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<b>14. ABSTRACT</b>  The overall goal of this work is to increase the availability of critical mouse models of human muscular dystrophy (MD) for both hypothesis testing and preclinical therapy development. Our multi-disciplinary team from The Jackson Laboratory (JAX) and the Children's National Medical Center (CNMC) has expertise in MD, repository management, mouse models, and preclinical testing. For Year 3 of funding, Drs. Lutz and Cox at JAX have added 7 new strains to the MD Repository (Aim1) to leverage JAX's considerable expertise and infrastructure to maintain and distribute MD mouse resources to the scientific community. In Aim 2 we have completed gene targeting of dystrophin transgenes into DBA/2J ES cell lines and are screening chimeric mice for germ-line transmission. These novel DMD transgenic mice, which model patients receiving successful exon-skipping therapies, will be crossed to mutant D2.B10-mdx to score for phenotypic rescue of each mutant and WT line to compare the functionality of resulting Dystrophin molecules containing in-frame deletions that are expected to arise by successful treatment of patient mutations. In Aim 3, we have completed generation of a DBA/2J congenic mdx strain that appears to better model the symptoms of the human disease. In addition, we are screening F2 crosses between B6 and D2 to identify genetic modifiers that can alter disease onset and severity. In Aim 4, Dr. Nagaraju at CNMC has performed a baseline phenotypic analysis of our new D2.B10-mdx model and we are combining the analysis from JAX and CNMC to write a manuscript detailing the advantages of this new model for preclinical testing. A no-cost extension for a fourth year of work was awarded to allow completion of our transgenic analysis of Becker-like muscular dystrophy rescue experiments, allow completion of the D2.B10-mdx characterization and preclinical evaluation of this improved model. Our DMD repository has greatly expand the accessibility and availability of mouse model resources for MD translational research and therapeutic development.					
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**Translational Research for Muscular Dystrophy W81XWH-11-1-0330**  
**Gregory A. Cox, Ph.D., Principal Investigator**

**A. INTRODUCTION**

The goal of this work is to increase the availability of critical mouse models of human muscular dystrophy (MD) for both hypothesis testing and preclinical therapy development. Our multi-disciplinary team from The Jackson Laboratory (JAX) and the Children's National Medical Center (CNMC) has expertise in MD, repository management, mouse models, and preclinical testing. At JAX, Drs. Lutz and Cox have established the MD Repository (Aim1) to leverage JAX's considerable expertise and infrastructure to maintain and distribute MD mouse and information resources to the scientific community. In Aim 2 we are developing novel DMD transgenic mice, which model patients receiving successful exon-skipping therapies. We propose to address the fundamental, but often overlooked question related to the functionality of resulting Dystrophin molecules containing in-frame deletions that are expected to arise by successful treatment of patient mutations. Our transgenic experiments will model the best-case-scenario outcome for AO-mediated therapy in which one assumes that a particular compound is capable of 100% effective exon-skipping to restore the reading frame. In Aim 3, we are generating congenic *mdx* mice to better model the symptoms of the human disease and to identify genetic modifiers that can alter disease onset and severity. In Aim 4, Dr. Nagaraju at CNMC is carrying out preclinical studies with promising therapeutic compounds initially in the C57BL10-*Dmd*<sup>*mdx*</sup> but is now completing the direct phenotypic comparison of the D2.B10-*Dmd*<sup>*mdx*</sup> mice so that the drug studies can be completed in this new model developed at JAX. Overall, this program will greatly expand the accessibility and availability of mouse model resources for MD translational research and therapeutic development.

**B. BODY**

**Aim 1. Develop a centralized repository for mouse models of MD. JAX.**

Our goal is to identify, import, and cryopreserve 3-5 biomedically significant models per year for the MD Repository and to disseminate mouse information resources to the scientific community. The repository is providing researchers with centralized access to high-priority DMD models imported from outside investigators and transgenic and congenic DMD models developed in **Aims 2 & 3**. The following lines have been identified as relevant models to the MD Repository at JAX. We have reached out to the investigators who engineered the mice, requesting that they deposit the models to the JAX MD Repository. A description of the models and the status of the request are outlined below:

Strains imported in year 3:

JAX stock 14563: STOCK *Utrn*<sup>*tm1Ked*</sup> *Dmd*<sup>*mdx*</sup>/J

Female mice that are homozygous for the *Utrn*<sup>*tm1Ked*</sup> allele and the *Dmd*<sup>*mdx*</sup> allele, and male mice that are homozygous for the *Utrn*<sup>*tm1Ked*</sup> allele and hemizygous for the *Dmd*<sup>*mdx*</sup> allele, exhibit a more severe phenotype than single *Dmd*<sup>*mdx*</sup> mutants: earlier onset of muscle dystrophy (degeneration, macrophage infiltration and necrosis), weight loss after weaning, joint contractures, kyphosis, dystrophy of extraocular muscles, abnormal electrocardiograms, infertility and premature death. Growth retardation onset is at weaning. By 4 of 6 weeks of age, the double mutants exhibit reduced body weight, reduced mobility, abnormal breathing pattern and slack posture. Muscle weakness and kyphosis (curvature of the spine) is progressive and the double mutant mice develop a waddling gait. Necrosis of the diaphragm muscle is observed in 6 day old double mutant mice. Muscle fibers with centralized nuclei are seen in 2

week old double mutants. 8 to 10 week old mutants exhibit a muscular dystrophy phenotype similar to mice homozygous for the *Dmdmdx* allele with variation in muscle fiber size, necrosis, fibrosis, macrophage infiltration and centrally nucleated muscle fibers.

JAX stock 18018: B10ScSn.Cg-*Prkdc*<sup>scid</sup> *Dmd*<sup>mdx</sup>/J

Like human patients who suffer from one of the most common neuromuscular diseases, Duchenne muscular dystrophy (DMD), the *Dmd*<sup>mdx</sup> mutants do not express dystrophin and therefore have been routinely used as an animal model of the disease even though the resultant myopathology is much less severe compared to the human disease course. When combined with the *Prkdc*<sup>scid</sup> allele, there is some amelioration of the mdx phenotype including a reduction in the rate of muscle fibrosis, higher endurance and decreased expression of active TGFβ1. However, MDX/SCID mice continue to exhibit necrosis, centrally located nuclei and the muscle degeneration characteristic of DMD. The MDX/SCID mouse may be used as a dystrophic model for the transplantation of human donor cells to evaluate skeletal muscle regeneration.

JAX stock 13141: D2.B10-*Dmd*<sup>mdx</sup>/J

The DBA/2-congenic *Dmd*<sup>mdx</sup> mouse may be a superior Duchenne muscular dystrophy model as it better recapitulates several of the human characteristics of DMD myopathology (lower hind limb muscle weight, fewer myofibers, increased fibrosis and fat accumulation, and muscle weakness).

JAX stock 23535: B6.Cg-*Terc*<sup>tm1Rdp</sup> *Dmd*<sup>mdx-4Cv</sup>/BlauJ

T *mdx/mTR*<sup>KO</sup> mouse model combines dystrophin-deficiency with telomere dysfunction/shortening, and may be a superior Duchenne muscular dystrophy model as it better recapitulates several of the human characteristics of DMD myopathology (progressive muscle weakness and damage, skeletal muscle fibrosis, diminished muscle stem cell regenerative capacity, dilated cardiomyopathy, heart failure and shortened life-span).

JAX stock 18305: MYD/Le-*Large*<sup>myd</sup>/J

The spontaneous autosomal recessive mutation myodystrophy (*myd*) is a deletion in exons 5-7 of the glycotransferase gene (*Large*) on chromosome 8; causing a frameshift and premature stop codon before the first two catalytic domains. This *Large*<sup>myd</sup> mutation results in abnormal glycosylation of its substrate α-dystroglycan. *Large*<sup>myd</sup> mice are a model of Congenital muscular dystrophy type 1D (MDC1D; also called human α-dystroglycan glycosylation-deficient muscular dystrophy). MYD/Le-*Large*<sup>myd</sup> homozygotes exhibit a progressive myopathy, abnormal posture, thoracic kyphosis, calcium deposits in muscle, loss of Schwann cells and myelin, central nervous system defects, and reduced growth.

JAX stock 17917: B6.Cg-*Dysf*<sup>prmd</sup> *Prkdc*<sup>scid</sup>/J

These Scid/blAJ mice are a C57BL/6-congenic line that is dysferlin-deficient and also lack B and T lymphocytes. Their attenuated muscle-damaging immune responses result in a dysferlinopathy that is less severe compared to A/J mice naturally carrying the dysferlin mutation. Scid/blAJ mice may be useful for studying limb-girdle muscular dystrophy type 2B (LGMD2B), Miyoshi myopathy, transplantation studies, complement system / membrane attack pathway / membrane attack complex, and how distinct subpopulations of macrophages can promote muscle injury or repair in muscular dystrophy.

These mice carry a floxed allele of *Fktn* (fukutin). When crossed with a Cre strain, tissue-specific knockouts of the gene can be generated. Crosses with *Myf5* (myogenic factor 5) and *Ckm* (creatine kinase, muscle) Cre strains generate dystroglycanopathy mice representative of a spectrum of mild to severe patient diseases.

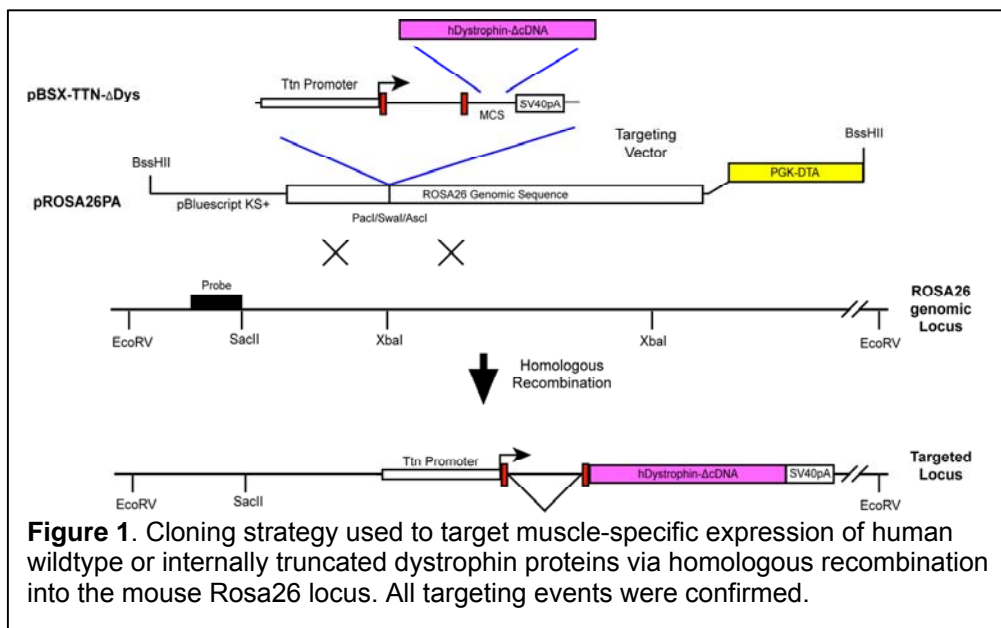
## Disseminate MD mouse and information resources to the scientific community through the MD Repository.

- a) All mice that are available from the MD repository at JAX are readily accessed from our public website at JAX. Each strain has its own public datasheet with a description of the mouse, the development of the model, links to genotyping protocols, and animal husbandry information. For example <http://jaxmice.jax.org/strain/013786.html>
- b) Dr. Lutz attended The Society for Neuroscience meeting in San Diego California where her lab presented a poster on the DBA/2J mdx model characterization.

## Aim 2. Engineer mice expressing in-frame deletions of the human dystrophin cDNA to model patients receiving successful exon-skipping therapies. JAX.

We have successfully generated the three human dystrophin cDNA clones containing in-frame mutations (deletion of exons 44-45, 49-51, 48-53) along with a full-length wildtype cDNA clone. The cDNAs have been fully sequence verified and plasmid vectors containing each clone are prepared. As shown in **Figure 1**, we have successfully cloned dystrophin cDNAs downstream of the mouse Titin

(Ttn) promoter to drive high-level skeletal and cardiac muscle expression of the transgene. The Ttn promoter construct contains the first non-coding exon 1, the entire first intron and the 5' half of exon 2 truncated just upstream of the start codon. We have had great success using this promoter to drive skeletal and cardiac-specific expression of transgenes in the past and expect that a single-copy insertion into the Rosa26 locus should



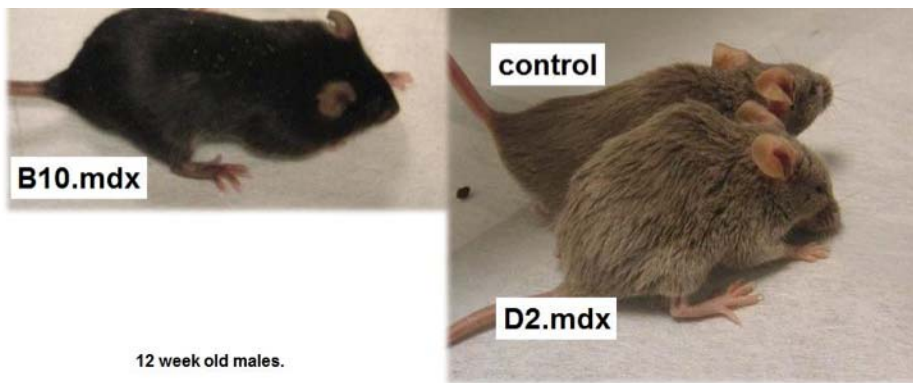
provide uniform expression between each of the independent lines of mice generated by homologous recombination. The promoter/cDNA construct was completed in step 1, and has been inserted into a Rosa26 vector containing 5' and 3' homology arms for homologous recombination into ES cell lines. To date, we have successfully generated the three human dystrophin cDNA clones containing in-frame mutations (deletion of exons 44-45, 49-51, 48-53) along with a full-length wildtype cDNA clone. Each construct has been successfully targeted into the ROSA26 locus of DBA/2J ES cells. These ES cell lines have been microinjected into host blastocysts and we are just now receiving chimeric mice

for analysis of germline transmission. Once germline transmission is confirmed, the mice will be crossed with our newly generated D2.B10-Dmd<sup>mdx</sup> congenic strain (produced in Aim 3) to evaluate the ability of each construct to rescue all or part of the Duchenne muscular dystrophy phenotype of the mice. Based on our phenotypic analysis of the DBA.B10-Dmd<sup>mdx</sup> congenic strain (Aim 3 and 4, below) we are confident that generating DMD genetic resources on the DBA/2J genetic background will be the strongest strategy with the largest biomedical relevance to the community.

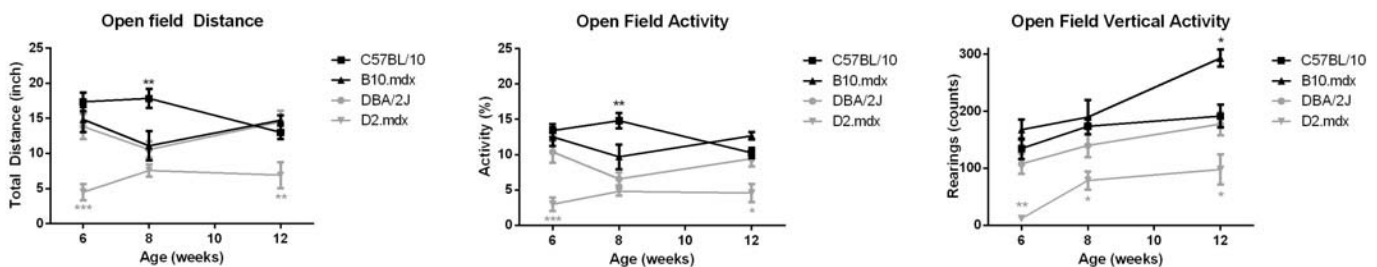
**Aim 3. Develop improved phenotypic mdx mouse models using genetic background variation in mice to map and identify genetic modifiers of disease severity. JAX.**

*3.1. Create a D2.B10-mdx congenic strain of mice on the DBA/2J genetic background.*

Based on descriptions that the DMD<sup>mdx</sup> mouse model had a more severe phenotype when crossed with a DBA mouse strain, we have completed the backcrossing of the mdx mutation onto the DBA/2J background using a speed congenic approach. The JAX DBA/2J substrain was chosen for this backcross as it was used by the Sanger Center for complete genomic sequencing to facilitate identification of genetic modifiers. The genetic quality control employed by the GRS at JAX during the marker-assisted backcross ensures that the resulting mice contain >99.9% of the DBA/2J background across the genome. Markers were chosen for genotyping out of 2,000+ markers in our set, spaced an average of approximately 1.5 Mb or 0.75cM apart and have been assayed on over 103 JAX® mouse strains, including virtually all of the most commonly used JAX® inbred and wild-derived inbred strains. Intercrosses between D2.B10-+/mdx heterozygous N5 congenic mice are currently being used to generate homozygous mdx/mdx mice on the DBA/2J genetic background followed for phenotypic analysis.



**Figure 2.** 12 week old males (pictured) show the absence of an overt phenotype in BL10.mdx mice. However, the muscle wasting and kyphosis of the D2.mdx mouse is evident at this early adult age.



**Figure 3.** Voluntary locomotor activity, Open Field (n=6-12 /group, males only) demonstrates that the D2.mdx mice display significantly reduced average track length covered in 30 min, % of time spent active and number of rearings during the 30 min in the open field compared with the B10.mdx strain and their respective parental strain controls.

3.2. *Assess the skeletal muscle regenerative capacity in a survey of 16 inbred background strains and create two congenic mdx strains on the backgrounds with the lowest regenerative capacity.*

Based on the significant increase in severity and the acceleration of disease symptoms we have discovered in the D2.B10-*Dmd*<sup>mdx</sup> congenic strain described above (**Figures 2 and 3**) and in **Figure 4** (below), we have modified our strategy for this sub-aim to more rapidly develop additional *mdx* mouse models for phenotypic analysis. We plan to utilize a CRISPER/Cas9 approach to target exon 23 of the mouse *Dmd* gene to create a series of DMD mouse models directly on eight additional genetically diverse mouse strains. The eight strains are A/J, C57BL/6J, 129S1/SvImJ, NOD/LtJ, NZO/HILtJ, CAST/EiJ, PWK/PhJ, and WSB/EiJ. These strains will allow us to capture nearly 90% of the known variation present in laboratory mice as potential genetic modifiers of disease. In addition, these strains have an additional advantage as they have been used to develop sophisticated genetic reference populations from an 8-way cross of these founder strains - the Collaborative Cross (CC) and Diversity Outcross (DO) populations. The use of CC and DO populations will facilitate genetic mapping and phenotypic confirmation of any potential modifier loci that might control disease pathology.

3.3. *Genetically map quantitative trait locus modifiers of mdx phenotypic severity derived from the DBA/2J background in an N2 backcross population of C57BL/6J x D2.B10-mdx mice.*

The mapping cross is currently underway but we have modified the strategy slightly to utilize an F2 intercross that will increase the genetic resolution of the map and speed the discovery of genetic recombinants. The significant enhancement of muscular dystrophy symptoms observed on the DBA/2J background (mild vs. severe) will allow us to better discriminate phenotypic variation such that additive effects that might be apparent in an intercross to aid in identifying additional modifier loci.

**Aim 4. Test three promising therapeutic compounds in the preclinical DBA/2J-*mdx* congenic model. K. Nagaraju, CNMC.**

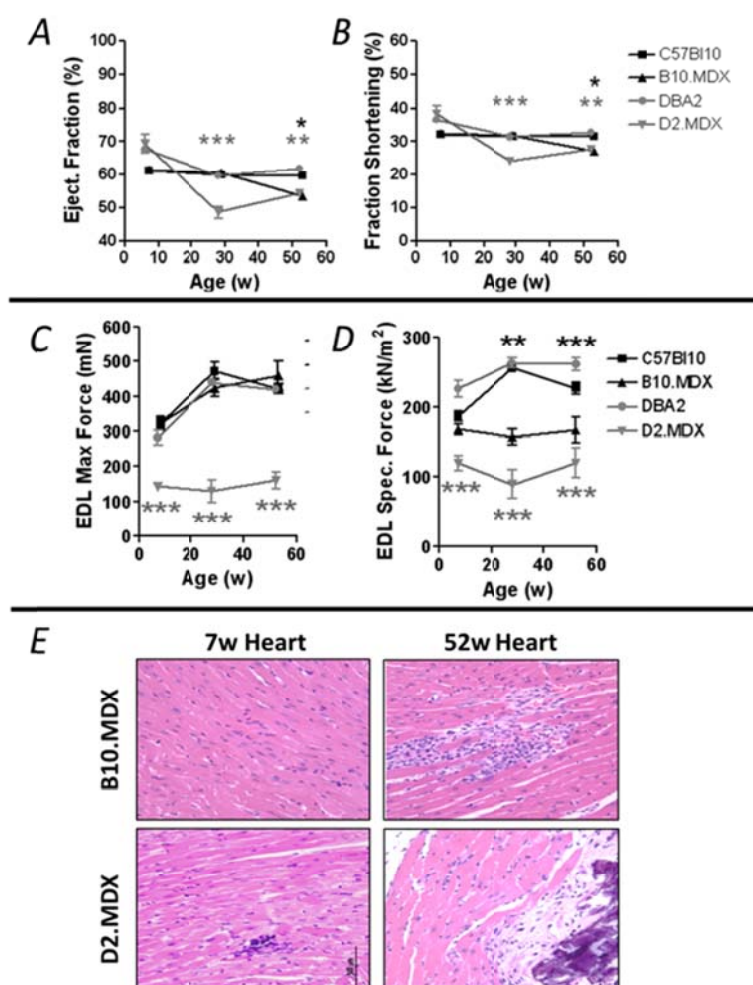
#### **Overview**

As outlined in Aim 4 of the grant proposal, we have established a D2.MDX colony and carried out an extensive characterization of this mouse using a battery of behavioral, functional, and histological measures. We have compared the D2.MDX mice to their B10.MDX counterparts, as well as both relevant control strains for up to one year. This extensive comparison allows us to state that the D2.MDX mice do indeed possess a more severe muscular dystrophy than the commonly used B10.MDX strain. Our work has also identified multiple measurable outcomes in D2.MDX that may be assessed at earlier time points. Consequently, the D2.MDX model of Duchenne muscular dystrophy (DMD) could potentially reduce the time required to carry out an animal study by half.

**Aim 4.1 Results:** For comparisons, D2.MDX mice were compared to B10.MDX mice and respective DBA2 and C57Bl10 control strains at three different time points (1.5 months, 7 months, and 12 months). The full results have been drafted into a manuscript for publication in a peer reviewed journal. The most novel findings were made during our assessment of cardiac performance, our assessment of muscle function, and our examinations of muscle histology.

*Cardiac performance:* The results from our echocardiography indicated that the D2.MDX mice suffer from a drop in cardiac performance as early as 7 months of age. This is an accelerated pathology compared to the common B10.MDX model that requires up to 12 months before any cardiomyopathy become apparent. Furthermore, the D2.MDX cardiomyopathy is already more severe at 7 months compared to B10.MDX mice. These striking results are seen in part in **Figure 4A and 4B**. This rapid heart damage makes the D2.MDX mouse a superior model for testing drugs that may improve heart performance.





**Figure 4.** A total of four mouse strains (C57Bl10, B10.MDX, DBA2, D2.MDX) were compared extensively at three time points (7, 28, and 52 weeks). Echocardiography revealed that the D2.MDX strain had significant deficits in cardiac performance at 28 and 52 weeks of age both in terms of the ejection fraction (A) and the shortening fraction (B). Skeletal muscle strength was also significantly reduced in the D2.MDX strain at all ages (C) although the specific force generated was not seen to be weaker until the animals were at least 28 weeks old (D). Representative histological findings from the heart of D2.MDX and B10.MDX mice at both 7 weeks and 52 weeks old are shown in panel E. The early appearance of damage in the D2.MDX heart is consistent with the loss in cardiac performance. In summary, the D2.MDX mice show an accelerated and more severe pathology compared to the commonly used B10.MDX strain.

**Skeletal muscle function:** Our quantitative assessment of muscle function is a very sensitive and powerful technique. Our results show that there is a dramatic loss of muscle strength in the D2.MDX mice compared to any other mouse strain at any age. A portion of these results are shown in **Figure 4C and 4D**. This weakness is measurable both in terms of the total force generated, and force generated per volume of muscle mass. This weakness also appears in the D2.MDX much earlier than in the B10.MDX mice. Again, the severity and accelerated course of disease symptoms in the D2.MDX mice makes this new model a superior choice for animal drug trials.

**Muscle histology:** The most striking feature visible in the muscle histology of D2.MDX mice is the appearance of calcified lesions within the skeletal muscle as early as 1.5 months of age. Similar lesions do not appear in B10.MDX animals until a year or later. A few representative images from the 1.5 month old animals are shown in **Figure 4E**. The majority of the data does not suggest that the inflammatory response is significantly different between the two strains, thereby suggesting that the appearance of these calcium deposits is due to innate differences in muscle fiber death and clearance within the muscle tissue. It must also be noted that at 1.5 months of age the D2.MDX23 mice show cardiac fiber degeneration and calcification centered over the right ventricle epicardium. This unusual pathology was not seen in B10.MDX animals even up to 12 months in age. Overall, these results reinforce the conclusion that compared to B10.MDX mice the D2.MDX cardiomyopathy is accelerated and more severe.

### Future directions

As mentioned previously, we are in the process of completing a manuscript based on the full results of our work on the D2.MDX strain. We are also maintaining the colony of D2.MDX mice in order to carry out additional experiments to discern the mechanisms that underlie the severe pathology seen in this particular strain. Our first two exploratory experiments will focus on two possible paths; whether or not the D2.MDX mice have a diminished capacity for muscle fiber regeneration, and whether or not the D2.MDX mice have a defective membrane repair mechanism.

## C. KEY RESEARCH ACCOMPLISHMENTS

- We have established an MD Repository at The Jackson Laboratory that is actively soliciting critical models of human muscular dystrophy.
- We have imported and re-derived seven additional lines of mice (Aim 1) in year 3 into the MD Repository to add to our over 20 models of muscular dystrophy currently available.
- Genetic and phenotypic information regarding all new lines of mice have been posted to the JAX website and will soon be consolidated into a dedicated page for muscular dystrophy models.
- We have generated full-length and internally truncated human dystrophin constructs for inclusion in targeted transgenic experiments that will test the potential efficacy of antisense oligonucleotide therapies to induce deletions of exons 44-45, 49-51, 48-53. Chimeric mice are breeding to determine germ-line transmission and expression of the transgenes will be tested in their offspring.
- We have created a D2.B10-*Dmd*<sup>mdx</sup> congenic strain of mice using a speed congenic protocol that shows significant muscle pathology.
- We have shown that Dantrolene treatment alone has minimal or no significant beneficial effects at the tested doses in mdx mice.

## D. REPORTABLE OUTCOMES

### Abstracts and presentations

- a) 2013 Dr. Lutz attended and presented at the Neuroscience Meeting in San Diego, CA
- b) 2013 Dr. Lutz and Dr. Nagaraju attended and participated in the workshop Developing Rigor in Congenital Muscle Disease Preclinical Testing in Washington DC
- c) 2013 Dr. Lutz and Dr. Nagaraju attended and presented talks at the Muscular Dystrophy Association Meeting in Washington DC
- d) Quinn, J., Huynh T., Uaesoontrachoon K., Tatem K., Phadke A., Vandermeulen J., Yu Q., Nagaraju, K. (2013) Effects of Dantrolene Therapy on Disease Phenotype in Dystrophin Deficient mdx Mice. MDA Scientific Conference, Washington DC

## E. CONCLUSION:

The development of an MD Repository will significantly increase the availability of high-demand strains of mice for research and will allow standardization of genetic background and genetic quality control to facilitate academic and pharmaceutical adoption of these strains for translational studies. We have established outreach into the muscular dystrophy research and clinical community (congenital muscle disease consortium) to determine the models most anticipated for preclinical research and we are actively soliciting those models for inclusion in the repository. In Aim 2, our transgenic experiments to express in-frame deleted forms of dystrophin will model the best-case-scenario outcome for AO-mediated therapy in which one assumes that a particular compound is

capable of 100% effective exon-skipping to restore the reading frame. By eliminating all of the caveats regarding the efficiency of delivery and pharmacodynamics of the particular therapeutic, we can provide a model that will allow functional analysis of the extent of phenotypic rescue for the three most common in-frame deletions for which clinical information from human Becker muscular dystrophy patients (deletion of exons 44-45, 49-51, 48-53) is lacking. In Aim 3, we have created a novel D2.B10-mdx congenic strain of mice that appears to be a better preclinical model of disease with an increased severity of myopathic symptoms. These congenic mice will also provide the starting point for a genetic modifier screen to identify genes and pathways that affect disease severity.