From Structure and Dynamics to Novel Therapeutic Development for Muscular Dystrophy.

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It is said that “you are only as good as who you surround yourself with,” and I’ve had the honor of meeting some of the most wonderful and inspiring people during my graduate training. Thank you all, because I am better for knowing you.
Dedication

This work is dedicated to the patients, friends and family coping with neuromuscular diseases: for your good will, moral support and enthusiasm for a better future.

And to all the researchers who have dedicated countless hours to push the forefront of medical advances.
Abstract

Dystrophin is defective in Duchenne (DMD) and Becker (BMD) muscular dystrophies, which are debilitating X-linked diseases that currently have no cure. Dystrophin links the actin cytoskeleton at its N-terminus and a glycoprotein complex (DGC) embedded in the sarcolemma at its C-terminus, apparently providing mechanical stability to the muscle during contraction (1-6). Due to the large size (427 kD) and filamentous nature of dystrophin, studies of its function and attempts to develop effective therapeutics have developed slowly, despite intensive efforts.

Utrophin (395 kD) is a homolog of dystrophin that has shown therapeutic promise in mdx mice, which lack dystrophin (7). Utrophin is endogenously expressed in the cytoskeleton of fetal and developing muscle but is replaced by dystrophin as the muscle matures (8-10). Both dystrophin and utrophin belong to the spectrin superfamily of actin-binding proteins, which carry out diverse functions in the cytoskeleton of most cells.

Of the many proteins included in this superfamily, dystrophin and utrophin are among the least studied in terms of structural dynamics, limiting the understanding of their function at the sarcolemma. In order to target the root of dystrophin malfunction in muscular dystrophy, we need to better understand the native functions of dystrophin and utrophin. Lack of structural information about dystrophin and its interactions adds to the complexity of tying clinical presentations to the diverse disease-causing mutations, and hinders therapeutic advancement in gene or drug therapy. There are numerous mouse-model studies, but there are varied results across several parameters tested, and no construct or drug has been found that restores normal muscle force in the mdx mouse (11,
12). Exon-skipping morpholinos are expensive to produce with variable delivery and efficacy to muscle groups and require a customized oligo design for each mutation, making it difficult to test them individually in mouse models (13, 14). In order to (a) understand disease mechanisms and (b) design better therapies rationally, we need more fundamental information about the structures and interactions of specific regions of dystrophin and utrophin (15). That is the goal of this project.
# Table of Contents

ACKNOWLEDGEMENTS ........................................................................................................................................ I
DEDICATION ........................................................................................................................................................ VI
ABSTRACT ............................................................................................................................................................ VII
TABLE OF CONTENTS ......................................................................................................................................... IX
LIST OF TABLES .................................................................................................................................................. XI
LIST OF FIGURES ................................................................................................................................................ XII
LIST OF EQUATIONS ........................................................................................................................................ XIV
LIST OF ABBREVIATIONS ................................................................................................................................... XV

## CHAPTER 1 – INTRODUCTION .................................................................................................................... 1

1.1 MUSCLE CYTOSKELETON AND DUCHENNE MUSCULAR DYSTROPHY ...................................................... 1
1.2 DYSTROPHIN AND THE DMD ...................................................................................................................... 2
  1.2.1 Causes of DMD ........................................................................................................................................ 3
  1.2.2 Defects in dystrophin leads to mechanical destabilization at the muscle sarcolemma .............................. 3
  1.2.3 The domains of dystrophin ..................................................................................................................... 5
  1.2.4 Disease-Causing mutations .................................................................................................................. 6
1.3 UTROPHIN AS A THERAPEUTIC SURROGATE FOR DMD ................................................................. 8
  1.3.1 A highly similar homolog protein – utrophin ....................................................................................... 8
  1.3.2 A fetal form of dystrophin ..................................................................................................................... 8
1.4 ANIMAL MODELS FOR DMD: .................................................................................................................. 9
1.5 EMERGING THERAPIES FOR DMD ........................................................................................................ 12
  1.5.1 Exon skipping ......................................................................................................................................... 13
  1.5.2 Utrophin up-regulation ....................................................................................................................... 14
  1.5.3 Gene therapy ........................................................................................................................................ 15
1.6 STRUCTURAL EVALUATIONS OF DYSTROPHIN AND UTROPHIN FOR THE ADVENT OF THERAPEUTIC
  DEVELOPMENTS ........................................................................................................................................ 16
1.7 AIMS ........................................................................................................................................................... 17
  AIM 1. Spectroscopic probes of actin structure and dynamics, as perturbed by utrophin, dystrophin and
  related constructs ........................................................................................................................................... 18
  AIM 2. Spectroscopic probes of utrophin and dystrophin structure and dynamics, as perturbed by
  actin ................................................................................................................................................................. 18

## CHAPTER 2 – PHOSPHORESCENCE, FLUORESCENCE AND EPR ...................................................... 19

2.1 THEORY OF FLUORESCENCE AND PHOSPHORESCENCE: .......................................................... 19
  2.1.1 Time-resolved Phosphorescence Anisotropy (TPA) ........................................................................... 21
  2.1.2 Fluorescence resonance energy transfer (FRET) .............................................................................. 22
  2.1.3 Time-resolved fluorescence vs. steady-state fluorescence ................................................................ 24
2.2 PRINCIPLES OF ELECTRON PARAMAGNETIC RESONANCE (EPR) ................................................... 25
  2.2.1 The hyperfine interaction ................................................................................................................... 26
2.3 DISTANCE MEASUREMENTS USING EPR: ............................................................................................. 27
  2.3.1 Dipolar broadening ............................................................................................................................ 27
  2.3.2 Dipolar CW-EPR and distance measurement ................................................................................... 29
  2.3.4 Determining distances from DEER waveforms ............................................................................. 33
List of Tables

**Table 1.** Table summary of the effects of individual domains in dystrophin (A) and utrophin (B) on actin rotational dynamics. ................................................................. 56

**Table 2.** Comparison of the distance and distance distributions (indicated as FWHM, full width at half maximum) of probed UtrABD1(195) and DysABD1 (Fig. 39). ......................... 103
List of Figures

FIG 1. DISTRIBUTION OF MUSCLE WEAKNESS IN DMD. ................................................................. 1
FIG 2. DYSTROPHIN LINKS THE ACTIN CYTOSKELETON TO THE EXTRACELLULAR MATRIX VIA THE
DYSTROGlycopROTEIN COMPLEX AT THE SARCOLEMMA MEMBRANE ...................................... 2
FIG 3. THE COMPLEXITY OF THE SIGNALING NETWORK AT THE COSTAMERE ........................................... 4
FIG 4. LOCATION OF THE IN-FRAME MUTATIONS CAN AFFECT DISEASE SEVERITY ........................... 7
FIG 5. DOMAINS OF DYSTROPHIN AND UTROPHIN ........................................................................ 8
FIG 6. COMMON ANIMAL MODELS FOR DMD ................................................................................. 9
FIG 7. A MULTIFACETED PROTOCOL PROPOSED FOR PRECLINICAL TESTING USING THE MDX MICE DURING
THE ACUTE PHASE OF MUSCLE DEGENERATION (3-6 MONTHS) ................................................. 12
FIG 8. VECTORS FOR GENE THERAPY DELIVERY ....................................................................... 15
FIG 9. DYSTROPHIN AND UTROPHIN TRITs ................................................................................. 16
FIG 10: ENERGY DIAGRAM OF FLUORESCENCE AND PHOSPHORESCENCE ............................. 19
FIG 11: SCHEMATIC OF TIME-RESOLVED PHOSPHORESCENCE ANISOTROPY INSTRUMENT ........ 21
FIG 12: RANGE OF FRET SENSITIVITY ......................................................................................... 23
FIG 13: TIME-RESOLVED FLUORESCENCE DETECTION SUPERIOR TO STEADY-STATE IN UNDERSTANDING
MOLECULAR STRUCTURE ........................................................................................................... 24
FIG 14: THE ZEEMAN EFFECT ........................................................................................................... 26
FIG 15. CYs-REACTIVE MaleimIDE SPIN LABEL (MSL) FOR CYs AND BIFUNCTIONAL SPIN LABEL (BSL) FOR
DI-CYs (CXXXC) .......................................................................................................................... 26
FIG 16. CHARACTERISTIC HYPERFINE SPLITTING FROM A NITROXIDE SPIN LABEL ..................... 27
FIG 17. DIPOLAR INTERACTIONS ..................................................................................................... 28
FIG 18. FOUR-PULSE DEER EXPERIMENT SEQUENCE ................................................................... 30
FIG 19. EPR ABSORBANCE SPECTRUM ........................................................................................ 31
FIG 20. DIAGRAM OF ACTIN FILAMENT ROTATIONAL MOTIONS IN COMPLEXES WITH DYSTROPHIN AND
UTROPHIN ..................................................................................................................................... 36
FIG 21. CONSTRUCTS EVALUATED BY TPA IN THIS STUDY .............................................................. 37
FIG 22: TPA RESULTS ON MINI-DYSTROPHIN (DysAH2-R19)............................................................ 39
FIG 23. TPA EFFECTS OF END-TRUNCATION CONSTRUCTS (LEFT DYSTROPHIN, RIGHT UTROPHIN) ON
ACTIN STRUCTURAL DYNAMICS ............................................................................................... 41
FIG 24: TPA OF ACTIN, SHOWING EFFECTS OF Micro-Utr, COMPARED WITH THOSE OF FULL-LENGTH
UTR AND Dys .................................................................................................................................. 43
FIG 25: THE RESILIENCE OF ACTIN .................................................................................................. 44
FIG 26.: CORRELATION OF RECOVERY SCORE (EQ. 27) WITH ACTIN RESILIENCE IN MDX MICE .... 48
FIG 27:. ACTIN FLEXIBILITY AND ORDER PARAMETER FROM TPA STUDIES ............................. 60
FIG 28: DEER DATA REVEALING THE STRUCTURAL DYNAMICS OF UTROPHIN CH DOMAINS AND A
CLOSED-TO-OPEN TRANSITION UPON ACTIN BINDING ........................................................... 64
FIG S29.: CD SPECTRUM OF (A) Utr261, (B) DOUBLE MUTANT V136C/L222C, AND (C) MSL-LABELED
V136C/L222C ................................................................................................................................ 66
FIG S30.: AFFINITY OF UTROPHIN CONSTRUCTS BINDING TO ACTIN ............................................. 68
FIG S31.: ANALYSIS OF DEER DATA .............................................................................................. 70
FIG 32.: DEER WAVEFORM (TOP) AND DISTANCE DISTRIBUTION (BOTTOM) BETWEEN THE TWO CH
DOMAINS OF UTR261, AS A FUNCTION OF THE MOLAR RATIO OF ACTIN TO UTR261 (SPIN LABELED
AT C136 AND C222). .................................................................................................................... 71
FIG 33.: DIPOLAR CW EPR SPECTRA OF SPIN-LABELED UTR261 ..................................................... 71
FIG 34.: MODEL-INDEPENDENT (TIKHONOV) FIT TO DEER WAVEFORM OF UTR261, LABELED AT C136
AND C222.................................................................................................................................... 73
FIG 35.: UTROPHIN BINDS TO ACTIN BY AN INDUCED-FIT MECHANISM ....................................... 74
FIG S36.: SECONDARY DISTANCE MEASUREMENT AT G75C/L222C ............................................. 75
FIG S37.: 21 SIMULATED MODELS OF THE ACTIN-BOUND UTR246 ............................................... 76
Fig. 38. The closed conformation (169) (A) is modeled directly from the dimeric crystal structure of DysABD1 (175) (B)................................................................. 85
Fig. 39. An open conformation of actin-binding in DysABD1 with marked similarities with UtrABD1........................................................................................................ 87
Fig. 40. Dimerization of DysABD1 with increasing salt concentrations.............................. 89
Fig. 41. FRET assay between two singly-labeled DysABD1 at residue 120 shows dimerization and not random self-association................................................................. 91
Fig. 42. The dimeric form of DysABD1 has lower affinity to actin........................................ 94
Fig. 43. In comparison, actin-bound UtrABD1 is not as sensitive to salt conditions ............ 95
Fig. 44. Binding to actin cannot protect against hydrophobic associations between DysABD1 in the presence of NaCl................................................................. 98
Fig. 45. Monomeric DysABD1 binding to actin exhibits no short distance components that would correspond to a closed model of binding............................................ 99
Fig. 46. Comparison of the actin-bound DysABD1 and UtrABD1 by simulations based on distance measurements................................................................. 101
Fig. 47. Structural flexibility of DysABD1 promotes intermolecular hydrophobic associations................................................................. 104
Fig. S48. Summary of FRET fits.......................................................................................... 113
Fig. S49. Summary of fits of Dys120/239.......................................................................... 114
Fig. S50. Summary of fits of Utr136/222.......................................................................... 114
Fig. S51. Dimerization in a doubly-labeled CH domains sample results in a faster DEER decay and dampened oscillations................................................................. 115
Fig. S52. Results from distance measurements between a secondary site 120/227 in DysABD1................................................................................................. 115
Fig. S53. Actin cosedimentation assays of native and spin-labeled DysABD1 at 120/239 and 120/227................................................................. 116
Fig. S54. Circular dichroism of native DysABD1, and spin-labeled 120/239 & 120/227........ 117
Fig. S55. Summary of fits of Dys120/227.......................................................................... 118
Fig. S56. A summary of actin mechanical properties.......................................................... 119
Fig 57.: Persistence length of actin filament bound to dystrophin or utrophin at 20% decoration................................................................. 121
Fig 58.: The bending flexibility of actin filaments modulated by dystrophin or utrophin................................................................. 122
Fig 59.: A comparison between the twist (defined by TPA) and bending (defined by persistence length)................................................................. 123
Fig 60.: Calculated resilience from twisting (TPA) versus bending (LP)................................ 124
Fig. 61. Diagram of entropic elastic recoil........................................................................ 125
Fig. 62. Correlation between observed structural disorder and protein function.............. 127
Fig. 63. Diagram of the relationship between hydrophobic association and entropic elastic force development in titin................................................................. 128
Fig 64.: Using utrophin as an example, models for how the non-actin-interacting CT region functions to increase cooperativity when present in the construct.............. 130
Fig. 65. Disease causing mutations in ABD1 of full length dystrophin............................... 132
Fig. 66. TPA results on disease-causing mutations in dystrophin....................................... 133
# List of Equations

<table>
<thead>
<tr>
<th>Eq.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phosphorescence Anisotropy Anisotropy</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>Overlap Integral of FRET Pairs</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>FRET Transfer Efficiency</td>
<td>22</td>
</tr>
<tr>
<td>4</td>
<td>Definition of R₀</td>
<td>23</td>
</tr>
<tr>
<td>5</td>
<td>Magnetic moment of electrons in EPR</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>Electron Spin Energy in EPR</td>
<td>25</td>
</tr>
<tr>
<td>7</td>
<td>Electron Spin Energy in EPR</td>
<td>25</td>
</tr>
<tr>
<td>8</td>
<td>Electron Spin Energy in EPR</td>
<td>26</td>
</tr>
<tr>
<td>9</td>
<td>Pake Pattern</td>
<td>28</td>
</tr>
<tr>
<td>10</td>
<td>Pake Pattern</td>
<td>28</td>
</tr>
<tr>
<td>11</td>
<td>Pake Pattern</td>
<td>29</td>
</tr>
<tr>
<td>12</td>
<td>Dipolar Broadening</td>
<td>29</td>
</tr>
<tr>
<td>13</td>
<td>Dipolar Evolution Function</td>
<td>30</td>
</tr>
<tr>
<td>14</td>
<td>Dipolar Modulation Frequency</td>
<td>31</td>
</tr>
<tr>
<td>15</td>
<td>DEER Echo Amplitude</td>
<td>32</td>
</tr>
<tr>
<td>16</td>
<td>Dipole Coupling in Pulsed EPR</td>
<td>32</td>
</tr>
<tr>
<td>17</td>
<td>DEER Waveform Function</td>
<td>33</td>
</tr>
<tr>
<td>18</td>
<td>Gaussian Function</td>
<td>33</td>
</tr>
<tr>
<td>19</td>
<td>Time-resolved Phosphorescence Anisotropy</td>
<td>51</td>
</tr>
<tr>
<td>20</td>
<td>Biexponential TR-Anisotropy Decay</td>
<td>51</td>
</tr>
<tr>
<td>21</td>
<td>Amplitude of Actin Rotational Motion</td>
<td>52</td>
</tr>
<tr>
<td>22</td>
<td>Rate of Actin Rotational Motion</td>
<td>52</td>
</tr>
<tr>
<td>23</td>
<td>Resilience (TPA)</td>
<td>52</td>
</tr>
<tr>
<td>24</td>
<td>Actin Binding</td>
<td>53</td>
</tr>
<tr>
<td>25</td>
<td>Simulated Fraction-Bound to Actin by Quadratic Binding Equation</td>
<td>53</td>
</tr>
<tr>
<td>26</td>
<td>Cooperativity Function</td>
<td>54</td>
</tr>
<tr>
<td>27</td>
<td>Recovery Score</td>
<td>55</td>
</tr>
<tr>
<td>28</td>
<td>Pearson’s Correlation Coefficient</td>
<td>55</td>
</tr>
<tr>
<td>29</td>
<td>Gaussian Distribution</td>
<td>82</td>
</tr>
<tr>
<td>30</td>
<td>Gaussian Distribution</td>
<td>110</td>
</tr>
<tr>
<td>31</td>
<td>CD Analysis</td>
<td>112</td>
</tr>
<tr>
<td>32</td>
<td>2D Correlation Function</td>
<td>121</td>
</tr>
</tbody>
</table>
List of Abbreviations

ABD, Actin-binding domain
AFM, Atomic force microscopy
AON, Antisense oligonucleotides
BMD, Becker muscular dystrophy
CD, Circular dichroism
CH, Calponin homology
CNS, Central nervous system
CT, C-terminal region
CW, Continuous wave
DABCYL, 4-((4-(dimethylamino)pheyll)azo)benzoic acid
DCM, Dilated cardiomyopathy
DEER, Dipolar electron-electron resonance
DGC, Dystroglycoprotein complex
DMD, Duchenne muscular dystrophy
Dys, Dystrophin
ECM, Extracellular matrix
EGTA, Ethyleneglycol-bis-(2-aminoethyl ether)N,N,N,N-tetraacetic acid
EM, Electron microscopy
EPR, Electron paramagnetic resonance
FRET, Fluorescence resonance energy transfer
FWHM, Full width at half maximum
IAEDANS, 5-(((2-idoacetyl) amino) ethyl) amino)naphthalene-1-sulfonic acid
IRF, Instrument response function
K_d, Dissociation constant
kDa, Kilodaltons
L_p, Persistence length
MSL, N-(1-oxy-2,2,5,5-tetrametyl-4-piperidinyl)maleimide
NaCl, Sodium chloride
NMR, Nuclear magnetic resonance
NOS, Nitric Oxide Synthase
PDB, protein data bank
PMO, Phosphodiameate morpholino oligomers
R_0, Forster distance
rAAV, Recombinant adeno-associated virus
RMSD, Root-mean-square deviation
SDM, Site-directed mutagenesis
SEM, Standard error of mean
STR, Spectrin-type repeats
T1, Spin-lattice or longitudinal relaxation time
T2, Spin-spin or transverse relaxation time
TA, Tibialis anterior
TPA, Time-resolved phosphorescence anisotropy
TRIS, 2-amino-2-hydroxymethyl-propane-1,3-diol
TRIT, Therapeutically-relevant internally truncated constructs
Utr, Utrohhin
WT, wild-type
CHAPTER 1 – Introduction

1.1 Muscle Cytoskeleton and Duchenne Muscular Dystrophy

The muscular dystrophies are characteristic myogenic disorders with signature progressive muscle weakness and wasting. They are classified by the origin of mutation and the distribution of pronounced muscle weakness (16). The most common of the muscular dystrophies is Duchenne muscular dystrophy, which was originally identified by Dr. Edward Meryon in 1851. (17) Meryon predicted an X-link genetic affliction which results in a break down of the sarcolemma in this type of muscular dystrophin from histological studies. He showed that the spinal cord was not affected, therefore the disease is purely myogenic. It wasn’t until the 1861 edition of “Paraplegie hypertrohique de l’enfance de cause cerebrale” was published by Guillaume Benjamin Amand Duchenne did the disease earn its name as Duchenne muscular dystrophy.

Duchenne (DMD) and the milder form Becker muscular dystrophies (BMD) are best recognized clinically due to the distinct regions of muscular weakness in the proximal before the distal limb muscles and lower before the upper extremities (Fig 1). This is classically reflected in the delayed development for affected boys to walk in the more severe cases. They often adapt an unusual waddling or steppage gait, where they maximizes the stronger distal calf muscles when they walk. Proximal weakness also triggers the hallmark Gower’s

![Fig 1. Distribution of muscle weakness in DMD. Adapted from (16)](image-url)
sign, in which affected boys will use their upper body strength to push themselves up from a sitting position on the floor due to weakness in the proximal hip muscles.

Though the predominant clinical presentation is progressive skeletal muscle weakness, there is also a later onset of cardiac involvements. The similarities in the ultrastructure between skeletal fascicles and the rectangular cardiomyocytes was proposed to be the reason for the shared defects due to mutations in dystrophin (18). While the skeletal muscles preserve a certain degree of regeneration capability from satellite cells, cardiac muscles most commonly develop connective tissue or scar from injuries due to its much more limited regenerative capability (18). Thus, in milder forms of BMD or in carriers, defects in dystrophin mostly cause a clinical presentation of X-linked cardiomyopathy (19), which was seen in all 328 patients over 18 years old surveyed in a ten-year longitudinal study (20). Cardiac involvement in DMD or BMD patients can be easily missed due to the low physical activity in these patients, but its significance cannot be overlooked. One pediatric study showed that 26% of the cases with dilated cardiomyopathy (DCM) was associated with neuromuscular diseases. (21).

1.2 Dystrophin and the DMD

Fig. 2. Dystrophin links the actin cytoskeleton to the extracellular matrix via the dystroglycoprotein complex at the sarcolemma membrane. (modified from (22)).
1.2.1 Causes of DMD

Duchenne (DMD) and Becker (BMD) muscular dystrophies are caused by mutations in dystrophin, a 427kD protein localized to the cytoskeletal lattice of the sarcolemma (23) (Fig. 2). The dystrophin gene is located on the X chromosome and is the largest gene identified in humans (24). This 2.3 mega bases gene on chromosome Xp21.2 produces a giant 427kD dystrophin, which is a structural protein in the muscle costamere that physically couples the sarcolemma with the Z disk in myofibrils (25). Defects in dystrophin increases costamere fragility and susceptibility to damage during muscle contraction, leading to myopathies seen in DMD and BMD (24).

1.2.2 Defects in dystrophin leads to mechanical destabilization at the muscle sarcolemma

The physical connection of dystrophin between cytoskeletal actin and the DGC allows a mechanical linkage to transduce lateral force and prevent damage to the sarcolemma during muscle contractions (26-28). Lack of functional dystrophin in skeletal muscles results in disarray of the cytoskeletal organization at key structural regions in striated muscles (1-3), disabling proper transmission or diffusion of lateral force (4, 5), and rendering the muscle prone to eccentric contraction damage (6). AFM studies showed a decrease in the stiffness of myocytes due to lack of dystrophin (29). A similar loss in stiffness was observed when cytochalasin D was applied to disrupt the actin
cytoskeleton, indicating that the dystrophin-actin interaction plays a central role in maintaining mechanical stability in the muscle cytoskeleton (29).

Dystrophin assists in the proper assembly of the DGC. In addition to providing mechanical buffering by transmitting lateral force, the DGC is also actively involved with key signaling processes vital to the health of the myocyte. (30-33). The signaling pathways at the DGC are complex, due to the multifaceted protein-protein interactions at that junction (Fig. 3).

Fig. 3. The complexity of the signaling network at the costamere, with dystrophin being at the very center of it (28).
1.2.3 The domains of dystrophin

Dystrophin is comprised of 4 domains, a very elongated protein that spans a length of 120nm to 125nm (34). Dystrophin’s N-terminus consists of a tandem calponin homology (CH) domains which bind to cytoskeletal γ-actin (35-37). The dystrophin C-terminus (CT) contains mainly the WW, EF hand and ZZ motifs which bind to the dystroglycoprotein complex (DGC) at the sarcolemma membrane (38).

The largest domain in dystrophin is the central 24 spectrin-type repeats (STR). These repeats are identified based on their homology to the repeats in spectrin, which is in the same actin-binding protein family (34, 39). The STRs bundle into triple helical coiled-coil motifs due to amphipathic moment and form cylinrdicals approximately 5nm long and 2nm in diameter. Investigation of the properties of spectrin repeats began with erythrocyte spectrin (34). The STRs were first identified as a major component in erythrocyte membrane cytoskeleton. Its unique arrangement with other members of the cellular matrix suggested a viscoelastic property for spectrin. Based on studies done on the repeats in spectrin (40) and α-actinin in red blood cells (41), there are several mechanisms proposed for the STRs, including a shock absorber, force transducer or a spacer for these elastomeric proteins. However, the STR domain is much longer in dystrophin, and how they contribute to its function as a mechanical stabilizer in muscle remains to be investigated.

In the central STR domain of dystrophin there are 4 structurally undetermined “hinge” regions which was proposed to provide flexibility or elasticity in the dystrophin molecule in addition to the triple-helical coils in the STRs (42, 43). There is an additional
actin-binding site located within STR 11-17, which contains basic residues that utilizes electrostatic interactions with actin to provide additional mechanical buffering (22, 44).

The C-terminal region of dystrophin assembles the DGC, which is comprised of an assembly of the dystroglycans, sarcoglycans, sarcospan, dystrobrevins, syntrophins and nNOS (45). The DGC binds to the laminin complex in the muscle extracellular matrix (ECM), and thus lateral force generated during muscle contractions is transmitted through the actin-dystrophin-DGC-ECM and preventing unwanted damage to the sarcolemmal membrane (24, 45).

### 1.2.4 Disease-Causing mutations

The majority of disease-causing mutations in dystrophin are deletions, occurring at approximately 72% in patients with DMD and 85% with BMD (46). The remaining 15% to 20% of the patients have point mutations in the coding sequence or at splice sites. These point mutations can result in central deletions or premature truncations, though single amino acid replacement throughout the dystrophin molecule have shown to be equally or more devastating than deletions (47). The reading-frame rule was first proposed in 1988 to explain why certain mutations cause a mild BMD while others a severe DMD (48). Mutations that maintain the correct reading frame tend to be less severe and result in BMD, while out of frame mutations lead to the more devastating DMD. This rule is consistent with 91% of the 4700 mutations in the Leiden DMD mutation database (46).
In addition, the location of in-frame mutations also impacts disease severity (46). Deletions in the N- or C-terminal regions are most severe while a large portion of the central spectrin-type repeats (STR) domain can be removed with only mild clinical phenotype (49-51). While there are several endogenous forms of dystrophin in the humans with variable N-terminal deletions due to an internal start site, there are no known natural deletions of the C-terminal regions (52). Mouse models demonstrated that deletions of the C-terminal regions are more severe when the β-dystroglycan binding site (exon 64-67) is compromised (52). Reported clinical cases also show that deletions in the C-terminal region that does not affect β-dystroglycan binding (exon 72-79) have minimal impact on the functionality of dystrophin or the health of the myocytes. Conversely, a deletion in the β-dystroglycan binding site is heavily severe, particularly if the mutation is within the dystrophin Cys-rich region (Fig 4) (52-55).

Fig 4. Location of the in-frame mutations can affect disease severity (46).
1.3 Utrophin as a therapeutic surrogate for DMD

1.3.1 A highly similar homolog protein – utrophin

Utrophin is a 395kD dystrophin homolog that is present at the subsarcolemmal region, with functions similar to dystrophin in fetal or regenerating muscles (3) (Fig 5). As muscle fibers mature, utrophin is replaced by dystrophin (8-10) and utrophin has been proposed as a viable therapeutic replacement for mutated dystrophin in muscular dystrophy (7, 58). Utrophin also contains a highly homologous N-terminal actin-binding domain (ABD1) consisting of tandem calponin homology (CH) motifs, followed by a central domain containing a series of triple-helical spectrin-type repeats (STR), and a C-terminal (CT) region (35, 59). However, dystrophin and utrophin have distinctly different lateral interactions with actin (57); Dystrophin’s second actin-binding domain (ABD2) is separated from ABD1 by 10 STR (22, 44), while utrophin’s ABD2 is adjacent to ABD1 (56, 60).

1.3.2 A fetal form of dystrophin.

The reason to for the change from utrophin to dystrophin as the muscle matures is largely unknown. There is some evidence to suggest that the utrophin-actin-DGC complex is more flexible, and therefore creates the optimal environment for a developing
muscle (61). The microenvironment for a myocyte to properly organize actomyosin striations require a very narrow range of cellular elasticity, which is centered around the elasticity of the muscle cell (62, 63). But in a mature contracting muscle, which sustains larger strain at the sarcolemma, a stiffer dystrophin-actin-DGC complex may be required. However, this argument is complicated by the fact that utrophin up-regulation in dystrophin-deficient mice was equally effective as wild-type dystrophin (7). Up-regulation of utrophin did not revert the mature myofibrils into myotubes. This is quite an interesting line of pursuit since there are no disease-causing mutations in utrophin, while dystrophin mutants affect predominantly the skeletal muscle and can involve CNS and cardiac muscles.

Table 2: Overview of animal models for Duchenne muscular dystrophy gene-therapy studies

<table>
<thead>
<tr>
<th>Model</th>
<th>Mutation/Region</th>
<th>Effect</th>
<th>Pathologic/Physiologic symptoms</th>
<th>Gene-therapy studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>mdx mouse</td>
<td>Nonsense mutation in exon 23 (3185C&gt;T)</td>
<td>Stop codon introduced, dystrophin synthesis aborted prematurely</td>
<td>Fibre degeneration (particularly from 2-3 weeks), replacement by centrally nucleated regenerating fibres, myopathy less severe later in life, normal lifespan</td>
<td>For use in strategies owing to its relative experimental simplicity</td>
</tr>
<tr>
<td>CVMD/GRMD</td>
<td>5’ splice-site point mutation in intron 5 (739-910+5)</td>
<td>Exon 7 skipped from transcript, frame shift, dystrophin synthesis aborted prematurely</td>
<td>Severe delay, massive muscle degeneration, resembles human DMD best compared with other models</td>
<td>Used in most strategies owing to its relative experimental simplicity</td>
</tr>
<tr>
<td>HFMD</td>
<td>Deletion of Cpa427q and Dnp427q promoter regions</td>
<td>No muscle dystrophin expression</td>
<td>Large areas of muscle-fibre degeneration and regeneration, mononuclear infiltration, hypertrophied fibres</td>
<td>None</td>
</tr>
</tbody>
</table>

Note: Further mouse mutants (mdx<sup>257</sup>-<sup>711</sup>) that have been generated by N-ethyl-N-nitrosourea (ENU) mutagenesis are not often used for therapeutic studies. 
CVMD, canine X-linked muscular dystrophy; DMD, Duchenne muscular dystrophy; GRMD, golden retriever muscular dystrophy; HFMD, hypertrophic false muscular dystrophy.

Fig 6. Common animal models for DMD. Adapted from (64)

1.4 Animal models for DMD:

There are several naturally occurring mutations in other organisms that produce quasi-equivalent myopathies akin to the human form of DMD. The most famous is the X-linked myopathy in golden retriever dog models (grmd), which was shown to be near
identical to the human form of DMD (65) (Fig 6). Although several invertebrate models are being established, their benefits are still unclear (66, 67). Several mouse lines have exhibited muscular pathologies that mimic DMD (68, 69), but there is a controversy over whether they are myogenic or neurogenic in origin (70, 71).

In vivo investigation into the pathophysiology of DMD was greatly advanced by the finding of a spontaneous mutation (mdx) in an inbred C57BL/10 colony (72) (Fig 6). A single nucleotide mutation in exon 23 results in a reduced dystrophin RNA levels and the absence of dystrophin (24, 73). Necrotic myofibers was seen as early as 5 days postnatal. Although the mdx mice have high serum levels and histological lesions, they do possess unique qualities that are dissimilar to DMD (74). Detailed examination of the tibialis anterior muscle by mechanical and histological testing indicates that there is an acute episode of muscle degeneration at 3-4 weeks of age, but transitions to a chronic low-level damage by 8 weeks and even experiences further improvement after 1 year of age (75, 76). Though 20-80% of the muscles can be affected during the acute necrotic phase around 21 days of age in mdx mice, the presence of reverent fibers and hypertrophy stabilizes the degeneration and regeneration process to ~4-6% active damage after 8 weeks of life (76, 77). The reason for the decline in degeneration after 3 weeks of age in the mdx mice is unknown. Studies have proposed that the up-regulation of utrophin (78), genes in creatine synthesis and proteins involved in excitation-contraction coupling (79, 80) are contributing factors.

Studies of the mdx mice show that fast-twitch fibers are most susceptible to damage from lack of functional dystrophin compared to other fiber types (81, 82).
Though quadriceps have an earlier onset (16-17 days), the tibialis anterior (TA) muscle suffers the most severe damage (~90% during the acute phase) (81, 82). This is reflected in the heightened susceptibility to lengthening contractions, or eccentric contractions in TA muscles (83, 84), a hallmark for dystrophic muscles that is shared between DMD and mdx mice (6, 85, 86). In contrast, extraocular muscles, masseter and laryngeal muscles appear largely spared from dystrophic degeneration (87-89).

There are also reports of gender bias, that female hormones affect the cytoskeletal function in the mdx mice (90). Although young female mdx mice have milder phenotype, older mice (1-2 years) and those that underwent ovariectomies showed increasing myonecrosis (90).

Despite the underlying dystrophic pathology in the mdx model, it is comparatively milder than the human form of DMD after the 8 months of life (76, 77), and this is in part attributed to the higher endogenous level of utrophin at the subsarcolemmal region (78). A dystrophin and utrophin-null mice (dko, mdx/utrn−/) was generated to clarify the contribution of the utrophin upreguation in stabilizing the phenotype in the mdx (91, 92). The dko model shows a much more severe phenotype that correlates better with findings in DMD patients. Unlike the mdx mice, which can have a lifespan similar to that of the wild-type (18 months), the dko model have a shortened average survival of 8.2 weeks with much more severe and progressive dystrophin presentations (93). Though more similar to the human form of DMD, there is comparatively less studies conducted using the dko model since it was only available in the past decade or so (93). Thus, studies to set up preclinical parameters have only been completed for the mdx model (66, 75, 94,
95) (Fig 7), despite the recent studies that show beneficial transformative potentials when using the dko model for therapeutic texting (93, 96, 97). Since studies using the mdx model are currently more prevalent, with more complete documentations, we have used the results from this model to compare with our *in vitro* analysis in Chapter 3.

**1.5 Emerging therapies for DMD**

There have been many promising revenues in terms of novel therapeutic developments for DMD. In the following section, I summarized the advances made in the three most promising areas to date.

![Flow chart for a typical pre-clinical efficacy drug trial](Image)

**Fig 7. A multifaceted protocol proposed for preclinical testing** using the mdx mice during the acute phase of muscle degeneration (3-6 months) (75).
1.5.1 Exon skipping

Approximately 50% of patients with DMD have sparingly distributed revertant fibers due to spontaneous frame-restoring skipping over a region of exons (48, 98). From this observation, it was proposed that severe DMD presentations can be alleviated by converting the mutant dystrophin into its nearest in-frame counterpart. One of the first constructs to head into clinical trial testing was PTC-124, which was specifically designed for this purpose. It is a small molecule that binds to the ribosome and desensitize it to the presence of stop codons (99, 100), effectively allowing the translational machinery to “read-through” non-sense mutations. Despite the low off-target potential with minimal side-effects, PTC-124 (Ataluren) was only able to slow ambulatory decline for specific non-sense mutations (Reports from Genzyme, 2010). In addition, the marginal benefits of PTC-124 did not have a linear dose-dependence, with high dose regimes toxic or non-effective to the muscle. Thus, other alternatives to induce exon skipping are also considered.

Other methods to “skip” over mutation-containing exons include the use of antisense oligonucleotides (AON) to sterically block transcription of that region (93, 101). Due to the relatively short half-life of AONs, a modified form of phosphodiamidate morpholino oligomers (PMOs) with a modified back bone was synthesized (102). The morpholino ring and dimethylamino phosphorodiamidate group prevents enzyme degradation and have been effectively used in embryonic systems (103-106). Despite its promise, PMOs have their distinctive drawbacks: (1) large doses that lead to toxicity are required for benefit (>100mg/kg) (11, 107, 108) (2) large variations in effect (11, 107),
even within a specific muscle (3) little efficacy in cardiac improvement (109) (4) and this form of patient-based treatment can prove to be costly.

1.5.2 Utrophin up-regulation

Ever since the first publication that utrophin is an effective therapeutic surrogate in dystrophic mice ($mdx$), utrophin became a popular homolog protein of study in DMD (7, 58). Most importantly, utrophin overexpression in non-muscle tissues is not detrimental, lowering possible side-effects from treatments (106). There are several ways to induce utrophin expression in vivo. Heregulin (110, 111), L-arginin (110, 111), RhoA (112) and biglycan (113) have all showed to be efficacious. A phase I clinical trial tested a promising small molecule, BMN195 (or SMT C1100) that upregulated utrophin in mouse models, but suffered decreased bioavailability humans and thus did not reach efficient plasma concentrations for effective treatment (114).
1.5.3 Gene therapy

One way to mitigate pathology caused by a defective protein is to replace it with an equally functional molecule through gene delivery. Many vectors for gene delivery of this purpose have been tested over the years (Fig 8). The vector with the highest transfection efficiency into skeletal muscles are derived from adeno-associated virus (rAAV) (115). However, there is a 4.8kb size limitation for delivery due to the capacity of the capsid for rAAV (116, 117). This necessitates using a internally truncated dystrophin or utrophin (therapeutically relevant internally truncated constructs, TRITs)(Fig 9). Some serotypes of rAAV can induce unwanted immune response to the vector due to its prevalence (118). This can be avoided by using alternative rAAV serotypes or further vector engineering (119, 120). Other studies have also suggested
applying immunosuppressant regimens during rAAV treatment is beneficial in large animal models (grmd) (121).

Alternatively, Ervasti et al. have also been successful in direct delivery of the utrophin construct proteins into skeletal muscle using the transactivator of transcription (TAT) protein derived from the HIV virus (97, 122).

1.6 Structural evaluations of dystrophin and utrophin for the advent of therapeutic developments.

Initial clinical trials or pre-clinical trials using either exon-skipping or rAAV gene therapy have so far been disappointing. Ataluren was not as effective as expected and did not meet its secondary endpoint goals in Phase IIb (reports from PTC Therapeutics at American Academy of Neurology Meeting 2010). Interestingly, those who were on a small dose regimen showed improvement in the 6 minute walk test, but those on high dose regimens showed no improvement. Although lose dose Ataluren improved ambulatory abilities in the DMD patients, they still experienced significant functional decline. The mini-dystrophins are limited by their size and efficient delivery (123). Studies in the dog model (grmd) was severely limited by unwanted immunoreaction (124,
and investigations in immunosuppression have presented with varied effects (121, 126). Therefore, there is a lack in functional studies to determine its restorative potential in large animal models. The mini-dystrophin was highly effective in mouse models, but expression levels as high as 10x the endogenous level were needed for complete recovery (127). Micro-dystrophin constructs have higher transfection efficiency, but do not restore mechanical strength despite impressive morphological improvements (117, 128, 129).

In comparison with physiological studies into these therapy constructs, the biochemical and biophysical characterizations of these proteins at a molecular scale lack in comparison. A more effective gene therapy construct is needed. It is also unclear as to how to better choose or define the boundaries of the “skipped” dystrophin region with exon-skipping to create a better functional protein (14). Thus, the study of the individual domains in dystrophin has strategic therapeutic value in the rational design of novel treatments for DMD and BMD.

1.7 Aims

Our long-term goal is to define the molecular structure and dynamics that influence the functions of dystrophin and utrophin at the muscle cytoskeleton. In comparison with physiological studies, structural evaluations of dystrophin and utrophin lax in comparison. Despite the current advances in physiological studies into the functions of dystrophin, utrophin and related gene products, we have yet to find a workable treatment for Duchenne (DMD) and Becker (BMD) muscular dystrophies. A biophysical blueprint would greatly aid in designing the next generation of muscular
dystrophy therapeutics. The goal of the current proposal is to elucidate the structural dynamics of dystrophin and utrophin, focusing on their interactions with actin. We aim to use site-directed spectroscopic probes (phosphorescence, fluorescence [FRET], and electron paramagnetic magnetic resonance [EPR]) to investigate the structure and dynamics of dystrophin and utrophin, and their complexes with actin in solution.

**AIM 1. Spectroscopic probes of actin structure and dynamics, as perturbed by utrophin, dystrophin and related constructs.**

Structural dynamics of actin will be analyzed as affected by utrophin and dystrophin. Smaller constructs of these proteins will also be tested, to determine which domains are most important in determining interactions with actin. This AIM also relates to physiological studies of therapeutic constructs and disease-causing mutations.

**AIM 2. Spectroscopic probes of utrophin and dystrophin structure and dynamics, as perturbed by actin.**

Site-directed Cys mutagenesis will be used in isolated actin-binding domain 1 of dystrophin and utrophin (DysABD1 and UtrABD1). These sites will be labeled with nitrooxide spin labels, and intramolecular distances and dynamics will be measured by EPR, in the absence and presence of actin. Distance distributions measured will be used to refine structural models based on EM.
2.1 Theory of Fluorescence and Phosphorescence:

Luminescence is the emission of photons when an electronically excited molecule returns to the ground state as described by a Jablonski diagram (Fig 10). This diagram illustrates the various electronic states of a molecule and its energy transitions: A singlet ground state (X) and two higher singlet excited states (S₁ and S₂). The triplet states (T₁) is the electronic state with parallel spins. While transitions between electronic states of the same spin multiplicity are allowed, transitions between states of different spin multiplicity are not.

![Energy diagram of fluorescence and phosphorescence](image)

**Fig 10:** Energy diagram of fluorescence and phosphorescence. A Jablonski diagram showing electronic transitions for fluorescence and phosphorescence processes. Straight arrows represent radiative transition (absorption or emission of photon). Dashed arrows represent non-radiative transitions (relaxation, quenching). Adapted with modifications from (130).
multiplicity are forbidden, but can occur by spin-orbit coupling or intersystem crossing.

Since thermal energy is not enough to populate the excited vibrational states under room temperature, so the excitation is typically from the lowest vibrational state of \( S_0 \). The rate constants for radiative and non-radiative processes (dashed boxes) are indicated in Fig 10. Fluorescence is the process of an excited molecule undergoing first an internal conversion \( (k_{IC} \sim 10^{-12} \text{ s}) \), then returning down to ground state \( S_0 \) with a rate constant \( k_F \sim 10^8 \text{ s} \) (Fig 10, green). Phosphorescence has an additional intersystem crossing \( (k_{IC} \sim 10^{-6} \text{ s}) \) to a triplet state \( T_1 \) before decaying to ground state. Due to the forbidden nature of this transition, the lifetime of phosphorescence is longer, on the scale of microseconds to seconds compared with the nanoseconds in fluorescence (131). Additional non-radiative processes, such as collisional quenching and fluorescence resonance energy transfer can also depopulate the excited state.

The emission spectra are independent of the excitation wavelength. When a fluorophore is excited from ground state, excess energy is quickly dissipated through vibrational relaxation \( (k_{VR} \sim 10^{-12} \text{ s}) \). Thus, nearly all emission waveforms are detected from the lowest excited state in \( S_1 \) to the ground state \( (S_0) \), eliminating any influence from the excitation wavelength (132).
2.1.1 Time-resolved Phosphorescence Anisotropy (TPA)

The TPA setup is similar to the time-resolved high-performance direct waveform recording instrument (Fig 11). The samples are excited with a vertically polarized laser (JDS Uniphase NanoLaser, 10 kHz pulse rate, 1ns FWHM). Phosphorescence emission passes through a sheet polarizer that is automated to filter both the vertical or horizontal positions before a band pass filter and finally to a photomultiplier (Hamamatsu R928P). The waveform output from the photomultiplier is compiled on board PCI digitizer (GaGe, CS14100 ), featuring 100M/s sampling rate, giving rise to 1 μs maximum temporal resolution. The IRF of TPA is negligible due to the narrow pulse width of the laser (1ns FWHM), which is minute compared to the experimental time-resolution.

Time-resolved phosphorescence anisotropy was calculated from (133):

\[ r(t) = \frac{(I_{VV} - GI_{VH})}{(I_{VV} + 2GI_{VH})} \]

Eq. 1

where G is an instrument response correction factor and \( I_{VV} \) and \( I_{VH} \) are the vertically and horizontally polarized components of the emission from signal averaging of 1000 laser pulses at each orientation.

Fig 11: Schematic of time-resolved phosphorescence anisotropy instrument. The overall system is very similar to the DWR fluorescence.
TPA will allow us to monitor changes in actin’s rotational motions (twisting) in the microsecond time range which we will demonstrate in Chapter 3.

2.1.2 Fluorescence resonance energy transfer (FRET)

FRET is a distance-dependent transfer of non-radiative energy from a donor molecule to an acceptor molecule. This energy transfer, or “resonance” occurs over greater than interatomic distances, without conversion to thermal energy or molecular collision. Several factors are required for FRET to occur: (1) the donor-acceptor distance should be within a certain range (typically 10-100Å), (2) the absorption spectrum or excitation spectrum of the acceptor fluorophore must overlap the fluorescence emission spectrum of the donor. This overlap in spectrum is also known as the overlap integral (J), which contributes to the $R_0$ parameter, which is the Forster distance at which half the energy is transferred between donor and acceptor.

The overlap integral $J$ is defined by:

$$J(\lambda) = \frac{\int F_D(\lambda) \varepsilon_A(\lambda) \lambda' d\lambda'}{\int F_D(\lambda) d\lambda}, \quad \text{Eq. 2}$$

Where $F_D(\lambda)$ is the donor emission spectrum and $\varepsilon_A(\lambda)$ is the acceptor absorption system.

The efficiency of the transfer process depends on the equation:

$$R = \frac{R_0^6}{R_0^6 + r^6}, \quad \text{Eq. 3}$$
Where \( r \) is the experimental distance between the donor and acceptor probes. \( R_0 \) dictates the sensitivity range of a donor-acceptor pair (Fig 12). Other parameters that affect \( R_0 \) include the orientation factor \( (\kappa) \), index of refraction \( (n) \) and the quantum yield of the donor \( (\phi) \) as summarized in:

\[
R_0 = 9.790 (j\kappa^2 n^{-4} \phi_D)^{-6},
\]

\text{Eq. 4}

The orientation factor \( \kappa^2 \) is dependent on the orientation vectors of the donor and acceptor transition dipoles and is approximated to 2/3 under isotropic conditions.

We applied FRET for several interdomain high resolution distance measurements, and is described in more detail in Chapter 5.

Fig 12: Range of FRET sensitivity. The highest sensitivity is centered at \( r = R_0 \). Adapted from Dr. David Thomas’ lecture notes and (134).
Though typically more complex and expensive, time-resolved fluorescence can preserve key structural information in its detection, while much molecular information is lost in the averaging process in steady-state detection. Steady-state fluorescence only provides information regarding changes in donor fluorescence intensity, while time-resolved measurements can provide additional information. As indicated in Fig 13, we can resolve an inhomogeneous sample due to identification of a multiexponential decay in

Fig 13: Time-resolved fluorescence detection superior to steady-state in understanding molecular structure. As indicated in the equations, we can clearly resolve different populations in an inhomogeneous sample in a time-resolved experiment. This is attributed to the ability to detect multiexponential decays within the fluorescence lifetime. Whereas, in the steady-state detection, the two population are averaged into a single output.

### 2.1.3 Time-resolved fluorescence vs. steady-state fluorescence

Though typically more complex and expensive, time-resolved fluorescence can preserve key structural information in its detection, while much molecular information is lost in the averaging process in steady-state detection. Steady-state fluorescence only provides information regarding changes in donor fluorescence intensity, while time-resolved measurements can provide additional information. As indicated in Fig 13, we can resolve an inhomogeneous sample due to identification of a multiexponential decay in
the fluorescence life-time experiment, but this information is lost in steady-state detection, which only indicates overall loss in average donor intensity.

2.2 Principles of Electron Paramagnetic Resonance (EPR)

EPR is a type of spectroscopy that is based on the interaction of electromagnetic radiation with the intrinsic magnetic moment of electrons arising from their spin. It is based on evaluation of the absorption of electromagnetic radiation in the microwave frequency region, by a paramagnetic sample within a magnetic field. Quantum theory describes the spin of an electron as having an angular momentum which contributes to an inherent magnetic moment ($\mu_s$). When subjected to a steady magnetic field $H_0$, the electron spin experiences a torque that aligns the spins with the magnetic moment of the field. This relation between the magnetic moment and the spin vector is:

$$\mu_s = -\frac{g\mu_B}{\hbar} S,$$

Eq. 5

Where $\mu_B$ is the Bohr magnetron and $g$ is the electron g factor. The spin vector is defined by $S$ and its components ($S_x$, $S_y$, $S_z$) are organized in a Cartesian frame.

When a magnetic field is applied to the spins, the electron spin energy will depend on the orientation of $\mu_s$ with respect to the magnetic field $B$:

$$E = -\mu_s \cdot B = g\mu_B S \cdot B,$$

Eq. 6

If we align the $S_Z$ with the magnetic field $B_0$, the equations is:

$$E = g\mu_B |S_Z| B_0,$$

Eq. 7
Since an electron spin can exist in two states, in which the z component of the spin is +1/2 and -1/2, there is a splitting of the electron spin energy into two levels in the magnetic field. This phenomenon is called the Zeeman effect (Fig 14). The difference in the electron energy of the two spins is:

$$\Delta E = h \nu = g \mu_B B_0$$  \hspace{1cm} \text{Eq. 8}$$

Where the $\nu$ is the radiation frequency.

In our experiments, a standard 3.5T magnetic field is used, which corresponds with a $\nu$ of 9.5 GHz (X-Band).

2.2.1 The hyperfine interaction

In biological systems, such as muscle proteins or fibers, thio-reactive paramagnetic spin labels are used to specifically probes sites within a protein or protein complex for structural investigations (Fig. 15). Nitroxides spin labels used for this purpose have a characteristic hyperfine interaction, which is the
interaction between the magnetic moment of an electron with the magnetic moment of the nucleus in its vicinity (Fig. 16).

![Diagram of hyperfine splitting](image)

**Fig. 16. Characteristic hyperfine splitting from a nitroxide spin label.** Adapted from lecture notes by Dr. David Thomas.

### 2.3 Distance measurements using EPR:

#### 2.3.1 Dipolar broadening

In a system where two spins are separated by a distance of \( r \), as depicted in Fig 17A, where the red vector is the interspin vector which is perpendicular to the applied magnetic field. As a result, the absorption peak from the applied magnetic field is split into two new peaks, with a separation distance that is twice the strength of the local magnetic field of the first spin \( (2H_{dipolar}) \). The presence of two but equal peaks is because the spins have equal probability of having either the same or opposite polarity, resulting in a symmetric doublet.
In a randomly oriented sample, the distribution interspin vector with relation to the applied magnetic field has a cosine dependence. Therefore the splitting in the Pake pattern can be described by:

$$H_{\text{dipolar}}(r, \theta) = \pm \frac{3g_e \beta_e}{4} \frac{1}{r^3} (3 \cos^2 \theta - 1),$$  
Eq. 9

$$= \pm 27.85 \cdot G \cdot nm^3 \frac{1}{2} \frac{1}{r^3} (3 \cos^2 \theta - 1),$$

Where $r$ is the interspin distance, $\theta$ is the angle between the interspin vector and the applied magnetic field, $\beta_e$ as the Bohr magnetron, and $g_e$ as the anisotropy value for the electrons. The resulting dipolar splitting is separated by $2H_{\text{dipolar}}$. From the equation, we can see that the maximal dipolar interaction occurs when the interspin vector is perpendicular ($\theta = 90^\circ$) or parallel ($\theta = 0^\circ$) to the applied magnetic field. In randomly oriented system, orientations of the interspin vector affect the local dipolar field with a $\sin \theta$ dependence.

$$H_{\text{dipolar}}(r, \theta) = \pm \frac{\alpha}{r^3} \frac{(3 \cos^2 \theta - 1)}{2},$$  
Eq. 10

Where $\alpha$ is defined as $\mu_0 g \beta / (4\pi) = 18.6 \text{ G} \cdot \text{nm}^3$. 

Fig 17. Dipolar interactions. (A) A diagram of spin-spin interactions with the interspin vector (red) perpendicular to the magnetic field. (B) A description of sum of magnetic moment due to spin-spin interactions. (C) An example of dipolar splitting as observed by NMR. (D) In EPR, we take the derivative spectrum, and in randomly oriented samples, the splitting is represented by the characteristic Pake pattern.
As a result, single “splitting” is not observed, but a “broadening” with relation to the Pake pattern is seen. Subsequently, the Pake pattern can be described by integrating Eq. 9 over \( \sin \theta \) (136):

\[
H_{\text{Bloch}}(r, \theta) = \pm \frac{\alpha}{r^3} \left( \frac{3 \cos^2 \theta - 1}{2} \right),
\]

Eq. 11

As a result, the broadened interacting spectrum \( V_{\text{int}} \) is a function of the non-interacting spectrum \( V_{\text{non}} \) and the Pake pattern (137).

\[
V_{\text{int}}(H) = \int V_{\text{non}}(H') P(H - H') dH',
\]

Eq. 12

To interpret a complex distribution of distances within a biological structures, we use a Gaussian-based sum of Pake patterns to simulate \( V_{\text{int}} \) from \( V_{\text{non}} \) to fit our acquired data (Chapter 4 & 5).

### 2.3.2 Dipolar CW-EPR and distance measurement

Continuous wave (CW) experiments for distance detection requires acquiring both the \( V_{\text{int}} \) and \( V_{\text{non}} \) spectra. To reduce rotational motion of the labeled protein, which can broaden the EPR spectrum due to slower motions, the samples are frozen in 10% glycerol as a cryoprotectant and experiments are carried out at 200K. CW-EPR can reliably detect distances between 0.8nm to 2.5nm (137, 138).
2.3.3 Pulsed Electron Double Resonance

ELDOR or pulsed electron-electron resonance (DEER) is a pulsed-EPR method that allows accurate distance detection between spin labels 2nm to 6nm apart. The DEER dipolar evolution function $V(t)$ for an isolated spin pair is given by:

$$V(t, \theta, r) = 1 - \lambda [1 - \cos(\omega_{\text{dipolar}}(\theta, r)t)]$$  \hspace{1cm} \text{Eq. 13}$$

Where $\lambda$ is the pump efficiency depending on the spin’s flip angle, band width, the position of the pump pulse in the spectrum, and mutual spin label orientation. The angle $\theta$ is defined as the angle between the interspin vector and the applied magnetic field (140,
ω_{dipolar} is the modulation frequency of the DEER signal which is correlated with the interspin distance, r. More specifically ω_{dipolar} is defined as:

\[ \omega_{dipolar} = \frac{(\mu_B \cdot g)^2}{(\hbar r^3)} \ast (3 \cos^2(\theta) - 1), \]

Eq. 14

In randomly oriented samples featuring macroscopic disorder, as such in frozen protein solutions, both Eq. 13 & Eq. 14 are averaged over all possible θ by using a weighing factor of \( \sin \theta \), similar to the case for CW-dipolar-EPR.

Fig. 19. EPR absorbance spectrum. The positions of the observation pulse (excitation of spins A, blue) and the pump-pulse (excitation of spins B, red) are denoted.

The four-pulse DEER sequence is arranged according to excitation of spins A (Fig. 18 & Fig. 19, \( \omega_A \), blue) or B (Fig. 18 & Fig. 19, \( \omega_B \), red) using short electromagnetic pulses. The two spins A and B are separated by a distance r in a magnetic field. First spins A are excited at frequency \( \omega_A \) using a π/2 and a π with a relaxation time \( \tau_1 \). Isolated excitations of spins A (no dipolar interactions) would form a refocused echo (Fig. 18, green). If a second population of spins (spins B) are excited independently at an alternative frequency (Fig. 18, \( \omega_B \), red), the refocused echo will be affected (echo modulation). The degree of this modulation will depend on the timing \( t \), which is the lag between the primary echo and the pump-pulse.
The echo modulation is affected by the transverse relaxation of spin A ($T_{2, A}$) in addition to the presence of excited B spins. The degree of echo attenuation is by a factor of $\exp[-2k(\tau_1 + \tau_2)]$. The decay rate constant $k = 1/T_{2, A} + k_{ID}$ depends on both the transverse relaxation of A spins ($T_{2, A}$) and the instantaneous diffusion rate $k_{ID}$, which is proportional to the concentration of A spins. The instantaneous diffusion rate $k_{ID}$ is also affected by the diffusion strength, which is inverse proportional to the length of the $\pi$ pulses at $\omega_A$, and is approximately $0.25\text{mM}^{-1}\text{µ}s^{-1}$ for a nitroxide spin label at X band frequencies with a 32ns $\pi$ pulse length.

The remainder of the spins (B spins) are excited by a 12ns pump pulse. At X-band frequencies, the inversion efficiency for nitroxide spin labels is approximately 50% of available spins. The inverted B spins subsequently changes the frequency of A spins by electron-electron coupling ($\omega_{ee}$). The echo amplitude is consequently affected as a function of time ($t$) by:

$$\nu(t) = \prod_{i} (1 - \frac{\lambda_i}{2}[1 - \cos(\omega_{eei}t)])$$

Eq. 15

In DEER, it is assumed that exchange coupling between electron spins can be neglected, and that both spins A and B are quantized along the applied magnetic field. The resulting magnetic dipole-dipole coupling is:

$$\omega_{eei} = \frac{e_i}{\gamma_i^2(\gamma_e^2)}[1 - 3\cos^2\theta]$$

Eq. 16

Supposing isotropic $g$ values of nitroxide spin labels ($g_A = g_B = 2.0055$), $C_i$ equals $52.2\text{MHz nm}^{-3}$. 32
Additionally, it is assumed that the homogenous spatial distribution in the DEER sample can be relaxed to a homogeneous distribution with a fractional dimension $D$ (142). For example, membrane proteins has a $D \approx 2$, and an proteins align along a filament axis has a $D \approx 1$. These assumptions further defines the DEER waveforms as:

$$\mathcal{N}(t) = (1 - \beta[1 - \int_{0}^{1} \cos\left(\frac{C}{\sqrt{r}} (1 - 3 \cos^2 \theta) d \cos \theta\right)]B(t),$$  \hspace{1cm} \text{Eq. 17}$$

$B(t)$ is defined as the background signal between clustered $B$ spins ($\exp(-c_BK_BD^3)$). In the case where more than one $B$ spin is within detection distance, the DEER waveform is further convoluted by the form factor $F(t)$, which is the product of all possible spin pair contributions to the waveform (143). According to \textbf{Eq. 17}, the DEER waveform will be oscillating with a cosine dependence.

### 2.3.4 Determining distances from DEER waveforms.

Practically, the background signal can be determined by recording the waveform of a singly-labeled sample, akin to the non-interactive spectrum in CW-dipolar EPR, and subsequently “subtract” this from the experimental data. In general, the distance distributions can be assumed to be Gaussian:

$$\rho(r) = \frac{1}{\sigma\sqrt{2\pi}} \exp\left(-\frac{(r - \mu)^2}{2\sigma^2}\right), \hspace{1cm} \sigma = \frac{\text{FWHM}}{2\sqrt{2\ln 2}},$$  \hspace{1cm} \text{Eq. 18}$$

We applied these EPR distance measurement techniques to do many intradomain measurements that is demonstrated in Chapters 4&5.
CHAPTER 3 – Impacts of dystrophin and utrophin domains on actin structural dynamics: implications for therapeutic design

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Chapter Summary:

We have used time-resolved phosphorescence anisotropy (TPA) of actin to evaluate domains of dystrophin and utrophin, with implications for gene therapy in muscular dystrophy. Dystrophin and its homolog utrophin bind to cytoskeletal actin to form mechanical linkages that prevent muscular damage. Because these proteins are too large for most gene therapy vectors, much effort is currently devoted to smaller constructs. We previously used TPA to show that dystrophin and utrophin both restrict the amplitude of microsecond rotational dynamics while increasing the rate, thus increasing resilience, with utrophin more effective than dystrophin. Here we evaluate individual domains of these proteins. We found that a “mini-dystrophin,” lacking one of the two actin-binding domains, is less effective than dystrophin in regulating actin dynamics, correlating with its moderate effectiveness in rescuing the dystrophic phenotype in mice. In contrast, we found that a “micro-utrophin,” with more extensive internal deletions, is as effective as full-length dystrophin in the regulation of actin
dynamics. Each of utrophin’s actin-binding domains independently promotes resilience in actin, while dystrophin constructs require the presence of both actin-binding domains and the CT domain for full function. This work supports the use of a utrophin template for gene or protein therapy designs. Resilience of the actin-protein complex, measured by TPA, correlates remarkably well with previous reports of functional rescue by dystrophin and utrophin constructs in mdx mice. We propose the use of TPA as an in vitro method to aid in the design and testing of emerging gene therapy constructs.

Keywords:

- time-resolved phosphorescence anisotropy
- TPA
- muscular dystrophy
- gene therapy

Introduction:

Duchenne (DMD) and Becker (BMD) muscular dystrophies are caused by mutations in dystrophin, a 427kD protein localized to the cytoskeletal lattice of the sarcolemma. (23) Dystrophin’s N-terminus binds to cytoskeletal actin, and the C-terminus (CT) binds to the dystroglycoprotein complex (DGC) at the sarcolemma membrane (Fig. 20). Lack of functional dystrophin in skeletal muscles results in disarray of the cytoskeletal organization at key structural regions in striated muscles, (1-3) disabling proper transmission or diffusion of lateral force, (4, 5) and rendering the muscle
susceptible to eccentric contraction damage. (6) AFM studies showed a decrease in myocyte stiffness due to lack of dystrophin. (29) A similar loss in stiffness was observed when cytochalasin D was applied to disrupt the actin cytoskeleton, indicating that the dystrophin-actin interaction play a central role in maintaining mechanical stability in the muscle cytoskeleton. (29) Our goal is to elucidate the molecular mechanism by which the dystrophin-actin interaction contributes to the molecular mechanics of force transmission, and to use this understanding to improve therapeutic strategies. muscle fibers mature, utrophin is replaced by dystrophin,(8-10) but utrophin has

Utrophin is a 395kD dystrophin homolog that is present at the subsarcolemmal region, with functions similar to dystrophin in fetal or regenerating muscle (Fig. 20). (3) As been proposed as a viable therapeutic replacement in dystrophin-deficient mice. (7, 58) Both proteins contain a highly homologous N-terminal actin-binding domain (ABD1)

**Fig. 20. Diagram of actin filament rotational motions in complexes with dystrophin and utrophin.** The homologous structures of the two proteins are indicated schematically. Each has an N-terminal actin-binding domain (ABD1, containing tandem CH domains) and a C-terminal domain (CT) that binds to the sarcolemma, with intervening spectrin-type repeats (STR, ovals) and hinges (H). On the TPA time scale (1 to 1000 µs), the detected motions are dominated by twisting,(144, 145) but the double-helical structure of the actin filament strongly couples its twisting and bending flexibility. (146) The ratio of rotational rate to amplitude, detected by TPA, defines the filament’s resilience (see Materials & Methods), which is increased by both dystrophin and utrophin. (147)
consisting of tandem calponin homology (CH) motifs, followed by a central domain containing a series of triple-helical spectrin-type repeats (STR), and a C-terminal (CT) region. However, dystrophin and utrophin have distinctly different lateral interactions with actin. Dystrophin’s second actin-binding domain (ABD2) is separated from ABD1 by 10 STR, while utrophin’s ABD2 is adjacent to ABD1 

We previously used time-resolved phosphorescence anisotropy (TPA) of actin to determine how dystrophin and utrophin affect the structural dynamics of the actin filament. We showed that the binding of dystrophin or utrophin decreases the amplitude (increases the order) of actin’s rotational flexibility while also increasing the rate, both in a highly cooperative manner, thus creating a more resilient complex. We found that utrophin is much more effective than dystrophin in increasing this resilience. The goal of the present study is to understand more clearly how the individual domains of dystrophin and utrophin influence these effects on the resilience of actin.

This goal is important for the rational design of gene therapy for muscular dystrophy, which is currently being developed using recombinant adeno-associated virus (rAAV) vectors. These vectors are size-limited, so mini- and micro- versions of dystrophin and

Fig 21. Constructs evaluated by TPA in this study. (a) Dystrophin. (b) Urophin. CT = non-actin-binding C-terminal domain. Actin-binding domains are depicted in black. This color scheme is used below for clarity.
utrophin have been developed with large deletions of the central STR domains, including all or part of ABD2. (96, 117, 122, 148) Since these therapeutically relevant internally truncated (TRIT) constructs rely on less than a third of the full-length protein, there is a need to evaluate how individual regions in dystrophin and utrophin affect their interaction with actin. The present study applies TPA to further evaluate the effects of isolated regions in dystrophin and utrophin through deletion constructs (Fig 21). (149, 150) Two TRIT constructs, referred to as mini-dystrophin (Mini-Dys in Fig 21a) and micro-utrophin (Micro-Utr in Fig 21b), were selected due to the availability of consistent physiological studies in dystrophic (mdx) mice. (12, 122, 123) Additional deletion constructs, with either N- or C-terminal truncations, were engineered to test specific domains within dystrophin and utrophin (Fig 21). (149, 150) Our goal is to use TPA to (a) create a biophysical blueprint that identifies key regions in dystrophin and utrophin required to regulate actin microsecond structural dynamics, (b) investigate why the most promising therapeutic constructs have limited effects on rescuing the dystrophic phenotype in mice, and (c) establish TPA as a rapid in vitro method for selecting potential therapeutic dystrophin and utrophin constructs according to their ability to mimic the effects of full-length dystrophin on actin dynamics.
Results:

Microsecond dynamics of actin in the presence of therapeutically promising mini-dystrophin

To provide motivational context for this study, we begin with the mini-dystrophin construct ("Mini-Dys" in Fig 1a and Fig 22a). This TRIT (designated DysΔH2-R19, because it lacks the portion from hinge H2 to STR19) is modeled after cases of mild Becker muscular dystrophy, in which up to 46% of the central STR region is missing but the patients can remain ambulatory past 60 years of age. Despite the extensive internal deletions, leaving only 8 STRs, this construct has shown high efficacy in rescuing the dystrophic phenotype in mdx mice. To compare the TPA results independently of the different actin-binding affinities of the constructs used in this study, we report results as a function of the fractional saturation of binding sites on actin (ν = y/Bmax, Eq. 25). Direct comparison of the TPA decays when
actin binding is 50% saturated ($\nu = 0.5$) shows that Mini-Dys is quite similar to full-length dystrophin (Dys) in its ability to regulate actin structural dynamics (Fig 22b). Indeed, Mini-Dys decreases the amplitude of rotational dynamics while decreasing the rate (Fig 22c&d), corresponding to increased actin resilience. This supports our hypothesis that increased actin resilience is key to the function of dystrophin or its therapeutic surrogates.

The TPA data were analyzed to determine amplitudes and rates (Eqs 19-24), which were plotted against $\nu$ and fitted with Eq. 26. This yielded the cooperativity $n$, which is the number of actin protomers whose amplitudes and rates are affected by the binding to a single actin protomer (Fig 22c,d). These plots show that Mini-Dys is less effective than Dys at low $\nu$ values, primarily because its effect on amplitude is four times less cooperative ($n = 2.5$) than that of Dys ($n = 9.6$) (Fig 22c). This result suggests that Min-Dys can restore normal actin resilience only at high expression levels, approaching that of Dys in WT mice.

Despite the efficacy of Mini-Dys in rescuing the dystrophic phenotype, its large size causes great challenges for delivery of gene or protein delivery into muscle using rAAV,(123) thus its development has not progressed beyond small animal testing.(117, 127, 129) “Micro” constructs (micro-TRITS, based on utrophin as well as dystrophin) which have larger internal truncations (typically leaving only 4 STRs instead of 8), are preferred for large-animal delivery.(117, 123, 126) To establish a rational basis for the design of micro-TRITs, we used several other deletion constructs (Fig 21) to investigate
systematically the impact of specific regions in dystrophin and utrophin on actin structural dynamics.

**Effects of end truncations in dystrophin and utrophin**

The constructs of dystrophin containing end truncations (Fig. 23a) were found to retain partial capacity to restrict actin rotational amplitude (Fig. 23b), but they have all lost the capacity to increase actin rotational rate (Fig. 23c). Dp260, which lacks the N-terminal half of Dys (thus lacking ABD1) but retains the second actin-binding domain (ABD2) and the rest of the C-terminal third of Dys, is as cooperative as full-length Dys ($n \sim 10$); but DNR17, which contains both actin-binding domains and lacks the C-
terminal third, shows much lower cooperativity ($n \sim 2$). Thus the dystrophin C-terminal third, which does not interact directly with actin, appears to play an allosteric role in regulating actin dynamics. The cooperativity of this effect is more pronounced when ABD2 is present (Dp260, Fig. 23b, $n \sim 10$) compared with ABD1 alone (minidystrophin, Fig 22c, $n \sim 3$). Isolated DysABD1 restricts actin rotational amplitude significantly at low $\nu$, but its propensity to bundle actin at higher concentrations(152) prevented a complete analysis.

End truncations in utrophin (Fig. 23d) also decrease its effectiveness on actin dynamics (Fig. 23e&f). UNR10, containing both actin-binding domains but lacking the C-terminal third of Utr, has effects at high $\nu$ similar to those of full-length Utr, but completely lacks cooperativity (Fig. 23e&f). The isolated ABD1 of Utr is about half as effective as UNR10 and also lacks cooperativity. These results suggest that (1) both actin-binding domains of Utr are important for full regulation of actin dynamics, and (2) the C-terminal portion of Utr appears to determine cooperativity even more clearly than in Dys.

Although deletions in both dystrophin and utrophin decrease their regulation of actin structural dynamics compared with full-length proteins, utrophin constructs retain more effectiveness in enhancing actin resilience. The truncated utrophins retain the capacity to enhance rate substantially, as well as to restrict amplitude (Fig. 23e&f), thus clearly enhancing resilience (rate/amplitude, Eq. 23), while the truncated dystrophins enhanced rate (and hence resilience) only slightly (Fig. 23b&c). These results support the use of a utrophin template for gene therapy designs to enhance actin resilience.
Effect of micro-utrophin on actin dynamics

To test the hypothesis that utrophin is a highly effective template for therapeutic designs, we evaluated a micro-utrophin therapy construct (Micro-Utr in Fig 21 and Fig. 24). This micro-TRIT (designated Utr∆R4-21, because the segment from STR4 through STR 21 has been deleted, leaving only 4 STRs) has previously been tested in mouse models.(97, 153) TPA data shows that Micro-Utr restricts actin rotational amplitude and increases rate with comparable cooperativity as full-length dystrophin or utrophin (Fig. 24b&c). Although it is not as effective as full-length utrophin, it is more effective than dystrophin at all values of v.

Effects of dystrophin and utrophin constructs on the resilience of actin.

As explained in Methods, resilience of the actin-protein complex is measured from TPA as the ratio of rate to amplitude of actin rotational dynamics, normalized to that of actin alone (Eq. 23).(147) Fig. 25 shows plots of resilience vs v, derived from data in Fig 22, Fig. 23, and Fig. 24. We compared different constructs with regard to their
maximum effect on actin resilience (Fig. 25c) and the cooperativity of this effect (Fig. 25d). Resilience was increased by all constructs, but utrophin constructs were consistently more effective than their homologous dystrophin counterparts (e.g., compare Utr with Dys, UNR10 with DNR17, Micro-Utr with Mini-Dys).

Utrphin’s greater capacity than dystrophin, for increasing actin’s resilience, correlates well with previous studies of the utrophin’s rescue of muscle mechanics in the dystrophin-null mdx mouse. Full mechanical function can be restored by upregulation of utrophin at half the level of dystrophin in wt muscle. (7, 57, 60, 127) Similarly AFM analysis of dystrophic myocytes showed that utrophin upregulation can restore cellular
stiffness to wild-type levels at 28% of the wt dystrophin level (29). This correlation between TPA-measured actin resilience and muscle function also extends to results from physiological tests in \textit{mdx} mice, as discussed below.

\textbf{Discussion:}

Deletions in dystrophin and utrophin decrease their effectiveness in regulating actin structural dynamics. However, since utrophin and its constructs are more effective than the dystrophin counterparts, large deletions in utrophin produced structural regulation similar to that of full-length dystrophin, showing great promise for utrophin as a therapeutic surrogate.

\textbf{The domains of dystrophin.}

TPA shows that both DNR17 (containing the N-terminal 2/3 of Dys, including both actin-binding domains) and Dp260 (containing the C-terminal 1/3 of Dys, including ABD2), restrict actin rotational amplitude (Fig. 23b) but have little effect on rate (Fig. 23c) and thus have little effect on resilience (Fig. 25a&c), while Mini-Dys (containing both N- and C- termini but lacking ABD2) has effects on both amplitude (Fig 22c) and rate (Fig 22d), thus enhancing resilience nearly as well as full-length Dys (Fig. 25a&c). However, the cooperativity of Mini-Dys on actin is less than that of Dys (Fig. 25d), and the highly cooperative restriction of amplitude by Dp260 (Fig. 23b) suggests that the C-terminal region, especially in conjunction with ABD2, is essential for cooperativity in
Dys-actin interactions. Thus, the two separated ABDs in dystrophin affect actin structural dynamics with distinctly different but interlacing effects.

**The domains of utrophin.**

Despite their high sequence homology, utrophin’s domains have effects on actin structural dynamics that are distinct from those of dystrophin. In general, utrophin’s effects are simpler – for example, all four utrophin constructs decrease amplitude to a similar extent that they increase rate (Fig. 23, Fig. 24), so it is sufficient to look at the effects on resilience (rate/amplitude) (Fig. 25). Another key difference is that utrophin does not require any of its C-terminal half after STR10 to enhance actin’s resilience, since the N-terminal UtrABD1 has substantial effects on its own, and UNR10 (lacking the C-terminal half of Utr) has nearly identical effects as full-length Utr at high \( \nu \) values (Fig. 25b&c). However, neither of these N-terminal constructs (UtrABD1, UNR10) regulates actin structural dynamics cooperatively (Fig. 25b&d). Since Micro-Utr (which does contain the C terminus of Utr) shows cooperativity comparable to that of Utr (Fig. 25b&d), it appears that the C-terminal region of Utr, like that of Dys, is essential for conferring cooperativity.

In contrast to dystrophin, the effects of the two ABDs in utrophin appear to be identical and additive. UtrABD1 enhances actin resilience by a factor of 2.3, UNR10 (containing both ABD1 and ABD2, but lacking the C-terminal half) by a factor of 5 (Fig. 25), and Micro-Utr (containing ABD1 plus 30% of ABD2) by a factor of 2.94 (30% more than UtrABD1). Thus, the regulation of actin structural dynamics by utrophin constructs
appears to be simpler and more predictable than for dystrophin constructs. Coupled with the higher efficacy of Utr construct compared with Dys constructs, this result argues for utrophin as a template for therapeutic design.

**TPA as a rapid *in vitro* measure of therapeutic efficacy.**

We surveyed previous physiological studies on *mdx* mice with transgenic expression of dystrophin and utrophin.(7, 127) For meaningful comparison of resilience with physiological findings, we considered only studies that tested the mechanical function (specific isometric force) of treated dystrophic muscles, reported levels of expression in the tested muscles, and had tested the animals at the same age. Despite large numbers of studies done on dystrophic mouse models, few studies meet these standards. However, we found two studies that consistently measured the isometric specific force in diaphragm muscles in *mdx* mice at 3-4 months of age(7, 127) (Fig. 26a). These studies included the *Fio* and *Fer* mouse lines which expressed full length utrophin at 54% and 27% of the endogenous dystrophin level, respectively, in a dystrophin-null background (*mdx*)(7, 37, 57). A recovery score with regard to the isometric specific force in these mice was calculated based on preclinical standard operating procedures established by TREAT-NMD (Eq. 27).(154-156) As explained in Methods, resilience values were calculated using data in Fig. 25a&b, to correct for the reported protein expression levels and actin affinities. Correlation plots relating recovery scores of wild type muscles (100% recovery with 100% dystrophin) and dystrophic muscles (0% recovery with 0% dystrophin) treated with different levels of dystrophin or utrophin
showed a distinctively high correlation to the relative resilience calculated by TPA studies ($R = 0.98$). The remarkably linear relationships between the resilience of the actin cytoskeleton and the observed specific force (Fig. 26a), suggests that the measurement of resilience by TPA is a potentially useful *in vitro* predictor of the therapeutic efficacy of dystrophin and utrophin constructs.

Our measured resilience also demonstrated excellent correlation with mechanical restoration of dystrophic muscles treated with Mini-Dys(123) and Micro-Dys(97, 122) (Fig. 26b, $R = 0.99$). Although the micro-utrophin construct demonstrated a larger effect on increasing actin resilience compared to wild type dystrophin at saturating levels (Fig. 25c), the *mdx* study did not achieve saturating expression levels in the tested muscles.(122) As a result, the resulting relative resilience calculated based the level of micro-utrophin expression reported and on our data (Fig. 25a&b) was lower than that expected for Dys (wt), in which actin is saturated with full-length dystrophin. This further supports the use of TPA as a rapid *in vitro* method to screen proposed gene therapy constructs for potential therapeutic efficacy.

Fig. 26.: Correlation of Recovery Score (Eq. 27) with actin resilience in *mdx* mice (a) in diaphragm of transgenic mice tested at 3-4 month of age (7, 127) and in (b) tibialis anterior. (122, 123) Values of Pearson’s correlation coefficient ($R$) (Eq. 28) are (a) 0.98 and (b) 0.99, respectively.
Our results suggest the feasibility of using utrophin as a therapeutic surrogate. Fig. 25c shows that utrophin constructs consistently provided higher resilience compared with dystrophin constructs. Even Micro-Utr provided higher resilience compared with full length dystrophin, despite extensive internal deletions. Thus, the same level of resilience can be achieved with lower concentrations of utrophin constructs compared with dystrophin constructs. This is consistent with the finding that expression of full-length utrophin at half the concentration of wild-type dystrophin was sufficient to fully restore muscle mechanics. Since improving the transfection efficacy of these therapeutic constructs is still an ongoing pursuit, our data suggest that it would be most beneficial to use a utrophin-derived construct. Coupled with possible immunogenicity in dystrophin-derived therapeutic constructs, these results argue for the use of utrophin as a template for gene therapy designs.

**Conclusion:**

We have used TPA to draw a biophysical blueprint of dystrophin and utrophin regarding their effects on actin structural dynamics and resilience. The domains of dystrophin and utrophin have different effects on actin, despite their high structural homology. Urophin constructs are generally more robust in making actin resilient, despite large deletions. Compared with dystrophin constructs, utrophin constructs are more effective in enhancing actin resilience, and the effects of their domains on actin resilience are simpler and more independent, facilitating rational design. We find a strong correlation between resilience of actin-protein complexes and functional restoration.
reported from previous studies in *mdx* mice. We propose to use TPA as a rapid *in vitro* screening method for designing and testing the next generation of gene therapy constructs for muscular dystrophy.

**Methods:**

**Protein preparation**

Larger dystrophin and utrophin constructs including DNR17, UNR10, Dp260 and DysΔH2-R19 were expressed using Sf9 insect cells infected with high-titer recombinant N-terminal FLAG-tags. (117, 149, 150) FLAG-Micro-Utr was engineered by recombinant PCR using FLAG-Utr cDNA as a template with identical primers as previously described.(122) Proteins were purified by Flag affinity chromatography and dialyzed against two changes of phosphate buffered saline pH 7.5 to remove excess flag peptide. DysABD1 and UtrABD1 are expressed in E. Coli BL21 AI cell line and purified using a cation exchange (HiTrap SP XL, GE) for DysABD1 and anion exchange (HiTrap Q XL, GE) for UtrABD1 followed by a size exclusion column (Sephadex S200, GE). Proteins were concentrated in Millipore Amicon Ultra centrifuge-based concentrators with cutoffs of either 100 kDa or 10 kDa depending on protein molecular weight. Concentrations were determined by Bradford protein assay with a BSA standard.

**Time-resolved phosphorescence anisotropy (TPA)**

Actin preparation and labeling with phosphorescent erythrosine-iodoacetamide (ErIA) (Anaspec) was as described in.(147) Phalloidin-stabilized ErIA-actin was diluted in U/D
buffer (100mM NaCl$_2$, 2mM MgCl$_2$, 0.2mM ATP, 1mM DTT, 10mM Tris pH 7.5) to 1μM. Increasing concentrations of dystrophin and utrophin constructs was added to bind to 1μM ErIA-actin. Oxygen removing system containing of glucose oxidase (55ug/ml), catalase (36ug/ml), and glucose (45ug/ml) was added to the sample prior to each experiment and incubated for 5 minutes to prevent photobleaching.(158, 159) The phosphorescent dye is excited at 532nm with a vertically polarized 1.2ns laser pulse from a FDSS 532-150 laser (CryLas) with a 100Hz repetition rate. Emission was detected through a 670nm glass cutoff filter (Corion) using a photomultiplier (R928; Hamamatus) and transient digitizer (CompuScope 14100; GaGe) with a resolution of 1us per channel. Time-resolved anisotropy is defined by

$$r(t) = \frac{I_v(t) - GI_h(t)}{I_v(t) + 2GI_h(t)}$$

Eq. 19

Where $I_v(t)$ and $I_h(t)$ are the vertically and horizontally polarized components of the detected phosphorescent emission, using a single detector at 90° and a rotating sheet polarizer alternating between the two orientations every 500 laser pulses. $G$ is a correction factor calibrated by detection of the signal with horizontally polarized excitation, and correcting so that the anisotropy is zero. TPA experiments were recorded with 30 cycles, each consisting of 500 pulses in each polarization.

Anisotropy decays were analyzed by fitting to the sum of two exponential terms.(144, 147)

$$r(t) = r_1 \exp(-t/\phi_1) + r_2 \exp(-t/\phi_2) + r_\infty.$$  

Eq. 20
Results were validated by comparison of residuals and chi-squared values of the fits at one, two and three exponential terms, with the best fit consistently requiring two exponential terms.

The overall angular amplitude of rotational motion was defined as the radius of a cone calculated from the wobble-in-cone model. (144)

\[
\text{Amplitude} = \theta_c = \cos^{-1}\left[-0.5 + \{0.5(1 + 8[r_\infty/r_0]^{1/2})\}^{1/2}\right]. \quad \text{Eq. 21}
\]

Thus a maximally flexible actin filament would exhibit a final anisotropy value of \( r_\infty = 0 \), yielding and a cone angle \( \theta_c = 90^\circ \), and a rigid filament would have \( r_\infty = r_0 \) (no decay) and \( \theta_c = 0^\circ \) (no detectable rotation).

The mean rate of actin filament rotational motions was defined as the inverse of the mean correlation time:

\[
\text{Rate} = (r_1 + r_2)/(\phi_1 r_1 + \phi_2 r_2). \quad \text{Eq. 22}
\]

Resilience is defined as the maximum amount of elastic energy per unit volume that can be stored without large structural distortions to the protein complex. (160) Highly resilient polymers can recover quickly from deformations. During this process, the system stores mechanical energy from deformation as elastic energy. (161) Resilience of the actin-bound complex is determined from TPA as:

\[
\text{Resilience} = \frac{\text{Rate}}{\text{Amplitude}}, \quad \text{Eq. 23}
\]

where Amplitude is calculated from Eq. 21 and Rate from Eq. 22. (147) The plotted resilience values of Fig. 25 are “Relative Resilience,” normalized to the value observed for actin alone in the same study.
TPA-derived parameters (Amplitude, Rate, or Resilience, are plotted against the fractional saturation of actin binding ($\nu = \text{bound protein molecules per binding site on actin}$). These $\nu$ values were determined from actin-binding assays of dystrophin and utrophin constructs using high-speed co-sedimentation.\(\cite{147, 162}\) Varying concentrations of dystrophin or utrophin constructs were added to 6$\mu$M labeled actin, incubated for 30 minutes at $20^\circ$C and centrifuged at 100,000 x g for 20 minutes. The resulting pellets and supernatants were analyzed by SDS-PAGE to determine the concentrations of free and actin-bound protein. Then $K_d$ (dissociation constant in $\mu$M) and $B_{\text{max}}$ (number of protein binding sites per actin protomer) were determined by fitting the data with

$$y = \frac{B_{\text{max}} [P]}{(K_d + [P])}, \quad \text{Eq. 24}$$

where $y = \text{moles of bound protein per mole of actin}$, and $[P]$ is the concentration of free protein. Thus $B_{\text{max}}$ and $K_d$ were determined for each construct. In TPA experiments, the concentration of free protein $[P]$ was not known, so $y$ was calculated from

$$y = \frac{B_{\text{max}} ([P_T] + B_{\text{max}} + K_d - \sqrt{([P_T] + B_{\text{max}} + K_d)^2 - 4[P_T]B_{\text{max}}})^{1/2}}{2B_{\text{max}}}, \quad \text{Eq. 25}$$

$$\nu = \frac{y}{B_{\text{max}}},$$

where $[P_T]$ is the total concentration of protein added to labeled actin, and $\nu$ is the fractional saturation of actin-binding sites used in the horizontal axes of Fig. 22 to Fig. 25.
Cooperativity

The degree of cooperativity was determined by fitting the plot of rotational Amplitude (Eq. 21), Rate (Eq. 22), or Resilience (Eq. 23) vs v (Eq. 25) to the expression.(145, 151)

\[ X(v) = X_{\text{max}} - (X_{\text{max}} - X_0)(1 - v)^n, \]  

Eq. 26

where \( X(v) \) is the observed value (amplitude, rate or resilience), \( X_0 \) is the value for actin only (\( v = 0 \)), \( X_{\text{max}} \) is the value when actin is fully saturated (\( v = 1 \)), \( v = y/B_{\text{max}} \) (Eq. 25) is the fraction of actin sites occupied by added protein, and \( n \) is the degree of cooperativity in the system, i.e., the number of actin protomers affected by the binding of a single actin protomer. In other words, the number of actin protomers affected dynamically by the binding of one protein molecule in each experiment is \( n B_{\text{max}} \).

Correlation with Preclinical Data (Fig. 26)

In order to correlate resilience, as measured by TPA, with specific force measured in mice (Fig. 26), resilience was normalized according to the level of expressed constructs, relative to dystrophin in the wild-type mouse, reported from each mouse study.(7, 122, 123) The concentration of dystrophin in wild-type mouse muscle was calculated from previous reports.(57, 163) Dystrophin comprises 0.026% of total muscle protein,(57) which amounts to 0.12µM with a cellular density of 0.2g/ml.(163) We assume a sarcolemmal surface area to cell volume ratio of 0.103µm²/µm³(164) and a length of 120nm in the z-dimension (length of the dystrophin molecule).(60) Thus, the estimated local concentration of dystrophin is 9.9 µM in the wild-type mouse. Similarly, the
concentration of cytoskeletal γ-actin at the subsarcolemmal region (using a total γ-actin concentration of 0.20µM reported in the whole cell), (163) was calculated to be 16µM. Since 1 dystrophin molecule interacts with 27 actin protoomers with submicromolar affinity, (57) we assume that the fraction of dystrophin-decorated actin at the costamere is 1 (ν = 1). To normalize the relative resilience of each construct to the reported % expression levels in each study, we use Eq. 25 to calculate the fractional saturation ν for each construct, based on their respective $K_d$ and $B_{max}$ values (57, 153). The resilience of the actin cytoskeleton was determined from this value of ν, using the plots in Fig. 25a&b.

To compare the mechanical function of treated muscles across studies, we used the preclinical standard operating procedure to calculate a recovery score, based on measurements of isometric specific force: (154-156)

$$\text{Recovery Score} = \frac{\text{treated} - \text{untreated}}{\text{wt-untreated}}, \quad \text{Eq. 27}$$

Pearson’s correlation coefficient ($R$) was calculated from

$$R = \frac{1}{m} \sum_{i=1}^{m} \left( \frac{x_i - \bar{x}}{\sigma_x} \right) \left( \frac{y_i - \bar{y}}{\sigma_y} \right) \quad \text{Eq. 28}$$

where $m$ is the number of samples, $x_i$ and $y_i$ are the values of specific force and actin resilience. $\bar{x}$ and $\bar{y}$ are mean values, and $\sigma_x$ and $\sigma_y$ are standard deviations.
**Additional Information:**

Results from the TPA studies also provided some additional insights into the dystrophin-actin and utrophin-actin interaction. In this section, I will summarize of these additional findings and alternative interpretation to the TPA data (Table 1).

<table>
<thead>
<tr>
<th>Dystrophin domains</th>
<th>Effect on actin rotational dynamics</th>
</tr>
</thead>
<tbody>
<tr>
<td>DysABD1 + C-terminal region</td>
<td>Cooperative increase on actin rotational rate ↓ actin rotational amplitude ↓ Dys-actin torsional flexibility</td>
</tr>
<tr>
<td>DysABD2 + C-terminal region</td>
<td>Cooperative restriction actin rotational amplitude</td>
</tr>
<tr>
<td>DysABD1 + DysABD2 + C-terminal region</td>
<td>Cooperative effect on both amplitude and rate of actin rotational motion</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Utraphin domains</th>
<th>Effect on actin rotational dynamics</th>
</tr>
</thead>
<tbody>
<tr>
<td>UtrABD1</td>
<td>↓ actin rotational amplitude by 50% ↑ actin rotational rate by 50% ↑ Utr-actin torsional flexibility by 50%</td>
</tr>
<tr>
<td>UtrABD2</td>
<td>↓ actin rotational amplitude by 50% ↑ actin rotational rate by 50% ↑ Utr-actin torsional flexibility by 50%</td>
</tr>
<tr>
<td>C-terminal region</td>
<td>Cooperative effect on both amplitude and rate of actin rotational motion</td>
</tr>
</tbody>
</table>

**Table 1.** Table summary of the effects of individual domains in dystrophin (a) and utrophin (b) on actin rotational dynamics.

**Dystrophin and utrophin constructs increase actin torsional flexibility.**

Since the function of the dystrophin-actin and utrophin-actin is to provide mechanical buffering at the sarcolemma (4-6, 22), it is important to evaluate changes in actin elasticity when bound to deletion or therapy constructs of dystrophin and utrophin. The continuous elasticity within the actin filament can be evaluated through fitting of our anisotropy data to the torsional twist model based on derivations of the Schurr’s theory
The model describes actin as a series of cylindrical elementary rods and allows global evaluation of the rigid-body tumbling and intrafilament twisting motions in the azimuthal plane. DysABD1 was not included in this analysis due to its inherent instability in its interaction with actin. All dystrophin and utrophin constructs tested increased actin’s torsional flexibility by 42-86%, with utrophin constructs more effective than their dystrophin counterparts (Fig. 25).

With larger deletions, dystrophin and utrophin constructs lose their ability to increase actin torsional flexibility (Fig. 25). Loss of the C-terminal region impacted dystrophin and utrophin differently. DNR17 loses approximately 52% of the torsional flexibility when calculated from the mean values, while UNR10 does not seem to be significantly affected by the C-terminal deletion. This further points out the differences in the C-terminal region of dystrophin and utrophin in terms of their impact on actin structural dynamics despite their high sequence homology.

While the C-terminal deletion in dystrophin (DNR17) is more devastating than the N-terminal deletion (Dp260) in terms of restricting actin rotational amplitude (Fig 5c), deletion of the dystrophin N-terminal region greatly decreased its ability to increase actin torsional flexibility (Fig. 25). ABD2 is required for cooperative restriction of actin rotational amplitude, but it is comparatively non-important in terms of increasing actin torsional flexibility. This is reflected in the dramatic deficit (92%) in the torsional flexibility of Dp260 bound actin compared to that of DNR217 or DysABD1. Results from DysΔH2-R19 also supports the relatively unimportance of dystrophin ABD2 in
increasing actin torsional flexibility since it demonstrates comparable results to full length dystrophin (Fig. 25).

Our TPA results show that DysΔH2-R19 binding induce similar levels of torsional flexibility in actin compared with dystrophin (Fig. 25). This finding explains why patients with internal deletions that mimic DysΔH2-R19 have mild dystrophic presentations compared with other disease-causing mutations (49-51). It also explains why DysΔH2-R19 is so effective at restoring mechanical functions of dystrophic muscles and providing protection from eccentric contraction damages in the mdx mice (117, 123, 166). The main difficulty in using DysΔH2-R19 as a gene therapy construct is its size. It is significantly more difficult to deliver mini-dystrophins with 8 central STRs into the muscle cells compared with micro-dystrophins with only 4 central STRs (117, 123). However, in transgenic mice that overexpress DysΔH2-R19 at 10 fold endogenous dystrophin levels restore protection from eccentric contraction damage up to approximate 90%, proving that DysΔH2-R19 can be a highly effective replacement for damaged dystrophin (117, 127).

Additionally, our results provide an explanation to the high level of expression (>10X) required for DysΔH2-R19 in order to revert dystrophic phenotype in mice (127). Compared with full length dystrophin, there is a 3 to 4 fold loss in cooperativity in the ability of DysΔH2-R19 to restrict actin rotational amplitude (Fig 22c). The loss in cooperative regulation of actin results in a 1 to 1 correlation between the level of expressed DysΔH2-R19 and the degree of functional recovery in the dystrophic mice (123). A 42% level of expression corresponded with a 40% recovery in specific force in
the *mdx* model (123). Coupled with the difficulty to deliver such a large genetic construct to muscle, the largest organ in the human body, it would be beneficial to use a construct that has an altogether larger effect or higher cooperativity on actin rotational dynamics, so a lower degree of transduction can yield substantial results.

Our TPA results suggest that to fully restore cooperative regulation of a dystrophin construct on actin rotational amplitude, ABD2 is required. Dp260 with ABD2 demonstrates cooperative restriction of amplitude to levels similar to full length dystrophin (*Fig. 23c*). However, we cannot simply replace ABD1 in DysΔH2-R19 with ABD2, since loss of ABD1 resulted in an ablation on the construct’s ability to increase actin rotational rate (*Fig. 23e*). It may be highly beneficial to replace the current 8 STRs (STR1-3, 20-24) in DysΔH2-R19 with the 8 STRs of ABD2 (STR11-17) optimize the function of the this mini-dystrophin so that a higher degree of rescue can be achieved with a lower amount of expression.

On the other hand, results from utrophin deletions further support our conclusion that the two ABDs in utrophin have more or less equivalent effects on actin. UtrABD1 loses approximately half of its ability to increase actin torsional flexibility compared with UNR10 or utrophin (*Fig. 25*). The micro-utrophin construct, UtrΔR4-21, and isolated UtrABD1 induce a 1.5 fold higher actin torsional flexibility than binding to full length dystrophin. All in all, UtrΔR4-21 has similar, if not larger, effects on actin rotational dynamics and flexibility as full length dystrophin. This suggests that UtrΔR4-21 is more effective than DysΔH2-R19 in restoring elastic buffering at the subsarcolemmal region.
Dystrophin and utrophin increased actin structural order.

DysABD1+2 and UtrABD1+2 (C-terminal deletions) cause a 15-20% loss in actin orientational order compared with the full-length proteins (Fig. 25a). This suggests a role for the non-actin interacting C-terminal region on stabilizing the structural orientation of actin filaments in both dystrophin and utrophin. Comparison of DysABD2+CT and the mini-dystrophin (ABD1+CT) show that deletion of one or the other ABDs in dystrophin does not significantly impact actin orientational order.

On the other hand UtrABD1 loses half of its ability to increase actin orientational order compared with UtrABD1+2, indicating that the two ABDs in utrophin share equivalent functions in stabilizing actin structural orientational order (Fig. 25a). In micro-utrophin, where 35% of the ABDs is lost, it demonstrated similar effects on structural orientation of bound actin as utrophin. Thus, when the utrophin C-terminal region is present, it enhances the ability of either utrophin ABD1 or ABD2 to stabilize the structural orientation of bound actin. In both dystrophin and utrophin constructs, those that have a C-terminal deletion dramatically loses cooperative effect on actin orientational order. Utrophin constructs that contain the C-terminal region fully restores

Fig 27.: Actin flexibility and order parameter from TPA studies. (a) Increases in actin orientational order and (b) resilience due to binding of dystrophin and utrophin constructs tested. (c) Corresponding degrees of cooperativity (n).

<table>
<thead>
<tr>
<th>Construct</th>
<th>Order (n)</th>
<th>Resilience (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dystrophin</td>
<td>21.1 ± 1.7</td>
<td>4.9 ± 1.4</td>
</tr>
<tr>
<td>Utrophin</td>
<td>19.7 ± 1.9</td>
<td>6.0 ± 1.7</td>
</tr>
<tr>
<td>DysABD1+2</td>
<td>2.7 ± 0.6</td>
<td>0.2 ± 0.3</td>
</tr>
<tr>
<td>UtrABD1+2</td>
<td>1.9 ± 1.7</td>
<td>2.6 ± 0.7</td>
</tr>
<tr>
<td>DysABD2+CT</td>
<td>21.2 ± 0.5</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>UtrABD1</td>
<td>5.5 ± 2.3</td>
<td>5.0 ± 0.6</td>
</tr>
<tr>
<td>UtrABD1+CT</td>
<td>2.7 ± 0.0</td>
<td>2.0 ± 0.7</td>
</tr>
<tr>
<td>Mini-Dys (ABD1+CT)</td>
<td>19.4 ± 3.2</td>
<td>5.5 ± 1.2</td>
</tr>
</tbody>
</table>
cooperativity to that observed in full-length protein, but dystrophin requires ABD2 and not ABD1 in conjunction with the CT for this effect (Fig. 25c). In summary, for both dystrophin and utrophin, ABD1 and ABD2 are highly effective in stabilizing actin orientational order, and the C-terminal region greatly enhances this ability albeit through different mechanisms.
CHAPTER 4 – Large-scale opening of utrophin’s tandem CH domains upon actin binding by an induced-fit mechanism

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Ava Yun Lin, Ewa Prochniewicz, Zachary M. James, Bengt Svensson, and David D. Thomas. Large-scale opening of utrophin’s tandem calponin homology (CH) domains upon actin binding by an induced-fit mechanism. Proc Nat Acad Sci USA, 2011; 108:12729-12733.

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Chapter Summary:

We have used site-directed spin labeling and pulsed electron paramagnetic resonance to resolve a controversy concerning the structure of the utrophin-actin complex, with implications for the pathophysiology of muscular dystrophy. Utrophin is a homolog of dystrophin, the defective protein in Duchenne and Becker muscular dystrophies, and therapeutic utrophin derivatives are currently being developed. Both proteins have a pair of N-terminal calponin homology (CH) domains that are important for actin binding. Although there is a crystal structure of the utrophin actin-binding domain, electron microscopy of the actin-bound complexes has produced two very different structural models, in which the CH domains are in open or closed conformations. We engineered a pair of labeling sites in the CH domains of utrophin and used dipolar electron-electron resonance (DEER) to determine the distribution of interdomain distances with high resolution. We found that the two domains are flexibly connected in solution, indicating a dynamic equilibrium between two distinct open structures. Upon actin binding, the two domains become dramatically separated and ordered, indicating a transition to a single
open and extended conformation. There is no trace of this open conformation of utrophin in the absence of actin, providing strong support for an induced-fit model of actin binding.

**Introduction:**

Utrophin is a homolog protein of dystrophin that has shown high therapeutic promise for the treatment of muscular dystrophy (7). It is endogenously found in fetal or regenerating muscle but is replaced by dystrophin, the defective protein in Duchenne and Becker muscular dystrophies, as the muscle matures (167). Upregulation of utrophin in *mdx* mice, which lack dystrophin, has been shown to rescue its dystrophic phenotype, improving muscle morphology and function (7, 113). The full-length protein is not required to improve dystrophic pathology in *mdx* mice; i.e., substantial internal truncations in utrophin can be tolerated (58). These internally truncated constructs for muscular dystrophy therapeutics support the importance of actin binding by the N-terminal portions of either dystrophin or utrophin (117). Utrophin (395 kD) and dystrophin (427 kD) both contain highly homologous N-terminal actin-binding domains (ABD1), consisting of a pair of calponin homology (CH) domains. Despite additional actin-binding regions identified in the central spectrin-type repeats (60), micro-utrophin constructs with high potential for clinical applications rely almost exclusively on the N-terminal CH domains for actin interaction (96, 122). Therefore, understanding the structural interaction between utrophin CH domains and actin has become crucial for the rational development of therapeutic constructs.
More generally, there is an urgent need for a structural blueprint of CH domain-actin complexes for the entire spectrin superfamily of actin binding proteins (e.g., fimbrin and α-actinin), of which dystrophin and utrophin are members. The diversity of crystal structures for these domains, despite high sequence homology, suggests a high degree of dynamics and flexibility and has prevented the development of a reliable structural model for any of these complexes. A major unresolved question concerns the relative disposition of the tandem CH domains (CH1 and CH2) (171, 172).
the tandem CH domains showed a closed conformation for fimbrin (173) and α-actinin (174), but an open conformation for both utrophin (Utr261) (Fig 28A) and dystrophin (Dys246) (175). The crystal structure of Utr261 suggests that the central helical region connecting CH1 and CH2 is highly flexible. Even for α-actinin, which has a closed crystal structure, computational analysis suggests the potential for a high degree of dynamic flexibility that facilitates actin binding (176). A method is needed that allows high-resolution detection of multiple structural states in the presence of flexibility and disorder, both free and bound to actin. The present study achieves this goal with site-directed spin-labeling and dipolar electron-electron resonance (DEER), to resolve the structures of both the actin-bound and unbound states of the utrophin CH domains (Utr261).

Structural analysis of utrophin-actin complexes has been difficult, since filamentous actin does not co-crystallize with its binding partners. Cryoelectron microscopy has been performed on the Utr261-actin complex, but resolution has not been sufficient to distinguish closed and open conformations of the tandem CH domains (171, 172). Sutherland-Smith et al. proposed that the CH domains (CH1 and CH2) are organized in a compact and closed conformation when bound to actin (169) (Fig 28B). However, based on similar EM data, Galkin et al. proposed an open conformation (170) (Fig 28C), although they could not distinguish between two possible open conformations – a “half-decorated model” (Open 1) in which every Utr261 contacts two actin protomer and a “singly-decorated model” (Open 2) in which every Utr261 contacts one actin protomer (Fig 28C). Thus it is clear that cryo-EM does not provide sufficient resolution
to distinguish between three very different structural models for the actin-utrophin complex. In the present study, we have resolved this controversy using site-directed spin labeling and high-resolution pulsed EPR spectroscopy (DEER) to measure distances between the CH domains, both free in solution and bound to actin.

**Results:**

![Graphs showing CD spectra](image)

![Table showing secondary structure](table)

**Fig S29.**: CD spectrum of (A) Utr261, (B) double mutant V136C/L222C, and (C) MSL-labeled V136C/L222C. (D) % \( \alpha \)-helix determined from molar ellipticity at 222nm with s.d. Neither the presence of the double cysteine mutations nor the MSL labeling disrupted the secondary structure.

**Site-directed labeling in the CH domains of utrophin.**

A series of DEER simulations was performed, assuming that spin labels were attached to different pairs of labeling sites on utrophin, to select an optimal pair of
labeling sites (Cys mutations) in the endogenously cysteine-free Utr261. The goal was to distinguish the crystal structure (Fig 28A) and the proposed EM-based atomic models of the closed (Fig 28B) and open (Fig 28C) conformations. The simulations show that the pair V136 and L222 is ideal for this study, since the predicted DEER waveforms are clearly distinguishable for all four models (Fig 28D). Therefore, we engineered a double-cysteine mutant (V136C/L222C) and attached a pair of thio-reactive spin labels to the Utr261 construct. As shown by DEER simulations, a 1.8 nm distance between residues V136C and L222C, as predicted by the closed model for the actin-bound complex (Fig 28B) should generate an extremely rapid oscillation in the DEER waveform (Fig 28D, magenta), compared with the slower oscillation (Fig 28D, red) predicted by the more open crystal structure (Fig 28A, 3.5 nm). In contrast, the open models for the actin-bound complex (Fig 28C, 4.3 or 5.1nm) should produce a much slower oscillation (Fig 28D, blue and green). Neither the presence of the double-cysteine mutations nor the spin labeling significantly perturbed the α-helical secondary structure, as determined by circular dichroism (Fig S29); or actin affinity, as determined from cosedimentation assays (Fig S30).
Utr261 has two resolved conformations when free in solution.

Inspection of the dipolar electron-electron resonance (DEER) waveform of Utr261 free in solution shows clearly the presence of two oscillations with distinct frequencies (Fig 28E, red), unambiguously indicating two distinct interspin distances; i.e., two coexisting structural (conformational) states. The slower of the two oscillations, corresponding to the longer distance, is in excellent agreement with the data predicted (Fig 28D, red) for the crystal structure (Fig 28A, 3.5nm). Quantitative analysis (Fig 28F),
based on least-squares minimization (Fig S31), yielded a bimodal distribution with mean distances of 2.7nm and 3.3nm, corresponding to mole fractions of 0.4 and 0.6, respectively. Thus the actin-binding domain of utrophin is in dynamic equilibrium between two structural states of nearly equal free energy, an open one (corresponding to the 3.3 nm distance) that is trapped in the crystal structure (Fig 28A), and another that is substantially more closed.
Fig S31: Analysis of DEER data, by nonlinear least-squares fit, reveals two resolved conformational states. The top row shows the model-independent Tikhonov fit, while lower rows show the results of fits assuming models of Gaussian distance distributions. The number of Gaussian components was determined to be $n$ (enclosed by boxes) when $n+1$ did not significantly improve the residual or $\chi^2$. (A) For Utr261 free in solution, a 2-Gaussian fit ($n = 2$) is sufficient, since 3 Gaussians does not improve the fit. (B) When Utr261 is bound to actin, $n = 1$ is sufficient. Note that $\chi^2$ values for the Tikhonov fits are virtually identical to those for the best Gaussian fits.
Utr261 binds to actin in an open conformation through an induced-fit mechanism.

Upon actin binding, the DEER waveform of Utr261 (Fig 28E, green) undergoes a dramatic change, showing a much slower oscillation, thus indicating a substantial increase in the interdomain distance. Indeed, the observed waveform is in excellent agreement with that predicted (Fig 28D, green) by an extremely open conformation of Utr261 in the actin-bound complex (Fig 28C). Quantitative analysis showed clearly that the interprobe distance increased to a well-defined 4.8nm with a narrow distribution.

Fig 32: DEER waveform (top) and distance distribution (bottom) between the two CH domains of Utr261, as a function of the molar ratio of actin to Utr261 (spin labeled at C136 and C222). When actin is absent (A) there is a bimodal distribution of short distances that is not observed when actin is in excess (C,D), but when actin is substoichiometric (B), both populations are observed.

Upon actin binding, the DEER waveform of Utr261 (Fig 28E, green) undergoes a dramatic change, showing a much slower oscillation, thus indicating a substantial increase in the interdomain distance. Indeed, the observed waveform is in excellent agreement with that predicted (Fig 28D, green) by an extremely open conformation of Utr261 in the actin-bound complex (Fig 28C). Quantitative analysis showed clearly that the interprobe distance increased to a well-defined 4.8nm with a narrow distribution.

Fig 33: Dipolar CW EPR spectra of spin-labeled Utr261. The spectrum of doubly labeled V136C/L222C (red) is overlaid on the spectrum of singly labeled L222C-Utr261 (black). Top: no actin. Bottom: 2 moles actin per mole Utr261. In each case, there is no difference between red and black spectra (i.e., no dipolar broadening), implying that there is no significant population having an interprobe distance less than 2.5nm.
(Fig 28F, green), which is consistently obtained with different actin content (Fig 32). As we titrated actin into the labeled Utr261 sample, we detected a distinct shift of population from the bimodal population free in solution (2.7 and 3.3nm) (Fig 32A) to the single actin-bound conformation (4.8nm) (Fig 32C&D). Under sub-saturating conditions, where the molar ratio of actin to Utr261 is less than 1, we found a mixture of the free and actin-bound populations (Fig 32B). When we saturated the sample with actin, only a single long distance of 4.8nm was observed; no conformations with distances less than 4.0nm were populated (Fig 32C&D). Indeed, dipolar CW EPR shows that no distances less than 2.5nm are detected, in either the presence or absence of actin (Fig 33), indicating that the closed conformation depicted in Fig 28B is not populated in the presence or absence of actin. Thus it is clear that the CH domains of Utr261 move apart substantially upon actin binding, corresponding very well to the open conformation proposed from some previous EM studies (Fig 28C), but not to the closed conformation proposed from other EM studies (Fig 28B). The detected distance of 4.8 nm was in best agreement with the singly-decorated open conformation model (170) (Fig 28C, “Open 2”).
With considerable precision, there is no overlap between the distance distributions determined for free and actin-bound Utr261 (Fig 28F). This is shown even more rigorously by model-independent analysis using Tikhonov regularization (TR, Fig 34). Because TR does not insist on a particular functional form, such as Gaussian, for the distance distribution, it is more likely to resolve a small population that deviates from the major component. The TR results agree remarkably well with those determined from Gaussian fits, speaking further to the integrity of the data and fitting analysis (Fig 28F, Fig 34B&D). We conclude that (a) when Utr261 is free in solution, there is no trace (less than 1%) of the open actin-bound conformation (Fig 34AB), and (b) when Utr261 is bound to actin, there is no trace of the closed conformation (Fig 34CD). These results clearly demonstrate that the structural change in Utr261 is driven by induced fit, not by conformational selection among pre-existing conformers (177).
To illustrate the key results of this study, we simulated models using our measured distances as constraints (Fig 35). We started from the crystal structure of Utr261 (PDB 1QAG) and performed rigid-body rotations of the two heads (CH1 and CH2) relative to one another around a pivot point set at the peptide bond between residues 149 and 150. In the case of the actin-bound state, a total of 21 models with no steric clashes that satisfied our distance constraints were generated, based on both the 4.8nm distance measured between labeled residues 136 and 222, and the 5.3nm distance measured between residues 75 and 222 (Fig S36, Fig S37). The structural model from our 21 simulated results that fitted best to prior EM data (Fig S37) was selected to represent the actin-bound state in Fig S37B, which strongly resembled that of the “Open 2” EM model (Fig 28C). The results clearly demonstrate an induced-fit mechanism, where Utr261 is structurally dynamic in solution (Fig S37A) and opens dramatically upon actin binding (Fig S37B). Future DEER measurements of distances between other pairs of labeling sites in Utr261, as well as measurements between specific sites on actin and Utr261 will be needed to obtain a detailed atomic model for the utrophin-actin complex.

Fig 35.: Utrophin binds to actin by an induced-fit mechanism. Model of Utr261 free in solution (A) and bound to actin (B), based on DEER data (see text). In the absence of actin, the Utr261 is in equilibrium between two distinct conformations, corresponding to the 2.7nm (red) and 3.3nm (green) distances observed by DEER (Fig 28F, Fig 34). Upon binding to actin, Utr261 dramatically opens up, corresponding to the 4.8nm distance observed by DEER (Fig 28F).
Fig S36: Secondary distance measurement at G75C/L222C. (A&B) DEER data from the G75C/L222C mutant of Utr261, confirming the much slower oscillation (longer interdomain distance) observed in the actin-bound state (5.3 nm mean distance, green) compared with the free state (2.2nm and 2.6nm mean distances, red. The populations are 49% and 51% respectively). The 5.3 nm distance was used as a second constraint in modeling the Utr261-actin complex shown in Fig 5. (C&D) A significant disruption in secondary structure (detected by CD) is induced by mutation and labeling G75C/L222C, compared with mutation and labeling V136C/L222C (Fig S1), which probably explains the broader distance distributions (and thus decreased resolution) observed by DEER (compare Fig S4B with Fig. 1F).
Discussion:

**Utr261 binds to actin in an open conformation, by an induced fit mechanism.**

We have used pulsed dipolar EPR (DEER) to show that the two CH domains of utrophin’s N-terminal actin-binding domain (Utr261) open dramatically upon actin binding, resolving a controversy regarding EM-based models (Fig 28). When free in solution, Utr 261 is in equilibrium between two distinct structural states, one consistent with the crystal structure, and one that is more closed, but neither of these conformations is found in the actin-bound structure. Thus, this binding event occurs by an induced-fit mechanism, not by selection of a pre-existing conformation (Fig 35). Both the bimodal distance distribution of free Utr261 and the large-scale structural change upon actin binding demonstrate the high flexibility of the tandem CH domains.
Implications for utrophin’s role in therapy for muscular dystrophy.

Utrophin constructs that include the N-terminal actin-binding domain are under intensive study for possible use in therapeutic replacement of dystrophin for treatment of muscular dystrophy (96), and its localization to the sub-sarcolemma suggests its essential role in replacing dystrophin’s interactions with cytoskeletal (non-sarcomeric) actin. A previous study showed that utrophin restricts the amplitude of actin’s microsecond torsional flexibility with high cooperativity, while increasing the rate of these flexing motions, and that utrophin is even more effective than dystrophin in this regulation of actin’s dynamic mechanical properties (147). It was proposed that this regulation of flexibility in cytoskeletal actin is crucial for the functions of both utrophin and dystrophin as mechanical stabilizers to diffuse lateral force at the surface of the muscle cell. Our results suggest a mechanism for these effects. The extensive opening of Utr261 upon actin binding (Fig 35) would bring the two CH domains in contact with adjacent actin protomers, providing a plausible structural explanation for the decreased amplitude of actin’s torsional flexibility. This open conformation probably extends and partially unwinds the central linker between the two CH domains, facilitating the observed increase in the rate of the restricted flexibility that remains in the actin-utrophin complex (147). ABD1’s ability to regulate actin’s torsional flexibility is of great potential interest, since current promising gene therapy constructs have large deletions in the central domain of either utrophin or dystrophin, and thus rely solely on the N-terminal ABD1 for actin interaction (117). Future structural studies with the N-terminal ABD1 of dystrophin,
analogous to the present study on utrophin, and additional distance measurements between these actin-binding domains to actin, will provide insight needed to continue this protein engineering project.

Structural implications for other tandem CH domains.

Based on crystal structures showing a variable linkage between CH domains, it has been proposed that tandem CH domains are highly dynamic in both structure and function (168, 176, 177). Our study provides high-resolution structural information on one of these tandem CH domains in solution, showing that it is in equilibrium between two distinct conformations. Thus we have obtained direct information in solution supporting the hypothesis that the linker between the CH domains is highly flexible (168), establishing the capacity for the versatile structural transitions required for other similar actin binding proteins such as \(\alpha\)-actinin (178) and fimbrin (179).

Structures of actin-bound CH domains have long been elusive. There are no crystal structures containing both actin and tandem CH domains. EM studies have generated controversy, with competing closed (compact) and open (extended) models proposed for Utr261 (Fig 28), \(\alpha\)-actinin (176, 178, 180), and fimbrin (179, 181). The present study provides the first direct high-resolution measurements on the relative disposition of tandem CH domains bound to actin, clearly resolving the controversy for utrophin and establishing powerful methodology that should be applicable to the other important members of the spectrin superfamily of actin-binding proteins. The extensively open conformation of the CH domains in actin-bound utrophin is a structure not found in
crystal or solution, demonstrating dramatically the plasticity of this actin-binding interaction (Fig 35).

Conclusions:

We report a new approach, involving site-directed spin-labeling and pulsed electron paramagnetic resonance (DEER), for defining the structural dynamics of actin-binding CH domains. DEER provides high-resolution distance measurements, showing clearly that the N-terminal actin-binding domain of utrophin is quite flexible. Its tandem CH domains are arranged in two resolved conformations in the absence of actin, one of which agrees with the crystal structure, and a single distinct conformation when bound to actin, corresponding to a much more open state that has not previously been observed (Fig 35), clearly indicating an induced-fit mechanism of binding. This approach will be powerful in future studies of utrophin and other actin-binding proteins, with important implications for the molecular pathology and therapy of muscular dystrophy (7).

Materials and Methods:

Protein purification and spin labeling.
Utr261 was cloned from mouse utrophin cDNA and ligated as a BamHI-XhoI fragment into pET23a vector. Utr261 contains no native cysteines. Site-directed mutagenesis (Stratagene Quikchange) was performed to engineer a pair of Cys residues into the construct for thioreactive spin label attachment at chosen sites (either V136C and L222C,
or G75C and L222C). The double-Cys mutant construct was transformed into the *Escherichia coli* BL21 AI cell line and grown at 37°C in LB media to an absorbance of 0.6-0.8 at 600nm. Cells were induced with 1mM IPTG and 0.2% L-arabinose and allowed to grow for 3-5hrs at 28°C. Cells were then harvested by centrifugation and lysed with lysozyme for 1 hour at 4°C in 25% sucrose, 1mM EDTA, 1mM PMSF, 50mM Tris (pH 8.0, 4°C), followed by a freeze-thaw procedure in a dry ice/Isopropanol bath. The lysate was treated with 2U/L DNase I and incubated with the addition of 10mM MgCl₂ and 10mM MnCl₂ for 1 hour, then centrifuged at 40,000g for 30 minutes. The supernatant was purified using an anion-exchange column (HiTrap Q XL, GE) equilibrated in 1mM EGTA, 1mM DTT, 20mM Tris (pH 8.0, 4°C). Protein was eluted with a linear gradient of NaCl from 0 to 0.5M. Fractions containing the target protein were verified using SDS-PAGE and pooled to run over a gel filtration column (Sephadex S200, GE) in 100mM NaCl, 2mM MgCl₂, 1mM DTT, 10mM Tris (pH 8.0, 4°C). Fractions containing the target protein were again verified using SDS-PAGE and concentrated. DTT was removed prior to labeling using Zeba desalting columns (Pierce). The protein was labeled with MSL [N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide] (Toronto Research Chemicals, North York, Ontario). Labeling efficiency was determined by spin-counting with using a Bruker E500 spectrometer at X-band (9.5 GHz) with an SHQ cavity at a microwave power where there was no saturation (0.03 mW). The molar ratio of spin labels per labeling site was determined to be 0.82 ± 0.02.
**Dipolar electron-electron resonance (DEER) and dipolar CW-EPR.**

We performed DEER to measure distances from 2 to 6nm, and dipolar CW-EPR for distances from 0.5 to 2.5nm. DEER experiments were performed and analyzed as described previously (182). The concentration of Utr261 was 100 µM. F-actin was prepared from rabbit skeletal muscle (151). Both proteins were dialyzed into a buffer containing 100mM NaCl, 2mM MgCl₂, 1mM DTT, 10mM Tris (pH 8.0, 4°C). The final actin concentration was 400 to 500µM. Actin was mixed with Utr261 at the desired molar ratio (typically 2 moles actin per mole Utr261), such that the final Utr261 concentration was 60 to 75µM. 100µL samples containing 10% glycerol (vol/vol) were flash-frozen in liquid nitrogen in a 5mm OD quartz NMR tube (Wilmad glass, Buena NJ). Samples were stored at -80°C. Pulsed EPR experiments were performed with an Elexsys E580 spectrometer (Bruker Biospin) containing a dielectric resonator (MD-5; Bruker Biospin). Spectra were acquired using a four-pulse sequence, with the pump frequency centered on the central resonance of the nitroxide and the observed frequency set at a low-field resonance >65 mHz away from the pump frequency. The π/2 pulse was 16ns and the ELDOR pulse was 40 to 44ns. Each waveform was recorded at 65K for 24 to 72hrs. The resulting spectra and spin-spin distances were analyzed using DeerAnalysis2008 software suite (183) and software developed in-house (WACY, Edmund Howard). Background subtraction of the DEER waveforms were done using singly-labeled Utr261 (183). To account for protein flexibility, fits to the data were done assuming that each conformational state of the protein corresponds to a Gaussian distribution of distances:
\[ \rho(r) = \frac{1}{\sigma \sqrt{2\pi}} \exp\left(-\frac{(r - R)^2}{2\sigma^2}\right), \quad \sigma = \frac{\text{FWHM}}{2 \sqrt{2 \ln 2}}. \] 

Eq 29

where \( \sigma \) is the standard deviation and FWHM is the full width at half maximum of the distribution.

The number of Gaussian components in the fit \( n \) was determined by varying \( n \) such that no improvement in the residual plot and \( \chi^2 \) value was obtained by increasing the number of components to \( n+1 \) (Fig S31). Distance distributions determined by Tikhonov regularization were consistent with the Gaussian fits (Fig 34).

Spin-spin distances were also determined by CW-EPR using a Bruker E500 spectrometer at X-band (9.5 GHz) with an SHQ cavity, as described previously (184). Spectra were acquired at 200K with a 200G field sweep to detect spectral broadening. The modulation amplitude was decreased to 1G to minimize modulation broadening, and all spectra were acquired under non-saturating conditions (0.03mW).
CHAPTER 5 – The more flexible dystrophin calponin homology (CH) domains also undergo large-scale opening upon actin-binding.

Chapter Summary:
We have used dipolar electron-electron resonance to detect conformational changes in the N-terminal calponin homology (CH) domains of dystrophin (DysABD1) when binding to actin. The actin-bound structure of the DysABD1 have been elusive, with a possible closed model of binding formulated based on its dimeric crystal structure despite the fact that dystrophin binds actin as a monomer. In this study, we provide high-resolution measurements that directly probe the free-in-solution and actin-bound conformations of DysABD1. Monomeric DysABD1 binds actin in a well-ordered single open conformation that is reminiscent to that of its utrophin homolog (UtrABD1). Though DysABD1 has long been proposed to have large structural flexibility, this study provides the first high-resolution evidence to the extent of its structural flexibility, which is larger than that observed for UtrABD1. DysABD1 is more sensitive to salt concentrations and forms stable dimers through hydrophobic interactions. Dimerization locks it in a closed conformation with lower actin affinity, further supporting a monomeric and open conformation of the actin-bound DysABD1. Structural flexibility combined with distinct hydrophobic interactions suggest that DysABD1 itself can behave as an entropic spring. However, excess hydrophobic interactions of isolated DysABD1s can also lead to unwanted aggregation. Our findings resolve a structural ambiguity to the actin-bound
state of DysABD1 and provide a new structural model for the pathophysiology of muscular dystrophy with implications towards therapy development.

**Introduction:**

Dystrophin is a key muscle cytoskeletal protein with protective effects against eccentric contraction damages by diffusing excess lateral force at the muscle subsarcolemmal region (4-6). Mutations within dystrophin lead to dystrophic muscle pathology in which there is inflammation, necrosis, degeneration and fibrosis of the muscle fibers (185, 186). This 427kDa protein relies on a stable interaction with cytoskeletal actin for its function as a mechanical stabilizer of contracting muscles (28, 61, 187). To replaced damaged dystrophin, various Therapeutically Relevant Internally Truncated constructs (TRITs) with large central deletions were proposed and tested in animal models (117, 127, 188). Though a second actin-binding domain in the central rod region of dystrophin have been identified (44, 162), it is mostly removed in the TRITs, forcing these gene therapy constructs to rely exclusively on the N-terminal calponin homology (CH) actin-binding domains (ABD1) for actin interaction (117). Overexpression of these TRITs were required to fully restore mechanical protection in muscles, and to date it is unclear why such a drastic overexpression is required (127). This prompts further understanding of the dystrophin CH domains (DysABD1)-actin interaction on a molecular scale.
Dystrophin belongs to the spectrin superfamily of actin binding proteins, which contain tandem CH domains at the N-terminus (189). Though most of the CH domains in this superfamily crystallized as a single compact monomer (173, 176, 190, 191), the CH domains in dystrophin (Dys246, i.e. DysABD1) and its homolog protein utrophin (Utr261, i.e. UtrABD1) uniquely crystallized in an extended conformation as an anti-parallel dimer (168, 175). This finding lead to the speculation that the central linker region for dystrophin and utrophin CH domains are more structurally dynamic (175). Due to the dynamic nature of these proteins, attempts to visualize their actin-bound structures in electron microscopy (EM) resulted in alternate interpretations with regards to the relative displacement of the two CH domains – compact (closed) or extended (opened) (171, 172).

Only one structural study has been successful in outlining a possible actin-bound model of DysABD1 (169). This singular study proposed a closed conformation for actin binding both DysABD1 and UtrABD1. This is largely due to the high structural similarity.
of the domain swapped DysABD1 and UtrABD1 to that of the monomeric and compact fimbrin CH domains. (Fig. 38). Indeed, the authors noted DysABD1 dimers decorating actin filaments in their EM report of a closed conformation of actin binding (169). This ambiguity between an open or closed conformation, monomeric or dimeric actin-binding is partially due to the low resolution (10-20Å) of EM and inadequate understanding of why DysABD1 dimerizes in solution when larger constructs are monomers (192). Thus, there is a need to evaluate structural changes within these domains upon actin interaction using a high-resolution structural technique.

Previously, we published our findings on the utrophin CH domains (UtrABD1), which dramatically opened upon binding to actin (10). Using a pulsed electron paramagnetic resonance method (dipolar electron-electron resonance, DEER), we obtained high-resolution (~1Å) structural information regarding the distance between the lobes in UtrABD1 both free-in-solution and when bound to actin. In the present study, we have employed a similar strategy to resolve the free in solution and actin-bound conformations of DysABD1. Our results detail some key structural features unique to the DysABD1-actin complex, which can provide an explanation to its effective mechanical buffering capability (4-6) and the propensity for missense mutations within DysABD1 to cause severe aggregation (193, 194).

**Results:**

The more structurally dynamic dystrophin CH domains binds actin in a distinct open conformation.
To probe the free-in-solution and actin-bound conformation of dystrophin CH domains (DysABD1), we performed site-directed spin labeling at homologous sites to those previously reported for utrophin CH domains (UtrABD1) (195) (Fig. 39A). Site-directed mutagenesis in the cys-null DysABD1 allowed us to engineer cysteines at residues V120/L239 for specific labeling using a thiol-reactive monofunctional spin label (MSL). For the free-in-solution data, the initial decay is faster in DysABD1 (Fig. 39B, red) than UtrABD1 (Fig. 39A, red), and the waveform oscillation is observably more dampened. This corresponds to a shorter distance between the labeled sites with a broader

Fig. 39. An open conformation of actin-binding in DysABD1 with marked similarities with UtrABD1. (A) A distinct shift from a partially closed to an open conformation of actin-binding previously reported for UtrABD1 (195). Labeled sites are shown in the crystal structure on left (1gvg) (168). Distances between the Cα-Cα of the labeled residues are denoted in red. (B) Labeling at homologous sites in DysABD1 (1dx1, 175)) also show a similar transition from partially closed to an open conformation upon actin-binding. However, isolated DysABD1 in solution is more structurally flexible (disordered) as is evident from dampened DEER waveform oscillation and the resulting broader distance distributions.
distribution, suggesting a more compact but disordered DysABD1 structure in solution compared to that of UtrABD1. Like UtrABD1, fits to the DEER waveform of DysABD1 show two distinct distances, but they are much less resolved. The predominant distance detected when DysABD1 is the free in solution (2.5nm) suggests a more closed conformation than that depicted in the crystal structure (Fig. 39B, red). A second distance centered at 3.5nm is also observed, which approximates to the distance in the crystal structure, but is much more disordered. Therefore, like UtrABD1, DysABD1 is also in an equilibrium between two conformational states when free in solution but has higher disorder and flexibility.

Though more disordered when free-in-solution, binding to actin effectively stabilized DysABD1 into a single distinct conformation (Fig. 39B, green). The DEER waveform shows slowing of the DEER decay in the presence of actin, which indicates an increase of interprobe distance between the two probed CH domains. This corresponds to a distinct open conformation of actin-binding. The actin-bound DEER waveforms of UtrABD1 and DysABD1 (Fig. 39, green) demonstrate highly similar decay and oscillation profiles. Fits to the DysABD1-actin waveform show a well-resolved single distance at 4.7nm when bound to actin (Fig. 39B, green, structural conclusions), reminiscent to that observed for UtrABD1 labeled at homologous sites (Fig. 39A, green, structural conclusions).
Sodium chloride concentrations effectively shift the monomer-dimer equilibrium in DysABD1.

A caveat in the investigation of the actin-bound structure of DysABD1 is its reported propensity to dimerize in solution (169, 175). In contrast, biochemical characterizations using analytical centrifugation have shown UtrABD1 to remain a stable monomer in solution (177). Since full length dystrophin interacts with actin as a monomer (147, 192, 196), it would be preferable to probe the actin-binding conformation of monomeric DysABD1. Thus, we investigated ways to shift the dimerization equilibrium to favor the monomeric state for DysABD1 for our study.

Two possible mechanisms have been proposed for DysABD1 dimer formation (175). There are 30 possible hydrogen bonds within the dimeric DysABD1. Secondly, hydrophobic associations within the linker region between the two CH domains were evident in the dimeric crystal structure. Since the dimeric form is presumably more bulky and will migrate slower in a native gel, we tested 15µM of DysABD1 incubated in a gradient of sodium chloride (NaCl) to see if we can perturb dimer formation with increasing or decreasing the ionic strength (Fig. 40). Though UtrABD1 remained monomeric under a wide range of NaCl concentrations as is evident by a single band in
the native gel, DysABD1 shows an additional band with slower migration when NaCl is present. The band with slower migration can be construed as the dimer population. This suggests hydrophobic associations between DysABD1s as a mechanism for dimerization as opposed to the formation of hydrogen bonds or salt bridges.

To verify that the two bands observed in the native gel is due to self-association of DysABD1s and not due to drastically different structural packing of monomeric but flexible DysABD1 (i.e. a more compact, well structured DysABD1 verses partially unfolded DysABD1), a method to directly probe interactions between DysABD1s was needed. Since monomer-dimer $K_{eq}$ reported in prior studies were approximately $4\text{-}10\mu\text{M}$ (169, 175), EPR methods to detect DysABD1 self-association would not be ideal since a minimum of $50\mu\text{M}$ of labeled protein is needed for sufficient signal-to-noise. Thus, we elected to use fluorescence resonance energy transfer (FRET) between DysABD1 differentially labeled at a single site with either a donor (IAEDANS) or acceptor (DABCYL) probe (Fig. 41). A single cysteine was introduced into the cys-null DysABD1 at residue V120, and specifically labeled with either the donor or acceptor fluorescent probes (Fig. 41A). The C$\alpha$-C$\alpha$ distance between adjacent V120 residues within the crystal dimer is 3.2nm, which suggested the use of IAEDANS to DABCYL as a donor-acceptor pair (Ro = 3.3nm).
We titrated a range of V120C-acceptor (0, 2, 4, 8, 10, 15, 25µM) to 2µM of V120C-donor and recorded changes within the donor fluorescence lifetime under 0mM, 91 µM V120C-acceptor titrated to 2 µM of V120C-donor. (B) No change in donor lifetime (<2%) was observed with as high as 25µM V120C-acceptor titrated to 2µM V120C-donor under 0mM NaCl (orange). Upon, addition of 500mM NaCl to the same sample, we see a dramatic decrease in donor lifetime from 12.0 ± 0.8ns to 3.3 ± 0.1ns (purple). This demonstrates significant self-association of DysABD1 under higher concentrations of NaCl. (C) Determining the monomer-dimer $K_{eq}$ under different [NaCl]. The fraction of dimeric DysABD1 is determined from global fitting of the donor lifetime changes when 2µM of V120C-donor is titrated against a range of V120C-acceptor (0, 2, 4, 8, 10, 15, 25µM). The dimeric fraction (the fraction of V120C-donor participating in FRET) is plotted against the concentration of free or monomeric V120C-acceptor. The concentration of monomeric DysABD1 is the concentration of added V120C-acceptor subtracted by the concentration of V120C-donor participating in FRET. $K_{eq}$ for dimerization is determined by fitting the data to a Michaelis-Menten plot. There is significant shift to favor dimerization at 500mM NaCl ($K_{eq} = 0.3 ± 0.1µM$) compared with 100mM NaCl ($K_{eq} = 10.1 ± 3.2µM$) and 0mM NaCl (no detectable dimers). (D & E) Example FRET data from titration experiments under 100mM and 500mM NaCl. Donor only lifetime is shown in black. Average distances and distributions obtained from the global fitting for both conditions are shown on the right. An increase in NaCl (500mM) results in a increasingly well-defined distance distribution which suggests a structured dimer formation and not random self association in DysABD1 in the presence of NaCl.
100mM and 500mM NaCl conditions (Fig. 41B,D,E). While no change in donor fluorescence lifetime was seen under 0mM NaCl throughout the titrations (Fig. 41B, Fig. S48), there were slight changes under 100mM NaCl (Fig. 41D) and a dramatic ~4 fold decrease under 500mM NaCl (Fig. 41E). The increasing FRET with higher NaCl conditions mirrors our findings in native gels (Fig. 40). Results from global fitting of the titration data also showed the best fit to a single Gaussian distribution with a mean distance of 2.2-2.4nm in the presence of NaCl (Fig. S48) (Fig. 41D&E, structural conclusions). An increase in NaCl from 100mM to 500mM resulted in a more well-defined distance distribution, which suggest a more stable and compact DysABD1 dimer. Thus, the upper bands observed in the native gel of DysABD1 is from self-association and not folded verses unfolded monomeric DysABD1.

Global fitting of the titration data showed a ~3 fold lower $K_{eq}$ under 500mM NaCl conditions compared with 100mM NaCl, favoring the dimeric state at higher salt conditions (Fig. 41C). $K_{eq}$ was calculated by plotting the fraction of V120C-donor participating in FRET (Dimeric fraction) versus the concentration of monomeric DysABD1 (added V120C-acceptor subtracted by the concentration of V120C-donor involved in FRET) and fitting the data to a Michaelis-Menton curve. The fraction of V120C-donor participating in FRET was obtained from global fitting of the donor fluorescence lifetime when 2µM was titrated with a range of V120C-acceptor (0, 2, 4, 8, 10, 15, 25µM). As is evident from a larger change in donor fluorescence lifetime in 500mM NaCl (Fig. 41E) compared with 100mM (Fig. 41D) and 0mM NaCl (Fig. 41B, Fig. S48), the $K_{eq}$ favors the dimeric state of DysABD1 with increasing concentrations.
of NaCl. One caveat in this experiment is that we are not be able to detect any donor to donor or acceptor to acceptor dimerization. Therefore, the $K_{eq}$ determined for dimerization in this assay is underestimated. To minimize this complication, we elected to use a concentration of V120C-donor that is below previously reported $K_{eq}$ for dimerization\cite{169, 175}. Even with underestimation, we still see a significant decrease (~3 fold) in $K_{eq}$ with increasing NaCl, demonstrating the high sensitivity of DysABD1 to the surrounding ionic strength.

In order to directly compare the actin-bound conformation of monomeric Dys/UtrABD1, the data for DysABD1 in 0mM NaCl and the previously reported data on UtrABD1 in 100mM NaCl was compared in Fig. 39.
Monomeric DysABD1 has higher affinity to actin.

Actin cosedimentation assays under varying NaCl concentrations show a ~4-6 fold higher affinity of DysABD1 in 0mM NaCl compared to higher NaCl conditions (Fig. 42). We obtained similar $K_d$ values in experiments that varied either the DysABD1 or actin concentrations, effectively removing variations of protein aggregation in our evaluations as suggested by previous studies (193). NMR of synthetic peptides have identified three individual actin-binding sites (ABS) within DysABD1 that directly interacts with actin. ABS1 is partially hidden in the closed conformation of CH domains (197), and further simulations have suggested a large-scale conformational change within the CH domains to allow proper exposure of all ABSs for actin interaction (176). This study is the first direct evidence of a stable DysABD1 dimer which has lower affinity to actin, possibly due to locking of DysABD1 and preventing key structural opening for high affinity actin binding.
Fig. 43. In comparison, actin-bound of UtrABD1 is not as sensitive to salt conditions. (A) Distance measurements between residues V136C/L222C of actin-bound UtrABD1 under varying NaCl concentrations. Note that only at extremely high salt (500mM NaCl, purple) conditions do we see a secondary shorter distance with a broad distribution that befits the presence of dimers (Fig. S49 & Fig. S50). (B) DysABD1 probed at homologous sites (V120C/L239C) show similar open conformation of binding to actin under 0mM NaCl (monomeric form, orange). Dimerization under 100mM (blue) and 500mM NaCl (purple) conditions restricts the structural opening of DysABD1, resulting in an increasing population of a shorter distance that correspond with DysABD1 dimers.
A comparison of the actin-bound UtrABD1 and DysABD1 under different NaCl conditions.

In DEER spectroscopy, the presence of dimers in doubly-labeled samples of UtrABD1 or DysABD1 will introduce a population with a short distance and broad distributions due to overlapping of multiple unresolved distances (Fig. S51). To ensure that we are detecting distances within actin-bound constructs, we mixed UtrABD1 or DysABD1 with actin in a 1 to 4 molar ratio with concentrations well above the measured $K_d$ (> 40µM). The actin-bound complex is then pelleted using ultracentrifugation and any unbound UtrABD1 or DysABD1 in the supernatant is removed. Since the final concentration of actin within the DEER samples are between 250µM and 400µM, it can be safely assumed that >90% of the labeled constructs (60µM-90µM in the DEER sample) are bound, even in the case of 500mM NaCl, where the $K_d$ is the highest (Fig. 42C).

Should UtrABD1 bind to actin as a dimer that is similar to that of the crystal structure, a shorter distance of approximately 3.0nm with a large distribution would be seen (Fig. S51). However, such a distance population is not seen until extreme NaCl concentrations (500mM, i.e. ionic strength of 514mM >> physiological 150mM) (Fig. 43A, purple). This corresponds with a faster initial decay and dampened oscillations as seen in the acquired DEER waveforms (Fig. 43A, compare DEER waveform blue and purple). The shorter distance population observed in actin-bound UtrABD1 at high salt conditions (3.1nm) corresponds well to the predicted mean distance in the dimeric crystal structure (3.0nm) (Fig. S51). These results suggest a lower sensitivity to changes in ionic
strength for UtrABD1 in its interaction with actin and that the dimeric form can be similar to that of the crystal structure.

In comparison, a significant actin-bound dimer population is observed at lower ionic strengths in DysABD1 compared with UtrABD1 (Fig. 43B, blue & purple). The average distance between spin-labels at sites V120C/L239C within a DysABD1 dimer is approximately 2.6nm based on the dimeric crystal structure (Fig. S51). A significant population of short distance corresponding to dimeric DysABD1 (2.9nm) is detected under conditions that is just approaching physiological ionic strength (100mM NaCl, i.e. ionic strength of 114mM ~150mM physiological ionic strength) (Fig. 43B, blue). It is notable that under conditions of 100mM NaCl, the measured $K_{eq}$ between monomers and dimers is 10.1µM in our FRET assay (Fig. 41C), which is comparable to its affinity with actin ($K_d = 11.2µM$) (Fig. 42). This matches our observation of a roughly 50:50 split in the population of actin-bound monomers (4.7nm) and actin-bound DysABD1 dimers (2.9nm) (Fig. 43B, blue). An increase in NaCl results in a larger shift to the dimeric state of actin-binding, which is indicated by the higher % population of a shorter 2.9nm distance distribution (Fig. 43B, purple).
Could actin-binding decrease the sensitivity of DysABD1 to NaCl? We made a parallel sample where DysABD1 is first bound to actin under 0mM NaCl, and 100mM NaCl is added just before freezing the sample for DEER spectroscopy (Fig. 44). Even with the short incubation time (< 5min) in 100mM NaCl prior to freezing in the presence of actin, we still saw the dimeric population in the resulting DEER waveform. The molar ratio between the dimeric and monomeric form is similar to that observed when DysABD1 was bound to actin under 100mM NaCl conditions (Fig. 43B, blue). The extent of DysABD1’s hydrophobic associations is staggering, as demonstrated by its dramatically heightened sensitivity to ionic strength.

Fig. 44. Binding to actin cannot protect against hydrophobic associations between DysABD1 in the presence of NaCl. (A) DEER waveform of a parallel actin-bound sample made under 0mM NaCl conditions. 100mM NaCl was added just prior to freezing the DEER sample. (B) Distance distributions from Gaussian fits. The best fit was determined to be two Gaussians, with the same distances seen in (Fig. 43B, blue) as well as similar mole fractions between the dimeric and monomeric states.
A uniform, ordered interaction between monomeric DysABD1 and actin.

Since DysABD1 displays larger structural flexibility when free-in-solution (Fig. 39B, red), we did a more rigorous evaluation of the actin-bound DysABD1 to rule out polymorphic binding on actin, i.e., Does the monomeric DysABD1 bind to actin in both an open and closed conformation? Since the initial decay in the DEER waveform for the actin-bound DysABD1 labeled at V120C/L239C is so slow, we did not observe any distances <4.0nm in our Gaussian fits (Fig. 39B, red). Model-independent analysis of DEER waveforms using Tikhonov regularization demonstrates this more clearly (Fig. 45A&B). Because Tikhonov regularization does not restrict the distance distributions to a set function, it is more likely to indicate small populations of distances that would otherwise be obscured in Gaussian fitting. Results from Tikhonov regularization to our DEER waveform is in excellent agreement with Gaussian fits, which speaks for the integrity of the DEER data analysis, and also rules out the presence of shorter distances between 2 to 4nm (Fig. 45A&B).

Fig. 45. Monomeric DysABD1 binding to actin exhibits no short distance components that would correspond to a closed model of binding. (A) Model-independent (Tikhonov) fit of the actin-bound DysABD1 DEER waveform labeled at V120C/L239C (Fig. 39B). (B) Distance distributions from model-independent fit (green) match that from Gaussian fits (cyan, Fig. 39B). No short distances < 4.0nm were detected the DEER data of actin-bound DysABD1 (C) Dipolar CW-EPR spectra also show no distances between 0.6-2.5nm. The spectrum of doubly-labeled DysABD1 (V120C/L239C) bound to actin (green) is overlaid with the singly-labeled actin-bound DysABD1 at V120C (non-interactive) (light green) in 0mM NaCl.
Though DEER cannot accurately resolve distances <2nm, the presence of such short distances would still cause a rapid initial decay in the DEER waveform, which is not seen (Fig. 39B, green). However, to confirm that there are no distances shorter than 2nm in the actin-bound DysABD1, we also evaluated the actin-bound monomeric DysABD1 labeled at V120C/L239C with dipolar CW-EPR. This allows us to accurately resolve distances that are within a range of 0.6-2.5nm (184). Similar to our findings in the UtrABD1-actin complex (195), the CW-EPR spectrum of monomeric DysABD1 binding to actin (Fig. 45C, green) overlays completely with its singly-labeled non-interactive counterpart (Fig. 45C, light green), indicating no distances <2.5nm present. Thus, we conclude that monomeric DysABD1 binds to actin in an ordered single opened conformation despite its structural flexibility when free in solution.

Discussion:

An open conformation of actin-binding for DysABD1.

The actin-bound structure of DysABD1 has been challenging to the field, with only a single report using electron microscopy (EM) with structural resolution of approximately 20Å (169). Though a closed conformation was determined from this EM study, the authors noted a bi-lobed mass decorating actin, with its density approximately twice that of the uni-lobed UtrABD1 in the same study. In conjunction with a reported $K_{eq}$ of 6.3µM between monomeric and dimeric forms of DysABD1, they concluded that the bi-lobed mass was in fact the binding of DysABD1 dimers to actin. This was not surprising since their experimental conditions included 200mM NaCl. We demonstrate in
Fig. 38 that DysABD1 dimers binding to actin can very well be construed as a closed conformation of binding. When DysABD1 binds actin as a monomer, no short distances corresponding to a closed conformation of actin is observed (Fig. 45B). Combined with a lower actin affinity under dimeric conditions (Fig. 42), our results support a monomeric, open conformation of the actin-bound DysABD1 (Fig. 39B).

![Diagram of actin-bound DysABD1 and UtrABD1](image)

**Fig. 46. Comparison of the actin-bound DysABD1 and UtrABD1 by simulations based on distance measurements.** (A) A model of actin-bound DysABD1 overlaid with our previously published actin-bound UtrABD1 model (195). The model was made through rigid body rotation with a hinge between residues 134/135 in DysABD1, similar to previous methods (195, 198). Though both DysABD1 and UtrABD1 binds actin in a open conformation where the two CH domains are far apart, the orientation of DysCH2 is different from that of UtrABD1 (195). This corresponds with the higher affinity and higher homology of CH1 compared with CH2 (189, 195). (B) If we force the crystal structure of monomeric DysABD1(175) to overlap with that of the actin-bound UtrABD1, then the distances between Cα-Cα of the labeled residues will be >1nm different than experimental data (Fig. 39).

To better compare structural similarities or differences between the actin-bound UtrABD1 and DysABD1, we simulated an actin-bound model of DysABD1 according to the method used to evaluate the actin-bound structure of UtrABD1 (195). Rigid body rotations of CH2 relative to CH1 of DysABD1 was performed using the peptide bond between residues 134 and 135 as a hinge. Distances measured between spin-labeled sites at V120C/L239C (Fig. 39), and between secondary sites at V120C/S227C (Fig. S52)
were used as constraints to make the simulated structure shown in **Fig. 46A** (green). The UtrABD1-actin complex shown in **Fig. 46A** (purple) was the simulated model that fits best with reported electron microscopy density (195). We chose the simulated actin-bound DysABD1 structure most similar to UtrABD1 from a series of 8 models to show in **Fig. 46A** (green). Though the CH1 domains of the actin-bound DysABD1 is well aligned with that of actin-bound UtrABD1 model, there are distinct differences in the relative disposition of CH2 with CH1 in DysABD1 and UtrABD1. The DysCH2 domain is comparatively closer to CH1 than its counterpart in UtrABD1. This allows DysABD1 be in comparatively bent conformation compared with UtrABD1 when bound actin. This “bent” conformation may allow the DysABD1-actin complex to have higher bending flexibility that can straighten under force, similar to the elastic α-solenoid proteins (199, 200). Our results reflect the highly conserved nature of CH1 while CH2 is much more diversified in these tandem CH domains (178, 189). When we forced the two CH domains in DysABD1 to match that of the proposed model for actin-bound UtrABD1 (**Fig. 46B**), the distances between Cα-Cα of the labeled residues would be drastically different from our experimental observations (compared the 3.8nm in **Fig. 46B** for V120C/L239C and the 4.7nm measured by DEER in **Fig. 39B**; the 3.8nm between V120C/S227C is also largely different from the 3.0nm measured by DEER in **Fig. S52**). The four main α-helices that constitutes each of the CH2 in DysABD1 and UtrABD1 have slightly different packing in the crystal structure (168, 175), which accounts for the slight misalignment in **Fig. 46B** that is most noticeable in CH2 domains. However, the root-mean-square-deviation of the DysABD1 and UtrABD1 structures shown in **Fig. 46B**
(0.3nm), is significantly smaller than the difference between our experimental measurements (Fig. 39B) and that observed in Fig. 46B. These results show that although DysABD1 also binds actin in an extended, open conformation, it has distinctive properties that differentiates it from UtrABD1.

**A flexible DysABD1.**

Comparison between the DysABD1 and UtrABD1 crystal structures lead to the initial prediction of a more structurally flexible DysABD1 (175). In the current study, we provide the first data showing the extent of this structural disorder (Table 2, Fig. 39). DysABD1 has larger distance distributions (FWHM) when free in solution compared to UtrABD1. Though DysABD1 also showed two structural states (indicated by the two Gaussian distances) similar to UtrABD1, the distributions in DysABD1 are comparatively wider and the two distributions relatively unresolved (Fig. 39B, red).

<table>
<thead>
<tr>
<th></th>
<th>Distance 1</th>
<th>FWHM</th>
<th>% Population</th>
<th>Distance 2</th>
<th>FWHM</th>
<th>% Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>UtrABD1 (136/222)</td>
<td>2.7nm</td>
<td>0.5nm</td>
<td>50</td>
<td>3.3nm</td>
<td>0.6nm</td>
<td>50</td>
</tr>
<tr>
<td>DysABD1 (120/239)</td>
<td>2.5nm</td>
<td>1.0nm</td>
<td>62</td>
<td>3.5nm</td>
<td>3.3nm</td>
<td>38</td>
</tr>
</tbody>
</table>

*Table 2. Comparison of the distance and distance distributions* (indicated as FWHM, full width at half maximum) of probed UtrABD1(195) and DysABD1 (Fig. 39).
Studies into elastomeric proteins or intrinsically disordered proteins show that hydrophobic interactions predominate protein-protein interactions of disordered domains (202, 203), which accurately describes DysABD1 (Fig. 40, Fig. 41, Fig. 44, Fig. 47A). In comparison, homologous hydrophobic residues in fimbrin or UtrABD1 does not exhibit any mechanistic effect to cause dimerization (Fig. 47C&D). In the case of UtrABD1, the homologous hydrophobic residues are aligned at the dimeric interface of the crystal structure (Fig. 47C). This is congruent with the hypothesis that the UtrABD1 dimer was mostly due to crystallization artifacts, unlike DysABD1 (168, 175). Our experimental data also show UtrABD1 remaining largely monomeric across a range of ionic strengths (Fig. 40 & Fig. 43A). Though fimbrin is known to dimerize between its naturally occurring dual tandem CH domains (2 conjoined ABDs), its dimer structure is very different from that of DysABD1 or UtrABD1 (Fig. 47D). The two ABDs of fimbrin are collapsed upon itself forming two
closed CH domains with the homologous hydrophobic patch (orange) buried within each monomer. Hydrophobic residues at the fimbrin dimer interface is also poorly aligned, (Fig. 47D, yellow), arguing against distinct hydrophobic association as the main mechanism for its dimerization (201). These evidence support a more structurally dynamic DysABD1 compared with other CH domains, with sufficient structural flexibility that would increase the probability for its large hydrophobic patches to associate and cause dimerization.

**Structural flexibility and powerful hydrophobic interactions in DysABD1, what does it mean?**

**(1) Inherent elastic properties.**

The striking hydrophobic force in DysABD1 interactions and its evident structural flexibility is also found in proteins with highly elastic behavior (204-207). Studies on elastomeric proteins have shown through both simulation and experimental data that hydrophobic associations and structural flexibility comprise the basis for an entropic spring (204-206). Elastin, a highly efficient entropic spring, utilize its propensity for hydrophobic associations and large-scale structural changes from a flexible entropic relaxed state to the ordered stretched state under force (204-206, 208, 209). Both hydrophobic forces and the large difference in structural entropy contribute to effective elastic recoil when elastin is under stress (205, 206, 210). Secondly, recent advances in tandem \(\alpha\)-helical solenoid structures also demonstrated that both hydrophobic interactions and structural flexibility within each core dominate their high elastic nature,
allowing extensions by nearly a factor of two can be rapidly and effectively reversed to its original native states (207). This study demonstrate that DysABD1, like other model elastomeric proteins, share fundamental structural and biochemical elements that would contribute to its function as a mechanical stabilizer at the muscle cytoskeleton (4-6).

(2) **A structurally fragile domain with a propensity to aggregate when perturbed by disease-causing point mutations.**

Accentuated hydrophobic interactions have also been implicated in aggregation of disordered proteins (211, 212). Indeed, disease-causing point mutations within DysABD1 induces extensive aggregative properties in full length dystrophin (193). This phenomenon is even more drastic in isolated DysABD1, which forms irreversible amyloid-like fibrils (194). Domains downstream of the disordered DysABD1 showed increasing thermodynamic stability towards the C-terminal end of dystrophin (149). Structurally labile domains gain stability when fused to a more elastic or stable protein that is sustained even under applied force (213). Thus, the more thermally stable downstream domains of dystrophin can reduce the aggregative propensity of mutated DysABD1.

**Conclusion:**

We have used dipolar electron-electron resonance (DEER) to demonstrate that monomeric DysABD1 binds actin in a single open conformation similar to that of UtrABD1 (Fig. 39 & Fig. 46) (195). Nevertheless, DysABD1 showed structural properties that distinct from UtrABD1. DysABD1 has larger structural flexibility when
free in solution (Fig. 39B, red). DysABD1 also has uniquely high sensitivity to ionic strength and is prone to hydrophobic interactions (Fig. 40, Fig. 41, Fig. 43B). Dimerization of DysABD1 locks this domain in the previously proposed closed conformation for actin-binding (169), but its 5-fold lower actin affinity in the dimeric form argues against a closed conformation of actin-binding (Fig. 42, Fig. 43B purple). DysABD1’s structural flexibility and propensity for hydrophobic interactions suggest a possible elastic nature of this domain and provide a possible mechanism for the aggregative nature of disease-causing point mutations.

**Materials and Methods:**

**Protein purification and spin labeling.**

Utr261 (UtrABD1) and Dys246 (DysABD1) was cloned from murine utrophin and dystrophin cDNA and ligated as a BamHI-XhoI fragment into pET23a vector (195). UtrABD1 contains no native cysteines, and the two native cysteines in DysABD1 was mutated to serines as described in the original crystal structure report (175). Site-directed mutagenesis (Stratagene Quikchange) was performed to engineer a pair of Cys residues into the construct for thio-reactive spin label attachment at chosen sites (136/222 in UtrABD1, 120/239 & 120/227 in DysABD1). The double-Cys mutant constructs were transformed into the *Escherichia coli* BL21 AI cell line and grown at 37°C in LB media to an absorbance of 0.6-0.8 at 600nm. Cells were induced with 1mM IPTG and 0.2% L-arabinose and allowed to grow for 3-5hrs at 28°C. Cells were then harvested by centrifugation and lysed with lysozyme for 1 hour at 4°C in 25% sucrose, 1mM EDTA,
1mM PMSF, 50mM Tris (pH 8.0, 4°C), followed by a freeze-thaw procedure in a dry ice/Isopropanol bath. The lysate was treated with 2U/L DNase I and incubated with the addition of 10mM MgCl₂ for 1 hour, then centrifuged at 40,000g for 30 minutes. The supernatant was purified using an anion-exchange column (HiTrap Q XL, GE) for UtrABD1 and a cation-exchange column (HiTrap Sp XL, GE) for DysABD1, which were equilibrated in 1mM EGTA, 1mM DTT, 20mM Tris (pH 8.0, 4°C). Protein was eluted with a linear gradient of NaCl from 0 to 0.5M. Fractions containing the target protein were verified using SDS-PAGE and pooled to run over a gel filtration column (Sephadex S200, GE) in 500mM NaCl, 1mM DTT, 50mM Tris (pH 8.0, 4°C). Fractions containing the target protein were again verified using SDS-PAGE and concentrated. DTT was removed prior to labeling using Zeba desalting columns (Pierce). The constructs were then labeled with MSL [N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide] (Toronto Research Chemicals, North York, Ontario). Labeling efficiency was determined by spin-counting with using a Bruker E500 spectrometer at X-band (9.5 GHz) with an SHQ cavity at a microwave power where there was no saturation (0.03 mW). The molar ratio of spin labels per labeling site was determined to be 0.92 ± 0.1.

**Dipolar electron-electron resonance (DEER) and dipolar CW-EPR.**

We performed DEER to measure distances from 2 to 6nm, and dipolar CW-EPR for distances from 0.6 to 2.5nm. DEER experiments were performed and analyzed as described previously (182). The concentration of UtrABD1 and DysABD1 were between 60-100 µM. F-actin was prepared from rabbit skeletal muscle following methods in
Both proteins were dialyzed into a buffer containing 2mM MgCl$_2$, 1mM DTT, 10mM Tris (pH 8.0, 4°C) with varying concentrations of NaCl (0mM, 100mM or 500mM). The final actin concentration was 400 to 500µM. Actin was mixed with UtrABD1 and DysABD1 at the desired molar ratio (4 moles actin per mole UtrABD1 or DysABD1) with concentrations of both proteins above 40µM (> measured $K_d$). The UtrABD1-actin and DysABD1-actin complexes are subsequently pelleted to remove any unbound proteins with high speed ultracentrifugation (100,000g, 20 minutes) and resuspended into 120µL with 10% glycerol (vol/vol). Final concentrations of UtrABD1 and DysABD1 in each sample concentration was 60 to 90µM. Samples were flash-frozen in liquid nitrogen in 5mm OD quartz NMR tubes (Wilmad glass, Buena NJ). Samples were stored at -80°C prior to data acquisition. Pulsed EPR experiments were performed with an Elexsys E580 spectrometer (Bruker Biospin) containing a dielectric resonator (MD-5; Bruker Biospin) at 65K. Spectra were acquired using a four-pulse sequence, with the pump frequency centered on the central resonance of the nitrooxide and the observed frequency set at a low-field resonance >65 mHz away from the pump frequency. The $\pi/2$ pulse was 16ns and the ELDOR pulse was 40 to 44ns. Each waveform was recorded at 65K for 24 to 72hrs. The resulting spectra and spin-spin distances were analyzed using DeerAnalysis2008 software suite (183) and software developed in-house (WACY, Edmund Howard). Background subtraction of the DEER waveforms were done using singly-labeled UtrABD1 or DysABD1 corresponding to each buffer condition (183). To account for protein flexibility, fits to the data were done assuming that each
conformational state of the protein corresponds to a Gaussian distribution of distances:
\[
\rho(r) = \frac{1}{\sigma \sqrt{2\pi}} \exp\left(-\frac{(r - R)^2}{2\sigma^2}\right),
\]
where \(\sigma\) is the standard deviation and FWHM is the full width at half maximum of the distribution.

The number of Gaussian components in the fit \(n\) was determined by varying \(n\) such that no improvement in the residual plot and \(\chi^2\) value was obtained by increasing the number of components to \(n+1\) (Fig. S49&Fig. S50) (184, 195). Distance distributions determined by Tikhonov regularization were consistent with the Gaussian fits (Fig. 45A&B).

Spin-spin distances were also determined by CW-EPR using a Bruker E500 spectrometer at X-band (9.5 GHz) with an SHQ cavity, as described previously (184). Spectra were acquired at 200K with a 200G field sweep to detect spectral broadening. The modulation amplitude was decreased to 1G to minimize modulation broadening, and all spectra were acquired under non-saturating conditions (0.03mW) (184, 195).

**Actin-cosedimentation Assay.**
Binding affinities of UtrABD1 and DysABD1 constructs were determined by mixing 4µM with a range of actin concentrations (0-50µM), then centrifuging at 100,000 x g for 20 minutes at 4°C or 6µM of actin with a range of UtrABD1 and DysABD1 concentrations (0-25µM). Concentrations of free UtrABD1 and DysABD1 were
determined using a Bradford assay using a BSA standard (Biorad) in addition to densitometry. Regression analysis was done fitting the data to $X_b = A/(A + K_d)$, where $A$ = concentration of free actin protomers, $X_b$ = the fraction of actin protomers with bound UtrABD1 or DysABD1, and $K_d$ = dissociation constant. The $K_d$ values determined for native UtrABD1 or DysABD1, unlabeled and MSL-labeled constructs (Fig. S53) were very similar. Thus, Cys-mutations or MSL attachment did not affect the affinity of the ABD1s to bind to actin.

Native gel.
Polyacrylamide gel electrophoresis was run in the absence of SDS to allow UtrABD1 or DysABD1 to migrate in their native conformation. 15 µL of UtrABD1 or DysABD1 was loaded in a 12% agarose gel under varying NaCl concentrations, and ran at 150V for 4 hours.

Fluorescence resonance energy transfer (FRET).
Fluorescence lifetime waveforms were acquired using a customized time-resolved fluorescence spectrometer with direct waveform recording built in collaboration with FluorescenceInnovation.Inc (214). We have previously demonstrated that this instrument allows $10^5$ times higher throughput than time-correlated single-photon counting with comparable signal-to-noise, accuracy and resolution (214). DysABD1-IAEDANS were excited by a Q-switched microchip YAG laser (NanoUV-355; JDS Uniphase) at 355nm with a pulse repetition frequency of 10kHz and a uniform narrow (~1ns full width at half
maximum) high-energy pulse (1 µJ/pulse). Photon emission was polarized to the magic angle (54.7°), filtered through an interference band-pass filter (Semrock 470/22 nm), detected with a photomultiplier tube module (H5773-20, Hamamatsu), and digitized (Acqiris DC252; time resolution, 0.125 ns). Time-resolved FRET waveforms were globally analyzed as described in (215) (Fig. S48).

**Supplementary Information:**

**Circular dichroism:**

DysABD1 and UtrABD1 constructs were prepared in PBS, at 20µM concentration. Proteins were clarified by ultracentrifugation at 100,000 x g for 15 min at 4°C prior to data acquisition. CD spectra were acquired using a JASCO J-815 spectrophotometer and a temperature-jacketed spectral cell with a path length of 0.1cm. Spectra were taken from 200nm to 260nm at intervals of 1nm, bandwidth of 1nm and a 1s averaging time with temperature set at 25°C. Data analysis were done assuming that the CD spectrum is a linear combination of the contributions from α-helix, β-sheet and random coil.

\[
E(\lambda) = x_\alpha E_\alpha (\lambda) + x_\beta E_\beta (\lambda) + x_R E_R (\lambda)
\]

Eq 31

The unit of E is molar ellipticity (deg*cm²dmol⁻¹) per residue. The % α-helix of the sample is similar between native, unlabeled and MSL-labeled DysABD1s (). This
indicated that the presence of the mutation or the attachment of MSL did not significantly alter the secondary structure of the CH domains in our studies (Fig. S54).
Fig. S49. Summary of fits of Dys120/239.

Fig. S50. Summary of fits of Utr136/222.
**Fig. S51.** Dimerization in a doubly-labeled CH domains sample results in a faster DEER decay and dampened oscillations. (A) Monomeric UtrABD1 and the Cα-Cα distance between the labeled sites. (B) Dimeric UtrABD1 in the crystal structure (1qag)(168). UtrABD1 dimers give 4 different distances measurements when probed at residues 136/222 simultaneously. This gives a predicted mean distance of 3.0nm within the sample. (C) Simulated DEER waveform based on distances within the monomeric and dimeric species based on the crystal structure. (D) Monomeric DysABD1 and Cα-Cα distance between homologous labeling sites as UtrABD1. (E) DysABD1 gives 3 different distance measurements when probed at homologous sites (120/239). The predicted mean distance is approximately 2.6nm. (F) Simulated DEER waveform based on distances within the monomeric and dimeric species within the crystal structure.

**Fig. S52.** Results from distance measurements between a secondary site 120/227 in DysABD1. These residues were selected based on structural similarities between labeling sites 136/222 in UtrABD1.
Fig. S53. Actin cosedimentation assays of native and spin-labeled DysABD1 at 120/239 and 120/227. Spin-labeling does not perturb actin binding.
Fig. S54. Circular dichroism of native DysABD1, and spin-labeled 120/239 & 120/227 under 0mM, 100mM and 500mM NaCl conditions. No significant perturbations in secondary structure was detected with spin-labeling. A slightly higher α-helicity is noted under conditions with higher NaCl, which presumably is due to stabilization of the α-helix structure of the linker region between the two CH domains.
Fig. S55. Summary of fits of Dys120/227.
CHAPTER 6 – Elucidating the Twist and Bend in Actin
Rotational Dynamics:

Introduction:

Actin network serves in a broad range of eukaryotic cellular functions with its complex mechanical properties and internal motions (217) (Fig 56). Depending on its function, either tracks for contractile myosin motors or crosslinked bundles in filopodia, actin can adapt a wide range of elastic properties that is dependent on the actin-bound proteins (195, 218-224). The variable twist of actin filament was shown to regulate its elastic properties (225). Isolated actin filament display highly polymorphic structures (226), which is accentuated when binding to different protein partners (227-229) or under tension (230, 231).

The very first experimentation to determine actin flexibility is through evaluation of its bending motions in the millisecond to seconds time-scale using fluorescence microscopy (232) or electron microscopy (152, 233). The bending, or flexural flexibility is described by the persistence length (L_p) of actin filaments (232, 234). Persistence length is measured by the length over which correlations in the direction of the tangent was lost. The flexibility of actin filaments were mainly evaluated by its bending motions, until advances in the Thomas lab have allowed evaluation of the actin filament twisting.
flexibility by applying the torsional twist model to evaluate time-resolved actin phosphorescence anisotropy decays (144, 158).

Simulations have shown that the bending and twisting properties of actin are highly coupled, with up to 60% of the filament subunit elastic free energy due to twist-bend coupling (146). In myosin VI, the twisting and bending flexibilities follow similar trends shown by actin phosphorescence anisotropy and fluorescent microscopy experiments, respectively (235). For a cytoskeletal mechanical stabilizer, the contribution of actin’s twist verses bend to overall resilience as described by time-resolved phosphorescence studies (Chapter 3) have yet to be elucidated (236).

In this Chapter, I will summarize preliminary findings from fluorescence microscopy to prove how full length dystrophin and utrophin affect the bending motions of actin in the millisecond to second time domain.

**Methods:**

**Sample preparation, image acquisition and analysis:**

Actin was prepared from rabbit skeletal muscle and labeled with phalloidin-rhodamine (Rh-Ph, Molecular Probes) at 1U per 0.1mg/ml F-actin. Final concentration of Rh-Ph actin is 12nM in a concentration of 15mM dextrose, 2mM MgCl₂, 100mM NaCl, 1mM DTT, 55 µg/ml glucose oxidase, 36 µg/ml catalase, 45 µg/ml glucose, 10mM Tris (pH 7.6). Final concentrations of dystrophin and utrophin in this trial experiment is 48nM which amounts to approximately 20% decoration on actin. Images of individual filaments were acquired on an Eclipse TE200 microscope (Nikon instruments) using excitation
filters 430nm/24 and emission filters 470/24. Images were acquired at 100x oil drop objective, an X-cite metal-halide lamp (EXFO) and a Cascade II CCD camera (Photometrics). A 100ms exposure time was used and emission wavelengths were acquired with 0.5 second intervals over a period of 2 minutes.

For analysis, the images were processed using ImageJ software and analyzed with a code written in Matlab (The Mathworks, Matic, MA) provided to us courtesy of Brannon McCullough, De La Cruz Lab. The bending persistence length \((L_p)\) was determined by fitting the average of >100 angular \((\theta)\) cosine correlation measurements \((C_s)\) of a segment length \((s)\)corrected for measurement variance, to a 2D correlation function:

\[
C_s = \cos[\theta(s) - \theta(0)] = e^{-s/2L_p }, \quad \text{Eq. 32}
\]

A: Actin  
B: Actin + Dystrophin  
C: Actin + Utrophin

**Fig 57.** Persistence length of actin filament bound to dystrophin or utrophin at 20% decoration. The bending amplitude does not visibly change, but the rate of actin fluctuation is higher when dystrophin binds, and slower when utrophin binds.

**Preliminary results:**
The persistence length ($L_p$) measured for actin-only is within the range of previously reported values (216, 237). A total of 10 samples were evaluated for actin, dystrophin-decorated actin, and utrophin-decorated actin (Fig 57). There was a slight increase in the persistence length of dystrophin or utrophin-bound actin, but the increase is not dramatic and is mostly within the range of persistence length reported from actin only samples (Fig 58) (216). However, the rate of actin flexural fluctuation was visibly different between actin, dystrophin-decorated actin and utrophin-decorated actin.

In this preliminary study the rates of fluctuation was determined by measuring the $L_p$ of individual images from a series acquired over 2 minutes with 0.5 second intervals (Fig 59). This allowed us to compare between the amplitude and rates of the actin bending to that of twisting (defined by TPA, Chapter 3). For preliminary comparison, we approximate TPA data to represent actin’s twisting motions, since the bending motions from millisecond to seconds time scale would have a comparatively smaller contribution, if any, in our TPA data since the phosphorescent dye used have a lifetime of 500-1000 microseconds (Chapter 3).
Overall evaluation showed that binding of dystrophin and utrophin reduces the amplitude of both bending and twisting, but they have very different effects on the rates (Fig 59). Dystrophin increases the rate of both bending and twisting, with an emphasis on bending. Utrophin mainly just increased the rate of actin filament twisting. These differences are rather distinctive and can help explain the different mechanical properties of the two proteins and why replacing utrophin with dystrophin in myocytes increased its cellular stiffness (61). Distance measurements within the ABD1 of dystrophin (Chapter 5) and utrophin (Chapter 4) indicated a larger structural flexibility in DysABD1 compared with UtrABD1 (Fig. 39, Table 1). Our results in Chapters 4 & 5 laid down the foundation for structural evaluations in other domains of dystrophin and utrophin. A structural blueprint of individual domains will help us understand possible functional significance of the bending or twisting properties of Dys-actin and Utr-actin complex.
Although TPA studies have determined that utrophin constructs are more effective in increasing actin’s resilience (Chapter 3), TPA studies are more biased towards the twisting of actin filaments rather than the bending. To refine our structural definition of resilience, I have preliminarily compared the resilience measured by TPA to that determined from persistence length ($L_p$) measurements. Resilience was calculated by Eq. 23 as described in Chapter 3 (Fig 60). It appears from this initial evaluation that dystrophin is more effective in increasing actin filament’s resilience from a bending perspective but utrophin is more effective from a twisting perspective.

This finding provides a glimpse into the reason behind replacing utrophin with dystrophin as myocytes matures (8-10). Additionally, a better algorithm is required for more accurate calculation of the bending rate of actin complete with error. In any case, this preliminary study sets up the basis for future studies to (1) evaluate the twist-bend correlation in dystrophin or utrophin-bound actin (2) refine the definition of actin’s resilience when bound to the two cytoskeletal proteins.
CHAPTER 7 – A comprehensive view of dystrophin-actin and utrophin-actin interaction: Future Directions

The more flexible utrophin is required for muscle maturation:

Although utrophin is present at the muscle cytoskeleton during the regenerative phase, it is replaced by the mutation prone dystrophin as the muscle matures (8-10). The higher resilience and flexibility seen in utrophin-actin complexes in TPA studies suggest a mechanism for replacing utrophin with dystrophin at the muscle cytoskeleton as the muscle matures (Fig. 25, Fig. 27). The microenvironment for a myocyte to properly organize actomyosin striations require a very narrow range of matrix elasticity which is less flexible than that required for myoblast differentiation (62, 63). Direct measurement of a skeletal myoblast over the course of its differentiation also showed an increase in cellular rigidity as the muscle matures (61). This coincides with a more flexible utrophin-actin complex detected by TPA, which is later swapped for the comparably more rigid dystrophin-actin complex in mature myocytes (Chapter 3, Fig. 25 & Fig. 27).

A disordered N-terminus and an ordered C-terminus in dystrophin. What is the benefit for these dual properties to be within one protein?

Elastomeric proteins are classified based on their ability to behave as a mechanical buffer in many systems, such as tendons, vascular walls, etc (206). They are capable of high deformation without rupture and returning to their original state when stress is removed. Though their energy storage/dissipation system differs between various
elastic proteins, they can be further classified as either a ordered (spectrin, collagen) or disordered (elastin, resilin) systems (Fig. 61). The elastic potential of a protein system consists of two components – the entropic energy (fs) and the internal energy (fe). In an ordered system such as collagen, its highly organized triple helical structure allows a capacity to store more than 10 times the elastic energy of steel (161, 238). In a disordered system, such as the case with the internally disordered protein elastin, are akin to rubber, storing elastic energy in the entropic difference between stressed and relaxed states (205, 206). The entropy of a relaxed state is higher than that of the deformed state, which results in elastic recoil within the protein system (210). In any case structural studies into disordered systems have given us insight into elastomeric properties in these proteins, but studies directed towards dystrophin and utrophin are still needed.

In some systems, we observe both types of elastomeric proteins working hand in hand to maintain mechanical stability. For example, in the aortic wall, collagen provides the strength required to prevent rupture due to high blood pressures, while the resilience and extensibility of the vessel wall is managed by the disordered elastin (239, 240).

It is highly intriguing that dystrophin appears to possess both types of elastomeric properties within a single protein. Its C-terminal region has higher thermal stability, and in many cases is shown to stabilize the structure or function of dystrophin as a whole.
Conversely, the dystrophin N-terminus is more structurally flexible. This variation in structural stability described by thermal melting experiments using circular dichroism is unique to dystrophin, whereas utrophin showed a highly homogenous thermodynamic stability through the entire molecule. Further biophysical characterization of the flexibility by spectroscopic probing in other domains of dystrophin and utrophin (similar to Chapter 4 & 5) complimented by viscoelastic measurements using AFM or optical tweezers will help unravel the mystery behind the fundamental functional similarities of dystrophin and utrophin in animal models despite their drastically different biochemical (57) and structural differences (147, 236) (195)(Chapter 4 & 5).

![Fig. 62. Correlation between observed structural disorder and protein function. Adapted from (241).](image)

**Flexibility is structural dynamics and structural disorder combined.**

When a large distance distribution is detected in DEER, it can be attributed to two structural phenomenon – (1) dynamics, where we observe large-scale motions of a well structured domain, and (2) disorder, where there is local unfolding of the labeled domains. Rarely in biology are these two phenomenon entirely separate. A
comprehensive review of spectroscopic results and simulations have suggested that a structure fluctuations larger than 1.0nm would have a significant unfolding component (241) (Fig. 62). A similar filamentous muscle cytoskeletal protein, titin, has been well described for its structural flexibility and disorders(242-245).

Coupled with evidence of the unfolding of Ig domains under force unfolding,suggestive of an entropic nature of its spring-like function, the stiffness of titin can be tuned based on changes in ionic strength suggesting it can behave as an enthalpic spring in addition to its function as a entropic spring (Fig. 63). This was determined to be due to both hydrophobic interactions as well as changes in electrostatic potentials in the PEVK domain (242, 246). Changes in charge pairing or chain configurations from hydrophobic interactions allows titin adapt a wide range of molecular elasticity. Our current study points to a similar elastic tuning potential in DysABD1 due to its strong hydrophobic associations and structural flexibility. Further structural and biophysical characterizations through intermolecular measurement between DysABD1 and actin, use of fluorescence

Fig. 63. Diagram of the relationship between hydrophobic association and entropic elastic force development in titin. Adapted from (204).
anisotropy or CW-EPR to probe regional structural flexibility under a range of ionic strength is required. Future directions also include repeating the measurements done on UtrABD1 and DysABD1 as described in Chapter 4 & 5 in larger constructs extending out to the central spectrin-type repeat regions to better define the structural regulation between the more thermally stable downstream domains on the flexible ABD1.

Though utrophin increases actin’s resilience more effectively than dystrophin (Fig. 25), dystrophin, at least in the ABD1, shows more structural flexibility than that of utrophin (Fig. 39, Table 2). This suggests that dystrophin’s mechanical buffering has at least two facets: (1) increase in actin’s resilience (2) dystrophin itself is an effective entropic spring. In contrast, utrophin mainly increases actin’s resilience but is a stiffer linker between the actin cytoskeleton and extracellular matrix through the DGC.

Although we currently only have structural information in DysABD1, other studies have suggested a flexible dystrophin molecule in other domain as well (247). AFM studies show that the central spectrin-type repeats in dystrophin can unfold under forces a few myosin molecules can provide during the crossbridge cycle (247). Unfortunately, the extensibility of homologous utrophin constructs were not investigated in the same study. Our results are the first to compare the structural flexibilities and possible roles as elastic buffers between dystrophin and utrophin and more biophysical studies of this nature are still needed. This information would be extremely important, since current methods for gene therapy are still restricted by the efficiency of construct delivery and prolonged expression of the delivered constructs (115, 126). Plus, even the best current gene therapy constructs cannot fully restore mechanical function in the
skeletal muscles of mouse models, which has a smaller loading force than that of dogs or humans (117, 126). To design the next generation of gene therapy constructs with higher mechanical restorative capacity, effectively minimizing the total concentration of therapy constructs required at the muscle cytoskeleton, we would need a more detailed biophysical perspective into the similarities or differences between dystrophin and utrophin, as well as the functions of their individual domains.

More than just a membrane linker – cooperativity of function is directed by the C-terminal (CT) region.

The CT region of both dystrophin and utrophin conveys cooperativity in how the ABDs restrict actin rotational amplitude and increase in rate (150, 236). This suggests an allosteric regulation of the CT region on the interactions between the ABDs and actin. However, the mechanism behind this long-range allosteric regulation is unknown.

I will use the contiguous ABDs of utrophin as an example to illustrate possible models for this allosteric regulation (Fig 64). One possibility is that the CT region serves
to orient the ABDs so that they will bind at a specific orientation along the actin filament (Fig 64, Model I). In this case, loss of the CT region will cause a disarray in orientation of the ABDs on actin leading to a breakdown in cooperative regulation. Another possibility is that UNR10 may be less wrapped around the long axis of actin compared with full-length utrophin (Fig 64, Model II). It is suggested that the central STRs wrap around actin along the inner and outer cleft of the longitudinal axis similar to that of tropomyosin (169). When the ABDs are wrapped around actin, it can convey cooperativity in its interaction with actin that is not present when aligned in a linear fashion. Finally, cooperativity can be due to the CT region inducing a propensity for utrophin to cluster when binding to actin (Fig 64, Model III). For all three models, allosteric structural changes within the actin filament itself may play a role in inducing cooperative regulation on its dynamic twisting and bending motions.

The story is more complex bimodal ABDs of dystrophin and how they affect actin structural dynamics. In either case, further investigations by labeling dystrophin and utrophin directly are required to test these possible models. Despite extensive investigations into mouse models of dystrophin and utrophin, structural work on the two proteins lax in comparison due to technical difficulties in evaluating protein complexes of this enormity. In this study, we have taken a stride forward to evaluate properties of the structural dynamics of dystrophin-actin and utrophin-actin complexes, but evidently more work is required. Nevertheless, our results demonstrate that the CT region is more than just a site for attachment to the extracellular matrix through the DGC; it can directly
affect the biophysical properties of dystrophin-actin and utrophin-actin interaction (150, 236).

**Disease-causing point mutations and their effect on actin’s resilience determined by TPA.**

Studies by Henderson et al., characterized the first biochemical effects of disease-causing point mutations in the ABD1 of full length dystrophin (193). Their sophisticated studies showed a surprising result – the presence of the point mutation in ABD1 did not drastically alter the actin-binding affinities of the dystrophin. The slight perturbations in $K_d$ to actin is disproportional to the severity of the disease presentation. Additionally, they note a higher aggregative behavior that seems to correlate better with the severity of the mutation. But this correlation does not extend to all of the mutations evaluated in the study, and the *in vitro* aggregation alone still could not fully explain the extent of the dystrophic presentation in patients. The most severe mutation investigated (L54R) showed a aggregation level approaching 50%, but only about 20% of dystrophin is seen in skeletal muscles containing this defect (47). In collaboration with Dr. Henderson and the Ervasti lab, we performed some preliminary evaluation of the effects of these point mutation constructs on actin rotational dynamic using TPA (for more details on method, see Chapter 3).
We acquired some preliminary data on three disease-causing point mutations that were addressed in Henderson et al (193). The most severe mutation is the L54R, which causes the Duchenne form of muscular dystrophy (47). Y231N causes a mild form of Becker’s muscular dystrophy (46) and K18N is the mildest (248), causing X-linked cardiomyopathy (Fig. 65). Preliminary studies on the amplitude and rates of actin rotational motion shows that all three mutations decreases its effect on actin rotational dynamics (Fig. 66A). However, there is poor correlation with the severity of disease presentations. When we calculate the relative resilience of these constructs at 60% decoration on actin (method described in Chapter 3, Eq. 23), there is only a slightly better correlation between “Relative Resilience of Bound Actin” with disease severity (Fig. 66B). Despite poor correlation with disease severity, all three dystrophin mutants decreased its effect on actin’s resilience. This suggests that the pathology of these disease-causing mutations are multifaceted, and that decrease in affinity, aggregation and control over actin’s rotational motion all are contributing factors. However, further evaluations of these mutations, including intradomain measurements as described in Chapters 4 &5 are still needed.

Fig. 66. TPA results on disease-causing mutations in dystrophin. (A) Amplitude and rates of actin rotational motion (B) Calculated relative resilience according to Eq. 23 (more information, see Chapter 3)
Concluding Remarks:

This project would never be possible without the pioneering efforts by Dr. Ewa Prochniewicz and members of Dr. James Ervasti’s lab. Results from this thesis have furthered our understanding of the molecular interactions of dystrophin and utrophin with actin, but its contribution is still modest in our pursuit for structural-based designs of next generation therapeutics for muscular dystrophy. Unlike other elastomeric proteins proposed to have similar functions, such as titin, there is still much work to be done to create a biophysical blueprint of dystrophin and utrophin. Furthermore, there is lack of studies directly comparing the molecular properties of dystrophin with utrophin. The Ervasti lab have opened this forefront with several landmark publications (22, 57, 147). As their collaborators, we look forward to accelerating these investigations through high-resolution structural studies.
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