

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

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22 **Running title:**

23 **Activin promotes cardiac aging**

24 **Abstract**

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

44

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

47 **Introduction**

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

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

70 increased mitochondria damage, and the production of reactive oxygen species (ROS) (Dai et al.,
71 2009; North and Sinclair, 2012).

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

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

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

94 **Materials and methods**

95 *Fly Stocks, Feeding Protocol, and Chloroquine Treatment*

96 UAS-Atg1 RNAi (HB387, BL26731), UAS-Trip atp40 (HB389, BL36304), UAS-Tsc
97 RNAi (HB359, BL52931), UAS-Tsc1 RNAi (HB361, BL54034), Daw RNAi (HB314,
98 BL50911), Daw RNAi (HB226, BL34974) were from the Bloomington Stock Center. Daw
99 RNAi (HB226, BL34974) were backcrossed to ywR in our lab. Heart specific drivers Hand4.2-
100 Gal4 and TinΔ4-Gal4 were kind gifts from Rolf Bodmer (Sanford Burnham Prebys Medical
101 Discovery Institute). Unless specialized food is needed, fly strains were maintained on standard
102 cornmeal molasses agar medium at room temperature (25°C). For aging experiments, fly stains
103 were transferred to fresh food every 2-3 days. For chloroquine treatments, 100 ul of 20 mM
104 chloroquine diphosphate salt (Sigma) was added onto the food.

105 *Fly heartbeat analysis*

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

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

122 *Immunostaining and imaging*

123 Antibodies for immunostaining included: anti-Atg8a (1:300) (generated in this study) and
124 anti-rat IgG 594 (Jackson ImmunoResearch). F-actin was visualized by Alexa Fluor 488-
125 conjugated Phalloidin (Invitrogen). Adult female flies were collected and dissected in PBS.
126 Hearts were fixed in 4% paraformaldehyde for 15 min at RT. After washing in PBS with 0.1%
127 Triton X-100 (PBST), the fixed hearts were blocked in 5% normal goat serum diluted in PBST
128 (5% NGS) for 1 hour at RT. Hearts were then washed with PBST and incubated overnight at 4 °C
129 with primary antibodies diluted in 5% NGS. After washing hearts with PBST, the samples were
130 incubated for 2 hours at RT with the appropriate fluorescence-conjugated secondary antibodies
131 (Jackson ImmunoResearch) diluted in PBST. Hearts were then washed again with PBST and
132 mounted in ProLong Gold antifade reagent (Invitrogen). Samples were examined under an
133 epifluorescence-equipped (Olympus) microscope. The number/area of positive immunostaining
134 was measured with the “Measure and Count” function provided by CellSens (Olympus).

135 *Western Blot*

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

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

146 *Statistical analysis*

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

152 **Results**

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

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

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

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

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

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

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

214 **Discussion**

215 Aging is an extremely complex, multifactorial process accompanied by accumulation of
216 deleterious changes in cells and tissues that are respond for a wide range of diseases and even
217 death. During the process, the heart undergoes complex phenotypic changes such as progressive
218 myocardial remodeling, declined myocardial contractile capacity, increased left ventricular wall
219 thickness and chamber size, prolonged diastole as well as increased arrhythmia (Lakatta & Levy,
220 2003; Strait & Lakatta, 2012). All of those biological changes can gradually alter the cardiac
221 functions and confer vulnerability of the heart to various cardiovascular stresses, thus increase
222 the chance of developing cardiovascular disease (CVD) dramatically. Even though the death
223 rates in United States caused by CVD have declined in the United States recent years due to
224 large amount of research on related field, it is still the leading cause of death, especially in elder
225 population. There are 30.8% deaths caused by CVD from 2003 to 2013 in United States, 65% of
226 them occurred after the age of 75 (Mozaffarian et al., 2016). In this regard, it is crucial to
227 understand the mechanism behind cardiac aging, it can help us not only prevent CVD but also
228 explore potential treatments for promoting cardiac health.

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

241 Autophagy is a highly conserved process that maintains tissue and cellular homeostasis
242 by degradation and recycling of damaged organelles, protein aggregates and other cytoplasmic
243 substances. Microautophagy, chaperon-mediated autophagy and macroautophagy are three
244 identified pathways of autophagy, macroautophagy (simply referred to autophagy hereafter) is
245 the pathway that will be the focus of our study. The autophagic process initiated with the
246 isolated membrane, or phagophore that elongates to form a double-membrane structure, the
247 autophagosome. Then the autophagosome fuses with a lysosome to form an autolysosome to
248 degrade the enclosed materials along with autophagosomal membrane (Shibutani & Yoshimori,
249 2014). In *Drosophila*, the components of autophagy can be divided into several function units,
250 they are: (1) the serine/threonine protein kinase complex Atg1 (ULK1, ULK2 in mammals),
251 commonly considered as the initiator of the autophagy machinery; (2) two ubiquitin-like

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

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

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

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

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

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303 fly stocks and cDNA clones. We thank Michael O'Connor for the kind advice, activin reagents,
304 and fly lines.

305 **Author Contributions Statement**

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

308 **Competing Interests**

309 The authors have declared that no competing interest exists.

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311 **References**

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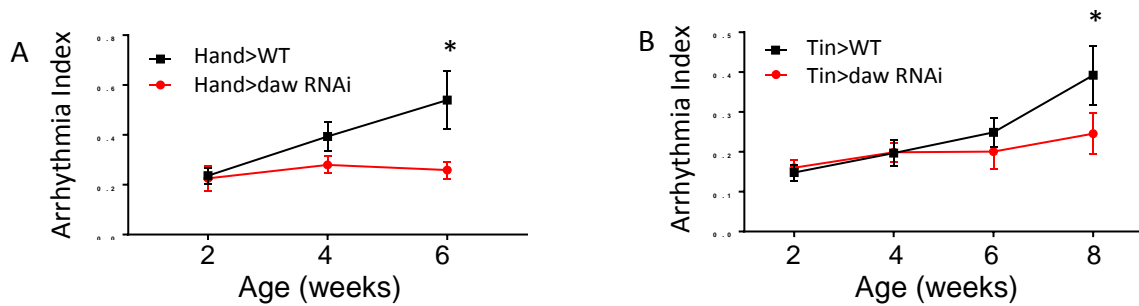


Figure 1. Arrhythmicity was measured and quantified as arrhythmia indexes across a 6-week and 8-week time courses (Mean \pm SEM, 16-38 flies/data points. Among those, larger sample size were used in 6-week and 8-week time points to control increased variance caused by aging). Cardiac performance were measured every 2 weeks. Adult progeny of Dawdle RNAi stains (daw 50911 and daw 34974) crossed with heart tissue drivers (Hand4.2-Gal4 and Tin Δ 4-Gal4) has a significantly lower arrhythmia index in old flies [6-week-old and 8-week-old, *P < 0.05 (unpaired t-test)].

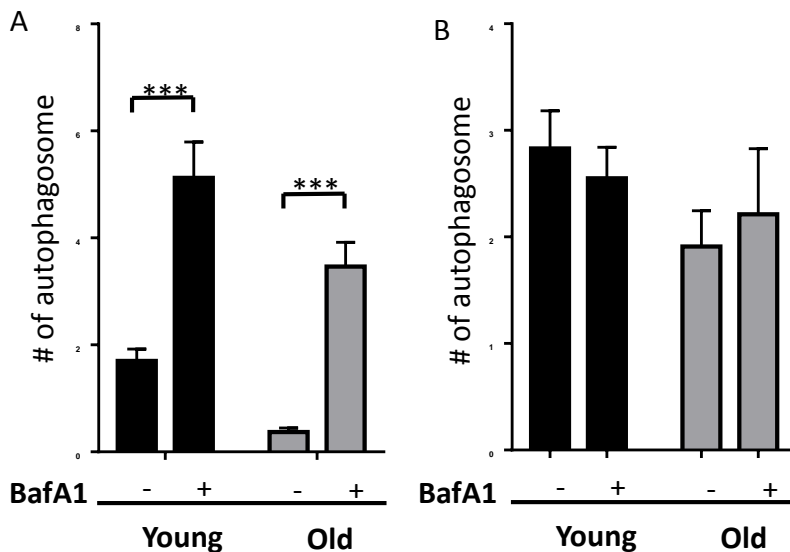


Figure 2. Knocking down Daw induced basal level autophagosome formation. (2A) The basal autophagosome number in young (2 week old) and old (8 week old) female flies was detected by Atg8a antibodies (Mean \pm SEM, 4 flies/data points). The regions of interested (ROI) selected from A1/A2 abdominal segments 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 \pm SEM, 4 flies/data points, P < 0.05 (student's t-test)].

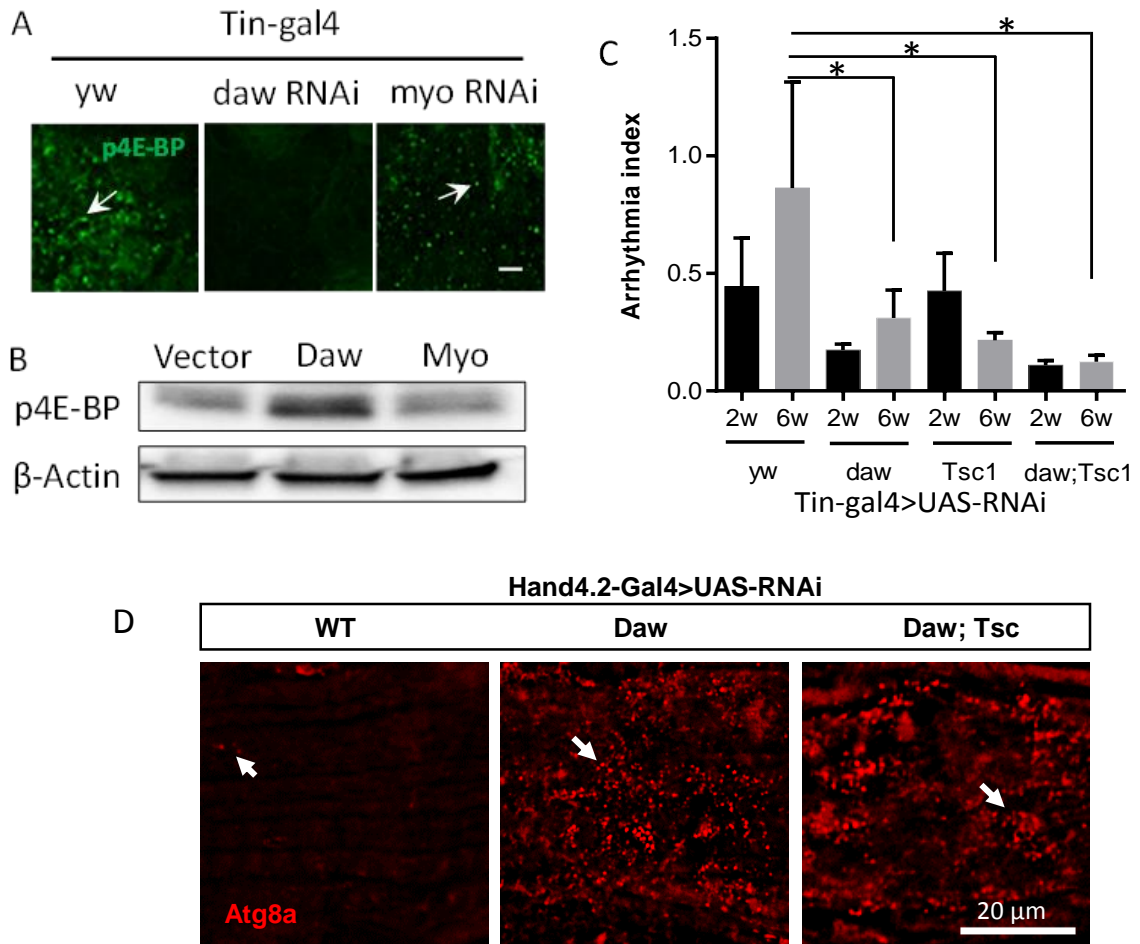


Figure 3. Activin/Daw signaling regulates cardiac aging through autophagy/lysosome system, independent of mTOR. (A) Western blots show Daw, not Myo treatment induces phosphorylation of 4E-BP in cultured Kc167 cells. (B) Daw RNAi induced autophagosome formation detected by Atg8a antibodies (C) and age-associated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

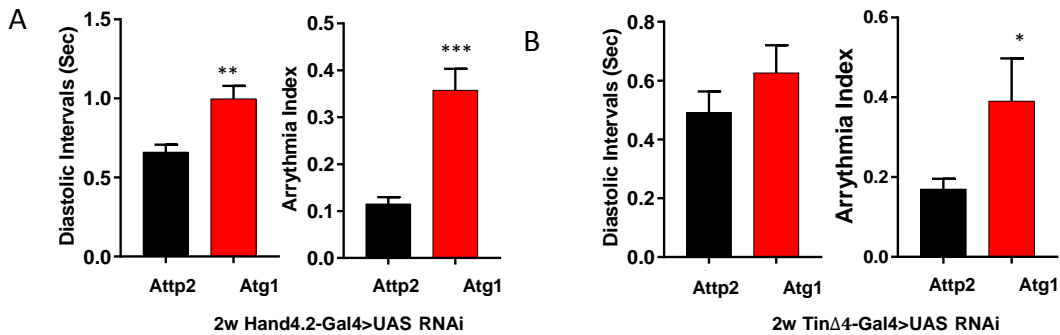


Figure 4. Autophagy disruption via Atg1 RNAi led to premature cardiomyopathy in young flies. High-speed video microscopy and SOHA were used to quantitatively analyze beating *D. melanogaster* hearts at 2 weeks (Mean \pm SEM, 16-20 flies/data points). Adult progeny of Atg1 RNAi stains crossed with heart 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)].

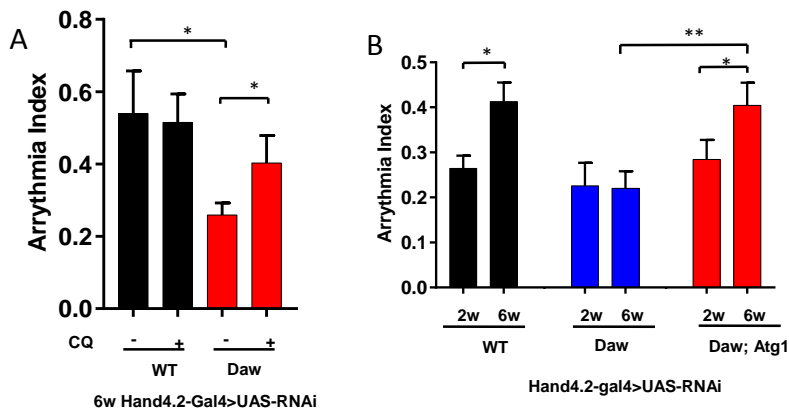


Figure 5. Autophagy disruption via CQ feeding in Daw RNAi old flies phenocopied wild type old flies cardiac performance. For chloroquine treatments, 100 μ l of 20 mM chloroquine diphosphate salt (Sigma) was added onto the food. High-speed video microscopy and SOHA were used to quantitatively analyze beating *D. melanogaster* hearts at 6 weeks (Mean \pm 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)].