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Dual Role of β 1-Integrin and Integrin-Linked Kinase in Modulating Cardiac Aging

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SUMMARY

Cardiac performance decreases with age, which is a major risk factor for cardiovascular disease and mortality in the aging human population, but the molecular mechanisms underlying cardiac aging are still poorly understood. Investigating the role of Integrin-Linked Kinase (*ilk*) and β 1-Integrin (*myspheroid*, *mys*) in *Drosophila*, which co-localize near cardiomyocyte contacts and Z-bands, we find that reduced *ilk* or *mys* function prevents the typical changes of cardiac aging seen in wildtype, such as arrhythmias. In particular, the characteristic increase in cardiac arrhythmias with age is prevented in *ilk* and *mys* heterozygous flies with nearly identical genetic background, and they live longer, in line with previous findings in *C. elegans* for *ilk* and *Drosophila* for *mys*. Consistent with these findings we observed elevated β 1-integrin protein levels in old compared to young wild-type flies, and cardiac-specific overexpression of *mys* in young flies causes aging-like heart dysfunction. Moreover, moderate cardiac-specific knockdown of integrin/ILK pathway-associated genes also prevented the decline in cardiac performance with age. In contrast, strong cardiac knockdown of *ilk* or ILK-associated genes can severely compromise cardiac integrity, including cardiomyocyte adhesion and overall heart function. These data suggest that *mys/ilk* function is necessary for establishing and maintaining normal heart structure and function, and appropriate fine-tuning of this pathway can retard the age-dependent decline in cardiac performance, and extend lifespan. Thus, integrin/ILK-associated signaling emerges as an important and conserved genetic mechanism in longevity, and as a new means to improve age-dependent cardiac performance, in addition to its vital role in maintaining cardiac integrity.

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Keywords

cardiomyopathy; arrhythmia; heart failure; senescence; *Drosophila*; *C. elegans*; *ilk*; *myspheroid*; *parvin*; *paxillin*; *pinch*; *talin*; cell adhesion

INTRODUCTION

With age, heart function declines and the prevalence of heart disease is dramatically increased. For example, the incidence of heart failure and atrial fibrillation is markedly increased in the elderly (Lakatta & Levy, 2003; Roger et al., 2011), which suggests that aging *per se* is a major risk factor for heart disease. It is well known that the heart undergoes many age-related functional and structural changes (Bernhard & Laufer, 2008; Khan et al., 2002). However, the control mechanisms of cardiac-intrinsic aging, and their coordination with organismal aging, remain elusive.

Cardiac decline with age and many specific age-related changes occurring in the human heart have also been observed in a variety of other species. Therefore, insights from model organisms, such as *Drosophila*, the simplest (genetic) model system with a heart (Bier & Bodmer, 2004), will likely provide valuable clues for understanding the cellular and molecular mechanisms involved in cardiac aging (Dai et al., 2010; Nishimura et al., 2011). *Drosophila* has a short lifespan that makes it an ideal model system for studying the genetic underpinnings of aging. Although the linear heart tube of *Drosophila* is much less complex than the mammalian heart, its development and functional characteristics are remarkably conserved (Bodmer, 1995; Bodmer & Frasch, 2010; Ocorr et al., 2007; Cammarato et al., 2008; Olson, 2006). With age, the fly heart also shows features similar to mammals, including humans, with respect to structural alterations and propensity towards arrhythmias (Ocorr et al., 2007; Cammarato et al., 2008; Taghli-Lamalle et al., 2008; Wessells et al., 2004; Fink et al., 2009). At young ages, surgically exposed fly hearts show a regular myogenic beating pattern. As flies age, these heartbeats become less regular and show increased arrhythmias, which are reminiscent of the increased incidence of atrial fibrillation in elderly humans. Thus, many fundamental aspects of cardiac aging seem to be conserved (Dai et al., 2010; Bodmer & Frasch, 2010), as is organismal aging (Kenyon, 2010).

Integrins are major adhesive transmembrane receptors that bind to the extracellular matrix (ECM). Their activation affects cytoskeletal remodeling and other intracellular signaling pathways (Delon & Brown, 2007). Integrin signaling and the link between integrins and the cytoskeleton are mediated by many proteins, including Integrin-Linked Kinase (ILK), Talin, and Focal Adhesion Kinase (FAK) (Geiger et al., 2001). Mammalian ILK is a putative serine/threonine kinase, originally identified as a binding partner for the cytoplasmic tail of β 1-integrin (Hannigan et al., 1996). ILK also binds to the adapter proteins Parvin, Pinch, and Paxillin (Pax), thereby providing an integrin signaling platform (Legate et al., 2006). *Drosophila* has single orthologues of ILK, Parvin, Pinch, and Pax (Legate et al., 2006). In *Drosophila*, *ilk* homozygous mutants are embryonic lethal and show severe muscle attachment defects (Zervas et al., 2001). ILK is also essential for development in mouse and *C. elegans* (Sakai et al., 2003; Mackinnon et al., 2002). Remarkably, RNAi-induced

reduction of ILK, or the ILK binding partner Parvin, causes increased longevity in *C. elegans* (Hansen et al., 2005; Curran & Ruvkun, 2007). These findings suggest that in contrast to complete loss of *ilk*, which is deleterious for organismal development, moderate *ilk* knockdown (KD) extends lifespan in *C. elegans*. Interestingly, heterozygous *Drosophila* mutants for β 1-integrin (*mys*) also have an increased lifespan (Goddeeris et al., 2003). Therefore, reduced β 1-integrin/ILK signaling may also be beneficial for cardiac-specific aging. Interestingly, overexpression of *ilk* in rat cardiac fibroblasts induces cellular senescence, whereas inhibition of *ilk* prevents senescence-related changes in these cells (Chen et al., 2006). In contrast, conditionally targeted knockout of *ilk* in the mouse heart causes left ventricle dilation, heart failure, disaggregation of cardiac tissue, leading to sudden death (White et al., 2006). Taken together, these results indicate a critical role for *ilk* in establishing and maintaining heart contractility. Thus, we hypothesize that *ilk* has a dual role in the heart, one that modulates cardiac aging and one that maintains the heart's structural integrity.

In this study, we demonstrate that reduced integrin/ILK ameliorates the effects of normal cardiac and organismal aging in *Drosophila*. *ilk* and *mys* heterozygotes not only live longer, but their hearts perform better at old age than wild-type controls, similar to young flies. Moreover, moderate cardiac-specific KD of integrin/ILK-associated genes, *pax*, *parvin*, *talin* and *pinch* also prevents the decline of heart performance with age. Conversely, cardiac overexpression of *mys* causes a senescent-like phenotype in young flies. These findings suggest that the accumulation of β 1-Integrin at an older age may mediate in part the declining heart function, and that a moderate reduction of integrin/ILK activity maintains youthful heart function with age. In contrast, more severe cardiac-specific KD of *ilk* and other ILK-associated components leads to a higher incidence of cardiac arrhythmia already in young flies, which is accompanied by defective cellular adherence of the cardiomyocytes. Thus, severely compromised integrin/ILK pathway function is detrimental for the heart, but fine-tuned moderate reduction maintains youthful cardiac performance, suggesting a dual role for this complex in regulating cardiac integrity and aging.

RESULTS

ilk heterozygous mutants have extended lifespan in *Drosophila*

Since the RNAi-mediated knock-down (KD) of *ilk* extends lifespan in *C. elegans* (Hansen et al., 2005; Curran & Ruvkun, 2007), we wondered if reduced *ilk* expression is also beneficial to longevity in *Drosophila*. Since lifespan can be significantly modulated by genetic background (Grandison et al., 2009), we first backcrossed *ilk*⁵⁴ mutants (premature stop codon; Zervas et al., 2011; see Experimental Procedures) to the wildtype control strain, *w*^{CS} (Cook-Wiens & Grotewiel, 2002) for six generations. The resulting backcrossed lines are referred to as *ilk*^{54-wCS}. We found both female and male *ilk*^{54-wCS} heterozygous mutants (*w*^{CS};*ilk*^{54-wCS/+}) show extended lifespan compared to their *w*^{CS} controls (Table 1, Fig. 1).

β 1-integrin interacts with ILK (Hannigan et al., 1996), and homozygous mutants (*mys*) have embryonic muscle phenotypes similar to *ilk* mutants (Zervas et al., 2001). In addition, *mys* heterozygotes had previously been reported to exhibit an extended mean lifespan (Goddeeris et al., 2003). Thus, we re-examined the lifespan of *mys*^{XG43} heterozygotes (also in the *w*^{CS}

genetic background, contains small deletion and premature stop codon; Goddeeris et al., 2003; see Experimental Procedures), and confirmed that *mys*^{XG43/w^{CS}} flies have a significantly extended lifespan, similar to *ilk*^{54-w^{CS}/+} flies (Table 1, Fig. 1), in terms of both maximum as well as mean life span (Fig. 1). Together, these data suggest a conserved role of the integrin/ILK pathway in organismal aging.

ILK is localized to cell-cell contact sites and Z-discs in adult hearts

To examine *ilk* expression in the *Drosophila* heart (Fig. 2A), we used a genomic *ilk-GFP* fusion rescue construct that contains the putative *ilk* promoter, enhancers, and *ilk* transcription unit. This transgene fully rescues the embryonic *ilk* mutant defects previously reported (Zervas et al., 2001). Similar to the abundant expression of *ilk* in human hearts (Hannigan et al., 1996), *ilk-GFP* was also expressed in the adult *Drosophila* heart and co-localized with β 1-integrin (Fig. 2A-C). Interestingly, ILK-GFP and β 1-Integrin prominently accumulated at or near cell-cell junctions at both dorsal and ventral sides of cardiomyocytes (Fig. 2B-C, arrows), where it coincides with the cytoskeletal protein α -Spectrin (Fig. 2D) (Pesacreta et al., 1989). This suggests that ILK and β 1-integrin concentrate at the plasma membrane that contacts adjacent cardiomyocytes within the heart tube (see Fig. 2A). ILK-GFP accumulation was also found to co-localize with α -actinin, a sarcomeric Z-disc marker (Fig. 2E). β 1-Integrin also co-localizes with Cypher-GFP, another Z-disc marker in myocardial cells (Fig. 2F).

***ilk* and *mys* heterozygotes do not exhibit age-related increases in arrhythmias**

The expression patterns of ILK and β 1-integrin in *Drosophila* hearts suggest a possible functional requirement for the β 1-integrin/ILK pathway in maintaining normal heart structure and function. To test this possibility we analyzed cardiac performance of *ilk* and *mys* heterozygotes using high-speed video imaging of semi-intact heart preparations (Ocorr et al., 2007). At young ages (1-week old), the control hearts show regular beating patterns (see M-modes in Fig. 3A), and consequently a low Arrhythmia Index (AI) (Fig. 3B). AI is the normalized heart period's standard deviation similar to the coefficient of variation, and is a quantification of the variability in heart period (defined as the period from the beginning of one contraction to the beginning of the next contraction; Fink et al., 2009). As wild-type (*w^{CS}*) flies age, the heart rhythm becomes progressively less regular, as exemplified by increases in AI (Fig. 3A,B; Ocorr et al., 2007). In contrast to wildtype, *ilk*^{54-w^{CS}/+} exhibit a much diminished increase in AI with age (Fig. 3A,B). To gain additional information about heart function, we also measured systolic and diastolic diameters of the hearts from the video images. Wildtype *w^{CS}* hearts exhibited a modest decrease in the diastolic as well as systolic diameters with age (Fig. 3C,D), as has previously been observed in other wild-type controls (Cammarato et al., 2008). This suggests a tendency of age-related diastolic dysfunction also in flies, as is the case in humans (Lakatta & Levy, 2003), although this parameter is somewhat variable. In contrast, aging *ilk*^{54-w^{CS}/+} flies exhibit no significant difference with age in heart tube diameters; however, hearts from these flies are already relatively constricted even at young ages (Fig. 3C,D). Fractional shortening, a measure of cardiac contractility, was preserved in these flies (Fig. 3E).

Similar to *ilk^{54-wCS/+}* flies, 5-week old *mys^{XG43-wCS/+}* heterozygotes also exhibited a more regular heart beat pattern, compared to age-matched *w^{CS}* controls, as manifest in a markedly lower AI, thus abolishing the typical wildtype age-related increase in AI (Fig. 3A,B). However, unlike *ilk^{54-wCS/+}* flies, old *mys^{XG43-wCS/+}* heterozygotes still showed a modest decrease in diastolic diameter, as *w^{CS}* controls (Fig. 3C), thus diastolic dysfunction was not prevented in this case. Moreover, young *mys^{XG43-wCS}* heterozygotes had larger systolic diameters (Fig. 3D) and thus lower fractional shortening (Fig. 3E) compared to controls, suggestive of systolic dysfunction at that age.

We replicated the above findings and confirmed similar trends in different genetic backgrounds: *ilk^{54/+}* flies as well as in *mys^{XG43/+}* and *mys^{1/+}* fly lines that were not backcrossed to the *w^{CS}* background, but were crossed out to *w¹¹¹⁸*, another laboratory wildtype strain (Supplementary Fig. 1). Together, the data suggest that reduction in *ilk* or *mys* gene dosage overall attenuates the normally age-dependent changes in heart performance, consistent with an increased lifespan of these flies.

Reduced *ilk* activity abolishes the age-dependent change in myocardial stiffness

As is observed in human cardiac aging (Lakatta & Levy, 2003), another feature of aging hearts in *Drosophila* is stiffening of the myocardium (measured in kiloPascals, kPa, upon applying external pressure and at the ventral midline of the heart; Kaushik et al., 2012). Because *ilk* heterozygous mutants attenuates or halts the age-dependent changes in heart performance (Fig. 3A,B) and ILK-GFP was found at cell-cell contact sites near the ventral and dorsal midline in the wild type animal (Fig. 2B-D), we speculated that ILK may be involved in the increase of myocardial stiffness with age. To test this idea, ventral midline stiffness was measured with nanoindentation in *ilk^{54-wCS/+}* and corresponding wildtype controls *w^{CS}*. Aged *w^{CS}* hearts exhibit a stiffening of approximately 65% with age from 1 to 5 weeks of age (Fig. 3F and Supplementary Fig. 1C). In contrast to *w^{CS}*, the *ilk^{54-wCS/+}* myocardium did not stiffening with age, but instead started out relatively stiff and then softened with age (Fig. 3F). These results suggest a lack of age-dependent myocardial stiffening in *ilk* heterozygous mutants. Moreover, *mys^{XG43-wCS/+}* myocardium also showed lack of stiffening with age, similar to the *ilk^{54-wCS/+}* myocardium (Fig. 3F). Thus, we find that *ilk^{54-wCS/+}* and *mys^{XG43-wCS/+}* hearts do not display several measures of cardiac aging, such as increasing AI, diastolic dysfunction (except for *mys^{XG43-wCS/+}*) and myocardial stiffening.

β 1-integrin protein levels are increased in old flies and overexpression in young fly hearts mimics the effects of cardiac aging

Since *ilk* and *mys* heterozygous mutants show a much-attenuated progression in several age-dependent characteristics (Fig. 3; Supplementary Fig. 1), we wondered whether *ilk* and *mys* expression levels would be increased in old wild-type flies. Neither *mys* nor *ilk* mRNA levels were increased in 5-week compared to 1-week old flies (Supplementary Fig. 2). However, when examining β 1-integrin protein levels, we found an almost 2-fold increase at 5 weeks compared to 1 week (Fig. 4A,B). This increase in β 1-integrin protein was attenuated in old *ilk* and *mys* heterozygotes (Fig. 4A,B). To test whether excess *mys* function can induce cardiac aging, we overexpressed *mys* in young hearts and found indeed that it

produced an increased AI, reminiscent of old control flies (Fig. 4C). In addition, we observed decreased diastolic diameters and fractional shortening (Fig. 4D,E), suggesting diastolic dysfunction, a characteristic of old flies (Cammarato et al., 2008). Although it is possible that overexpression of *mys* may cause dominant-negative effects, the observed phenotype did not resemble a *mys* loss-of-function cardiac phenotype (see also below), but rather the cardiac aging phenotype observed in wildtype flies (Fig. 3B-E). Thus, we suggest that *mys* overexpression in young hearts may possibly accelerate the cardiac aging phenotype.

Heart-specific *ilk* knockdown causes arrhythmias and impaired adhesion between cardiomyocytes

Given the mixed results with *ilk* manipulations elsewhere (Chen et al., 2006; White et al., 2006), it is possible that *ilk* has multiple functions. Thus, we hypothesize *ilk* reduction could have detrimental or beneficial effects depending on the conditions. To ask whether *ilk* is also critical for maintaining heart function in *Drosophila*, we examined the effect of cardiac RNAi-mediated knockdown of *ilk* (using two different drivers, the strong heart-specific *hand-Gal4* (that includes some pericardial cells) and the weaker cardiomyocyte-specific GMH5 (Han & Olson, 2005; Wessells et al., 2004). Strong cardiac reduction of *ilk* leads to high arrhythmias in young flies, which was further elevated at old age (orange bars in Fig. 5A). In contrast, with weaker *ilk* KD in cardiomyocytes (see also qPCR in Supplementary Fig. 3) prevented an increase in the level of arrhythmias with age (yellow bars in Fig. 5A), unlike in wildtype controls. This suggests that a moderate reduction of *ilk*, as in *ilk* heterozygotes (Fig. 3), averts an age-dependent increase in AI, but a more substantial reduction disrupts the regular beating patterns, including at young ages.

To investigate this notion further, we examined the structural integrity of heart with strong cardiac KD of *ilk*. With *hand-Gal4* mediated KD we found severe morphological defects in *ilk*-RNAi hearts, including gaps between cardiomyocytes and abnormal patterns of β 1-integrin staining, which were not seen in wildtype controls or *ilk* heterozygous mutants (Fig. 5B-C, Supplementary Fig. 4A-E). This suggests that ILK may be required for adhesion between cardiomyocytes, consistent with ILK localization at cell-cell contact sites in wild-type hearts (Fig. 2A-C). These results are in contrast to our finding with *ilk* heterozygous mutants, which showed improved cardiac performance at older age (Fig. 3). Thus, cardiac *ilk* KD with *hand-Gal4* is likely to cause more severe diminution of *ilk* function than 50% reduction in function of *ilk* heterozygous mutants or with GMH5-mediated KD (see Supplementary Fig. 3). Interestingly, heart-specific KD of *pinch*, encoding an ILK binding partner, results in similar intercellular adhesion defects (Supplementary Fig. 4F-F''). In addition, heart-specific KD of *mys* or *talin*, the latter encoding a critical integrin-actin linker essential for integrin activation, also compromised cardiomyocyte adhesion to their neighbors (Supplementary Fig. 4G-J). This finding supports the hypothesis that the integrin complex is essential for adhesion between adult cardiomyocytes.

Dual effects of *ilk/pat-4* RNAi knockdown in *C. elegans* longevity

In order to investigate whether the dual - beneficial vs. detrimental - role of scaled reduction of *ilk* signaling in aging is conserved over a substantial evolutionary distance, we turned to

the model aging organism, *C. elegans*. Similar to *ilk* or *mys* null mutations in *Drosophila*, *C. elegans ilk/pat-4* null mutants are also embryonic lethal (MacKinnon et al., 2002). However, post-embryonic RNAi KD results in viable animals with extended lifespan, suggesting *ilk/pat-4* as an important and conserved longevity gene (Hansen et al., 2005; Curran & Ruvkun, 2007). However, long-lived animals subjected to *pat-4*/ILK since hatching are paralyzed, because of detached cytoskeleton of muscle cells ('muscle foci'), which was visualized by GFP-tagged myosin heavy chain A/MYO-3 (Fig. 5E,F; Campagnola et al., 2002; Kumsta et al., manuscript submitted). This detachment phenotype is reminiscent of *Drosophila ilk* homozygous embryos (Zervas et al., 2001). To test whether the lifespan extension was linked to the paralysis phenotype, we grouped populations of *C. elegans* with either mild or severe muscle cytoskeleton detachment in early adulthood (Fig. 5E,F), and then carried out lifespan analysis. Interestingly, we found that the population with many MYO-3-positive muscle foci was shorter-lived, whereas the group that had either no or few muscle foci was longer lived (Fig. 5D). To address whether this difference in the lifespan of animals correlated with the severity of the reduction of *ilk/pat-4*, we conducted qPCR to measure *ilk/pat-4* transcript levels in these animals. Indeed, animals with few muscle foci did not exhibit as dramatic a reduction of *ilk/pat-4* RNA levels as compared to animals with many muscle foci (Fig. 5G). Taken together, these observations suggest that a substantial reduction of *ilk/pat-4* is detrimental for whole organismal physiology and lifespan, whereas a milder reduction improves longevity of *C. elegans*, thus underlining a dual role in aging and muscle integrity that is evidently conserved in evolution.

Dual effects of β 1-integrin/ILK pathway on heart function

Given the beneficial or detrimental effects upon *ilk* reduction, we wondered if strong versus weak inhibition of ILK binding partners also has opposing phenotypic effects on cardiac function in *Drosophila*. One of ILK's binding partners is Parvin, an adaptor protein containing two calponin homology domains (Tu et al., 2001). In *C. elegans*, RNAi KD of *parvin* (*pat-6*) had been shown to significantly extend lifespan, similar to *ilk* KD (Hansen et al., 2005). Thus, an appropriate reduction of *parvin* may also have beneficial effects on cardiac aging. When *parvin* was knocked down in the fly's heart using *hand-Gal4*, the flies survived to adulthood and displayed modestly elevated arrhythmias at 1 week of age suggesting a slightly detrimental effect at that age (Fig. 6). However, at 5 weeks of age the incidence of arrhythmias with *parvin* KD hearts was low, similar to the 1-week time point of wildtype control (*hand-Gal4* alone) and comparable to long-lived *ilk* and *mys* heterozygotes (Fig. 3 and Supplementary Fig. 1) This suggests a beneficial effect of *parvin* KD on old hearts by preventing an age-dependent increase in AI. Examining *pax*, *talin* and *pinch*, coding for other ILK-interacting proteins, we found that *hand-Gal4*-mediated KD exhibited high AI levels for *talin* and *pinch* already at young ages (Fig. 6), comparable to strong *ilk* KD (Fig. 5A), and consistent with the observed structural defects (Supplementary Fig. 4). In contrast, moderate cardiomyocyte KD (using *GMH5*) of *pax*, *talin*, *pinch*, as well as *mys*, exhibited a low AI, typical of 1-week control heart, and importantly failed to show any significant age-dependent elevation of AI, unlike their wildtype controls (Fig. 6), thus strongly suggesting a similar beneficial effect of fine-tuned reduction in gene function as with *ilk* or *mys*. Interestingly, weak *talin* KD did not show increased arrhythmias at young

ages, but already resulted in some disorganization in myofibrillar structure (Supplementary Fig. 4I,J).

In order to further substantiate that moderate reduction of ILK pathway components in the myocardium contribute better cardiac performance at old ages, we examined heterozygous mutants of *parvin*, *pinch (stck)* and *talin (rhea)* (see Experimental Procedures). All three heterozygotes failed to show an increase in AI or decrease in diastolic diameter at old ages (Supplementary Fig. 5), thus corroborating the conclusion that they indeed participate in the beneficial modulation of cardiac aging, similar to moderate *ilk* and *mys* attenuation (Fig. 3&5A). Together, these results further support the notion that moderately reduced levels of β 1-integrin/ILK signaling components within the heart prevents the increase in cardiac arrhythmias with age, whereas strong reduction causes severe functional and structural defects often already at young ages.

DISCUSSION

Our data demonstrate that the β 1-integrin/ILK pathway is a critical genetic modulator of cardiac and organismal aging. We have shown that reduction of β 1-integrin/ILK levels is beneficial for *Drosophila* longevity and cardiac performance at older ages. In addition, severe reduction of ILK pathway components in the heart causes loss of cardiomyocyte cell adhesion, along with structural and functional deficits. We also find that β 1-integrin protein levels increase with age in wild-type flies and that overexpression of *mys* seems to be detrimental to the heart, and perhaps causes a premature aging-like phenotype in the heart at young ages. Whether this is a bona fide progeric phenotype awaits further study. However, considering that *ilk* KD also extends *C. elegans* lifespan (Hansen et al., 2005; Curran & Ruvkun, 2007), mutation in *mys* increases *Drosophila* lifespan (Goddeeris et al., 2003), and that the inhibition of *ilk* expression prevents senescence in old rat fibroblasts (Chen et al., 2006), we suggest that the role of integrin/ILK pathway components in (cardiac/organismal) aging is likely conserved across species.

Similar to our findings, it has been shown that β 1-integrin is significantly higher in old compared to young monkey vascular smooth muscle cells (VSMCs) (Qiu et al., 2010). Therefore, the accumulation of β 1-integrin might be a common phenomenon underlying both myocardial and vascular aging. Interestingly, applied mechanical force appears to regulate the assembly of focal contacts and integrin turnover (Riveline et al., 2001; Pines et al., 2012), consistent with the idea that with age this regulation is no longer as finely-tuned leading to excess integrin accumulation and consequent heart defects.

In contrast to the cardioprotective effects of moderate reductions in integrin/ILK complex levels, more drastic interference with complex function/levels can lead to severe abnormalities in cardiac structure and function, suggesting a critical requirement of this complex across species. In mice, *ilk* ablation causes dilated cardiomyopathy, heart failure, and sudden death (White et al., 2006). In flies, strong cardiac *ilk* complex KD causes severe arrhythmia and loss of cardiac integrity, including gaps between adjacent cardiomyocytes. In contrast, the moderate reduction in *ilk* complex function attenuates the age-dependent

changes in cardiac performance, suggesting that alterations in this pathway need to be finely tuned to have beneficial effects, and this seems to be the case across species.

Interestingly, similar observations have been made for ion channel function: expression of KCNQ, encoding a K⁺ channel responsible for repolarizing the cardiac action potential, is reduced at old age (Nishimura et al., 2011). Heart-specific overexpression of KCNQ in old wildtype flies reverses the age-dependent increase in arrhythmia, whereas overexpression of KCNQ in young flies increases the incidence of arrhythmias (Nishimura et al., 2011; Ocorr et al., 2007). Thus, precise control of signaling via KCNQ channels, as well as via the integrin/ILK pathway investigated here, seems to be critical for tipping the balance between beneficial and detrimental effects.

Integrins can mediate both “outside-in” and “inside-out” signaling and interactions (Legate et al., 2006). For “outside-in” signaling, integrin activation recruits a large variety of proteins that interface with the actin cytoskeleton and affect many diverse signaling pathways, including ILK-mediated phosphorylation of target genes, such as AKT. However, the requirement for the kinase activity of ILK has been questioned (Wickström et al., 2010), and the longevity effect of *ilk/pat-4* RNAi appears to be largely *foxo/daf-16* independent in *C. elegans* (Hansen et al., 2005; Curran & Ruvkun, 2007). Interestingly, the longevity effect of *ilk/pat-4* RNAi in *C. elegans* instead seems to be dependent on heat shock factor HSF-1 (Kumsta et al., submitted). Further investigation is necessary to address whether this is also the case in *Drosophila*.

With “inside-out” signaling, integrin activation might modulate the assembly of ECM ligands on the cell surface. Excessive deposition of ECM is associated with ventricular fibrosis that leads to stiffening of the ventricular wall causing ventricular dysfunction and heart failure (Pellman et al., 2010). In *Drosophila*, β 1-Integrin protein levels are increased with age (Fig. 4A,B) and *mys* heterozygous mutants lack the age-dependent increase in myocardial stiffness (Fig. 3F). Therefore, it is possible that increased levels of β 1-integrin during aging causes excessive ECM deposition leading to stiffening of the heart, which results in diastolic dysfunction and an elevated incidence of arrhythmias with age.

Although in the aggregate our results show that a moderate decrease of several components of β 1-Integrin/ILK signaling can reverse several characteristics of cardiac aging, it is interestingly that there are notable exceptions. For example, *mys* heterozygotes in the same genetic background as *ilk* do not reverse the age-dependent decrease in diastolic diameter (Fig. 3C), thus still show an age-dependent diastolic dysfunction. This may reflect a difference in the functional spectrum of the different signaling components that is not only and completely dedicated to this signaling pathway. Alternatively, an occasional inconstancy may also be due to experimental variability inherent in these types of physiological experiments.

In conclusion, our data provide novel insights into cardiac-specific aging involving a recently identified pathway that modulates organismal aging: the integrin/ILK complex. We find that in order to maintain a youthful cardiac and organismal physiology, the pathway's activity needs to be finely tuned and maintained at specific activity levels, and this set point

appears to be altered or deregulated during aging. Thus, the integrin/ILK pathway emerges as a key modulator of cardiac homeostasis and aging.

EXPERIMENTAL PROCEDURES

Fly strains

ilk⁵⁴ (Zervas et al., 2011; The allele *ilk⁵⁴* has a premature stop codon instead of a codon for W7), ILK-GFP;*ilk⁵⁴* (ILK-GFP is previously described in Zervas et al., 2001), UAS-*mys^{wt}*, and UAS-*ilk^{wt}* are generous gifts from Y. Inoue. *ilk^l* (Zervas et al., 2001) and *mys^l* (Bunch et al., 1992) were obtained from the *Drosophila* stock center. *w^{CS}* (Cook-Wiens & Grotewiel, 2002) and *mys^{XG43}* (Goddeeris et al., 2003) were kindly provided by M. Grotewiel. The *mys^{XG43}* allele is a 113 bp deletion in exon 5 causing a frame shift and premature truncation of *mys*, and introgressed into the *w^{CS}* genetic background for six generation (Goddeeris et al., 2003). *ilk⁵⁴* was introgressed into the *w^{CS}* genetic background for six generation in this study. The following RNAi lines were obtained from Vienna Drosophila RNAi Center (VDRC): UAS-*parvin^{RNAi}* (#11670), UAS-*pinch^{RNAi}* (#52538), UAS-*mys^{RNAi}* (#29619), UAS-*talⁱⁿRNAi* (#40399) and UAS-*paxillin^{RNAi}* (#107789). UAS-*ilk^{RNAi}* (originally from VDRC, #16062) was kindly given by F. Schnorrer. The effectiveness of *ilk*, *mys*, *parvin* and *talⁱⁿ* RNAi lines had been tested in Schnorrer et al. (2010) and found lethal when crossed to the mesodermal driver *mef2-Gal4*. We have also tested ourselves the *pinch* and *parvin* RNAi lines when crossed to *mef2-Gal4* and found them lethal as well. In addition, Perkins et al (2010) tested *mys* and *talⁱⁿ* RNAi KD effectiveness. Cardiac-specific driver *hand-Gal4* and cardiomyocyte-specific driver *GMH5*, have been previously described (Han & Olson, 2005; Wessells et al., 2004). *parvin⁶⁹⁴* (Vakaloglou et al., 2012), *stck^{T2}* (Zervas et al., 2011), and *rhea^{79A}* (Brown et al., 2002) were kindly provided by C. Zervas.

Immunostaining

Adult female flies were dissected and immunostained as previously described (Taghli et al. 2008). Images were acquired using ApoTome (Zeiss) or FV1000 (Olympus). The following primary antibodies were used: rabbit anti-GFP (1:250, Invitrogen), mouse anti- α -PS (1:20, CF.6G11; DSHB Iowa), mouse anti- α -actinin (1:20, kindly provided by J. Saide), and mouse anti- α -Spectrin (1:50, 3A9; DSHB Iowa).

Lifespan assays

Virgin female and male progeny were collected for three days. Then they were briefly anaesthetized and separated in groups of 25 flies in each vial. The flies were kept at 25°C and the dead flies were counted every three days after transfer. Each experiment was performed twice: first on the smaller scale (100~150 flies) and then on larger scale (200~250 flies). Data were analyzed using Prism 5.0 (GraphPad Software).

C. *elegans* RNAi treatment and lifespan assay

The *C. elegans* strain RW1596: *myo-3(st386)V; stEx30[myo-3p::gfp::myo-3 + rol-6]* (Campagnola et al., 2002) used in this study was maintained and cultured under standard conditions at 20°C using *E. coli* OP50 as a food source, except when subjected to RNAi

treatment. The *pat-4* RNAi clone was obtained from the Ahringer RNAi library. RNAi treatment was carried out as previously described (Hansen et al., 2005). Muscle detachment was scored on day 1 and day 2 of adulthood under a fluorescent stereoscope and animals were grouped into two groups based on the efficiency of the RNAi treatment – one group had 0-3 detached muscle cells per animal, whereas the other group had a high degree of detachment (>80% detached muscle cells per animal). Lifespan assays were carried out as previously described (Hansen et al., 2005). For statistical analysis, Stata software was used (StataCorp). P values were calculated with the log-rank (Mantel-Cox) method.

Heartbeat analysis

Flies were anesthetized with fly nap (Carolina Biol., Corp.) and dissected as previously described (Ocorr et al., 2007). Movies were taken at rates between 140-160 frames per second for 30 seconds using a Hamamatsu CCD digital camera (McBain Instr.) on a Leica DM LFSa microscope with a 10x water immersion lens and HCImage imaging software. The images were analyzed and M-modes were generated using Semi-automatic Optical Heart Beat Analysis software (Fink et al., 2009).

Drosophila heart indentation

Adult female flies were anesthetized and immobilized on 25mm glass coverslips with a thin layer of vacuum grease ventral-side-up. The heart tube was exposed via microsurgery as previously described (Ocorr et al., 2007) with additional micropipette aspiration to remove all ventral tissue proximal to the conical chamber. Each coverslip is mounted on a Fluid Cell Lite coverslip holder (Asylum Research) with 1 mL of hemolymph. Hearts are checked for regular contractions to ensure they are in good health and then resubmerged in 10 mM ethylene glycol tetraacetic acid (EGTA)-treated hemolymph to arrest contraction. Prior to indentation, probes were calibrated via thermal noise method in MFP-3D Bio software (Asylum Research). Nanoindentation was performed on an MFP-3D Bio Atomic Force Microscope (Asylum Research) mounted on a Ti-U fluorescent inverted microscope (Nikon Instruments) with 120 pN/nm silicon nitride cantilevers with pre-mounted, 2 μ m radius borosilicate glass spheres (Novascan Technologies). All indentation curves were analyzed to calculate myocardial stiffness using previously-published, automated software custom-written in MATLAB (MathWorks)(Kaushik et al., 2012).

Statistical Methods

Statistical Analyses were performed using Prism 6.0 (Graph Pad Software, Inc.). All data was checked prior to analysis using the D'Agostino & Pearson omnibus normality test to determine if the data violated the assumption of a Gaussian distribution. We employed a one-way Analysis of Variance (ANOVA) when comparing more than two groups (e.g. Fig. 4D,E) followed by Tukey's multiple comparisons post hoc test. If the data was not normally distributed (e.g. Fig. 4C) a Kruskal Wallis test was performed followed by Dunn's multiple comparisons post hoc test. A two-way ANOVA was employed when comparing two or more groups at more than one age (e.g. Fig. 3 B-E). Data that did not exhibit a Gaussian distribution (the Arrhythmia Indices) were first normalized before applying a two-way ANOVA (e.g. Fig. 3B). Post hoc comparisons of two-way ANOVA analyses were made

using a Dunnett's multiple comparisons test. Comparisons of Arrhythmia Indices within a single genotype between two ages were made using the Mann Whitney test for non-parametric data (e.g. Fig. 5A). Life span analyses were performed using a log-rank analysis (Mantel-CoxTest). Specific tests used are indicated in the figure legends. In all cases P values less than 0.05 were taken as significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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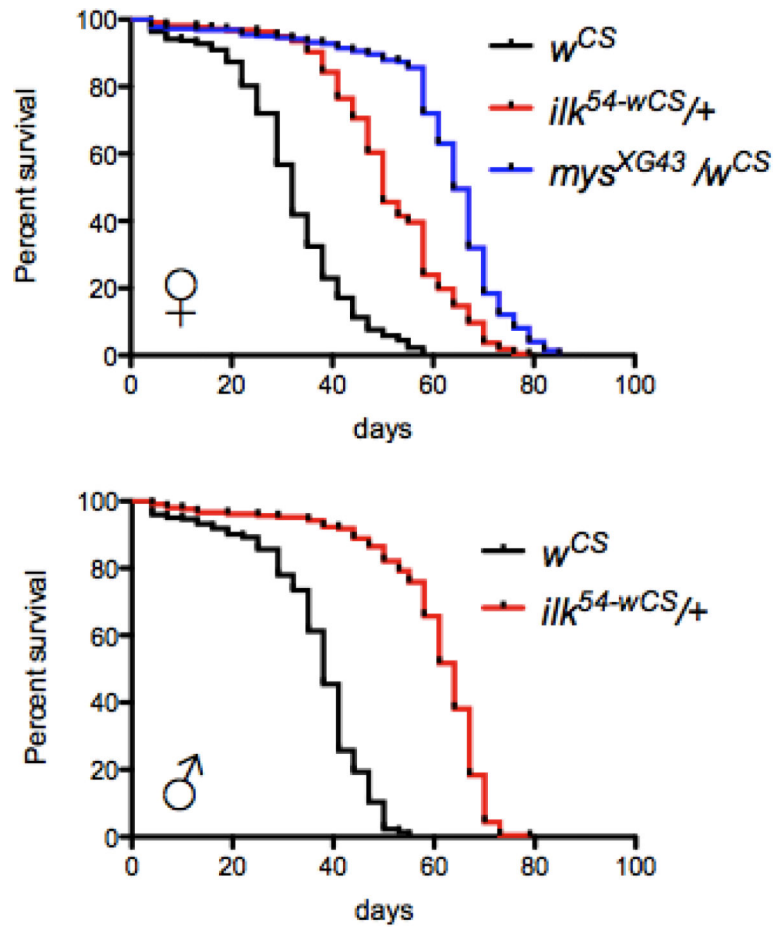


Figure 1. *ilk* heterozygous mutants have increased life span

The lifespan of $ilk^{54-wCS/+}$ (red), $mys^{XG43/wCS}$ (blue) and w^{CS} (control, black) flies were examined. ilk^{54} and mys^{XG43} were introgressed into the w^{CS} wildtype background for six generations. Top, the survival curves of female w^{CS} (N=222), $ilk^{54-wCS/+}$ (N=217), and $mys^{XG43/+}$ (N=222) flies. Bottom, the survival curves of male w^{CS} (N=222) and $ilk^{54-wCS/+}$ (N=207) flies. Since *mys* is X-linked and hemizygous *mys*/Y flies are lethal, males could not be examined. Both male and female $ilk^{54-wCS/+}$ flies, and female $mys^{XG43/+}$ flies lived longer than the controls (w^{CS}). For statistics, see Table 1. Survival curves of trial 2 in Table 1 are shown.

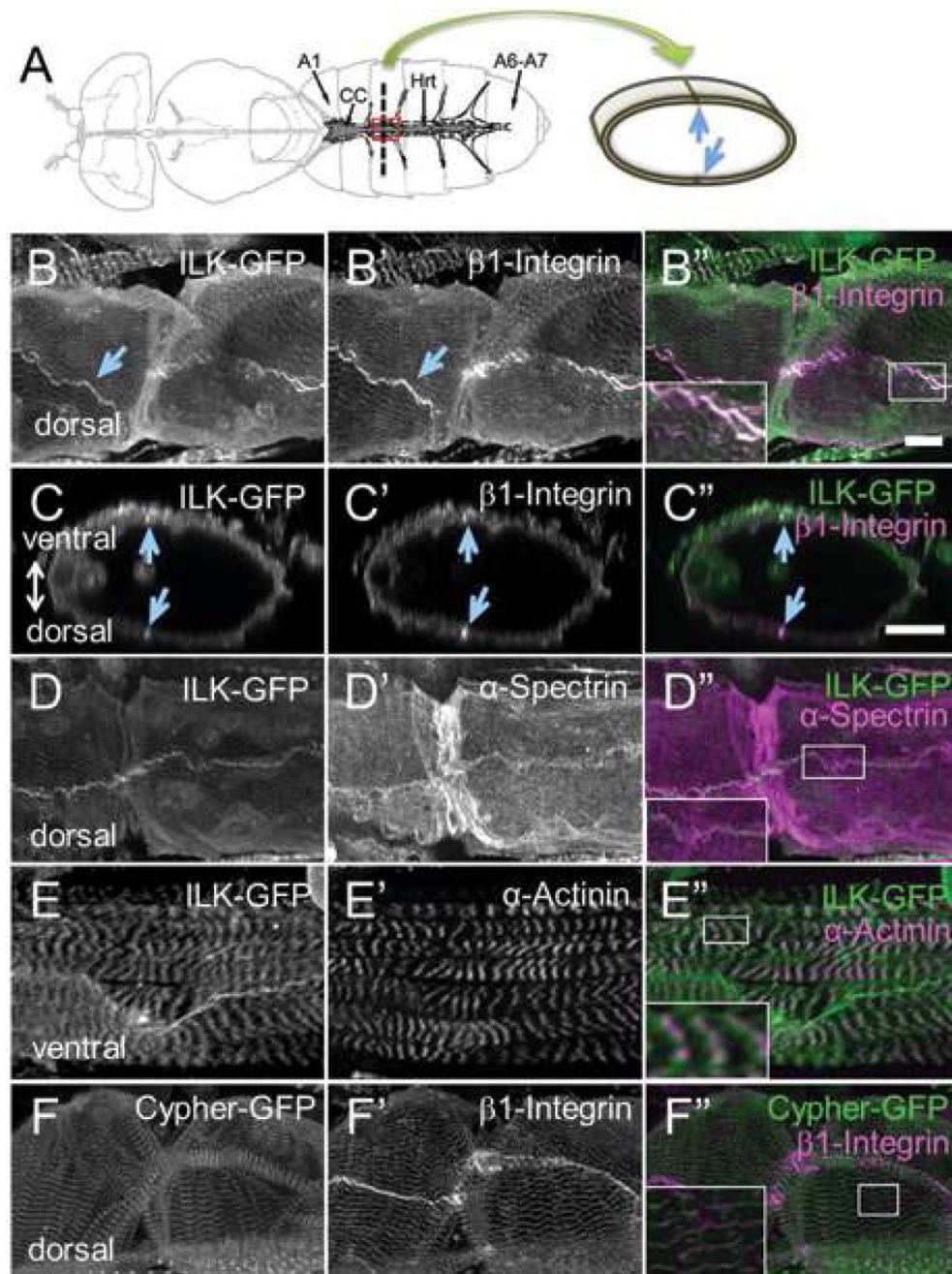


Figure 2. ILK and β 1-integrin are localized to cell-cell contact sites and Z-discs in fly hearts (A) Left, a schematic diagram of the adult fly heart (adopted from Cammarato et al., 2008). Hrt, fly heart tube; CC, conical chamber; A1, abdominal segment 1; A6-A7, abdominal segments 6 and 7. The region outlined in red corresponds to the area shown in B, D, E, and F. Right, a schematic diagram of a cross section of the heart tube along the dotted line in the left panel. The circumference of the heart tube is composed of two myocardial cells. Myocardial cell-cell contact sites are pointed out by arrows. (B-B'') Dorsal views of the heart tube. Both ILK (B and green in B'') and β 1-integrin (B' and magenta in B'') are

expressed in adult hearts and co-localize. **(C-C'')** Optical cross sections from B-B'' show circumference of the heart tube. The ventral side is to up. Prominent signals of ILK and β 1-integrin are indicated by arrows in B-C''. **(D-D'')** ILK (D and green in D'') was found at the cell membrane labeled with α -Spectrin (D' and magenta in D''). **(E-E'')** Ventral views of the heart tube. ILK (E and green in E'') was also localized to Z discs marked by α -actinin (E' and magenta in E''). **(F-F'')** Dorsal views of the heart tube. β 1-integrin (F and magenta in F'') are also associated with Z discs marked by Cypher-GFP (F' and green in F''). All the images in B-F'' are in abdominal segment 3 region. Insets in B'', D'', E'', and F'' are higher magnification images corresponding to the each rectangle in the respective figures. Bars: 20 μ m.

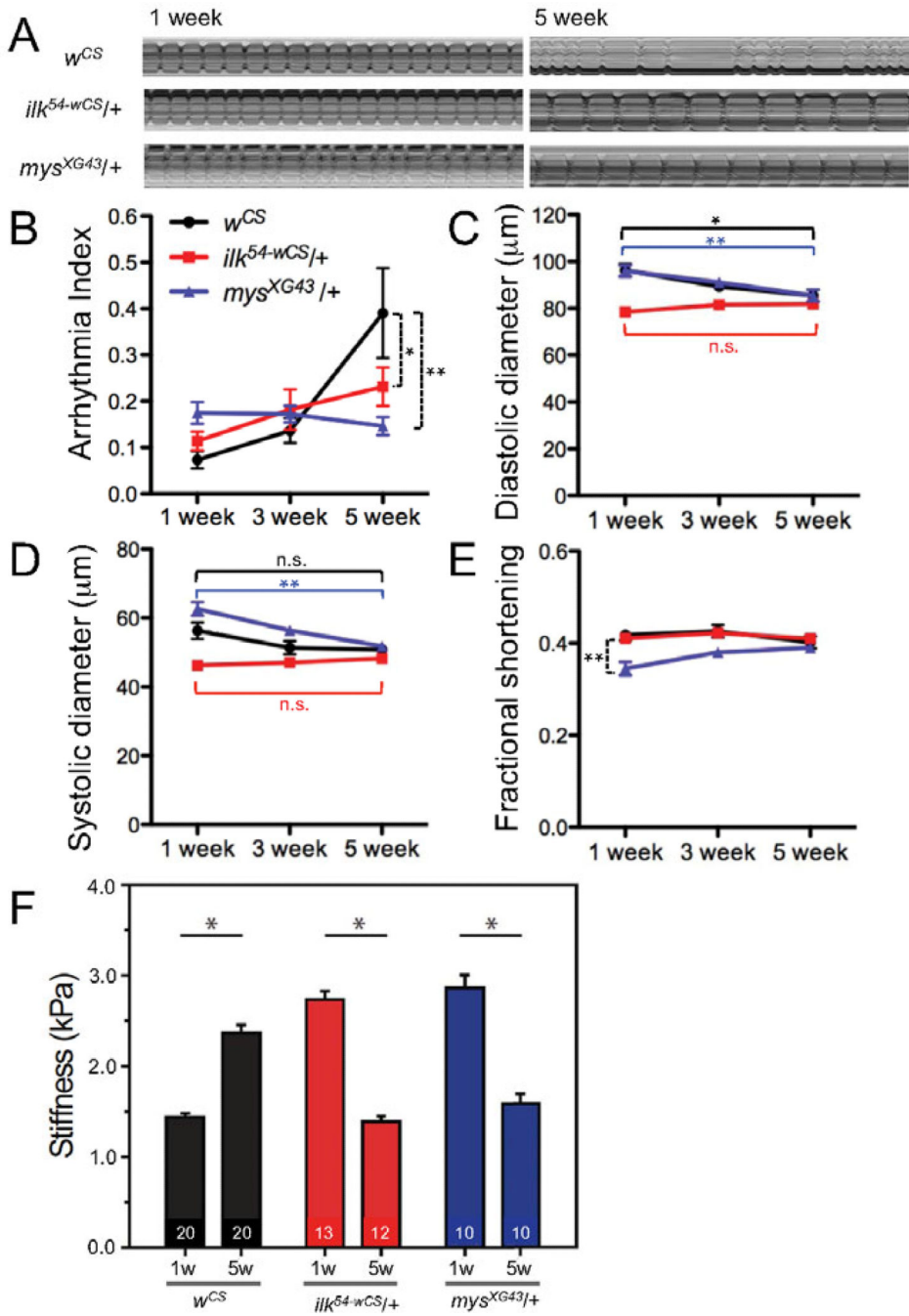


Figure 3. Changes in heart function with age are attenuated in *ilk* and *mys* heterozygous mutants (A) M-mode traces from 10 sec movies show heart wall movements in 1 week old (left) and 5-week old (right) hearts. Top, *w^{CS}*. Middle, *ilk^{54-wCS/+}*. Bottom, *mys^{XG43/+}*. The incidence of arrhythmia is increased with age in wildtype controls (top panels, see also Ocorr et al., 2007a,b), but 5-week old *ilk^{54-wCS/+}* hearts (middle right) and *mys^{XG43/+}* hearts (bottom right) present more regular beating patterns than 5-week old *w^{CS}* hearts (top right). (B-E) Plots of physiological parameters of *w^{CS}* (●), *ilk^{54-wCS/+}* (■), and *mys^{XG43/+}* (▲) at 1, 3, and 5 weeks of age. 20-30 flies were used for each data point. (B) Incidence in heartbeat

irregularities indicated by the Arrhythmia Index (AI) represents the standard deviation of heart period normalized to the median value for each fly (Fink et al., 2009). AI increases with age in wildtype w^{CS} hearts ($P < 0.001$ for both 3 & 5 weeks), as previously reported for other wildtype strains (Ocorr et al., 2007). Notably, 5-week $ilk^{54-w^{CS}/+}$ and $mys^{XG43}/+$ flies had lower AI compared to the age-matched w^{CS} flies. $*P < 0.01$, $**P < 0.001$, two-way ANOVA, see Experimental Procedures. **(C, D)** Diastolic diameter decreases with age in w^{CS} , and diastolic and systolic diameters decrease $mys^{XG43}/+$ flies, but not in $ilk^{54-w^{CS}/+}$ flies. $*P < 0.01$, $**P < 0.001$, two-way ANOVA. **(E)** Plots of fractional shortening for w^{CS} , $ilk^{54-w^{CS}/+}$, and $mys^{XG43}/+$ flies. 1-week old $mys^{XG43}/+$ flies had a significantly lower fractional shortening compared to w^{CS} and $ilk^{54-w^{CS}/+}$. $**P < 0.001$, two-way ANOVA. **(F)** Nanoindentation was performed to determine the stiffness (measured in kiloPascal, kPa) of fly heart tubes at the indicated ages and genotypes. Averages and standard errors are shown along with statistical significance, indicated by a Wilcoxon rank sum test. Number of flies analyzed per condition is indicated at bottom of each bar. $* P < 10^{-7}$. Error bars indicate SEM.

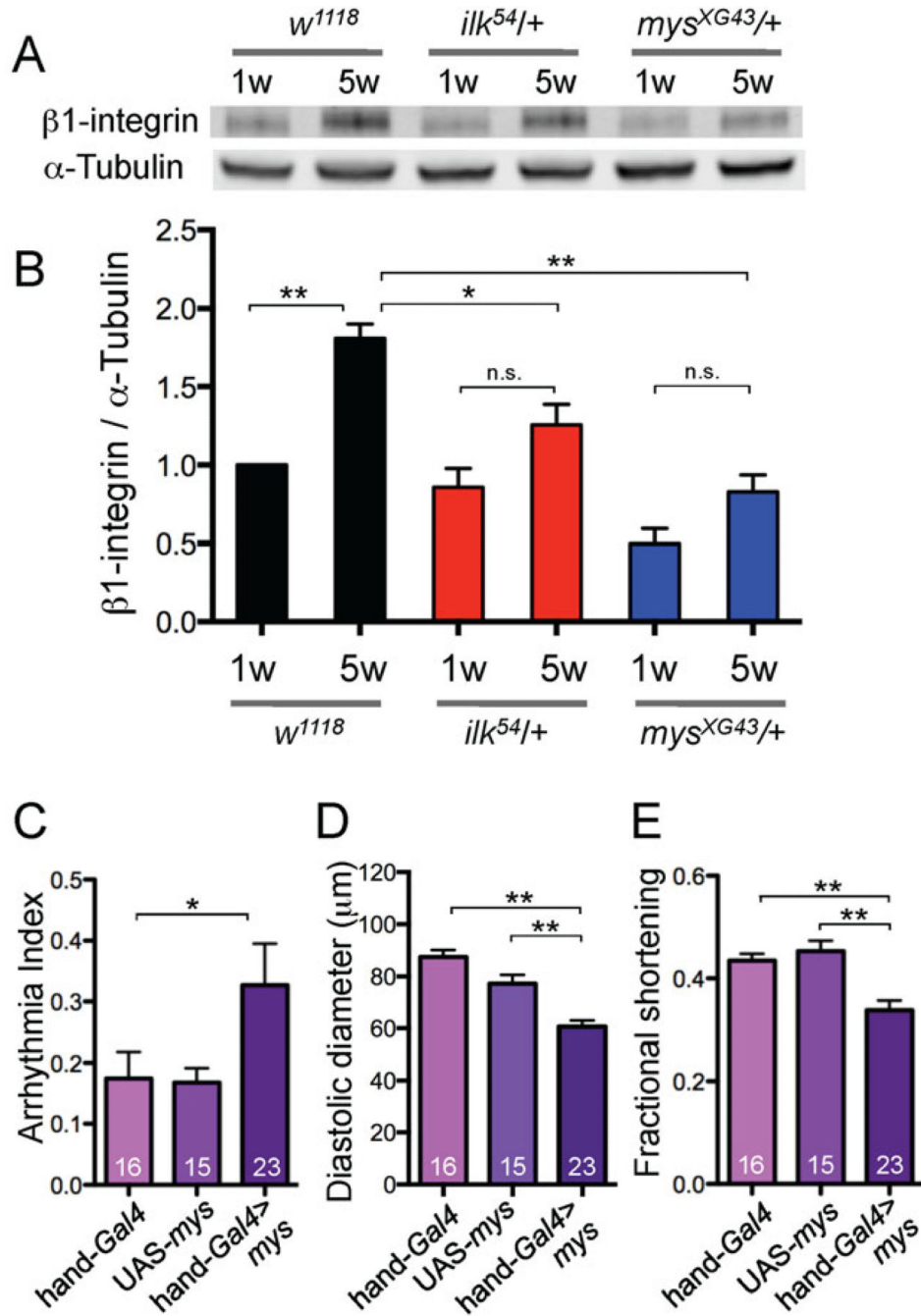


Figure 4. β1-integrin accumulates in old flies and overexpression of *mys* impairs heart function (A, B) Western blot analysis was carried out with whole fly preps of 1-week and 5-week old *w¹¹¹⁸*, *ilk^{54/+}*, and *mys^{XG43/+}* flies, using anti-β1-integrin antibody and anti-β3Tubulin antibody. β1-integrin was almost two-fold higher in 5-week old wildtype control (*w¹¹¹⁸*) compared to 1 week (**P<0.001, two-way ANOVA). In contrast, β1-integrin accumulation was suppressed in 5-week old *ilk^{54/+}* and *mys^{XG43/+}* flies, compared to 5-week old *w¹¹¹⁸* (*P<0.01, **P<0.001, two-way ANOVA). (B) Triplicate biological samples were analyzed by qPCR and normalizing to Tubulin. All data was normalized to the value for *w¹¹¹⁸* at 1-

week. **(C-D)** Cardiac overexpression of *mys* using *hand-Gal4* driver significantly increased arrhythmia compared to *hand-Gal4* **(C)**, decreased diastolic diameter compared to the controls (*hand-Gal4* and *UAS-mys*) **(D)**, and decreased fractional shortening compared to the controls (*hand-Gal4* and *UAS-mys*) **(E)** in relatively young flies (3-week old). Sample numbers are indicated at the bottom of each bar. * $P < 0.05$, ** $P < 0.001$, Kruskal-Wallis test (AI) and one-way ANOVA (DD & FS). Error bars are SEM.

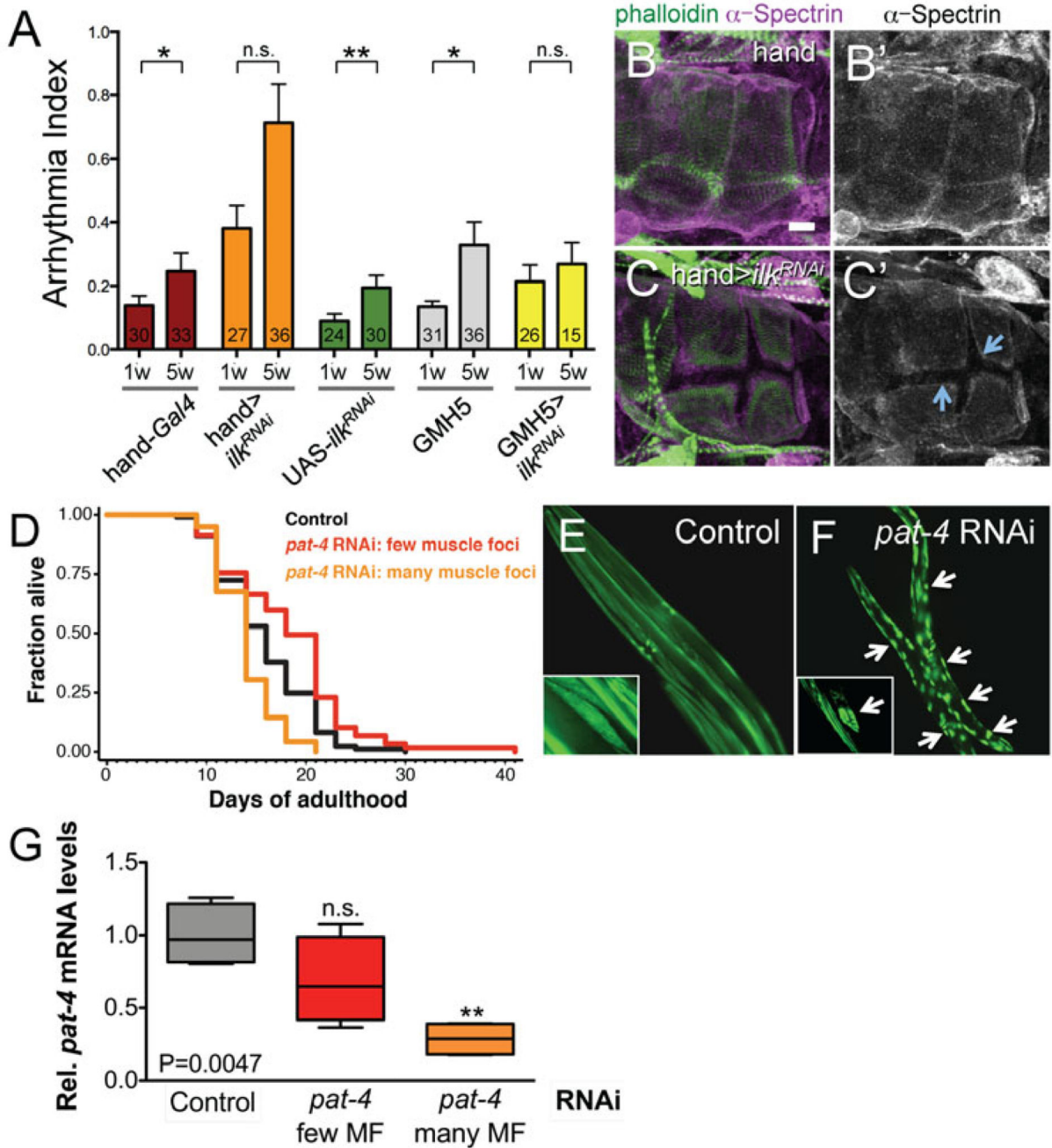


Figure 5. Effect of *ilk* RNAi KD in *Drosophila* hearts and of *ilk/pat-4* RNAi KD in *C. elegans* lifespan

(A) Bar graph representations of Arrhythmia Index (AI) of 1-week and 5-week old *ilk* RNAi hearts. Wildtype controls (hand-Gal4, UAS-*ilk*RNAi and GMH5) show the expected age-dependent increase in AI. Heart-specific *ilk* KD hearts (with hand-Gal4, orange bars) have an elevated AI at 1 week, which seemed to worsen with age (not statistically significant). In contrast, cardiomyocyte-specific *ilk* KD hearts (with GMH5, yellow bars) have a slightly elevated AI at 1 week, which did not exhibit a further increase at 5 weeks. Each sample number is indicated at the bottom of each bar. *P<0.05, **P<0.01, ***P<0.001, Mann-

Whitney test. Error bars are SEM. **(B-C')** Heart structure in conical chamber region visualized by phalloidin staining (green in B and C) and anti- α -Spectrin antibody (magenta in B and C, gray-scale in B' and C'). Heart-specific *ilk* RNAi caused gaps between cardiomyocytes (C, C', arrows). Bar, 20 μ m. **(D-F)** *C. elegans* expressing a GFP-tagged body-wall muscle myosin reporter (MYO-3::GFP) were fed control bacteria (vector only) or bacteria expressing *pat-4* dsRNA from hatching. **(D)** On day 1 of adulthood, animals were grouped according to either a low or a high number of detached cytoskeleton in muscle cells (referred to as few or many 'muscle foci', respectively, see Experimental Procedures), and their lifespan was assayed. While control animals had a mean lifespan of 16 days (N=104), *pat-4(RNAi)* animals with a low number of detached muscle cells had a mean lifespan of 18 days (N=114), thus displaying a ~15% increase in mean lifespan ($P < 0.001$, log-rank test). In contrast, *pat-4(RNAi)* animals in which almost all muscle cells were detached displayed a ~11% lifespan reduction (mean lifespan 14 days, N=114, $P < 0.005$, log-rank test). This experiment was repeated twice with similar results. **(E-F)** Animals subjected to **(E)** control RNAi with few 'muscle' foci, or to **(F)** *pat-4* RNAi with many visible 'muscle foci' were imaged on day 2 of adulthood at 50x magnification. Arrows point to examples of muscle cells with detached MYO-3::GFP structures, and inserts show higher magnification of single muscle cells. **(G)** The *ilk/pat-4* transcript levels of animals with a low or a high number of 'muscle foci' was determined using qRT-PCR. Only the animals with a high number of 'muscle foci' show a significant reduction in *ilk/pat-4* transcript levels (n.s. $P > 0.05$, ** $P < 0.005$, one-way ANOVA).

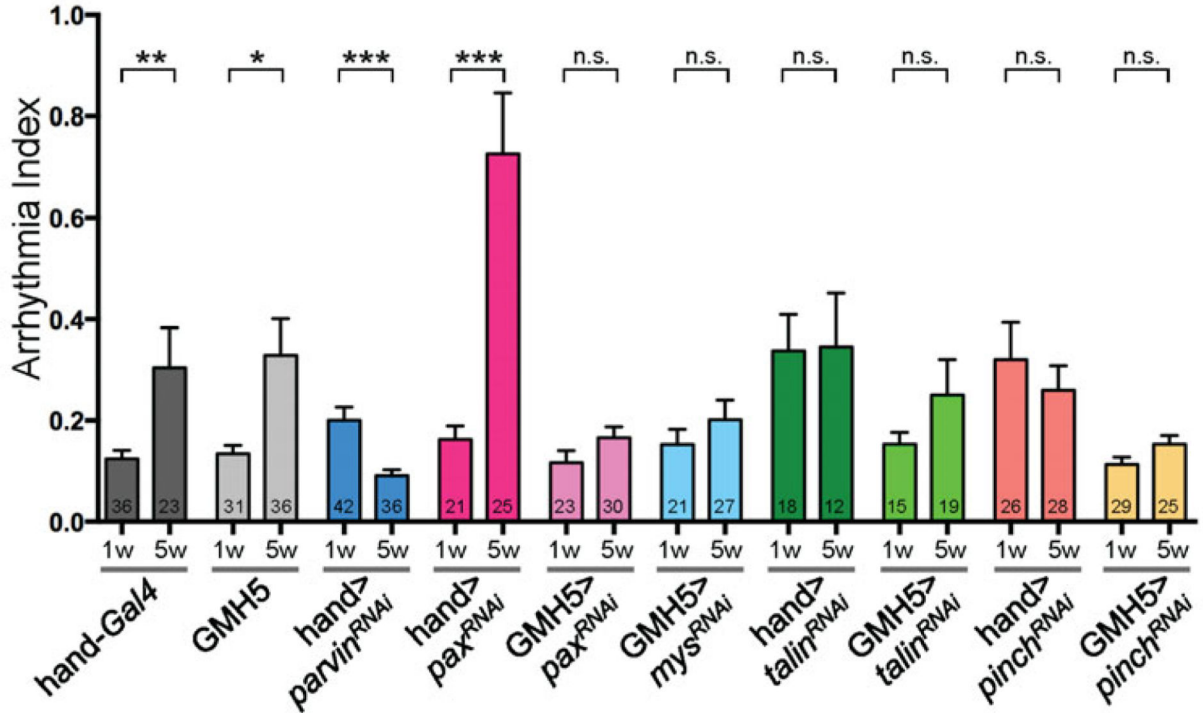


Figure 6. RNAi KD analysis of of β 1-integrin/ILK pathway components

Bar graph representations of the Arrhythmia Index (AI) of 1- and 5-week old hearts with *hand-Gal4* and *GMH5* mediated KD of *parvin*, *pax*, *mys*, *talin* and *pinch*. Wildtype controls (*hand-Gal4* and *GMH5* drivers alone) show the expected age-dependent increase in AI. In contrast none of the experimental KDs exhibited an age-dependent increase in AI, except for *hand>pax-RNAi*, which was much elevated at 5 weeks. Moreover, strong *hand*-driven KD of *parvin*, *talin* and *pinch* was causing a high AI already at 1 week. Each sample number is indicated at the bottom of each bar. Error bars indicate SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Mann Whitney test.

Table 1Life span extension in *ilk^{54-wCS/+}* and *mys^{XG43/wCS}* flies.

	Sexes	Genotypes	N	Median survival	% extension	P
Trial1	Female	<i>w^{CS}</i>	153	45		
		<i>ilk^{54-wCS/+}</i>	110	66	37	<0.0001
		<i>mys^{XG43/wCS}</i>	128	66	44	<0.0001
	Male	<i>w^{CS}</i>	150	45		
		<i>ilk^{54-wCS/+}</i>	107	66	56	<0.0001
Trial2	Female	<i>w^{CS}</i>	222	32		
		<i>ilk^{54-wCS/+}</i>	217	54	60	<0.0001
		<i>mys^{XG43/wCS}</i>	222	64	95	<0.0001
	Male	<i>w^{CS}</i>	222	38		
		<i>ilk^{54-wCS/+}</i>	207	64	63	<0.0001

Lifespan was examined in *ilk^{54-wCS/+}* and *mys^{XG43/wCS}* flies first on the smaller scale (Trial 1) and then on larger scale (Trial 2). *mys* gene is on X chromosome, thus only female heterozygotes with null *mys* mutation (*mys^{XG43}*) can be tested. % extension of mean life span compared to *w^{CS}* is shown. p values were obtained from Log-rank analysis (Mantel-Cox Test).