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## Dystrophin deficiency in *Drosophila* reduces lifespan and causes a dilated cardiomyopathy phenotype

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### Summary

A number of studies have been conducted recently on the model organism *Drosophila* to determine the function of genes involved in human disease, including those implicated in neurological disorders, cancer and metabolic and cardiovascular diseases. The simple structure and physiology of the *Drosophila* heart tube together with the available genetics provide a suitable *in vivo* assay system for studying cardiac gene functions. In our study, we focus on analysis of the role of dystrophin (Dys) in heart physiology. As in humans, the *Drosophila dys* gene encodes multiple isoforms, of which the large isoforms (*DLPs*) and a truncated form (*Dp117*) are expressed in the adult heart. Here, we show that the loss of *dys* function in the heart leads to an age-dependent disruption of the myofibrillar organization within the myocardium as well as to alterations in cardiac performance. *dys* RNAi-mediated knockdown in the mesoderm also shortens lifespan. Knockdown of all or deletion of the large isoforms increases the heart rate by shortening the diastolic intervals (relaxation phase) of the cardiac cycle. Morphologically, loss of the large *DLPs* isoforms causes a widening of the cardiac tube and a lower fractional shortening, a phenotype reminiscent of dilated cardiomyopathy. The dilated *dys* mutant phenotype was reversed by expressing a truncated mammalian form of *dys* (*Dp116*). Our results illustrate the utility of *Drosophila* as a model system to study dilated cardiomyopathy and other muscular-dystrophy-associated phenotypes. Key words: aging; arrhythmia; cardiac function; diastole; heart; heart rate; muscle fibers; muscular dystrophy; myofibrillar disarray; systole.

### Introduction

Muscular dystrophy (MD) involves over 30 different inherited diseases, all causing progressive weakness and degeneration of skeletal muscle (Emery, 2002). Dystrophin (Dys) was the first mutant protein shown to cause MD. Mutations in the *dystrophin* gene (*dys*), the largest gene in the human genome, cause Duchenne MD (DMD) as well as the milder phenotype of Becker

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MD (BMD) (Koenig *et al.*, 1988). Duchenne MD is caused by a nonfunctional or absent Dys protein, whereas BMD has been associated with a reduction of Dys protein levels. In humans, *dys* is expressed in skeletal, smooth and cardiac muscles, as well as in the brain (Holder *et al.*, 1996). Dystrophin protein is enriched in the sarcolemma, known to link the extracellular matrix to the actin cytoskeleton via interactions with the Dys glycoprotein complex (DGC), and therefore, has an important structural role during muscle contraction and muscle stretch (Petrof *et al.*, 1993; Straub *et al.*, 1997). The absence of Dys destabilizes the DGC components and results in membrane fragility and disruption during contraction. Besides the mechanical role, Dys is involved in signaling cascades via their DGC protein partners (dystroglycan and syntrophin): DGC components seem to be able to interact with growth factor receptor-bound protein 2, calmodulin and neuronal nitric oxide synthase (Rando, 2001). Furthermore, Dys may be involved in the aggregation or organization of ion channels in the membrane because its absence causes abnormalities in channel function (Franco & Lansman, 1990).

The *Drosophila dys* gene is as complex as its mammalian counterparts. It encodes three large isoforms called Dys-like products (DLPs) and three truncated products sharing with DLPs the carboxy-terminal and cysteine-rich domains driven by separate internal promoters (Greener & Roberts, 2000; Neuman *et al.*, 2001, 2005). The three DLPs consist of an N-terminal actin-binding domain, numerous spectrins repeats, a dystroglycan-binding domain (cysteine-rich CR domain) and a carboxy-terminal domain involved in binding to other DGC proteins. The three shorter isoforms Dp186, Dp205 and Dp117 have a unique N-terminal region appended to the common C-terminal region (Neuman *et al.*, 2005). As in mammals, the fly *dys* transcripts are expressed in distinct tissue-specific patterns (Neuman *et al.*, 2001, 2005; Dekkers *et al.*, 2004; Van der Plas *et al.*, 2006; Shcherbata *et al.*, 2007).

Despite the evolutionary divergence between flies and humans, the genomic sequences indicate that around 70% of the genes involved in human disease are also found in *Drosophila* (Adams *et al.*, 2000; Fortini & Bonini, 2000; Bier, 2005). Fly models have been generated for a wide spectrum of human diseases such as developmental disorders, neurological disorders, cancer, metabolic disorders and cardiovascular disease (Bonini & Fortini, 2003; Sutcliffe *et al.*, 2003; Bier & Bodmer, 2004; Bier, 2005). Among these disease genes, a number of them are implicated in cardiac function: mutations in *myosin*, *tropomyosin 1*, *tropomyosin 2* and  *$\delta$ -sarcoglycan* cause dilated cardiomyopathy (Wolf *et al.*, 2006; Cammarato *et al.*, 2008), and mutations in a potassium channel  $\alpha$ -subunit for *KCNQ1* cause arrhythmias in humans as well as in flies (because of prolonged duration of contractions phases – ‘long QT’) (Ocorr *et al.*, 2007).

In this study, we investigate the role of Dys in maintaining heart morphology and function, using the fly heart as an *in vivo* assay system. Using reverse transcriptase–polymerase chain reaction (RT–PCR), we determined that both the long DLPs and the short DP117 *dys* isoforms are expressed in the adult *Drosophila* heart. The *dys* mutant, haploinsufficiency or knockdown flies show shortened lifespan and develop age-dependent cardiac abnormalities, reminiscent of *mdx* mice. We characterized the cardiac properties in *dys* mutants by measuring changes in its dynamic parameters, including heart rate, rhythmicity, systolic and diastolic diameters and intervals and fractional shortening. The *dys* mutant flies have dilated and abnormally performing hearts consistent with the mammalian phenotype of dilated cardiomyopathy. Deletion of the long DLP isoforms also causes progressively disorganized myofibrillar arrangements with age that may contribute to the altered performance. We discuss the potential of the fly heart model in studying the molecular basis of MD effects in the heart.

## Results

### Dys protein distribution in the *Drosophila* heart

The *dys* gene products are expressed in a tissue-specific manner; the long-form DLP1 and DLP2 are predominantly found during development in the visceral mesoderm, in the gut and throughout muscle fibers, while Dp186 is present at high levels in the central nervous system (Neuman *et al.*, 2001; Dekkers *et al.*, 2004; Van der Plas *et al.*, 2006; Shcherbata *et al.*, 2007). After germ band retraction, two rows of cardiac mesoderm fuse at the dorsal midline to form the linear heart tube in *Drosophila* (Bodmer, 1995; Bodmer & Frasch, 1999; Haag *et al.*, 1999). Dystrophin expression is detected in the myocardial cells shortly before fusion (data not shown) and continuously throughout embryogenesis (Fig. 1A). During metamorphosis, the heart undergoes remodeling forming a functional adult heart that contains inflow tract openings (ostia) and interval valves (Molina & Cripps, 2001; Monier *et al.*, 2005). In the adult, the cardiac tube remains a simple linear tube that pumps the hemolymph through the organism in an open circulatory system. Dystrophin accumulates in the cell membranes outlining the membranes of the myocardial cells similar to discs large (Dlg) (Fig 1B,C). Using RT-PCR with RNA isolated from adult hearts, we detect the long *DLPs dys* isoforms and a short form, *Dp117* (Fig. 1D). *Dp186* and *Dp205* appear to be absent from the adult heart.

Staining of the cardiac tube with the sarcomeric Z-line marker,  $\alpha$ -actinin, reveals two kinds of myofibrillar organization: the myofibrils of the contractile Dmef2-positive ‘working’ myocardium (Fig. 1E), which are also Tinman positive, are organized in a transverse or spiral fashion (Fig. 1F,F’; Molina & Cripps, 2001); and the longitudinally oriented myofibrils of syncytial muscles containing small Dmef-2 positive nuclei (without Tinman) are associated with the myocardium along the ventral side (Fig. 1G,G’).

### *dys* Knockdown and deletion mutants decrease lifespan

To investigate *dys* function on organism longevity, we examined flies with reduced *dys* expression. A small deletion *Dys*<sup>8-2</sup> has been reported to affect some of the long *DLP* forms, whereas *Df(3R)KX43* (*Dyskx43*) (Shcherbata *et al.*, 2007) and *Df(3R)Exel6184* (*DysExel6184*) are larger deficiencies of the *dys* locus. We first checked the isoform expression in each transheterozygous deficiencies by RT-PCR analysis (Fig. 2A). Using specific primers for each isoform, we determined that in the transdeficiency *DysExel6184/Dyskx43* flies, all isoforms were absent except the short-form *Dp117*, whereas the *Dyskx43/dys*<sup>8-2</sup> or *DysExel6184/dys*<sup>8-2</sup> combinations show a modest decrease in most isoforms (Fig. 2A). We also examined the effects of *dys-RNAi* transgenic flies targeting all *dys* isoforms (Shcherbata *et al.*, 2007). To determine to what extent the *mRNA* levels of *dys* isoforms in the *dys-RNAi* line are affected, we performed quantitative RT-PCR (qRT-PCR) on heart RNA, using primers specific for all isoforms. Quantitative RT-PCR analysis shows an approximately 60% reduction in all isoforms when knockdown was achieved with the mesodermal *24B-Gal4* driver, and an approximately 40% reduction with a cardiac-specific driver *GMH5-Gal4* (Fig. 2B,C; Wessells *et al.*, 2004).

We observed a moderate lifespan reduction in the mesodermal *dys* knockdown flies (13%), but not when exclusively targeted to the heart (Fig. 2D). In contrast, half of the transheterozygous *DysExel6184/Dyskx43* deficiency flies died at 29 days compared to 63 days in controls (Fig. 2D). Because losing one copy of a gene usually causes a corresponding reduction in the RNA level, we also tested the single heterozygous deficiencies for *dys*, which showed a moderate lifespan reduction similar to the *dys* knockdown with *24B-Gal4*. Interestingly, a 40% knockdown in *dys* RNA levels specifically in the heart was not sufficient to impair adult viability. This suggests that a moderate *dys* loss-of-function in all muscles, but not in just the heart, reduces the fly's normal lifespan. Therefore, *dys* influences longevity in

*Drosophila* reminiscent of the observed reduction in lifespan of *dys*-deficient *mdx* mice (Chamberlain *et al.*, 2007).

### Age-dependent abnormalities in myofibrillar organization in *dys* mutant hearts

Examination of the myocardium structure in *dys*-deficient *mdx* mice shows considerable muscle degeneration that is aggravated by age (Chamberlain *et al.*, 2007). Defects in the thoracic muscles of *dys* mutant flies have also been reported (Shcherbata *et al.*, 2007). We assessed the heart structure in flies by staining with  $\alpha$ -actinin, a structural element and component of the contractile machinery of muscles localized to the Z-bands. The  $\alpha$ -actinin staining revealed a tight and well-aligned spiral arrangement of cardiac myofibrils in *wild-type* (*wt*) flies at 1 week and 3 weeks of age (Fig. 3A,B',G,I). Older hearts (at 5 weeks) begin to show disruptions and abnormalities in the parallel alignment of myofibrils (Fig. 3C,C',I). In contrast, *dys* deficiency flies *dysExel6184/dyskx43* revealed a less organized and less compact arrangement of the myocardial myofibrils already at 1 week of age (Fig. 3D,D',H,I). This phenotype becomes progressively more pronounced in older *dys* deficiency flies, in that the myocardial myofibrils are increasingly disorganized with more gaps devoid of myofibrils (Fig. 3E-F',I). Thus, *dysExel6184/dyskx43* mutants show age-dependent disruption of the heart myofibril integrity. The gaps in myofibrillar  $\alpha$ -actinin staining were quantified by measuring the size of these areas using image J software from confocal stacks (illustrated in Fig. 3G,G',H,H'). The percentage area devoid of myofibrils from seven to eight hearts was plotted as according to age (Fig. 3I). *Wild-type* flies at 5 weeks show gaps covering approximately 3% of the heart tube, whereas in *dysExel6184/dyskx43* hearts the gaps cover about 13% of the total myocardium. These data suggest that *dys* is required for the physical integrity of the heart muscle throughout the fly's life.

### Characterization of cardiac function in *dys* mutants

While the development of the *Drosophila* heart involves similar genetic pathways and molecular mechanisms as the vertebrate system, the fly has not yet been extensively exploited in heart physiology studies, with notable recent exceptions (Johnson *et al.*, 2002; Wessells *et al.*, 2004; Wolf *et al.*, 2006; Ocorr *et al.*, 2007). To study heart function, we dissected adult flies in artificial hemolymph to record cardiac contractions with a high-speed digital video camera (Ocorr *et al.*, 2007); see Materials and Methods. M-mode traces of movie clips provided details in the heart wall edge positions (y-axis) over time (x-axis), illustrating the rhythmicity and the dynamics of heart contractions. The exposed and largely denervated heart of *wt Drosophila* shows regular rhythmic contractions (Fig. 4A, 1 week *wt*), which become progressively more irregular with age (Fig. 4A-C; Supplementary Fig. S1). *Drosophila dys*-deficient hearts lacking DLPs function (*dysExel6184/dyskx43*) also become more irregular with age (Fig. 4A-C), but in addition exhibit a significantly increased heart rate that corresponds to a reduced heart period (HP, defined diastolic plus systolic interval) at 3 weeks and 5 weeks of age compared to *wt* (Fig. 4B). The distribution of all the measured heart periods for all flies of a specific genotype and age is represented in histogram format (Fig. 4B). The majority of the *wt* flies show a relatively tight clustering of the heart period lengths at 1-3 weeks, and this distribution broadens in 5-week-old flies because of the increased variability in heart period with age (see also Ocorr *et al.*, 2007).

The variability in the heart periodicity can be quantified using the heart period standard deviation as an 'arrhythmicity index' (Fig. 4C, AI; Ocorr *et al.*, 2007). *Wild-type* flies show a low value for this AI at 1 week, and this value increased with age (Fig. 4C, see also Ocorr *et al.*, 2007). The AI for *dys* mutants is elevated at 1 week compared to *wt*, but is reduced at progressively older ages in *dys* mutants (Fig. 4C). The shorter heart period/increased heart rate is mainly because of a shorter diastolic interval (relaxation period; Fig. 4D). This elevated heart rate may explain the more regular heartbeat observed in older *dys* flies. Another parameter of

rhythm abnormalities studied in *dys* mutants is the distribution of asystoles within the 1-min movies. An asystolic event is a prolonged relaxation we defined as a diastolic interval longer than 1 s. We find that *dyskx43/dys<sup>8-2</sup>* and *dysExel6184/dyskx43* flies show a reduced incidence of asystolic events with age than do the control flies (Fig. 4E). These results suggest that partial or total lack of DLP function causes shorter diastolic intervals, resulting in increased heart rates and fewer asystoles, and producing a more regular heartbeat with age than *wt*.

We also tested the overall cardiac performance of *dys* mutants by pacing the heart to a higher rate (6 Hz) using external electrical pacing (as described in Wessells *et al.*, 2004). *dys-RNAi/24B-Gal4* or heterozygous *dysExel6184/+* deficiency flies display a similar age-dependent increase in the heart failure rate immediately after pacing compared to *wt* (Wessells *et al.*, 2004) or the age-matched *dysRNAi/+* control without the driver (Supplementary Fig. S1E). The reason why a reduced *dys* function does not cause an elevated susceptibility to electrical pacing may be that they already have an increase in heart rate, making them less sensitive to this stress.

### ***dys* Mutant flies exhibit a ‘dilated cardiomyopathy’ phenotype**

Our image analysis of heart contractions also provides cardiac chamber parameters, including end-diastolic and end-systolic diameters. In addition, the proportional decrease in heart wall diameter during contraction, referred to as the fractional shortening, provides an indication of the cardiac output. *Wild-type* flies display an average diastolic diameter of about 60  $\mu\text{m}$  and a systolic diameter of 40  $\mu\text{m}$ , which do not change much with age (Fig. 5A–D), and the fractional shortening in *wt* flies was 35–40% (Fig. 5E). In contrast, *dyskx43/dys<sup>8-2</sup>* and *dysExel6184/dyskx43* have a significantly wider diastolic (80–90  $\mu\text{m}$ ) and systolic (60  $\mu\text{m}$ ) diameter (Fig. 5A–D; see also Supplementary Fig. S1). In addition, the *dys* mutant fractional shortening was also reduced compared to *wt* (25–30%; Fig. 5E). Interestingly, some of the heterozygous *dys* mutants also show enlarged tube diameters (see Supplementary Fig. S2A,B). Three situations of phenotype are found according to the mutant severity: the *dys* mutant heterozygotes *dysExel6184/+* and *Dyskx43/+* show a dilated phenotype without reducing a fractional shortening, *dyskx43/dys<sup>8-2</sup>* in addition have reduced fractional shortening later in life and *dysExel6184/dyskx43* already from week 1 on (Fig. 5; Suppl. movies). This phenotype of cardiac chamber enlargement and impaired systolic function in *dys* mutants recapitulates the dilated cardiomyopathy phenotype observed in DMD patients or the mouse model *mdx* remarkably well (Quinlan *et al.*, 2004; Wehling-Henricks *et al.*, 2005).

### **A truncated mammalian Dys isoform (Dp116) rescues the dilated phenotype of *Drosophila dys* mutants**

Next, we attempted to rescue the dilated cardiomyopathy phenotype characteristic of DLP-deficient *dys* mutant flies (*dysExel6184/dyskx43*) by expressing a truncated version of mouse *dys*. We sought to determine if any of the shorter isoforms of Dys, which assemble and localize the DGC but do not bind to the actin cytoskeleton (Yue *et al.*, 2003; Judge *et al.*, 2006), might be able to rescue any of the abnormalities in the fly hearts. Thus, we first expressed *Dp116*, a short C-terminal isoform of mammalian *dys* (Judge *et al.*, 2006), in a *wt* background under the control of the mesodermal *24B-GAL4* driver and found no difference compared to *wt* (data not shown). In contrast, when we expressed mouse *Dp116* in the mesoderm of *dysExel6184/dyskx43* flies, we observed a smaller systolic diameter and a higher fractional shortening than *dysExel6184/dyskx43* flies, which was comparable to *wt* and the other controls (Fig. 5F,G). This result suggests that introducing the short mammalian *Dp116* transgene to the heart of *dys* mutant flies significantly restores the *wt* heart diameters and fractional shortening, and hence ameliorates abnormal systolic function. This would also be consistent with the idea that the functionally important Dys domains that rescue the dynamic cardiac properties include two spectrin repeats, WW-, CR- and C-terminal domains, and human exon 55S unique to *Dp116* (Judge *et al.*, 2006). The ability to rescue the abnormalities observed in *dysExel6184/*



*dyskx43* mutants with a *dys* transgene indicates strongly that *dys* is required for normal heart function.

## Discussion

*Drosophila* has been used extensively to study cardiac development (Bodmer, 1995; Bodmer & Frasch, 1999; Prall *et al.*, 2002; Zaffran & Frasch, 2002). More recently, the *Drosophila* model has also been adopted to investigate cardiac physiology, performance and aging (Wessells *et al.*, 2004; Sanyal *et al.*, 2006; Wolf *et al.*, 2006; Ocorr *et al.*, 2007; Mery *et al.*, 2008) using assays to determine the contractility, rhythmicity and other performance parameters, for example, stress assay, in which the cardiac performance is tested by using external electrical pacing of the heart rate (Paternostro *et al.*, 2001; Wessells & Bodmer, 2004), ultrasound-like image analysis of the heart in intact flies [using optical coherence tomography (OCT); Wolf *et al.*, 2006], digital high-speed video imaging of semi-intact adult fly heart, which in addition allows characterizing the contractility and rhythmicity of the heart (Ocorr *et al.*, 2007). Using these assays, it was found that the fly homologue of the *KCNQ1* potassium channel gene, which is associated with human cardiac arrhythmias and long QT, causes heart rhythm abnormalities also when mutated in flies, and they become more severe with age (Ocorr *et al.*, 2007). Moreover, mutations of genes encoding sarcomeric contractile proteins (tropomyosin II, troponin I,  $\delta$ -sarcoglycan) showed dilated cardiomyopathy in flies (Wolf *et al.*, 2006). Our studies of *dys*-dependent heart function abnormalities further validate the utility of *Drosophila* for studying the genetic basis of cardiac physiology.

The data described here explore the function of the cytoskeletal protein Dys in the fly heart. We find that: (i) mesodermal expression of *Drosophila* Dys is required for 'normal' lifespan; (ii) is necessary for the integrity of the heart structure (dilated heart phenotype and myofibril disorganization in *dys* mutants); and (iii) plays a physiologically important function in directly or indirectly modulating heart rate, rhythmicity and pumping efficiency (systolic dysfunction in *dys* mutants). These phenotypes are reminiscent of the 'dystrophic' heart phenotypes in mammals (Quinlan *et al.*, 2004; Wehling-Henricks *et al.*, 2005).

### Lifespan analyses and age-dependent heart muscle disruption

Model organisms have been used extensively for studying the MD pathology (*mdx*-mouse, dog, hamster, *Caenorhabditis elegans* and zebra fish (see Gieseler *et al.*, 2000; Watchko *et al.*, 2002; Bassett & Currie, 2004). These animal models of muscular dystrophies have been complicated by gene redundancy, which can substitute at least in part for the deleted or mutated gene (e.g. *dys*-related *utrophin*; Grady *et al.*, 1997). The single *dys* gene in *Drosophila* simplifies studies of functional requirements, and together with the fly's efficient genetic tools makes *Drosophila* a powerful model for probing dystrophic mechanisms. To study the effect of *dys* function on the fly's lifespan, we conducted longevity assays with *dys* mutants, including mesodermal and heart-specific *dys* knockdown. We found that mesodermal knockdown (*dys-RNAi/24B-Gal4*) reduces lifespan similar to heterozygote deficiencies (*dysExel6184/+* and *dyskx43/+*), by about 13%. The decrease in lifespan of *dys-RNAi/24B-Gal4* flies is likely a consequence of skeletal muscle deterioration (Shcherbata *et al.*, 2007), as opposed to cardiac abnormalities, because (moderate) cardiac *dys*RNAi knockdown did not affect longevity.

Interestingly, a recent study shows that *mdx* mice show an approximately 20% reduction in lifespan compared to *wt* mice (Chamberlain *et al.*, 2007). This mild reduction in *mdx* mice lifespan could be attributed to a partially compensatory up-regulation of the *dys* relative *utrophin* (reviewed in Blake *et al.*, 2002). In old *mdx* mice, muscle histopathology is also more extensive and pronounced than what has been observed in young *mdx* mice (Chamberlain *et al.*, 2007), which is consistent with our observations in the fly heart: *dysExel6184/dyskx43* mutants show a strongly age-dependent disorganization of cardiac myofibrils. In *mdx* mice,

fibrous connective tissue is observed in cardiomyocytes that is likely the consequence of the damage (Quinlan *et al.*, 2004; Williams & Allen, 2007). In contrast to the vertebrate *mdx* heart, the fly myocardium does not show an increase in collagen gene expression (collagen types IV and XVIII), as determined by RT-PCR (data not shown).

### ***Drosophila* as a model for dilated cardiomyopathy**

The lack of Dys in cardiac muscle leads to progressive cardiomyocyte degeneration and fibrosis (Rudge & Duncan, 1988; Williams & Allen, 2007). In the late phase of the disease, when extensive fibrosis is present, the conduction system is also perturbed. Among the DMD patients, 50% will have cardiac abnormalities by 18 years of age (Nigro *et al.*, 1990). A progressive dilated cardiomyopathy occurs in all boys with DMD (Nigro *et al.*, 1990). In BMD, cardiac disease is seen in over 70% of men by the age of 40 (Nigro *et al.*, 1990; Saito *et al.*, 1996). A severe dilated cardiomyopathy occurs in BMD with relatively preserved skeletal muscle function (Grain *et al.*, 2001). Duchenne MD gene therapy has been mainly focused on correcting the skeletal muscle pathology in *mdx* mice (Chamberlain, 2002), with little progress to treat the cardiomyopathy (Koh *et al.*, 1995; Yue *et al.*, 2003). A notable exception is the recent finding that adenoviral-mediated delivery of a micro-Dys to *mdx* mice ameliorated the hemodynamic abnormalities and pump dysfunction of the heart under stress (Townsend *et al.*, 2007). The paucity of cardiac gene therapy attempts in the *mdx* mice is mainly caused by our incomplete understanding of the role of Dys in this tissue. Using physiological assay in *Drosophila* may thus facilitate the role of cardiac *dys* function.

Analyzing the effects of *dys* mutations (reduced or complete loss of the long DLP form), we observed that loss of DLP function causes dilated hearts and lower fractional shortening, accompanied by increasing myofibril disorganization with age. *dyskx43/dys<sup>8-2</sup>* Mutants show dilated heart, but the systolic function was not affected in young flies, only at more advanced ages. In contrast, the mutant DLP-deficient *dysExel6184/dyskx43* flies exhibit a more severe phenotype already in young flies. Heterozygous *dysExel6184/+* flies have been reported to also show dilated cardiomyopathy, using OCT (Wolf *et al.*, 2006), which is consistent with our results using high-speed transmitted light imaging (Supplementary Fig. S2A,B). It is important to note that in our semi-intact preparation, heart physiology is recorded under drastically reduced neuronal or hormonal influences, thus assessing the myogenic properties of the heart. The observed phenotypes suggest a haploinsufficiency for *dys* in cardiac physiology even in the absence of obvious structural abnormalities, supporting the idea of a conserved role for *dys* in maintaining heart function from flies to humans (this study; Nigro *et al.*, 1990; Goodwin & Muntoni, 2005).

We also observed an increased heart rate with age in *dys* mutant flies, which is mainly because of shorter diastolic phases, and is accompanied by a more regular heart rhythm and less asystolic episodes. Interestingly, a recent report shows that *mdx* mice have increased heart rate apparently because of a shortened PR interval (Bostick *et al.*, 2008). Moreover, the inactivation of the *C. elegans dys-1* gene does not show muscle degeneration, perhaps because of their short lifespan, but these animals display body wall muscle hypercontraction and are hyperactive (Bessou *et al.*, 1998; Carre-Pierrat *et al.*, 2006). This phenotype has been linked to the BK (Slo) calcium-activated large conductance potassium channels. The hyperactivity phenotype in *C. elegans dys-1* is because of the down-regulation of the SLO-1 channel activity in muscles but not in neurons (Carre-Pierrat *et al.*, 2006). Further studies are required to determine whether the increased heart rate in flies has a similar cause as the worm hypercontractility. Interestingly, other Dys models also seem to exhibit ion channel misregulation (e.g. slo, Kir4.1 and calcium channels; Mallouk & Allard, 2000; Connors & Kofuji, 2002). Abnormalities in calcium handling could also be a mechanism that contributes to the observed increase in heart rate in flies, hypercontractility in worms and hypercontraction and death of mouse *mdx* myocardial

cells (see Yasuda *et al.*, 2005). Furthermore, *mdx* hearts have been shown to have defects in  $\text{Ca}^{2+}$  handling proteins, including decreased cardiac sarcoplasmic reticulum  $\text{Ca}^{2+}$  *ATPase 2* (*SERCA2*) mRNA and delayed normalization of intracellular  $\text{Ca}^{2+}$  concentration (Rohman *et al.*, 2003; Williams & Allen, 2007).

*Drosophila* has been proven to be a valuable model to dissect the DMD pathogenicity (Shcherbata *et al.*, 2007), including that of the heart (this study). Importantly, we have shown that a truncated human form of *dys* (*Dp116*) is able to rescue the dilated cardiac phenotype of DLP-deficient fly mutants. This is in contrast to the finding that expression of *Dp116* in skeletal vertebrate muscles acts as a dominant negative, aggravating the dystrophic phenotype (Judge *et al.*, 2006). Even though *Dp116* expression does not rescue dystrophic skeletal muscles of *mdx* mice, it does ameliorate the severe *dystrophin:utrophin* double mutant phenotype (L. Judge and J.S.C., unpublished). The rescue of the fly heart function by *Dp116* may thus be caused by the assembling and the restoration of higher levels of the DGC and increased signaling functions of the DGC, as recently shown in skeletal muscles of *dystrophin:utrophin* double knockout mice (L. Judge and J.S.C., unpublished). Notably, both flies and humans naturally have a short C-terminal *dys* isoform expressed in the heart (*Dp71* in mammals and *Dp117* in flies; Muntoni *et al.*, 2003; present data). Thus, it may be that *Dp116* has a positive effect on muscle functionality in the heart but not in skeletal muscle. Alternatively, the fly system may be simpler and thus be able to use this truncated form to ameliorate (heart) muscle function, which in mammals may only become evident if both *dys* and *utrophin* are compromised. In any case, our data indicate that *Drosophila* can be used as a genetic tool to probe for suppressors or enhancers of (dilated) ‘cardiomyopathy’, thus allowing us to elucidate the mechanism of *Dys*-dependent disease.

## Materials and methods

### *Drosophila* strains

Exelixis stock *dysExel6184* was obtained from the *Drosophila* Stock Center (Bloomington, IN, USA; <http://flystocks.bio.indiana.edu>). *Df(3R)Dl-X43* referred to as *dyskx43* and the *dys*<sup>8-2</sup> deletion mutant flies were a generous gift from Hannele Ruohola-Baker (University of Washington, Seattle, WA, USA) (Shcherbata *et al.*, 2007). *dys RNAi* transgenic *Drosophila* against all isoforms was generously provided by Uri Nudel (Weizmann Institute of Science, Rehovot, Israel; Shcherbata *et al.*, 2007). The heart-specific driver *GMH5-Gal4* is a 900 bp heart enhancer fragment 73 from the *tinman* gene (Bodmer, 1993; Venkatesh *et al.*, 2000) that was cloned into the P{*GaWB*} vector upstream of the *Gal4* sequences. This driver was enhanced with multiple copies of a *UAS-Gal4* element allowing stronger myocardial expression when activated in late embryonic or during adult stages (detailed description in Wessells *et al.*, 2004). *24B-GAL4*, also known as P{*GawB*}*how*[24B], which drives expression in all muscles was described previously in Brand & Perrimon (1993) (see also Zaffran *et al.*, 1997 and full description on the flybase Web site <http://flystocks.bio.indiana.edu/Browse/misc-browse/gal4.htm>).

### Micro-Dys *Dp116* transgenic flies

The mouse short C-terminal isoform *Dys Dp116* (containing the first exon from the human *Dp116* isoform) was generated by Judge *et al.* (2006). *Dp116* contains two spectrin repeats, the *ww* domain (dystroglycan-binding domain) and a carboxy-terminal domain involved in binding to other DGC proteins like syntrophin and dystrobrevin (for details of the construct, see Judge *et al.*, 2006). For the generation of transgenic *Dys* flies, *Dp116* cDNA construct was subcloned into the *Gal4*-inducible vector *pUAST* at the *NotI* site. The *UAS-Dp116* construct was injected into *w*<sup>1118</sup> embryos, and transgenic lines (two) were established (according to standard procedures). *UAS-Dp116* transgenic flies were crossed to the heterozygous deficient



flies *dyskx43/+* to generate a stock Dp116/cyo, *dyskx43/TM3*. A stock of 24B-Gal4, *dysExel6184/TM3* was generated by recombining the driver 24B-Gal4 and the deficiency line *dysExel6184/TM3* in the same line. Thus, the flies Dp116/cyo, *dyskx43/TM3* were crossed to 24B-Gal4, *dysExel6184/TM3* and Dp116/+; *dyskx43/24BGal4-dysExel6184* were generated and tested for heart function.

### Immunostaining of embryos and adult *Drosophila* hearts

The embryo staining was performed as described previously (Kosman *et al.*, 2004). The following primary antibodies were used: rabbit anti-Dys antibody was a gift from Andreas Wodarz (University of Göttingen, Göttingen, Germany) used at 1/1000 (see Schneider *et al.*, 2006), mouse anti  $\beta$ -galactosidase 1/500 (Sigma, St Louis, MO, USA), mouse anti  $\alpha$ -actinin (a gift from J. Saide; Saide *et al.*, 1989) used at 1/40, rabbit anti-Dmef2 at 1/100 (Lilly *et al.*, 1995) and mouse anti-Dlg 1/100 (Hybridoma Bank, University of Iowa, Iowa City, IA, USA). The secondary antibodies donkey anti-rabbit and anti-mouse conjugated with Alexa Fluor 555 dye and Alexa Fluor 488 dye used at 1/500 (Molecular Probes, Eugene, OR, USA), respectively. Adult flies were dissected to expose the heart, fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) for 20 min, washed three times in PBT (PBS/0.1% Triton X-100). The preparations were incubated with primary antibodies in PBT/Western blocking reagent 1 $\times$  [Roche (Roche Applied Science, Indianapolis, IN, USA) #1921673] for 2 h, washed three times 10' and then incubated with secondary antibodies for 1 h at room temperature. After washing in PBT/Western blocking reagent 1 $\times$  three times 10', they were mounted onto slides in DABCO/Tris/glycerol [2.5% DABCO (Sigma #D-2522)/50 mM Tris pH 8.0/90% glycerol] and analyzed using a single-photon laser scanning microscope. The quantification of the areas devoid from myofibrils was performed by measuring the dark areas not stained with  $\alpha$ -actinin with mage J software. The average and the standard error was calculated by Microsoft Excel software.

### Reverse transcription reaction and qRT-PCR analysis

Total RNA was extracted from whole flies or isolated hearts using Trizol reagent (Promega, Madison, WI, USA). The samples were treated with DNAase (Invitrogen, Carlsbad, CA, USA) to eliminate any remaining DNA. First-strand cDNA synthesis was performed using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen #18080-051). Polymerase chain reaction was performed with *actin* or ribosomal protein RP49 as controls, and *dys*-specific primers for Dlps, Dp186, Dp205 and Dp117. The oligonucleotides used were as follows: *actin* forward ATCCGCAAGGATCTGTATGC, *actin* reverse ACATCTGCTGGAAGGTGGAC; Rp49 forward GACGCTTCAAGGGACAGTATCTG, Rp49 reverse AAACGCGGTTCTGCATGAG; 281 bp Dlps product forward AGCGTTGGCCGCGCAGCTTCG, Dlps reverse CCGCTGAACAGTGCAGGTGCT; 294 bp product of Dp186 forward AGGAACTTCCGGTTCGCAGC, Dp186 reverse AGCTGGGTTTTCGTCGTGCGA; 263 bp Dp205 product forward CAAGTGCTCGGAGGCCCTGC, Dp205 reverse ACAGCCGCATGCGATCGTGC; 263 bp product for Dp117 forward TCGCTGAAGCGTGCAGTTCG, Dp117 reverse TGCGAGTAACTCAGGCTCAG. To determine the extent of RNAi knockdown of all *dys* transcripts, we used primers in the common sequence for all isoforms and outside the dsRNA sequence: forward primer CAAGTGGCCTAGTGACCGTAA; reverse primer CGTCGTCGTCGTGGTGTTCGT corresponding to a 104 bp fragment. For qPCR, we used the LightCycler FastStart DNA Master<sup>PLUS</sup> SYBR Green I kit (Roche Applied Science).

### Lifespan analysis

Procedures for lifespan studies were as described in Wessells *et al.* (2004). Flies were kept at 25 °C transferred to fresh food on alternate days and scored the deaths after each transfer.

## Heart physiological analysis

Anesthetized flies with fly nap (Carolina Biol., Corp., Burlington, NC, USA) for 2 min were aligned dorsal down on a dish and cutting off the head and the ventral parts of the body (thorax + abdomen). These semi-intact preparations of flies were cleaned from fat and other tissues except the heart, and kept for 20 min equilibration before taking movies. Movies were taken at rates 100–130 frames per second by using Simple PCI software (Compix, Sewickley, PA, USA). Cardiac parameter measurements were obtained as output from the MatLab-based program (Mathworks, Natick, MA, USA). For further description, see Ocorr *et al.* (2007).

External electrical pacing of the fly's heart rate was carried out as previously described (Wessells & Bodmer, 2004; Wessells *et al.*, 2004).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank Martina Schneider and Hannele Ruohola-Baker for generously providing *dys* RNAi transgenic, *dys*<sup>8-2</sup> and *dyskx43* flies. We are grateful to Judith Saide for sending anti  $\alpha$ -actinin antibodies, and to Andrea Wodarz for sending anti-Dys antibodies. We thank Katherine Kling for helping with the preparation of the manuscript, Anthony R. Cammarato for the myofibril quantification and Laurent Perrin for critically reading the manuscript. This research was supported by grants from NIA and NHLBI of the National Institutes of Health. Uri Nudel and David Yaffe receive support from AFM, MDA and the Israel Science Foundation.

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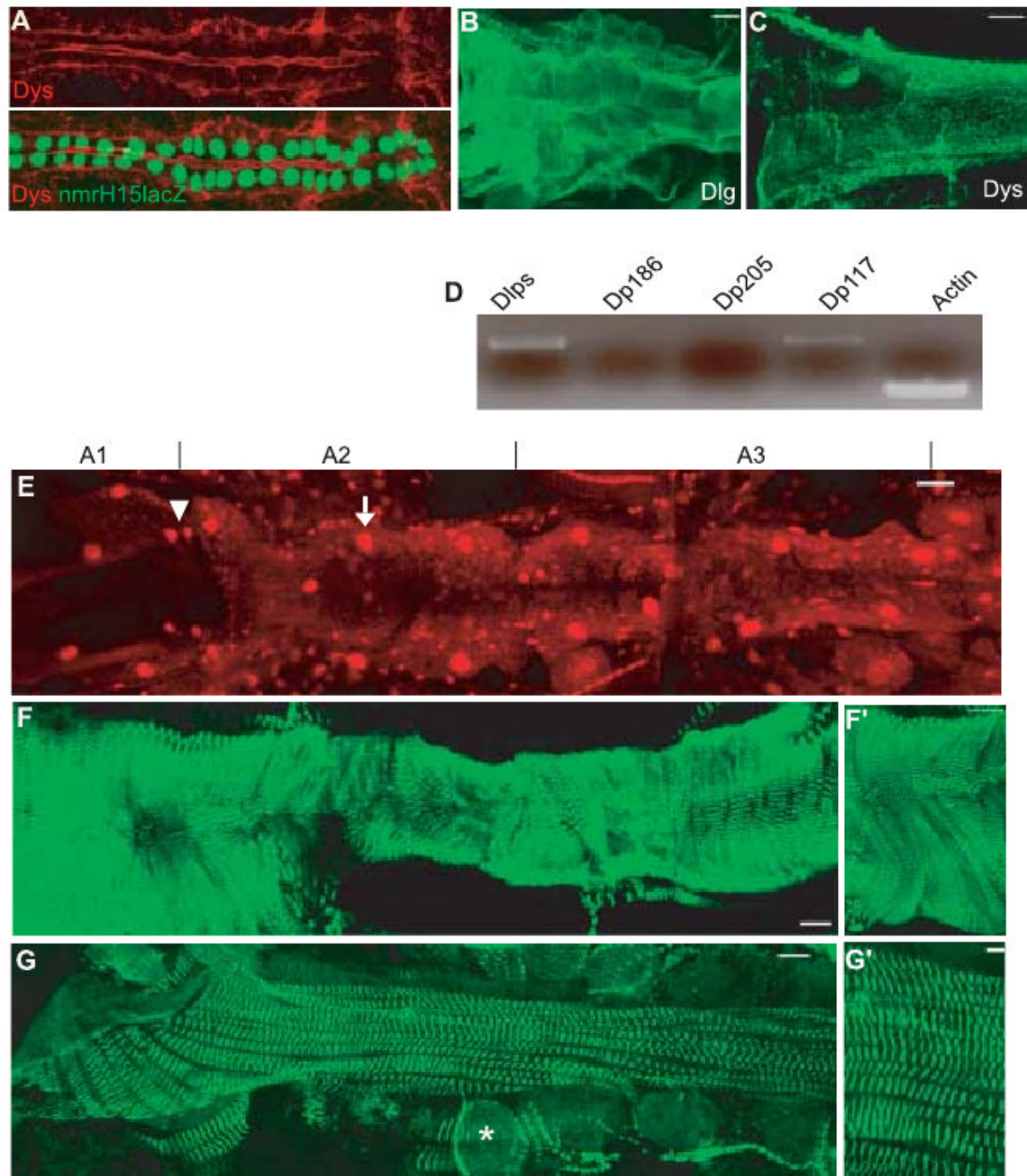
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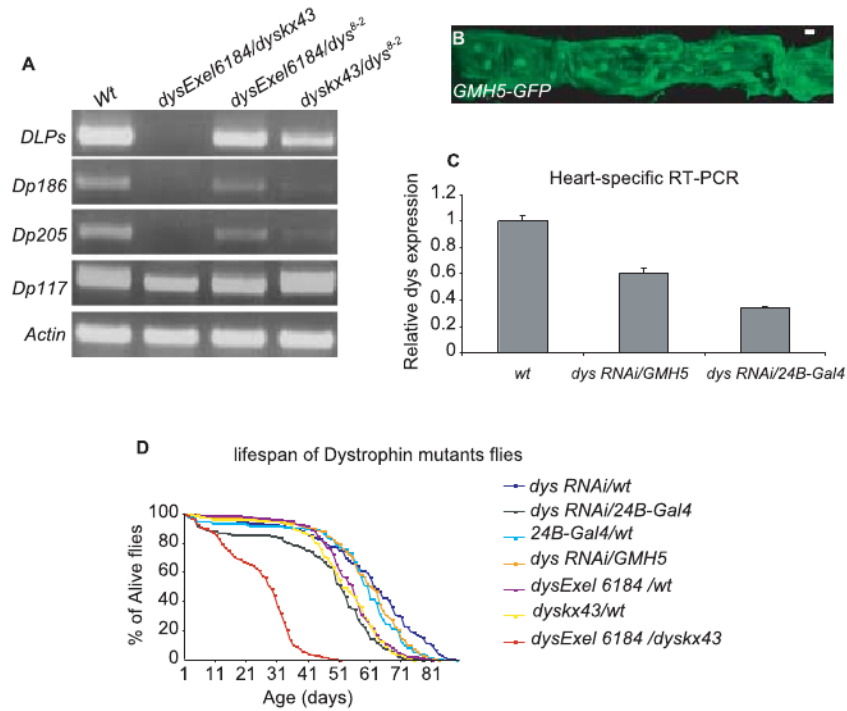
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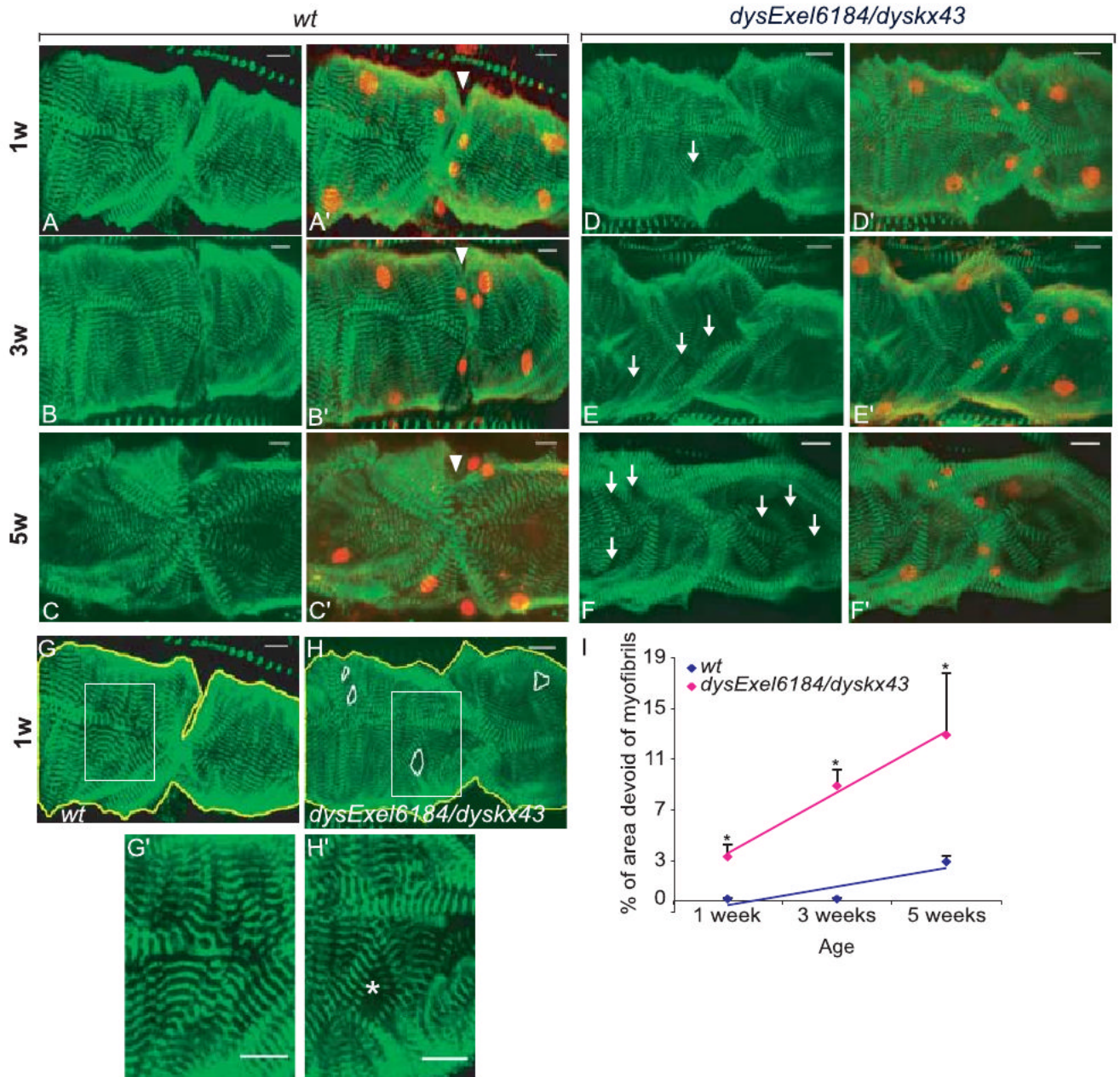
**Fig. 1.** Protein distribution in *Drosophila* embryo and adult heart. (A) Stage 16 *wild-type* (*wt*) embryo double-labeled for Dystrophin (Dys, red) and nuclear *nmr<sup>H15</sup>-lacZ* (green; see Qian *et al.*, 2005) Dystrophin (Dys) shows dorsal and ventral depositions in the myocardial cells (green nuclei) (B, C, E–G) Adult cardiac tube stained for disc large, (B) Dys, (C) protein outlining the myocardial cell membranes (bar = 25  $\mu$ m). Note that Dys is localized at the Z-lines in the longitudinal muscles on the side of the myocardial cells. (D) Reverse transcriptase–polymerase chain reaction (RT–PCR) of *wt* adult heart using specific primers for Dys-like products (DLPs), Dp186, Dp205 and Dp117 compared to control actin (30 PCR cycles). Note that only DLPs and Dp117 are expressed in the adult heart. (E) *Dmef2* expression in the adult cardiac tube

(A1–A3). Dmef2 is a muscle-specific transcription factor, expressed in all Tinman (arrow, big nuclei) and Seven-up (Svp) expressing myocytes (arrowhead, smaller nuclei and localizes to the ostia) (for further details, see Molina & Cripps, 2001). (F, G)  $\alpha$ -Actinin expression in the adult cardiac tube (abdominal segments A1–A3 are shown). (F) Sarcomeric Z-line marker  $\alpha$ -actinin shows a spiral or transverse organization of the myofibrils of the contractile myocardium. (F') High magnification of the myocardium shows the orientation of the myofibrils. (G)  $\alpha$ -Actinin reveals a second type of myofibrillar organization in longitudinal non-Tinman-expressing muscles associated with the ventral part of the heart (Molina & Cripps, 2001) (\* indicates pericardial cells). (G') High magnification. For E–G, the bar = 38  $\mu$ m; for F', bar = 106  $\mu$ m; and for G' bar = 100  $\mu$ m.



**Fig. 2.** Loss of *dys* function decreases the lifespan in flies. (A) Whole fly reverse transcriptase–polymerase chain reaction indicates the abolishment or reduction in *dys* isoform transcripts. *dysExel6184/dyskx43* flies abolish all but *Dp117* transcripts, whereas *dysExel6184/dys<sup>8-2</sup>* and *dys<sup>8-2</sup>/dyskx43* mutants have moderately reduced *DLPs* transcript levels. (B) Adult expression pattern of *GMH5-Gal4* driving with GFP expression specifically in the heart (see also Wessells *et al.*, 2004) (bar = 19  $\mu$ m). (C) Relative expression of *dystrophin* in cardiac tube of 1-week-old adults presented as ratio of *dys* to *rp49* mRNA. The bar graph shows that the knockdown of all *dys* isoforms in *dysRNAi/24B-Gal4* flies causes a reduction of ~60%, and ~40% with the heart-specific *dysRNAi/GMH5-Gal4* knockdown. Each value represents the average  $\pm$  standard error of the mean of four independent experiments. (D) Survival curves of *dys* mutant females. The knockdown of all *dys* isoforms in the mesoderm (*dysRNAi/24B-Gal4*) shows a moderate reduction in longevity similar to the heterozygous deficiencies *dysExel6184/+* and *dyskx43/+*. In contrast, the heart-specific *dys* knockdown does not have an effect on lifespan. Dystrophin-like-product-deficient *dysExel6184/dyskx43* mutants show dramatically shortened lifespan. It is possible that this transdeficiency deletes additional genes that are contributing to a normal lifespan.

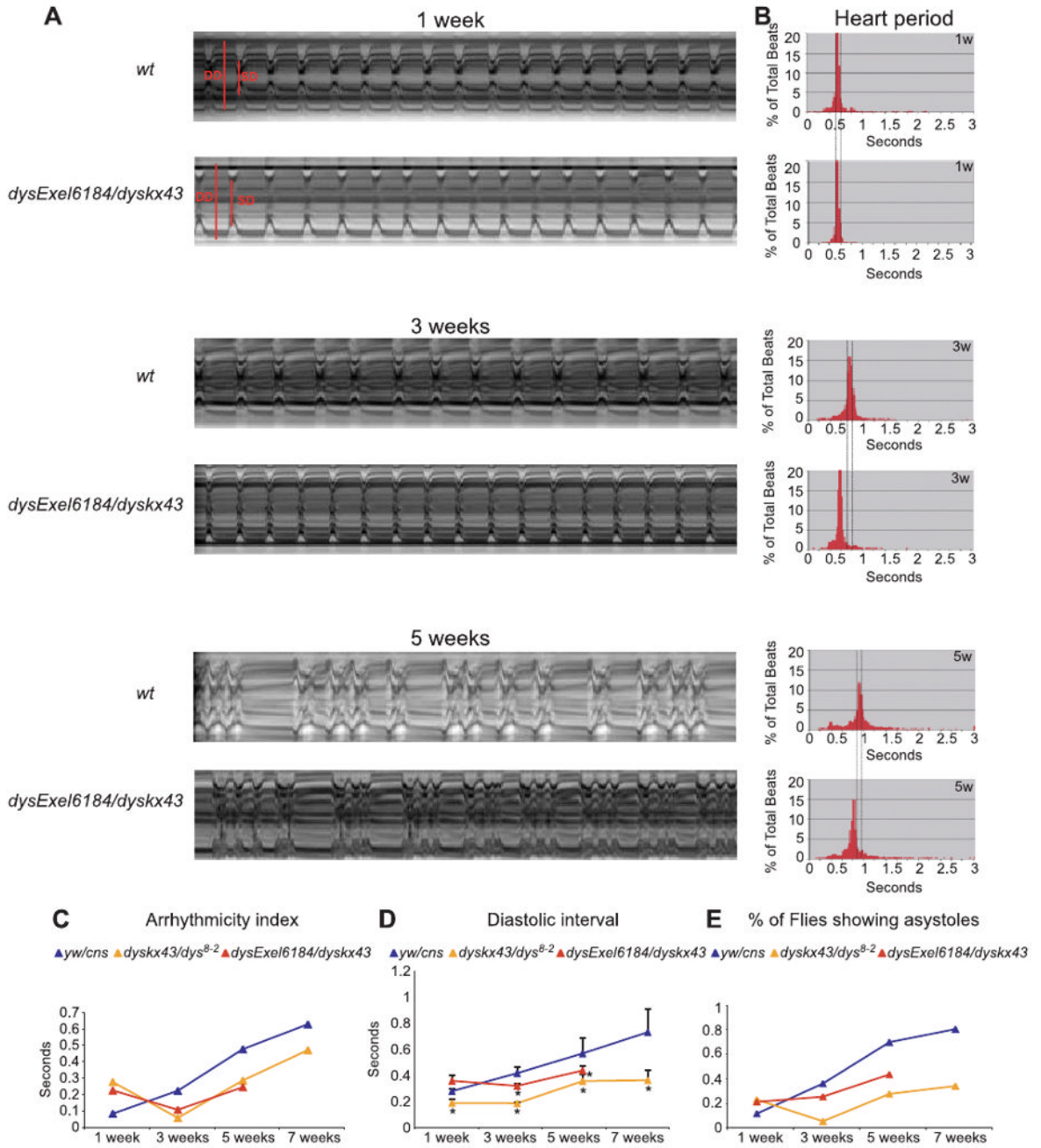




**Fig. 3.** *dysExel6184/dyskx43* Mutant flies exhibit age-dependent abnormalities in myofibrillar organization. Representative confocal stacks of adult hearts (posterior A2-anterior A3 segment) stained with  $\alpha$ -actinin (green) and anti-Dmef2 (red, right panels) antibodies revealing detail of heart structure. (A-C') *Wild-type* (*wt*) heart structure shows myofibrillar organization in a spiral fashion shown at progressively older different age (A, A' 1-week-old: 1w; B, B' 3-week-old: 3w; C, C' 5-week-old hearts: 5w). The *wt* hearts at 5w reveal some disruptions of the myocardial myofibrils (C, C'). Ostia at the segmental boundaries of the myocardium were identified as an opening in the heart wall, associated with two pairs of smaller Dmef2-positive nuclei (A'-F', arrowhead). (D-F') The *dysExel6184/dyskx43* mutant heart structure at 1w (D,

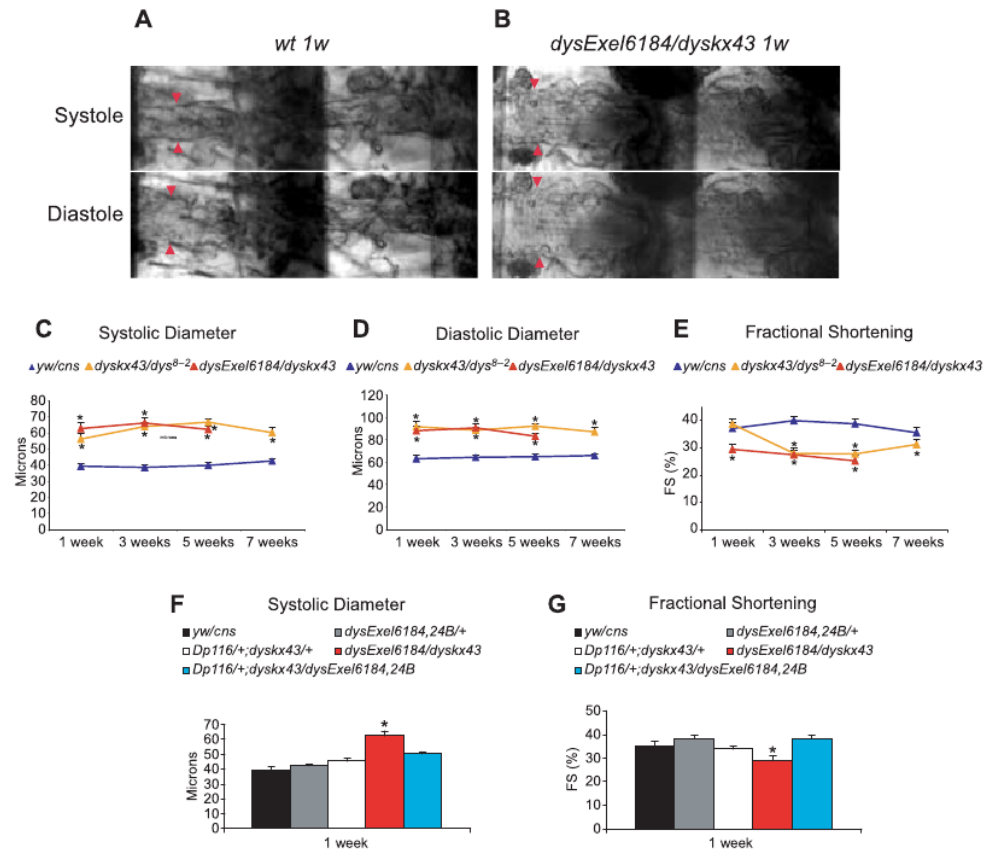
D'), 3w (E, E') and 5w (F, F'), respectively, shows age-dependent disruption of the heart myofibril integrity (D–F arrow pointing out to more spacing between myofibrils). (G) *Wild-type* and (H) *dysExel6184/dyskx43* mutant hearts showing outlined areas devoid of myofibrils in the confocal stacks (H, white circled area). Areas without myofibrils were measured and normalized to the total area of heart examined (outlined in yellow) using Image J software. See G' and H' for a close-up of the myofibril arrangement and that in H' the asterisk points to a gap between myofibrils (I) Plot of quantification by age of areas devoid of myofibrils (dark areas not stained with  $\alpha$ -actinin). Note that *dysExel6184/dyskx43* mutant hearts show much more disorganization by this measure than *wt*. Each data point was from six to eight hearts.  $P < 0.005$  at 1 week;  $P < 0.0001$  at 3 weeks;  $P < 0.01$  at 5 weeks between *wt* and *dysExel6184/dyskx43* mutants. Scale bar = 12  $\mu\text{m}$ .





**Fig. 4.** Reduced level or loss of the long dystrophin (Dys)-like product isoforms of Dys results in faster heart rate by shortening the diastolic intervals. (A) Representative M-mode traces (10 s) from high-speed movies of semi-intact flies. *Wild-type* (*wt*) flies show rhythmic heart beating at 1-week-old to 3-week-old of age, but moderate arrhythmicity at 5 weeks. *dysExel6184/dyskx43* hearts show increased heart rate in 3-week-old and 5-week-old flies. (B) Heart period histograms obtained from 1 min movies plotted as individual data points illustrating the variability of the heart period within a group of flies for controls and *dysExel6184/dyskx43* mutants. At 1 week, the mean heart period for *wt* and *dysExel6184/dyskx43* flies is about 0.5 s, which increases in *wt* to 1 s at 5 weeks. In contrast, aging *dysExel6184/dyskx43* shows less

of an increase, to only 0.8 s at 5 weeks (bottom panel in B). (C) Standard deviation of the heart period was used as a measure of irregularity in heart rhythm ('arrhythmicity index'). All time points (except 1 week) show less arrhythmicity for *dys* mutants compared to *wt* flies. Differences were estimated by *t*-test  $P < 0.05$  and are indicated by \*. (D) Mean diastolic interval ( $\pm$  SEM) for *wt* and *dys* mutant flies obtained from 1 min high-speed movies at the indicated ages. Significant differences were determined by two-tailed independent samples *t*-test.  $P$  values  $< 0.05$  were considered significant ( $*P < 0.01$ ). (E) Percentage of total flies showing asystoles (prolonged diastolic phases of more than 1 s) in 1-min movie clips. Differences were estimated by *t*-test  $P < 0.05$  and are indicated by \*.



**Fig. 5.** Reduced or no dystrophin-like products (DLPs) causes dilated cardiomyopathy in the adult *Drosophila* heart. (A) Two-segment image of a 1-week-old *wild-type* (*wt*) heart in systole (top) and diastole (bottom). (B) 1-week-old *dysExel6184/dyskx43* heart in systole and diastole. Note that both diastolic and systolic diameters are wider in the DLPs-deficient mutants compared to *wt*. Arrowheads indicate the heart wall in both phases of the cardiac cycle. (C–E) Heart parameters in *wt* and *dys* mutants. (C,D) *dysExel6184/dyskx43* and *dys<sup>8-2</sup>/dyskx43* mutants have a larger systolic (C) and diastolic diameters (D) than the *wt* at all ages. Significant differences were determined by *t*-test. *P* values < 0.05 were considered significant (*\*P* < 0.0001). (E) Plot of fractional shortening for the *wt* and *dys* mutants. *dysExel6184/dyskx43* shows lower fractional shortening at all ages, whereas *dys<sup>8-2</sup>/dyskx43* mutants only at 3 weeks or older (*\*P* < 0.001, except *dys<sup>8-2</sup>/dyskx43* at 7 weeks is *P* < 0.05). Movies were taken from 15 to 20 flies for each genotype. (F, G) Rescue of *dysExel6184/dyskx43* mutants by the mammalian short *dys* isoform *Dp116*. Mesodermal expression of *Dp116* restores the dilated systolic diameter (F) and the reduced fractional shortening to near normal (G). The *Dp116* transgene was driven by the mesodermal driver *24B-GAL4* in the *dys* mutant background (*Dp116/+; dyskx43/dysExel6184,24B*). Cardiac function evaluated in 1-week-old rescue *dys* mutants shows a normalization of the systolic dysfunction (F, G) (*\*P* < 0.01 by *t*-test assuming equal variances). For the genotype *Dp116/+; dyskx43/dysExel6184,24B* measurements ( $\pm$  standard error) were derived from movies of 20 flies. Similar rescue as with 1-week-old flies was observed in 3-week-old flies (data not shown).