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A dual role for integrin-linked kinase and β1-integrin 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 β1-integrin (myospheroid, mys) in Drosophila, which colocalize 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 mys flies. Consistent with these findings, we observed elevated β1-integrin protein levels in old compared with 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-linked kinase (ILK)/integrin 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 ILK/mys 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, ILK/integrin-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.

Key words: Drosophila; Caenorhabditis elegans; arrhythmia; cardiomyopathy; cell adhesion; heart failure; senescence; ilk; myospheroid; parvin; paxillin; pinch; talin.

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 (Khan et al., 2002; Bernhard & Laufer, 2008). 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; Olson, 2006; Ocorr et al., 2007; Cammarato et al., 2008, Bodmer & Frasch, 2010). With age, the fly heart also shows features similar to mammals, including humans, with respect to structural alterations and propensity toward arrhythmias (Wessells et al., 2004; Ocorr et al., 2007; Cammarato et al., 2008, Taghil-Lamallem et al., 2008; 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 (Bodmer & Frasch, 2010; Dai et al., 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 β1-integrin (Hannigan et al., 1996, 2007). 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 Caenorhabditis elegans (MacKinnon et al., 2002; Sakai et al., 2003). Remarkably, RNAi-induced reduction in ILK, or the ILK

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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 knock-down (KD) extends lifespan in C. elegans. Interestingly, heterozygous Drosophila mutants for β1-integrin (mys) also have an increased lifespan (Goddegeois et al., 2003). Therefore, reduced β1-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 β1-integrin at an older age may mediate in part the declining heart function and that a moderate reduction in 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

As the RNAi-mediated KD of ilk extends lifespan in C. elegans (Hansen et al., 2005; Curran & Ruvkun, 2007; Kumsta et al., 2014), we wondered whether reduced ilk expression is also beneficial to longevity in Drosophila. As lifespan can be significantly modulated by genetic background (Grandison et al., 2009), we first backcrossed ilk54 mice (premature stop codon; Zervas et al., 2011; see Experimental procedures) to the wild-type control strain, w1118 (Cook-Wiens & Grotewiel, 2002) for six generations. The resulting backcrossed lines are referred to as ilk54-wCS. We found that both female and male ilk54-wCS heterozygous mutants (w1118/ilk54-wCS+) show extended lifespan compared with their w1118 controls (Table 1, Fig. 1).

β1-integrin interacts with ILK (Hannigan et al., 1996, 2007), and homozygous mutants (mys) have embryonic muscle phenotypes similar to ilk mutants (Zervas et al., 2001). In addition, mys heterozygotes have previously been reported to exhibit an extended mean lifespan (Goddegeois et al., 2003). Thus, we re-examined the lifespan of mysXG43 heterozygotes (also the wCS genetic background contains small deletion and premute stop codon; Goddegeois et al., 2003; see Experimental procedures) and confirmed that mysXG43/wCS flies have a significantly extended lifespan, similar to ilk54-wCS flyes (Table 1, Fig. 1), in terms of both maximum and mean lifespan (Fig. 1). Together, these data

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>N</th>
<th>Median survival</th>
<th>% extension</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female w1118</td>
<td>153</td>
<td>45</td>
<td>37</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ilk54-wCS/+/w1118</td>
<td>110</td>
<td>66</td>
<td>27</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>mysXG43/wCS</td>
<td>128</td>
<td>66</td>
<td>44</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Male w1118</td>
<td>150</td>
<td>45</td>
<td>56</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ilk54-wCS/+/w1118</td>
<td>107</td>
<td>66</td>
<td>27</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>mysXG43/wCS</td>
<td>122</td>
<td>64</td>
<td>95</td>
<td>&lt;0.0001</td>
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<tr>
<td>Trial 2</td>
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<tr>
<td>Female w1118</td>
<td>222</td>
<td>32</td>
<td>44</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ilk54-wCS/+/w1118</td>
<td>217</td>
<td>54</td>
<td>37</td>
<td>&lt;0.0001</td>
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<tr>
<td>mysXG43/wCS</td>
<td>222</td>
<td>64</td>
<td>95</td>
<td>&lt;0.0001</td>
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<tr>
<td>Male w1118</td>
<td>222</td>
<td>38</td>
<td>27</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ilk54-wCS/+/w1118</td>
<td>207</td>
<td>64</td>
<td>63</td>
<td>&lt;0.0001</td>
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Lifespan was examined in ilk54-wCS/+ and mysXG43/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 (mys043) can be tested. % extension of mean lifespan compared with w1118 is shown. P values were obtained from log-rank analysis (Mantel–Cox test).
Vilk rescues the embryonic ilk promoter, enhancers, and green in D“) was found at the cell membrane labeled with Z-disks marked by cypher-GFP (F“ and green in F“). All the images in B–E″ show circumference of the heart tube. The expression patterns of ILK and β1-integrin in Drosophila hearts suggest a possible functional requirement for the β1-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). Arrhythmia index 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 (wCS) flies age, the heart rhythm becomes progressively less regular, as exemplified by increases in AI (Fig. 3A,B). In contrast to wild-type, ilk54-wCS+ flies 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. Wild-type wCS 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 ilk54-wCS+ 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 ilk54-wCS+ flies, 1-week-old mysXG43-wCS+ heterozygotes also exhibited a more regular heart beat pattern, compared with age-matched wCS controls, manifested as a markedly lower AI, thus abolishing the typical wild-type age-related increase in AI (Fig. 3A,B). However, unlike ilk54-wCS+ flies, old mysXG43-wCS+ heterozygotes still showed a modest decrease in diastolic diameter, as wCS controls (Fig. 3C); thus, diastolic dysfunction was not prevented in this case. Moreover, young mysXG43-wCS heterozygotes had larger systolic diameters (Fig. 3D) and thus lower fractional shortening (Fig. 3E) compared with controls, suggestive of systolic dysfunction at that age.

We replicated the above findings and confirmed similar trends in different genetic backgrounds: ilk+ flies as well as in mysXG43+ and mys+ fly lines that were not backcrossed to the wCS background, but were crossed out to w1118, another laboratory wild-type strain (Fig. S1, Supporting Information). 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.

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

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Fig. 3 Changes in heart function with age are attenuated in ilk and mys heterozygous mutants. (A) M-mode traces from 10 s movies show heartwall movements in 1-week-old (left) and 5-week-old (right) hearts. Top, W\textsuperscript{CS}. Middle, ilk\textsuperscript{54-wCS/+}. Bottom, mys\textsuperscript{XG43/+}. The incidence of arrhythmia is increased with age in wild-type controls (top panels, see also Ocorr et al., 2007), but 5-week-old ilk\textsuperscript{54-wCS/+} hearts (middle right) and mys\textsuperscript{XG43/+} hearts (bottom right) present more regular beating patterns than 5-week-old W\textsuperscript{CS} hearts (top right). (B–E) Plots of physiological parameters of W\textsuperscript{CS}, ilk\textsuperscript{54-wCS/+}, and mys\textsuperscript{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). Arrhythmia index increases with age in wild-type W\textsuperscript{CS} hearts (P < 0.001 for both 3 and 5 weeks), as previously reported for other wild-type strains (Ocorr et al., 2007). Notably, 5-week-old ilk\textsuperscript{54-wCS/+} and mys\textsuperscript{XG43/+} flies had lower AI compared with the age-matched W\textsuperscript{CS} flies. *P < 0.01, **P < 0.001, two-way ANOVA, see Experimental Procedures. (C, D) Diastolic diameter decreases with age in W\textsuperscript{CS}, and diastolic and systolic diameters decrease mys\textsuperscript{XG43/+} flies, but not in ilk\textsuperscript{54-wCS/+} flies. *P < 0.01, **P < 0.001, two-way ANOVA. (E) Plots of fractional shortening for W\textsuperscript{CS}, ilk\textsuperscript{54-wCS/+}, and mys\textsuperscript{XG43/+} flies. 1-week-old mys\textsuperscript{XG43/+} flies had a significantly lower fractional shortening compared to W\textsuperscript{CS} and ilk\textsuperscript{54-wCS/+}. **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\textsuperscript{-2}. Error bars indicate SEM.

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 kiloPascal, kPa, upon applying external pressure and at the ventral midline of the heart; Kaushik et al., 2011, 2012). Because ilk heterozygous mutants attenuate or halt 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 in myocardial stiffness with age. To test this idea, ventral midline stiffness was measured with nanoindentation in ilk\textsuperscript{54-wCS/+} and corresponding wild-type controls W\textsuperscript{CS}. Aged W\textsuperscript{CS} hearts exhibit a stiffening of approximately 65% with age from 1 to 5 weeks of age (Fig. 3F and Fig. S1B). In contrast to W\textsuperscript{CS}, the ilk\textsuperscript{54-wCS/+} myocardium did not stiffen 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\textsuperscript{XG43-wCS} myocardium also showed lack of stiffening with age, similar to the ilk\textsuperscript{54-wCS/+} myocardium (Fig. 3F). Thus, we find that ilk\textsuperscript{54-wCS/+} and mys\textsuperscript{XG43-wCS} hearts do not display several measures of cardiac aging, such as increasing AI, diastolic dysfunction (except for mys\textsuperscript{XG43-wCS}) and myocardial stiffening.

\(\beta1\)-integrin protein levels are increased in old flies and overexpression in young fly hearts mimics the effects of cardiac aging

As ilk and mys heterozygous mutants show a much attenuated progression in several age-dependent characteristics (Fig. 3; Fig. S1), 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-old flies compared with 1-week-old flies (Fig. S2, Supporting Information). However, when examining \(\beta1\)-integrin protein levels, we found an almost two-fold increase at 5 weeks compared with 1 week (Fig. 4A,B). This increase in \(\beta1\)-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 wild-type 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 GMR-Gal4 (Wessels et al., 2004; Han &
1-week and 5-week-old heart function. (A, B) Western blot analysis was carried out with whole fly preps of antibody and anti-

myosin driver significantly increased arrhythmia compared with hand-decreased fractional shortening compared with the controls (hand-

ª

Olson, 2005). Strong cardiac reduction in young flies, which was further elevated at old age (orange bars in Fig. S3, Supporting information) prevented an increase in the level of mutants, which showed improved cardiac performance at older age.

To investigate whether the dual – beneficial versus detrimental – role of scaled reduction in ilk signaling in aging is conserved over a substantial evolutionary distance, we turned to another 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; Kumsta et al., 2014). However, long-lived animals exposed to pat-4/Ilk RNAi 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; Campagnola et al., 2002; Kumsta et al., 2014). This detachment phenotype is reminiscent of what is observed in 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 in 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 in ilk/pat-4 RNA levels as compared with animals with many muscle foci (Fig. 5G). Taken together, these observations suggest that a substantial reduction in 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 ilk/pat-4 RNAi knockdown on C. elegans longevity

To investigate this notion further, we examined the structural integrity of hearts 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 β1-integrin staining, which were not seen in wild-type controls or ilk heterozygous mutants (Fig. S8–C, Fig. S4A–E, Supporting information). 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 the expected 50% reduction in function of ilk heterozygous mutants or with GMH5-mediated KD (see Fig. S3). Interestingly, heart-specific KD of pinch, encoding an ILK binding partner, results in similar intercellular adhesion defects (Fig. S4F–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 (Fig. S4G–J). This finding supports the hypothesis that the integrin complex is essential for adhesion between adult cardiomyocytes.

Dual effects of β1-integrin/ILK pathway on heart function

Given the beneficial or detrimental effects depending on the extent of ilk reduction, we wondered whether strong versus weak inhibition of ilk binding partners also could have 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) significantly extends lifespan, similar to ilk KD (Hansen et al., 2005). Thus, an appropriate reduction in 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 wild-type

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control (hand-Gal4 alone) and comparable to long-lived ilk and mys heterozygotes (Fig. 3 and Fig. S1) 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 (Fig. S4). 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 wild-type 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 (Fig. S4I,J).

To further substantiate that moderate reduction in ILK pathway components in the myocardium contributes to better cardiac performance at old ages, we examined heterozygous mutants of parvin, pinch (stick), and talin (rhea; see Experimental procedures). All three heterozygotes failed to show an increase in AI or decrease in diastolic diameter.
at old ages (Fig. S5, Supporting information), thus corroborating the conclusion that they indeed participate in the beneficial modulation of cardiac aging, similar to moderate ilk and mys attenuation (Figs 3 and S5A). Together, these results further support the notion that moderately reduced levels of β1-integrin/ILK signaling components within the heart prevent 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 β1-integrin/ILK pathway is a critical genetic modulator of cardiac and organismal aging. We have shown that reduction in β1-integrin/ILK levels is beneficial for Drosophila longevity and cardiac performance at older ages. In addition, severe reduction in ILK pathway components in the heart causes loss of cardiomyocyte cell adhesion, along with structural and functional deficits. We also find that β1-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; Kumsta et al., 2014), 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 β1-integrin is significantly higher in old compared with young monkey vascular smooth muscle cells (VSMCs; Qiu et al., 2010). Therefore, the accumulation of β1-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 wild-type flies reverses the age-dependent increase in arrhythmia, whereas overexpression of KCNQ in young flies increases the incidence of arrhythmias (Ocorr et al., 2007; Nishimura et al., 2011). 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/dafl-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., 2014). 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, β1-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 β1-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 in several components of β1-integrin/ILK signaling can reverse several characteristics of cardiac aging, it is interesting 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) and 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 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

ilk^54 (Zervas et al., 2011; the allele ilk^54 has a premature stop codon instead of a codon for W7), ILK-GFP/ilk^54 (ILK-GFP is previously described in Zervas et al., 2001), and UAS-mys^54 were generous gifts from Y. Inoue. ilk^1 (Zervas et al., 2001) and mys^1 (Bunch et al., 1992) were obtained from the Drosophila stock center. w^CS (Cook-Wiens & Grotewiel, 2002) and mys^VGA3 (Goddereis et al., 2003) were kindly provided by M. Grotewiel. The mys^VGA3^ 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 (Goddereis et al., 2003). ilk^54 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 (VDRD): UAS-parvin^{RNAi} (#11670), UAS-pinch^{RNAi} (#52538), UAS-mys^{RNAi} (#29619), UAS-talin^{RNAi} (#40399) and UAS-paxillin^{RNAi} (#107789). UAS-ilk^{RNAi} (originally from VDRD, #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 mel2-Gal4. We have also tested ourselves the pinch and parvin RNAi lines when crossed to mel2-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 GMHS have been previously described (Wessells et al., 2004; Han & Olson, 2005). parvin^{GFP} (Vakaloglou et al., 2012), stck^{22} (Zervas et al., 2011), and rhea^{TM} (Brown et al., 2002) were kindly provided by C. Zervas.

#### Immunostaining

Adult female flies were dissected and immunostained as previously described (Taghlil-Lamallem et al. 2008). Images were acquired using ApoTome (Carl Zeiss Microscopy, Thornwood, NY, USA) or FV1000 (Olympus, Center Valley, PA, USA). The following primary antibodies were used: rabbit anti-GFP (1:250, Invitrogen, Grand Island, NY, USA), mouse anti-α5 (1:20, Cell Signaling), mouse anti-α-actinin (1:20, kindly provided by J. Saide), and mouse anti-α-spectrin (1:50, 3A9; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA).

#### Lifespan assays

Virgin female and male progeny were collected for 3 days. Then, they were briefly anesthetized and separated in groups of 25 flies in each vial. The flies were kept at 25 °C, and the dead flies were counted every 3 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, La Jolla, CA, USA).

#### 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 Escherichia 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, College Station, TX, USA). P values were calculated with the log-rank (Mantel–Cox) method.

#### Heartbeat analysis

Flies were anesthetized with fly nap (Carolina Biological Supply Co., Burlington, NC, USA) and dissected as previously described (Ocorr et al., 2007). Movies were taken at rates between 140 and 160 frames per second for 30 s using a Hamamatsu CCD digital camera (McBain Instruments, Chatsworth, CA, USA) on a Leica DM LFS 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 25-mm 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, Goleta, CA, USA) 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, Melville, NY, USA) with 120 pN nm⁻¹ silicon nitride cantilevers with premounted, 2-μm radius borosilicate glass spheres (Novascan Technologies, Ames, IA, USA). All indentation curves were analyzed to calculate myocardial stiffness using previously published, automated software custom-written in MATLAB (MathWorks, Natick, MA, USA; Kaushik et al., 2011, 2012).

Statistical methods

Statistical Analyses were performed using Prism 6.0 (Graph Pad Software, Inc.). All data were checked prior to analysis using the D’Agostino & Pearson omnibus normality test to determine whether 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 were 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. 3B). 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 nonparametric data (e.g., Fig. 5A). Lifespan analyses were performed using a log-rank analysis (Mantel-Whitney test for nonparametric data (e.g., Fig. 5A). Lifespan analyses within a single genotype between two ages were made using the Mann-Whitney test. Comparisons of two-way ANOVA analyses were made using a one-way analysis of variance (ANOVA) when comparing more than two groups (e.g., Fig. 3B). All data were checked prior to analysis using the D’Agostino & Pearson omnibus normality test to determine whether 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 were 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. 3B). Data that did not exhibit a Gaussian distribution (the arrhythmia indices) were first normalized before applying a two-way ANOVA (e.g., Fig. 3B). 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 nonparametric data (e.g., Fig. 5A). Lifespan analyses were performed using a log-rank analysis (Mantel–Cox test). Specific tests used are indicated in the figure legends. In all cases, P values less than 0.05 were taken as significant.

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Conflict of interest

None declared.
Integrin-linked kinase modulates longevity and thermostolerance in \textit{C. elegans} through neuronal control of HSF-1. \textit{Aging Cell}, in press.


**Supporting Information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.

**Fig. 51** *ilk* heterozygotes and *mys* heterozygotes retard cardiac decline with age.

**Fig. 52** mRNA levels of *ilk* and *mys* do not change with age.

**Fig. 53** *ilk* expression is reduced by cardiac RNAi knockdown.

**Fig. 54** *pinch*, *mys*, and *talin* RNAi but not *ilk* heterozygous mutants induce gaps between cardiomyocytes.

**Fig. 55** Heterozygous mutants for *parvin*, *pinch* and *talin* lack age-dependent heart function changes.