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# Mechanical and non-mechanical functions of *Dystrophin* can prevent cardiac abnormalities in *Drosophila*

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## Abstract

*Dystrophin*-deficiency causes cardiomyopathies and shortens the life expectancy of Duchenne and Becker muscular dystrophy patients. Restoring *Dystrophin* expression in the heart by gene transfer is a promising avenue to explore as a therapy. Truncated *Dystrophin* gene constructs have been engineered and shown to alleviate dystrophic skeletal muscle disease, but their potential in preventing the development of cardiomyopathy is not fully understood. In the present study, we found that either the mechanical or the signaling functions of *Dystrophin* were able to reduce the dilated heart phenotype of *Dystrophin* mutants in a *Drosophila* model. Our data suggest that *Dystrophin* retains some function in fly cardiomyocytes in the absence of a predicted mechanical link to the cytoskeleton. Interestingly, cardiac-specific manipulation of nitric oxide synthase expression also modulates cardiac function, which can in part be reversed by loss of *Dystrophin* function, further implying a signaling role of *Dystrophin* in the heart. These findings suggest that the signaling functions of *Dystrophin* protein are able to ameliorate the dilated cardiomyopathy, and thus might help to improve heart muscle function in micro-*Dystrophin*-based gene therapy approaches.

### Keywords

Micro-*Dystrophins*; Heart period; Myofibrils; Nitric oxide synthase; Aging



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**Conflict of interest**
The authors declare that they have no conflict of interest.

## 1. Introduction

Dystrophinopathies are due to mutations in the *Dystrophin* (*Dys*) gene causing Duchenne and Becker muscular dystrophy (DMD and BMD, respectively) and X-linked dilated cardiomyopathy. In all these pathologies, the heart muscle is affected to different degrees, depending on the type of the mutation and the progression of the disease. Cardiac disease in both DMD and BMD manifests itself as a dilated cardiomyopathy (DCM) and/or cardiac arrhythmia (Corrado et al., 2002). Cardiomyopathy is present in about 90% of DMD/BMD patients, and progressively leads to heart failure, causing the death of 20% DMD and 50% BMD patients (Bushby et al., 2003).

Currently, there are no effective treatments of DCM besides transplantation and pharmacological intervention, such as with angiotensin-converting enzyme inhibitors and beta blockers, which only ameliorate heart symptoms, without correcting the underlying pathology (Kaspar et al., 2009). Inevitably, the DCM worsens as the patient becomes older and as the disease progresses. New therapy alternatives to manage the DCM are thus needed. Viral-based gene therapies, including the adeno-associated virus (AAV) system have recently drawn considerable attention for exploring the potential utility of a modified, but functionally active *Dys* gene in ameliorating the dystrophic skeletal and heart muscle phenotypes (Bostick et al., 2011; Gregorevic et al., 2006).

Two essential functions have been attributed to *Dys* protein. The first one is referred to as a mechanical role: *Dys* links to F-actin via its N-terminal and central actin-binding domains, and to Dystroglycan (Dg) via its WW and cysteine-rich (CR) domains, thus enabling force transduction from the inside to the outside of the cell, and stabilizing the sarcolemma. The second is a signaling role: *Dys* assembles signaling molecules, including neuronal nitric oxide synthase (nNos), growth factor receptor-bound protein 2 (Grb2), Calmodulin, and Calmodulin-dependent kinases (Anderson et al., 1996; Brenman et al., 1996).

Previous work in skeletal muscle of the mouse DMD model ("*mdx*") showed that expressing *Dys* isoforms preserving its mechanical function is beneficial, by improving *mdx* muscle function and preventing dystrophy (Gregorevic et al., 2006; Harper et al., 2002). Other studies suggest that restoring the *Dys*-glycoprotein complex (DGC) by expressing the Dp71 or the Dp116 isoforms in skeletal muscle of *mdx* mice, which lack the mechanical function of *Dys*, does not ameliorate the dystrophic phenotypes (Greenberg et al., 1994; Rafael et al., 1996). These data demonstrate that the mechanical role of *Dys* protein is the major contributor to the *mdx* dystrophic pathology. However, Dp116 expression in mouse mutants lacking both *Dys* and Utrophin (*mdx:utrn*$^{-/-}$) increases the muscle mass and improves growth, mobility and lifespan (Judge et al., 2011). Dp116 is a non-muscle isoform specifically expressed in Schwann cells of the peripheral nervous system that binds to the DGC components Dg, syntrophin (Syn) and dystrobrevin (Dbr), but does not provide a link to F-actin (it lacks the actin-binding domains). Thus Dp116 allows the assessment of the functional characteristics of the *Dys* 'signaling domain' in the absence of the 'mechanical domain' function.

While many studies have focused on structural–functional analysis of *Dys* in skeletal muscle to develop gene therapy [reviewed by (Blankinship et al., 2006)], little effort has so far been directed to correcting the heart pathology (Bostick et al., 2009, 2011; Hainsey et al., 2003; Townsend et al., 2007; Yue et al., 2003). To differentiate between the 'mechanical' and 'signaling' roles of *Dys* in cardiac muscle function, we generated flies that express either the *Dys* constructs ΔH2-R19/ΔCT and ΔR4-23/ΔCT, which can bind the F-actin, but lack the C-terminal domain that interacts with Syn and Dbr (predicted *Dys* with 'mechanical function') or the Dp116 with the C-terminal domains only, thus lacking the F-actin-binding domains (predicted *Dys* with 'signaling function'). We note that probably none of these *Dys* constructs are involved in nNos signaling, since they lack repeat 16 and 17 of the rod domain implicated in the interaction between nNos and *Dys* (Lai et al., 2009). We provide evidence that both the predicted 'mechanical' (ΔH2-R19/ΔCT, ΔR4-23/ΔCT) and 'signaling' (Dp116) functions of *Dys* are able to ameliorate dilated cardiomyopathy and improve myofibrillar organization. Manipulating *Dp116* and *Nos* in $Dys^{-/-}$ mutants provides further evidence of a signaling role of *Dys* in modulating the heart function. We conclude that both 'mechanical' and 'signaling' roles of *Dys* are important for cardiac muscle function.

## 2. Materials and methods

### 2.1. Drosophila strains

The flies $Dys^{-/-}$ are transheterozygous for *DysExel6184* and *Df(3R) Dl-X43* (Taghli-Lamallem et al., 2008). The *DysEP(3)3397* and the Dystroglycan mutants were a generous gift from R. Ray. GAL4 drivers were: *24B-GAL4* (Brand and Perrimon, 1993) and *Hand-Gal4* (from A. Paululat laboratory) kindly offered by L. Perrin. *UAS-Nos*$^{RNAi}$ from VDRC (transformant ID 27725) and the *UAS-Nos* flies were generously offered by S.A. Davies and P.H. O'Farrell.

### 2.2. Dystrophin transgenic flies

The murine *Dys* cDNAs Δ*H2-R19ΔCT*, Δ*R4-23ΔCT* and *Dp116* have already been described (Harper et al., 2002; Judge et al., 2006). The ΔH2-R19ΔCT and ΔR4-23ΔCT lack a portion of the rod domains (spectrin repeats) and the C-terminal region of *Dys*. Dp116 is a short C-terminal isoform, containing two and a half spectrin repeats, the WW domain, the cysteine-rich domain, and the carboxy-terminal domain involved in binding to other DGC proteins like Syn and Dbr (for details of the construct, see Judge et al., 2006). For the generation of transgenic flies, Δ*H2-R19ΔCT*; Δ*R4-23ΔCT*; and *Dp116* cDNA constructs were sub-cloned into the Gal4-inducible vector pUAST at the NotI site, injected into $w^{1118}$ embryos, and transgenic lines established. The transgenic flies were crossed to the heterozygous deficient flies *Df(3R) Dl-X43* to generate a stock *UAS-*Δ*H2-R19ΔCT* (or *UAS-*Δ*R4-23ΔCT* or *Dp116*)/cyo, *Df(3R)Dl-X43/TM3*. These lines were crossed to *Hand-Gal4*, *DysExel6184/TM3* flies to generate the transgenic rescue flies with the truncated *Dys*.

### 2.3. Immunostaining of adult Drosophila hearts

Staining of adult flies was performed as described previously (Taghli-Lamallem et al., 2008). Primary antibodies: rabbit anti-Dystroglycan diluted at 1/1000 (gift from A. Wodarz);

phalloidin-cy3 diluted at 1/1000, and two rabbit NOS antibodies diluted at 1/400 (gift from P.H. O'Farrell) and 1/100 (Thermo-Scientific, PA1-039). Secondary antibodies: donkey anti-rabbit conjugated to CY3 (Jackson ImmunoResearch) at 1/300. Immunostained preparations were visualized on Olympus FV300 or Zeiss LSM 510 laser scanning confocal microscopes.

### 2.4. Heart physiological analysis

Flies anesthetized with fly nap (Carolina Biol., Corp.) were aligned on a dish (dorsal down) and dissected to expose the heart for optical recording by previously described protocols (Fink et al., 2009; Ocorr et al., 2007a). Beating heart images were acquired at rate of about 130 frames per second using Simple PCI software (Compix, Sewickley, PA). Cardiac parameter measurements were quantified and generated using the MatLab-based image analysis program (Fink et al., 2009). M-modes illustrate movements of the heart tube edges in the y-axis over time in the x-axis, generated by excising and aligning a single pixel-wide image from successive frames. Heart periods (HPs) are defined as the time between the ends of two consecutive diastolic intervals. Single HPs were plotted in the histograms to see overall distribution and clustering of HPs. We used Prism software, one-way ANOVA analysis and a Tukey test to process statistics on 20 flies for each genotype.

## 3. Results

### 3.1. Dystrophin proteins carrying either the 'mechanical' or 'signaling' domains ameliorate Dystrophin-deficient heart dysfunction

The large size of the *Dys* transcript (14 Kb) presents a major challenge for successful gene transfer with viral vectors. This limitation has led to the construction of *mini*- and *micro-Dys* genes (Scott et al., 2002). Among these are the micro-*Dys* constructs ΔH2-R19/ΔCT and ΔR4-23/ΔCT, both maintaining the N-terminal, some of the rod, and cysteine-rich domains, but lacking the C-terminal domain that directly binds to Dbr and Syn, also components of the DGC. These truncated proteins are therefore expected to retain the capacity of force transduction in the sarcolemma, but they may have some 'signaling function' (Fig. 1A) (Scott et al., 2002). By contrast, Dp116 *Dys* contains intact CR and carboxy-terminal (CT) domains, but it lacks the N-terminal and most of the rod domains, preventing its link with the actin cytoskeleton and the mechanical reinforcement at the sarcolemma (Fig. 1A) (Judge et al., 2006).

We had previously found that the heart of $Dys^{-/-}$ mutant flies *(Df(3R)Dl-X43*/*DysExel6184*) was dilated and exhibited abnormal heart performance and contractility, reminiscent of dilated cardiomyopathy in mammals (Taghli-Lamallem et al., 2008). To probe potential differences between *Dys* mechanical and signaling roles, we generated transgenic flies that express *Dys* constructs Δ*H2-R19/*Δ*C*T, Δ*R4-23/*Δ*C*T, or *Dp116* in all muscles (24B-Gal4 driver) or specifically in the heart (Hand-Gal4 driver). Our choice of constructs was made to differentiate between portions of *Dys* that may or may not confer the mechanical reinforcement at the cell membrane, based on mammal studies in skeletal muscles. First, we tested the heart- and muscle-specific effects of these micro-*Dys* constructs in a wildtype background, and found that their overexpression in mesodermal tissues does not induce

cardiac abnormalities (Fig. S1). We then expressed Δ*H2-R19/*Δ*C*T, Δ*R4-23/*Δ*C*T, or *Dp116* in all muscles or heart alone, to attempt to restore heart function in $Dys^{-/-}$ flies. Cardiac physiology was assessed by analyzing high-speed optical recordings of beating hearts in young and old flies (1 and 5 weeks old) (Fink et al., 2009). Expression of the truncated *Dys* constructs in $Dys^{-/-}$ caused a significant decrease in systolic diameters, and a corresponding increase in fractional shortening (Fig. 1B, C). This suggests a robust rescue of systolic function and heart contractility by both 'mechanical' and 'signaling' *Dys* constructs. We note that the pan-mesodermal driver 24B-GAL4 showed a stronger effect than the Hand-GAL4. This difference could arise because the 24B-GAL4 expression in the ventral muscle layer containing the longitudinal myofibrils (skeletal muscle-like associated with cardiomyocytes) could influence the heart function. M-mode traces of 5-week-old $Dys^{-/-}$ and rescue hearts illustrate the dynamics of heart wall movements (Fig. 1D).

$Dys^{-/-}$ mutant hearts exhibit progressively uncompact and disorganized transverse myofibrils with age (Fig. 1E) (Taghli-Lamallem et al., 2008). Examining whether expression of *Dys* constructs can restore the cytoarchitecture of $Dys^{-/-}$ cardiomyocytes, we found that compared with $Dys^{-/-}$ mutant alone, hearts expressing the *Dys* constructs displayed a more compact myofibrillar arrangement as revealed with actin–phalloidin staining (Fig. 1E). Importantly, we observed that in some rescued cardiomyocytes the array of the sarcomeric units was oriented longitudinally (Fig. 1E, arrows indicate orientation of nearby myofibrils), indicating that micro-*Dys* and Dp116 proteins do not have full capacity to restore normal myofibril orientation. Together, these results suggest that like the micro-*Dys*, Dp116 is able to restore myofibrillar integrity and contractility.

### 3.2. Dystroglycan does not play a critical role in Drosophila heart function

It is unclear whether in heart tissue the integrity of the Dg–*Dys* interaction is critical for heart function. This prompted us to determine Dg localization in *Dys*-deficient hearts, in $Dys^{-/-}$ hearts expressing the truncated *Dys* constructs, and whether Dg null mutants showed heart phenotypes similar to $Dys^{-/-}$. Similar to *Dys*, Dg protein delineates the plasma membranes of the cardiomyocytes (Fig. 2A, B). $Dys^{-/-}$ mutant hearts show a moderately reduced amount of Dg protein (Fig. 2C). Expression of *Dys* constructs in $Dys^{-/-}$ mutant hearts does not seem to appreciably restore Dg protein (Fig. 2D; Fig. S2), consistent with the idea that rescue of $Dys^{-/-}$ mutant hearts by micro-*Dys* constructs is not achieved by increasing sarcolemma Dg localization.

To address this further, we characterized the fly heart's contractile properties of *Dg* null mutants ($Dg^{055}/Dg^{086}$: $Dg^{-/-}$) (Christoforou et al., 2008). We found that $Dg^{-/-}$ flies and double heterozygotes for *Dys* and *Dg* did not exhibit a dilated heart phenotype nor reduced fractional shortening (Fig. 2E–G), thus preserving systolic function compared with *Dys* mutants ($Dys^{-/-}$ and *DysEP3397*/*DysExel6184*). Conversely, *Dg* overexpression in cardiomyocytes caused an increase in systolic and diastolic diameters without significantly affecting the fractional shortening (Fig. 2E–G), suggesting that cardiac contractility was not compromised. Taken together, these results suggest that *Dg* is not required for *Drosophila* adult heart function.

### 3.3. Dp116 Dystrophin increases the heart period in Dystrophin mutant flies

We showed previously that $Dys^{-/-}$ mutants display an increase in heart rate with age (Taghli-Lamallem et al., 2008). To investigate the effects of truncated *Dys* constructs on heartbeat length, we characterized the cardiac wall dynamics by measuring the time intervals of systolic and diastolic phases at 1 and 5 weeks of age. The mean HPs of wildtype and of $Dys^{-/-}$ both increased with age, but less so in $Dys^{-/-}$ flies (Fig. 3A). Cardiac-driven *Dp116* expression in $Dys^{-/-}$ mutants, however, resulted in significantly prolonged HPs compared with $Dys^{-/-}$ flies at both ages (Fig. 3A), mainly due to increased diastolic intervals (Fig. 3B, C). The HP distribution is represented in histograms, showing a tight clustering at a young age, which expands because of increased age-dependent arrhythmias, but does not significantly change with genotype (Fig. 3D). This suggests that the C-terminal domain of *Dys* likely plays a role in regulating heart rate, in addition to contractility.

### 3.4. Nitric oxide synthase (Nos) maintains youthful heart function of $Dys^{-/-}$ flies during aging

The findings with the micro-*Dys* genes Δ*H2-R19/*Δ*CT*, Δ*R4-23/*Δ*CT* and the *Dp116* suggest that the mechanical function of *Dys* is important to normalize heart diameters and systolic function, whereas the signaling function of *Dys*, in addition to restoring the diameters and fractional shortening, modulates the heart rate when expressed in $Dys^{-/-}$ mutant background. To further investigate the signaling function of *Dys* in heart performance, we decided to investigate the role of nitric oxide synthase (Nos) in regulating heart function in wildtype and $Dys^{-/-}$ mutant backgrounds. Nos produces nitric oxide (NO), a signaling molecule known to modulate cardiac function (Balligand et al., 1993). In mammals, the NO role in cardiac function is more complex, since there are three *Nos* genes with autocrine and paracrine effects (Barouch et al., 2002; Vila-Petroff et al., 1999).

To study the relationship between *Dys* and Nos signaling function in heart performance and with aging, we have determined the dynamic heart properties of single and combined mutations of *Nos* and *Dys*. First, we used RNAi knockdown of transcripts for the single *Drosophila Nos* gene ($Nos^{RNAi}$) in cardiomyocytes. We found an increase in the HPs at 1 and 5 weeks of age (Fig. 4A), primarily due to prolonged diastolic intervals in young and old flies and to systolic intervals in aged individuals (Fig. 4B, C). Next, we overexpressed wildtype *Nos* cDNA and found a decrease in HPs, compared with controls (Fig. 4A), mainly due to decreased diastolic intervals (Fig. 4B, C). Taken together, these data suggest that *Nos* modulates the *Drosophila* heart rate. To further determine *Nos* effect in $Dys^{-/-}$ mutants, we made genetic combinations of both genes, i.e. modulating Nos function in *Dys* deficiency background ($Dys^{-/-}$; *Hand*-Gal4 > $Nos^{RNAi}$ or $Dys^{-/-}$; *Hand*-Gal4 > *Nos*). We found that either homozygous or heterozygous *Dys* deficiency reversed the HP increase due to cardiac *Nos* knockdown in young and old flies, by shortening both diastolic and systolic intervals (Fig. 4A–C). However, the double mutant $Dys^{-/-}$; *Hand-Gal4* > $Nos^{RNAi}$ does not survive to 5 weeks of age, indicating that *Nos* expression is important for long-term survival of $Dys^{-/-}$ flies. Interestingly, the shorter heart periods observed with *Nos* overexpression tended to be further reduced in a *Dys* deficiency background ($Dys^{-/-}$; *Hand-Gal4* > *Nos*), due to decreased diastolic and systolic intervals (Fig. 4A–C). *Nos* overexpression is beneficial for the $Dys^{-/-}$ mutant, so that the heart performance of old $Dys^{-/-}$ flies was similar to that of

young flies. The variability in the heart periodicity is quantified using the HP standard deviation as an arrhythmia index (AI) (Supplemental Fig. S3A). The AI for *Dys*$^{-/-}$ was significantly reduced at an older age due to overexpression of *Nos* (Supplemental Fig. S3A). In addition, in old flies *Nos* knockdown worsened the heart systolic diameters of *Dys*$^{+/-}$, and *Nos* gain of function normalized the fractional shortening of 5 week old *Dys*$^{-/-}$ (Fig. S3B), implying that gain of Nos function is cardioprotective upon loss-of-*Dys* function. However, further studies are needed to determine the specific effects of Nos manipulation on cardiac contractility. Taken together, these results demonstrate that Nos is a key regulator of heartbeat, and acts in a cardiomyocyte-autonomous and age-dependent manner.

Using two different anti-Nos antibodies we could detect weak Nos staining in the adult heart cardiomyocytes and strong Nos expression in body wall muscles and neurons innervating cardiac cells (Fig. S3C–E). Also, upon overexpressing *Nos* we observed a striated sarcomeric pattern organized in a doublet of bands on either side of the Z-lines stained with α-actinin antibodies (Fig. 4D–D″), which may explain increased heart rate in *Hand-Gal4 > Nos* (Fig. 4A). These observations are evidence that Nos protein is associated with sarcomeres, and might thus regulate the contractility of cardiac muscle, improving heart performance in aged *Dys*$^{-/-}$ mutant flies.

## 4. Discussion

Cardiomyopathy is a major health threat to DMD and BMD patients. Focusing on the gene therapy strategy to redress cardiac pump failure is of great importance to alleviate the severity of this heart disease. In the present study, we evaluated truncated *Dys* genes for their potential to rescue the heart phenotypes in *Drosophila Dys*$^{-/-}$ mutants. We found that both micro-*Dys* constructs with a predicted mechanical role and Dp116-*Dys* with a predicted signaling role can markedly reverse morphological and functional features of dystrophic hearts. Specifically, we highlight the beneficial effect of Dp116 in rescuing the *Dys*$^{-/-}$ heart abnormalities even in the absence of a predicted actin cytoskeleton link (Fig. 4E).

The expression of micro-*Dys* in *mdx* mice results in cardiac histopathology correction and partial normalization of heart function (Bostick et al., 2009, 2011; Townsend et al., 2007), consistent with our data, which also demonstrate partially normalized heart contractility of young and aged dystrophic flies. Both micro-*Dys* proteins fail to correct the increased heart rate of old *Dys*$^{-/-}$ flies, again consistent with ΔH2-R19 also having no effect on tachycardia in *mdx* mice (Bostick et al., 2009). This suggests that micro-*Dys* functions in the fly heart mirror in many aspects their functional capacities in the *mdx* mouse. Interestingly, in *Drosophila*, the micro-*Dys* rescue of heart function also includes restoration of cardiomyocyte myofibril integrity. Whether a residual myofibrillar mis-orientation is a similar phenomenon to that of micro-*Dys* ΔR4-23/ΔCT generating ringed fibers in skeletal muscle of *mdx* mice (Banks et al., 2010) remains to be established.

The expression of Dp116 does not seem to ameliorate the dystrophic phenotypes of *mdx* mice or the extensive muscle degeneration in *mdx; utrn*$^{-/-}$ double knockout mice, but it improves their mobility and lifespan (Judge et al., 2006, 2011), suggesting that predicted non-mechanical functions of *Dys* are indeed important. Also, *Dys* isoform Dp71 (predicted

to have a non-mechanical function) is not sufficient to prevent the *mdx;utrn*$^{-/-}$ cardiomyopathy, probably because Dp71, but not Dp116, lacks the entire rod domain as well as a functional WW domain, and only weakly associates with the sarcolemma (Greenberg et al., 1994; Hainsey et al., 2003). We showed that the Dp116 ameliorates the *Dys*$^{-/-}$ flies' cardiomyopathy and is effective in restoring the cytoarchitectural myofibrils, also suggesting an important signaling role of *Dys*. It will be interesting to examine the rescue abilities further in the future with other assays, for example the atomic force microscopy-based analysis to measure the passive mechanical stiffness of the cardiomyocytes (Kaushik et al., 2011).

To further explore the non-mechanical role of *Dys*, we analyzed *Nos*, known to be involved in *Dys*-complex signaling and the progression of myopathy. In vertebrate heart muscle, *Nos* modulates excitation–contraction coupling and thus myocardial function (Ziolo et al., 2008), but its role in regulating heart rhythm remains controversial (Barouch et al., 2002; Khan et al., 2003; Sears et al., 2003). Unexpectedly, genetic disruption of all *Nos* genes [neuronal (*nNos*), inducible (*iNos*) and endothelial (*eNos*)] results in viable mice with left ventricular hypertrophy and diastolic dysfunction, consistent with previous studies indicating that No affects cardiac remodeling (Janssens et al., 2004; Shibata et al., 2010). We found that cardiac-specific RNAi knockdown of *Nos* resulted in lower heart rates, due to enlarged relaxation periods. Recent studies revealed that myocardial nNos promotes the $[Ca^{2+}]_i$ decay and relaxation by stimulating sarcoplasmic reticulum (SR) $Ca^{2+}$ reuptake (Tong et al., 2010; Zhang et al., 2008), possibly in close proximity to SR and Z-lines as our data suggest (Fig. 4D). One of the Nos targets could be the sarcoplasmic/endoplasmic reticulum $Ca^{2\pm}$ ATPase (SERCA). In intact arteries, NO induced post-translational modifications such as S-glutathiolation of SERCA, thus increasing SERCA activity and enabling $Ca^{2\pm}$ uptake by the SR (Adachi et al., 2004). Alternatively, *Nos* may also directly affect transcriptional activation of effector genes (Caceres et al., 2011). Human and mice ventricular myocardial sections show a similar SR-related pattern of nNos (Ramachandran et al., 2013; Xu et al., 1999). Interestingly, the slower heart rate of *Nos*$^{RNAi}$ flies was reversed in *Dys*$^{-/-}$ mutant background, so that *Dys*$^{-/-}$; *Hand > Nos*$^{RNAi}$ flies exhibited a significant decrease in both diastolic and systolic intervals. These results allow the hypothesis that *Dys* and Nos differentially regulate a common but still unknown target involved in $Ca^{2+}$ homeostasis and in modulating heart periods.

In conclusion, our findings reveal an age-related cardioprotective effect of truncated *Dys* proteins, including Dp116 lacking the actin cytoskeleton link, raising intriguing possibilities of *Dys* signaling functions that warrant further investigation in a less complex cardiac model such as *Drosophila*. Our study also supports the importance of micro-*Dys* based gene therapy approaches in improving cardiac performance in DMD patients.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| ***Dys*** | *Dystrophin* |
| **DMD** | Duchene muscular dystrophy |
| **BMD** | Becker muscular dystrophy |
| **XLDCM** | X-linked dilated cardiomyopathy |
| **DCM** | Dilated cardiomyopathy |
| **AAV** | Adeno-associated virus |
| **Dg** | Dystroglycan |
| **CR** | Cysteine-rich domain |
| **nNos** | Neuronal nitric oxide synthase |
| **Grb2** | Growth factor receptor-bound protein 2 |
| **DGC** | *Dystrophin*-glycoprotein complex |
| **Utrn** | Utrophin |
| **Syn** | Syntrophin |
| **Dbr** | Dystrobrevin |
| **Dg** | Dystroglycan |
| **HPs** | Heart periods |
| **CT** | Carboxy-terminal domain |
| **NO** | Nitric oxide |
| **AI** | Arrhythmia index |
| **iNos** | Inducible Nos |
| **eNos** | Endothelial Nos |
| **SR** | Sarcoplasmic reticulum |

## References

Adachi T, Weisbrod RM, Pimentel DR, Ying J, Sharov VS, Schoneich C, Cohen RA. S-glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. Nat Med. 2004; 10:1200–1207. [PubMed: 15489859]

Anderson JT, Rogers RP, Jarrett HW. Ca2+-calmodulin binds to the carboxyl-terminal domain of dystrophin. J Biol Chem. 1996; 271:6605–6610. [PubMed: 8636075]

Balligand JL, Kelly RA, Marsden PA, Smith TW, Michel T. Control of cardiac muscle cell function by an endogenous nitric oxide signaling system. Proc Natl Acad Sci U S A. 1993; 90:347–351. [PubMed: 7678347]

Banks GB, Judge LM, Allen JM, Chamberlain JS. The polyproline site in hinge 2 influences the functional capacity of truncated dystrophins. PLoS Genet. 2010; 6:e1000958. [PubMed: 20502633]

Barouch LA, Harrison RW, Skaf MW, Rosas GO, Cappola TP, Kobeissi ZA, Hobai IA, Lemmon CA, Burnett AL, O'Rourke B, Rodriguez ER, Huang PL, Lima JA, Berkowitz DE, Hare JM. Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. Nature. 2002; 416:337–339. [PubMed: 11907582]

Blankinship MJ, Gregorevic P, Chamberlain JS. Gene therapy strategies for Duchenne muscular dystrophy utilizing recombinant adeno-associated virus vectors. Mol Ther. 2006; 13:241–249. [PubMed: 16361117]

Bostick B, Yue Y, Long C, Marschalk N, Fine DM, Chen J, Duan D. Cardiac expression of a mini-dystrophin that normalizes skeletal muscle force only partially restores heart function in aged Mdx mice. Mol Ther. 2009; 17:253–261. [PubMed: 19066599]

Bostick B, Shin JH, Yue Y, Duan D. AAV-microdystrophin therapy improves cardiac performance in aged female mdx mice. Mol Ther. 2011; 19:1826–1832. [PubMed: 21811246]

Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development. 1993; 118:401–415. [PubMed: 8223268]

Brenman JE, Chao DS, Gee SH, McGee AW, Craven SE, Santillano DR, Wu Z, Huang F, Xia H, Peters MF, Froehner SC, Bredt DS. Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. Cell. 1996; 84:757–767. [PubMed: 8625413]

Bushby K, Muntoni F, Bourke JP. 107th ENMC international workshop: the management of cardiac involvement in muscular dystrophy and myotonic dystrophy. 7th-9th June 2002, Naarden, The Netherlands. Neuromuscul Disord. 2003; 13:166–172. [PubMed: 12565916]

Caceres L, Necakov AS, Schwartz C, Kimber S, Roberts IJ, Krause HM. Nitric oxide coordinates metabolism, growth, and development via the nuclear receptor E75. Genes Dev. 2011; 25:1476–1485. [PubMed: 21715559]

Christoforou CP, Greer CE, Challoner BR, Charizanos D, Ray RP. The detached locus encodes Drosophila Dystrophin, which acts with other components of the dystrophin associated protein complex to influence intercellular signalling in developing wing veins. Dev Biol. 2008; 313:519–532. [PubMed: 18093579]

Corrado G, Lissoni A, Beretta S, Terenghi L, Tadeo G, Foglia-Manzillo G, Tagliagambe LM, Spata M, Santarone M. Prognostic value of electrocardiograms, ventricular late potentials, ventricular arrhythmias, and left ventricular systolic dysfunction in patients with Duchenne muscular dystrophy. Am J Cardiol. 2002; 89:838–841. [PubMed: 11909570]

Fink M, Callol-Massot C, Chu A, Ruiz-Lozano P, Izpisua Belmonte JC, Giles W, Bodmer R, Ocorr K. A new method for detection and quantification of heartbeat parameters in Drosophila, zebrafish, and embryonic mouse hearts. Biotechniques. 2009; 46:101–113. [PubMed: 19317655]

Greenberg DS, Sunada Y, Campbell KP, Yaffe D, Nudel U. Exogenous Dp71 restores the levels of dystrophin associated proteins but does not alleviate muscle damage in mdx mice. Nat Genet. 1994; 8:340–344. [PubMed: 7894483]

Gregorevic P, Allen JM, Minami E, Blankinship MJ, Haraguchi M, Meuse L, Finn E, Adams ME, Froehner SC, Murry CE, Chamberlain JS. rAAV6-microdystrophin preserves muscle function and extends lifespan in severely dystrophic mice. Nat Med. 2006; 12:787–789. [PubMed: 16819550]

Hainsey TA, Senapati S, Kuhn DE, Rafael JA. Cardiomyopathic features associated with muscular dystrophy are independent of dystrophin absence in cardiovasculature. Neuromuscul Disord. 2003; 13:294–302. [PubMed: 12868498]

Harper SQ, Hauser MA, DelloRusso C, Duan D, Crawford RW, Phelps SF, Harper HA, Robinson AS, Engelhardt JF, Brooks SV, Chamberlain JS. Modular flexibility of dystrophin: implications for gene therapy of Duchenne muscular dystrophy. Nat Med. 2002; 8:253–261. [PubMed: 11875496]

Janssens S, Pokreisz P, Schoonjans L, Pellens M, Vermeersch P, Tjwa M, Jans P, Scherrer-Crosbie M, Picard MH, Szelid Z, Gillijns H, Van de Werf F, Collen D, Bloch KD. Cardiomyocyte-specific

overexpression of nitric oxide synthase 3 improves left ventricular performance and reduces compensatory hypertrophy after myocardial infarction. Circ Res. 2004; 94:1256–1262. [PubMed: 15044322]

Judge LM, Haraguchiln M, Chamberlain JS. Dissecting the signaling and mechanical functions of the dystrophin-glycoprotein complex. J Cell Sci. 2006; 119:1537–1546. [PubMed: 16569668]

Judge LM, Arnett AL, Banks GB, Chamberlain JS. Expression of the dystrophin isoform Dp116 preserves functional muscle mass and extends lifespan without preventing dystrophy in severely dystrophic mice. Hum Mol Genet. 2011; 20:4978–4990. [PubMed: 21949353]

Kaspar RW, Allen HD, Montanaro F. Current understanding and management of dilated cardiomyopathy in Duchenne and Becker muscular dystrophy. J Am Acad Nurse Pract. 2009; 21:241–249. [PubMed: 19432907]

Kaushik G, Fuhrmann A, Cammarato A, Engler AJ. In situ mechanical analysis of myofibrillar perturbation and aging on soft, bilayered Drosophila myocardium. Biophys J. 2011; 101:2629–2637. [PubMed: 22261050]

Khan SA, Skaf MW, Harrison RW, Lee K, Minhas KM, Kumar A, Fradley M, Shoukas AA, Berkowitz DE, Hare JM. Nitric oxide regulation of myocardial contractility and calcium cycling: independent impact of neuronal and endothelial nitric oxide synthases. Circ Res. 2003; 92:1322–1329. [PubMed: 12764022]

Lai Y, Thomas GD, Yue Y, Yang HT, Li D, Long C, Judge L, Bostick B, Chamberlain JS, Terjung RL, Duan D. Dystrophins carrying spectrin-like repeats 16 and 17 anchor nNOS to the sarcolemma and enhance exercise performance in a mouse model of muscular dystrophy. J Clin Invest. 2009; 119:624–635. [PubMed: 19229108]

Ocorr K, Reeves NL, Wessells RJ, Fink M, Chen HS, Akasaka T, Yasuda S, Metzger JM, Giles W, Posakony JW, Bodmer R. KCNQ potassium channel mutations cause cardiac arrhythmias in Drosophila that mimic the effects of aging. Proc Natl Acad Sci U S A. 2007; 104:3943–3948. [PubMed: 17360457]

Rafael JA, Cox GA, Corrado K, Jung D, Campbell KP, Chamberlain JS. Forced expression of dystrophin deletion constructs reveals structure–function correlations. J Cell Biol. 1996; 134:93–102. [PubMed: 8698825]

Ramachandran J, Schneider JS, Crassous PA, Zheng R, Gonzalez JP, Xie LH, Beuve A, Fraidenraich D, Peluffo RD. Nitric oxide signaling pathway in Duchenne muscular dystrophy mice: upregulation of L-arginine transporters. Biochem J. 2013; 449:133–142. [PubMed: 23009292]

Scott JM, Li S, Harper SQ, Welikson R, Bourque D, DelloRusso C, Hauschka SD, Chamberlain JS. Viral vectors for gene transfer of micro-, mini-, or full-length dystrophin. Neuromuscul Disord. 2002; 12(Suppl. 1):S23–S29. [PubMed: 12206791]

Sears CE, Bryant SM, Ashley EA, Lygate CA, Rakovic S, Wallis HL, Neubauer S, Terrar DA, Casadei B. Cardiac neuronal nitric oxide synthase isoform regulates myocardial contraction and calcium handling. Circ Res. 2003; 92:e52–e59. [PubMed: 12623875]

Shibata K, Yatera Y, Furuno Y, Sabanai K, Morisada N, Nakata S, Morishita T, Yamazaki F, Tanimoto A, Sasaguri Y, Tasaki H, Nakashima Y, Shimokawa H, Yanagihara N, Otsuji Y, Tsutsui M. Spontaneous development of left ventricular hypertrophy and diastolic dysfunction in mice lacking all nitric oxide synthases. Circ J. 2010; 74:2681–2692. [PubMed: 20966596]

Taghli-Lamallem O, Akasaka T, Hogg G, Nudel U, Yaffe D, Chamberlain JS, Ocorr K, Bodmer R. Dystrophin deficiency in Drosophila reduces lifespan and causes a dilated cardiomyopathy phenotype. Aging Cell. 2008; 7:237–249. [PubMed: 18221418]

Tong X, Evangelista A, Cohen RA. Targeting the redox regulation of SERCA in vascular physiology and disease. Curr Opin Pharmacol. 2010; 10:133–138. [PubMed: 20045379]

Townsend D, Blankinship MJ, Allen JM, Gregorevic P, Chamberlain JS, Metzger JM. Systemic administration of micro-dystrophin restores cardiac geometry and prevents dobutamine-induced cardiac pump failure. Mol Ther. 2007; 15:1086–1092. [PubMed: 17440445]

Vila-Petroff MG, Younes A, Egan J, Lakatta EG, Sollott SJ. Activation of distinct cAMP-dependent and cGMP-dependent pathways by nitric oxide in cardiac myocytes. Circ Res. 1999; 84:1020–1031. [PubMed: 10325239]

Xu KY, Huso DL, Dawson TM, Bredt DS, Becker LC. Nitric oxide synthase in cardiac sarcoplasmic reticulum. Proc Natl Acad Sci U S A. 1999; 96:657–662. [PubMed: 9892689]

Yue Y, Li Z, Harper SQ, Davisson RL, Chamberlain JS, Duan DR. Microdystrophins gene therapy of cardiomyocytes restores dystrophin-glycoproteins complex and improves sarcodemma integrity in the mouse. Heart Circ. 2003; 108:1626–1632.

Zhang YH, Zhang MH, Sears CE, Emanuel K, Redwood C, El-Armouche A, Kranias EG, Casadei B. Reduced phospholamban phosphorylation is associated with impaired relaxation in left ventricular myocytes from neuronal NO synthase-deficient mice. Circ Res. 2008; 102:242–249. [PubMed: 18007024]

Ziolo MT, Kohr MJ, Wang H. Nitric oxide signaling and the regulation of myocardial function. J Mol Cell Cardiol. 2008; 45:625–632. [PubMed: 18722380]

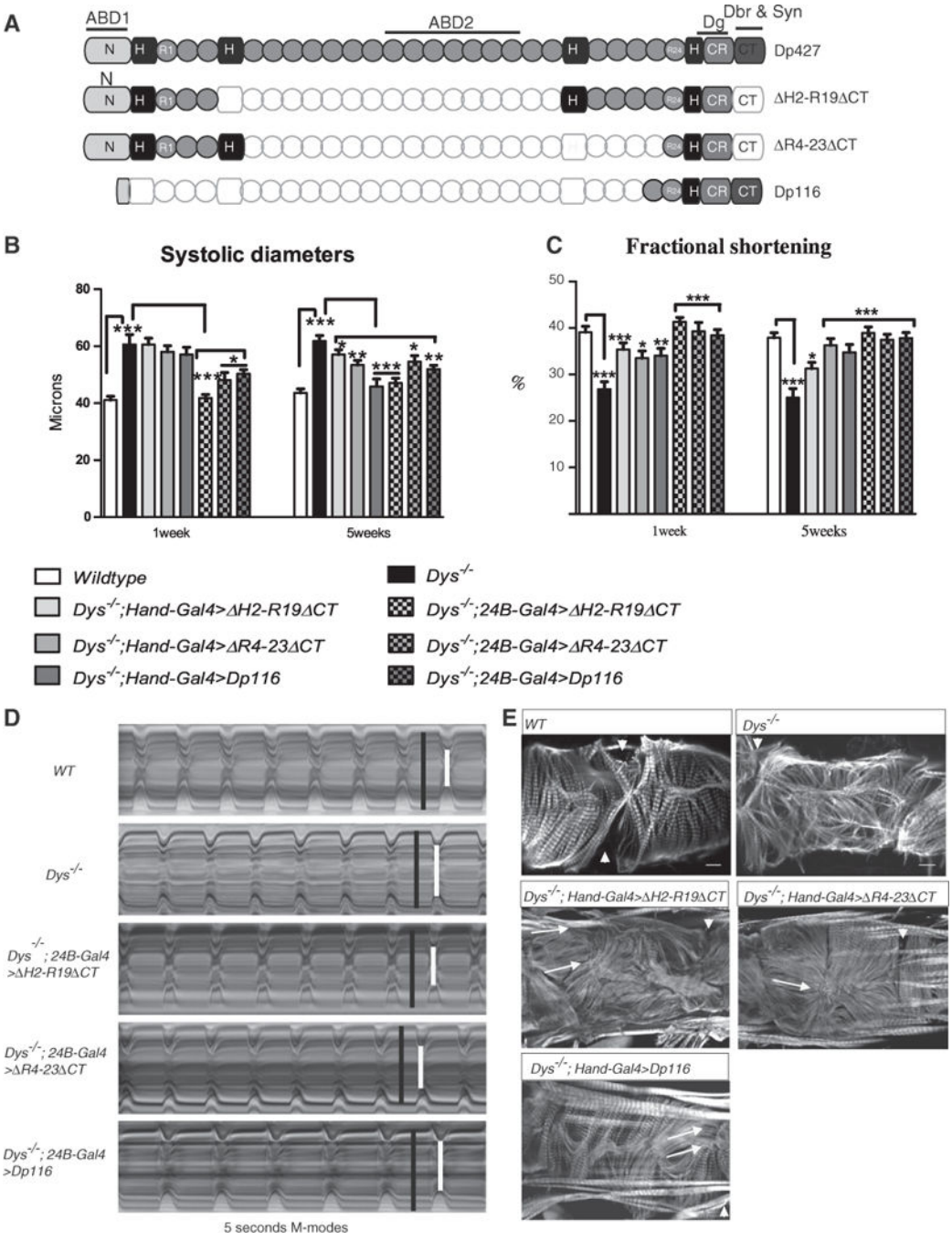


**Fig. 1.**

*Dystrophin* (*Dys*) mechanical and signaling functions prevent dilated cardiomyopathy. (A) Scheme illustrating the structure of *Dys* and the truncated proteins tested in $Dys^{-/-}$ flies. N: NH2-terminal actin-binding domain (ABD1); H: hinge; R: spectrin-like repeat; actin-binding domain 2 (ABD2); CR: cysteine-rich domain; CT: carboxy-terminal domain; Dg: Dystroglycan; Dbr: dystrobrevin; Syn: syntrophin. Domains in white are deleted domains. The ΔH2-R19ΔCT and ΔR4-23ΔCT lack the CT and are deleted from the rod domain hinge 2 to repeat 19 and repeat 4 to 23, respectively. Dp116 lacks the actin-binding domains and

retains only two repeats (R23-R24) with intact CR and CT domains (Judge et al., 2006). (B) Heart systolic diameters for 1- and 5-week-old flies. (C) Percent fractional shortening (%FS) represents an estimation of the heart tube contractility. Significant differences were determined by one-way ANOVA (*$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$). Error bars indicate SEM. N = 20 flies for each genotype. (D) Representative M-mode traces (5 s) illustrating movements of heart tube walls (y-axis) over time (x-axis). Diastolic (black) and systolic (white) diameters were indicated in each M-mode trace. (E) Representative confocal images (stacks) of adult hearts (A3 segment) stained with phalloidin, showing myofibrillar organization. The sarcomeric units are oriented longitudinally as indicated by arrows, and are seen in 57% of $Dys^{-/-}$; *Hand-Gal4* > Δ*H2-R19*Δ*CT*, in 71% of $Dys^{-/-}$; *Hand-Gal4* > Δ*R4-23*Δ*CT* and in 50% of $Dys^{-/-}$; *Hand-Gal4* N *Dp116*. Ostia in the myocardium are inflow tracks indicated by arrowheads. Scale bar 10 μm.

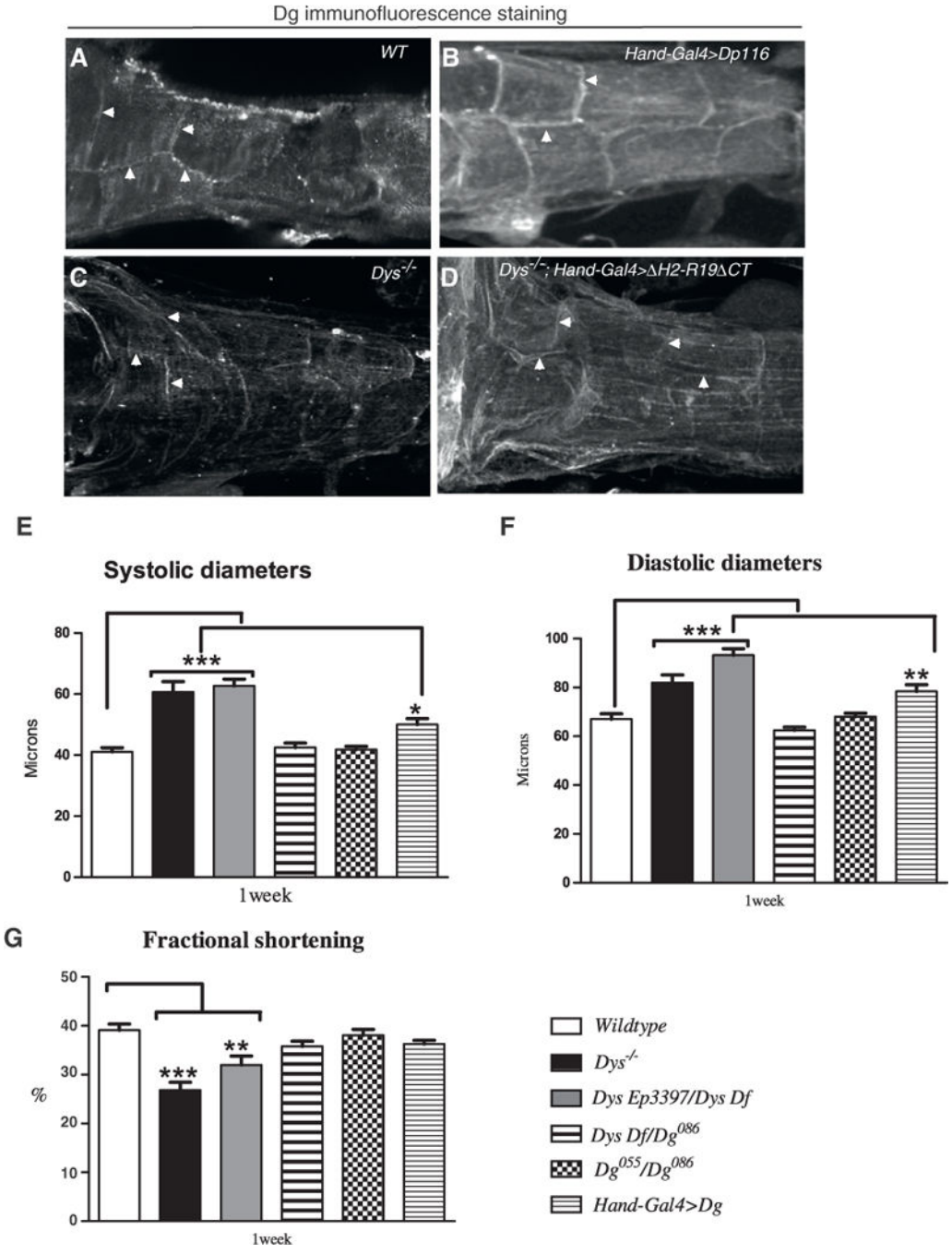


**Fig. 2.**

Dystroglycan (Dg) null mutants do not affect heart function. (A–D) Representative confocal stacks of adult hearts stained with Dg antibody. Dg is found at the cell membrane of the cardiomyocytes. Arrowheads indicate Dg localization. (E–G) Bar graph representations of changes in heart chamber dimensions. Note that *Dg* null mutants do not show decreased fractional shortening. N = 20–30 flies per genotype. One-way ANOVA analysis was used for statistics and p values <0.05 were considered significant (*p < 0.05; **p < 0.005; ***p < 0.0005). Error bars indicate SEM.

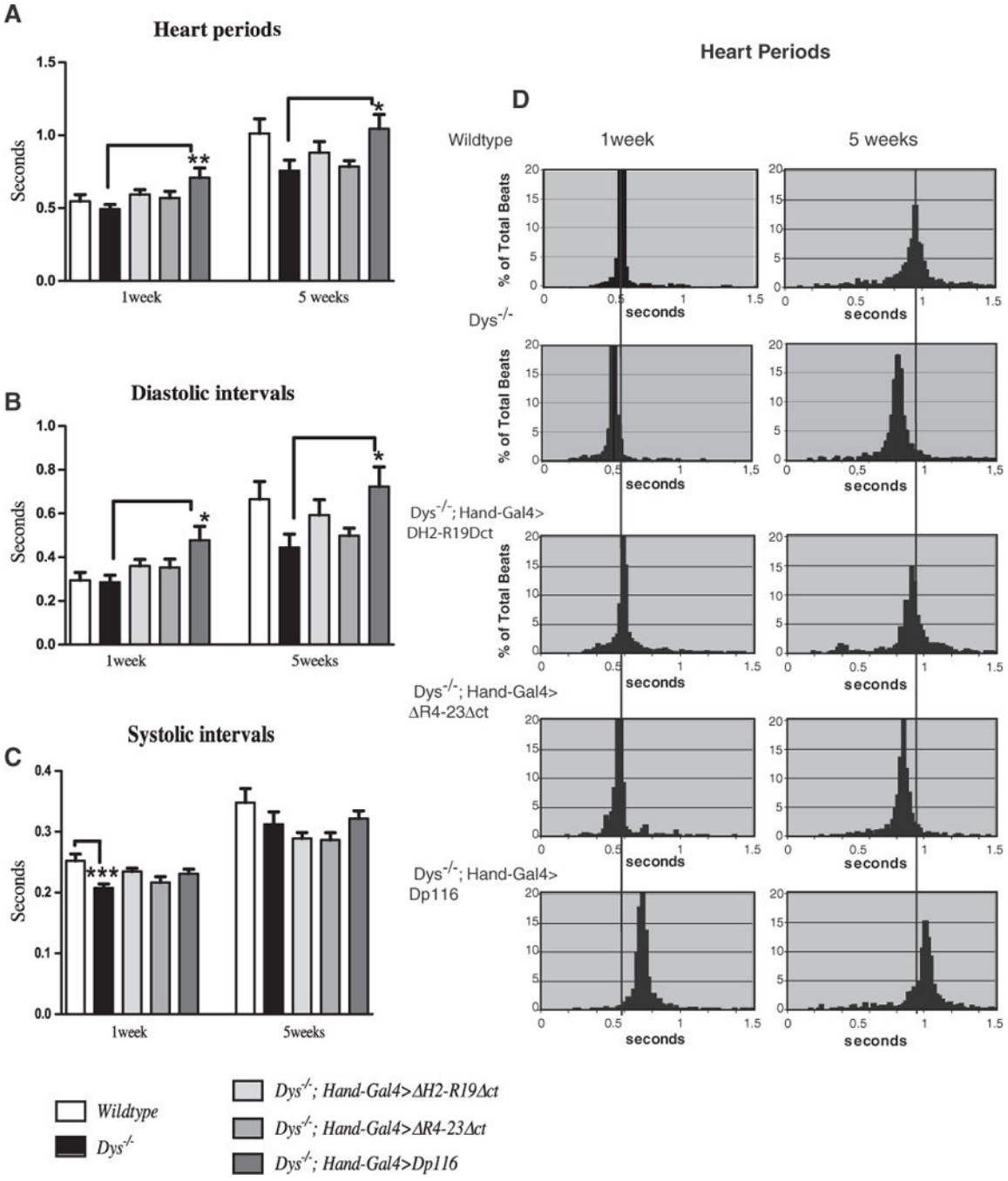


**Fig. 3.**

Dp116 prolongs the heart periods (HPs). (A–C) Bar graph representations of changes in HPs, systolic and diastolic intervals of young 1- and 5-week-old flies. Note that Dp116 increased the HPs, mainly by prolonging the diastolic interval. Error bars are SEM, N = 20 flies per genotype and age. (D) HP histograms obtained from 30 s optical recordings indicate the distribution of HPs for each fly. Individual data points are plotted, illustrating HP variability for wildtype, *Dys*$^{-/-}$ and the rescued *Dys*$^{-/-}$ hearts. The vertical line through the peak of the heart period distributions is to facilitate comparison between the different plots.

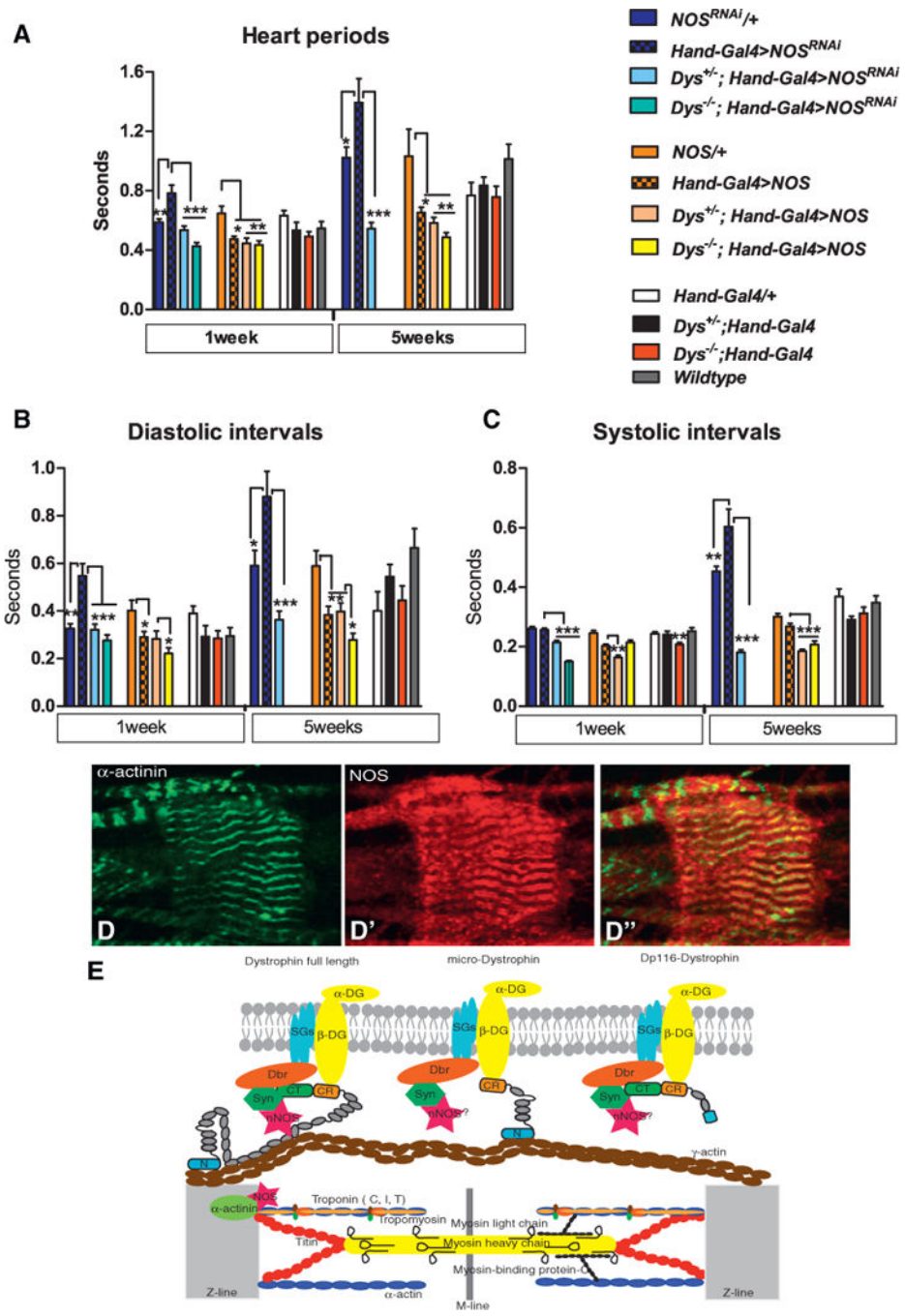


**Fig. 4.**

Nitric oxide synthase (Nos) regulates the *Drosophila* heart beat. (A–C) Heart beat parameters in 1- and 5-week-old flies. Cardiac *RNAi* knockdown of *Nos* shows significant increase in HPs and diastolic intervals. An effect reversed in $Dys^{-/-}$ mutants. Error bars are SEM. N = 20 flies per genotype and age. Differences were estimated by one-way ANOVA analysis (*$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$). (D) Representative confocal image of adult cardiomyocyte stained with α-actinin (D) and NOS antibody (D′) from hearts overexpressing *Nos*. Nos protein is found in a doublet of bands on each side of α-actinin (D

″). (E) Schematic outline of the full-length *Dys*, micro-*Dys* and Dp116 and their interaction with other subcellular components. *Dys* full-length binds to F-actin and to the DGC complex. The micro-*Dys* binding to F-actin and not interacting with Nos is functional and rescues the heart cell contractility. Similarly, the Dp116 which does not bind F-actin but links the other DGC proteins is efficient in reinstating the cardiac function. Note that Nos in the sarcolemma is what is known from vertebrate cardiomyocyte biochemistry. Note the new Nos pattern close to the Z-lines. Dg: Dystroglycan, SGs: sarcoglycan complex, Dbr: dystrobrevin, Syn: syntrophin, N: NH2-terminal actin-binding domain, CR: cysteine-rich domain, CT: carboxy-terminal domain.