studies of lens induction in mice have suggested that Pygo2 may have a Wnt-independent function.\(^{17}\)

In this study, we investigated the role of pygo in Drosophila heart model. We show that pygo is specifically expressed in adult myocardial cells and cardiac-specific knockdown of pygo drastically compromises heart function. In contrast to this strong pygo loss-of-function phenotype, knockdown of other canonical Wnt signaling components, such as β-cat and TCF, caused only mild defects in heart function. Furthermore, pygo mutants failed to show significant genetic interaction with other molecules involved in canonical Wnt signaling, including the TCF target gene Ubx and components of the Wnt signaling-associated mediator complex, skd and ktn. We also show that in an isoproterenol (ISO)-induced cardiac hypertrophy model, Pygo1 protein itself is upregulated. Thus, we conclude that pygo function is critical for maintaining optimal adult heart performance and acts independently of canonical Wnt signaling, and increases in Pygo1 expression may mediate some of the effects of cardiac hypertrophy.

Methods
Semi-intact Drosophila hearts were prepared as described previously.\(^{13,19}\) Movies of beating hearts were recorded for 30 seconds with a high-speed EM-CCD camera (Hamamatsu) at 130 frames/s. Data were captured using HC Image software (Hamamatsu). Movies were analyzed with Semi-automatic Optical Heartbeat Analysis software to quantify heart periods, systolic and diastolic intervals, systolic and diastolic diameters, fractional shortening (FS), and arrhythmia indexes (defined as the SD of the heart period normalized to the median of each fly) and to produce M-mode records.\(^{18,20,21}\) Immunohistochemistry was performed as previously described.\(^{22}\)

Cardiac hypertrophy was induced in male BALB mice (8–12 weeks of age) by continuous intraperitoneal injection of ISO (0.9% sodium 5 mg/kg per day) for 14 days as previously described.\(^{23}\) Sham-operated animals were submitted to intraperitoneal injection of vehicle (0.9% sodium solution) for the same amount of time. Mice were kept for an additional 14 days after treatment and then were euthanized by cervical dislocation and hearts were surgically removed. The heart weight:body weight ratio was calculated, and hearts were then frozen in liquid nitrogen before real-time quantitative polymerase chain reaction or Western analysis, or fixed for immunohistochemistry (see Methods in the online-only Data Supplement).

Drosophila and mouse stocks and the methods for real-time quantitative polymerase chain reaction, Western blotting, and determination of lifespan are described in the Methods in the online-only Data Supplement.

Statistical Analysis
All statistical analyses were performed using Prism Statistical Software (Graph Pad, Inc, version 6). A 1-way ANOVA was used when comparing the effects of different genetic manipulations in flies of the same age. Data sets were tested for normal (Gaussian) distributions using the D’Agostino and Pearson omnibus normality test. For data sets that passed this test, we used a regular 1-way ANOVA followed by multiple comparisons post hoc tests (specific tests indicated in figure legends). Data sets that did not show a normal distribution were analyzed for significance using a Kruskal–Wallis test followed by a Dunn multiple comparison test post hoc test. When analyzing heart function in flies with both differing genetic manipulations and differing ages, we used a 2-way ANOVA followed by a Tukey multiple comparisons post hoc test of significance. In all cases, \(P<0.05\) were taken as significant.

Figure 1. pygo knockdown causes heart dysfunction. (A), Heart period, (B) systolic interval, and (C) diastolic interval were measured for hearts from 1-week-old wild-type control Drosophila (hand\(^{+}\)), flies with cardiac-specific pygo knockdown (hand\(\rightarrow UAS\)-pygo-RNAi), pygo rescue (hand\(\rightarrow UAS\)-pygo-RNAi; UAS-stinger), β-cat/arm knockdown (hand\(\rightarrow UAS\)-arm-RNAi), TCF knockdown (hand\(\rightarrow UAS\)-TCF-RNAi), and overexpression of dominant-negative (DN) TCF (hand\(\rightarrow TCF\)-DN). Note the significant systolic interval and diastolic interval interval prolongation with pygo knockdown and the partial rescue with pygo overexpression. (D), Cardiac arrhythmia index and (E) contractility, quantified as fractional shortening. In all measures the pygo knockdown phenotype is more severe than that in response to arm/β-cat or TCF knockdown. Significance was determined using a 1-way ANOVA and Tukey multiple comparisons post hoc test (for normally distributed data: systolic interval, fractional shortening, and diameters) or Kruskal–Wallis with Dunn multiple comparisons test (for non-Gaussian data: heart period, diastolic interval, and arrhythmia index). Differences relative to the Hand4.2/+/control are indicated by individual asterisks; significance between experimental groups is indicated by the capped lines; * \(P<0.05\), ** \(P<0.01\), and *** \(P<0.001\). Sample size was 20 to 30 flies per genotype.
Results

pygo Is Expressed in the Adult Heart

Immunostaining of Drosophila adult hearts revealed that Pygo protein accumulates in the nuclei of cardiomyocytes but not in heart-associated pericardial cells (Figure 1 in the online-only Data Supplement). We examined animals with heart-specific pygo knockdown achieved using RNA interference (RNAi) to inhibit gene expression; Pygo immunoreactivity in cardiomyocyte nuclei was reduced greatly or abolished, compared with the expression in wild-type hearts (Figure 1E–IH in the online-only Data Supplement). These results were corroborated by quantitative polymerase chain reaction, which showed that pygo mRNA expression in the pygo KD hearts was only 19% of the levels expressed in control hearts (Figure II in the online-only Data Supplement).

pygo Is Required to Maintain Normal Heart Physiology

We characterized the effects of pygo loss of function on adult heart function using a semiautomated optical heartbeat analysis protocol.16–20 Cardiac-specific pygo KD prolonged the heart period (ie, reduced heart rate) in 1-week-old flies (Figure 1A), and this was because of increases in both the systolic and diastolic intervals (Figure 1B and 1C). We also observed a significant increase in the incidence of arrhythmias (arrhythmia index) in pygo KD hearts compared with controls (Figure 1D). The cardiac phenotype induced by pygo KD is also illustrated in the M-mode traces obtained from high-speed movies, which show heart wall movements over time.18,19 M-Mode frames from pygo KD hearts exhibited long pauses between beats (asytoles), prolonged or multiple (fibrillatory) contractions, and wider systolic heart wall dimensions (Figure 2A).

Knockdown of pygo caused a dramatic reduction in cardiac contractility at young ages, as demonstrated by diminished FS, a classic measure of cardiac output (Figure 1E). The reduced FS was mainly attributable to systolic dysfunction because the diameters of pygo KD hearts during systole were markedly increased compared with the controls, whereas there was little effect on diastolic diameters (Figure 3H; see 1-week time point).

We confirmed these observations in an independent pygo KD line, which displayed a similar, albeit slightly less severe, phenotype (Figure III in the online-only Data Supplement). Furthermore, transgenic overexpression of a wild-type pygo cDNA construct rescued the pygo KD phenotype; both systolic and diastolic intervals were significantly shortened in the hearts of pygo KD, pygo-overexpressing animals compared with the pygo KD hearts (Figure 1B and 1C). The decrease in FS was also rescued partially in these animals but not the increased arrhythmia index (Figure 1D and 1E).

pygo Is Required for Normal Heart Morphology

The observed reduction in contractility suggested that cardiac structure might be altered in pygo KD hearts. Normal adult hearts contained densely packed, circumferentially organized myofibrils within the cardiomyocytes (Figure 2B). In contrast, all of the pygo KD hearts examined showed extensive myofibrillar disorganization and reorientation, as well as less densely packed myofibrils with obvious gaps (Figure 2B–2E; Figure IV in the online-only Data Supplement). These morphological defects were rescued partially by cardiac coexpression of the wild-type pygo transgene, with only 25% of hearts exhibiting myofibrillar defects (Figure 2E). Our observation that cardiac pygo overexpression itself in wild-type flies caused some mild structural heart defects (data not shown) may explain the inability of overexpression to rescue completely this pygo KD phenotype (Figures 2B–2E; Figure IV in the online-only Data Supplement). Overall, these data indicate that pygo expression in cardiomyocytes is required for the structural integrity and normal function of the heart.
Cardiac pygo KD Causes an Accelerated Age-Dependent Deterioration of Heart Function

Several studies have shown that heart structure and function deteriorate with age in both flies and humans.\textsuperscript{1,2,24-31} The average lifespan of different laboratory wild-type strains of\textit{Drosophila} ranges between 40 and 80 days at 25°C, suggesting that each week of age in a fly is equivalent to \(\approx\) 1 decade in human age. In flies, cardiac senescence increases steadily after eclosion.\textsuperscript{21,33-35} Thus, in the current study, we examined the role of pygo in aging adult flies at 1, 3, and 5 weeks as representative of young, middle, and late middle ages, respectively, but prior to ages where significant mortality occurs (see also Figure VI in the online-only Data Supplement). We found that pygo-depleted hearts exhibited dramatic age-associated changes in most of the cardiac parameters measured (Figure 3). The percentage of unusually long systolic intervals (\(>0.4\) seconds, twice the average length in wild-type flies)\textsuperscript{18,20,33} was markedly elevated in pygo KD flies compared with age-matched controls (Figure 3E and Figure V in the online-only Data Supplement) and are reminiscent of the fibrillatory contractions observed in hearts from KCNQ mutant flies.\textsuperscript{18} The percentage of unusually long diastolic intervals (\(>1\) seconds) also increased with age in pygo KD hearts (Figure 3F). Unexpectedly, pygo KD caused a reduction in contractility (FS) in flies at 1 and 3 weeks but not at 5 weeks (Figure 3G and 3H). This increase in contractility with age may be the result of increased calcium influx that would be expected to occur during the prolonged systolic intervals observed in older flies (Figure 3B and 3E; see also Figure V in the online-only Data Supplement; 5-week time point).

Examination of heart morphology in the pygo KD flies revealed an increase in the age-related disorganization of the normally compact myofibrillar structure of the myocardium (Figure 4). Moreover, cardiac pygo KD decreased the median survival time of the flies by 24% (Figure VI in the online-only Data Supplement). These data suggest that pygo plays a critical role in protecting the adult heart from age-related dysfunction, and that compromised heart function may significantly reduce the normal lifespan.

pygo Is Required in the Adult Heart to Maintain Its Function

To determine whether pygo is required for maintenance of cardiac performance in the adult fly, we disrupted pygo

Figure 3. pygo loss of function exacerbates age-associated cardiac dysfunction. Heart period (A), systolic interval (B), diastolic interval (C), and arrhythmia (D) are increased by heart-specific pygo knockdown compared with controls (hand4.2/+ and pygo-RNAi+/+) and are further exacerbated with age. (E), pygo knockdown hearts exhibit a dramatically increased prevalence of prolonged systoles (\(>0.4\) seconds, indicative of unsustained fibrillations) and (F) diastoles (\(>1.0\) seconds, asystolic events), and these phenotypes are exacerbated with age. (G), Contractility (fractional shortening) is reduced in 1- and 3-week-old pygo knockdown hearts. (H), Heart diameters during diastole (dashed lines) are unchanged in pygo knockdown hearts, but systolic diameters are increased significantly in response to pygo knockdown at 1 and 3 weeks. For each data point (ie, at 1, 3, and 5 weeks), 20 to 30 individual hearts from flies of the specified genotype were analyzed; data are displayed as mean±SEM. Significance at each age was determined using a 1-way ANOVA and Tukey multiple comparisons post hoc test (for normally distributed data: systolic interval [SI], fractional shortening, and diameters) or Kruskal–Wallis with Dunn multiple comparisons test (for non-Gaussian data: heart period, diastolic interval [DI], and arrhythmia index). Significant differences with respect to both pygo-RNAi+/+ and hand4.2/+ controls are indicated by individual asterisks; capped lines represent differences between specified groups (*\(P<0.05\), **\(P<0.01\), and ***\(P<0.001\)).
expression during the adult stage using the Gene Switch system in conjunction with a hand-Gal4 driver (hand/GS-Gal4; see Materials and Methods). This driver is expressed specifically and conditionally in the heart on dietary supplementation with the antiprogestin compound, RU486. Hearts from RU486-fed flies exhibited increased heart periods, diastolic intervals, and arrhythmias, and reduced FS because of systolic dysfunction (Figure VII in the online-only Data Supplement), which is similar to the phenotype of temporally unrestricted cardiac pygo KD animals (Figure 1). These results indicate that pygo function is required specifically in the adult myocardium to prevent premature deterioration of heart function.

**pygo Functions Independently of Canonical Wnt Signaling in the Adult Heart**

Pygo was identified originally as a critical component of canonical Wnt signaling that associates with β-Cat, BCL9/Lgs, and TCF in the nucleus to activate transcription of Wnt-responsive genes. Therefore, we examined whether the heart-specific role of pygo was associated with canonical Wnt signaling. First, we determined the efficiency of RNAi KD was less than that of the β-Cat/arm cardiac KD of and using these lines should rescue (Figure 5A). These findings demonstrate that the efficiency of pygo KD was less than that of the β-Cat/arm and TCF RNAi lines that we used.

Thus, if Wnt signaling plays a role in the adult heart, then cardiac KD of TCF and β-cat/arm using these lines should affect heart function to a greater extent than pygo KD. Surprisingly, we found that cardiac-specific KD of β-cat/arm only modestly altered the cardiac parameters (Figure 1, pink bars), and TCF KD had an even weaker effect (Figure 1, blue bars). Therefore, disruption of β-cat/arm or TCF function had remarkably little effect on cardiac function compared with the striking pygo KD phenotype, which is in stark contrast to their relative potencies in the canonical Wnt signaling eye size assay (Figure 5).

In contrast to the weak effect of TCF KD, we found that a dominant-negative form of TCF (TCF-DN) induced a cardiac phenotype similar to that observed for β-cat/arm KD, with the exception that the reduction in FS was more pronounced (Figure 1, pink and purple bars). It should be noted that DN constructs often induce stronger phenotypes than genetic loss of function. The weak effect of TCF KD on cardiac contractility contrasted with the strong effect of TCF DN suggests a gain-of-function phenotype.

This possibility is supported by the observation that TCF-DN hearts showed significant myofibrillar abnormalities, as...
previously observed, compared with the β-cat/arm and TCF KD hearts, which contained few cardiomyocytes with myofibrillar abnormalities (Figure 2E). However, the TCF-DN phenotype consisted mainly of a reorientation of myofibrils, which is quite different from the effects of pygo KD (Figure 2E). Finally, an equally mild adult cardiac phenotype was observed on overexpression of activated GSK3β/shaggy (sgg), a negative regulator of Wnt signaling (data not shown). Taken together, these data indicate that canonical Wnt signaling does not play a major role in maintaining the structure and function of the adult heart, and thus it suggests that the cardiac role of pygo is largely independent of canonical Wnt signaling.

**pygo Does Not Interact Genetically With β-Cat/TCF Complex Genes to Regulate Adult Heart Function**

If pygo functions in a Wnt signaling–independent manner in the adult heart, we would predict that pygo mutants would not interact genetically with β-cat/arm or TCF/pangolin (pan) mutants with respect to heart function. To test this, we generated double-heterozygous combinations of pygo<sup>S123</sup> with arm<sup>2</sup> or pan<sup>1</sup>. We found little difference in the function of the pygo<sup>S123</sup>/arm<sup>2</sup> or pygo<sup>S123</sup>/pan<sup>1</sup> hearts compared with control hearts (Figure VIII in the online-only Data Supplement). These findings are consistent with our finding that the β-Cat/TCF complex plays only a minor role in adult heart function and further support a canonical Wnt signaling–independent requirement for pygo in the adult heart.

To test this hypothesis further, we examined whether pygo genetically interacts with other components of the β-Cat/TCF transcriptional complex or with the known TCF target gene, Ubx. Ubx is involved in cardiomyocyte reprogramming in the Drosophila heart during metamorphosis, and overexpression causes longitudinal reorientation of the normally circumferentially oriented myofibrils. Interestingly, cardiac overexpression of pygo caused a similar myofibrillar reorientation (data not shown). Double-heterozygous pygo<sup>S123</sup>/Ubx<sup>22</sup> animals showed only slight changes in cardiac parameters compared with the single heterozygotes (Figure IX in the online-only Data Supplement). A similarly weak interaction was observed between pygo and kohtholo (kto) and skuld (skd), which encode that the mediator complex genes Med12 and Med13, that in the wing disk, are recruited by Pygo to regulate the **hand**<sup>4.2</sup>-Cat/TCF complex. Therefore, both genetic and expression analysis support a model in which the TCF complex plays only a minor role in adult heart function and further support a model in which pygo functions in a Wnt signaling–independent manner in regulating heart function.

**Genetic Interaction Between pygo and CaMKII in Regulating Heart Function**

Because sustained, fibrillatory contractions, and impaired contractility are prominent defects in pygo KD hearts, we tested whether pygo interacts with genes involved in mediating intracellular calcium signaling. Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) is critical for mediating the effects of increased intracellular calcium in contracting cardiomyocytes and is a known modulator of contractility. Although cardiac KD of CaMKII alone did not affect heart function, we found that CaMKII KD significantly increased heart dysfunction in the pygo KD flies (Figure 7). This genetic interaction suggests that pygo may function in conjunction with CaMKII to maintain adult heart performance.
Pygo1 Is Expressed in the Adult Heart and Is Upregulated in Response to Isoproterenol-Induced Cardiac Hypertrophy

We used Western blots to establish the embryonic and adult temporal expression pattern of Pygo1 in the mouse heart. Our results show that Pygo1 is relatively weakly expressed during embryonic stages, increases at birth, and is still present at significant levels in hearts from 6-week-old adults (Figure XA in the online-only Data Supplement), consistent with previous findings that Pygo1 is exclusively expressed in the adult mouse heart. To determine whether Pygo1 levels are altered under pathological conditions, we used the ISO-induced hypertrophy mouse model. BALB mice were infused for 14 days with ISO, and 14 days later, animals were euthanized. Hearts from ISO-treated mice showed a 25% increase in the heart weight:body weight ratio compared with sham-treated controls (Figure XB and XC in the online-only Data Supplement). Pygo1 mRNA expression was increased in ISO-infused mice as was expression of the cardiac hypertrophy marker atrial natriuretic factor compared with controls, suggesting that Pygo1 expression is upregulated during hypertrophy (Figure XD in the online-only Data Supplement). Immunohistochemistry using α-Pygo1 antibody and Diaminobenzidine showed increased staining in transverse sections from ventricles of ISO-treated mice (Figure 8A and 8B), and Western blot analysis confirmed the increased protein levels of both Pygo1 and atrial natriuretic factor (Figure 8C) in hypertrophic hearts.

**Discussion**

The results described here provide strong evidence that pygo function is specifically required in the adult myocardium to maintain cardiac function and structural integrity and to counteract age-associated cardiac deterioration. The age-dependent changes in heart rhythm observed here, which are aggravated by pygo deficiency, are reminiscent of the increased incidence of atrial fibrillation in elderly humans. It will be interesting to determine whether an age-related reduction in pygo function contributes to the development of arrhythmias in humans.

In contrast to the effects of pygo deficiency, we found that disruption of other Wnt pathway components, such as β-cat and TCF, had relatively mild effects on cardiac physiology and morphology. This was not because of inefficacy of the
described here provide strong evidence that Pygo1 function for Drosophila to stress. and suggest that it is involved in the hypertrophic response. Data indicate a role for Pygo1 in mammalian adult heart tissue marker atrial natriuretic factor in the mouse model. These to ISO-induced hypertrophy along with the hypertrophy Pygo1 protein levels were significantly increased in response hypothesis. Unfortunately, the fly heart does not provide not bind wild-type TCF (Figure XI in the online-only Data TCF KD, in particular the reduction of FS (Figure 1E). Therefore, it is possible that Pygo might serve as an adaptor for a TCF-like transcription factor that binds to consensus TCF DNA-binding sites. In this scenario, TCF-DN (which retains DNA-binding ability) might interfere with the Wnt-independent function of pygo by occupying TCF consensus sites that normally do not bind wild-type TCF (Figure XI in the online-only Data Supplement). Unfortunately, the fly heart does not provide sufficient material to perform chromatin immunoprecipitation or RNAseq experiments, which would be required to test this hypothesis.

Consistent with the results in the fly heart, we found that Pygo1 protein levels were significantly increased in response to ISO-induced hypertrophy along with the hypertrophy marker atrial natriuretic factor in the mouse model. These data indicate a role for Pygo1 in mammalian adult heart tissue and suggest that it is involved in the hypertrophic response to stress.

In summary, we propose a novel Wnt pathway–independent function for pygo in the adult Drosophila heart. The results described here provide strong evidence that pygo function is specifically required in the adult myocardium to maintain cardiac function and structural integrity and to counteract age-associated cardiac deterioration. The fact that cardiac Pygo1 expression increases at birth in the mouse model suggests that Pygo1 may play a similar maintenance function in vertebrates, and that increases in pygo1 expression may underlie cardiac hypertrophic responses. The age-dependent changes in heart rhythm observed here, which are aggravated by pygo deficiency, are reminiscent of the increased incidence of atrial fibrillation in elderly humans. Because Pygo1 and Pygo2 are not obligatory for cardiogenesis in mammals, it will be interesting to determine whether an age-related reduction in pygo function contributes to the development of arrhythmias in humans and whether they are critical for maintaining normal heart structure and function under stress conditions or during aging.

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**Disclosures**

None.

**References**

The nuclear adaptor protein encoded by pygoopus (pygo), a core component of canonical Wnt signaling, is specifically expressed in the mammalian heart. Although Wnt signaling pathways are known to play important roles in embryological development, reductions in pygo expression do not affect heart development. The results presented in this study indicate that pygo plays a role in maintaining adult heart function. Cardiac-specific pygo knockdown in Drosophila results in significant increases in arrhythmia and cardiac dilation and reduced contractility with concomitant disruption of myofibrillar organization. Cardiac-specific knockdown of other Wnt signaling components, including β-catenin/arm and TCF/pangolin, has fewer deleterious effects on cardiac function, suggesting that pygo may function in the adult heart independently of canonical Wnt signaling. Double knockdown of pygo and Ca<sup>2+</sup>/calmodulin-dependent protein kinase II in the fly heart caused additional increases in arrhythmia compared with pygo knockdown alone, suggesting an interaction between pygo and Ca<sup>2+</sup> signaling. In the adult mouse heart, we also show that Pygo1 protein levels are increased in response to isoproterenol-induced cardiac hypertrophy, indicating that a role for pygo in adult cardiac function is likely conserved. Thus, we have identified new signaling components involved in the maintenance of adult heart function that should provide novel targets for therapeutic intervention in age-related heart disease.
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Supplemental Figure 1

**Pygopus is specifically expressed in adult cardiomyocytes.** Triple staining of adult *Drosophila* hearts showing Nmr in green, Pygo in red, and DAPI in blue. Ventral view, in all images anterior is up. (A-D) Heart from wildtype fly; anti-Nmr staining identifies small ostial cell nuclei (B, top panel) and larger myocardial cell nuclei (B, bottom panel). (C) Anti-Pygo staining shows nuclear localization in both small and large myocardial nuclei (A) Anti-pyro, anti-Nmr and DAPI staining are coincident. (E-H) Hearts from cardiac pygo KD using hand4.2-Gal4. (F) Anti-Nmr staining identifies nuclei of ostial and myocardial cells. (G) Anti-Pygo staining is absent in these cells. (I-L) Pericardial cells do not express pygo. Dashed outlines indicate the pericardial cell nuclei and yellow arrows indicate the myocardial cell nucleus. (J) Nmr reactivity identifies myocardial cell nuclei. (K) Pygo staining is concentrated in the nuclei of myocardial cells but not of pericardial cells. (I) The merged images showed co-localization of Pygo and Nmr staining in the myocardial, but not pericardial cell nuclei.
Supplemental Figure 2

qPCR of *pygo* RNA from cardiac *pygo* KD hearts. Relative expression of *pygo* in 1-week-old adult hearts was normalized to ribosomal *rp49* expression. Control (hand4.2-Gal4 X UAS-KK-control) was set as one. Cardiac-specific *pygo* KD showed 81% reduction compared to control, indicating that the *pygo* RNA level was efficiently knocked down. Significance was determined using a Student's T-test; **p<0.01.
Supplemental Figure 3

Cardiac-specific expression of a second pygo RNAi line also causes cardiac dysfunction. Pygo RNAi line (v19693) was crossed to the cardiac-specific hand4.2-Gal4 driver. (A-C) Cardiac KD with this RNAi line also causes prolongation of the heart period (A) due to increases in both (B) systolic and (C) diastolic intervals. (D) Hearts also exhibit increases in arrhythmia. (E) Fractional shortening is also significantly decreased due to (F) an increased systolic diameter and slight decrease in diastolic diameter. Note that cardiac pygo KD with this v19693 line causes similar cardiac dysfunction as with v100724 but the phenotype with v100724 (Fig. 1&2) is more severe than with the v19693 RNAi line.

Each bar (genotype) represents data from 20-30 hearts and data is shown as mean ± SEM. Significant differences between means were determined using a one-way ANOVA and Tukey’s multiple comparisons post-hoc test (for normally distributed data: SI, FS and diameters) or Kruskal-Wallis with Dunn’s multiple comparisons test (for non-Gaussian data: HP, DI and AI; *p < 0.5, **p < 0.01, ***p < 0.001).
Supplemental Figure 4

Cardiac-specific pygo KD results in a loss of myofibrils. Hearts were stained with phalloidin (green) to identify actin filaments and anti-Trol (red) to mark the cell membrane. **Top panel** shows a wildtype heart with a circumferential myofibrillar arrangement. **Bottom Panel** shows pygo KD hearts with regions that lack myofibrils (gaps between myofibrils). **Right hand panels**: Cross sections (Y-Z) taken at the locations indicated by red arrows show that the myocardial cells are intact (red staining) in the region where myofibrillar gaps are observed in the pygo KD heart (blue arrow, no green staining).
Supplemental Figure 5

Age-dependent deterioration in cardiac function is revealed by M-modes. Representative 10 sec M-mode traces from semi-intact *Drosophila* heart preparations reveal the movements of the heart walls (y axis) over time (x axis). **1 week** - *pygo* KD hearts are significantly slower and show incomplete relaxations (arrow) and a reduction in fractional shortening (the extent to which the heart walls come together during a contraction). **3 weeks** - the heart rate is further reduced in *pygo* KD hearts and the beating pattern has become more irregular. **5 weeks** - the heart exhibits significant bouts of unsustained fibrillation compared to controls (red line).
Supplemental Figure 6

Cardiac-specific *pygo* KD reduces life span. The median survival of *pygo* KD flies (*hand4.2-Gal4* > *pygo* RNAi, v100724 line) was 39 days compared to 51 days for control. This reduction was significant (p<0.0001, Mandel-Cox log rank test). Graph plots % survival (n=250) versus time (in days) post-eclosion.
Supplemental Figure 7

**Adult-specific cardiac requirement for pygo.** Temporal and spatial disruption of pygo in the adult heart was controlled using the Gene Switch system [1] in conjunction with a hand-Gal4 driver (handGS-Gal4, gift from L. Perrin, see Supplemental Methods). (A-C) pygo KD specifically in the adult heart induced by RU486 resulted in prolongation of the (A) heart period which was due to prolonged (C) diastolic intervals. (D) The incidence of arrhythmia was also increased in RU486 fed flies (+RU) compared to controls (-RU). (E) Fractional shortening also decreased significantly in the presence of RU486. (F) This decrease was due to systolic dysfunction as systolic diameter (S) is significantly increased relative to the diastolic diameter (D). Reduced fractional shortening was also seen in the absence of RU486, which may be due to some leakiness of the hand GS driver.

Each bar represents data from 20-30 hearts from the indicated genotypes and data are displayed as mean ± SEM. Significant differences were determined using a one-way ANOVA and Tukey’s multiple comparisons post-hoc test (for normally distributed data, SI, FS, and diameters) or Kruskal-Wallis with Dunn’s multiple comparisons test (for non-Gaussian data: HP, DI and AI). Individual asterisks indicate significant differences with respect to both controls (hand GS/+ and pygo RNAi/+); capped lines indicate significant differences between specified groups (*p < 0.5, ***p < 0.001).
Supplemental Figure 8

**Genetic interactions between pygo and genes involved in canonical Wnt signaling in maintaining adult heart function.** Double heterozygotes were generated for pygo and β-catenin/arm or TCF/pan. (A) Only pygo/pan double heterozygotes show a slight synergism with respect to the effect on systolic interval (and compare to pygo KD, Fig. 1). (B) arm/+ single heterozygotes showed a significant increase in diastolic interval that was not further increased in the pygo/pan double heterozygotes. (C) The small increases in arrhythmias for arm/+ and pygo/arm were not statistically significant. (D) Contractility (measured as Fractional Shortening) was relatively unaffected with a small increase in both pan3/+ heterozygotes. (E) This increased contractility was due to a small increase in diastolic (D) relative to systolic (S) diameters.

Each bar represents data from 20-30 hearts from the indicated genotypes and data are displayed as mean ± SEM. Significant differences from the pygo heterozygote (pygo6123/+, indicated by individual asterisks) were determined using a one-way ANOVA and Dunnett’s multiple comparisons post-hoc test (for normally distributed data: SI, FS, and diameters) or Kruskal-Wallis with Dunn’s multiple comparisons test (for non-Gaussian data: DI and AI). Capped lines indicate significant differences between specified groups (*p < 0.5, **p < 0.01, ***p < 0.001).
Supplemental Figure 9

**Pygo genetically interacts with mediators and targets of canonical Wnt signaling to maintain adult heart function.** Double heterozygotes for *pygo*^{s123}/kto^{1} and *pygo*^{s123}/Ubx^{9.22} showed (A) moderately prolonged systolic and (B) diastolic intervals. (C) *pygo*^{s123}/Ubx^{9.22} double heterozygotes show a small increase in arrhythmia (compare to pygo KD in Fig. 1). (D) Both skd^{2} heterozygotes showed a decrease in fractional shortening which was due to an (E) increase in systolic diameter (S) relative to diastolic diameter (D).

Each bar (genotype) represents data from 20-30 hearts and data is shown as mean ± SEM. Significant differences from the *pygo* heterozygote (*pygo*^{s123}/+) were determined using a one-way ANOVA and Dunnett’s multiple comparisons post-hoc test. For non-Gaussian data (DI) the Kruskal-Wallis with Dunn’s multiple comparisons test was employed. Capped lines indicate significant differences between specified groups (*p < 0.5, **p < 0.01, ***p < 0.001).
Pygo1 expression in adult mouse hearts is increased by isoproterenol-induced hypertrophy. (A) Western blot analysis of hearts isolated from BALB mice at the indicated ages shows weak embryonic expression of pygo1 that increased dramatically after birth. (B) Hearts from sham-treated (left) and isoproterenol-infused mice (right). (C) Heart weight normalized to total body weight shows a significant increase in isoproterenol-infused mice (25.1% **p<0.005, N=8) compared to controls (N=5 mice). (D) mRNA levels of pygo1 and the cardiac hypertrophy marker Atrial Natriuretic Factor (ANF) in hearts from sham-treated (N=3) and isoproterenol-infused (N=3) mice. mRNA levels are expressed relative to GADPH, normalized to the sham-infused control and are displayed as mean ± SEM. Significance was determined using a Student’s t-test; *p<0.05.
Supplemental Figure 11

Canonical Wnt signaling and proposed TCF/β-Cat-independent function of Pygo in maintaining adult cardiac performance. **(A)** The canonical Wnt signaling-dependent transcriptional complex activates gene transcription. The mediator complex genes *skd* and *kto* also contribute to the transcription of canonical Wnt signaling targets. **(B)** The proposed adult cardiac function of Pygo is independent of canonical Wnt signaling. Pygo may interact with TCF-like factors that bind to TCF-related sites, which normally do not normally bind TCF. Dominant-negative *TCF* (*TCF DN*), however, could occupy these sites and block this Wnt-independent function of *pygo*. 
Material and Methods

Drosophila stocks and Mouse stocks

\( w^{1118}, \) UAS-Stinger-GFP, UAS-\textit{TCF}^{DN} and mutants (\textit{pygo}^{s123}, \textit{arm}^{2}, \textit{pan}^{2}, \textit{skd}^{6}, \textit{kto}^{1}, \textit{Ubx}^{9,22}) \) were obtained from the Bloomington stock center. UAS-\textit{pygo}-RNAi lines (v100724 and v19693), UAS-\textit{arm}-RNAi (v107344), and UAS-\textit{TCF}-RNAi (v3014) were obtained from the Vienna \textit{Drosophila} RNAi Center (VDRC)\(^1\). UAS-\textit{pygo} was a generous gift from Konrad Basler. The heart-specific driver \textit{hand}4.2-Gal4\(^2\), expressed in myocardial as well as pericardial cells, and the eye-specific, constitutively-activated \( \beta\text{-cat} \), GMR;UAS-\textit{arm}\(^{+}\), were kindly provided by Eric Olson, Laurent Perrin and Kenneth Cadigan, respectively. The RU486 inducible \textit{hand}-Gene-Switch-Gal4 driver (\textit{handGS}), expressed in myocardial and pericardial cells, was generated and kindly provided by Laurent Perrin (unpublished).

RNAi lines were crossed to \textit{hand}4.2-Gal4 flies and incubated at room temperature throughout development. Female F-1 progeny were collected and aged at 25°C. The \textit{handGS} system\(^4\) was used to control the temporal and spatial expression pattern. Cardiac-specific expression of RNAi was induced in the presence of the anti-progestin RU486, which activated Gal4 production. We crossed \textit{handGS} with \textit{pygo}-RNAi flies (v100724) and the progeny of this cross were collected at eclosion and fed RU486. A \textit{pygo} null allele (\textit{pygo}^{s123}/TM6B) was crossed with different mutants listed above for analysis of double heterozygous interactions.

BALB Mice were specific pathogen-free (SPF) animals obtained from Hunan SJA laboratory Animal Co., Ltd. They were housed 5 mice per cage and maintained according to the guidelines approved by the Ethics Committee of Hunan Normal University and international guidelines outlined in the “Act on Welfare and Management of Animals. The study was approved by the Ethics Committee of Hunan Normal University

Lifespan analysis

Male and virgin female progeny from \textit{hand}4.2-Gal4 x UAS control (KK) and \textit{hand}4.2-Gal4 x UAS-\textit{pygo}-RNAi (v100724) crosses were collected and housed separately in groups of 25 flies per vial. Flies were kept at 25°C, transferred to fresh food on every 3rd day and survival was scored following each transfer. We collected a total of approximately 250 flies from each cross and the study was performed in duplicate. Data were analyzed using Prism 6.0 (Graphpad Software) and the results were comparable between the duplicate experiments.

Immunocytochemistry

Semi-intact \textit{Drosophila} hearts were fixed \textit{in situ} with 4% paraformaldehyde in phosphate buffered saline (PBS, Sigma) for 20 min. Then the hearts were washed three times in PBST (0.1% Triton X-100 in PBS). Primary antibodies (in PBST) were incubated at 4°C overnight, washed, then incubated with secondary antibodies for 2 hours and washed again. Hearts were mounted in VectaShield (Vector Laboratories). Images were acquired with an Apotome microscope (Zeiss). The following primary antibodies were used: rabbit or guinea pig anti-Nmr (1: 3000, \(^5\)), rabbit anti-Pygopus (1:30, \(^6\)), anti-Trol (1:1000, \(^7\)). Fluorescent labeled Alexa 488-phalloidin (1:1000, Chemicon, Temecula, CA), anti-rabbit-Cy3 (1:250) and anti-guinea pig-FITC (1:150) (Jackson Immunoresearch) were used.
Mouse hearts were fixed in 4% PFA at 4°C overnight, embedded in paraffin and cut into 5μM sections. Sections were stained with Pygo1 antibody according to the manufacturer’s recommendations (Beijing Zhong Shan-Golden Bridge Biological Technology).

**RQ-PCR**

RNA was extracted from 20-30 fly hearts using PicoPure™ RNA Isolation Kit (ARCTURUS). The samples were treated with DNaseI (Qiagen) to remove DNA. First strand cDNA was synthesized using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). For polymerase chain reaction, we used LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche applied science).

Total RNA was extracted from mouse cardiac tissue using Trizol reagent (Invitrogen) according to the manufacturer’s recommendations and reverse transcribed using mRNA Selective PCR Kit (TaKaRa). RT-PCR analysis were performed. cDNA was added to make 20 μl to PCR buffer containing 1.25 U of Taq DNA polymerase and 2.5 mM dNTP. The mixture was subjected to 30 cycles of PCR (94°C, 1 min; 60°C, 30 s; 72°C, 1 min). Primers used are listed below. All determinations were repeated at least three times.

**Primer name**

<table>
<thead>
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<th>Primer name*</th>
<th>Primers</th>
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<td>Pygo1-A</td>
<td>5’-ATGCAGGTTCAGGGTTAG-3’</td>
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<td>RT-ANF-S</td>
<td>5’-TAAGCCCTTGTTGTTGCTCA-3’</td>
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*S=Sense, A= Antisense

**Western Blot Analysis**

Western blot analysis of heart extracts (see above) was performed as previously described [8]. Protein was extracted from sequential embryonic development stages (E12.5, E16.5, P1 and P42). All experiments were repeated at least three times.

**Supplemental References**


