# Microdystrophin with an extended C-Terminal domain protects against pharmacologically induced cardiac damage and remodeling in mdx mice

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## Introduction

The most common cause of mortality in Duchenne muscular dystrophy (DMD) is cardiopulmonary failure. Various clinical and commercial gene therapies aim to alter DMD disease trajectory with adeno-associated virus- (AAV) mediated delivery of expression constructs for microdystrophins (μDys), truncated dystrophin proteins that retain key functional elements of the full-length protein. We recently showed that the dystrophin C-Terminal (CT) domain enhances the effectiveness of a μDys construct in skeletal muscle. However, the impact of the dystrophin CT domain on cardiac health and function remains unknown. Although dystrophin-deficient *mdx* mice do not fully phenocopy human DMD, repeated isoproterenol injections induce a dilated cardiac phenotype that resembles DMD-associated cardiomyopathy.

We compared μDys constructs with or without the CT domain (termed μDysCT194 and μDysCT48, respectively) for their abilities to resist pharmacologically induced cardiac damage in *mdx* mice. Both μDysCT194 and μDysCT48 were packaged into AAVhu32 and administered intravenously at a dose of $2x10^{13}$ GC/kg. 4 weeks after AAV.μDys administration, we performed daily isoproterenol injections for 10 days and assessed cardiac function and remodeling through echocardiography and histology. Cardiac damage, as measured by serum cardiac troponin, was increased in *mdx* hearts after the first injection and prevented by μDys (39.7 ng/ml, *mdx* Vehicle vs. 0.7, C57Bl/10, 2.9, μDysCT194, and 9.7, μDysCT48). Repeated isoproterenol administration dilated *mdx* left ventricles (LVEDd 4.3mm vs. 3.9mm, C57Bl/10), induced fibrosis (35.2% vs. 18.9%, C57Bl/10), reduced fractional shortening (0.24 vs. 0.33, C57Bl/10) and reduced ejection fraction (43.0% vs. 54.4% in C57Bl/10). Both μDys provided ameliorated these effects, with μDysCT194 providing a stronger rescue on LVEDd (3.9mm, μDysCT194 vs. 4.1mm, μDysCT48), fibrosis (9.8%, μDysCT194 vs. 16.4%, μDysCT48), fractional shortening (0.33, μDysCT194 vs. 0.29, μDysCT48), and ejection fraction (54.6%, μDysCT194 vs. 49.4%, μDysCT48).

As a complementary analysis, we assessed whether μDys protects against acute injury induced by isoproterenol co-administered with atropine, an enhancer of its chronotropic activity. Injured cardiomyocytes were identified by uptake of cell-impermeant Evans blue dye. A single injection of isoproterenol/atropine produced a minor injury in C57Bl/10 mice (4.2% EBD+ cardiomyocytes) which was markedly increased in *mdx* mice (16.3%). Both μDys significantly protected against this effect (4.7% and 3.5%, μDysCT194 and μDysCT48, respectively). Single isoproterenol/atropine and repeated isoproterenol injections induced damage-associated B-type natriuretic peptide expression in *mdx* mice, which was attenuated by μDysCT194 but not μDysCT48. This suggests that μDysCT48 and μDysCT194 may protect against rupture of cardiomyocyte sarcolemma equally but nevertheless provide different qualities of response to stress. Overall, the studies presented here offer evidence that the dystrophin CT domain may enhance protection against pathological remodeling in dystrophin-deficient hearts.

## Materials and Methods

***AAV Vectors.*** Transgene cassettes encoding μDys were packaged into AAVhu32 and included the muscle-specific Spc5-12 promoter. Vectors were administered to 5-week-old *mdx* mice by tail vein injection at a dose of $2x10^{13}$ GC/kg.

***Echocardiography.*** Transthoracic echocardiography was performed on mice using a Visual Sonics Vevo 2100 ultrasound imaging system equipped with an MS400 linear-array transducer. Mice were anesthetized with isoflurane and maintained at physiological heart rates on a heated platform to preserve body temperature. Two-dimensional, M-mode, and Doppler images were acquired in parasternal long- and short-axis views to assess cardiac structure and function. All measurements were averaged over multiple cardiac cycles and analyzed by an investigator blinded to experimental groups.

***Isoproterenol, atropine, and Evans blue administration.*** Isoproterenol was injected at 2 mg/kg subcutaneously beginning 4 weeks after AAV administration. In the case of repeated isoproterenol injection (Study 1, Fig. 1), heart rate was monitored beginning immediately before administration through 5 minutes post-administration with a toe-clip heart rate monitor (Kent Scientific). The maximum heart rate throughout this period was noted. Evans blue dye was formulated as 1% w/v solution in isotonic saline solution and injected at 0.5% bodyweight into the tail vein (Study 2, Fig. 1). On the next day, atropine (0.2 mg/kg) was given intraperitoneally, followed immediately by isoproterenol (2 mg/kg). Tissue was harvested one day later.

***Immunofluorescence.*** 10 μm transverse cryosections were incubated with primary antibodies against dystrophin/μDys (Leica, 1:50), counterstained with anti-laminin (Sigma, 1:400) overnight and detected with fluorescent secondary antibodies. Whole tissue 20x tiled images were acquired on a Zeiss AxioScan 7 slide scanner. Evans blue was detected on the same sections using excitation/emission filters compatible with far red fluorescence. Evans blue-positive area was determined using ImageJ software as follows: a region of interest was drawn around each heart section (excluding internal chambers), then a second region of interest was generated by thresholding signal from the dye. The ratio of dye-positive to total area was multiplied by 100 yield values reported as a percentage of total area.

***Histology.*** Sirius red (Abcam) staining was performed according to the manufacturer's protocol on 10 μm transverse cryosections. Sections were dehydrated, cleared, and coverslips were mounted with Permount (Fisher) mounting medium. Trichrome staining was performed on hearts drop-fixed in 10% neutral buffer formalin, bisected in the transverse plane, and embedded in paraffin. Whole tissue 20x tiled images were acquired on a Zeiss AxioScan 7 slide scanner. Quantification of total fibrotic area was performed on Sirius Red-stained sections for both studies in ImageJ by setting a color threshold to identify red areas.

***Capillary Electrophoresis.*** Total protein was extracted from heart in SDS/Tris buffer. 0.25μg of protein per sample were loaded onto plates for analysis by capillary electrophoresis (Jess, bio-techne). Purified His-tagged μDysCT194 was generated by LakePharma. Dystrophin/μDys were detected with a primary antibody recognizing the dystrophin N-Terminus (Leica, 1:400) and HRP-conjugated anti-mouse secondary antibody.

***Cardiac troponin I ELISA.*** Blood was collected from tail vein on Study Day 2 (after 1 isoproterenol injection) or via cardiac puncture (at terminus, after 10 isoproterenol injections). Serum was separated by centrifugation and assessed for circulating cardiac troponin I using a high-sensitivity ELISA kit according to manufacturer's instructions (Life Diagnostics).

***RNA Extraction and ddPCR.*** Total RNA was extracted from tissues using the Kingfisher Apex via the MagMAX mirVana Total RNA kits (Thermo). RNA was converted to cDNA using the Maxima First Strand cDNA Synthesis Kit (Thermo). TaqMan assays (Thermo) were used to evaluate *Nppb, Myh6,* and *Myh7* gene expression. All values were normalized to TATA binding protein (*Tbp*) copies.

***Statistics.*** All datasets were assessed by Shapiro-Wilk test for normality. If all groups followed normal distribution, they were compared by one-way ANOVA with Dunnet's multiple comparisons correction. If any group followed a non-normal distribution, all groups were compared via Kruskal-Wallis test with Dunn's correction for multiple comparisons. A p-value < 0.05 was considered statistically significant in all cases.

## Results

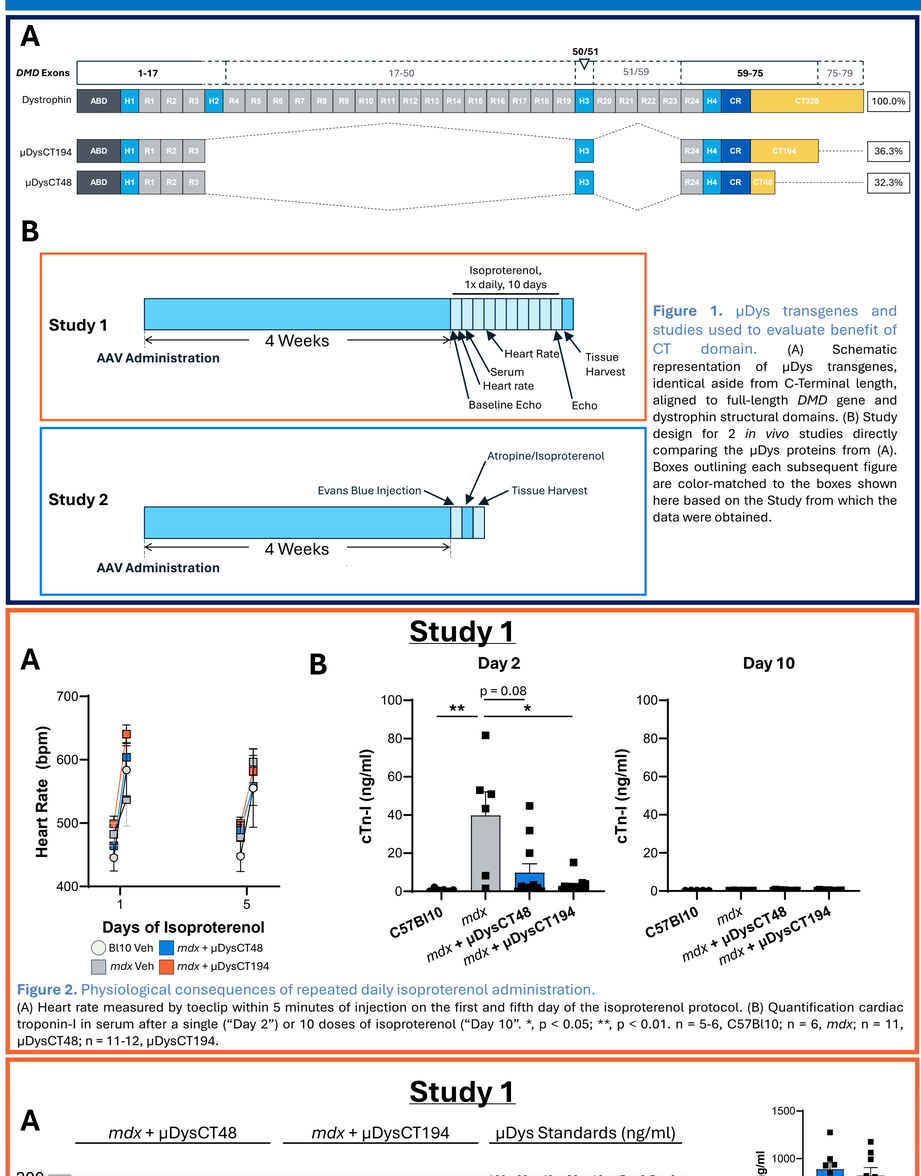


**Figure 1.** μDys transgenes and studies used to evaluate benefit of CT domain. (A) Schematic representation of μDys transgenes, identical aside from C-Terminal length, aligned to full-length *DMD* gene and dystrophin structural domains. (B) Study design for 2 *in vivo* studies directly comparing the μDys proteins from (A). Boxes outlining each subsequent figure are color-matched to the boxes shown here based on the Study from which the data were obtained.

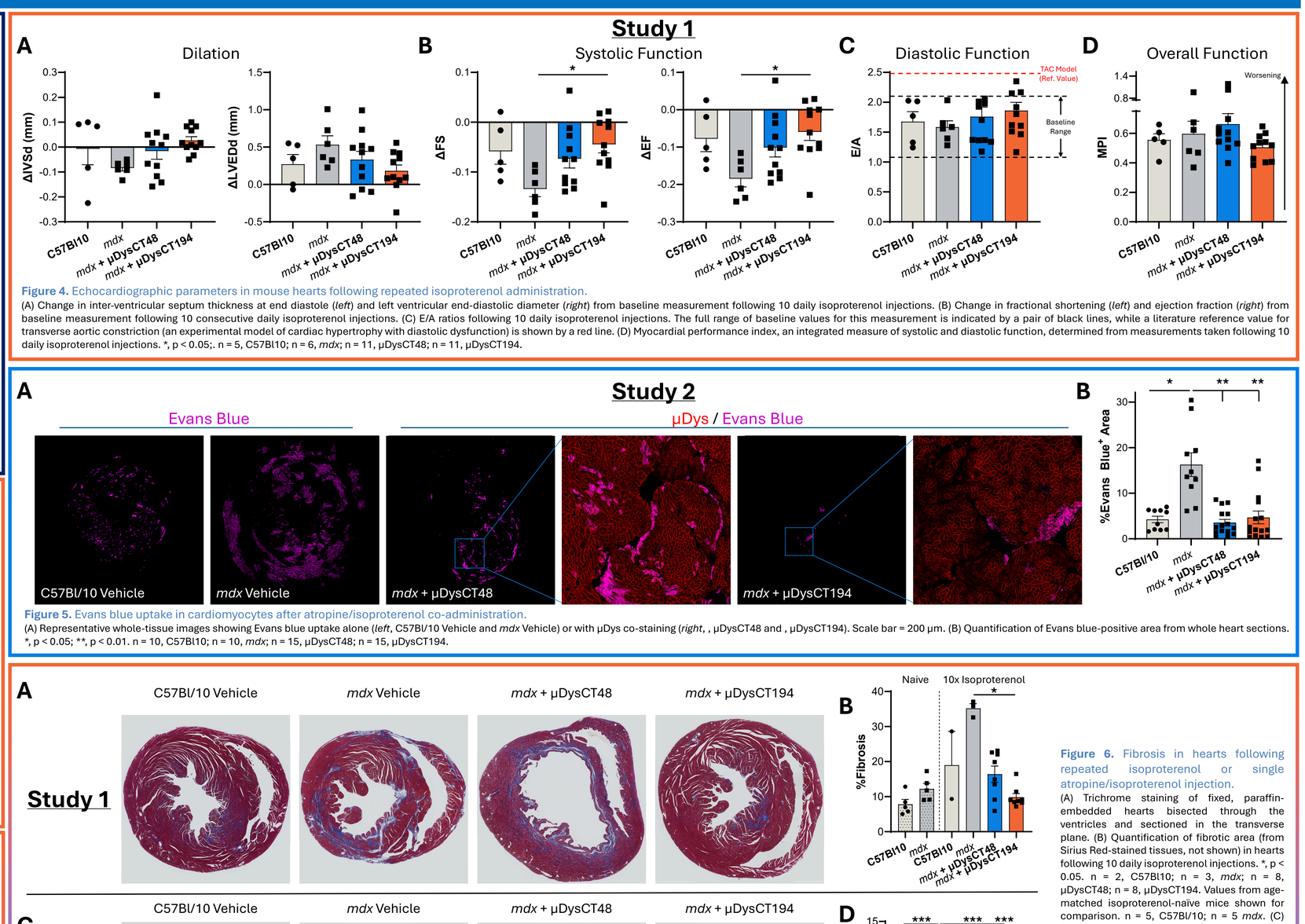


**Figure 4.** Echocardiographic parameters in mouse hearts following repeated isoproterenol administration.
(A) Change in inter-ventricular septum thickness at end diastole (*left*) and left ventricular end-diastolic diameter (*right*) from baseline measurement following 10 daily isoproterenol injections. (B) Change in fractional shortening (*left*) and ejection fraction (*right*) from baseline measurement following 10 consecutive daily isoproterenol injections. (C) E/A ratios following 10 daily isoproterenol injections. The full range of baseline values for this measurement is indicated by a pair of black lines, while a literature reference value for transverse aortic constriction (an experimental model of cardiac hypertrophy with diastolic dysfunction) is shown by a red line. (D) Myocardial performance index, an integrated measure of systolic and diastolic function, determined from measurements taken following 10 daily isoproterenol injections. *, $p < 0.05$;. n = 5, C57Bl10; n = 6, *mdx*; n = 11, μDysCT48; n = 11, μDysCT194.




**Figure 5.** Evans blue uptake in cardiomyocytes after atropine/isoproterenol co-administration.
(A) Representative whole-tissue images showing Evans blue uptake alone (*left*, C57Bl/10 Vehicle and *mdx* Vehicle) or with μDys co-staining (*right*, , μDysCT48 and , μDysCT194). Scale bar = 200 μm. (B) Quantification of Evans blue-positive area from whole heart sections. *, $p < 0.05$; **, $p < 0.01$. n = 10, C57Bl10; n = 10, *mdx*; n = 15, μDysCT48; n = 15, μDysCT194.




**Figure 2.** Physiological consequences of repeated daily isoproterenol administration.
(A) Heart rate measured by toeclip within 5 minutes of injection on the first and fifth day of the isoproterenol protocol. (B) Quantification cardiac troponin-I in serum after a single ("Day 2") or 10 doses of isoproterenol ("Day 10". *, $p < 0.05$; **, $p < 0.01$. n = 5-6, C57Bl10; n = 6, *mdx*; n = 11, μDysCT48; n = 11-12, μDysCT194.




**Figure 3.** Broad cardiac μDys expression is achieved from a single low-dose administration of AAVhu32.μDys vectors.
(A) Representative images and quantification of actinin-normalized μDys protein levels assessed via capillary Western analysis from hearts ~5.5 weeks post administration. n = 8, μDysCT48; n = 8, μDysCT194 (Study 1). (B) Whole-tissue immunofluorescent images showing broad, properly localized μDys protein in treated hearts. (C) Representative images and quantification of actinin-normalized μDys protein levels assessed via capillary Western analysis from hearts ~4 weeks post administration. n = 15, μDysCT48; n = 14, μDysCT194. (D) Whole-tissue immunofluorescent images showing broad, properly localized μDys protein in treated hearts (Study 2).




**Figure 7.** Expression of mRNAs associated with cardiac damage and remodeling.
(A) Quantification of B-Type natriuretic peptide (*Nppb*), α-myosin heavy chain (*Myh6*) and β-myosin heavy chain (*Myh7*) mRNA normalized to copies of *Tbp* following 10 daily isoproterenol injections. **, $p < 0.01$; ***, $p < 0.001$. n = 2, C57Bl10; n = 3, *mdx*; n = 8, μDysCT48; n = 8, μDysCT194. (B) Quantification of B-Type natriuretic peptide (*Nppb*), α-myosin heavy chain (*Myh6*) and β-myosin heavy chain (*Myh7*) mRNA normalized to copies of *Tbp* following a single injection of atropine/isoproterenol. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. n = 10, C57Bl10; n = 10, *mdx*; n = 15, μDysCT48; n = 15, μDysCT194.

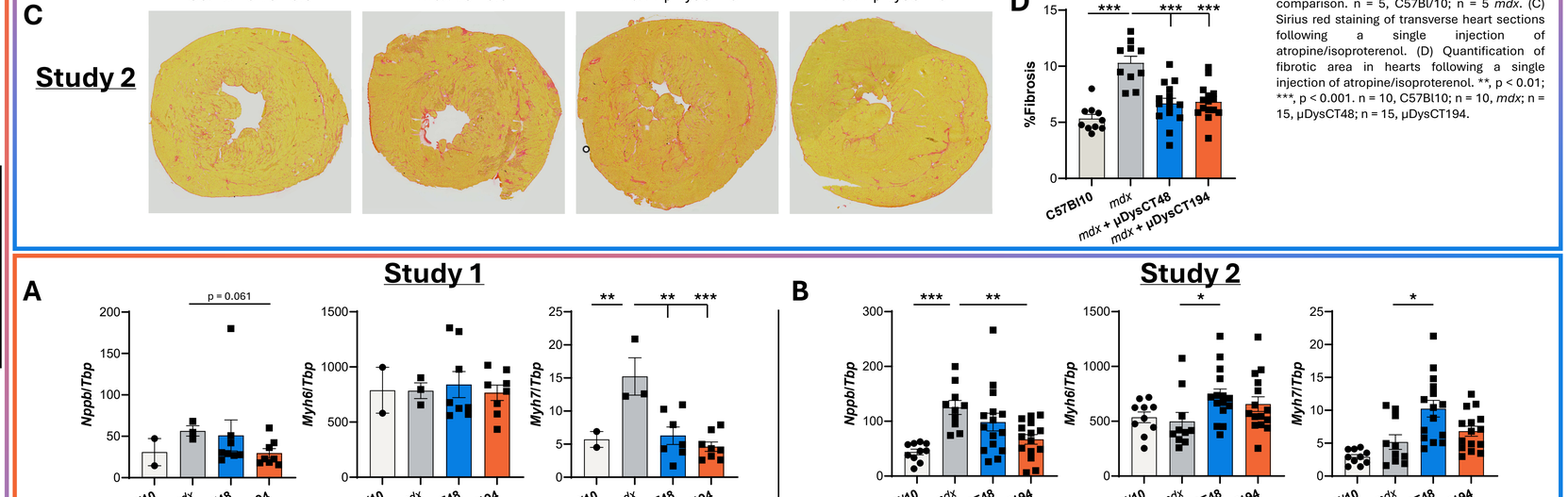


**Figure 6.** Fibrosis in hearts following repeated isoproterenol or single atropine/isoproterenol injection.
(A) Trichrome staining of fixed, paraffin-embedded hearts bisected through the ventricles and sectioned in the transverse plane. (B) Quantification of fibrotic area (from Sirius Red-stained tissues, not shown) in hearts following 10 daily isoproterenol injections. *, $p < 0.05$. n = 2, C57Bl10; n = 3, *mdx*; n = 8, μDysCT48; n = 8, μDysCT194. Values from age-matched isoproterenol-naïve mice shown for comparison. n = 5, C57Bl/10; n = 5 *mdx*. (C) Sirius red staining of transverse heart sections following a single injection of atropine/isoproterenol. (D) Quantification of fibrotic area in hearts following a single injection of atropine/isoproterenol. **, $p < 0.01$; ***, $p < 0.001$. n = 10, C57Bl10; n = 10, *mdx*; n = 15, μDysCT48; n = 15, μDysCT194.

## Discussion and Conclusions

Results from these preclinical studies provide evidence indicating that μDys protects the heart from pharmacologically-induced damage and that incorporation of the **dystrophin CT** domain may enhance this protection.

- μDysCT48 and μDysCT194 are well expressed in *mdx* hearts following low-dose administration of AAVhu32.Spc5-12.μDys vectors. The two μDys proteins accumulate to equivalent levels.
- Isoproterenol induces cardiac damage specifically in *mdx* hearts after a single administration, while repeated administration leads to remodeling and reduced systolic function.
- μDysCT194 provides superior preservation of systolic function, relative to μDysCT48, following 10 isoproterenol injections.
- Expression of μDysCT194 allows *mdx* heart to resist ventricle dilation and development of fibrosis in response to repeated isoproterenol administration.
- Atropine enhances the effect of isoproterenol and coadministration of these agents induces significant cardiomyocyte damage in *mdx* hearts, as evidenced by infiltration of Evans blue dye.
- μDysCT48 and μDysCT194 reduce levels of Evans blue dye uptake in cardiomyocytes to C57Bl/10 levels following atropine/isoproterenol coadministration.
- μDysCT194 prevents induction of B-type natriuretic peptide, a clinically relevant biomarker for cardiac damage and failure, after atropine/isoproterenol injection and shows a similar, albeit not statistically significant, trend following 10 isoproterenol injections.
- RGX-202, an investigational μDys therapy for DMD featuring μDysCT194, is being evaluated in a late-stage clinical trial (AFFINITY DUCHENNE®, NCT05693142).

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