Heart-Specific Activin Signaling Promotes Cardiomyopathy and Organismal Aging through Autophagy Inhibition

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Running title:

Activin promotes cardiac aging

Abstract

Age-dependent loss of cardiac tissue homeostasis largely impacts heart performance and contributes significantly to cardiovascular diseases later in life. Cellular quality control machinery, such as autophagy/lysosome system, plays a crucial role in maintaining cardiac health and preventing age-induced cardiomyopathy and heart failure. However, how aging alters autophagy/lysosome system to impact cardiac function remain largely unknown. Here using Drosophila model system, we show that cellular autophagic flux and lysosome number decrease in aging heart, which is associated with increased cardiomyopathy and cardiac arrhythmias. Among many known autophagy regulators, activin signaling (a member of TGF-beta superfamily) was identified in our recent study as a negative factor of autophagy and protein homeostasis in flight muscle. In this study, we find that cardiac-specific knockdown of Daw, an activin-like protein in Drosophila, prevents age-dependent increases in cardiac arrhythmias and diastolic dysfunction. Furthermore, cardiac-specific expressed activin type I receptor Babo results in pre-matured cardiac aging phenotypes at young ages. Similar to our previous flight muscle study, Daw silencing strongly promotes early step of autophagy process (i.e. autophagosome formation), and shows less impacts on autophagosome-lysosome fusion. Flies with Daw knockdown also maintain robust autophagic flux in aged fly hearts. Interestingly, reduction in cardiac activin signaling significantly prolongs lifespan and improves the functions of distal tissues (such as age-dependent climbing ability). Thus, our findings highlight the emerging role of activin signaling in autophagic regulation, cardiac aging, as well as systemic control of longevity.

Key words: Activin ligand, dawdle, myoglianin, Smad2, Atg8a, Lamp1, mTOR, TSC1, Bafilomycin A1

Introduction

Aging is associated with an exponential increase of the incidence of cardiovascular diseases (CVD) (Dai et al., 2012; North and Sinclair, 2012). Resolving the contributing mechanisms of cardiovascular diseases is a pressing goal of basic and translational aging research. Among many plausible mechanisms underlying age-related diseases, the impact of inter-organ communication on tissue aging, including cardiac aging, has recently become an important topic in the field of aging research. Several recently discovered blood-borne factors, such as growth differentiation factor 11 (GDF11), have been linked to systemic aging control and age-associated pathologies (e.g., cardiac hypertrophy) (Castellano et al., 2017; Demontis et al., 2014; Loffredo et al., 2013). However, due to their signal complexity, the precise regulatory role of these circulating factors (especially hormones and cytokines) in tissue aging remains largely unknown. To develop effective strategies targeting humoral factors to treat age-associated diseases (e.g., CVD), it is crucial to decipher the mechanisms underlying systemic aging regulation. These include how systemic factors interact with their receptors, how tissue-specific regulation is achieved, and how they mediate inter-organ communication to maintain organismal homeostasis.

Age-related changes in cardiovascular structure and output have been linked to increased risk of coronary heart disease, sudden cardiac death and stroke in aging population (Lakatta and Levy, 2003). During normal aging, the left ventricular wall of human hearts becomes thickened and the diastolic filling rate of left ventricle gradually decreases with age. On the other hand, the left ventricular systolic performance at rest remains less or shows no change with age (Lakatta and Levy, 2003). Several mechanisms underlying age-associated changes in cardiovascular structure and function are proposed, for example changes in growth factor signaling, decreased cellular quality control, altered calcium handling, elevated extracellular matrix deposition or fibrosis,

increased mitochondria damage, and the production of reactive oxygen species (ROS) (Dai et al., 2009; North and Sinclair, 2012).

Cellular quality control systems, such as macroautophagy (hereafter autophagy), are essential to maintain tissue homeostasis during aging (Quarles et al., 2015). Disruption of autophagy pathways by Atg5 knockout in the mouse heart accelerates cardiac aging, including an increase in left ventricular hypertrophy and decrease in factional shortening (Taneike et al., 2010). Although many longevity interventions activating autophagy can greatly preserve cardiac function during aging (North and Sinclair, 2012), no evidence has indicated that autophagy activation alone can delay the aging process in animal hearts. Furthermore, how aging negatively impacts and modulates autophagy is largely unknown (Quarles et al., 2015). Therefore, there is an urgent need to fully understand the regulation of cellular autophagy during cardiac aging, in order to develop effective ways to activate autophagy to prevent age-associated tissue damage and cardiac dysfunction.

We recently identified that activin signaling acts on hearts to regulate autophagy and age-induced cardiomyopathy in *Drosophila*. Additionally, the regulation of cardiac aging by activin signaling is ligand-dependent. RNAi against activin-like protein Daw preserved cardiac function with age, while reduction in GDF11-like protein Myo promotes cardiac aging. We found that Daw negatively regulates cardiac autophagy, while autophagy inhibition attenuates the positive effects of Daw RNAi on cardiac aging. Interestingly, despite the positive relationship between Daw and mTOR (mechanistic target of rapamycin, a major autophagy regulator), we found that activation of mTOR through TSC1 RNAi did not block the beneficial effects of Daw knockdown during cardiac aging, suggesting that Daw might regulate cardiac aging through mTOR-independent

pathways. Our findings suggest that Drosophila activin signaling may regulate autophagy and age-induced cardiomyopathy through novel mTOR-independent mechanisms.

Materials and methods

Fly Stocks, Feeding Protocol, and Chloroquine Treatment

UAS-Atg1 RNAi (HB387, BL26731), UAS-Trip attp40 (HB389, BL36304), UAS-Tsc RNAi (HB359, BL52931), UAS-Tsc1 RNAi (HB361, BL54034), Daw RNAi (HB314, BL50911), Daw RNAi (HB226, BL34974) were from the Bloomington Stock Center. Daw RNAi (HB226, BL34974) were backcrossed to ywR in our lab. Heart specific drivers Hand4.2-Gal4 and TinΔ4-Gal4 were kind gifts from Rolf Bodmer (Sanford Burnham Prebys Medical Discovery Institute). Unless specialized food is needed, fly strains were maintained on standard cornmeal molasses agar medium at room temperature (25℃). For aging experiments, fly stains were transferred to fresh food every 2-3 days. For chloroquine treatments, 100 ul of 20 mM chloroquine diphosphate salt (Sigma) was added onto the food.

Fly heartbeat analysis

A semi-intact Drosophila adult fly heart was prepared according to previously described protocols (Ocorr, Fink, Cammarato, Bernstein, & Bodmer, 2009; Vogler & Ocorr, 2009) in order to measure cardiac function parameters. High-speed 3000 frames movies were taken around the rate of 100 fps using a HAMAMATSU C11440 camera on an Olympus BX51WI microscope with a 10X water immersion lens. The live images were processed using HCI imaging software (Hamamatsu Corp). M-modes and quantitative data were generated using SOHA, a MatLab-based application that was published in K Occor et al., 2007 (Ocorr et al., 2007). The M-mode provides a snapshot of movement of heart tube wall edges of abdominal A3 segment in Y-axis over time in the X-axis. Diastolic Interval is the heart relaxation time, and systolic interval is the

heart contraction time. Heart Period is the duration between ends of two consecutive diastolic intervals. Arrhythmias observed in M-mode can be indicated as an arrhythmia index, which is obtained by normalizing the standard deviation of all heart periods in each record to the median heart period for each fly. The above measurements were made in abdominal A3 segment as well and then analyzed by GraphPad Prism 7 (GraphPad Softwore, Inc). The outliers were identified using Robust regression and Outlier removal (ROUT) method (Q=1%), and statistical significances were evaluated by t-test and one-way ANOVA analyses using GraphPad Prism 7.

Immunostaining and imaging

Antibodies for immonustaining included: anti-Atg8a (1:300) (generated in this study) and anti-rat IgG 594 (Jackson ImmunoResearch). F-actin was visualized by Alexa Fluor 488-conjugated Phalloidin (Invitrogen). Adult female flies were collected and dissected in PBS. Hearts were fixed in 4% paraformaldehyde for 15 min at RT. After washing in PBS with 0.1% Triton X-100 (PBST), the fixed hearted were blocked in 5% normal goat serum diluted in PBST (5% NGS) for 1 hour at RT. Hearts were then washed with PBST and incubated overnight at 4 ℃ with primary antibodies diluted in 5% NGS. After washing hearts with PBST, the samples were incubated for 2 hours at RT with the appropriate fluorescence-conjugated secondary antibodies (Jackson ImmunoResearch) diluted in PBST. Hearts were then washed again with PBST and mounted in ProLong Gold antifade reagent (Invitrogen). Samples were examined under an epifluorescence-equipped (Olympus) microscope. The number/area of positive immunostaining was measured with the “Measure and Count” function provided by Cellsens (Olympus).

Western Blot

Antibodies for western blot included: beta-Actin antibody (1:2000) (Cell Signaling Technology# 4967S), phospho-4E-BP1 antibody (1:1000) (Cell Signaling Technology# 2855S),

Atg8a antibody (generated from our lab), and HRP conjugated secondary antibodies, anti-Rat-IgG-HRP (1:5000) (Company), anti-Rabbit-IgG-HRP (1:5000) (Company). KC167 cells were homogenized in lysis buffer with leupeptin, benzamidine, antipain, PMSF and 2-Mercaptoethonal. Supernatant was denatured at 95 ℃ for 5 min. About 30ug of denatured protein was separated on Mini-PROTEAN precast gels (Bio-Rad) and then transfer to PVDF membrane (Bio-Rad). Following incubation with primary and secondary antibodies, the blots were visualized with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). Band intensity was quantified with Image Lab software (Bio-Rad).

Statistical analysis

GraphPad Prism 6 (GraphPad Software, La Jolla, CA) was used for statistical analysis. To compare the mean value of treatment groups versus that of control, either student t-test or one-way ANOVA was performed using Dunnett's test for multiple comparison. The effects of mutants during aging was analyzed by two-way ANOVA, including Tukey multiple comparisons test.

Results

In order to investigate Activin signaling regulation on cardiac aging, heart-specific drivers were crossed with ywR wild type and daw mutant flies (RNAi). The female flies from the offspring of above crosses were used to analyze heart performance on their 2-week-old, 4-week-old, 6-week-old and 8-week-old. On each time point, semi-intact Drosophila adult fly hearts were prepared according to previously described protocols (Ocorr, Fink, Cammarato, Bernstein, & Bodmer, 2009; Vogler & Ocorr, 2009) in order to measure cardiac function parameters. The heart movement was captured in high-speed 3000 frames movies taken at around the rate of 100 fps using a HAMAMATSU C11440 camera on an Olympus BX51WI microscope with a 10X

water immersion lens. The movies were processed using HCI imaging software (Hamamatsu Corp.) M-modes and quantitative data were generated using SOHA, a MatLab-based application that was published in K Occor et al., 2007 (Ocorr et al., 2007). The M-mode provides a snapshot of movement of heart tube wall edges in Y-axis over time in the X-axis. Diastolic Interval is the heart relaxation time, and systolic interval is the heart contraction time. Heart Period is the duration between ends of two consecutive diastolic intervals. Arrhythmias observed in M-mode can be indicated as an arrhythmia index, which is obtained by normalizing the standard deviation of all heart periods in each record to the median heart period for each fly. In this study, we found that cardiac-specific Daw knockdown preserves age-dependent increases in cardiac arrhythmia and diastolic dysfunction (Figure 1).

To investigate Activin signaling on autophagy, Atg8a (homolog of mammals LC3) antibody generated from our lab was used to detect the autophagosome. In young flies, the intensity of basal autophagosome levels were very low in wildtype hearts, but much higher in hearts expressing Daw RNAi. In old flies, even though the autophagosome number in hearts expressing Daw RNAi was lower than the young hearts, it was still significantly higher than the autophagosome number in wild type hearts (Figure 2A). In order to verify whether the high level autophagosome in daw RNAi line is due to elevated upstream autophagosome formation level, or defect downstream lysosomal activity, the BafA1 was used to inhibit the lysosomal activity. The accumulation of autophagosome after 1 hour BafA1 incubation was captured to represent the autophagy flux. Based on my results, the basal autophagy flux was very low and showed no significant difference between wild type and Daw RNAi hearts of either young or old flies (Figure 2B). There are two explanations for these results: (1) Activin signaling stimulates cardiac

autophagosome formation but does not affect basal autophagy flux. (2) The basal autophagy flux was too low to detect any difference between wild type and Daw RNAi flies.

It is well-known that nutrient-sensing pathways, such as mTOR, negatively regulate the activation of autophagy machinery. We then tested whether Activin/Daw signaling regulates autophagosome formation through mTOR. We found that reduced expression of Daw decreases the phosphorylation of 4E-BP, one of the major targets of mTOR signaling, suggesting that Daw positively regulates mTOR activities (Figure 3A). However, we found that mTOR signaling is not required for the activation of autophagosome formation by Daw RNAi, indicated by the high levels of autophagosome in Daw and TSC1 double knockdown flies (Fig. 3B). These results suggest that Daw regulates autophagosome formation through an unknown mTOR-independent mechanism. As we observed an unconventional regulation of autophagy by Activin/Daw signaling, we wonder whether or not Daw might regulate cardiac aging through mTOR. In contrast to previous reports (Lee et al., 2010), activation of mTOR by Tsc1 RNAi did not affect cardiac arrhythmicity or accelerated cardiac aging, and mTOR is not required for Daw-mediated cardiac aging (Fig. 3C). Altogether, Activin/Daw signaling regulates cardiac aging through autophagy/lysosome system, independent of mTOR.

Atg1 is essential to initiate autophagy machinery in Drosophila, our next step was to test whether autophagy plays any role in cardiac aging. Autophagy disruption via silencing Atg1 in heart using two tissue-specific drivers Tin-Delta4-Gal4 and Hand4.2-Gal4 both showed premature cardiac phenotypes: in 2-week-old female hearts, the Arrhythmia index and diastolic intervals increased to aged hearts' level (Figure 4). Knocking-down Atg1 in Hand4.2-Gal4 tissue driver had stronger phenotype than in Tin-Delta4-Gal4 because Hand4.2-Gal4 is more robust than the other.

To further test whether autophagy plays any role in Daw-regulated cardiac aging, cardiac tissue-specific drivers Hand4.2-gal4 were crossed with ywR wild type and Daw mutant flies (RNAi), the adult fly progeny was then fed with lysosomal inhibitor chloroquine (CQ) for 24 hours before analyzing cardiac function by SOHA. CQ treatment increased arrhythmia index of Daw RNAi flies at advanced age, suggesting disruption of autophagy/lysosome system abolishes the beneficial effects of Daw knockdown on cardiac arrhythmicity (Figure 5A). Similarly, autophagy disruption via Atg1 RNAi blocked the beneficial effects of Daw RNAi on age-dependent cardiac arrhythmicity (Figure 5B).

Discussion

Aging is an extremely complex, multifactorial process accompanied by accumulation of deleterious changes in cells and tissues that are respond for a wide range of diseases and even death. During the process, the heart undergoes complex phenotypic changes such as progressive myocardial remodeling, declined myocardial contractile capacity, increased left ventricular wall thickness and chamber size, prolonged diastole as well as increased arrhythmia (Lakatta & Levy, 2003; Strait & Lakatta, 2012). All of those biological changes can gradually alter the cardiac functions and confer vulnerability of the heart to various cardiovascular stresses, thus increase the chance of developing cardiovascular disease (CVD) dramatically. Even though the death rates in United States caused by CVD have declined in the United States recent years due to large amount of research on related field, it is still the leading cause of death, especially in elder population. There are 30.8% deaths caused by CVD from 2003 to 2013 in United States, 65% of them occurred after the age of 75 (Mozaffarian et al., 2016). In this regard, it is crucial to understand the mechanism behind cardiac aging, it can help us not only prevent CVD but also explore potential treatments for promoting cardiac health.

So far there are large amount of theories for cardiac aging process. It was generally accepted that age-related CVD results from the accumulation of cholesterol and fatty acids in tissues, which will further induce the production of inflammatory cytokines and reactive oxygen species (ROS) (North & Sinclair, 2012). Recent years, a number of longevity genes and their related pathways have been identified involved in regulating fundamental process of cardiac aging. For instance, it has been proposed that calories restriction can increase health span and reduce the incidence of most age-related diseases (i.e. CVDs) by promoting expression of longevity genes (Ahmet et al., 2011). Even though enriched knowledge of molecular mechanism of cardiac aging has been revealed, the precise aging process, which cannot explain in a unifying manner still remains largely unknown. As more novel mechanisms behind cardiac aging being explored, the unique role of autophagy has been depicted in regulation of biological aging process.

Autophagy is a highly conserved process that maintains tissue and cellular homeostasis by degradation and recycling of damaged organelles, protein aggregates and other cytoplasmic substances. Microautophagy, chaperon-mediated autophagy and macroautophagy are three identified pathways of autophagy, macroautophagy (simply referred to autophagy hereafter) is the pathway that will be the focus of our study. The autophagic process initiated with the isolated membrane, or phagophore that elongates to form a double-membrane structure, the autophagosome. Then the autophagosome fuses with a lysosome to form an autolysosome to degrade the enclosed materials along with autophagosomal membrane (Shibutani & Yoshimori, 2014). In Drosophila, the components of autophagy can be divided into several function units, they are: (1) the serine/threonine protein kinase complex Atg1 (ULK1, ULK2 in mammals), commonly considered as the initiator of the autophagy machinery; (2) two ubiquitin-like

conjugation systems, Atg12 (covalently conjugated to Atg5), and Atg8 [(LC3 in mammals) conjugated to the lipid molecule phosphatidylethanolamine (PE)]; and (3) a protein complex containing the class III phosphatidylinositol 3-kinase Vps34 (PIK3C3 in mammals), that is required for autophagosome formation (Neufeld, 2012; Wong, Puente, Ganley, & Jiang, 2013). There are evidence supports that basal autophagy plays essential roles in maintaining normal heart homeostasis and morphology. Disruption of autophagy by Atg5 knockout in mouse heart has shown premature phenotype, such as increased left ventricular hypertrophy and decreased fractional shortening (Taneike et al., 2010). Thus manipulation of autophagy machinery could potentially help to develop the therapeutic approaches to treat CVDs. However, the most fundamental questions about autophagy, how cardiac aging regulates autophagy, and what is the cause of age-associated autophagy alterations are still unsolved. Therefore in my study, I aim to gain deeper understanding of the fundamental relationship between cardiac aging and autophagy.

Previous literature performed lifespan screening and proposed that the activin signaling of TGF-b family is involved in regulating muscle aging and autophagy (Bai, Kang, Hernandez, & Tatar, 2013). Based on my study, the Activin signaling also plays a role in cardiac aging and cardiac basal autophagosome formation. Besides, the transforming growth factor b (TGF-b) family signaling is essential in regulating cell growth, differentiation and developmental process in wide range of biological systems (Massague, 2012). Thus the Activin signaling of TGF-b family could be a good start point to explore the mechanism of cardiac aging process. Moreover, the reverse genetics screening of Actin signaling downstream targets can be an alternative method to identify more candidate genes that are potentially involved in Activin signaling/cardiac aging mechanism. In Drosophila, fewer signaling components are present which can simplify mechanistic study of this pathway. As in mammals, TGF-b family signaling

pathway has two branches in Drosophila, they are bone morphogenetic protein (BMP) and Activin signaling pathway. In both pathways, signaling starts with ligand binding to a receptor complex composed of type I and type II receptor kinases. Activation of type II receptor kinase by ligand binding causes phosphorylation of type I receptor kinase, which enables appropriate phosphorylation of receptor-activated Smad (R-Smad) substrate. R-Smad phosphorylation causes formation of complex with the Drosophila common-Smad (co-Smad), Medea, which can translocate to the nucleus and regulate its downstream transcriptional activity. Within the Activin subfamily, three ligands, activin-b (Actb), Dawdle (Daw), and Myoglianin (Myo) signal through the type I receptor Baboon (Babo), type II receptor Wit or Punt to activate transcriptional factor dSmad2, the activated form of dSmad2 then forms a complex with its cofactor Medea and translocates to nucleus to regulate downstream transcription (Upadhyay, Moss-taylor, Kim, Ghosh, & Connor, 2017).

Here we uncovered an important role of activin signaling in the regulation of cardiac aging in Drosophila. Our findings of mTOR-independent autophagy suggests that there are diverse signaling pathways controlling autophagy processes and each of them might play distinct roles in tissue homeostasis and aging. This work will contribute to advance our understanding of the molecular and genetic mechanisms underlying cardiac aging. Activin signaling is one of the important targets of therapeutic drug development for the treatment of lung cancer and muscle dystrophy (especially cancer cachexia) (Tsuchida et al., 2009; Zhou et al., 2010). A number of clinical trials are currently investigating treatments for cancer and muscle wasting using activin inhibitors (Cohen et al., 2015). Thus, our work is expected to significantly advance the field at both the basic and applied levels. Due to the signal complexity of activin signaling (for example, multiple ligands act on a few receptors), the tissue-dependent action of each activin ligand

remains to be carefully examined. Using sophisticated Drosophila genetic tools and tissue-specific control of gene expression, we anticipate the identification of distinct mechanisms by which activin signaling regulates tissue homeostasis during aging.

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Author Contributions Statement

H.B., R.B., and K.O. planned research and prepared manuscript. K.C., P.K., Y.L., K.H., E.T., H.B. performed research. All authors reviewed the manuscript and approved.

Competing Interests

The authors have declared that no competing interest exists.

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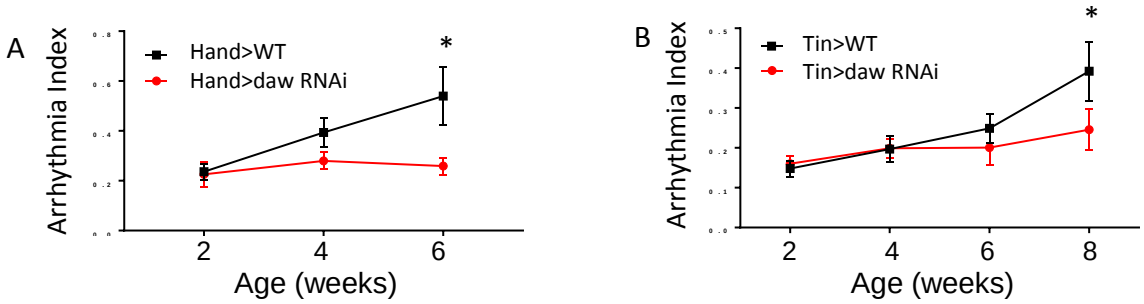

Figure 1. Arrhythmicity was measured and quantified as arrhythmia indexes across a 6-week and 8-week time courses (Mean ± SEM, 16-38 flies/data points. Among those, larger sample size were used in 6-week and 8-week time points to control increased variance caused by aging). Cardiac performance were measured every 2 weeks. Adult progeny of Dawdle RNAi stains (daw 50911 and daw 34974) crossed with heart tissue drivers (Hand4.2-Gal4 and TinΔ4-Gal4) has a significantly lower arrhythmia index in old flies [6-week-old and 8-week-old, *P < 0.05 (unpaired t-test)].

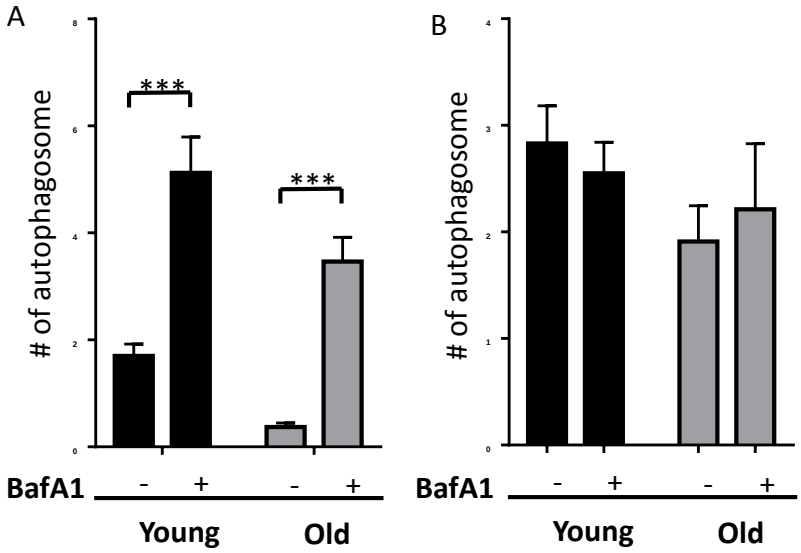

Figure 2. Knocking down Daw induced basal level autophagosome formation. (2A) The basal autophagosome number in young (2 week old) and old (8 week old) female flies was detected by Atg8a antibodies (Mean ± SEM, 4 flies/data points). The regions of interested (ROI) selected from A1/A2 abdominal segments of adult fly heart were used for *measure and count* analysis provided by CellSens (Olympus Corp.) [**P < 0.01, ***P < 0.001 (student's t-test)]. (2B) Quantification of autophagy flux by autophagosome number fold change after BafA1 treatment in young (2 week old) and old (8 week old) female flies [Mean ± SEM, 4 flies/data points, P < 0.05 (student's t-test)].

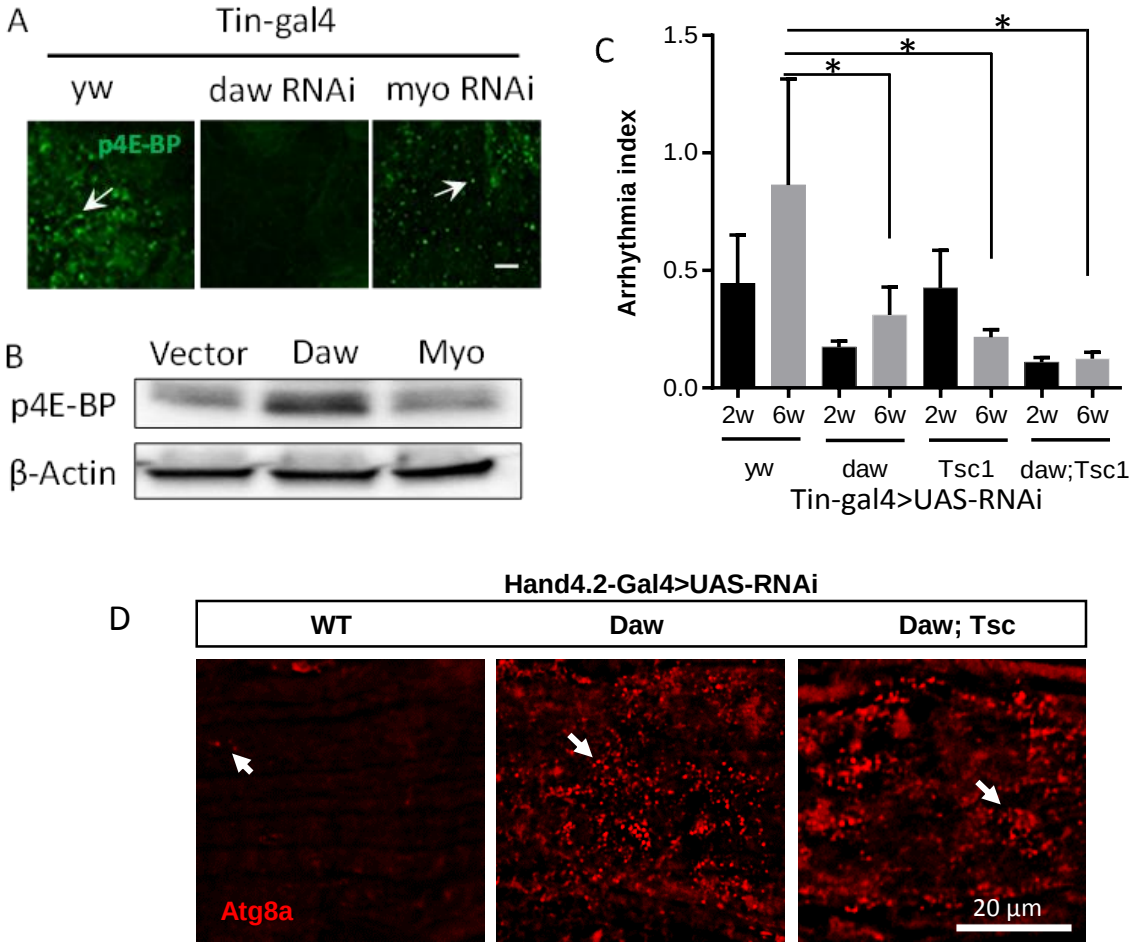

Figure 3. Activin/Daw signaling regulates cardiac aging through autophagy/lysosome system, independent of mTOR. (A) Western blots show Daw, not Myo treatment induces phosphorylation of 4E-BP in cultured Kc167 cells. (B) Daw RNAi induced autophagosome formation detected by Atg8a antibodies (C) and age-associated arrhythmicity independent of mTOR/TSC1. [Scale bar: 50 µm. *P < 0.05 (Student's t-test)].

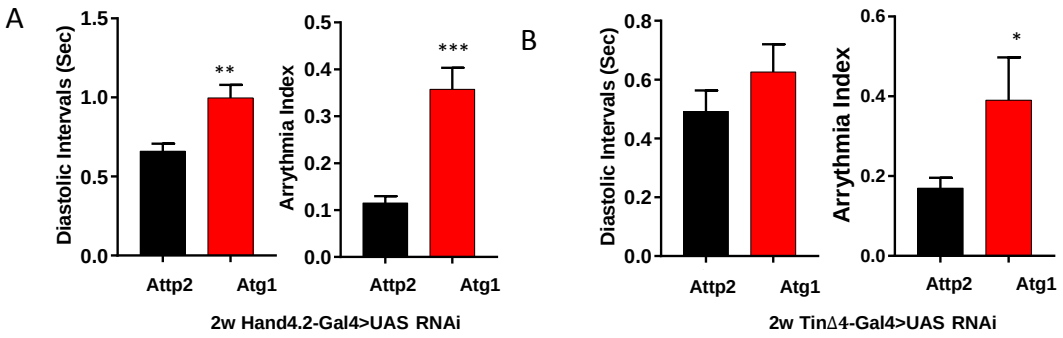

Figure 4. Autophagy disruption via Atg1 RNAi led to premature cardiomyopathy in young flies. High-speed video microscopy and SOHA were used to quantitatively analyze beating D. melanogaster hearts at 2 weeks (Mean ± SEM, 16-20 flies/data points). Adult progeny of Atg1 RNAi stains crossed with heart tissue drivers (Hand4.2-Gal4 and TinΔ4-Gal4) has a significantly higher arrhythmia index. The diastolic interval also elevated significantly in adult progeny crossed with Hand4.2-Gal4 ([*P < 0.05, **P < 0.01, ***P < 0.001 (unpaired t-test)].

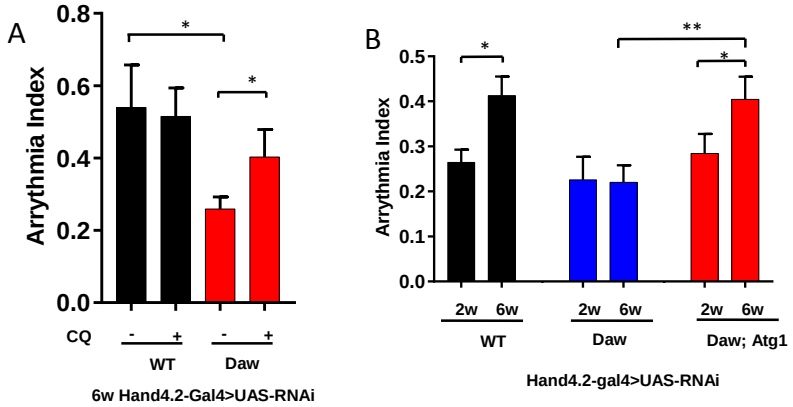

Figure 5. Autophagy disruption via CQ feeding in Daw RNAi old flies phenocopied wild type old flies cardiac performance. For chloroquine treatments, 100 ul of 20 mM chloroquine diphosphate salt (Sigma) was added onto the food. High-speed video microscopy and SOHA were used to quantitatively analyze beating D. melanogaster hearts at 6 weeks (Mean ± SEM, 14-20 flies/data points). After 24 hour chloroquine (CQ) treatment, the arrhythmia index for adult progeny of Daw RNAi stains crossed with heart tissue drivers (Hand4.2-Gal4) was brought back to wild type level. [*P < 0.05, (unpaired t-test)].